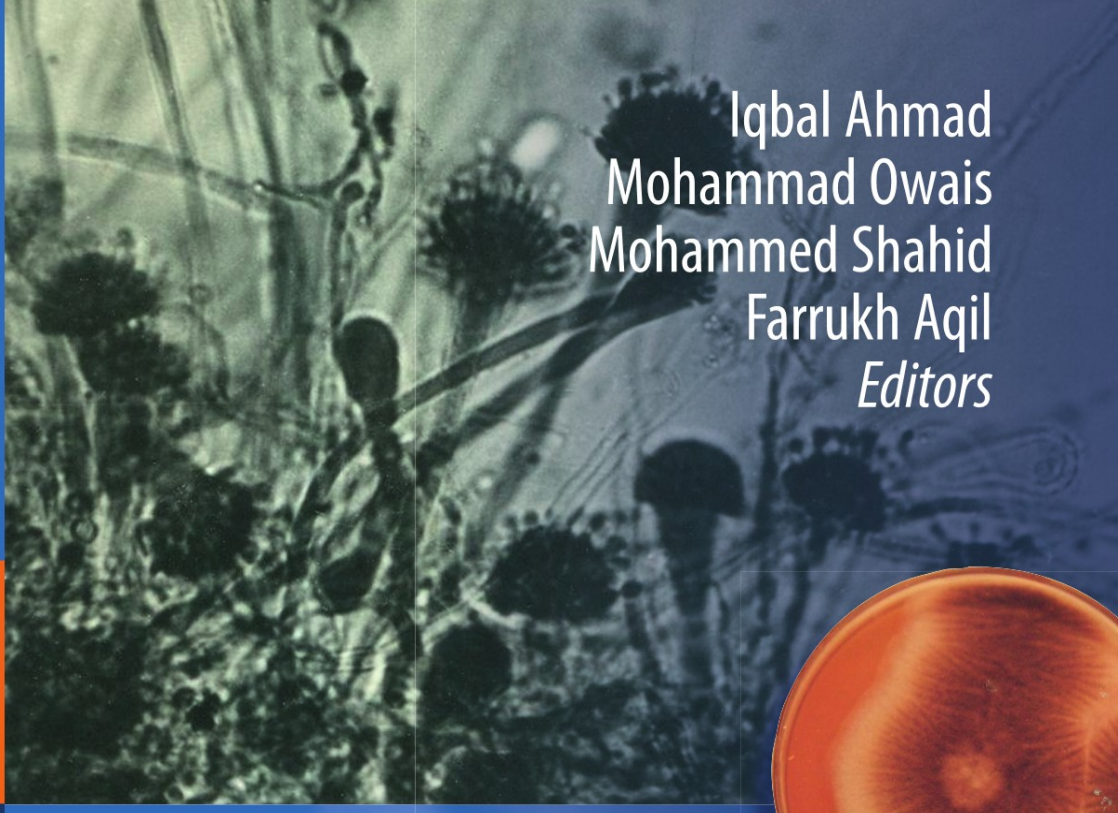

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Editors

Combating Fungal Infections

Problems and Remedy

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Preface

Fungi are eukaryotic microorganisms that are closely related to humans at cellular level. Human fungal pathogens belong to various classes of fungi, mainly zygomycetes, ascomycetes, basidiomycetes, and deuteromycetes. In recent years, fungal infections have dramatically increased as a result of improved diagnosis, high frequency of catheterization, instrumentation, etc. However, the main cause remains the increasing number of immunosuppressed patients, mostly because of HIV infection and indiscriminate usage of antineoplastic and immunosuppressive agents, broad-spectrum antibiotics and prosthetic devices, and grafts in clinical settings. Presently available means of combating fungal infections are still weak and clumsy compared to control of bacterial infection. The present scenario of antifungal therapy is still based on two classes of antifungal drugs (polyenes and azoles).These drugs are effective in many cases, but display toxicity and limited spectrum of efficacy. The recent trend towards emergence of drug-resistant isolates in the clinic is an additional problem. In recent years, a few new antifungal drugs have entered the clinics, but they are expected to undergo same fate as the older antifungal drugs.

The application of fungal genomics offers an unparalleled opportunity to develop novel antifungal drugs. However, it is too early to expect any novel drugs, as the antifungal drug discovery program is in the stage of infancy. Interestingly, several novel antifungal drug targets have been identified and validated. In contrast to the classical and genomic approaches to drug discovery, other traditional knowledge derived from natural products and phytomedicine has ample opportunity to provide an alternative mode of action for combating fungal infections which include superficial, cutaneous, and invasive and life-threatening systemic mycoses. Since a lot of scientific literature and data have been generated in recent decades on various aspects of fungi such as biology, taxonomy, drug resistance, and mode of action of antifungal drugs, on the molecular basis of virulence and pathogenicity, and on therapeutic approaches. However, little effort has been made to discuss various issues in a holistic way and to present them in the form of a book to provide more interrelated, interdisciplinary work on combating fungal infections with different strategies.

We have discussed these issues, and decided to edit a book in the larger interest of students and researchers, to fill the gap in book publication. Here we have worked to bring together experts in the field to contribute a series of chapters spanning a cross-section of the field. It is our hope and intent that the outcome of these efforts in the form of *Combating Fungal Infections: Problems and Remedy* will serve as a valued resource to the entire scientific/academic community. We hope that this text not only encapsulates the recent literature in the field, but will also illuminate related issues for the benefits of teaching, research and drug discovery.

This book consist of 20 chapters which address various aspects pertaining to the pathogenesis of fungi, their management, and recent advancement in their treatment. Chapter 1 provides a glimpse of various types of fungal infections, with special reference to immunocompromised hosts. Chapter 2 discusses virulence and pathogenicity of fungal pathogens. Chapters 3 and 4 deal with the reservoirs of animal and ocular fungal pathogens. Recent developments in antifungal drugs and their mode of action are the subject of review in Chap. 5. As presently available, antifungals, in particular polyene-based anti-fungals, manifest various side-effects, especially nephrotoxicity. Chapter 6 discusses these issues, especially in children under the age of 12. The development of resistance in fungal pathogens, including multidrug resistance and its mechanism, is dealt with in Chaps. 7 and 8. Diverse diagnostic approaches for fungal infections are reviewed in Chap. 9. Chapters 10 and 11 discuss antifungal therapy, and combination strategies used in combating invasive fungal infections. Chapter 12 deals with the management of pulmonary mycoses in stem-cell transplantation. Novel antifungal drugs (synthetic and herbal), fungal vaccines, and metabolic pathways as drug targets are discussed in detail in Chaps. 13, 14, 18, and 19. The role of innate immunity in management of dermatomycosis is elaborated in Chap. 15. Cytokine therapy and its application in the management of fungal infections are the topic of discussion in Chap. 16. Chapter 17 reviews the potential role of immunomodulators in the treatment of systemic fungal infections. As novel drug delivery systems have a great potential for modifying the pharmacokinetics of medicaments, Chap. 20, taking this fact into consideration, covers state-of-the-art delivery systems for controlling fungal infections.

We have made our sincere efforts to provide good scientific content in this book. It is our hope that this book will be useful to students, teachers, researchers and clinicians. However, there may also be some shortcomings. We invite you to communicate your experiences with the book to us.

We thank all the contributors/experts for timely submission of their excellent contributions and for their overall cooperation. We also thank many leading scientists in this field who may not contributed directly, but have encouraged or guided us towards successful completion of this project.

We express deep gratitude to Dr. J.N.S. Yadava FNA, Dr. C.M. Gupta FNA, Dr. G.P. Datta FNA, Prof. Shamim Jairajpuri FNA, Prof. M. Saleemuddin, and Dr. Shahid Jameel FNA, who have all been a great source of inspiration. We also thank all our respected teachers, colleagues, and friends for their cooperation

and critical suggestions. Assistance provided by research students, especially Mr. M. Sajjad Ahmad Khan (SRF, ICMR) is greatly appreciated. The technical and scientific advice received from learned reviewers and the Springer book editorial team, especially from Dr. Britta Settmacher and Anette Lindqvist (Springer, Germany), is thankfully acknowledged. Finally we acknowledge the Almighty God, who provided all the positive thoughts and channels needed for completion of this book project.

Aligarh, Louisville
July 2010

Iqbal Ahmad
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Editors

Dr. Iqbal Ahmad, M. Phil., Ph.D. is a senior faculty of Microbiology in the Department of Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University (AMU), India, Principal Investigator of the UGC research project, and supervises an Indian Council of Medical Research (ICMR) Project on essential oils efficacy against drug-resistant pathogenic fungi. He is actively involved in teaching as well as research activities, guiding students in their M.Sc. and Ph.D. theses programs. His major research training is in the area of *Escherichia coli* plasmid biology, transferable and mobilizable plasmids, virulence and pathogenicity, drug resistance, characterization of enterotoxins, and anti-secretory drug screening in animal models. Trained at the Central Drug Research Institute, Lucknow (1989–1993) and Himalaya Drug Company India, Dr Ahmad joined AMU in 1995, where he conducts research in the area of soil microbial ecology, plant–microbe interactions, metal & drug resistance and distribution of extended spectrum beta lactamases among environmental bacteria, and metal–fungi interactions. Screening of traditionally used Indian medicinal plants for the various biological activities (antibacterial, antifungal, antimutagenic, antioxidant, and antiplasmid) of plant extracts, essential oils and natural products is an ongoing research activity since 1995 in his laboratory. His works on medicinal plants have received high acclaim in the international scientific community. Another area of his research interest is in developing effective PGPR and fungal-based biosorbents for heavy metal removal from aqueous systems and wastewater.

Dr. Ahmad has published more than 60 research papers/review articles, edited four books, and guided several M.Sc/Ph.D students. His recent research interest includes bacterial quorum sensing and biofilm modulation by natural products against microbial pathogens, for the purpose of discovering novel antipathogenic compounds to combat fungal and or bacterial infections. Dr Ahmad is currently a member of many scientific bodies, both national and international. He is also a member of the reviewer panel of various international scientific journals, and a founder member of the Association of Professional Biotechnologists, India.

Dr. Mohammad Owais, M. Pharm., Ph. D., is Associate Professor in the Interdisciplinary Biotechnology Unit, AMU, Aligarh. He is actively involved in teaching, as well as in research activities guiding students for their PhD programs. He is also jointly running a research venture in collaboration with Cadilla Pharmaceuticals Ltd., Ahmedabad under the PRDSF program of DST, Govt. of India. As is evident from recent studies in Dr. Owais's laboratory, the efficacy of nanoparticle-based formulations was increased several-fold by co-entrapment of potential immunomodulators such as tetrapeptide tuftsin, protein A and various analogs of muramyl peptide, etc., in combination with antifungal agents.

Trained at CDRI, Lucknow and IMTECH, Chandigarh for his graduate program, Dr. Owais got the opportunity to work in various state-of-the-art research laboratories. During his post-doctoral training, he worked for Dr. R. C. Gallo's group at NIH, USA, where he developed HIV-2-based gene therapy vectors and also elucidated functional domain mapping of chemokines for their anti-HIV activity. After joining Aligarh Muslim University in 1998, he extended his work on drug targeting, in which nanoparticle/liposome/microsphere/erythrocyte-based novel drug-delivery systems were developed for treatment of various infectious diseases in model animals.

At present, he is mainly focusing on the development of antigen delivery systems to develop vaccines against various infectious diseases of fungal as well as bacterial origin. He has successfully demonstrated the potential of fusogenic liposomes in activation of antigen-specific CTL response. He has also demonstrated that co-administration of immunomodulators alongside chemotherapy can be employed in the treatment of both drug-sensitive and drug-resistant strains of pathogens. He is also screening various herbal drugs for their potential to treat drug-resistant infections.

Dr Owais has received a national award from DBT India. He is well-established in his area of research, and has guided many Ph.D students, edited books, and published several papers in high impact journals.

Dr. M. Shahid M.D., Ph.D., is Associate Professor in the Department of Microbiology of J.N. Medical College & Hospital, Aligarh Muslim University, India, He has recently been awarded the Young Scientist Award by the Department of Science & Technology, Ministry of Science & Technology, Govt. of India. He was also been awarded a Commonwealth Academic Fellowship by the British Council and the Association of Commonwealth Universities, U.K., during 2005–2006 to work with Prof. Peter Hawkey's group on the antibiotics resistance problem. His field of interest and research is confined to mechanism and resistance to beta-lactam antibiotics, with special interest in CTX-M and AmpC β-lactamases, plasmid-mediated drug resistance, and indigenous drugs. He also has research experience on human fungal pathogens such as *Aspergillus* and *Candida* species. His works on Aspergillosis in patients suffering from bronchogenic carcinoma has received high acclaim and recognition in international scientific community.

Dr. Shahid has published 50 research papers in reputed journals, and has two books to his credit. He is presently the member of many scientific bodies, both

international and national. He has also been the member of the reviewer panel and editorial board of various international journals/publication houses, including those of Bentham Science Publications, USA, and Global Science Books, UK. Currently, he is working as an Associate Professor in College of Medicine, King Faisal University, Al-hassa, Kingdom of Saudi Arabia.

Dr. Farrukh Aqil, M.S., Ph. D. is a postdoctoral fellow at the J.G. Brown Cancer Center, University of Louisville, Louisville, USA. He completed his graduate studies in the Department of Microbiology, Aligarh Muslim University, India under the supervision of Dr Iqbal Ahmad, an authority on applied microbiology and prospecting natural products. Dr Aqil's work primarily involved screening the biological activities of medicinal plants against multidrug-resistant bacterial and fungal pathogens. He has also reported the antimutagenic and antioxidant activity of many Indian medicinal plants. He has authored several research publications, book chapters and review articles. In addition to this, he has to his credit the co-authorship of two reference books of international repute. He has also served as an Assistant Professor in the Department of Biotechnology, Integral University, Lucknow, India. He has presented his research findings in many national and international conferences and symposia, and is a member of many research bodies.

Presently, Dr Aqil is working in the area of cancer chemoprevention, where he exploits the natural products, medicinal plants and their metabolites against several cancer types. He aims to develop modern phytomedicine for the prevention and treatment of cancer and other chronic diseases.

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Chapter 1
Mould Infections: A Global Threat to Immunocompromised Patients

Ricardo Araujo, Cidália Pina-Vaz, and Acácio Gonçalves Rodrigues

Abstract The increase in critical care and immunosuppressed patients all over the world during recent decades is due to a general medical concern with regard to fungal infections, particularly regarding patients admitted at intensive care, haematological, stem cell and solid organ transplant and burn units. The wide use of intense suppressive therapies which affect patients' immune status and frequently cause neutropenia represents an enormous risk of mould infections. *Aspergillus*, *Fusarium*, *Scedosporium,* and zygomycetes, particularly *Rhizopus* and *Mucor*, are invariably responsible for high morbidity and mortality in patients, even after the administration of proper antifungal therapy. These infections are often associated with longer stays in critical care units and extremely high healthcare costs. Other therapeutic drugs administered to high-risk patients may considerably promote or reduce the fungal growth and affect the development of the invasive disease. Thus, it is recommended that drug interactions be taken into account in patients receiving multiple medications.

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1.1 Introduction

Over the last decade, there have been changes in the epidemiology of fungal infections as well as dramatic improvements regarding antifungal therapeutic options. *Candida* infections, once a major cause of death in patients with leukaemia and recipients of stem cell or solid organ transplants, are now seen more often in patients admitted at intensive care units. In turn, mould infections continue to occur predominantly among highly immunosuppressed patients, such as those with acute leukaemia and patients submitted to haematopoietic stem cell or solid organ transplantation, causing devastating diseases. *Aspergillus* species remain the most common mould that causes invasive infection, but other infections caused by environmental filamentous fungi, such as *Scedosporium*, *Fusarium*, and various zygomycetes, appear to be increasing in some medical centers. These moulds are angioinvasive, and lead to extensive tissue infarction and widespread dissemination. The mortality associated with invasive mould infections is far greater than that associated with yeast-invasive infections. Many of the emerging moulds are resistant to amphotericin B and fluconazole, the most used antifungals empirically. With regard to prescription criteria for an appropriate antifungal agent, some parameters have to be taken into consideration: the underlying disease, kind and level of immunosuppression, previous prolonged exposure to antifungal drugs, microorganism identification, and its characteristic susceptibility pattern.

Attending to the complexity of the host (population of risk) and the characteristics of those fungi, the diagnosis and treatment of infection with moulds, particularly of its invasive forms, remains problematic, and raises considerable challenges. The diagnosis of invasive fungal infection, including cultural methods, antigen testing, nucleic acid detection, and radiological imaging, has improved greatly in recent years, although up to 75% of patients are only diagnosed during autopsy (Chamilos et al. 2006). Several factors are able to influence the performance of these diagnostic tests such as underlying disease, concomitant drugs, prevalence of fungal infection in particular populations, and prophylactic antifungal drug strategies. Fungi recovery from surgically removed tissue or from sterile sites accompanied by positive histopathology should always be considered significant, although these invasive procedures are in some cases impossible to perform due to, for example, severe thrombocytopenia. Mould organisms are ubiquitous in the environment so that a culture that yields one of these moulds from sputum or bronchoalveolar lavage fluid requires closer scrutiny. It may merely reflect contamination or colonization (Horvath and Dummer 1996). On the other hand, in a high-risk host, a positive culture from sputum or bronchoalveolar lavage fluid is highly associated with invasive disease and provides firm evidence for initiating therapy. Research on fungal biological markers or molecular methods is in progress but still remains controversial or has not yet been clinically validated. Caution should be taken with the diagnostic value of antigen detection tests, and these should be evaluated in conjunction with the results of other diagnostic procedures. High-resolution chest computed tomography (CT) scans have assumed increasing importance in the

diagnosis of angioinvasive fungal infection in high-risk groups of patients. The presence of the so-called halo sign, nodular lesions that are surrounded by a ground glass appearance, is an early finding in pulmonary mould infections. Cavitation is a late sign of infection and not helpful for early diagnosis (Caillot et al. 2001). In order to establish the extent of invasion into the orbit or the brain, CT scans of the sinuses and magnetic resonance imaging scans of the brain are helpful.

Four strategies for prevention and treatment of mould diseases have been described: (1) the prophylactic, with antifungal administration to high-risk patients without disease evidence, (2) empirical therapy, with antifungal administration to neutropenic patients with persistent refractory fever, (3) pre-emptive therapy, using clinical, radiological and/or laboratory markers to determine a possible disease, and (4) treatment of established fungal infection. Some of the mould organisms are nonsusceptible to standard antifungal therapy and may involve the use of alternative antifungal agents, as well as surgical management and recovery of host defenses.

1.2 Spectrum of Mould Diseases

A group of people are commonly exposed to pathogenic fungi, especially those involved in outdoor activities, with consequent increased risk of invasive fungal disease (IFD). The most common fungal diseases are caused through minor skin lesions or by inhalation. Different lifestyle practices, such as smoking tobacco or marijuana, use of illicit intravenous drugs, and pet ownership, diverse outdoor relaxation activities, and travelling to endemic areas have showed association with an increased risk of IFD (Sipsas and Kontoyiannis 2008).

A wide range of diseases can be originated by moulds, from localized non-invasive diseases (with a much defined region being affected), to invasive (when an internal organ is affected and the risk of fungaemia is high) and disseminated diseases (when the fungal agent is present in the blood and multiple organs are affected). The noninvasive form is clinically the least problematic. Localized diseases can affect distinct tissues: (1) the skin and soft tissue (cutaneous disease), (2) the internal parts of the eyes (endophthalmitis), (3) the nose and/or paranasal sinus (rhinitis and/or rhinosinusitis), (4) *Aspergillus* conidia or hyphae, which may develop into the lungs forming an aspergilloma (a pulmonary ball in a free cavity) or causing allergic bronchopulmonary aspergillosis (ABPA), (5) the subcutaneous tissues involving the foot or leg (mycetoma), and (6) the bone, muscle and/or joint (osteomyelitis, discitis and arthritis) (Cortez et al. 2008; Nucci and Anaissie 2007; Singh and Paterson 2005). Fungal noninvasive diseases usually involve an inflammatory reaction at a specific region (redness and heat at the site) and the patient (immunocompetent or not) may complain of local pain. Depending on the fungus, the patient may receive antifungal treatment or/and be recommended for surgery (Chandrasekar 2009).

Most invasive fungal diseases are acquired from air and the adoption of preventive measures may reduce the incidence of mould infections in clinical units.

The most common invasive form is the pulmonary fungal disease (Cortez et al. 2008; Nucci and Anaissie 2007; Singh and Paterson 2005). If not rapidly contained, invasive fungal diseases may disseminate to other parts of the body. When the central nervous system (including the cerebral region) is affected, the fungal infection becomes very difficult to treat and it is normally fatal (Genzen and Kenney 2009; Lin et al. 2001). A few fungal agents are usually detected in blood samples, e.g., *Fusarium* spp. (detected in more than 70% of the cases), while others, such as *Aspergillus* spp., are rarely found in these products and the diagnosis is much more difficult to achieve (Nucci and Anaissie 2007). The consensus criteria for definition of proved, probable and possible IFD have recently been reviewed (De Pauw et al. 2008).

1.3 Risk Factors for Mould-Disseminated Diseases

IFDs have recently become a growing problem in immunosuppressed patients, being increasingly reported in patients with haematological malignancies, recipients of bone marrow and solid organ allografts, and patients with AIDS. The use of anti-*Candida* prophylaxis with azoles on medical practice might also contribute to a shift in the epidemiology of these infections from *Candida* spp. to *Aspergillus* and other filamentous moulds. Moreover, new risk factors for IFD have been identified. The numbers and types of mould diseases became more recognized in high-risk patients when the population of immunosuppressed individuals increased.

The immunocompromised population is heterogeneous (see Table 1.1). Mould-disseminated diseases are a major threat to patients treated for acute leukaemia.

Table 1.1 Risk factors of invasive fungal diseases

Immunosuppressive condition	Haematological malignancy
	Transplantation (bone marrow, blood stem cells, solid organ)
	AIDS
	Neutropenia
	Intensive care unit patients[a]
	Burn patients[a]
	Neonates[a]
	Diabetes mellitus with ketoacidosis
	Collagen disease (treated with immunosuppressive drugs)
	Iron chelation
	Chronic granulomatous disease
Immunosuppressive therapy	Corticosteroids
	Anticancer chemotherapy
	Cyclosporine
	Tacrolimus

[a]In particular if carriers of medical indwelling devices like central venous catheter, receiving total parenteral nutrition, broad-spectrum antibacterials and/or antifungals, or showing multisystem organ failure

Patients undergoing remission induction therapy are considered to be at high risk of IFD (Araujo et al. 2008b; Pagano et al. 2006). Also at risk are severely ill patients, such as those with multiorgan failure and the ones staying for long periods of time in intensive care units or burn units. Aggressive surgical and medical treatment are contributing iatrogenic factors, with special evidence of the potential impact of concomitant medications administered to critically ill patients (Costa-de-Oliveira et al. 2008a; Rodrigues et al. 2005; Stergiopoulou et al. 2009).

Cytopenias such as lymphopenia, neutropenia, and/or monocytopenia have been identified as risk factors of IFD (Costa-de-Oliveira et al. 2008b; Marr et al. 2002). Antifungal defenses involve more than neutrophil killing. *Aspergillus*-specific CD4+ T cell responses have long been described as relevant for the regulation of pulmonary inflammation (Rivera et al. 2006; Beck et al. 2006). Corticosteroids increase the risk of IFD by affecting the host immune response to *Aspergillus* species (Marr et al. 2002). According to some studies, there is evidence of iron overload impacting on the risk of zygomycosis (Kauffman 2004; Roden et al. 2005).

The practice of haematopoietic stem cell transplantation (HSCT) has undergone many changes that affect the likelihood that a given patient would develop an IFD. Over the time course after transplantation, the risk and the types of IFD occurring in patients are not always the same. IFDs vary with the events that occur during the pre-engraftment neutropenic period, the early post-engraftment period (until approximately day 100 posttransplant), and those in the late post-engraftment period (after day 100). A number of well-recognized factors play a role in the likelihood of IFD. Important recipient-related factors include age, state of the underlying disease for which the HSCT is being done, and treatment-related history (Murray et al. 2009). Factors related to transplant procedure range from the type of transplant (autologous or allogeneic), the antifungal strategy, the protection of the patient to mould conidia present in the environment, the choice of conditioning (myeloblastic or nonmyeloblastic), and the source and doses of stem cells. Complications related to transplant include the duration of pre-engraftment period of neutropenia, graft failure or rejection, the administration of corticosteroids, and the occurrence of graft-versus-host disease (GvHD), as well as the presence of cytomegalovirus infection.

Due to the development of powerful immunosuppressant drugs, survival following solid organ transplantation has significantly improved during the past decade, with rejection rates dramatically reduced. Aggressive immunosuppression associated with exposure to antibiotics and antifungals had the consequence of increased incidence of rare filamentous fungal infections (Berger et al. 2006; Uçkay et al. 2007). Personal characteristics such as genetic polymorphisms may also represent an increased risk of development of IFD (Ben-Ami et al. 2009; Zaas et al. 2008). With the awareness of this complexity, patients at risk presenting specific and nonspecific symptoms should be followed by proper diagnostic approach, biopsy, identification of the causing organism at the species level, and antifungal susceptibility testing. Few characteristics are common to all mould diseases, but there are important unique features that should be acknowledged.

1.4 Aspergillosis

Aspergillosis is the most common mould disease and *A. fumigatus* is responsible for over 90% of the total clinical disturbances (Cornely 2008). Other species, such as *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, *A. versicolor*, *A. ustus,* and *A. glaucus*, may also be related to infections in immunocompromised patients or other chronic disturbances (Abbasi et al. 1999; Cornely 2008; Krishnan-Natesan et al. 2008); *A. flavus* and *A. ustus* are more prone to cause cutaneous and paranasal sinus diseases (Cornely 2008; Krishnan-Natesan et al. 2008). More recently, new fungal species were identified and included in the sections *Fumigati* or *Usti*, some of them being described as responsible for cases of cutaneous, cerebral, peritoneal or pulmonary aspergillosis (Ghebremedhin et al. 2009; Sutton et al. 2009; Varga et al. 2008; Vinh et al. 2009). *Aspergillus* species are usually related to a wide spectrum of disease forms ranging from chronic pulmonary aspergillosis (fibrocavitary aspergillosis or chronic necrotizing pulmonary aspergillosis) to the most severe disseminated infections reaching the central nervous system (Genzen and Kenney 2009; Segal 2009). The most usual is the invasive pulmonary aspergillosis that generally occurs in immunocompromised hosts; the degree and duration of immunosuppression is a critical risk factor for developing aspergillosis in such patients. Aspergilloma is a fungal ball that develops in lung cavities, usually solved by surgical removal (Lee et al. 2009). Allergic sinusitis, ABPA, or asthma may also be found associated with *Aspergillus* colonization in immunocompetent individuals (Singh and Paterson 2005; Segal 2009).

The immunocompromised population has dramatically increased in recent decades and the number of deaths related to aspergillosis has followed the same tendency, as registered in the United States autopsy record (McNeil et al. 2001). The incidence of invasive aspergillosis is particularly high among allogeneic and autologous stem cell transplants, haematological patients, and solid organ transplants, as seen in Table 1.2 (Pagano et al. 2006; Paterson and Singh 1999; Segal 2009; Singh and Paterson 2005; Singh et al. 2003). In addition, patients with other pathologies, such as advanced acquired immunodeficiency or chronic granulomatous disease, are frequently associated with diverse forms of aspergillosis. Disseminated *Aspergillus* disease in more likely to occur in bone marrow transplant recipients (Lin et al. 2001). The mortality rate from aspergillosis ranges from 30% to 100%, the highest values being found in patients receiving stem cell (allogeneic or autologous), liver, or pancreas transplant recipients (Table 1.2) (Singh and Paterson 2005). Among the most relevant risk factors for aspergillosis are the decrease of neutrophil function or quantity, severe and chronic GvHD, cytomegalovirus disease, and immunosuppressive corticosteroid therapy (Cornely 2008; Lin et al. 2001; Singh and Paterson 2005). The patients tend to stay for longer periods at clinical units and the healthcare costs associated with the treatment of the patients are extremely high (Araujo et al. 2008b; Dasbach et al. 2000; Zaoutis et al. 2006).

The diagnosis of aspergillosis is difficult and most cases are achieved without confirmation by microbiology; it remains very difficult to recover *A. fumigatus*

Table 1.2 Incidence and mortality rates of invasive fungal diseases

Invasive fungal disease	Type of transplant	Incidence (%)	Disseminated infection (%)	Mortality (%)
Aspergillosis[a]	Autologous stem cell	2–6	10–20	78–92
	Allogeneic stem cell	5–26	27–30	78–92
	Solid organ			
	Liver	1–8	50–60	87
	Lung	3–14	15–20	68
	Heart	1–15	20–35	78
	Kidney	0–4	9–36	77
	Pancreas	1–3	–	100
Fusariosis	Autologous stem cell	0.2	70–90	80
	Allogeneic stem cell	2	70–90	80
	Solid organ	0.7	66	80
Zygomycosis[b,c]	Haematopoietic stem cell transplant	1	17–28	65–90
	Solid organ	0.9	2–17	33–66
Scedosporiosis[b]	Haematopoietic stem cell transplant	0.5–1	60–69	70–100
	Solid organ	0.9	8–46	58–80

[a]Aspergillosis data was adapted from Singh and Paterson (2005)
[b]Zygomycosis and scedosporiosis in stem cell transplants refer mostly to allogeneic transplants
[c]Disseminated zygomycosis is more frequent among lung transplant patients, while the highest mortalities are registered in liver transplants

from blood cultures (Nucci and Anaissie 2007). Most of the time, the first sign of aspergillosis comes from CT scan of the chest, particularly by identification of the halo sign (Segal 2009). The early diagnosis of aspergillosis has been improved with the introduction of the galactomannan serum detection assay. A few false positive results have been verified in patients receiving antibiotics such as piperacillin–-tazobactam, and cross-reactivity with other fungi has also been described. But the overall results of galactomannan are very good; the method showed good sensitivity and excellent specificity (Pfeiffer et al. 2006). Other diagnostic tests, such as the detection of β-D-glucan or PCR, may also help to diagnose aspergillosis (Hachem et al. 2009; Klingspor and Loeffler 2009).

A few antifungal agents are now available for the treatment of aspergillosis. The first line antifungal is now voriconazole, being amphotericin B formulations and caspofungin available as salvage therapy (Denning et al. 2002; Erjavec et al. 2009; Herbrecht et al. 2002). In paediatrics, voriconazole has shown good results, but specific pharmacokinetic studies are still needed, as children have been treated with the adult dose (Segal 2009; Walsh et al. 2002). Itraconazole is commonly used in patients with ABPA in combination with corticosteroid therapy (Stevens et al. 2000). For prophylaxis, posaconazole is the most recommended, although itraconazole is effective in patients with chronic granulomatous disease (Naeger-Murphy and Pile 2009). The available antifungals are active *in vitro* against most *Aspergillus* spp., particularly *A. fumigatus* (Araujo et al. 2007; Lionakis et al. 2005). A group of non-fumigatus *Aspergillus* have emerged recently as causes of aspergillosis, most

of them resistant *in vitro* to the antifungals (e.g., *A. terreus* versus amphotericin B, *A. lentulus* and *A. viridinutans* versus voriconazole, itraconazole and posaconazole) (Alcazar-Fuoli et al. 2008; Araujo et al. 2007; Balajee et al. 2005; Steinbach et al. 2004).

1.5 Zygomycosis

The zygomycetes are divided into two orders, the Mucorales and the Entomophthorales. *Rhizopus* spp. (responsible for around 50% of the total cases), *Absidia* spp., *Mucor* spp., *Rhizomucor* spp., and *Cunninghamella* spp. are the most common fungi responsible for zygomycosis (Chayakulkeeree et al. 2006; Kontoyiannis et al. 2005; Roden et al. 2005; Stelzmueller et al. 2008). All these genera belong to the order Mucorales. Zygomycetes are found in soil, vegetation, and in a variety of foodstuffs, being infections acquired by contact with any contaminated material or by inhalation of airborne conidia (Kauffman 2004). Cutaneous, sinus, and pulmonary infections are the most frequently caused by Mucorales, but zygomycosis can also reach internal organs and disseminate, depending on the underlying patient condition (Cornely 2008; Roden et al. 2005). The cutaneous form occurs in patients suffering trauma (e.g., automobile accidents) affecting tendons, muscles or bones, or with burn injuries, while pulmonary or disseminated zygomycosis is more frequent in immunocompromised patients. The most relevant risk factors for developing infection by zygomycetes are diabetes, haematological malignancy (mainly associated with neutropenia), immunosuppressive treatments, deferoxamine therapy (zygomycetes use iron as a siderophore), and transplantation procedures (Table 1.2) (Kauffman 2004; Roden et al. 2005). The diabetics are particularly prone to the acquisition of rhinocerebral zygomycosis which, if not rapidly treated, may present devastating consequences to the patient (Nucci 2003; Zaoutis et al. 2006). Additionally, there are reports of increased risk of zygomycosis among patients receiving voriconazole prophylaxis — zygomycetes are resistant to this antifungal (Siwek et al. 2004). Thus, it is recommended that voriconazole be carefully used for prophylaxis as to date no clinical trials exist to support this strategy. Changes in environmental conditions, particularly epidemiological variation of zygomycosis through changing seasons, have also been described (Al-Ajam et al. 2006; Gómez-López et al. 2001; Kontoyiannis et al. 2005).

No outbreaks of zygomycosis have been described to date in contrast with other mould infections, e.g., aspergillosis. It is possible that the patients developing zygomycosis be colonized before arriving in clinical units; this may explain the genetic diversity and reduced genetic clustering of zygomycetes found in samples collected from sick patients from the same place (Abzug et al. 1992). The diagnosis of zygomycosis is difficult and several cases are diagnosed postmortem (Stelzmueller et al. 2008). The culture of such fungi is not frequent; thus, biopsy, histopathology, and CT scan are commonly used for diagnosis. PCR may represent a valid alternative for the earlier diagnosis in the near future (Kasai et al. 2008).

Zygomycetes may represent the next challenge for fungal infection treatment. *Candida* infections have been drastically reduced in the last two decades with the introduction of fluconazole for prophylaxis and empirical use, and it is probable that a similar reduction of aspergillosis will happen worldwide now that more antifungals have been made available with high activity against *Aspergillus* spp. However, there are still a very limited number of antifungal agents with *in vitro* activity against zygomycetes and the incidence of those infections is increasing annually (Kauffman 2004). Amphotericin B and posaconazole have shown *in vitro* activity against *Rhizopus* spp., *Absidia* spp., *Mucor* spp., and *Cunninghamella* spp. (Alastruey-Izquierdo et al. 2009; Almyroudis et al. 2007; Gómez-López et al. 2001). Amphotericin B is recommended for the primary treatment of zygomycosis, and posaconazole is an alternative for salvage therapy (Naeger-Murphy and Pile 2009). The echinocandins do not present *in vitro* activity against zygomycetes (Almyroudis et al. 2007). Nevertheless, early diagnosis and a combination of diverse options, such as antifungal treatment, surgery, use of iron chelators, and/or adjunctive therapies (recombinant cytokines, hyperbaric oxygen, or granulocyte transfusions), may be the best alternative for treating zygomycosis (Roden et al. 2005; Spellberg et al. 2009).

1.6 Fusariosis

Commonly classified as plant pathogens, *Fusarium* species have become highly significant as opportunist fungi, causing serious disease in humans during recent decades. These fungi are commonly found in soil, plants, and contaminated waters. *Fusarium solani* (responsible for around 50% of the total cases), *Fusarium oxysporum*, and *Fusarium moniliforme* are the species mainly associated with infections in humans (Al-Abdely 2004; Nucci and Anaissie 2007). *Fusarium* species may cause a wide spectrum of diseases in humans that can range from localized diseases (keratitis, onychomycosis, or mycetoma) to pulmonary and disseminated infections (Nucci and Anaissie 2007). The skin may represent the primary site of infection as 70% of 259 published cases of fusariosis showed skin involvement (Nucci and Anaissie 2002). The existence of a large incidence of fusariosis in trauma cases and burns, and the development of onychomycosis or cellulitis, confirm that the skin participates as a decisive barrier for protection against *Fusarium* spp. Airborne conidia may also represent a relevant portal of entry, as well as the ingestion of water or food with *Fusarium* conidia by immunocompromised individuals. Waterborne fusariosis was reported as associated with nosocomial hospital infections in the United States, and the genetic analysis of the isolates showed that all tested strains were from the same clonal lineage (O'Donnell et al. 2004). Prevention of infection by using high-efficiency particulate air (HEPA) filters and water filtration systems, together with effective cleaning procedures of showers and toilet walls and superficies, represent the best strategy for reducing the number of infections among immunocompromised patients at clinical units (Nucci and Anaissie 2007).

Invasive and disseminated fusariosis are highly lethal fungal infections, particularly in haematological cancer and neutropenic patients (see Table 1.2) (Girmenia et al. 2000; Nucci et al. 2003, 2004). Fusariosis has become one of the most relevant mould infections in the United States, usually supplanted only by aspergillosis. The M. D. Anderson Cancer Center of Houston reported an increased number of cases in the period 1986–1995 (Boutati and Anaissie 1997), while in Europe the number of infections also increased particularly in France and Italy (Girmenia et al. 2000). The duration of neutropenia and the large administration of corticosteroids are the most important factors for the highest mortality in cancer patients (Nucci et al. 2003). A study in nine hospitals from North and South America confirmed that fusariosis was lethal in allogeneic haematopoietic stem cell transplant recipients, although this infection could also occur in patients submitted to autologous stem cell transplant (Nucci et al. 2004); the rate of proven infection was high (92%) in these patients. Simultaneously, it has been reported that fusariosis may show a greater propensity to appear in cold months (from autumn to spring) (Girmenia et al. 2000; Nucci et al. 2004). A hypothesis for explaining seasonal distribution is that the wind and rain play a crucial role in the dispersion of *Fusarium* conidia in the environment (Girmenia et al. 2000).

The diagnosis of fusariosis is usually easier than previously seen for aspergillosis. Skin lesions represent the single source of diagnosis in the majority of patients with these lesions (Nucci and Anaissie 2007). *Fusarium* conidia are normally recovered from two sites (blood and skin) and the microbiological culture of the organism is much more common than for aspergillosis – around 70% of the blood cultures are positive for *Fusarium* spp. versus less than 5% for *Aspergillus* spp. (Nucci et al. 2004). *Fusarium* spp. present reduced *in vitro* susceptibility to amphotericin B formulations and azoles (Azor et al. 2008, 2009; Tortorano et al. 2008). Echinocandins show no activity against these fungi. In fact, critical difficulties are faced today for the treatment of fusariosis. There are reports of infections treated with posaconazole, but the recommended antifungal treatment is a high dose of amphotericin B (conventional or lipid formulation), or voriconazole if the organism is not *F. solani* or *F. verticilloides* (Nucci and Anaissie 2007). Localized fusariosis may be better solved with surgery. Terbinafine proved to be the most effective *in vitro* against *Fusarium* spp. and may be used in a few cases (Azor et al. 2008, 2009; Tortorano et al. 2008). White blood cells growth factor, granulocyte–macrophage colony-stimulating factor, and granulocyte transfusion represent valid alternatives in neutropenic patients (Al-Abdely 2004).

1.7 Scedosporiosis

Scedosporium apiospermum (and its teleomorph or sexual form *Pseudallescheria boydii*) and *Scedosporium prolificans* are the most important species responsible for scedosporiosis. The first fungus is a relevant mould causing mycetoma and invasive and disseminated infections in immunocompromised patients, while *S. prolificans* is

a less frequent disease-related filamentous fungus that was included with the group of phaeohyphomycosis (Castón-Osorio et al. 2008). These ubiquitous fungi are usually present in the soil, sewage and polluted waters, particularly in temperate climates, although they can also be found in tropical regions (Cortez et al. 2008). It is possible to define a geographic world distribution for both fungi, *S. apiospermum* being found worldwide and *S. prolificans* in more restricted areas, such as the Iberian Peninsula, Australia and southern United States (Cortez et al. 2008; Tintelnot et al. 2009).

The overall frequency of scedosporiosis is low in most geographic areas, but during recent years it has become more frequent worldwide (Cortez et al. 2008; Kantarcioglu et al. 2008; Tintelnot et al. 2009). Patients with haematological malignancies, stem cell transplant recipients, patients under immunosuppressive treatments, patients with advanced HIV infection, or those suffering from chronic granulomatous disease are the main target of the opportunistic *Scedosporium* moulds (Table 1.2) (Cortez et al. 2008). Scedosporiosis was responsible for 14% of the non-*Aspergillus* mould infections found in stem cell transplant recipients, showing a very poor outcome – all nine patients identified with this infection died (Marr et al. 2002). *S. prolificans* has been described as more pathogenic than *S. apiospermum* due to its pigmentation (Cortez et al. 2008).

The most relevant risk factors are steroid treatment, lymphopenia, low level of serum albumin, neutropenia, diabetes, and cytomegalovirus infection (Lamaris et al. 2006). *S. apiospermum* or *P. boydii* may colonize the respiratory tract of individuals, particularly persons exposed to high environmental inocula, as is the case for people working in agricultural settings. In cystic fibrosis patients, colonization with *S. apiospermum* may cause an even greater deterioration in the respiratory function of these patients [the fungus was found in 9% of 128 cystic fibrosis patients (Cimon et al. 2000)] and can be fatal in patients admitted for transplantation (Symoens et al. 2006). Patients undergoing corticosteroid therapy for rheumatoid arthritis are also at high risk of developing scedosporosis (Lake et al. 1990; Miller et al. 1993). There are reports of outbreaks of *S. prolificans* infections in hospitals under reconstruction (Alvarez et al. 1995).

The diagnosis of such infections is based on cytology and histopathology, confirmed by the culture of the fungus. Radiology, serology, and molecular biology methods may help in the diagnosis of scedosporiosis (Cortez et al. 2008). The treatment of scedosporiosis is very difficult since these moulds are highly resistant to antifungals. *S. apiospermum* is resistant *in vitro* to amphotericin B and itraconazole, being more susceptible to voriconazole (Alastruey-Izquierdo et al. 2007; Cuenca-Estrella et al. 2008). *S. prolificans* is resistant to all the antifungals available in clinic, with few strains presenting reduced susceptibility to voriconazole (Cuenca-Estrella et al. 2008). Echinocandins show no activity on this group of moulds. Voriconazole is the recommended antifungal treatment for scedosporiosis (Cortez et al. 2008; Naeger-Murphy and Pile 2009), but the combination of antifungal treatment, surgery, and the recovery of patient immune status may be the best clinical strategy for treating scedosporiosis (Chandrasekar 2009; Cortez et al. 2008). Meanwhile, it is highly recommended that *in vitro* susceptibility testing be performed in clinical isolates of *Scedosporium* (Alastruey-Izquierdo et al. 2007).

1.8 Other Mould Infections

1.8.1 Histoplasmosis and Coccidioidomycosis

For the endemic mycoses, exposure is limited to certain geographic areas, and an individual who remains outside the organism's ecological niche is not at risk of infection. Patients with extensive immunosuppression may present a more severe form of disease, and reactivation of prior infection can occur in those who previously resided in the endemic area for histoplasmosis or coccidioidomycosis. *Histoplasma capsulatum* is an intracellular pathogen commonly found in soil containing high concentrations of bird or bat guano (Kauffman 2006). Patients at most risk of severe disease are those with cellular immune defects (especially AIDS), transplant recipients, and those receiving corticosteroids. In USA, *Coccidioides immitis* is common in the Lower Sonoran desert of Arizona and California. A determining factor for the severity of infection in both healthy and immunosuppressed hosts is the extent of environmental exposure. Coccidioidomycosis appears to be more severe in those immunosuppressed patients who have received transplants or have AIDS (Ampel 2009). Dissemination to lungs, meninges, skin, and other tissues is common.

1.8.2 Emergent and Uncommon Fungi

Immunosuppressed patients may develop infection with a variety of uncommon fungi, the severity of the disease being associated with the compromising of host defenses. Exceptional conditions may be responsible for such mould diseases. Disseminated infection with *Sporothrix schenckii* has been described in AIDS patients, particularly the ones involved in outdoor activities that make contact with contaminated soil (Kauffman 2006). *Penicillium marneffei* represents another opportunistic pathogen systematically responsible for infections in AIDS patients. Disseminated diseases caused by this mould are clinically indistinguishable from disseminated histoplasmosis (Mootsikapun and Srikulbutr 2006). *P. marneffei* infections can be seen in patients living in or travelling to Southeast Asia, the area endemic for this fungus (Murray et al. 2009).

Phaeohyphomycosis is an infection that usually follows traumatic accidents (Naggie and Perfect 2009). Dematiaceous or dark-walled fungi are the environmental saprophytes responsible for phaeohyphomycosis and may cause a variety of different diseases in immunosuppressed individuals. Solid organ transplant recipients develop subcutaneous nodules and the organisms *Exophiala*, *Alternaria*, or *Phialophora* are usually implicated (Murray et al. 2009). Disseminated infection with the dematiaceous fungi also occurs in transplant recipients and patients with hematologic malignancies. All the fungi that can cause this clinical picture appear identical in tissue. Therefore, they must be grown in culture and examined

histologically. For many of these fungi, the most effective antifungal therapy is not known and the mortality rate for disseminated infection is high. *Alternaria* is a very large and complex genus that encompasses hundreds of species, although specific data are difficult to obtain because of the proliferation of nomenclature of dubious taxonomic validity (Pastor and Guarro 2008). *Alternaria* is common in soil and air and represents ubiquitous agents sometimes described as plant pathogens. *Alternaria* infects mainly immunocompromised hosts. Immunosuppression is frequently associated with cutaneous and subcutaneous infections and rhinosinusitis. The most important risk factors for cutaneous and subcutaneous infections are solid organ transplantation and Cushing's syndrome, and those for rhinosinusitis are bone marrow transplants (Pastor and Guarro 2008).

Paecilomyces lilacinus has been linked to fungal keratitis in wearers of soft contact lenses (Pastor and Guarro 2006). Deep invasive infections due to this organism occur more frequently in immunocompromised patients. The clinical importance of other filamentous fungal, such as the genus *Trichoderma*, has been recognized in the last decade. Several species have been indicated as potential opportunistic pathogens in immunocompromised hosts, particularly among adult and paediatric neutropenic patients (Kantarcioglu et al. 2009; Lagrange-Xélot et al. 2008). *Curvularia* infections in humans are uncommon although the fungus shows a ubiquitous presence in the environment (soil and air). *Curvularia* spp. may cause respiratory tract, corneal, and cutaneous infections (Fan et al. 2009). A few cases of central nervous system involvement by *Curvularia* have been documented in the medical literature. Interestingly, infections with *Curvularia* do not appear to require an immunosuppressed host (Rinaldi et al. 1987).

1.9 Future Perspectives and Conclusions

It still remains critical to find promising diagnostic tools that combine good results for sensitivity and specificity with an inexpensive technology. Benefits from early diagnosis include reduced drug acquisition costs and reduced morbidity and mortality from drug-related adverse events. In addition, it may result in shorter hospital stay for patients. It is possible that newer prophylactic protocols reduce disease prevalence to levels where the performance of surveillance diagnostic tests becomes suboptimal (Ben-Ami et al. 2009). The resolution of the host immune status is also crucial for full recovery of patients and for the decrease in incidence of mould diseases.

The challenge for developing vaccines against fungal diseases has increased in recent years because of the high incidence rates of fungal diseases worldwide. Prototypic antigens have been identified and they may represent excellent alternatives for inducing protective immunity (Cutler et al. 2007). Major developments have been conducted in this field and the development of vaccines targeting human fungal pathogens is now a feasible goal. Nevertheless, many doubts with regard to the safety and efficacy of fungal vaccines will be raised in the near future.

Mould conidia cannot be eliminated from indoor environments. Normal buildings contain a diversity of materials and substrates that provide nutrition for many species of moulds, where they can grow and amplify. Exposure to moulds is inevitable except in the presence of air filtration, isolation, and when environmental protective measures are adopted, for example, in organ transplant isolation units or burn units. A few strategies can be used to reduce the indoor air fungal load in wards receiving high-risk patients, particularly by adding HEPA filters and positive air flow rate, providing an anteroom, and wearing protective clothes, hair and shoe covers, and facial mask (Araujo et al. 2008a; Muñoz et al. 2001). Therefore, routine mycological investigations of environmental reservoirs, namely air and water, should be constantly made in hospitals or at institutions where immunosuppressed individuals are treated.

References

Abbasi S, Shenep JL, Hughes WT, Flynn PM (1999) Aspergillosis in children with cancer: a 34-year experience. Clin Infect Dis 29:1210–1219

Abzug MJ, Gardner S, Glode MP, Cymanski M, Roe MH, Odom LF (1992) Heliport-associated nosocomial mucormycoses. Infect Control Hosp Epidemiol 13:325–326

Al-Abdely HM (2004) Management of rare fungal infections. Curr Opin Infect Dis 17:527–532

Al-Ajam MR, Bizri AR, Mokhbat J, Weedon J, Lutwick L (2006) Mucormycosis in the Eastern Mediterranean: a seasonal disease. Epidemiol Infect 134:341–346

Alastruey-Izquierdo A, Cuenca-Estrella M, Monzón A, Rodriguez-Tudela JL (2007) Prevalence and susceptibility testing of new species of *Pseudallescheria* and *Scedosporium* in a collection of clinical mould isolates. Antimicrob Agents Chemother 51:748–751

Alastruey-Izquierdo A, Castelli MV, Cuesta I, Zaragoza O, Monzón A, Mellado E, Rodríguez-Tudela JL (2009) In vitro activity of antifungals against zygomycetes. Clin Microbiol Infect 15:71–76

Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Cuenca-Estrella M, Rodriguez-Tudela JL (2008) *Aspergillus* section Fumigati: antifungal susceptibility patterns and sequence-based identification. Antimicrob Agents Chemother 52:1244–1251

Almyroudis NG, Sutton DA, Fothergill AW, Rinaldi MG, Kusne S (2007) In vitro susceptibilities of 217 clinical isolates of zygomycetes to conventional and new antifungal agents. Antimicrob Agents Chemother 51:2587–2590

Alvarez M, Lopez Ponga B, Rayon C, Garcia Gala J, Roson Porto MC, Gonzalez M, Martinez-Suarez JV, Rodriguez-Tudela JL (1995) Nosocomial outbreak caused by *Scedosporium prolificans* (inflatum): four fatal cases in leukemic patients. J Clin Microbiol 33:3290–3295

Ampel NM (2009) Coccidioidomycosis: a review of recent advances. Clin Chest Med 30:241–245

Araujo R, Pina-Vaz C, Rodrigues AG (2007) Susceptibility of environmental versus clinical strains of pathogenic *Aspergillus*. Int J Antimicrob Agents 29:108–111

Araujo R, Cabral JP, Rodrigues AG (2008a) Air filtration systems and restrictive access conditions improve indoor air quality in clinical units: *Penicillium* as a general indicator of hospital indoor fungal levels. Am J Infect Control 36:129–134

Araujo R, Carneiro A, Costa-Oliveira S, Pina-Vaz C, Rodrigues AG, Guimaraes JE (2008b) Fungal infections after haematology unit renovation: evidence of clinical, environmental and economical impact. Eur J Haematol 80:436–443

Azor M, Gené J, Cano J, Sutton DA, Fothergill AW, Rinaldi MG, Guarro J (2008) In vitro antifungal susceptibility and molecular characterization of clinical isolates of

Fusarium verticillioides (*F. moniliforme*) and *Fusarium thapsinum*. Antimicrob Agents Chemother 52:2228–2231

Azor M, Cano J, Gené J, Guarro J (2009) High genetic diversity and poor *in vitro* response to antifungals of clinical strains of *Fusarium oxysporum*. J Antimicrob Chemother 63:1152–1155

Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA (2005) *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot Cell 4:625–632

Beck O, Topp MS, Koehl U, Roilides E, Simitsopoulou M, Hanisch M, Sarfati J, Latgé JP, Klingebiel T, Einsele H, Lehrnbecher T (2006) Generation of highly purified and functionally active human TH1 cells against *Aspergillus fumigatus*. Blood 107:2562–2569

Ben-Ami R, Lewis RE, Kontoyiannis DP (2009) Invasive mould infections in the setting of hematopoietic cell transplantation: current trends and new challenges. Curr Opin Infect Dis 22:376–384

Berger N, Wirmsberger R, Kafka R, Margreiter C, Ebenbichler C, Stelzmueller I, Margreiter R, Steurer W, Mark W, Bonatti H (2006) Infectious complications following 72 consecutive enteric-drained pancreas transplants. Transpl Int 19:549–557

Boutati EI, Anaissie EJ (1997) *Fusarium*, a significant emerging pathogen in patients with hematologic malignancy: ten years' experience at a cancer center and implications for management. Blood 90:999–1008

Caillot D, Couaillier JF, Bernard A, Casasnovas O, Denning DW, Mannone L, Lopez J, Couillault G, Piard F, Vagner O, Guy H (2001) Increasing volume and changing characteristics of invasive pulmonary aspergillosis on sequential thoracic computed tomography scans in patients with neutropenia. J Clin Oncol 19:253–259

Castón-Osorio JJ, Rivero A, Torre-Cisneros J (2008) Epidemiology of invasive fungal infection. Int J Antimicrob Agents 32:S103–S109

Chamilos G, Luna M, Lexis GE, Bodey GP, Chemaly R, Tarrand JJ, Safdar A, Raad II, Kontoyiannis DP (2006) Invasive fungal infections in patients with hematologic malignancies in a tertiary care center: an autopsy over a 15 year period (1989–2003). Haematologica 91:986–989

Chandrasekar P (2009) Invasive mould infections: recent advances in management approaches. Leuk Lymphoma 50:703–715

Chayakulkeeree M, Ghannoum MA, Perfect JR (2006) Zygomycosis: the re-emerging fungal infection. Eur J Clin Microbiol Infect Dis 25:215–229

Cimon B, Carrère J, Vinatier JF, Chazalette JP, Chabasse D, Bouchara JP (2000) Clinical significance of *Scedosporium apiospermum* in patients with cystic fibrosis. Eur J Clin Microbiol Infect Dis 19:53–56

Cornely OA (2008) *Aspergillus* to zygomycetes: causes, risk factors, prevention, and treatment of invasive fungal infections. Infection 36:296–313

Cortez KJ, Roilides E, Quiroz-Telles F, Meletiadis J, Antachopoulos C, Knudsen T, Buchanan W, Milanovich J, Sutton DA, Fothergill A, Rinaldi MG, Shea YR, Zaoutis T, Kottilil S, Walsh TJ (2008) Infections caused by *Scedosporium* spp. Clin Microbiol Rev 21:157–197

Costa-de-Oliveira S, Araujo R, Silva-Dias A, Pina-Vaz C, Rodrigues AG (2008a) Propofol lipidic infusion promotes resistance to antifungals by reducing drug input into the fungal cell. BMC Microbiol 8:9

Costa-de-Oliveira S, Pina-Vaz C, Mendonça D, Gonçalves Rodrigues A (2008b) A first Portuguese epidemiological survey of fungaemia in a university hospital. Eur J Clin Microbiol Infect Dis 27:365–374

Cuenca-Estrella M, Alastruey-Izquierdo A, Alcazar-Fuoli L, Bernal-Martinez L, Gomez-Lopez A, Buitrago MJ, Mellado E, Rodriguez-Tudela JL (2008) In vitro activities of 35 double combinations of antifungal agents against *Scedosporium apiospermum* and *Scedosporium prolificans*. Antimicrob Agents Chemother 52:1136–1139

Cutler JE, Deepe GS Jr, Klein BS (2007) Advances in combating fungal diseases: vaccines on the threshold. Nat Rev Microbiol 5:13–28

Dasbach EJ, Davies GM, Teutsch SM (2000) Burden of aspergillosis-related hospitalizations in the United States. Clin Infect Dis 31:1524–1528

de Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Muñoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE, European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group (2008) Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 46:1813–1821

Denning DW, Ribaud P, Milpied N, Caillot D, Herbrecht R, Thiel E, Haas A, Ruhnke M, Lode H (2002) Efficacy and safety of voriconazole in the treatment of acute invasive aspergillosis. Clin Infect Dis 34:563–571

Erjavec Z, Kluin-Nelemans H, Verweij PE (2009) Trends in invasive fungal infections, with emphasis on invasive aspergillosis. Clin Microbiol Infect 15:625–633

Fan YM, Huang WM, Li SF, Wu GF, Li W, Chen RY (2009) Cutaneous phaeohyphomycosis of foot caused by *Curvularia clavata*. Mycoses 52:544–546

Genzen JR, Kenney B (2009) Central nervous system *Aspergillus* infection after epidural analgesia: diagnosis, therapeutic challenges, and literature review. Diagn Microbiol Infect Dis 65(3):312–318. doi:10.1016/j.diagmicrobio.2009.06.013

Ghebremedhin B, Bluemel A, Neumann KH, Koenig B, Koenig W (2009) Peritonitis due to *Neosartorya pseudofischeri* in an elderly patient undergoing peritoneal dialysis successfully treated with voriconazole. J Med Microbiol 58:678–682

Girmenia C, Pagano L, Corvatta L, Mele L, del Favero A, Martino P (2000) The epidemiology of fusariosis in patients with haematological diseases. Gimema Infection Programme. Br J Haematol 111:272–276

Gómez-López A, Cuenca-Estrella M, Monzón A, Rodriguez-Tudela JL (2001) In vitro susceptibility of clinical isolates of Zygomycota to amphotericin B, flucytosine, itraconazole and voriconazole. J Antimicrob Chemother 48:919–921

Hachem RY, Kontoyiannis DP, Chemaly RF, Jiang Y, Reitzel R, Raad I (2009) Utility of galactomannan enzyme immunoassay and (1, 3) beta-D-glucan in diagnosis of invasive fungal infections: low sensitivity for *Aspergillus fumigatus* infection in hematologic malignancy patients. J Clin Microbiol 47:129–133

Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, Kern WV, Marr KA, Ribaud P, Lortholary O, Sylvester R, Rubin RH, Wingard JR, Stark P, Durand C, Caillot D, Thiel E, Chandrasekar PH, Hodges MR, Schlamm HT, Troke PF, de Pauw B, Invasive Fungal Infections Group of the European Organisation for Research and Treatment of Cancer and the Global Aspergillus Study Group (2002) Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 347:408–415

Horvath JA, Dummer S (1996) The use of respiratory-tract cultures in the diagnosis of invasive pulmonary aspergillosis. Am J Med 100:171–178

Kantarcioglu AS, Guarro J, de Hoog GS (2008) Central nervous system infections by members of the *Pseudallescheria boydii* species complex in healthy and immunocompromised hosts: epidemiology, clinical characteristics and outcome. Mycoses 51:275–290

Kantarcioglu AS, Celkan T, Yücel A, Mikami Y, Kurugoglu S, Mitani H, Altas K (2009) Fatal *Trichoderma harzianum* infection in a leukemic pediatric patient. Med Mycol 47:207–215

Kasai M, Harrington SM, Francesconi A, Petraitis V, Petraitiene R, Beveridge MG, Knudsen T, Milanovich J, Cotton MP, Hughes J, Schaufele RL, Sein T, Bacher J, Murray PR, Kontoyiannis DP, Walsh TJ (2008) Detection of a molecular biomarker for zygomycetes by quantitative PCR assays of plasma, bronchoalveolar lavage, and lung tissue in a rabbit model of experimental pulmonary zygomycosis. J Clin Microbiol 46:3690–3702

Kauffman CA (2004) Zygomycosis: reemergence of an old pathogen. Clin Infect Dis 39:588–590

Kauffman CA (2006) Endemic mycoses: blastomycosis, histoplasmosis, and sporotrichosis. Infect Dis Clin North Am 20:645–662

Klingspor L, Loeffler J (2009) *Aspergillus* PCR formidable challenges and progress. Med Mycol 47:S241–S247

Kontoyiannis DP, Lionakis MS, Lewis RE, Chamilos G, Healy M, Perego C, Safdar A, Kantarjian H, Champlin R, Walsh TJ, Raad II (2005) Zygomycosis in a tertiary-care cancer center in the era of *Aspergillus*-active antifungal therapy: a case-control observational study of 27 recent cases. J Infect Dis 191:1350–1360

Krishnan-Natesan S, Chandrasekar PH, Manavathu EK, Revankar SG (2008) Successful treatment of primary cutaneous *Aspergillus ustus* infection with surgical debridement and a combination of voriconazole and terbinafine. Diagn Microbiol Infect Dis 62:443–446

Lagrange-Xélot M, Schlemmer F, Gallien S, Lacroix C, Molina JM (2008) *Trichoderma* fungaemia in a neutropenic patient with pulmonary cancer and human immunodeficiency virus infection. Clin Microbiol Infect 14:1190–1192

Lake FR, Tribe AE, McAleer R, Froudist J, Thompson PJ (1990) Mixed allergic bronchopulmonary fungal disease due to *Pseudallescheria boydii* and *Aspergillus*. Thorax 45:489–491

Lamaris GA, Chamilos G, Lewis RE, Safdar A, Raad II, Kontoyiannis DP (2006) *Scedosporium* infection in a tertiary care cancer center: a review of 25 cases from 1989–2006. Clin Infect Dis 43:1580–1584

Lee JG, Lee CY, Park IK, Kim DJ, Chang J, Kim SK, Chung KY (2009) Pulmonary aspergilloma: analysis of prognosis in relation to symptoms and treatment. J Thorac Cardiovasc Surg 138:820–825

Lin SJ, Schranz J, Teutsch SM (2001) Aspergillosis case-fatality rate: systematic review of the literature. Clin Infect Dis 32:358–366

Lionakis MS, Lewis RE, Torres HA, Albert ND, Raad II, Kontoyiannis DP (2005) Increased frequency of non-*fumigatus Aspergillus* species in amphotericin B- or triazole-pre-exposed cancer patients with positive cultures for aspergilli. Diagn Microbiol Infect Dis 52:15–20

Marr KA, Carter RA, Crippa F, Wald A, Corey L (2002) Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin Infect Dis 34:909–917

McNeil MM, Nash SL, Hajjeh RA, Phelan MA, Conn LA, Plikaytis BD, Warnock DW (2001) Trends in mortality due to invasive mycotic diseases in the United States, 1980–1997. Clin Infect Dis 33:641–647

Miller MA, Greenberger PA, Amerian R, Toogood JH, Noskin GA, Roberts M, Patterson R (1993) Allergic bronchopulmonary mycosis caused by *Pseudallescheria boydii*. Am Rev Respir Dis 148:810–812

Mootsikapun P, Srikulbutr S (2006) Histoplasmosis and penicilliosis: comparison of clinical features, laboratory findings and outcome. Int J Infect Dis 10:66–71

Muñoz P, Burillo A, Bouza E (2001) Environmental surveillance and other control measures in the prevention of nosocomial fungal infections. Clin Microbiol Infect 7:38–45

Murray PR, Rosenthal KS, Pfaller MA (2009) Medical Microbiology, 6th edn. Mosby, Philadelphia

Naeger-Murphy N, Pile JC (2009) Clinical indications for newer antifungal agents. J Hosp Med 4:102–111

Naggie S, Perfect JR (2009) Molds: hyalohyphomycosis, phaeohyphomycosis, and zygomycosis. Clin Chest Med 30:337–353

Nucci M (2003) Emerging moulds: *Fusarium*, *Scedosporium* and Zygomycetes in transplant recipients. Curr Opin Infect Dis 16:607–612

Nucci M, Anaissie E (2002) Cutaneous infection by *Fusarium* species in healthy and immunocompromised hosts: implications for diagnosis and management. Clin Infect Dis 35:909–920

Nucci M, Anaissie E (2007) *Fusarium* infections in immunocompromised patients. Clin Microbiol Rev 20:695–704

Nucci M, Anaissie EJ, Queiroz-Telles F, Martins CA, Trabasso P, Solza C, Mangini C, Simões BP, Colombo AL, Vaz J, Levy CE, Costa S, Moreira VA, Oliveira JS, Paraguay N, Duboc G,

Voltarelli JC, Maiolino A, Pasquini R, Souza CA (2003) Outcome predictors of 84 patients with hematologic malignancies and *Fusarium* infection. Cancer 98:315–319

Nucci M, Marr KA, Queiroz-Telles F, Martins CA, Trabasso P, Costa S, Voltarelli JC, Colombo AL, Imhof A, Pasquini R, Maiolino A, Souza CA, Anaissie E (2004) *Fusarium* infection in hematopoietic stem cell transplant recipients. Clin Infect Dis 38:1237–1242

O'Donnell K, Sutton DA, Rinaldi MG, Magnon KC, Cox PA, Revankar SG, Sanche S, Geiser DM, Juba JH, van Burik JA, Padhye A, Anaissie EJ, Francesconi A, Walsh TJ, Robinson JS (2004) Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. J Clin Microbiol 42:5109–5120

Pagano L, Caira M, Candoni A, Offidani M, Fianchi L, Martino B, Pastore D, Picardi M, Bonini A, Chierichini A, Fanci R, Caramatti C, Invernizzi R, Mattei D, Mitra ME, Melillo L, Aversa F, Van Lint MT, Falcucci P, Valentini CG, Girmenia C, Nosari A (2006) The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica 91:1068–1075

Pastor FJ, Guarro J (2006) Clinical manifestations, treatment and outcome of *Paecilomyces lilacinus* infections. Clin Microbiol Infect 12:948–960

Pastor FJ, Guarro J (2008) *Alternaria* infections: laboratory diagnosis and relevant clinical features. Clin Microbiol Infect 14:734–746

Paterson DL, Singh N (1999) Invasive aspergillosis in transplant recipients. Medicine 78:123–138

Pfeiffer CD, Fine JP, Safdar N (2006) Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. Clin Infect Dis 42:1417–1427

Rinaldi MG, Phillips P, Schwartz JG, Winn RE, Holt GR, Shagets FW, Elrod J, Nishioka G, Aufdemorte TB (1987) Human *Curvularia* infections. Report of five cases and review of the literature. Diagn Microbiol Infect Dis 6:27–39

Rivera A, Hohl T, Pamer EG (2006) Immune responses to *Aspergillus fumigatus* infections. Biol Blood Marrow Transplant 12:47–49

Roden MM, Zaoutis TE, Buchanan WL, Knudsen TA, Sarkisova TA, Schaufele RL, Sein M, Sein T, Chiou CC, Chu JH, Kontoyiannis DP, Walsh TJ (2005) Epidemiology and outcome of zygomycosis: a review of 929 reported cases. Clin Infect Dis 41:634–653

Rodrigues AG, Araujo R, Pina-Vaz C (2005) Human albumin promotes germination, hyphal growth and antifungal resistance by *Aspergillus fumigatus*. Med Mycol 43:711–717

Segal BH (2009) Aspergillosis. N Engl J Med 360:1870–1884

Singh N, Paterson DL (2005) *Aspergillus* infections in transplant recipients. Clin Microbiol Rev 18:44–69

Singh N, Avery RK, Munoz P, Pruett TL, Alexander B, Jacobs R, Tollemar JG, Dominguez EA, Yu CM, Paterson DL, Husain S, Kusne S, Linden P (2003) Trends in risk profiles for and mortality associated with invasive aspergillosis among liver transplant recipients. Clin Infect Dis 36:46–52

Sipsas NV, Kontoyiannis DP (2008) Occupation, lifestyle, diet, and invasive fungal infections. Infection 36:515–525

Siwek GT, Dodgson KJ, de Magalhaes-Silverman M, Bartelt LA, Kilborn SB, Hoth PL, Diekema DJ, Pfaller MA (2004) Invasive zygomycosis in hematopoietic stem cell transplant recipients receiving voriconazole prophylaxis. Clin Infect Dis 39:584–587

Spellberg B, Walsh TJ, Kontoyiannis DP, Edwards J Jr, Ibrahim AS (2009) Recent advances in the management of mucormycosis: from bench to bedside. Clin Infect Dis 48:1743–1751

Steinbach WJ, Benjamin DK Jr, Kontoyiannis DP, Perfect JR, Lutsar I, Marr KA, Lionakis MS, Torres HA, Jafri H, Walsh TJ (2004) Infections due to *Aspergillus terreus*: a multicenter retrospective analysis of 83 cases. Clin Infect Dis 39:192–198

Stelzmueller I, Lass-Floerl C, Geltner C, Graziadei I, Schneeberger S, Antretter H, Mueller L, Zelger B, Singh N, Pruett TL, Margreiter R, Bonatti H (2008) Zygomycosis and other rare filamentous fungal infections in solid organ transplant recipients. Transpl Int 21:534–546

Stergiopoulou T, Meletiadis J, Sein T, Papaioannidou P, Tsiouris I, Roilides E, Walsh TJ (2009) Comparative pharmacodynamic interaction analysis between ciprofloxacin, moxifloxacin and levofloxacin and antifungal agents against *Candida albicans* and *Aspergillus fumigatus*. J Antimicrob Chemother 63:343–348

Stevens DA, Schwartz HJ, Lee JY, Moskovitz BL, Jerome DC, Catanzaro A, Bamberger DM, Weinmann AJ, Tuazon CU, Judson MA, Platts-Mills TA, DeGraff AC Jr (2000) A randomized trial of itraconazole in allergic bronchopulmonary aspergillosis. N Engl J Med 342:756–762

Sutton DA, Wickes BL, Romanelli AM, Rinaldi MG, Thompson EH, Fothergill AW, Dishop MK, Elidemir O, Mallory GB, Moonnamakal SP, Adesina AM, Schecter MG (2009) Cerebral aspergillosis caused by *Aspergillus granulosus*. J Clin Microbiol 47(10):3386–3390. doi:10.1128/JCM.00588-09

Symoens F, Knoop C, Schrooyen M, Denis O, Estenne M, Nolard N, Jacobs F (2006) Disseminated *Scedosporium apiospermum* infection in a cystic fibrosis patient after double-lung transplantation. J Heart Lung Transplant 25:603–607

Tintelnot K, Just-Nübling G, Horré R, Graf B, Sobottka I, Seibold M, Haas A, Kaben U, de Hoog GS (2009) A review of German *Scedosporium prolificans* cases from 1993 to 2007. Med Mycol 47:351–358

Tortorano AM, Prigitano A, Dho G, Esposto MC, Gianni C, Grancini A, Ossi C, Viviani MA (2008) Species distribution and *in vitro* antifungal susceptibility patterns of 75 clinical isolates of *Fusarium* spp. from northern Italy. Antimicrob Agents Chemother 52:2683–2685

Uçkay I, Chalandon Y, Sartoretti P, Rohner P, Berney T, Hadaya K, van Delden C (2007) Invasive zygomycosis in transplant recipients. Clin Transplant 21:577–582

Varga J, Houbraken J, van der Lee HA, Verweij PE, Samson RA (2008) *Aspergillus calidoustus* sp. nov., causative agent of human infections previously assigned to *Aspergillus ustus*. Eukaryot Cell 7:630–638

Vinh DC, Shea YR, Sugui JA, Parrilla-Castellar ER, Freeman AF, Campbell JW, Pittaluga S, Jones PA, Zelazny A, Kleiner D, Kwon-Chung KJ, Holland SM (2009) Invasive aspergillosis due to *Neosartorya udagawae*. Clin Infect Dis 49:102–111

Walsh TJ, Lutsar I, Driscoll T, Dupont B, Roden M, Ghahramani P, Hodges M, Groll AH, Perfect JR (2002) Voriconazole in the treatment of aspergillosis, scedosporiosis and other invasive fungal infections in children. Pediatr Infect Dis J 21:240–248

Zaas A, Liao G, Chien J, Weinberg C, Shore D, Giles SS, Marr KA, Usuka J, Burch LH, Perera L, Perfect JR, Peltz G, Schwartz DA (2008) Plasminogen alleles influence susceptibility to invasive aspergillosis. PLoS Genet 4:e1000101

Zaoutis TE, Heydon K, Chu JH, Walsh TJ, Steinbach WJ (2006) Epidemiology, outcomes, and costs of invasive aspergillosis in immunocompromised children in the United States, 2000. Pediatrics 117:e711–e716

Chapter 2
Virulence and Pathogenicity of Fungal Pathogens with Special Reference to *Candida albicans*

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Abstract The frequency of severe systemic fungal diseases has increased in the last few decades. The clinical use of broad spectrum antibacterial drugs and immunosuppressive agents after organ transplantation, cancer chemotherapy, and advancements in surgery are associated with increasing risk of fungal infection. Despite the effectiveness of available antifungals in combating such infections, the emergence of drug resistance to antifungals, and problems of toxicity and poor delivery of drugs at the target site in systemic infections, have necessitated a systematic approach to the study of fungal pathogens, host–fungi interactions, and identification of virulence factors. Characterization of virulence factors is expected to improve understanding of fungal pathogenesis and to help explore new drug targets. In this article we discuss the process of fungal infections, virulence factors and pathogenicity of fungal pathogens, with special reference to *Candida albicans*. Adherence, dimorphism, phenotypic switching, secretion of hydrolytic enzymes, biofilm formation,

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and ability to adapt at host body temperature are some of the well-known virulence factors among pathogenic fungi and are discussed in relation to *C. albicans*.

2.1 Introduction

Fungi are eukaryotic microorganisms that are more closely related to humans than bacteria at cellular level. They belong to the group Eumycota, and are chemoheterotrophs with a chitinous cell wall. More than 100,000 species have been described. Most species grow as multicellular filaments called hyphae-forming mycelium such as molds; some species also grow as single cells like yeasts. Some groups of fungi are pathogenic to humans and require control measures. Human fungal pathogens belong to four main groups, namely zygomcetes, ascomycetes, deuteromycetes, and basidiomycetes. Fungi can cause significant number of human diseases represented by pathogens such as *Trichophyton* sp, *Epidermophyton* sp, *Histoplasma* sp, *Blastomyces* sp, *Sporothrix* sp, *Coccidioides* sp, and *Paracoccidioides* sp, capable of infecting healthy people, or opportunistic invaders such as *Aspergillus* sp, *Candida* sp, *Cryptococcus* sp, *Fusarium* sp, and *Rhizopus* sp, which are normally avirulent in healthy people but could be disseminated to deep tissue and cause fatal disease in unhealthy people (Chakrabarti 2005; Reedy et al. 2007). The morbidity and mortality rates caused by fungal species such as *Candida, Aspergillus, Fusarium, and Trichosporum* are relatively higher (Fluckiger et al. 2006). In Europe, fungal infections account for 17% cases associated with intensive care units (Rupp 2007), while in the USA it has become the seventh most common cause of deaths among hospitalized patients (Martin et al. 2003). About 15% of allogenic haemopoietic stem cell transplant recipients and 20% of lung transplant recipients suffered fungal infections (Ribaud et al. 1999). Approximately 60% and 20% of AIDS patients present with pneumonia and esophageal candidiasis respectively (Moore and Chaisson 1996).

Data from the late 1950s and early 1960s indicate that invasive fungal infections were extremely rare, even in immunocompromised cancer patients (Chakrabarti 2005). Now, fungal infections have dramatically increased in the past two decades as a result of improved diagnostics, high frequency of catheterization, instrumentation and an increasing number of immunosuppressed patients. Particularly, invasive fungal infections are showing extremely high mortality rate. The use of antineoplastic and immunosuppressive agents, broad-spectrum antibiotics, prosthetic devices and grafts, and more aggressive surgery have led to the development of complicated infections, including invasive fungal infections. Furthermore, patients with burns, neutropenia, and HIV infections are now seriously exposed to fungal infections (Kuleta et al. 2009).

Fungal infections have now also become more common in the healthy population. The National Nosocomial Infections Surveillance System has reported *Candida* spp. as the fourth most common bloodstream isolates in nosocomial infections in USA. Over 95% of all fungal infections have been associated with *Candida albicans, Aspergillus fumigates*, and *Cryptococcus neoformans* (Richardson 2005).

2.2 Diseases Caused by Human Pathogenic Fungi

Fungal diseases can be broadly classified on the basis of causative agents as: (a) dermatophytosis, (b) histoplasmosis, (c) blastomycosis, (d) coccidiomycosis, (e) candidiasis, (f) cryptococcosis, (g) aspergillosis, (h) hyalohyphomycosis, and (i) zygomycosis, as described by many authors (Sullivan et al. 2005). These diseases differ in their nature, causative agents, and distribution. Description of such fungal diseases, their causative agents and major organs involved etc are given in Table 2.1. However, candidiasis is described here briefly. Candidiasis encompasses secondary or opportunistic infections ranging from acute, sub-acute, and chronic to life-threatening mycoses. Infections are localized to mouth, throat, skin, vagina, fingers, bronchi, lungs, and gastrointestinal tract, or sometimes become

Table 2.1 Examples of commonly caused fungal diseases

Fungal diseases	Causative agent	Site of infection	Transmission
Dermatophytosis	*T. rubrum, T. mentagrophytes, E. floccosusm, M. gypseum, M. canis*	Skin, hair, nails, feet	Soil, contact with arthrospores or conidia from contaminated animals and humans
Histoplasmosis	*H. capsulatum*	Lungs	Soil, inhalation of microconidia
Blastomycosis	*B. dermatididis*	Lungs, skin, genitourinary tract, brain	Soil, inhalation of conidia
Coccidioidomycosis	*C. immitis, C. posadasii*	Lungs, bones, joints, meninges	Soil, inhalation of arthroconidia
Candidiasis	*C. albicans, C. tropicalis, C. glabrata, C. dubliniensis, C. krusei*	Intestinal tract, vaginal tract, skin, fingers, oral cavity	Endogenous flora, contact with secretions from infected person
Cryptococcosis	*C. neoformans*	Lungs, meninges, kidney, liver, prostate, bones	Soil, contamination with bird feces
Aspergillosis	*A. fumigatus, A. flavus, A. niger*	Lungs	Soil, inhalation of spores
Hyalohyphomycosis	*Fusarium* sp., *Phecilomyces* sp, *Scedosporium* sp, *Scopulariopsis* sp, and *Acremonium* sp	Keratin, nails, lungs	Soil, plant debris, ingestion of toxin contaminated plant parts
Zygomycosis	*Rhizopus* sp, *Mucor* sp and *Absidia* sp	Skin, cerebral, blood, lungs, genitourinary and gastrointestinal system	Soil, decaying plant material, inhalation or percutaneous contact of spores

Adapted from Weitzman and Summerbell (1995), Hogan et al. (1996), Pommerville (2004), Rappleye and Goldman (2006), Willey et al. (2008)

systemic as candidemia, endocarditis, and meningitis. A number of *Candida* spp are encountered in candidiasis such as *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. dubliniensis, C. parapsilosis* (Hayens and Westerneng 1996). *C. albicans* is a member of the commensal microflora of the intestine. It is pleomorphic and undergoes reversible morphogenic transitions between budding yeast, pseudohyphal, and hyphal growth forms. Healthy persons generally encounter superficial infections but in immunocompromised patients invasive infections could also occur. Approximately 70% of woman experience vaginal candidiasis once in a life, and 20% suffer from recurrence (Fidel et al. 1999). Among other *Candida* spp, *C. glabrata* has emerged as a frequent pathogen due to increased use of immune suppressive agents. *C. krusei* is a pathogen of importance in patients with hematological malignancies and transplants. *C. parapsilosis* is frequently isolated from blood cultures due to insertive medical devices. *C. tropicalis* is one of the causative agents of candidemia and isolated from patients with leukemia and those who have undergone bone-marrow transplantation. *C. dubliniensis* is found associated with systemic infections in AIDS patients.

2.3 Host–Fungi Interaction: The Process of Infection

Like any other microbial pathogen, fungal infection also involves some basic steps such as (1) entry and adherence to the host tissue, (2) invasion of the host tissue, (3) multiplication, colonization and dissemination in the tissues, and (4) evasion of the host immune system and damage to the tissues.

2.3.1 Entry or Adherence to the Host Tissue

Humans are first exposed to fungus *C. albicans* when passing through the vaginal canal during birth. In this course the fungus colonizes the buccal cavity, and upper and lower parts of the gastrointestinal tract of the newborn, where it becomes commensal (Khan and Gyanchandani 1998; Cladernone and Fonzi 2001). Other fungi of human diseases come from exogenous sources of soil and decaying vegetation as saprophytes. Generally, they enter through respiratory portals. Fungi rarely cause disease in immunocompetent hosts, though often exposed to infectious spores. Disease results when fungi accidentally penetrate host barriers or when immunologic defects or other debilitating conditions exist that favor fungal entry and growth (Hogan et al. 1996). Infection of a host starts with the adherence of fungi at epithelial surface layers and further dissemination to different host sites. Invasion of various tissues and resistance to attack by the host immune system is necessary for a pathogen to establish infection.

2.3.2 Adaptation and Propagation

For a fungus to survive in its niche it has to adapt to constantly changing parameters. Therefore, fungi respond to change in a specific environmental component by inducing transcriptional and translational changes that promote survival under the newest environmental conditions. When fungi enter the mammalian host their lifestyle changes from saprophytic to parasitic. As saprophytes, fungi survive in an environment with a moderate ambient temperature and pH, essential nutrients such as carbon and metal ions, and atmospheric concentrations of carbon dioxide and oxygen. Once having invaded a human host, these environmental factors are suddenly replaced by drastic changes. In the different niches of a host, completely different nutrient compositions may exist and specialized features of fungal pathogens may be involved in the establishment, dissemination, and manifestation of an infection (Brock 2009). For example, ambient temperature is replaced by the high temperature of the human body. Fungal survival at the elevated temperature of a human host is essential for virulence. The fungal pathogens *C. neoformans* and *A. fumigatus* are simply better able to survive at 37°C than their nonpathogenic counterparts (Hogan et al. 1996). Fungi often develop morphogenetic virulence mechanisms, e.g., formation of yeasts, hyphae, and spherules that facilitate their multiplication within the host at higher temperature. Yeast cells of many *Candida* species form filamentous pseudohyphae and hyphae in tissues, whereas *C. neoformans* yeasts become coated with a capsule, and *Coccidioides immitis* develops swollen, septated spherules in the host. Other fungi such as *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Penicillium marneffei* form filamentous mycelia in the environment, but convert to yeast morphology upon contact with the human host (Rappleye and Goldman 2006). Hyphae that grow in the skin or nail as dermatophytes can fragment into arthroconidia or other conidial types. On the other hand, ambient pH is replaced with acidic conditions of mucosal surfaces or neutral to slightly alkaline pH of blood and tissues. One pathway used by fungi in response to changing pH involves activation of the transcription factors such as PacC in *A. nidulans* and Rim101 in *C. albicans* (De Bernardis et al. 1998). Carbon and metal ions are lacking in host tissues; iron is sequestered from microbes by iron carrier proteins in tissues, creating an iron-limited environment. In order to survive, fungi encode certain mechanisms by employing siderophores, high affinity iron chelators, to efficiently bind host iron into fungal cytoplasm (Haas et al. 2008). Also, fungi have to face hypoxia and high levels of carbon dioxide in tissues. In *C. albicans*, the response to hypoxia is dependent on coordination of specific transcriptional regulators; for example, transcription factor Ace2 represses oxidative metabolic processes and promotes filamentation (Mulhern et al. 2006).

All these specialized adaptations help fungi in sustaining infection at the host site. Most of the free-living pathogenic fungi possess an extremely versatile metabolism which allows them to adapt immediately to changes in the environmental conditions during life in the soil. Therefore, success of infection depends on rapid adaptation to changing micro-environments.

2.3.3 Dissemination

Dissemination of fungi in the host body is facilitated by severe endocrinopathies and immune disorders. A fungus utilizes various mechanisms to deceive or destroy the immune cells and spread to various organs. Dissemination depends on interactions of factors from host and fungi, as described by several authors (Casadevall and Pirofski 2001; Latge and Calderone 2002).

2.3.3.1 Host Factors

Considering the interaction between host and pathogen, immune cells are the major antagonists to the survival of fungal pathogens inside the host. However, primary resistance to fungal invasion and colonization is contributed by cutaneous and mucosal physical barriers. The non-specific host defenses include (1) the antifungal activity of saliva and sweat, (2) the competition for space and nutrients by the normal microbiota of the skin and mucous membranes, which limits the growth of potential pathogens, and (3) the mechanical barrier of the skin and mucous membranes which prevent entry of fungi. Inflammatory systems to combat fungal proliferation involving the action of neutrophils, mononuclear phagocytes, and other granulocytes are also considered to be nonspecific. The specific host defenses or acquired immunity consist basically of the cell-mediated immunity regulated by T-lymphocytes. In humans, mycoses acquired by exposure to fungal spores through the respiratory tract are checked primarily by the first line of defense, i.e., mucociliary clearance. Remaining spores are ingested and killed by monocytes or macrophages through phagocytosis as adaptive innate immunity (Wanner et al. 1996). In addition, healthy individuals employ a second line of defense formed by neutrophilic granulocytes. They mainly attack hyphae, which are too large for ingestion. These in turn are killed by oxidative and non-oxidative mechanisms, including different defensins. Each of these two defense systems alone is able to protect the host against large spores over long time periods. Fungal pathogens can cause invasive disease only if both protective lines are surpassed (Murphy 1991). Overall, severity of disease depends on factors such as inoculum size of the attacking pathogen, magnitude of tissue damages, ability of fungi to multiply in the tissue, and the immune status of the host cells.

2.3.3.2 Fungal Factors

Production of extracellular enzymes such as keratinases, collagenases, gelatinases, phospholipases, lipases, and acid proteinases by dermatophytes, *Aspergillus* sp, *Candida* sp, and *Cryptococcus* sp is considered to be the fungal-associated factor that helps fungi in nutrient uptake, tissue invasion, adherence, and dissemination inside the host. In some fungi such as *C. neoformans*, the presence of capsule may be

an important factor. Similarly, the ability to grow at 37°C, dimorphism, and other factors contribute to fungal pathogenesis, which involves a complex interplay of many fungal and host factors.

2.4 Virulence and Pathogenicity

Pathogenesis is the ability of a microorganism to infect the host and produce disease resulting from interaction of pathogen with host via expression of certain factors on both sides. Pathogenicity of a fungus depends on its ability to adapt to the tissue environment and to withstand the lytic activity of the host's defenses. Several determinants including genes or gene products such as enzyme molecules known as virulence factors are involved in this relationship, producing superficial to invasive infections in humans. Virulence refers specifically to a property of the pathogen and, according to modern definitions, virulence is the ability of a pathogen to multiply and cause harm to its host (Casadevall 2007). For a fungus to produce disease in a patient, it must be actively invading tissues. Diseases caused by fungi without invasion of live tissues include mould allergies and cutaneous dermatophyte infections (ringworm), in which fungi invade and damage only the nonviable epidermis. Further, potentially lethal mycoses involving deep tissues result from fungal dissemination and invasion throughout the body (Fluckiger et al. 2006). Many human fungal pathogens are dimorphic (capable of reversible transitions between yeast and hyphal forms), and the morphogenetic transition between these forms is often stimulated by growth in the host and correlated with host invasion. However, the nature of association between fungal morphogenesis and host invasion is a highly debated aspect of fungal virulence (Molero et al. 1998; Klein and Tebbets 2007).

Determinants of pathogenicity are called virulence factors. Pathogenic microbes often possess a number of virulence factors and mechanisms. These factors determine whether the organism (the host) lives or dies during host–microbe interactions. The factors can be inducible or constitutive, the direct product of genetic elements (proteins), or the products of complex biosynthetic pathways such as polysaccharides or lipid mediators. The virulence factor can be assessed by comparing biological response in fungi with and without the factor. The most convincing evidence for a factor to be considered as a virulence determinant is the simultaneous loss of the factor and loss of virulence, and the regaining of virulence when the factor is restored. Virulence factors must help the pathogen to grow at elevated temperatures, facilitate adherence, penetration and dissemination, or assist in resistance against innate immune defenses, e.g., phagocytosis and complement, evasion from adaptive immune defenses, or nutritional and metabolic factors, necrotic factors, or morphology variation. The ability of a fungus to grow at 37°C and physiological pH is a virulence factor for fungi that invade deep tissue, and the transition to parasitic form is essential for the pathogenicity of dimorphic fungi. A size compatible with alveolar deposition is a virulence factor for fungi producing

infections by inhalation of airborne spores (Tomee and Kauffman 2000). Some kinds of virulence factors are commonly required for all pathogens, such as the ability to recognize and adhere to host tissues, to respond rapidly to changes in the external environment and to secrete hydrolases; all are thought to be important in virulence. But the complex nature of the host–fungus interaction has resulted in some factors that are absolutely required for fungal virulence. Some properties are frequently associated with pathogenesis across all fungal pathogens and others have been found to be important for specific pathogens. Because pathogenesis is complex phenomenon, possession of a single putative virulence factor is not sufficient for a fungus to become pathogenic; rather, a complex combination of properties is usually required. Several kinds of processes are thought to be involved in virulence in a wide range of fungal pathogens. Virulence factors associated with certain well-characterized fungi have been described in the literature, and we have listed some of them associated with medically important fungi in Table 2.2.

2.5 *Candida albicans*: An Opportunistic Fungus

Candida spp are asexual yeasts of the genus ascomycetes and genetically diploid with the presence of eight chromosomes. Some species have shown phenotypic switching, variant colony morphology and dimorphism, and transition from yeast to filamentous form. Out of more than 200 species, the most commonly encountered in medical practices are *C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis,* and *C. tropicalis*. About 8%–15% of nosocomial blood stream infections are reported to be caused by *Candida* spp (Pfaller and Diekema 2002). Candidal infections are a serious problem in individuals with weakened immune defense. Interestingly, *C. albicans* differs from other medically important fungi such as *H. capsulatum, A. fumigatus,* and *C. neoformans* in rarely being isolated from soil. Therefore, infections caused are categorized as endogenous and not exogenous as with others. *C. albicans* and related spp have been isolated from several body locations as a carrier in the oral cavities, gastrointestinal tract, anus, groin, vaginal canal, and vulva of healthy people, and may attain sufficiently high density without symptoms of disease. Among these, *C. albicans* was predominant at all body locations (70%), *C. glabrata* and *C. tropicalis* (7%) (Odds 1988). In normal conditions, it exists with other normal microbial flora of host organs; about 50% of a healthy population is supposed to be a benign carrier of *Candida* spp, but in immunocompromised patients who have undergone chemotherapy, bone-marrow transplantations, or diabetic treatment, it behaves like an opportunistic pathogen and produces superficial to systemic infection. Broad-spectrum antibiotic therapy may also alter the population of normal bacterial flora, resulting in *Candida* sp taking over the niche and assisting in flourishing and establishing secondary infections. Oral and vaginal thrushes are very common even in individuals with slightly weakened immunity (Soll 2002a; Fluckiger et al. 2006; Odds et al. 2006). The ability of *C. albicans* and other *Candida* spp to colonize and survive at different

Table 2.2 Role of virulence factors in pathogenic fungi

Fungal pathogen	Type of virulence factors	Role in pathogenicity
Dermatophytes	Keratinase	Damage of keratinous layer in epidermis
	Elastase	Destruction of elastin in tissues
	Acid proteinase	Cleavage of peptide bonds in host cells to obtain nutrients and invasion
	Phospholipase	Cleavage of phosphodiester bond in membrane lipids for invasion
Aspergillus spp	Cell wall component, β-1,3 glucan	Cell adhesion
	Conidial size (2–3 μ)	Escape from mucocilliary extrusion
	Adhesin, tronchin	Binding of conidia to lung tissue
	cAMP, rasA, rasB	Nutritional uptake and growth of pathogen, germination of conidia, branching of hyphae
	Elastase-alkaline serine proteinase	Degradation of elastin in lung tissue
	Phospholipase C (plb1,2,3)	Tissue damage and penetration
	Catalases (catA, catB and cat2), Superoxide dismutase	Prevention from oxidative damage in macrophages
	Gliotoxin, helvolic acid	Immunosuppressive properties, prevention from oxidative burst of macrophages
	Ribotoxin	Cleavage of phosphodiester bond in eukaryotic 28s rRNA
	Siderophore (sidA gene)	Uptake of iron from blood heme
	Growth at 37°C, hsp1, cgrA	Ability to invade host tissue and survival at elevated temperature of host
Histoplasma capsulatum	Dimorphism	Altered cell surface adhesion, tissue invasion by hyphal phase, dissemination by yeast phase
	α-1,3-glucan in cell wall	Required for adhesion
	Growth inside macrophage	Evasion from immune cells, dissemination to other organ tissues
	Catalase	Protection from oxidative killing
Coccidiodes immitis	Dimorphism	Sheer size of spherule is required for dissemination, hyphal phase tolerate pH 2–12
	Elastase	Destruction of lung insertium and blood vessels
	Estrogen binding protein	Acceleration of spherule maturation and endospore release
Blastomyces dermatitidis	Dimorphism	Tissue invasion and dissemination
	Adhesin (BAD1)	Suppression of immune response
	α-1,3-glucan in cell wall	Adhesion and masking of cell surface receptors being recognized by immune cells
Cryptococcus neoformans	Capsule	Inhibition to phagocytosis
	Melanin	Prevention from oxidative damages
	Mannitol	Scavenging of hydroxyl radical during respiratory burst
	Phospholipases A,B,C,D (plb1,2,3)	Tissue invasion and adherence
	Acid proteinases	Tissue invasion and dissemination
Candida albicans	Adhesin (Als family, HWP1)	Adherence to epithelial cells, fibronectin, biofilm establishment

(*continued*)

Table 2.2 (continued)

Fungal pathogen	Type of virulence factors	Role in pathogenicity
	Dimorphism (phr1, hyr1, chs2, chs3, rbf1)	Hyphal phase required for invasion and adherence, yeast phase for dissemination
	Phenotypic switching (efg1)	Conversion to more virulent forms showing increased ASP and adhesion production, evasion from host response
	Secreted aspartyl proteinases (SAPs 1–10)	Nutrient uptake, tissue invasion, adherence and dissemination
	Phosphlipases A,B,C,D (plb1,2,3)	Tissue invasion and adherence
	Farnesol	Quorum sensing, Biofilm formation
	Catalase, Superoxide dismutase	Prevention from oxidative damages
	SUN41, GCN4, MKc1p	Biofilm establishment

Adapted from Hogan et al. (1996), Ghannoum 2000, Cladderone and Fonzi (2001), Yang (2003), Blankenship and Mitchell (2006), Kuleta et al. (2009)

anatomic sites of the host has made them more harmful than other commensals of the human body.

Microorganisms colonizing the gut can normally cause dysfunctions of intestine such as *E. coli* or *Salmonella* sp, but opportunistically *Candida* cells can disseminate from gut to oral, vaginal mucosa, or skin as superficial infections and to the bloodstream as systemic infections. *Candida* makes this spread possible by expressing tissue site-specific metabolites. For example, in host tissue or bloodstream having neutral or slightly alkaline pH, it expresses the gene PHR1 whose products are involved in cell wall synthesis and work at neutral pH. On the other hand, in vaginal mucosa where pH is acidic, this gene is switched off and another gene, PHR2, is expressed with similar functions (Saporito-Irwin et al. 1995; Muhlschlegel and Fonzi 1997).

As a commensal, *Candida* resides in yeast form and multiplies by budding into blastospores, but during weakened immunity of the host it transforms into the hyphal form as the start of pathogenesis. In vivo study has revealed that change in pH, oxygen, carbon dioxide, or glucose concentration in host tissue triggers this transition (Cladderone and Fonzi 2001; Haynes 2001). Filamentous forms are more adhesive due to increased expression of adhesins on the surface, and also secretion of a higher amount of hydrolytic enzyme enhances the invasiveness. Moreover, the pathogenic stage has to resist recognition by the immune system or damaging macrophages and neutrophils. This interaction with host tissue in favor of *Candida* results in deep tissue penetration and the establishment of infections. It is speculated that host (tissue environment and immune system) alone determines the balance between commensalism and pathogenicity (Soll 2002a; Hube 2004).

The work of several researchers has shown that certain genes such as pH-regulated PHR1 and PHR2, genes encoding secreted aspartyl proteinases (SAPs 1–9), and genes encoding phospholipases (PHL A–D) are expressed differentially in specific tissues at different stages of infections (Yang 2003; Naglik et al. 2004).

Expression or modulation of these genes on the same mucosal surfaces only during transition from the commensal to the parasitic stage reflects a weakness in the immune system responsible for this shift (Casadevall and Pirofski 2001). *Candida* survives and proliferates as commensal in competition with other microbial flora and is affected by epithelial cell proliferation and the immune system. Proliferation of epithelial cells constitutively forces *Candida* to attempt deeper invasion into tissues. Prolonged antibiotic therapy provides more available nutrients and space for *Candida* to multiply as other commensal microbial flora are diminished (Senet 1998). Immune suppression in HIV patients and inhibition of epithelial cell proliferation such as in cancer therapy changes the tissue environment in terms of pH, osmolarity, and oxidative stress. This changed condition is perceived by the candidal cell and subsequently down- or up-regulation of certain genes provokes *Candida* to switch over from commensal to opportunistic pathogens (Claderone and Fonzi 2001; Hube 2004).

Advanced medical equipment and surgery has also led to the increased spread of commensal *Candida* to tissues as pathogens. Medical devices such as catheter, dental implants, artificial joints, pacemakers, central nervous system shunts, and others have provided the opportunity to form biofilms, a stage more resistant to drugs and capable of greater invasion to tissues. These devices are easily colonized by candidal cells from mucosal surfaces and blood stream, and frequently get spread from one tissue site to another. Further, candidal cells can also migrate via blood flow to all inner organs, leading to septicemia and life-threatening diseases (Douglas 2003; Hall-Stoodley et al. 2004). Biofilm-forming cells have been reported to be more virulent than planktonic cells (Ramage et al. 2005; Seneviratne et al. 2007). Recently, several workers have reported increased production of proteinases, phospholipases, and adhesins in biofilm compared to planktonic cells (Chandra et al. 2001; Al-Fattani and Douglas 2006; Seneviratne et al. 2007). All these collectively aid in establishing infections by heightening the adherence and invasion of tissues, leading to increased virulence. Further, genetic changes in biofilms result in elevated drug resistance, pronounced quorum sensing and regulated carbohydrate synthesis, thereby influencing the pathogenicity of *Candida*. Therefore, biofilms-forming capacity has greatly increased the potency of *Candida* to convert from the commensal stage into a virulent pathogen.

2.6 Virulence Factors Involved in Pathogenicity of *Candida albicans*

Like other fungal pathogens, *C. albicans* also regulates expression of certain genes and their products as virulence factors to produce disease. This is the most common opportunistic pathogen, utilizing several kinds of virulence factors. Some of the commonly studied virulence factors in *C. albicans* are briefly described here.

2.6.1 Adhesion

Adherence of candidal cells to host tissues is a complex multifactorial phenomenon utilizing several types of adhesins expressed on morphogenetically changing cell surfaces. But the striking feature of *Candida* cells is the formation of biofilms in host tissue, resulting in enhanced adherence. Ramage et al. (2006) have reported that in the last few decades, *Candida*-related infections have been found associated with biofilm-forming capacity. Well-known adhesins are agglutinin-like sequences (ALS) that are members of a family of seven glycosylated proteins. Als1p, Als3p and Als5p (Ala1p) on the cell surface of hyphae adhere to human buccal epithelial cells (HBEC) and fibronectin, collagen, laminin, and endothelial cells (Hawser and Douglas 1994; Hoyer 2001). Als6p and Als9p bind to collagen and laminin respectively. Als4p binds to endothelial cells and Als5p is additionally needed for cell aggregation. However, the role of Als7p is unclear (Filler et al. 2006; Kuleta et al. 2009). Another 34 kDa adhesin molecule, Hwp1 (hyphal wall protein), encodes an outer surface mannoprotein on the hyphal wall; the amino terminal sequences of this adhesin are recognized as mammalian transglutaminase substrate (TGase) and form covalent binding with HBEC. Studies with hwp1$^-$ knockout mutant and HWP1$^-$ deficient mutant of *C. albicans* have shown reduced adherences and mortality in murine models (Chaffin et al. 1998; Staab et al. 1999). An integrin-like protein (Int1p) which is a plasma membrane receptor and antigenic functionally similar to human complement receptors 3 and 4, has been isolated from *C. albicans* and found to bind with extracellular matrix (ECM) ligands such as fibronectin, laminin, and collagen I and IV, and induce morphological changes in response to extracellular signals (Cladera one and Fonzi 2001; Ruiz-Herrera et al. 2006).

2.6.2 Morphogenesis

Morphogenesis in *C. albicans* is defined as transition from unicellular yeast form to filamentous form (pseudohyphae or hyphae). Of all the species only *C. albicans* and *C. dubliniensis* are able to undergo morphogenesis. Transition from yeast form to hyphal form is facilitated by nutrients, near-neutral pH, temperature of 37°C–40°C, CO_2 concentration about 5.5%, and presence of *N*-acetyl-D-glucosamine, serum, some amino acids, and biotin. Reverse transition from hyphal to yeast form is provoked by lower temperatures, acidic pH, absence of serum, and higher concentration of glucose (Corner and Magee 1997; Eckert et al. 2007). This transition is strongly required for pathogenesis. Yeast forms are more suited for dissemination in tissues and to other hosts, whereas hyphal forms are required for tissue damage and invasion. For example, the yeast cell, when phagocytosed by macrophages, produces hyphae and secretes hyphae-associated proteinases that kill macrophages; these factors also prevent hyphal cells from being killed by neutrophils. In addition, hyphal cells have been shown to induce phagocytosis by endothelial cells, helping

Candida cells to escape from the bloodstream (Molero et al. 1998; Gow et al. 2002; Hube 2004). Further, hyphal cells have stronger adherence capacity due to expression of ALS adhesins and also exhibit greater invasiveness to tissues. Increased expression of superoxide dismutase (SOD) antagonizes oxidative burst of phagocytic cells. Several genes have been identified which regulate phase transition, namely PHR1, ECE1, HYR1, RBF1, CHS2, CHS3 which are differentially expressed during morphogenesis (Haynes 2001; Claderone and Fonzi 2001). Of these, ECE1 correlates with hyphal elongation although ECE1 null mutants displayed no morphological alterations. Similarly, null mutants for expression of CHS2, CHS3 and HYR1 did not show any obvious morphological type. But disruption of RBF1 demonstrated alteration in cell morphology and strongly involved in yeast–hypha transition (Yang 2003). Studies with homozygous null mutants for Hst7p, Cph1p and Cst20p have shown defective hyphal formation (Leberer et al. 1996); in addition, three genes *TUP1*, *EFG1*, *CLA4* were found to be regulating candidal morphogenesis (Liu 2001). Transcription factors such as Tup1 and Rbp1 are negative regulators of filamentation (Braun and Johnson 2000). A tup1 mutant strain resulted in constitutive filamentous growth under all conditions, indicating a role in filament formation. Deletion of homozygous allele of Ste20 encoded by CLA4 showed impaired hyphal formation in a wide range of medium, and decrease in virulence in a murine model (Braun and Johnson 1997; Celera and Claderone 2001). A protein of bHLH class encoded by EFG1 acts as transcriptional activator as well as repressor, and is required for pseudohyphal and hyphal morphogenesis (Liu 2001; Noffiz et al. 2008). A study with homozygous mutants cfg1 and cph1 showed failure of germ tube and hyphae production in a murine model (Noffiz et al. 2008).

2.6.3 Phenotypic Switching

Unlike other pathogens, phenotypic switching in *Candida* is pleitropic by affecting several phenotypic and metabolic parameters, with subsequently a number of virulence traits such as SAP gene regulation. This allows *Candida* to adapt to a different host environment during infection (Soll 1992; Soll 2002b). Colonies of *C. albicans* show morphological variation, including smooth, rough, star, stippled, hat, wrinkle, and fuzzy at high frequency. This switching is reversible, occurs spontaneously in stress, and results in changes in cell surface behavior, colony appearance, and metabolic, biochemical and molecular attributes to become more virulent and effective during infection (Soll 2002b; Odds et al. 2006). Strains isolated from vaginitis or systemically infected patients showed higher frequencies of switching, indicating a strong role for the switching phenomenon in establishing diseases (Kvaal et al. 1999). In the case of yeast–hypha transition, all cells of a population express the same phenotype under the same environmental conditions, whereas in the case of switching, some cells of a population express different phenotypes under the same set of environmental conditions. Earlier research had

reported that laboratory isolate 3153A, grown on amino acid rich agar which was limiting for zinc and incubated at 25°C, showed a smooth phenotype as dominant, while variant colonies of star, ring, irregular, and wrinkle occurred spontaneously. Such types of variation were also observed with cells of strain 3153A treated with low doses of UV irradiation (Soll 1992). At present, of all the phenotypes described, the white-opaque system in strain WO-1 is the most studied. This system is characterized by transition from smooth, white colonies to flat, gray opaque colonies. White cells are round ovoid while opaque cells are elongated or bean shaped (Soll 2002b). Study of gene expression with the WO-1 system revealed an association of OPA1 (SAP1) and SAP3 in opaque cells, in contrast to SAP2, WH11and EFG1 in white cells (Soll 1997; Miller and Johnson 2002). Study with efg1 null mutants exhibited no involvement of EFG1 in switching, but rather control of phenotypic characteristics. It has been reported that white cells in WO-1 hardly form hyphal stages, but this was achieved by opaque cells (Staib et al. 2002). There is good evidence that opaque cells are more virulent than white cells in several murine models (Yang 2003).

2.6.4 Phospholipases

Phospholipases are enzymes that hydrolyze ester linkages of glycophospholipids and hence impart tissue invasiveness to *Candida* cells. In *C. albicans*, four types of phospholipases are classified by researchers on the basis of the ester bond they cleaved, viz., phospholipase A, B, C, and D. All types possess hydrolase activity, but PLB in addition also possesses lysophospholipase transacylase activities; therefore, it is able to release fatty acids from phospholipids and the remaining fatty acid from lysophospholipids, and then transfer a free fatty acid to lysophospholipids, producing phospholipids. Of these, only PLB1, a 84 kDa glycoprotein isolated from hyphal tip in the course of tissue invasion, has been shown to be required for virulence in a murine model of candidiasis (Ghannoum 2000; Yang 2003; Theiss et al. 2006). A study conducted by Ibrahim et al. (1995) revealed an increased level of phospholipase production in blood isolates compared to commensal isolates.

2.6.5 Proteinases

Secretion of proteinases by pathogen is mandatory in order to degrade the tissue barriers and obtain nutrition at the infection site. Secreted aspartyl proteinases (SAPs) from *Candida* have been reported that hydrolyze many proteins such as albumin, hemoglobin, keratin, collagen, laminin, fibronectin, mucin, salivary lactoferin, interleukin1β, cystatin A, and Immunoglobulin A (Hube et al. 1998). To date, ten proteins have been recognized as SAP family (SAPs 1–10) and found to be responsible for tissue invasion. Several researchers have reported that production of SAPs is also correlated with hyphal formation, adherence, and phenotypic switching

(Monod and Zepelin 2002; Naglik et al. 2003). Such researches have highlighted the complex role played by SAPs in the pathogenicity of *C. albicans*. Several models using SAP inhibitors such as pepstatin A and SAP-disrupted or over-expressing mutants demonstrated the need for these factors in candidal pathogenesis. In vitro studies have reported that SAPs 1, 2 and 3 are expressed by the yeast phase, only while SAPs 4, 5 and 6 are expressed in the hyphal phase (Hube et al. 1998; Schaller et al. 1999; Naglik et al. 2004). Whereas, SAPs 9 and 10 are expressed by both forms (Albrecht et al. 2006). Structural analysis revealed that SAPs 1–8 are secreted extracellularly, but that SAPs 9 and 10 are anchored to the cell wall by glycophosphotidyinositol (GPI) protein (Naglik et al. 2003; Albrecht et al. 2006). Models of epidermal and vaginitis candidiasis revealed involvement of SAPs 4–6 in invasive systemic disease whereas SAP 7 was never detected *in vitro*. The role of SAPs 1–3 is associated with early adherence, invasion, and cutaneous infections as studied in the WO1 strain, whereas SAP8 is associated with extensive penetration. SAPs 6 and 9 were found expressed in later stages of hyphal growth (Hube et al. 1998; Schaller et al. 1999; Kvaal et al. 1999). Different properties of SAPs are exploited in the pathogenicity of *Candida*. For example, SAPs are active across a broad range of pH 2.0–7.0, as SAPs 1–3 are active at pH 3.5, SAPs 4–6 at pH 5.0–7.0, and therefore make *Candida* capable of colonizing and invading different tissue sites of varying pH. In addition, SAPs show varied levels of protein specificity, as SAPs 1, 2, 3 and 6 cleave peptide bonds in larger hydrophobic amino acids; SAPs 1, 2 and 6 act on phenylalinine, whereas SAP 3 attacks leucine and SAPs 9 and 10 hydrolyze yapsin and kexins (Naglik et al. 2004). This attribute enables *Candida* to obtain nitrogen at different tissue makeups, and aids pathogenicity by revealing potential binding sites from tissue for adhesion of candidal cells, and also dissemination via circulatory systems. In vivo studies have confirmed the role of SAPs in colonization, increased adhesion and tissue penetration (Naglik et al. 2004; Hube and Naglik 2001). Disruption of SAPs 1, 2 and 3 have resulted in decreased virulence in mouse models (Hube et al. 1997). Several reports have supported functional role of SAP2 in invasion and dissemination of systemic infections (Kvaal et al. 1999; Naglik et al. 2004; De Bernardis et al. 1999). Further research data have indicated increased expression of SAP genes, especially SAPs 5, 6 and 9 mRNA transcripts, in biofilm rather than planktonic cells (Green et al. 2004; Naglik et al. 2008). Recently, in addition to SAPs, a 60 kDa metallopeptidase and 50 kDa serine peptidases have also been isolated, and reported to hydrolyze extracellular matrix proteins and serums (dos Santos et al. 2006). Expression of SAPs has been found to be correlated with other virulence determinants to enhance the pathogenicity of *C. albicans*. Correlation of SAPs with other virulence factors in *C. albicans* is illustrated in Fig. 2.1.

2.6.6 Biofilm Formation

Biofilms are the organized structures involving microbial communities that are attached to some inanimate surfaces or tissues and circumvented in a matrix of

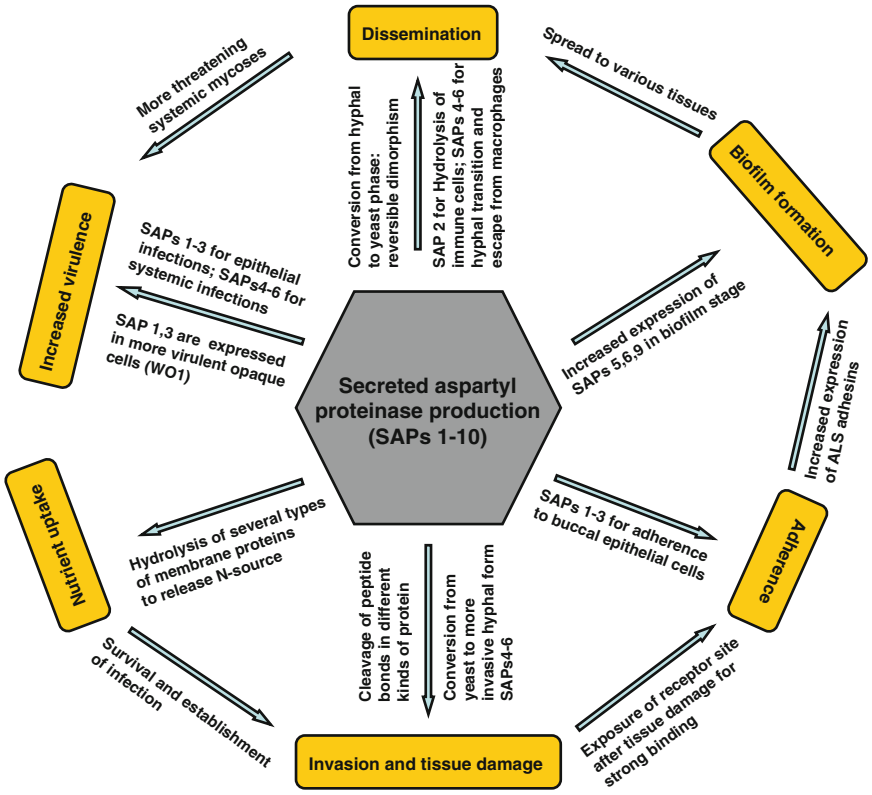

Fig. 2.1 Correlation of secreted aspartyl proteinases with other virulence attributes in *Candida albicans*. Partially adapted from Naglik et al. (2004)

exopolymeric materials. Biofilm formation is initiated by irreversible adherence of microbial cells to tissues or devices and followed by growth and maturation to form a mesh of cells with altered phenotype, growth rate, and gene expression compared to planktonic cells. Studies with scanning electron microscopy of biofilms revealed the presence of both adherent yeast cells and invasive hyphal forms constructing basal and upper layers respectively, enclosed in an extracellular polymer matrix consisting of polysaccharides and proteins and forming a three-dimensional structures with water channels (Dominic et al. 2007). These forms differ in ultrastructure, physiological behavior and composition of cell walls, and are required for candidal pathogenicity, as mutants lacking genes for any one became less virulent both *in vitro* and *in vivo* (Chandra et al. 2001). Heterogeneity of these biofilms depends on the substrate composition, environmental conditions, and type of strains involved. Although yeast–hypha transition is necessary for full maturation of the biofilm, strains that are unable to grow as yeasts or to form hyphae can still form biofilms but are easily detachable (Baille and Douglas 1999). In addition, some authors have reported a change in biofilm-forming ability of candidal cells, with

alteration in variants produced during phenotypic switching (Brown and Gow 1999; Berman and Sudbery 2002).

The ability of *Candida* to form biofilms on catheters, endotracheal tubes, pacemakers and other prosthetic devices has contributed to its predominant prevalence in nosocomial infections (Douglas 2003; Ramage et al. 2005). Such devices, in addition to providing a platform for candidal cells to form biofilm, grow and develop, provide a route through host barrier defenses for dissemination. During weakened immunity, hematogenous dissemination of candidal cells from biofilms to deep-seated organs could occur, resulting in candidemia and septicemia. Recent studies have confirmed biofilm growth in the majority of diseases caused by *Candida* spp (Chandra et al. 2001; Douglas 2003; Chandra et al. 2005). Dental plaque is a well-known example of biofilm formation from *Candida* cells, and is responsible for oral candidiasis. Biofilm formation on such tissues is favored by a high concentration of glucose, serum, and other proteins. Biofilm formation was found to be linked to dimorphism and phenotypic switching, well-known virulence traits for candidal cells (Baille and Douglas 1999; Chandra et al. 2001). Also, alerted phenotypes exhibited reduced susceptibility to the host immune system and to antifungal drug therapy (Chandra et al. 2001). These biofilm-specific cell properties are an indicator for virulence, and have prompted much recent interest in *C. albicans* biofilm structure, physiology, and regulation. Therefore, knowing the ability of *C. albicans* to populate a surface and produce a biofilm as a virulence trait, exhaustive research is being focused on the prevention of biofilm infection by *Candida* cells.

Adherence is the critical property for biofilm-forming cells and is mediated by hydrophobic interactions, electrostatic forces, and adhesion–ligand interactions; multiple adhesion molecules function in the successful establishment of biofilm. A variety of genes are involved in adhesion, and penetrations are associated deeply with the biofilm-forming capability of *C. albicans*. Here, we would discuss some of them for their role in pathogenicity. A number of adhesins, termed glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWPs), encoded by ALS1, ALS2, ALS4, ALS5 (ALA1), HWP1, and EAP1, mediate adhesion to organic and inorganic surfaces, extracellular matrix proteins, human endothelial cells, and epithelial cells (Blankenship and Mitchell 2006; Filler et al. 2006; Zhao et al. 2006). Experimental results showed upregulated ALS family gene expression in biofilm-forming cells compared with planktonic cells (Hoyer et al. 1998; Chandra et al. 2001; Nobile and Mitchell 2005; Green et al. 2004) and ALS 3 was also found necessary for biofilm formation on silicone substrates *in vitro* (Nobile et al. 2006a). ALS1, ALS3, and HWP1 are regulated by transcription factor BCR1 (biofilm and cell wall regulator), a zinc finger protein, which is under the control of transcription factor Tec1. An als3/als3 mutant strain was found defective in biofilm formation *in vitro*, and overexpression of ALS3 permitted biofilm formation by a bcr1/bcr1 mutant *in vitro* and *in vivo*. Studies with the bcr gene revealed involvement of BCR1 in governing the mechanism of biofilm formation only and not the filamentation (Nobile and Mitchell 2005; Lopez-Ribot 2005; Nobile et al. 2006a; Nobile et al. 2006b). Hwp1 is a cell surface protein covalently linked to the cell wall glucan

through a remnant of its GPI anchor. Functional analysis showed its requirement for tight adherence to oral epithelial cells (Chaffin et al. 1998; Staab et al. 1999; Mendes-Giannini et al. 2008). A role for Hwp1 in *C. albicans* cell–cell adherence is exhibited from the finding that it is induced by mating factor and is deposited on the surface of the bridge between mating partners (Staab et al. 1999; Hoyer et al. 1998). Recent studies showed that Hwp1is required as first cell surface protein *in vivo* for biofilm formation (Nobile et al. 2006a; Nobile et al. 2006b). Recently, work from Granger et al. (2005) described the role of Ywp1 (Yeast cell wall specific protein) as anti-adhesin. The mutant for Ywp1 led to enhanced adherence of yeast cells, therefore highlighting its negative role in biofilm establishment. In addition, studies with mutants for transcription factor Ace2 (activation of CUP1 expression 2) resulted in inhibition of biofilm formation (Kelly et al. 2004). A study conducted by Li et al. (2007) showed eap1 mutants exhibiting reduced adhesion to plastic surfaces and epithelial cells, and that Eap1p was able to mediate adhesion to yeast cells. The same study also showed of the need for eap1 gene expression in biofilm formation under shear flow *in vitro* and in central venous catheter biofilm model *in vivo*. In a study, another cell wall protein Ecm33 was found to be necessary for cell wall integrity and yeast-to-hypha transition (Martinez-Lopez et al. 2004), and Mp65 (Norice et al. 2007) is also required for full virulence in a disseminated infection model, illustrating that cell wall proteins may have diverse functions that are relevant to infection. Norice et al. (2007) also showed that protein SUN41 plays major roles in biofilm formation, cell wall integrity, and virulence in both oropharyngeal and disseminated candidiasis.

Richard et al. (2005) showed involvement of genes sun3, nup85, mds3, kem1 in hyphal development and biofilm formation. Some studies have also demonstrated that the hyphal regulatory gene efg1 is required for normal biofilm growth, and efg1/efg1 and efg1/efg1 cph1/cph1 mutants have exhibited defective biofilms and also adhered poorly to the substrate (Lewis et al. 2002; Watamoto et al. 2009). Gcn4, a general amino acid control regulatory gene, was shown to be required for full biofilm biomass production (Blankenship and Mitchell 2006). A contact-activated protein kinase, Mkc1p, is also required for biofilm development, suggesting that *C. albicans* may respond uniquely to surface contact during biofilm formation.

Further, an experiment by Ramage et al. (2002) showed that biofilms are organized communities under tight regulation of gene expression controlled through quorum sensing which in turn is regulated by farnesol and tyrosol molecules. Several workers have observed these organized communities under the control of a signaling molecule (Hogan 2006). This cell-to-cell communication prevents and controls unnecessary overpopulation and nutritional competition, and has implications in dissemination and establishment of infection at the distal site from old biofilm (Alem et al. 2006). Farnesol, a quorum-sensing molecule that inhibits *C. albicans* biofilm formation by inhibiting yeast-to-hypha transition, decreases HWP1 expression in biofilms (Ramage et al. 2002). Farnesol acts on yeast cells to prevent filamentation but elongated hyphae continue to form biofilms. A report highlighted differential expression of genes associated with hyphae formation in farnesol-treated biofilms, such as genes involved in cell wall maintenance,

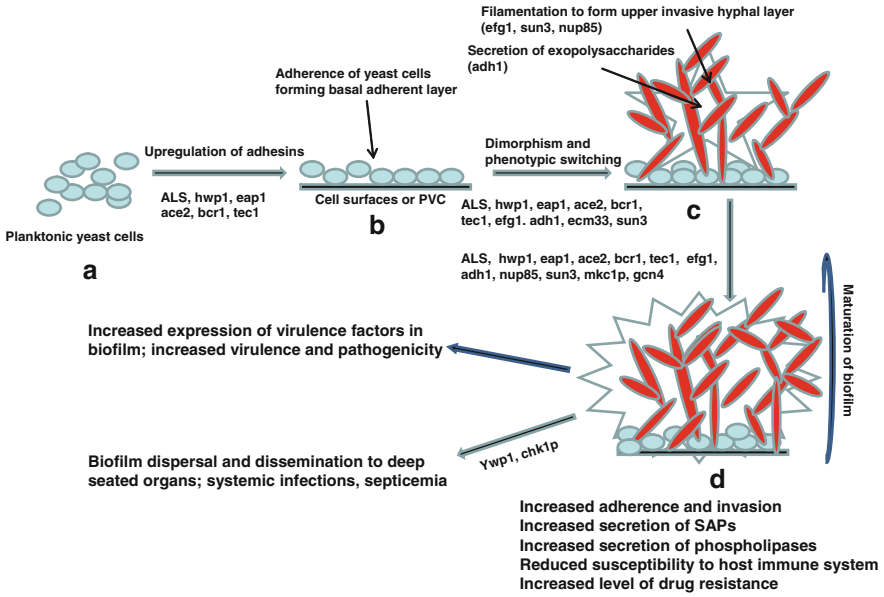

Fig. 2.2 Illustration of virulence factors involved in establishing biofilm and associated pathogenicity in *Candida albicans*: (**a**) planktonic yeast cells, (**b**) initiation of biofilm formation on living tissues or inert object like polyvinyl chloride (PVC) by adherence of yeast cells forming basal layer, (**c**) development of biofilm by initiation of upper invasive hyphal layer and production of exoploysaccharide, and (**d**) maturation of biofilm by more filamentation and exopolysaccharide production. Partially adapted from Blankenship and Mitchell (2006)

iron transport, stress response and upregulation of TUP1 and downregulation of CSH1 protein associated with cell surface hydrophobicity. Farnesol also prevents induction of Tup1-regulated filament specific genes hwp1, rbt1, cph1 and hst7 (Cao et al. 2005; Dominic et al. 2007). In contrast to farnesol, another quorum-sensing molecule, tyrosol, induces filamentation under conditions conducive to germ tube formation, but its role in biofilms has not been much investigated (Alem et al. 2006). However, a recent study highlighted a two-component signal transduction protein Chk1p regulating both quorum sensing and biofilm formation by negatively regulating hyphal development in *C. albicans*. However, it is not clear whether chk1p is directly involved in response to farnesol or not (Kruppa et al. 2004; Blankenship and Mitchell 2006). Involvement of different virulence factors in forming biofilms and associated pathogenicity is depicted in Fig. 2.2.

2.7 Conclusion

Based on the review of the literature on fungal infection, virulence, and pathogenicity, it is clear that at present the frequency of fungal infection rate is increasing, up to 90% for patients with disseminated candidiasis, aspergillosis or

cryptococcosis. Fungal pathogenesis is a multifactorial phenomenon; therefore, the nature of fungal pathogens, their virulence factors, and their interaction with host defense mechanisms need to be explored for the development of more effective antifungal therapy. Although phenomenal progress has been made on molecular characterization of various virulence factors and host–fungi interactions; this issue needs further investigation in order to know the exact contribution of each virulence factor under different disease conditions.

References

Albrecht A, Felk A, Pichova I, Naglik JR, Schaller M, De Groot P, MacCallum D, Odds FC, Schafer W, Klis F, Monod M, Hube B (2006) Glycosylphosphatidylinositol anchored proteases of *Candida albicans* target proteins necessary for both cellular process and host pathogen interactions. J Biol Chem 281:668–694

Alem MAS, Oteef MDY, Flowers TH, Douglas LJ (2006) Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. Eukaryotic Cell 5:1770–1779

Al-Fattani MA, Douglas LJ (2006) Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. J Med Microbiol 55:999–1008

Baille GS, Douglas LJ (1999) Role of dimorphism in the development of *Candida albicans* biofilm. J Med Microbiol 48:671–679

Berman J, Sudbery PE (2002) *Candida albicans*: a molecular revolution built on lessons from budding yeast. Nat Rev Genet 3:918–930

Blankenship JR, Mitchell AP (2006) How to build a biofilm: a fungal perspective. Curr Opin Microbiol 9:588–594

Braun BR, Johnson AD (1997) Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1. Science 277:105–109

Braun BR, Johnson AD (2000) TUP1, CPH1 and EFG1 make independent contributions to filamentation in *Candida albicans*. Genetics 157:57–67

Brock M (2009) Fungal metabolism in host niches. Curr Opin Microbiol 12:371–376

Brown AJ, Gow NA (1999) Regulatory networks controlling *Candida albicans* morphogenesis. Trends Microbiol 7:333–338

Cao YY, Cao YB, Xu Z, Ying K, Li Y, Xie Y, Zhu ZY, Chen WS, Jiang YY (2005) cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. Antimicrob Agents Chemother 49:584–589

Casadevall A (2007) Determinants of virulence in the pathogenic fungi. Fungal Biol Rev 21:130–132

Casadevall A, Pirofski LA (2001) Host-pathogen interactions: the attributes of virulence. J Infect Dis 184:337–344

Celera JA, Claderone R (2001) Signalling and the biology of human fungal pathogens. In: Claderone R, Cihlar R (eds) Fungal pathogenesis: principles and clinical applications. Marcel Dekker, New York, pp 115–137

Chaffin WL, Lopez-Ribot Jl, Casanova M, Gozalbo D, Martinez JP (1998) Cell wall and secreted proteins of *Candida albicans*: identification, function and expression. Microbiol Mol Biol Rev 62:130–180

Chakrabarti A (2005) Microbiology of systemic fungal infection. J Postgrad Med 51(Suppl1): S16–S20

Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormik T, Ghannoum MA (2001) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture and drug resistance. J Bacteriol 18:5385–5394

Chandra J, Patel JD, Li J, Zhou G, Mukherjee PK, McCormick TS et al (2005) Modification of surface properties of biomaterials influences the ability of *Candida albicans* to form biofilms. Appl Environ Microbiol 71:8795–8801

Claderone RA, Fonzi WA (2001) Virulence factors of *Candida albicans*. Trends Microbiol 9:327–336

Corner BE, Magee PT (1997) Candida pathogenesis: unraveling the threads of infection. Curr Biol 2:R691–R694

de Bernardis F, Muhlschlegel FA, Cassone A, Fonzi WA (1998) The pH of the host niche controls the gene expression in and virulence of *Candida albicans*. Infect Immun 66:3317–3325

de Bernardis F, Arancia S, Morelli L, Hube B, Sanglard D, Schafer W, Cassone A (1999) Evidence that members of aspartyl proteinase gene family, in particular SAP 2, are virulence factors for *Candida* vaginitis. J Infect Dis 179:201–208

Dominic RM, Shenoy S, Baliga S (2007) Candida biofilms in medical devices: evolving trends. Kathmandu Univ Med J 5:431–436

Dos Santos AL, de Carvalno IM, daSilva BA, Portela MB, Alviano CS, de Aroujo Soares RM (2006) Secretion of serine peptidase by a clinical strain of *Candida albicans*: influence of growth condition and cleavage of human serum proteins and extracellular matrix components. FEMS Immunol Med Microbiol 46:209–220

Douglas LJ (2003) Candida biofilms and their role in infection. Trends Microbiol 11:30–36

Eckert SE, Sheth CC, Muhlschlegel FA (2007) Regulation of morphogenesis in *Candia* species. In: d'Enfert CH, Hube B (eds) *Candida*. Comparative and functional genomics. Caister Academic, Norfolk, pp 263–291

Fidel PL Jr, Vanquez JA, Sobel JD (1999) *Candida glabrata*: a review of epidemiology, pathogenesis and clinical disease with comparison to *Candida albicans*. Clin Microbiol Rev 12:80–96

Filler SG, Sheppard DC, Edwards JE Jr (2006) Molecular basis of fungal adherence to endothelial and epithelial cells. In: Heitman J, Filler SG, Edwards JE Jr, Mitchell AP (eds) Molecular principles of fungal pathogenesis. ASM, Washington, DC, pp 187–196

Fluckiger U, Marchetti O, Bille J, Eggiman P, Zimmerli S, Imhof A, Garbino J, Ruef C, Pittet D, Tauber M, Glauser M, Calandra T (2006) Treatment options of invasive fungal infections in adults. Swiss Med Wkly 136:447–463

Ghannoum MA (2000) Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 13:122–143

Gow NAR, Brown AJP, Odds FC (2002) Fungal morphogenesis and host invasion. Curr Opin Microbiol 5:366–371

Granger BL, Flenniken ML, Davis DA, Mitchell AP, Cutler JE (2005) Yeast wall protein 1 of *Candida albicans*. Microbiol 151:1631–1644

Green CB, Cheng G, Chandra J, Mukherjee P, Ghannoum MA, Hoyer LL (2004) RT-PCR detection of Candida albicans ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. Microbiol 150:267–275

Haas H, Eisendle M, Turgeon BG (2008) Siderophores in fungal physiology and virulence. Ann Rev Phytopathol 46:149–187

Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2:95–108

Hawser SP, Douglas LJ (1994) Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. Infect Immun 62:915–921

Hayens KA, Westerneng TJ (1996) Rapid identification of *Candida albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei* by species specific PCR of large subunit ribosomal DNA. J Med Microbiol 44:390–396

Haynes K (2001) Virulence in *Candida* species. Trends Microbiol 9:591–596

Hogan DA (2006) Talking to themselves: autoregulation and quorum sensing in fungi. Eukaryotic Cell 5:613–619

Hogan LH, Klein BS, Levitz SM (1996) Virulence factors of medically important fungi. Clin Microbiol Rev 9:469–488

Hoyer LL (2001) The ALS gene family of *Candida albicans*. Trends Microbiol 9:176–180

Hoyer LL, Payne TL, Bell M, Myers AM, Scherer S (1998) *Candida albicans* AL S3 and insights into the nature of the ALS gene family. Curr Genet 33:451–459

Hube B (2004) From commensal to pathogen: stage and tissue specific gene expression of *Candida albicans*. Curr Opin Microbiol 7:336–341

Hube B, Naglik J (2001) *Candida albicans* proteinases resolving the mystery of a gene family. Microbiol 147:1997–2005

Hube B, Sanglard D, Odds FC, Hess D, Monod M, Schafer W, Brown AJ, Gow NA (1997) Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2 and SAP3 of *Candida albicans* attenuates virulence. Infect Immun 65:3529–3538

Hube B, Ruchel R, Monod M, Sanglard D, Odds FC (1998) Functional aspects of secreted *Candida* proteinases. Adv Exp Med Biol 436:339–344

Ibrahim AS, Mirbod F, Filler SG, Banno Y, Cole GT, Kitajima Y, Edwards JE Jr, Nozawa Y, Ghannoum MA (1995) Evidence implicating phospholipase as a virulence factor of *Candida albicans*. Infect Immun 63:1993–1998

Kelly MT, MacCallum DM, Clancy SD, Odds FC, Brown AJ, Butler G (2004) The *Candida albicans* CaACE 2 gene affects morphogenesis, adherence and virulence. Mol Microbiol 53:969–983

Khan ZK, Gyanchandani A (1998) Candidiasis: a review. PINSA 64:1–34

Klein BS, Tebbets B (2007) Dimorphism and virulence in fungi. Curr Opin Microbiol 10:314–319

Kruppa M, Krom BP, Chauhan N, Bambach AV, Cihlar RL, Calderone RA (2004) The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. Eukaryotic Cell 3:1062–1065

Kuleta JK, Kozik MR, Kozik A (2009) Fungi pathogenic to humans: molecular basis of virulence of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Acta Biochim Pol 56:211–224

Kvaal C, Lachke SA, Srikantha T, Daniels K, McCoy J, Soll DR (1999) Misexpression of the opaque phase specific gene PEP1 (SAP1) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. Infect Immun 67:6652–6662

Latge JP, Calderone R (2002) Host–microbe interactions: fungi Invasive human fungal opportunistic infections. Curr Opin Microbiol 5:355–358

Leberer E, Harcus D, Broadbent ID, Clark KL, Dignard D, Ziegelbauer K, Schmidt A, Gow NAR, Brown AJP, Thomas DY (1996) Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. Proc Natl Acad Sci USA 93:13217–13222

Lewis RE, Lo HJ, Raad II, Kontoyiannis DP (2002) Lack of catheter infection by the *efg1/efg1 cph1/cph1* double-null mutant, a *Candida albicans* strain that is defective in filamentous growth. Antimicrob Agents Chemother 46:1153–1155

Li F, Svarovsky MJ, Karlsson AJ, Wagner JP, Marchillo K, Oshel P, Andes D, Palecek SP (2007) Eap1p, a adhesin that mediates *Candida albicans* biofilm formation *in vitro* and *in vivo*. Eukaryotic Cell 6:931–939

Liu H (2001) Transcriptional control of dimorphism in Candida albicans. Curr Opin Microbiol 4:728–735

Lopez-Ribot Jl (2005) *Candida albicans* biofilms: more than filamentation. Curr Biol 15: R453–R455

Martin GS, Mnnino DM, Eaton S, Moss M (2003) The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med 348:546–1554

Martinez-Lopez R, Monteoliva L, Diez-Orejas R, Nombela C, Gil C (2004) The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*. Microbiol 150:3341–3354

Mendes-Giannini MJS, Da Silva JLM, Da Silva JF, Donofrio FC, Miranda ET, Andreotti PF, Soares CP (2008) Interactions of *Paracoccidioides brasiliensis* with host cells: recent advances. Mycopathologia 165:237–248

Miller MG, Johnson AD (2002) White opaque switching in *Candida albicans* is controlled by mating type locus homeodomain proteins and allows efficient mating. Cell 110:293–302

Molero G, Dies-Oreja R, Navarro-Garcia F, Monteoliva L, Pla J, Gill C, Sanchez-Perez M, Nambela C (1998) *Candida albicans*: genetics, dimorphism and pathogenicity. Int J Microbiol 1:95–106

Monod M, Zepelin MB (2002) Secreted proteinases and other virulence mechanisms of *Candida albicans*. Chem Immunol 81:114–128

Moore RD, Chaisson RE (1996) Natural history of opportunistic disease in an HIV infected urban clinical cohort. Ann Intern Med 124:633–642

Muhlschlegel FA, Fonzi WA (1997) PHR2 of *Candida albicans* encodes a functional homolog of the pH regulated gene PHR1 with an inverted pattern of pH dependent expression. Mol Cell Biol 17:5960–5967

Mulhern SM, Logue ME, Butler G (2006) *Candida albicans* transcription factor Ace2 regulates metabolism and is required for filamentation in hypoxic conditions. Eukaryotic Cell 5:2001–2013

Murphy JW (1991) Mechanisms of natural resistance to human pathogenic fungi. Annu Rev Microbiol 45:509–538

Naglik JR, Challacombe SJ, Hube B (2003) *Candia albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 67:400–428

Naglik JR, Albercht A, Bader O, Hube B (2004) *Candida albicans* proteinases and host pathogen interactions. Cellular Microbiol 6:915–926

Naglik JR, Moyes D, Makwana J, Kanzaria P, Tsichlaki E, Weindl G, Tappuni AR, Rodgers CA, Woodman AJ, Challacombe SJ, Schaller M, Hube B (2008) Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. Microbiol 154:3266–3280

Nobile CJ, Mitchell AP (2005) Regulation of cell surface genes and biofilm formation by the *Candida albicans* transcription factor Bcr1. Curr Biol 15:1150–1155

Nobile CJ, Andes DR, Nett JE, Smith FJ, Yue F, Phan QT, Edwards JE, Filler SG, Mitchell AP (2006a) Critical role of Bcr1- dependent adhesins in *Candida albicans* biofilms formation *in vitro* and *in vivo*. PLoS Pathog 2:636–649

Nobile CJ, Nett JE, Andes DR, Mitchell AP (2006b) Function of *Candida albicans* adhesin Hwp1 in biofilm formation. Eukaryotic Cell 5:1604–1610

Noffiz CS, Liedschulte V, Lengeler K, Ernst JF (2008) Functional mapping of the *Candida albicans* Efg1 regulator. Eukaryotic Cell 7:881–893

Norice CT, Smith FJ Jr, Solis N, Filler SG, Mitchell AP (2007) Requirement for *Candida albicans* Sun41 in biofilm formation and virulence. Eukaryot Cell 6:2046–2055

Odds FC (1988) Candida and Candidiasis: a review and bibliography. Bailliere Tindall, London, UK, p 67

Odds FC, Gow NAR, Brown AJP (2006) Toward a molecular understanding of *Candida albicans* virulence. In: Heitman J, Filler SG, Edwards JE Jr, Mitchell AP (eds) Molecular principles of fungal pathogenesis. ASM, Washington, DC, pp 305–319

Pfaller MA, Diekema DJ (2002) Role of sentinel surveillance of candidemia: trends in species distribution and antifungal susceptibility. J Clin Microbiol 40:3551–3557

Pommerville JC (2004) Alcamo's fundamentals of microbiology, 7th edn. Jones and Bartlett, Sudbury, MA

Ramage G, Saville SP, Wickes BL, Lopez-Ribot Jl (2002) Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum sensing molecule. Appl Environ Microbiol 68:5459–5463

Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL (2005) Candida biofilms: an update. Eukaryotic Cell 4:633–638

Ramage G, Ghannoum MA, Lopez-Ribot JL (2006) Fungal biofilms: agents of disease and drug resistance. In: Hetman J, Filler SG, Edwards JE Jr, Mitchell AP (eds) Molecular principles of fungal pathogenesis. ASM, Washington, DC, pp 177–185

Rappleye CA, Goldman WE (2006) Defining virulence genes in the dimorphic fungi. Annu Rev Microbiol 60:281–303

Reedy JL, Bastidas RJ, Heitman J (2007) The virulence of human pathogenic fungi: notes from the South of France. Cell Host Microbe 2:77–83

Ribaud P, Chastang C, Latge JP, BAffroy-Lafitte C, Parquet N, Devergie A, Esperou H, Selini F, Rocha V, Derouin F, Socie G, Gluckman E (1999) Outcome and prognostic factors of invasive aspergillosis after allogenic bone marrow transplantation. Clin Infect Dis 28:322–330

Richard ML, Nobile CJ, Bruno VM, Mitchell AP (2005) *Candida albicans* biofilm-defective mutants. Eukaryote Cell 4:1493–1502

Richardson MD (2005) Changing pattern and trends in systemic fungal infections. J Antimicrob Chemother 56:5–11

Ruiz-Herrera J, Elorza MV, Valentin E, Sentandreu R (2006) Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. FEMS Yeast Res 6:14–29

Rupp S (2007) Interactions of the fungal pathogen *Candida albicans* with the host. Future Microbiol 2:141–151

Saporito-Irwin SM, Birse CE, Sypherd PS, Fonzi WA (1995) PHR1, a pH regulated gene of *Candida albicans*, is required for morphogenesis. Mol Cell Biol 15:601–613

Schaller M, Hube B, Ollert MW, Schafer W, Borg-Von ZM, Thoma-Greber E, Korting HC (1999) In vivo expression and localization of *Candida albicans* secreted aspartyl proteinases during oral candidiasis in HIV infected patients. J Invest Dermatol 112:383–386

Senet JM (1998) Candida adherence pheneomenon, from commensalisms to pathogenicity. Int Microbiol 1:117–122

Seneviratne CJ, Jin L, Samaranayke LP (2007) Biofilm lifestyle of *Candida*: a mini review. Oral Dis 14:582–590

Soll DR (1992) High frequency switching in *Candida albicans*. Clin Microbiol Rev 5:183–203

Soll DR (1997) Gene regulation during high frequency switching in *Candida albicans*. Microbiol 143:279–288

Soll DR (2002a) Phenotypic switching. In: Claderone R (ed) Candida and candidiasis. ASM, Washington, DC, pp 123–142

Soll DR (2002b) Candida commensalism and virulence: the evolution of phenotypic plasticity. Acta Trop 81:101–110

Staab JF, Bradway SD, Fidel PL, Sundstrom P (1999) Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. Science 283:1535–1538

Staib P, Kretschmar M, Nichterlein T, Hof H, Morschhauser J (2002) Transcriptional regulators cph1p and Efg1p mediate activation of the *Candida albicans* virulence gene SAP5 during infection. Infect Immun 70:921–927

Sullivan D, Moran G, Coleman D (2005) Fungal diseases of humans. In: Kavanagh K (ed) Fungi: biology and applications. Wiley, Chichester, UK, pp 171–190

Theiss S, Ishdorj G, Brenot A, Kretschmar M, Lan CY, Nichterlein T, Hacker J, Nigam S, Agabian N, Kohler GA (2006) Inactivation of the phospholipase B gene PLB5 in wild type *Candida albicans* reduces cell associated phospholiupase A2 activity and attenuates virulence. Int J Med Microbiol 296:405–420

Tomee JFCH, Kauffman HF (2000) Putative virulence factors of *Aspergillus fumigatus*. Clin Exp Allergy 30:476–484

Wanner A, Salathe M, O'Riordan TG (1996) Mucociliary clearance in the airways. Am J Respir Crit Care Med 154:1868–1902

Watamoto T, Samaranayake LP, Jayatilake JAMS, Egusa H, Yatani H, Seneviratne CJ (2009) Effect of filamentation and mode of growth on antifungal susceptibility of *Candida albicans*. Int J Antimicrob Agents 34:333–339

Weitzman I, Summerbell RC (1995) The dermatophytes. Clin Microbiol Rev 8:240–259

Willey JM, Sherwood LM, Woolverton CJ (2008) Prescott, Harley and Klein's microbiology, 7th edn. McGraw Hill, Singapore

Yang YL (2003) Virulence factors of *Candida* species. J Microbiol Immunol Infect 36:223–228

Zhao X, Daniels KJ, Oh SH, Green CB, Veater KM, Soll DR, Hoyer LL (2006) *Candida albicans* Als3p is required for wild type biofilm formation on silicone elastomer surface. Microbiology 152:2287–2299

Chapter 3
Animal as Reservoir of Fungal Diseases (Zoonoses?)

Jose L. Blanco and Marta E. Garcia

Abstract Considering the term zoonoses in a wide sense, it should include not only the traditional concept of diseases transmitted from vertebrate animals to humans, but also the concept of diseases that are common to both animals and humans. In the first case, animals are essential to the transmission of the disease to humans, while in the second animals are essential as reservoirs for the pathogen in nature, developing the contagion through mixed sources: soil, water, invertebrates, etc. Many fungal diseases could be included within this concept, and, due to the generalized use of molecular methodologies, its study has advanced during the last years. This new approach has led to the description of new fungal species, most of them almost impossible to differentiate by following the traditional criteria of fungal classification, which rely on taxonomical keys based on phenotypic characteristics. Moreover, complete epidemiological studies can be now performed, and used to establish the similarities and/or differences between fungal isolates. In the present chapter, we review the habitats of the main fungal genera and the possibilities of considering the diseases caused by these organisms as zoonoses.

3.1 Mycoses as Zoonoses

From a traditional point of view, and according to the "Dictionary of Veterinary Epidemiology," zoonoses are considered as diseases or infections that can be transmitted naturally between humans and other vertebrate animals (Toma et al. 1999).

Following this criterion, diseases can be classified according to the source of infection as anthroponoses (when the source is an infectious human; interhuman

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transfer is typical), zoonoses (the source is an infectious animal; interhuman transfer is uncommon), and sapronoses (the source is an abiotic substrate; interhuman transfer is exceptional). The source of infection is often the reservoir or, in ecological terms, the habitat where the etiologic agent of the disease normally thrives, grows, and replicates. A characteristic feature of most zoonoses and sapronoses is that once transmitted to humans, the epidemiological chain is usually aborted, but the clinical course might be sometimes quite severe, even fatal (Hubalek 2003).

From this classification were derived a high number of related terms, which, depending on the case, have been applied with major or minor success. For instance, diseases transmissible from living animals to humans were often referred as anthropozoonoses, and the diseases transmissible from humans to animals were called zooanthroponoses. Unfortunately, many scientists used these terms in the reverse sense or indiscriminately. In view of this, an expert committee decided to abandon these two terms and recommended "zoonoses" as "diseases and infections which are naturally transmitted between vertebrate animals and man" (Hubalek 2003). Finally, sapronoses are human diseases transmissible from an abiotic environment (soil, water, decaying plants, or animal corpses, excreta, and other substrata). The ability of the agent to grow saprophytically and replicate in these substrata (i.e., not only to survive or contaminate them secondarily) are the most important characteristics of a sapronotic microbe. For these diseases the term "sapro-zoonoses" can be applied, defined as "having both a vertebrate host and a non-animal developmental site or reservoir (organic matter, soil and plants)" (Hubalek 2003).

Following this classification, candidiasis, ringworm (*Trichophyton rubrum*), and *Pneumocystis* pneumonia would be considered as anthroponoses. Like zoonoses transmitted by direct contact, alimentary (foodborne and waterborne), or aerogenic (airborne) routes, the only fungal zoonotic disease would be ringworm. The rest of the mycoses should be considered as sapronoses. Here could be cited the following processes: dermatophytosis (*Microsporum gypseum*), histoplasmosis (*Histoplasma capsulatum; H. duboisii*), blastomycosis (*Blastomyces dermatitidis*), emmonsiosis (*Emmonsia crescens; E. parva*), paracoccidioidomycosis (*Paracoccidioides brasiliensis*), coccidioidomycosis (*Coccidioides immitis*), sporotrichosis (*Sporothrix schenckii*), cryptococcosis (*Cryptococcus neoformans*), aspergillosis (*Aspergillus fumigatus*, and other species from the genus *Aspergillus*), mucormycosis (*Absidia corymbifera* and some other *Mucorales*), entomophthoromycosis (*Basidiobolus; Conidiobolus;* and *Entomophthora* spp.), maduromycetom (*Madurella mycetomatis; M. grisea; Pseudoallescheria boydii; Leptosphaeria senegalensis; Neotestudina rosatii*), chromoblastomycosis (*Phialophora verrucosa; Exophiala jeanselmei; Fonsecaea compacta; F. pedrosoi; Cladosporium carionii; Rhinocladiella aquaspersa*), phaeohyphomycosis (*Wangiella dermatitidis; Dactylaria gallopava; Exophiala spinifera*), and fusariosis (*Fusarium oxysporum; F. solani*) (Hubalek 2003).

Nevertheless, there are many authors, including us, who consider the term zoonoses in a wide sense that includes not only the traditional concept of diseases

transmitted from vertebrate animals to humans, but also the concept of diseases that are common to animals and humans. In the first case, animals are essential to the transmission of the disease to humans, while in the second animals are essential as reservoirs for the pathogen in nature, developing the contagion through mixed sources: soil, water, invertebrates, etc. It is evident that in both cases the role of the animals is essential in the maintenance, distribution and transmission of the infection.

On the other hand, it is important that when we talk about zoonoses, we should not be restricted to domestic animals, but also emphasise the high importance of the wild animals in the spread of this kind of diseases, including of course the fungal diseases. Wildlife is the reservoir of numerous infections that affect domestic animals and/or man, and an important potential source of new and emerging zoonotic diseases. Thus, the maintenance of biodiversity will always be associated with the maintenance of reservoirs for potential zoonoses (Pastoret et al. 2000). In this sense, human pathogenic fungi have been isolated from pigeon droppings, the cloacae of migratory birds, and birds of prey, suggesting that these animals could be potential carriers and spreaders of fungal infection (Cafarchia et al. 2008). Because of birds resting during migration in and around towns (e.g. in small artificial lakes in urban parks), migratory birds as well as Passeriformes and birds of prey are of importance for human health. The resting sites of these birds are commonly frequented by children, the elderly and immunocompromised people who are at increased risk of contracting opportunistic diseases (Cafarchia et al. 2008).

Diseases transmitted between human, wild and domestic animal species have an important impact on public health, livestock economy and wildlife conservation. According to Cleaveland et al. (2001), nearly two-thirds (61%) of human diseases are actually zoonotic, that is they can also infect animals. These authors also report a total of 1,922 species of infectious agents, of which 499 (26%) are fungi.

3.2 Importance of Molecular Tools in the Study of Fungal Infections

Until the 1980s, systemic mycoses in humans were reduced to sporadic episodes with a low incidence, or most probably, with a low level of diagnosis. With the arrival of the human immunodeficiency virus (HIV) infection, and the generalized use of immunosuppressive therapies, fungi began to acquire an increasing importance. With this fact, the study and the knowledge about fungal diseases were clearly increased. In recent years, the generalization of molecular studies has led to a significant advance in clinical mycology, mainly in two aspects:

a. Description of new fungal species. With the traditional criteria of fungal classification, following the taxonomical keys based on phenotypic characteristics, it was really difficult to differentiate in a clear way the different species within a

genus. *Pneumocystis* or *Cryptococcus* can be cited as two clear examples of genera of complex taxonomy, as we will describe below.

b. Epidemiological studies. Without the aid of molecular tools, it was practically impossible to find the origin of a specific fungal disease in a human or animal. Nowadays, different molecular methodologies are available that can be used to perform a complete genotypic study of the different fungal strains isolated, and establish the similarities or differences between those isolates. An illustrative example is the complete study performed about the cryptococcosis outbreak in Vancouver that we will discuss below.

3.3 Mycoses

Mycoses, conditions in which fungi pass the resistance barriers of animals and establish themselves in the form of infections, are a group of diseases with very varied clinical manifestations. For example, in the case of aspergillosis, with effects on very diverse organs, there is a variety of responses, such as local (aspergilloma), systemic (renal, lung, nervous central system, etc.) or even allergic (allergic bronchopulmonary aspergillosis in humans). For this reason, diagnosis of these diseases can be problematic, mostly because of the difficulty of interpreting the very different clinical pictures in individuals in the presence of colonization, infection and/or disease (Garcia and Blanco 2000). If the lesions can occur in very different sites, they can be accompanied by very varied symptoms.

In this era of widespread use of broad-spectrum antibiotics, corticosteroids, cytotoxic agents and other immunosuppressants, coupled with the increasingly stressful life styles demanded by modern civilization, it is hard to draw a sharp line between pathogenic and non-pathogenic fungi (Mishra et al. 1992).

In the same way, a high number of fungal diseases have been described in the last years in veterinary medicine. These diseases are related to many different factors, such as the ingestion of mould-infected feedstuff, the massive inhalation of conidia produced in that kind of feedstuff, the preventive or curative extended antibiotic treatments, the intensive method of breeding, changes in habitat or nutrition, and vaccination and anti-parasitic treatments. All of these factors could favor in some way the appearance of stress in the animal, and, in consequence, an immunosuppressive condition that contributes to the development of a fungal disease.

Despite the important advances achieved during recent years, there are still a lot of things to learn about mycoses. For instance, the exact role of animals in human mycoses is unknown. Furthermore, with certain exceptions, it is unknown which animal species can be vectors, reservoirs, intermediate hosts or carriers of fungi. On the other hand, at present there are only a reduced number of fungal diseases for which the direct transmission animal–human has been clearly established. Finally, the environmental habitats of many pathogenic fungi remain to be discovered.

3.4 Dermatophytosis

Dermatophytosis (ringworm) is an infection of the keratinized superficial tissues of man and animals (the corneum stratum, hair and skin) that is caused by fungal species belonging to the genera *Microsporum, Trichophyton* and *Epidermophyton* (Sparkes et al. 1993).

Dermatophytes are not part of the normal flora of the skin. Due to their keratinophilic and keratinolytic nature, they are able to use cutaneous keratin as a nutrient, in this way producing the infection (Garcia and Blanco 2000).

Mating strain experiments and recent works in molecular taxonomy have shown that the *T. mentagrophytes* species belongs to a complex which has teleomorph states (i.e., these species may develop sexual fructifications) (Fréalle et al. 2007; Gräser et al. 2006). Thus, *Arthroderma benhamiae* is the teleomorph obtained by mating strains of the *T. mentagrophytes* complex isolated from rodents, and *Arthroderma vanbreuseghemii* is the teleomorph obtained in crosses of isolates from humans, mice and chinchillas (Drouot et al. 2009).

Three broad ecological groups of dermatophyte species are recognized, namely anthropophilic, zoophilic and geophilic. In general, zoophilic and geophilic species cause lesions in humans that are more inflammatory than those induced by anthropophilic species (Weitzman and Summerbell 1995).

In humans, dermatophytosis is usually a highly inflammatory disease involving scalp, beard or exposed areas of the body such as nails and skin (Papini et al. 2009). *T. verrucosum* is still the most common cause of Tinea barbae in man, and remains a significant health problem (Papini et al. 2009). *A. vanbreuseghemii, M. canis* and *A. benhamiae* can all cause highly inflammatory tinea corporis, tinea faciae and tinea capitis in humans. In cases of such human mycosis, it is important to identify with certainty the precise aetiological agent, and to examine pets as the possible source of infection, as has been demonstrated in different studies (Drouot et al. 2009).

Clinically, it may present as a well-circumscribed erythematous plaque studded with vesicles, papules, and pustules. Other presentations include scaly erythematous patches with well-circumscribed edges having a typical “ringworm” appearance but limited central clearing, a boggy inflammatory form covered with crusts and draining purulent material, and lastly, a nodular presentation due to chronicity. It is important to note that these lesions may become secondarily infected with bacteria. Spontaneous resolution may occur, further confounding the clinical diagnosis (Silver et al. 2008).

In animals, dermatophytosis is important in the dog, not because of the severity of the process, which is never life-threatening, but fundamentally for its zoonotic character. On the contrary, dermatophytosis is much more frequent in domestic cats, which are the main source of infection for humans. This mycosis is also important in cattle, since there are a great number of these animals affected; in fact, in North and Central Europe dermatophytosis is considered one of the most important zoonoses, and fully effective vaccines, at least in principle, are of common use in many countries (Garcia and Blanco 2000).

Microsporum canis followed by *Trichophyton mentagrophytes* are the dermatophytes most frequently isolated from dogs and cats (Cabañes et al. 1997), whereas *Trichophyton verrucosum* and *T. mentagrophytes* are the most important species isolated from cattle and horses (Garcia and Blanco 2000). This fact has a clear consequence in the zoonotic transmission of these processes: whereas in urban areas human ringworm is mainly produced by *M. canis* or *T. mentagrophytes*, the zoonotic transmission of *T. verrucosum* is more often found in rural environments, where it can be considered as an occupational disease for farm workers that results from direct contact with cattle or infected fomites (Papini et al. 2009).

Pets infected with species of the *T. mentagrophytes* complex can also be a source of inflammatory dermatophytoses in people (Drouot et al. 2009). Ninety-three percent of cats with dermatophytoses caused by *A. vanbreuseghemii* were strictly outdoors and presumably hunters. In contrast, the vast majority of cats from which *M. canis* was isolated were strictly indoor cats. Therefore, it can be suspected that the feline infections with *A. vanbreuseghemii* occurred from a soil and/or a rodent prey source during hunting (Drouot et al. 2009).

The occurrence of infection in humans without a history of contact with cattle has also been observed (Mantovani and Morganti 1977), including a case of deep and invasive dermatophytosis with lymph-node involvement in an immunocompetent patient (Tejasvi et al. 2005).

M. canis is the dermatophyte most frequently recovered from canine and feline ringworm cases. The household environment can be contaminated both by symptomatic animals and through asymptomatic *M. canis* carriage, resulting in a potential human health risk. In a complete study, the load of *M. canis* arthrospores was determined in households harboring infected pets, in order to evaluate the infectivity of the animals versus the environment. The environment inhabited by symptomatic cats and dogs infected by *M. canis* was examined by sampling both surfaces and indoor air. Environmental contamination was detected in all households with cats, while only four out of nine houses harboring dogs were found positive. Heavily infected environments only harbored kittens. Infected owners were observed in eight households, where at least one infected cat was present. No history of human dermatophytosis in households harboring dogs was found. In conclusion, infected cats appear to cause substantial environmental contamination, and provoke a substantial presence of viable airborne fungal elements. Dogs seem to be of lower importance in the spread of *M. canis*: although they contaminated surfaces, they never contaminated the air. The results of this study confirm the potential leading role of the feline species in the environmental spread of *M. canis*. The arthrospores are very resistant, and can remain infectious in the environment for 12–24 months; therefore, the potential for human exposure from the environment is high (Mancianti et al. 2003).

Direct contact with infected hairs and scales or fungal arthrospores and hyphae on fomites and contact with a contaminated environment are the modes of transmission of the disease. The minimum infective dose is unknown, and the natural defences of the host and the invasiveness of the dermatophyte are likely to play a role. Furthermore, combing, ectoparasites, pruritus and disorders of keratinisation

may disrupt the integrity of the skin barrier, predisposing the host to the infection. Dermatophytes are thus both contagious diseases among animals and important zoonoses, especially with the increasing number of dogs and cats being kept as pets. Even the presence of a very low number of spores in the environment has practical relevance, as a minimum number of spores (not yet defined) along with other factors is required to cause infection and establish clinical disease (Mancianti et al. 2003).

Finally, it has to be emphasized that severe complications have been described. In this sense, an outbreak of nosocomial ringworm can be cited that affected five infants in a neonatal intensive care unit. The index case was a nurse infected with *M. canis* by her cat (Drusin et al. 2000).

3.5 Aspergillosis

Of all the fungal diseases, aspergillosis has been the subject of the most intensive study. It has been described in many species of mammals and birds, where cellular characteristics have been described that might predispose to respiratory aspergillosis (Tell 2005).

Human invasive aspergillosis is a disease that mainly affects highly immunocompromised subjects. It is a rapidly progressive, often fatal, disease characterized by tissue destruction associated with abundant hyphae in necrotic tissue (Bozza et al. 2002). Host defects that result in predisposition to invasive aspergillosis include neutropenia, defective neutrophil function (such as that seen in patients with chronic granulomatous disease), receipt of corticosteroid therapy, immunosuppresive agents for solid organ transplant and late-stage HIV infection (Feldmesser 2005).

Various authors have tried to relate these processes to some form of immunosuppression in animals, as is the case with humans, but up to now it has not been possible to establish this relationship with neutrophils, T lymphocytes or macrophage function, levels of complement compounds, or skin test reactivity (Day et al. 1985; Day and Penhale 1998; Garcia et al. 2001).

In humans, lungs are the most common location of *Aspergillus* infections. On the contrary, in domestic animals the situation is different and varies between species. For example, in cows, the forestomachs, especially the omasum, are mostly affected, from which the infection may spread to other organs, including the placenta (Garcia et al. 2001). In the dog, however, there is a symptomatology that is not usually accompanied by pulmonary affectation, unlike the pulmonary clinical picture seen in humans. Therefore, they differ from the conventional disease pattern that appears in human medicine (Clercx et al. 1996; Guerin et al. 1993).

With the increased knowledge about canine aspergillosis, it is clear that the clinical picture in the literature does not apply to all cases or species; for example, lesions can occur in very different sites, and can be accompanied by very varied symptoms. Until the 1980s, it seemed clear that the fungus involved in these processes was *Aspergillus terreus* (Berry and Leisewitz 1996; Day and Penhale

1991). However, an increasing number of studies were reported in which a different fungus was identified as the cause of the disease, including *Aspergillus deflectus, Aspergillus flavus,* and *Aspergillus flavipes*, as well as members of other genera, such as *Acremonium, Penicillium, Paecilomyces*, etc. (Gene et al. 2003; Jang et al. 1986; Watt et al. 1995; Zanatta et al. 2006). For this reason, some years ago we proposed that these cases should not be called aspergillosis but should be described as systemic mycosis or disseminated mycosis, at least until the causal agent is identified (Garcia and Blanco 2000).

The entrance pathway of fungi into the host dog is still not clear, although it has been suggested as being possibly through old wounds, medium or inner chronic otitis, or a urinary pathway (Garcia and Blanco 2000; Littman and Goldschmidt 1987; Mullanery et al. 1983; Starkey and McLoughlin 1996). A digestive pathway of entrance has also been proposed, since on some occasions lesions have been observed as mesenteric lymphadenitis with fungal hyphae (Pastor et al. 1993). Finally, the transuterine transmission of *A. terreus* was recently described (Elad et al. 2008). Once the fungus has gained entry, it can spread to different locations, mainly the rachis, where the disease can develop slowly, with dissemination to the brain, liver, spleen and abdominal lymph nodes. A clinical disease would be detected only after several years.

Theories about the primary spread of the fungal agent in the host suggest that it is intimately related to the ability of members of the genus *Aspergillus* to synthesize substances, such as elastase, that are able to invade non-necrotic tissues and penetrate into the bloodstream (Blanco et al. 2002; Markaryan et al. 1994). This capacity is associated with the production of certain types of lateral spores, named aleuriospores, which are usually observed when the fungus grows *in vivo* (Butterworth et al. 1995; Dallman et al. 1992; Gelatt et al. 1991; Kauffman et al. 1994; Perez et al. 1996; Wilson and Odeon 1992). Once the fungus has penetrated into the bloodstream, it spreads to the organs. Therefore, because the entrance is not through mucous membranes, but directly into the bloodstream, the first line of defence that is so effective in immunocompetent individuals with lung infection is eluded, and it raises the possibility that the disease occurs in immunocompetent individuals.

Clinical pictures of disseminated aspergillosis similar to those described in the dog have been reported in other animal species. For instance, in bovine mycotic abortion and in ovine *Aspergillus* mastitis the process is similar to that described in the dog: the rupture of the defensive barrier of skin and mucous membrane allows the fungus to enter directly into the bloodstream, where the immune system is unable to stop its development (Garcia et al. 2004; Jensen et al. 1989; Las Heras et al. 2000).

Aspergillus spp. have been recognized since the early 1800s as an important cause of pulmonary disease in birds. In contrast to mammals, birds are highly susceptible to invasive aspergillosis (Feldmesser 2005; Garcia et al. 2007).

Mould-infected feedstuff can have a dual role in the process; by dissemination of the fungus, and by containing small quantities of mycotoxins that would lead to immunodepression and, consequently, favor the appearance of the disease.

To date, a single case of person-to-person transmission of *A. fumigatus* has been described, with the exception of direct donor-to-recipient transmission, by aerosolization of conidiophores associated with surgical dressing changes and wound debridement (Pegues et al. 2002). If person-to-person transmission is possible, animal-to-person transmission might also be possible. Meanwhile, isolation of the same genotype from different patients is thought to result from exposure to the same fungal population in the environment (Alvarez-Perez et al. 2009a,b; Chazalet et al. 1998).

We are sure that in the next few years the application of molecular methodologies will increase our knowledge about episodes currently considered as sporadic, but that probably are more common than we think.

3.6 Candidiasis

Candida albicans is part of the normal microbial flora in human beings and domestic animals, and is associated with the mucous surfaces of the oral cavity, gastrointestinal tract and vagina. Immune dysfunction can allow *C. albicans* to switch from a commensal to a pathogenic organism capable of infecting a variety of tissues and causing a possibly fatal systemic disease (Romani 1999; Stevens et al. 1998; Traynor and Huffnagle 2001).

Although fungi require a host with predisposing factors to produce the disease, it is known that saprophytic colonization of the mucous membrane by *C. albicans* does not need the host to be immunocompromised, since it is detected in immunocompetent individuals (Garcia and Blanco 2000; Hostetter 1994).

In cattle, as a consequence of the abundant use, and occasional abuse, of antibiotics in the treatment of mastitis, there is a selection of flora, mainly members of the genus *Candida*, that are new etiological agents of these processes, which are initially difficult to diagnose because their presence is not expected. Candidiasis in birds is related to malnutrition and stress, generally produced by the same strains that are found naturally on the food plants of these animals. Arthritis caused by yeasts in horses is relatively frequent as a consequence of contamination of wounds or after surgical treatment. In pigs, candidiasis usually takes the form of digestive alterations in young animals, and is usually related to problems that predispose to the disease, like treatment with antibiotics (Garcia and Blanco 2000). *C. albicans* is a common causative agent of stomatitis in the dog (Jadhav and Pal 2006).

Person-to-person transmission is usually assumed in human candidiasis. In the same way, if the animals are considered as carriers of the yeast, its transmission to humans would also be possible, constituting a true zoonosis.

Finally, we have to emphasize that traditionally (especially in human medicine) *C. albicans* has been considered the sole pathogenic species in its genus. Nevertheless, in recent years different species of *Candida* have been implicated in different animal pathologies: *C. humicola, C. colliculosa, C. inconspicua, C. tropicalis,* etc. . . (Blanco et al. 1996).

3.7 Cryptococcoses

Cryptococcoses are mycoses caused by fungi of the genus *Cryptococcus*. During recent years, the study of this genus has been facilitated by the incorporation of molecular tools in clinical mycology. For instance, molecular characterization of *Cryptococcus* isolates has led to the differentiation of two species within the *C. neoformans* complex: *C. neoformans* and *C. gattii*. This differentiation was made on the basis of genetic variation and a lack of evidence for genetic recombination between both species (Duncan et al. 2006a, b). These genetic differences are also consistent with differences in habitat, geographical distribution and, most importantly, pathogenicity and the effectiveness of the response of the host immune system. *C. neoformans* infects predominantly immunocompromised hosts, while *C. gattii* has not been associated with a suppressed immune system (Duncan et al. 2006a, b). This can explain many historical contradictions about the pathogenicity of this fungus, and especially the need for a state of immunosuppression in the host for the disease to succeed (Blanco and Garcia 2008).

C. neoformans is a widespread fungus found in environmental niches such as soil and avian excreta, whereas *C. gattii* is mainly found on eucalyptus trees and in the soil (Duncan et al. 2006a; Traynor and Huffnagle 2001).

The primary route of entry for *Cryptococcus* is via the lungs, where the fungus may establish a primary infection. If the initial pulmonary infection is not controlled, the fungus can disseminate to other organs and the central nervous system, resulting in fatal cryptococcal meningoencephalitis (Aguirre et al. 2004; Chen et al. 2007; Traynor and Huffnagle 2001).

Clinical cryptococcosis has been reported worldwide in many animal species. It is the most common systemic fungal infection in cats, and is often described in dogs (Duncan et al. 2006b). In cats, the disease takes the form of rhinitis when the process is primary, and is systemic, with alterations mainly of the central nervous system and with important affectation of lymph nodes, when it is secondary to the infection by feline immunodeficiency virus (FIV). In the majority of these studies, the causative agent was assumed to be *C. neoformans* and not *C. gattii* (Duncan et al. 2006a).

In other animal species, cryptococcosis appears to be related to immunodepression, although not always. For instance, outbreaks of ovine and caprine cryptococcosis with respiratory signs in apparently non-immunodepressed animals have been described (Garcia and Blanco 2000). In these cases, respiratory symptoms associated with cachexia were the predominant clinical picture; liver and brain involvement has also been documented (Baro et al. 1998).

Cryptococcosis may occur in horses as a disseminated infection with osteomyelitis of both the axial and appendicular skeleton produced by *C. gattii*. The administration of corticoids has led to clinical deterioration due to immunomodulating effects (Lenard et al. 2007).

The study developed about the human cryptococcosis outbreak in Vancouver island (British Columbia, Canada) is very interesting. There, asymptomatic cats and dogs infected by *C. gattii* or with nasal colonization spread the yeast, and then, they

could be possible transmitters of the disease to humans. The objectives of the study were to ascertain the environmental source of the outbreak infections, survey the molecular types of the outbreak and environmental cryptococcal isolates, and determine the extent of genetic diversity among the isolates. PCR-fingerprinting and amplified fragment length polymorphism (AFLP) were used to examine the genotypes. All outbreak and environmental isolates belonged to *C. gattii*. The vast majority of clinical and veterinary infections were caused by isolates of the molecular type VGII/AFLP6, but two were caused by molecular type VGI/AFLP4. All environmental isolates belonged to molecular type VGII/AFLP6. Two or three subtypes were observed within VGII/AFLP6 among outbreak and environmental isolates (Kidd et al. 2004).

From these studies, it was hypothesized that the yeast had lived for a long time on the island, but that an unknown factor may have triggered its multiplication and subsequent spread. A possible cause might have been climatic change, in view of the temperature increasing since 1998. Warm weather and the specific microclimate on the island would favor the human and animal colonization by the yeast (Kidd et al. 2004; Duncan et al. 2005).

Zoonotic transmission of *C. neoformans* from bird's excreta to immunocompromised or immunocompetent humans has been demonstrated in a few cases, or has been seriously considered in others (Bovers et al. 2008). In this sense, pigeon and bat droppings are the most important reservoir of *C. neoformans* in nature. This fungus is not easily isolated from recent droppings, but can be found in the dry guano that accumulates in pigeon lofts, buildings, deserted houses, etc. (Rosario et al. 2008), Therefore, it is important to remove the accumulation of droppings, because a high level of environmental contamination by *C. neoformans* may pose a serious public health hazard. People working in the care, breeding, and clearing of pigeons and pigeon lofts, and those who have to remove soil contaminated with pigeon droppings are at particularly high risk (Rosario et al. 2008).

Molecular analyses have shown that one strain responsible of human cryptococcosis is nearly related (or even identical) to a strain isolated from droppings or the digestive tube of pet birds (Rosario et al. 2008).

3.8 *Malassezia pachydermatis*

The lipophilic yeast *M. pachydermatis* is part of the normal cutaneous microflora of most warm-blooded vertebrates. The opportunistic nature of this yeast has been demonstrated, and confirmed by the excellent response to specific antifungal therapy (Akerstedt and Vollset 1996; Ashbee 2006; Ashbee 2007; Guillot and Bond 1999).

Yeasts of the genus *Malassezia* can cause life-threatening fungemia and other nosocomial infections in immunocompromised humans, especially in preterm neonates. While disease in humans is most commonly caused by *M. furfur* and other *Malassezia* species, a commensal of human skin, it has also resulted from *M. pachydermatis*, for which dogs are a natural host (Morris et al. 2005).

M. pachydermatis is the only species in the genus that does not require an exogenous source of lipid for growth (Ashbee 2006; Blanco et al. 2000), but its growth is enhanced by the addition of lipid substrates to culture media (Gueho et al. 1996). Other *Malassezia* species are lipid-dependent, and appear to be more anthropophilic. In animals, *Malassezia* yeasts can be isolated from various mucosal sites (mouth, rectum, anus, anal sacs or vagina) but deep infections have not been reported. On the contrary, systemic *Malassezia* diseases are regularly diagnosed in immunocompromised humans (Pier et al. 2000). Thus, animals could sometimes be suspected as a source of *Malassezia* yeasts for humans (Pier et al. 2000).

M. furfur may be isolated from the skin and feathers of birds, *M. slooffiae* from pigs and herbivores, *M. globosa* from cats and cattle, and *M. sympodialis* from cats (Pier et al. 2000).

In dogs, this yeast acts as an opportunistic secondary pathogen within the ear canal. Otitis externa associated with *M. pachydermatis* is often characterized by a waxy, moist, brown or yellow exudate with variable erythema and pruritus (Griffin 1993; Guillot and Bond 1999).

In cats, dermatitis associated with *Malassezia* spp. is frequently reported in those with endocrine and metabolic diseases, neoplasia, and infection with feline leukemia virus (FLV) and FIV. Some reports suggest that *Malassezia* spp. overgrowth may be related to feline allergic skin diseases (Ahman et al. 2007; Ordeix et al. 2007).

M. pachydermatis has been documented to cause fungemia in human patients receiving nutritional infusions by catheter (Chang et al. 1998; vanBelkum et al. 1994). In this sense, it has been hypothesized that mechanical transfer of *M. pachydermatis* from the inflamed skin of dogs with *M. pachydermatis* infection to the healthy skin of humans occurs commonly. We also hypothesized that atopic dermatitis of dogs, which is a widely documented risk factor for *M. pachydermatis* infection, would be a risk factor for human carriage (Morris et al. 2005).

Chang et al. (1998) suggested that pet dogs owned by nursing staff who worked in the neonatal intensive care unit were the source of an outbreak in an intensive care nursery. A single strain of *M. pachydermatis*, as determined by pulsed-field gel electrophoresis, was isolated from infants, the hands of a nurse, and from three dogs owned by other healthcare workers in the unit. *M. pachydermatis* was introduced into the intensive care nursery on health care workers' hands after being colonized from pet dogs at home. The organism persisted in the nursery through patient-to-patient transmission. This observation suggested that *M. pachydermatis* could represent an emerging zoonotic pathogen (Chang et al. 1998).

3.9 *Pneumocystis*

Pneumocystis is a genus that has had an interesting tenure on the scientific stage. The first documentation of the existence of the organism known as *Pneumocystis* was as a part of the trypanosome life-cycle. Several years later, *Pneumocystis* was identified as an organism separate from trypanosomes. This includes a life-cycle

similar to that of protozoans, based on the identification of a small trophic form; the larger cyst form may include five to eight progeny within the cyst. However, the disease caused by *Pneumocystis* was not thoroughly reported until World War II, where it was observed to be associated with pneumonia in malnourished children. Thereafter, *Pneumocystis* infections became increasingly evident in the immunocompromised patient population but it was the AIDS epidemic that brought *Pneumocystis* to the forefront of lethal, opportunistic fungal infections. During this time, another interesting observation roused the *Pneumocystis* field with the report that *Pneumocystis* was more closely related to fungi than to protozoan. Actually, *Pneumocystis* is clearly within the fungal kingdom, falling between ascomycetes and basidiomycetes (Edman et al. 1988; Steele et al. 2005). Although *P. carinii* had been isolated from many animal species, including man, traditionally a great specificity of the isolates has been demonstrated for the species that could infect. Then, *P. carinii* was considered as a group of heterogeneous populations, genetically isolated from each other, that have undergone a prolonged process of genetic and functional adaptation to each mammalian species (Dei-Cas 2000). Further genetic analysis has shown that *Pneumocystis* isolates from different species have significant differences in their gene sequence and chromosomes. This has prompted nomenclature changes, and *P. carinii* is now reserved for the rat pathogen, *P. carinii* sp. *muris* for the murine pathogen, and *P. jiroveci* for the human pathogen (Steele et al. 2005; Stringer et al. 2002). It may be that this situation will be repeated in different animal species, like the description of the genetically different *P. canis* in dogs (English et al. 2001). But this situation does not mean that at a specific moment this fungus could infect a different specie of its natural host. *Pneumocystis* infects hosts by a respiratory route, and animal-to-animal airborne transmission has been clearly established (Dumoulin et al. 2000). This suggests the possibility of it being a zoonosis.

Pneumocystis pneumonia is a well-recognized major opportunistic infection in HIV-positive individuals, and is growing in importance in HIV-negative patients undergoing immunosuppressive treatment for malignancy, connective tissue disease, or organ transplantation.

In humans, the persistence of *Pneumocystis* in the lung is a limited-time phenomenon inversely related to immunological improvement (Dei-Cas 2000).

In the veterinary field, *Pneumocystis* has been described as producing pneumonia in dogs, causing serious pulmonary alterations. *Pneumocystis* is important in horses, where treatment with immunosuppressive drugs, like corticoids, is a relatively frequent practice in animals dedicated to sport, originating a pneumonic process with a bronchoneumonic diffuse image. In pigs, this fungus gives rise to pneumonic processes, affecting animals 7–11 weeks old, with lung damage that includes a decrease in the size of the pulmonary septa, with infiltration of mononuclear cells and appearance of exudates in the alveoli. This focal pneumonia evolves toward a diffuse pneumonia, very similar to that observed in children with this disease (Cavallini et al. 2007; Garcia and Blanco 2000; Kondo et al. 2000).

It may be that infection by *Pneumocystis* in domestic animals always needs an immunocompromised situation in the affected organism, as observed in humans (MacNeill et al. 2003).

3.10 Dimorphic Fungi

The systemic dimorphic fungi represent a family of six phylogenetically related ascomycetes: *B. dermatitidis, C. immitis, H. capsulatum, P. brasiliensis, Sporothrix schenkii,* and *Penicillium marneffei*. These primary pathogens are capable of converting from a non-pathogenic mold in the soil to a pathogenic yeast (or spherule in *C. immitis*) after infectious spores are inhaled into the lungs of human or other mammalian hosts. The morphologic conversion of the dimorphic fungi from mold to yeast is required for virulence. During conversion to the pathogenic form, the dimorphic fungi express other phase-specific products that have been shown to be essential in virulence (Klein and Tebbets 2007).

These organisms are considered true pathogens, as they are capable of causing infection in otherwise healthy persons. The severity of infection is determined by both the extent of the exposure to the organism and by the immune status of the patient (Laniado-Laborin 2007).

Most systemic endemic mycoses are a consequence of inhaling the fungus, while subcutaneous mycoses are acquired through the inoculation of vegetable matter or soil containing the organism (Laniado-Laborin 2007).

3.10.1 Blastomycosis

Blastomyces is a thermally dimorphic fungus, existing in the mycelial and yeast forms. The infectious form in the environment and soil is the mycelial phase. It produces microscopic conidia that mammals can inhale (Arceneaux et al. 1998).

Blastomycosis is a systemic fungal disease, most commonly diagnosed in dogs and humans, but also reported in cats, horses, and other mammals (Bromel and Sykes 2005a).

Infection in dogs with *B. dermatitidis* occurs when conidia produced from the mycelial phase in soil or decaying matter are inhaled into the lungs. The increase in temperature within the body causes conversion from the spore phase to a large broad-based budding yeast cell. Local pulmonary disease ranges from a self-limiting infection to severe pyogranulomatous. The organism may disseminate via the vascular or lymphatic system, causing granulomatous or pyogranulomatous inflammation in many organs, including lymph nodes, eyes, bones, brain, meninges, kidneys, liver, spleen, skin, and subcutis. Dogs are commonly presented for signs related to a single organ system, but further diagnostics usually reveal multisystemic involvement. In cats, the affected organs are similar to dogs, and clinical signs includes dyspnea, coughing, anorexia, lethargy, and weight loss (Bromel and Sykes 2005a).

Canine blastomycosis shares many common clinical features with blastomycosis in humans (Matwichuk et al. 1999).

There is no risk of aerosol transmission of the yeast phase of the organism directly from infected animals to humans. However, bandaging of blastomycosis

skin lesions is considered a health hazard because it may promote mycelialization of the organism (Bromel and Sykes 2005a).

Cutaneous inoculation of blastomycosis in veterinarians and veterinary technicians has been reported by accidental laceration during necropsy of a dog infected by *B. dermatitidis* (Graham and Callaway 1982), by puncture with a needle containing an aspirate from an infected dog (Ramsey 1994), as well as via a dog bite in an immunocompromised renal transplant recipient, resulting in dissemination to the lungs (Butka et al. 1984). Transmission of *B. dermatitidis* to humans during routine microbial culture of infected material from dogs and subsequent pulmonary blastomycosis also is possible (Cote et al. 1997).

Furthermore, a common source environmental exposure may result in disease of both animals and humans. A case-control study in Virginia identified a common-source outbreak of blastomycosis in hunters and their dogs (Armstrong et al. 1987).

3.10.2 Histoplasmosis

Histoplasmosis is a disease caused by the dimorphic fungus *H. capsulatum*, which is endemic to the temperate zones of the world, including America, Asia and Africa (Kurowski and Ostapchuk 2002; Murata et al. 2007).

There are two varieties of *H. capsulatum* that are pathogenic to humans (*H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii*) and a third variety that is an equine pathogen, *H. capsulatum* var. *farciminosum* (Kauffman 2007).

It took decades to prove that *H. capsulatum* is a dimorphic fungus, that histoplasmosis is primarily a pulmonary disease, and that the environmental reservoir is soil (Kauffman 2007).

The mycelial form of *H. capsulatum* thrives in organic, nitrogen-rich soils, such as those contaminated with bird or bat excrements. The most common route of infection is via inhalation of microconidia, which are small enough to reach the lower respiratory tract. At body temperature, the organism is transformed into the yeast phase, and phagocytosed by alveolar macrophages, which can then disseminate the infection to other organs in the body. Most canine patients suffering from clinical disease demonstrate gastrointestinal signs without concurrent respiratory involvement. There are three main manifestations of histoplasmosis: asymptomatic disease, respiratory disease, and disseminated disease. Asymptomatic disease is considered to be the most common manifestation in dogs and humans. The respiratory form can be benign and self-resolve, or it can cause major pulmonary disease. Disseminated disease affects multiple organs and carries a more guarded prognosis, not only pulmonary system and related lymph nodes but also the liver, spleen, intestine and bone marrow (Murata et al. 2007). Fifty percent of dogs suffering from disseminated disease have clinical signs related to gastrointestinal disease: diarrhea, intestinal blood loss, hypoalbuminemia, anemia, and weight loss (Kurowski and Ostapchuk 2002; Tyre et al. 2007). The host range for *H. capsulatum* includes

humans, dogs, cats, cattle, horses, rats, skunks, opossums, foxes, and many other animal species (Tyre et al. 2007).

H. capsulatum var. *farciminosum* affects the skin and subcutaneous lymph nodes and is found mainly in horses. In Japan, human cases of histoplasmosis have been described, caused by the same genotypes of *H. capsulatum* var. *farciminosum* and *H. capsulatum* var. *capsulatum* found in dogs and horses, and these varieties might cause not only cutaneous but also systemic histoplasmosis, regardless of their host species (Murata et al. 2007).

Outbreaks of human acute histoplasmosis have often been reported. These outbreaks were associated with disturbances of accumulations of bird or bat droppings (Laniado-Laborin 2007). The exposures to droppings have typically occurred while visiting caves, or following cleaning and construction activities at infested sites in endemic areas (Cano and Hajjeh 2001).

In cats with deep mycotic infections, histoplasmosis was the second most commonly reported fungal disease after cryptococcosis (Bromel and Sykes 2005b).

Genetic polymorphisms between *H. capsulatum* strains isolated from humans, animals and soil in the same geographic area were 100% similar, suggesting that an environmental microniche could be acting as a source of infection for animals and humans (Bromel and Sykes 2005b).

Cultures of *H. capsulatum* represent a severe biohazard to laboratory personnel, and must be handled with extreme caution in an appropriate pathogen-handling cabinet (Laniado-Laborin 2007).

3.10.3 Coccidioidomycoses

There are two nearly identical species of *Coccidioides*: *C. immitis*, which is found in California (USA), and *C. posadasii*, formerly known as non-California *C. immitis*, which is found primarly in Texas, Arizona, and the areas of endemicity in Mexico, Central and South America (Viriyakosol et al. 2005). These two species of fungi are genetically different, but at this time they cannot be distinguished phenotypically, nor is the disease or immune response to the organism distinguishable (Catanzaro 2004).

Coccidioides spp. grow as a mold in the soil and develop hyphae in their saprobic form, producing arthroconidia. As the soil dries or nutrients become limiting, the fungus reproduces asexually by disarticulating the hyphae into small, environmentally-resistant arthroconidia. These propagules are easily aerosolized when the soil is disturbed by wind or human activities. Consequently, it is the inhalation of the dust-borne arthroconidia that leads to infection in both human and domestic or wild mammals. Coccidioidomycoses is not contagious: reports of human-to-human spread are extremely rare (Laniado-Laborin 2007).

Coccidioidomycosis is a recognized opportunistic infection among persons infected with HIV. Early in the HIV epidemic, most cases presented as overwhelming diffuse pulmonary disease with a high mortality rate (Ampel 2005).

Approximately 60% of exposures to the fungus result in asymptomatic infection, or have symptoms so mild that medical attention is not sought. In the rest of infected individuals, who have symptomatic disease, there are protean manifestations that range from a primary (usually benign) pulmonary infection, also known as "Valley Fever", to a progressive pulmonary or extrapulmonary disease (Hector and Laniado-Laborin 2005). Fortunately, most patients with primary disease recover spontaneously and retain lifelong immunity to exogenous infection.

People with compromised immune systems are particularly susceptible to chronic forms of pulmonary disease, characterized by complications such as pleural empyema and bronchopleural fistulas (Hector and Laniado-Laborin 2005).

Extrapulmonary coccidioidomycosis is almost always the result of hematogenous spread from an initial pulmonary focus. Symptomatic extrapulmonary disease develops in about one of 200 people infected with *Coccidioides*. The most frequently affected sites are the skin, joints, bones and meninges (Galgiani 1993).

It must be remembered that *Coccidioides* spp. cultures pose a risk to laboratory personnel because the mycelial form is highly infectious. Cultures should be processed only within a biological containment cabinet by experienced personnel (Laniado-Laborin 2007).

In dogs and cats, the primary route of infection is inhalation of infective arthrospores. Primary cutaneous infection can occur by direct inoculation of damaged skin, but this is uncommon. Dissemination occurs when endospores spread via lymphatics and blood to distant sites in the body (Graupmann-Kuzma et al. 2008).

Although infection is less frequent in cats than in dogs, it may be more severe by the time it is recognized (Graupmann-Kuzma et al. 2008).

3.10.4 Sporotrichosis

This is a disease produced by *S. schenckii*. Generally, infection results from inoculation of the fungus through thorns, splinters, scratches and small traumas during activities such as floriculture, horticulture, gardening, fishing, hunting, farming and cattle-raising, mining, and wood exploration. It can also be acquired through bites or scratches from infected animals (cats, armadillos) (Kauffman 1999).

Sporotrichosis has diverse clinical manifestations. The most frequent clinical form is the lymphocutaneous form. It starts with a nodular or ulcerated lesion at the site of fungal inoculation, and follows a regional lymphatic trajectory characterized by nodular lesions that ulcerate, fistulate, and heal, representing true gummae. The disseminated cutaneous forms have mainly been observed among immunosuppressed patients, especially HIV-positive individuals. Among the extracutaneous forms, osteoarticular and pulmonary involvement are the most common (Lopez-Bezerra et al. 2006).

Laboratory professionals can be infected accidentally while manipulating *S. schenkii* cultures (Cooper et al. 1992).

There have been several reports of epidemics of sporotrichosis due to zoonotic transmission, especially from domestic cats (Barros et al. 2004; Gutierrez et al. 2008).

Starting in 1998, a sporotrichosis epidemic affecting humans, cats, and dogs has been described in the metropolitan region of Rio de Janeiro, mainly in areas with underprivileged socio-economic conditions and precarious health services. Between 1998 and 2004, a total of 759 humans, 1,503 cats and 64 dogs were diagnosed with sporotrichosis, with isolation of *S. schenkii* from different types of samples. The most frequently affected individuals were homemakers taking care of cats with sporotrichosis. The high percentage of genetic similarity demonstrated among these isolates suggests the possibility that only one virulent genetic population was present in this micro environment (Gutierrez et al. 2008).

3.11 Pythiosis

Pythium insidiosum is a pseudofungus, as it differs from the true fungi in the production of biflagellate zoospores and in the composition of its cell wall (Pier et al. 2000).

The common cycle of this fungus is based on the colonization of aquatic plants that serve as substrata for its development and reproduction, forming the zoosporangia. The zoospores move in the water until they find another plant or animal where they can encyst and emit the germinative tube, beginning a new mycelium and completing their cycle (Miller 1983).

Pythiosis is a granulomatous disease that occurs in tropical, subtropical, and temperate areas, affecting horses, cats, dogs, cattle and humans, which is caused by the oomycete *P. insidiosum*. The equine species is the most commonly affected: cutaneous, subcutaneous and gastrointestinal lesions form, characterized by the formation of eosinophilic granuloma, with the presence of necrotic masses called "kunkers" (Mendoza et al. 1996).

This disease has many common names, including hyphomycosis, zygomycosis, granular dermatitis, "bursattee," Florida "leeches," phycomycotic granuloma, and "swamp cancer" (Pier et al. 2000).

There are no reports of direct contagion among animals or between animals and human beings (Pier et al. 2000).

3.12 Conclusions

Most mycoses are included in the group of diseases named saprozoonoses. These are produced by fungi that can affect human and animals, and the infection is produced through soil or fomites. The fact that human and animals may be hosts for the same fungus is the reason that these diseases are considered as zoonoses.

Traditionally, ringworm has been defined as the only true zoonosis among the mycoses. Nevertheless, there have been some recent descriptions of outbreaks with

direct animal–person transmission of infections caused by other fungi, such as *Cryptococcus* or *Malassezia*. We are sure that this kind of description will increase in the future.

References

Aguirre K, Crowe J, Haas A, Smith J (2004) Resistance to *Cryptococcus neoformans* infection in the absence of CD4+ T cells. Med Mycol 42:15–25

Ahman S, Perrins N, Bond R (2007) Carriage of *Malassezia* spp. yeasts in healthy and seborrhoeic Devon Rex cats. Med Mycol 45:449–455

Akerstedt J, Vollset I (1996) *Malassezia pachydermatis* with special reference to canine skin diseases. Br Vet J 152:269–281

Alvarez-Perez S, Blanco JL, Alba P, Garcia ME (2009a) Mating type and invasiveness are significantly associated in *Aspergillus fumigatus*. Med Mycol. doi:doi: 10.1080/13693780903095414

Alvarez-Perez S, Garcia ME, Bouza E, Pelaez T, Blanco JL (2009b) Characterization of multiple isolates of *Aspergillus fumigatus* from patients: genotype, mating type and invasiveness. Med Mycol 47:601–608

Ampel NM (2005) Coccidioidomycosis in persons infected with HIV type 1. Clin Infect Dis 41:1174–1178

Arceneaux KA, Tabeada J, Hosgood G (1998) Blastomycosis in dogs: 115 cases (1980–1995). J Am Vet Med Assoc 213:658–664

Armstrong CW, Jenkins SR, Kaufman L, Kerkering TM, Rouse BS, Miller GB Jr (1987) Common-source outbreak of blastomycosis in hunters and their dogs. J Infect Dis 155:568–570

Ashbee HR (2006) Recent developments in the immunology and biology of *Malassezia* species. FEMS Immunol Med Microbiol 47:14–23

Ashbee HR (2007) Update on the genus *Malassezia*. Med Mycol 45:287–303

Baro T, Torres-Rodriguez JM, Hermoso de Mendoza M, Morera Y, Alia C (1998) First identification of autochthonous *Cryptococcus neoformonans* var. *gattii* isolated from goats with predominantly severe pulmonary disease in Spain. J Clin Microbiol 36:458–461

Barros MBL, Schubach A, Francesconi-Dovalle AC, Gutierrez MC, Conceiçao-Silva F, Schubach TMB, Reis RS, Marzochi KBF, Wanke B, Conceiçao MJ (2004) Cat-transmitted sporotrichosis epidemic in Rio de Janeiro, Brazil: description of a series of cases. Clin Infect Dis 38:529–535

Berry WL, Leisewitz L (1996) Multifocal *Aspergillus terreus* discospondylitis in two German shepherd dogs. J South Afr Vet Assoc 67:222–228

Blanco JL, Garcia ME (2008) Immune response to fungal infections. Vet Immunol Immunopathol 125:47–70

Blanco JL, Guedeja-Marron J, Blanco I, Garcia ME (2000) Optimum incubation conditions for the isolation of yeasts from canine otitis externa. J Vet Med B 47:599–605

Blanco JL, Guedeja-Marron J, Hontecillas R, Suarez G, Garcia ME (1996) Microbiological diagnoses of chronic otitis externa in the dog. J Vet Med B 43:475–482

Blanco JL, Hontecillas R, Bouza E, Blanco I, Pelaez T, Perez-Molina J, Garcia ME (2002) Correlation between the elastase activity index and invasiveness of clinical isolates of *Aspergillus fumigatus*. J Clin Microbiol 40:1811–1813

Bovers M, Hagen F, Boekhout T (2008) Diversity of the *Cryptococcus neoformans–Cryptococcus gattii* species complex. Rev Iberoam Micol 25:S4–S12

Bozza S, Gaziano R, Spreca A, Bacci A, Montagnoli C, di Francesco P, Romani L (2002) Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. J Immunol 168:1362–1371

Bromel C, Sykes JE (2005a) Epidemiology, diagnosis, and treatment of blastomycosis in dogs and cats. Clin Tech Small Anim Pract 20:233–239

Bromel C, Sykes JE (2005b) Histoplasmosis in dogs and cats. Clin Tech Small Anim Pract 20:227–232

Butka BJ, Bennett SR, Johnson AC (1984) Disseminated inoculation blastomycosis in a renal transplant recipient. Am Rev Respir Dis 130:1180–1183

Butterworth SJ, Barr FJ, Pearson GR, Day MJ (1995) Multiple discospondylitis associated with *Aspergillus* species infection in a dog. Vet Rec 136:38–41

Cabañes FJ, Abarca ML, Bragulat MR (1997) Dermatophytes isolated from domestic animals in Barcelona, Spain. Mycopathologia 137:107–113

Cafarchia C, Romito D, Coccioli C, Camarda A, Otranto D (2008) Phospholipase activity of yeasts from wild birds and possible implications for human disease. Med Mycol 46:429–434

Cano MVC, Hajjeh RA (2001) The epidemiology of histoplasmosis: a review. Sem Respir Dis 16:109–118

Catanzaro A (2004) Coccidioidomycosis. Sem Respir Care Med 25:123–128

Cavallini EM, Pescador C, Rozza D, Spanamberg A, Borba MR, Ravazzolo AP, Driemeier D, Guillot J, Ferreiro L (2007) Detection of *Penumocystis* spp. in lung samples from pigs in Brazil. Med Mycol 45:395–399

Chang HJ, Miller HL, Watkins N, Arduino MJ, Ashford DA, Midgley G, Aguero SM, Pinto-Powell R, von Reyn CF, Edwards W, McNeil MM, Jarvis WR (1998) An epidemic of *Malassezia pachydermatis* in an intensive care nursery associated with colonization of health care workers' pet dogs. N Engl J Med 338:706–711

Chazalet V, Debeaupuis JP, Sarfati J, Lortholary J, Ribaud P, Shah P, Cornet M, Vu Thien H, Gluckman E, Brücker G, Latgé JP (1998) Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. J Clin Microbiol 36:1494–1500

Chen GH, Olszewski MA, McDonald RA, Wells JC, Paine R III, Huffnagle GB, Toews GB (2007) Role of granulocyte macrophage colony-stimulating factor in host defense against pulmonary *Cryptococcus neoformans* infection during murine allergic bronchopulmonary mycosis. Am J Pathol 170:1028–1040

Cleaveland S, Laurenson MK, Taylor LH (2001) Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philos Trans R Soc Lond B 356:991–999

Clercx C, McEntee K, Snaps F, Jacquinet E, Coignoul F (1996) Bronchopulmonary and disseminated granulomatous disease associated with *Aspergillus fumigatus* and *Candida* species infection in a Golden Retriever. J Am Anim Hosp Assoc 32:139–145

Cooper CR, Dixon DM, Salkin IF (1992) Laboratory-acquired sporotrichosis. J Med Vet Mycol 30:169–171

Cote E, Barr SC, Allen C (1997) Possible transmission of *Blastomyces dermatitidis* via culture specimen. J Am Vet Med Assoc 210:479–480

Dallman MJ, Dew TL, Tobias L, Doss R (1992) Disseminated aspergillosis in a dog with diskospondylitis and neurologic deficits. J Am Vet Med Assoc 200:511–513

Day MJ, Penhale WJ (1991) An immunohistochemical study of canine disseminated aspergillosis. Aust Vet J 68:383–386

Day MJ, Penhale WJ (1998) Humoral immunity in disseminated *Aspergillus terreus* infection in the dog. Vet Microbiol 16:283–294

Day MJ, Eger CE, Shaw SE, Penhale WJ (1985) Immunologic study of systemic aspergillosis in German shepherd dogs. Vet Immunol Immunopathol 9:335–347

Dei-Cas E (2000) *Pneumocystis* infections: the iceberg? Med Mycol 38:23S–32S

Drouot S, Mignon B, Fratti M, Roosje P, Monod M (2009) Pets as the main source of two zoonotic species of the *Trichophyton mentagrophytes* complex in Switzerland, *Arthroderma vanbreuseghemii* and *Arthroderma benhamiae*. Vet Dermatol 20:13–18

Drusin LM, Ross BG, Rhodes KH, Krauss AN, Scott RA (2000) Nosocomial ringworm in a neonatal intensive care unit: a nurse and her cat. Infect Control Hosp Epidemiol 21:605–607

Dumoulin A, Mazars E, Seguy N, Gargallo-Viola D, Vargas S, Cailliez JC, Aliouat EM, Wakefield AE, Dei-Cas E (2000) Transmission of *Pneumocystis carinii* disease from immunocompetent contacts of infected hosts to susceptible hosts. Eur J Clin Microbiol Infect Dis 19:671–678

Duncan CG, Stephen C, Campbell J (2006a) Evaluation of risk factors for *Cryptococcus gattii* infection in dogs and cats. J Am Vet Med Assoc 228:377–382

Duncan CG, Stephen C, Campbell J (2006b) Clinical characteristics and predictors of mortality for *Cryptococcus gattii* infection in dogs and cats of southwestern British Columbia. Can Vet J 47:993–998

Duncan CG, Stephen C, Lester S, Barlett KH (2005) Follow-up study of dogs and cats with asymptomatic *Cryptococcus gattii* infection or nasal colonization. Med Mycol 43:663–666

Edman JK, Kovacs JA, Masur H, Santi DV, Elwood HJ, Sogin ML (1988) Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the fungi. Nature 334:519–522

Elad D, Lahav D, Blum S (2008) Transuterine transmission of *Aspergillus terreus* in a case of disseminated canine aspergillosis. Med Mycol 46:175–178

English K, Peters SE, Maskell DJ, Collins ME (2001) DNA analysis of Pneumocystis infecting a Cavalier King Charles Spaniel. J Eukaryot Microbiol 48(Suppl):106S

Feldmesser M (2005) Prospects of vaccines for invasive aspergillosis. Med Mycol 43:571–587

Fréalle E, Rodrigue M, Gantois N, Aliouat CM, Delaporte E, Camus D, Dei-Cas E, Kauffmann-Lacroix C, Guillot J, Delhaes L (2007) Phylogenetic análisis of *Tricophyton mentagrophytes* human and animal isolates based on MnSOD and ITS sequence comparison. Microbiology 153:3466–3477

Galgiani JN (1993) Coccidioidomycosis. West J Med 159:153–171

Garcia ME, Blanco JL (2000) Principales enfermedades fúngicas que afectan a los animales domésticos. Rev Iberoam Micol 17:S2–S7

Garcia ME, Caro A, Fragio C, Blanco I, Blanco JL (2001) A clinical case of canine mycotic pneumonia. J Vet Med A 48:501–506

Garcia ME, Duran C, Cruzado M, Andrino M, Blanco JL (2004) Evaluation of molecular and immunological techniques for the diagnosis of mammary aspergillosis in ewes. Vet Microbiol 98:17–21

Garcia ME, Lanzarot P, Lopez-Rodas V, Costas E, Blanco JL (2007) Fungal flora in trachea of birds from a wildlife rehabilitation centre in Spain. Vet Med Czech 52:464–470

Gelatt KN, Chrisman CL, Samuelson DA, Shell LG, Buergelt CD (1991) Ocular and systemic aspergillosis in a dog. J Am Anim Hosp Assoc 27:427–431

Gene J, Blanco JL, Cano J, Garcia ME, Guarro J (2003) The new filamentous fungus *Sagenomella chlamydospora* responsible for a disseminated infection in a dog. J Clin Microbiol 41:1722–1725

Graham WR, Callaway JL (1982) Primary inoculation blastomycosis in a veterinarian. J Am Acad Dermatol 7:785–786

Gräser Y, De How S, Summerbell RC (2006) Dermatophytes: recognizing species of clonal fungi. Med Mycol 44:199–209

Graupmann-Kuzma A, Valentine BA, Shubitz LF, Dial SM, Watrous B, Tornquist SJ (2008) Coccidioidomycosis in dogs and cats: a review. J Am Anim Hosp Assoc 44:226–235

Griffin CE (1993) Otitis externa and otitis media. In: Griffin CE, Kwochka KW, MacDonald JM (eds) Current veterinary dermatology. Mosby Year Book, St Louis, pp 244–262

Gueho E, Midgley G, Guillot J (1996) The genus *Malassezia* with description of four new species. Antonie Van Leeuwenhoek 69:337–355

Guerin SR, Walker MC, Kelly DF (1993) Cavitating mycotic pulmonary infection in a German shepherd dog. J Small Anim Pract 34:36–39

Guillot J, Bond R (1999) *Malassezia pachydermatis*: a review. Med Mycol 37:295–306

Gutierrez MC, Zancope RM, Francesconi AC, De Almeida R, Morais P, Monzon A, Mellado E, Rodríguez-Tudela JL, Cuenca-Estrella M (2008) Molecular epidemiology and antifungal

susceptibility patterns of *Sporothrix schenckii* isolates from a cat-transmitted epidemic of sporotrichosis in Rio de Janeiro, Brazil. Med Mycol 46:141–151
Hector RF, Laniado-Laborin R (2005) Coccidioidomycosis — A fungal disease of the Americas. PLoS Med 2:2e
Hostetter MK (1994) Adhesins and ligands involved in the interaction of *Candida* spp. with epithelial and endothelial surfaces. Clin Microbiol Rev 7:29–42
Hubalek Z (2003) Emerging human infectious diseases: anthropozoonoses, zoonoses and sapronoses. Emerg Infect Dis 9:403–404
Jadhav VJ, Pal M (2006) Canine mycotic stomatitis due to *Candida albicans*. Rev Iberoam Micol 23:233–234
Jang SS, Dorr TE, Biberstein EL, Wong A (1986) *Aspergillus deflectus* infection in four dogs. J Med Vet Mycol 24:95–104
Jensen HE, Basse A, Aalbaek B (1989) Mycosis in the stomach compartments of cattle. Acta Vet Scand 30:409–423
Kauffman CA (1999) Sporotrichosis. Clin Infect Dis 29:231–237
Kauffman CA (2007) Histoplasmosis: a clinical and laboratory update. Clin Microbiol Rev 20:115–132
Kauffman AC, Green CE, Selcer BA, Styles ME, Mahaffey EA (1994) Systemic aspergillosis in a dog and treatment with hamycin. J Am Anim Hosp Assoc 30:132–136
Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, MacDougall L, Boekhout T, Kwon-Chung KJ, Meyer W (2004) A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc Natl Acad Sci USA 101:17258–17263
Klein BS, Tebbets B (2007) Dimorphism and virulence in fungi. Curr Opin Microbiol 10:314–319
Kondo H, Hikita M, Ito M, Kadota K (2000) Immunohistochemical study of *Pneumocystis carinii* infection in pigs: evaluation of *Pneumocystis* pneumonia and a retrospective investigation. Vet Rec 147:544–549
Kurowski R, Ostapchuk M (2002) Overview of histoplasmosis. Am Fam Physician 66:2247–2252
Laniado-Laborin R (2007) Coccidioidomycosis and other endemic mycoses in Mexico. Rev Iberoam Micol 24:249–258
Las Heras A, Domínguez L, Lopez I, Paya MJ, Peña L, Mazzucchelli F, Garcia LA, Fernández-Garayzabal JF (2000) Intramammary *Aspergillus fumigatus* infection in dairy ewes associated with antibiotic dry therapy. Vet Rec 147:578–580
Lenard ZM, Lester NV, O'Hara AJ, Hopper BJ, Lester GD (2007) Disseminated cryptococcosis including osteomyelitis in a horse. Aust Vet J 85:51–55
Littman MP, Goldschmidt MH (1987) Systemic paecilomycosis in a dog. J Am Vet Med Assoc 191:445–447
Lopez-Bezerra LM, Schubach A, Costa RO (2006) *Sporothrix schenkii* and sporotrichosis. Ann Brazil Acad Sci 78:293–308
MacNeill AL, Alleman AR, Fanklin RP, Long M, Giguere S, Uhl E, Lopez-Martinez A, Wilkerson M (2003) Pneumonia in a Paso-Fino mare. Vet Clin Pathol 32:73–76
Mancianti F, Nardoni S, Corazza M, D'Achille P, Ponticelli C (2003) Environmental detection of *Microsporum canis* arthorspores in the households of infected cats and dogs. J Feline Med Surg 5:323–328
Mantovani A, Morganti L (1977) Dermatophytozoonoses in Italy. Vet Res Commun 1:171–177
Markaryan A, Morozova I, Yu H, Kolattukudy PE (1994) Purification and characterization of an elastinolytic metalloprotease from *Aspergillus fumigatus* and immuno-electron microscopic evidence of secretion of this enzyme by the fungus. Infect Immun 62:2149–2157
Matwichuk CL, Daniel GB, Bowman LA, Legendre AM, Smith GT (1999) Fluorine-18 fluorodeoxyglucose accumulation in *Blastomyces dermatitidis*-associated inflammation in a dog. Clin Positron Imaging 2:217–221
Mendoza L, Ajello L, McGinnis MR (1996) Infections caused by the oomycetous pathogen *Pythium insidiosum*. J Mycol Med 6:151–164

Miller RL (1983) Investigations into the biology of three "phycomycotic" agents pathogenic for horses in Australia. Mycopathologia 81:23–28
Mishra SK, Ajello L, Ahearn DG, Burge HA, Kurup VP, Pierson DL, Price R (1992) Environmental mycology and its importance to public health. J Vet Med Mycol 30:287–305
Morris DO, O'Shea K, Shofer FS, Rankin S (2005) *Malassezia pachydermatis* carriage in dog owners. Emerg Infect Dis 11:83–88
Mullanery TP, Levin S, Indrieri RJ (1983) Disseminated aspergillosis in a dog. J Am Vet Med Assoc 182:516–518
Murata Y, Sano A, Ueda Y, Inomata T, Takayama A, Poonwan N, Nanthawan M, Mikami Y, Miyaji M, Nishimura K, Kamei K (2007) Molecular epidemiology of canine histoplasmosis in Japan. Med Mycol 45:233–247
Ordeix L, Galeotti F, Scarampella F, Dedola C, Bardasí M, Romano E, Fondati A (2007) *Malassezia* spp. overgrowth in allergic cats. Vet Dermatol 18:316–323
Papini R, Nardoni S, Fanelli A, Mancianti F (2009) High infection rate of *Trichophyton verrucosum* in calves from central Italy. Zoonoses Public Health 56:59–64
Pastor J, Pumarola M, Cuenca R, Lavin S (1993) Systemic aspergillosis in a dog. Vet Rec 132:412–413
Pastoret PP, Bennett M, Brochier B, Akakpo AJ (2000) Animals, public health and the example of cowpox. Rev Sci Tech Off Int Epiz 19:23–32
Pegues DA, Lasker BA, McNeil MM, Hamm PM, Lundal JL, Kubak BM (2002) Cluster of cases of invasive aspergillosis in a transplant intensive care unit: evidence of person-to-person airborne transmission. Clin Infect Dis 34:412–416
Perez J, Mozos E, Chacon F, Paniagua J, Day MJ (1996) Disseminated aspergillosis in a dog: an immunohistochemical study. J Comp Pathol 115:191–196
Pier AC, Cabañes J, Chermette R, Ferreiros L, Guillot J, Jensen HE, Santurio JM (2000) Prominent animal mycoses from various regions of the world. Med Mycol 38:47S–58S
Ramsey DT (1994) Blastomycosis in a veterinarian. J Am Vet Med Assoc 205:968
Romani L (1999) Immunity to *Candida albicans*: Th1, Th2 cells and beyond. Curr Opin Microbiol 2:363–367
Rosario I, Acosta B, Colom F (2008) La paloma y otras aves como reservorio de *Cryptococcus* spp. Rev Iberoam Micol 25:S13–S18
Silver S, Vinh DC, Embil JM (2008) The man who got too close to his cows. Diagn Microbiol Infect Dis 60:419–420
Sparkes AH, Stokes R, Gruffydd-Jones TJ (1993) Humoral immune response in cats with dermatophytosis. Am J Vet Res 54:1869–1873
Starkey RJ, McLoughlin MA (1996) Treatment of renal aspergillosis in a dog using nephrostomy tubes. J Vet Intern Med 10:336–338
Steele C, Shellito JD, Kolls JK (2005) Immunity against the opportunistic fungal pathogen *Pneumocystis*. Med Mycol 43:1–19
Stevens DA, Walsh TJ, Bistoni F, Cenci E, Clemons KV, Del Sero G, Fe d'Ostiani C, Kullberg BJ, Mencacci A, Roilides E, Romani L (1998) Cytokynes and mycoses. Med Mycol 36:174S–182S
Stringer JR, Beard CB, Miller RF, Wakefield AE (2002) A new name (*Pneumocystis jiroveci)* for *Pneumocystis* from humans. Emerg Infect Dis 8:891–896
Tejasvi T, Sharma VK, Sethuraman G, Singh MK, Xess I (2005) Invasive dermatophytosis with lymph node involvement in an immunocompetent patient. Clin Exp Dermatol 30:506–508
Tell LA (2005) Aspergillosis in mammals and birds: impact on veterinary medicine. Med Mycol 43:71S–73S
Toma B, Vaillancourt JP, Dufour B, Eloit M, Moutou F, Marsh W, Benet JJ, Saana M, Michel P, Kass P, Bigras-Poulin M (1999) Dictionary of veterinary epidemiology. Iowa State University Press, Ames
Traynor TR, Huffnagle GB (2001) Role of chemokines in fungal infections. Med Mycol 39:41–50

Tyre E, Eisenbart D, Foley P, Burton S (2007) Histoplasmosis in a dog from New Brunswick. Can Vet J 48:734–736

vanBelkum A, Boekhout T, Bosboom R (1994) Monitoring spread of *Malassezia* infections in neonatal intensive care unit by PCR-mediated genetic typing. J Clin Microbiol 32:2528–2532

Viriyakosol S, Fierer J, Brown GD, Kirkland TN (2005) Innate immunity to the pathogenic fungus *Coccidioides posadasii* is dependent on toll-like receptor 2 and Dectin-1. Infect Immun 73:1553–1560

Watt PR, Robins GM, Galloway AM, O'Boyle DA (1995) Disseminated opportunistic fungal disease in dogs: 10 cases (1982–1990). J Am Vet Med Assoc 207:67–70

Weitzman I, Summerbell RC (1995) The dermatophytes. Clin Microbiol Rev 8:240–259

Wilson SM, Odeon A (1992) Disseminated *Aspergillus terreus* infection in a dog. J Am Anim Hosp Assoc 28:447–450

Zanatta R, Miniscalco B, Guarro J, Gene J, Capucchio MT, Gallo MG, Mikulicich B, Peano A (2006) A case of disseminated mycosis in a German shepherd dog due to *Penicillium pururogenum*. Med Mycol 44:93–97

Chapter 4
Fungi Associated with Eye Infections with Special Reference to Corneal Keratitis and Their Possible Reservoir

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Abstract Ocular fungal infections, or ophthalmic mycoses, are being increasingly recognized as an important cause of morbidity and blindness; certain types of ophthalmic mycoses may even be life-threatening in particular endogenous and exogenous fungal endophthalmitis. In this chapter, a review of literature is given on the types of common ocular fungal infections, with recent trends in corneal fungal keratitis and factors influencing such infections. Occurrence of both pathogenic and opportunistic fungal pathogens is described for both normal and diseased human eyes. Diagnoses of fungal infection are most critical. Occurrence of such fungi in other habitats, especially in soil and the hospital environment, suggests that soil is the main reservoir of ocular filamentous fungi which may be transmitted to human eyes by different routes.

4.1 An Introduction to Ocular Fungal Infection

In the last few decades, the association of fungi with eye infections has again received increased attention in ophthalmology. Our means of combating bacterial infections are good but defenses against fungal pathogens are relatively weak. Fungal infection is generally classified as endogenous or exogenous oculomycosis and extension oculomycosis. Various types of defenses are imparted naturally for the eye to be protected from pathogen attacks and may be classified as (1) anatomical defenses, (2) mechanical defenses (by eye lashes), (3) immunological

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defenses and (4) defenses provided by tear film, conjunctiva etc. For example, the nonkeratinized squamous epithelium of the conjunctiva and cornea serves as a protective anatomic barrier against pathogens. The tear film comprises three layers, namely oil, aqueous, and mucous, and is produced by the meibomian glands, the lacrimal glands, and the goblet cells of the conjunctiva respectively. The aqueous layer comprises the majority of the 7 mm thick tear film. The tear pH, 7.14–7.82, probably contributes to the neutralization of toxic substances. Tear flow mechanically bathes the anterior surface of the eye, preventing the adherence of microorganisms, and flushes allergens and foreign particles into the lacrimal excretory system. In addition, the mucous layer of the tear film entraps foreign material, which facilitates its removal (Klotz et al. 2000)

A first report of keratitis in humans was published by Prof. Theodor Laber in 1879. With the help of Prof. Reincke, a botanist, he identified the organism as *Aspergillus glaucus*. Since then several workers have reported occurrence of fungi from diseased eyes. In the occurrence and distribution pattern of fungi in the eye reported up to 1972, there were more than 83 species belonging to 49 genera. It has been found that various fungi isolated from an infected eye could also be recovered from a normal eye (Seegal and Khorazo 1972; Ahmad 1988). Common fungi isolated from adult normal eyes belong to various genera such as *Aspergillus, Candida, Cephalosporium, Cladosporium, Curvularia, Fusarium, Helimenthosporium, Mucor, Rhizopus, Penicillium, Trichophyton*, etc. (Tables 4.1 and 4.2). The great majority of these fungi are considered to have their normal habitat in soil and vegetable matter. Due to exposure of the eye to the atmosphere and its close continuity with skin, it is not surprising to recover various types of fungi from otherwise normal conjunctiva. At least 95 species out of 44 genera have been isolated from corneal ulcers. A few of them may be potential pathogens of humans, but the great majority of these fungi had normal habitat either in the soil or vegetable matter as saprophytes.

Wilson et al. (1991) listed about 105 species from 35 genera of fungi as causative agents of keratitis and other ophthalmic mycoses; however, the criteria by which these fungi were considered to be genuine ophthalmic pathogens, and not simply contaminants inadvertently introduced into specimens during or after collection, were not clearly delineated. An evaluation made by McGinnis (1980) of more than 300 reports pertaining to human fungal infections published in the literature from the late 1940s to the beginning of 1979 encountered similar difficulties. This assessment included reports on 30 genera (60 species) of fungi isolated from ophthalmic infections, principally keratitis; some reports pertaining to 32 species in 19 genera of fungi satisfied strict criteria of acceptability. Criteria recommended include an adequate clinical history that suggests a mycotic infection, the association of fungus in the clinical specimens, and also that the morphology of the fungus in the clinical specimens should be consistent with the reported etiologic agent.

In the case of fungal infections of the eye, it is erroneous to make a distinction between pathogenic and saprophytic fungi. A decreased host resistance, local lesion, incidental to the primary diseases and disturbances brought about by modern

Table 4.1 Genera and species of fungi isolated from adult normal eyes (Seegal and Khorazo 1972)

Genera	Species
Acremonium	*Acremonium* sp.
Alternaria	*Alternaria* sp.
Aspergillus	*A. flaviceps, A. fumigates, A. glaucus, A. niger, A. terreus, Aspergillus* sp.
Aureobasidium	*A. pullulans*
Botrytis	*B. cinera, Botrytis* sp.
Candida	*C. albicans, C. guilliermondii, C. kruseii, C. parapsilosis, C. pseudotropicalis, C. pulcherima*
Cephalosporium	*Cephalosporium* sp.
Cladosporium	*C. herbarum, C. mansoni, Cladosporium* sp.
Cryptococcus	*C. albidus, C. laurentii, Cryptococcus* sp.
Curvularia	*C. geniculata, C. lunata, Curvularia* sp.
Fusarium	*Fusarium* sp.
Geotricum	*Geotricum* sp.
Gliocladium	*Gliocladium* sp.
Helminthosporium	*Helminthosporium* sp.
Hormodendrum	*H. algeriensis, Hormodendrum* sp.
Monilia	*M. sitophila*
Mucor	*Mucor* sp.
Nigrospora	*Nigrospora* sp.
Paecilomyces	*P. varioti, Paecilomyces* sp.
Penicillium	*P. citrinum, P. commune, Penicillium* sp.
Rhizopus	*Rhizopus* sp.
Rhodotorula	*R. mucilaginosa, R. rubra, Rhodotorula* sp.
Saccharomyces	*S. cerevisiae, S. fragilis, Saccharomyces* sp.
Torulopsis	*T. glabrata, Torulopsis* sp.
Trichoderma	*T. viride, Trichoderma* sp.

therapy, including broad-spectrum antibiotics and corticosteroids, permits the growth of usually saprophytic fungi (Ahmad 1988).

Fungal infection of the eye may be endogenous endophthalmitis or exogenous endophthalmitis. Other infections are to cornea and adjacent structures (eyelids, conjunctiva, and lacrimal system). The term endogenous endophthalmitis implies that blood-borne spread of micro-organisms to the eye has occurred. Therefore, infection in the eye is the result of metastatic spread of infection from a distant site, for example, infected heart valves or the urinary tract. In this manner the eye becomes the site of numerous micro-abscesses. The epidemiology of endogenous endophthalmitis reflects both the natural habitats of the involved fungi and the habits and health status of the patients.

Endogenous endophthalmitis may be caused by *Candida*, *Aspergillus* as a direct result of the success of modern medical practice that sustains patients' lives with broad-spectrum antibiotics, indwelling central venous lines, parenteral nutrition, abdominal surgery, and cytotoxic chemotherapy. Virtually any intravascular prosthesis or device may become contaminated by blood-borne opportunistic fungi, and fungemia arising from such infection may lead to endogenous endophthalmitis. Exogenous endophthalmitis occurs through introduction of micro-organisms into

Table 4.2 Genera and species of fungi isolated in cases of keratomycosis (Seegal and Khorazo 1972)

Genera	Species
Absidia	*A. Corymbifera, A. ramose, Absidia* sp.
Acremonium	*Acremonium* sp.
Aspergillus	*A. flavus, A. fumigates, A. glaucus, A. nidulans, A. niger, A. niveus, A. ochraceus, A. versicolor, Aspergillus* sp.
Candida	*C. albicans, C. guilliermondii, C. kruseii, C. mycoderma, C. parapsilosis, C. pseudotropicalis, C. tropicalis, Candida* sp.
Cephalosporium	*C. arcuatum, C. curtipes, C. serrae, Cephalosporium* sp.
Cryptococcus	*C. laurentii, C. neoformans*
Curvularia	*C. geniculata, C. lunata, Curvularia* sp.
Fusarium	*F. conglutinans, F. oxysporum, F. nivale, F. solani, Fusarium* sp.
Fusidium	*F. terricola*
Helminthosporium	*Helminthosporium* sp.
Hormodendrum	*Hormodendrum* sp.
Microsporum	*M. canis*
Mucor	*M. cornealis, M. mucedo, M. racemosus, Mucor* sp.
Paecilomyces	*Paecilomyces* sp.
Penicillium	*P. citrinum, P. columnaris, P. crustaceum, P. chrysogenum, P. glacum, P. lilacidum, P. notatum, P. spinulosum, Penicillium* sp.
Phycomyces	*Phycomyces* sp.
Rhizopus	*R. equinus, R. nigricans, R. parasiticus, Rhizopus* sp.
Sporothrix	*S. schenkii*
Tricoderma	*Tricoderma* sp.
Tricophyton	*T. schoenleinii, T. tonsurans, Tricophyton* sp.
Ustilago	*U. zeae*
Verticillium	*V. graphii, V. rubrum, Verticillium* sp.

the eye from trauma or surgery. It can also be the end result of pre-existing scleritis or keratitis (Borderie et al. 1997; Klotz et al. 2000).

The number of fungi implicated in various exogenous and endogenous ophthalmic infections has been increasingly reported in recent years, such as yeasts, principally *Candida* sp., including *Candida glabrata* (Cameron et al. 1998), *Candida famata* (Rao et al. 1991), and *Fusarium* sp. (Pflugfelder et al. 1988). Furthermore, patients with AIDS may contact many different fungal infections of the eye and adjacent structures. Various common fungi in eye infections associated with AIDS patients have been reported, such as *Cryptococcus* sp., *Pneumocystis* sp., *Candida* sp., *Histoplasma* sp., *Aspergillus* sp., *Fusarium* sp., and *Bipolaris* sp. (Table 4.3).

Table 4.3 Site of occurrence and associated fungal pathogens in eyes of AIDS patients

Location or nature of lesions fungus	Organism	References
Eyelid nodules	*Cryptococcus* sp.	Coccia et al. (1999)
Conjunctivitis, colonization of the conjunctiva	*Pneumocystis* sp.	Ruggli et al. (1997)
Cornea	*Candida* sp.	Aristimuno et al. (1993)
Anterior chamber, limbus	*Cryptococcus* sp.	Aristimuno et al. (1993), Muccioli et al. (1995)
	Histoplasma sp.	Font et al. (1995)
Choroiditis	*Cryptococcus* sp.	Saran and Pomilla (1996)
	Pneumocystis sp.	Whitcup et al. (1992)
	Histoplasma sp., *Aspergillus* sp., *Candida* sp.	Morinelli et al. (1993)
Retinitis	*Cryptococcus* sp., *Histoplasma* sp.	Specht et al. (1991)
Endogenous endophthalmitis	*Aspergillus* sp.	Petersen et al. (1997)
	Fusarium sp.	Glasgow et al. (1996)
	Bipolaris sp.	Pavan and Margo (1993)
Optic neuropathy	Zygomycetes	Lee et al. (1996)
	Histoplasma sp.	Shmookler and Kolsky (1996)
	Cryptococcus sp.	Garrity et al. (1993)
Sino-orbital disease	*Aspergillus* sp.	Johnson et al. (1999)

Paecilomyces lilacinus is a ubiquitous soil saprophyte implicated in cases of keratitis and endophthalmitis after trauma (Westenfeld et al. 1996; Okhravi et al. 1997). However, a large outbreak of *P. lilacinus* exogenous endophthalmitis followed intraocular lens implantation; the lenses had been contaminated by a bicarbonate solution used to neutralize the sodium hydroxide sterilant added to the lenses. *Acremonium kiliense* (Fridkin et al. 1996) has caused infections following lens surgery. Fungal pathogens in posttraumatic endophthalmitis are legion and similar to those causing fungal keratitis. Some reports have identified *Fusarium moniliforme* (Srdic et al. 1993), *Exophiala jeanselmei* (Hammer et al. 1983), *Pseudo-Allescheria boydii* (Carney et al. 1996), *Aspergillus niger* (Jager et al. 1994), *Scytalidium dimidiatum* (Al-Rajhi et al. 1993), *Helminthosporium* spp. (Das et al. 1994), *S. schenckii* (Witherspoon et al. 1990), *Penicillium chrysogenum* (Eschete et al. 1981), and *L. theobromae* (Borderie et al. 1997).

Thomas (2003a) has extensively reviewed various types of ocular pathogenic fungi and grouped them as (1) hyaline filamentous fungi, (2) dematiaceous (phaeoid) fungi, (3) yeast and zygomycetious fungi, and (4) thermally dimorphic fungi, implicated in ophthalmic infections and other specific pathogenic fungi.

Ophthalmologists and optometrists in particular, and clinicians in general, need to be aware of the pathogenesis of fungal eye infections. More than 70 species representing 40 genera of fungi have been reported to cause fungal keratitis. Filamentous fungi form the major etiologic agents of fungal keratitis. *Fusarium* sp. (37%–62%) and *Aspergillus* sp. (24–30%) have been implicated as main pathogens.

Dematiaceous fungi are the cause of 8–16.7% of cases of fungal keratitis (Bharathi et al. 2003). Most filamentous fungi associated with corneal ulceration in the tropics are found widely within the environment. Yeast can also cause keratitis. The most common causes of fungal keratitis are *Fusarium solani* and other *Fusarium* sp., *Aspergillus* sp., and *Curvularia* sp. (Thomas 1994). There may be a hierarchy of fungi capable of producing keratitis, e.g., from most to least capable, *Fusarium*, *Acremonium*, and *Phialophora* spp. This hierarchy is predicated upon their individual ability to invade and destroy the cornea (Liesegang 1998).

4.1.1 Predisposing Risk Factors for Fungal Keratitis

Ocular fungal infections, or ophthalmic mycoses, are being increasingly recognized as an important cause of morbidity and blindness, and even certain types of ophthalmic mycoses may be life-threatening (Yohai et al. 1994; Levin et al. 1996). Keratitis (corneal infection) occurs most frequently (Srinivasan et al. 1991), but the orbit, lids, lacrimal apparatus, conjunctiva, sclera, and intraocular structures may also be involved (Fig. 4.1). However, here we will discuss infections related to corneal keratitis. A comprehensive review of fungal diseases of the eye, in particular endogenous and exogenous fungal endophthalmitis, has been published by different workers (Klotz et al. 2000; Thomas 2003a, b; Nayak 2008). A number of factors are known to promote mycotic ocular infections, as elaborated in Fig. 4.2.

Predisposing factors include trauma, contact lenses, and topical steroids. Trauma is the most important predisposing cause, followed by ocular and systemic defects, and prior application of corticosteroids. Previous history of ocular trauma (especially if organic matter is involved), agricultural occupations, age, recurrent ocular disease, exposure keratopathy, chronic keratitis, chronic use of steroids, systemic immunosuppressive disease also plays a role in fungal keratitis.

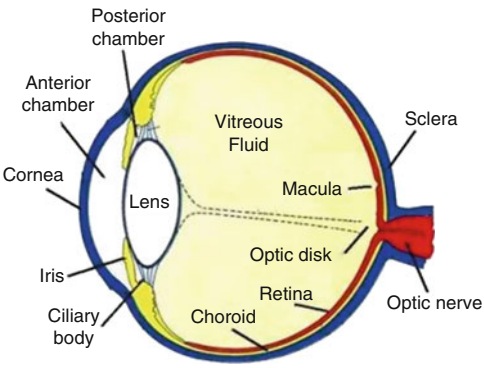

Fig. 4.1 Parts of the eye that may be infected from fungal pathogens

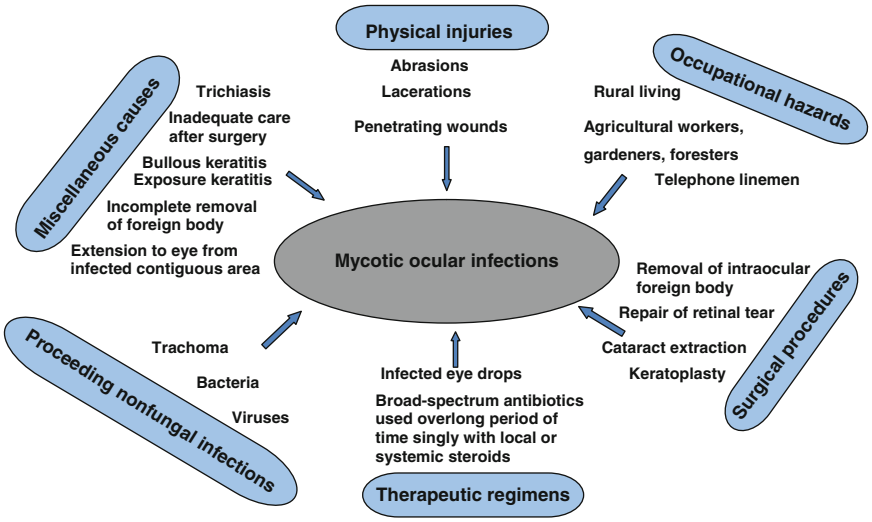

Fig. 4.2 Schematic illustration of factors that may promote mycotic ocular infections

4.1.1.1 Corneal Trauma

Injury to the cornea is the leading cause of microbial keratitis, particularly fungal keratitis. The nature of injury is often vegetative in origin, which may consist of trauma with plant twigs, rice-husk, cotton plant, etc. A history of corneal trauma with vegetable matter or organic matter is reported in 55%–65% of fungal keratitis (Bharathi et al. 2003). Trauma leads to destruction of the epithelium and Bowman's membrane, impairing the barrier to infection. The underlying stroma becomes excessively hydrated and possibly altered in such a way as to constitute a more favorable site for fungus to grow. Keratomycosis caused by filamentous fungi is an occupational hazard of farmers and agricultural workers. The seasonal variation noted in most series most probably represents occupational injuries associated with harvesting. On the other hand, mycotic infections, especially *Candida* spp., may develop in pre-existing lesions such as herpetic scars or neurotrophic keratitis, which alter local ocular immune defense (Sudan and Sharma 2003). Micro-organisms do not usually penetrate intact corneal epithelium. However, following trauma opportunistic fungi and bacteria may complicate the underlying disease, especially if caused by vegetative material. This is also the case with corneal diseases with epithelial disintegration, such as exposure keratitis, chronic corneal epithelial erosions, corneal degeneration, herpesvirus keratitis, and/or suppressed local immune system (corticosteroid treatment; Richter et al. 2003). A study from the northern United States reported trauma as the inciting event in only 8.3% of cases, whereas, in the southern United States, trauma was identified as a principal risk factor in 44% of children who had microbial keratitis and in 27% among 227 cases of microbial keratitis reported in a nonreferral county practice in southern California (Srinivasan 2004).

4.1.1.2 Contamination Through Contact Lenses

Several published case reports have identified contact lens wear as a risk factor for fungal keratitis in industrialized countries (29%; Hoflin-Lima and Roizenblatt 2002). Patients wearing any type of contact lens can get fungal keratitis. Most of the pathologies related to contact lens wearing occur in the anterior segment of the eyeball and clinically can be seen as serious inflammation. Nevertheless, using contact lenses without any coexisting symptoms does not guarantee their sterility, and may also be a source of infection. The factors causing infectious complications are connected with hygiene negligence, such as: improper disinfecting of contact lenses, usage of contaminated lenses, contact lens containers, and cleaning solutions, wearing contact lenses during eye infections, and contamination of contact lenses through the introduction of micro-organisms from the environment (Buczek-Kalakucka and Polz-Dacewicz 2006)

4.1.1.3 Use of Contaminated Topical Steroids

Many ophthalmologists identify topical steroids as the principal risk factor in enhancing ocular fungal growth. Steroid use as initial therapy was reported in 1%–30% of patients having microbial keratitis. Topical corticosteroid therapy has been associated with increased incidence and worsening of fungal keratitis (Bharathi et al. 2003).

The increased incidence is probably due to altered local immune response and increased rates of conjunctival colonization by fungi. Additionally, it indirectly promotes fungal replication and corneal invasion by interfering with the host's inflammatory response. The systemic use of corticosteroids may predispose to fungal keratitis by causing immunosuppression. *Candida* spp. commonly colonizes conjunctive and eye lid margins of normal individuals. However, it may produce keratitis in patients with impaired immune response. The local ocular resistance may be lowered by atopic disease, eyelid malposition, dry-eye conditions, and neurotrophic or herpetic keratitis predisposing to fungal keratitis (Sudan and Sharma 2003).

4.1.1.4 Other Factors

Other disorders, including corneal surface disorders, dry eye, bullous keratopathy, and exposure keratitis, are associated with the development of suppurative keratitis (Thomas 2003b). Several case reports of fungal keratitis after photorefractive keratectomy and Lasik have been published (Periman et al. 2003).

4.2 Infections of the Cornea

Fungal infection of the cornea, e.g., mycotic keratitis or fungal keratitis, was first observed in 1879 in Germany by Leber, and is now a major public health problem in the tropical parts of many developing nations, including India (Agrawal et al. 1994; Gunawerdena et al. 1994; Hagan et al. 1995; Thomas 2003a, b). Scarring of the cornea as a result of keratitis is one of the preventable causes of blindness and usually carries an unfavorable prognosis due to its protracted course and need for specific therapy (Baradkar et al. 2008). Corneal blindness is a major public health problem worldwide and infectious keratitis is one of the predominant causes. According to the World Health Organization, corneal diseases are a major cause of vision loss and blindness, second only to cataract in overall importance. It is estimated that ocular trauma and corneal ulceration result in 1.5–2 million new cases of corneal blindness annually (Whitcher et al. 2001). The incidence of ocular fungal infections has increased in the last few years due to the improvement in microbiologic diagnostic techniques and because of the introduction of new therapeutic measures such as widespread use of broad-spectrum antibiotics, immunosuppressive drugs, and corticosteroids (Saha and Das 2006).

The fungi most often isolated from human corneal ulcers are also known to inhabit normal eyes, and also commonly occur as saprobes in the soil and vegetable matter. Due to the abundance of fungi in human surroundings, it is difficult to assign a pathogenic role to them when they are isolated from the diseased eyes. The problem is further aggravated by the presence of predisposing factors such as the use of immunosuppressive agents, antibiotics, and physical injury to the eye. It was therefore thought that a simultaneous study in infected human eyes, normal human eyes, and the surroundings would be of help in elucidating some aspects of fungal infections of the eye (Sandhu et al. 1981).

If not treated early, this condition may lead to corneal blindness. The causal agent is largely filamentous fungi, although yeasts, particularly *Candida*, may also be responsible in a small number of cases. Fungal infections of the cornea (fungal keratitis or keratomycosis) may constitute 6%–53% of all cases of ulcerative keratitis, depending upon the country of origin of the study (Thomas 1994). Recently, Nayak (2008) has extensively reviewed such fungal infections of the eye with special focus on keratomycosis. The major risk factors for mycotic keratitis are trauma, systemic illness, previous eye surgery, contact lenses, and diabetes (Rosa et al. 1994; Gunawerdena et al. 1994; Panda et al. 1997; Srinivasan et al. 1997; Tanure et al. 2000; Gopinathan et al. 2002; Basak et al. 2005; Chowdhary and Singh 2005; Ula et al. 2009).

Most fungal keratitis occurs after trauma to the cornea in agricultural workers, usually, but not always, with fungus-contaminated plant material (leaves, grain, branches, or wood). The disease may also occur in gardeners and, following corneal trauma, from indoor plants as well. Occasionally the object striking the cornea is metal. The trauma to the cornea may be so slight as to be forgotten by the patient. Furthermore, fungal keratitis may also occur with contact lens wear. Trauma to the

cornea with vegetable matter either introduces the fungus directly into a corneal epithelial defect or, alternatively, the defect may become infected following the trauma (Klotz et al. 2000).

Keratomycosis is a suppurative, usually ulcerative, corneal disease. Infection is exogenous, with the organism entering through the corneal epithelium. The vast majority of cases of fungal keratitis are due to septate, filamentous and saprophytic fungi, mainly *Aspergillus* sp. and *Fusarium* sp.: others may include *Alternaria* sp., *Curvularia* sp., *Penicillium* sp., *Cladosporium* sp., and *Acremonium* sp., etc. and occasionally zygomycetes such as *Absidia* or *Rhizopus* spp. (Table 4.4; Schwartz et al. 1978; Marshall et al. 1997; Doczi et al. 2004). On the other hand, the abnormal or compromised cornea, e.g., chronic dry eye, is subject to infection with yeasts, usually *Candida* sp. Such uncommon *Candida* sp. as *Candida lipolytica* and *Candida humicola* have, however, been reported to cause posttraumatic keratitis (Nitzulescu and Niculescu 1975, 1976; Doczi et al. 2004) and *Candida guilliermondii* after corneal transplant (Ainbinder et al. 1998, Doczi et al. 2004). Fungal keratitis is recognizable by the presence of a coarse granular infiltration of the corneal epithelium and anterior stroma (Fig. 4.1). The corneal defect usually

Table 4.4 Fungi associated with keratitis

Fungal species	Ophthalmic infections (in which implicated)	References
Fusarium solani *F. oxysporum* *F. dimerum*	Keratitis, introcular infection	Tanure et al. (2000), Gopinathan et al. (2002)
Aspergillus fumigatus *A. flavus* *A. terreus*	Keratitis	Stevens et al. (2000), Gopinathan et al. (2002)
Paecilomyces lilacinus *P. variotii*	Keratitis, endopthalmitis	Rosa et al. (1994), Okhravi et al. (1997)
Acremonium kiliense *A. potronii*	Keratitis, endopthalmitis	Guarro et al. (1997), Read et al. (2000)
Curvularia lunata *C. geniculata* *C. senegalense*	Keratitis	Guarro et al. (1999), Garg et al. (2000)
Rhizopus arrhizus *Mucor* *Absisdia corymbifera*	Rhino-orbitocereberal, zygomycosis, keratitis, scleritis	Marshall et al. (1997), Locher et al. (1998)
Candida albicans *C. parasilosis*	Keratitis, inocular lesion	Rosa et al. (1994), Tanure et al. (2000)
Cryptococcus neoformans	Keratitis, chronic rhinitis	Morinelli et al. (1993), Tanure et al. (2000)
Paracoccus brasiliensis	Eyelids, cornea, conjuctiva	Burnier and Sant'Anna (1997)
Coccidioides immitis	Various	Rodenbiker and Ganley (1980)
Blastomyces dermatitidis	Lesion of eyelids, cornea, conjuctiva	Slack et al. (1992), Bartley (1995)
Histoplasma capsulatum	Endogenous, exogenous, retinis, optical neuritis	Yau et al. (1996), Gonzales et al. (2000)

becomes apparent within 24–36 h after the trauma. Some of the reports with multiple numbers of cases published in the last decade are briefly presented here.

Tanure et al. (2000) reported the spectrum of fungal keratitis at Wills Eye Hospital, Philadelphia from 24 eyes (24 patients) from 1991 to 1994. Predisposing factors included chronic ocular surface disease (OSD; 41.7%), contact lens wear (29.2%), atopic disease (16.7%), topical steroid use (16.7%), and ocular trauma (8.3%). Early identification of fungal elements was achieved by staining of corneal scrapings in 18 cases (75%). Half of the cases (12 eyes) had corneal infections caused by yeast, and the other half by filamentous fungi. *Candida albicans* was the most commonly isolated organism (45.8%), followed by *Fusarium* sp. (25%). They found *Fusarium* sp. the most commonly isolated filamentous fungus and *C. albicans* was the most frequent cause of fungal keratitis, and a past history of ocular trauma was uncommon.

Gopinathan et al. (2002) reported the retrospective laboratory results of 1,352 cases of fungal keratitis diagnosed at the L.V. Prasad Eye Institute (LVPEI) in south India from January 1991 to December 2000. Ocular trauma predisposed to infection in 736 (54.4%) of 1,354 eyes. A fungal cause was established by smears of corneal scrapings in 1,277 (95.4%) eyes. *Fusarium* (506, 37.2%) and *Aspergillus* sp. (417, 30.7%) have predominated the hyaline fungal spectrum (1,133) and *Curvularia* sp. (39, 2.8%) were the highest among the dematiaceous isolates (218).

Fungal keratitis is an uncommon complication in contact lens wearers: however, a study highlights use of contact lens cleaning solution as being responsible for causing a *Fusarium* outbreak from 2005 to 2006 in Singapore (Khor et al. 2006). During the study period, 66 patients (68 affected eyes) were diagnosed with *Fusarium* keratitis associated with contact lens wear; the estimated annual national incidence is 2.35 cases per 10,000 contact lens wearers. Patients ranged in age from 13 to 44 years (27.1 $\pm$ 8.4 years), of which 32 (48.5%) were men. The vast majority (65 patients; 98.5%) wore using soft, disposable contact lenses; 62 patients (93.9%) reported using one brand of contact lens cleaning solution (ReNu, Bausch & Lomb, Rochester, NY, USA), including 42 patients (63.6%) who recalled using ReNu with MoistureLoc. Most patients (81.8%) reported poor contact lens hygiene practices, including overnight use of daily wear contact lenses (19.7%), and use of contact lenses past the replacement date (43.9%). Because no predisposing factors, such as ocular trauma, pre-existing ocular diseases, topical steroid usage, or an exposure to Bausch & Lomb ReNu with the MoistureLoc cleaning solution, were involved in this case, poor contact lens hygiene was very probably the reason for this rare fungal infection. This study suggests that physicians and eye care practitioners worldwide need to be aware of the likelihood of similar outbreaks emerging among contact lens wearers.

Srinivas et al. (2007) presented a study over a period of 1 year in which they treated 12 patients with unilateral *Fusarium* keratitis. All patients were contact lens users who used ReNu MostureLoc contact lens solution and had no other specific predisposing conditions. Microbiological examination yielded growth of *Fusarium* spp. in seven patients from corneal scrapings at presentation and from three patients in subsequent corneal specimens. For two other patients, fungi were not detected from corneal scrapings, but *Fusarium* spp. was isolated from their contact lenses. The infections were treated with topical natamycin and amphotericin B eye drops,

and with systemic itraconazole in eight patients. The infection resolved with medical treatment in eight eyes, a conjunctival flap in one eye, and a therapeutic corneal graft in one eye. Two eyes required tectonic corneal grafts for perforation. Two of the three corneal grafts failed because of graft rejection. Final visual acuities ranged from counting fingers to 1.0. This cluster of *Fusarium* keratitis seems to be related to the use of the ReNu MoistureLoc contact lens solution. The cure rate with medical therapy was 66%. However, corneal scarring limited visual recovery. This episode highlights the need for clinical vigilance when dealing with corneal infiltrates in contact lens users.

Chowdhary and Singh (2005) reported the epidemiologic features and laboratory results of 191 consecutive cases of fungal keratitis presenting to a tertiary-level super-specialty teaching hospital (Department of Guru Nanak Eye Center, Maulana Azad Medical College, New Delhi) from January 1999 to June 2001. Diagnosis of mycotic keratitis was established in 191 (39%) out of the total study group of 485 cases. Direct microscopic examination of KOH mounts and Gram-stained smears revealed the presence of fungal elements in the corneal scrapings in 119 (62.3%) and 114 (60%) of the subsequently fungal culture-positive cases respectively. Predisposing risk factors were noted in 79%, with corneal trauma 42%, contact lens wear 25%, and topical corticosteroids in 21% patients. The spectrum of fungi isolated included *Aspergillus* sp. in 78 cases (41%) followed by *Curvularia* sp. in 55 cases (29%).

A prospective study of corneal ulcer was conducted by Shokohi et al. (2006) between May 2004 to March 2005 in Sari, North Iran. Patients who presented with clinically suspected corneal ulcer to the Ophthalmology Department of Bou-Ali Sina University Hospital in Sari were included in this study. A total of 22 patients met the inclusion criteria of this study, among whom ten (45.5%) were females and 12 (54.5%) were males. The investigation highlighted that infections of the cornea due to filamentous fungi are frequent causes of corneal damage. In direct microscopy, branching, and septate hyphae were identified in seven patients (31.8%). Two fungi (*Aspergillus fumigatus* and *Fusarium* spp.) were isolated (28.6%). Five patients (31.8%) with fungal keratitis were males and two (28.6%) were females. Three (42.85%) patients with fungal keratitis were farmers. Trauma with plant debris and straws were noted in two patients (28.6%) with fungal keratitis. Five patients (71.4%) received topical antibiotics.

A study was carried out by Saha and Das (2006) on patients attending tertiary care hospital, East Delhi. In this study, of the 346 patients with corneal ulcers investigated, in 77 of cases (22.25%) fungal etiology was identified. Males were more commonly affected, and were mostly in the age group of 31–40 years. It was seen that trauma was the most common predisposing risk factor especially in the agriculturists and the farmers. *Aspergillus flavus* was the most common fungus isolated in 31.16% of cases, followed by *A. fumigatus* (16.88%) and *Fusarium* spp. (7.79%). Yeasts were also isolated in 21.62% of cases. Both yeasts and mycelial fungi were isolated in 6.5% of cases.

David et al. (2007) reported an extensive study fungal keratitis seen at Moorfields Eye Hospital over a 13-year period to January 2007. There were 66 isolates from 65 patients. Forty (60.6%) of the isolates were subspecies of *Candida*.

Prior OSD or a penetrating keratoplasty (PK) was present in 38 patients (97.4%) with *Candida* infection, and 29 patients (74.4%) with *Candida* infection were using topical steroid at the time of diagnosis. The principal risk factors for filamentary fungal infection were trauma (eight cases, 30.8%) or cosmetic contact lens wear (eight cases, 30.8%), with OSD or a prior PK each present in five cases (19.2%). *Candida* was the principal isolate, usually from eyes with OSD or a prior PK treated with topical steroids.

A study conducted by Chander et al. (2008), carried out jointly by the Departments of Microbiology and Ophthalmology, Government Medical College Hospital, Chandigarh, over a period of 5 years from January 1999 to December 2003, revealed incidence of keratomycosis in and around Chandigarh. Out of 154 suspected patients, 64 cases were positive for agents. Most common fungal isolates found were *Aspergillus* sp. (41.18%) followed by *Fusarium* sp. (23.53%), *Candida* sp. (8.82%), *Curvularia* sp. (5.88%), and *Bipolaris* sp. (5.88%). This study highlighted hyaline filamentous fungi as most common etiological agents and mechanical trauma with vegetative matter as the most common predisposing factor (37.75%); other predisposing factors were chronic antibiotic usage (25%) and use of topical corticosteroids (7.81%).

Furthermore, a study by Baradkar et al. (2008) to identify aetiological agents and predisposing risk factors in mycotic keratitis, was conducted at Department of Microbiology, Lokmanya Tilak Municipal Medical College and General Hospital, Sion, Mumbai. Fungi were isolated in 13.07% of cases. Correlation between microscopy and culture was present in 58% of cases. The male:female ratio observed was 3:1. Filamentous fungi were isolated in 79.41% of cases and yeasts were isolated in 20.58% of cases. Predominant fungal isolates were *Aspergillus* sp. 17.64% (16/34), *Fusarium* sp. 14.7% (5/34). *Curvularia* sp., *Penicillium* sp., and *C. albicans* were isolated in three cases each (8.82% each); *Cladosporium* sp., *Dreschleria* sp., *Acremonium* sp., *Aureobasidium* sp. were isolated in two cases each (5.88% each), and *Alternaria* sp. and *Bipolaris* sp. in one each.

Ula et al. (2009) presented a report involving patient demographics, clinical and laboratory findings, and treatment and outcomes of 46 cases of culture-proven fungal keratitis diagnosed from January 2004 to November 2007, being compared with 23 cases of fungal keratitis previously collected over a similar period from January 1999 to November 2002 at the Massachusetts Eye and Ear Infirmary. They showed that during 2004–2007, the rate of fungal keratitis was 1.0 cases per month, an increase from the baseline rate of 0.5 cases per month during 1999–2002. The proportion of cases caused by filamentous fungi increased from 30% (1999–2002) to 65% (2004–2007; $p = 0.01$). Soft contact lens wear accounted for 41% of fungal keratitis cases in 2004–2007, as compared with 17% in 1999–2002. Patients with a history of corneal transplant had the highest rate of therapeutic keratoplasties (67%) and had the poorest visual outcome (40% counting fingers or less). In the contact lens group, 94% of patients maintained vision of at least 20/40 and only 12% required surgery to control the infection. The study concluded that there has been an increase in fungal keratitis in the Boston area and a change in the causative pathogens and risk factors for infection. Filamentous fungi now account for the majority of fungal

keratitis cases, whereas yeasts were the predominant pathogen in the past. Soft contact lens wear is currently the most common risk factor for development of fungal keratitis.

Elmer (2009) reviewed the cases of *Alternaria* keratitis diagnosed and treated at the University of Illinois Eye and Ear Infirmary from 1999 to 2007 for clinical presentation, antifungal therapy, and final visual acuity. They found that *Alternaria* keratitis has a varied clinical presentation and may present without macroscopic pigmentation.

A study carried out by Saha et al. (2009) at Priyamvada Birla Arvind Eye Hospital, West Bengal, from January to December 2008 highlighted *Aspergillus* sp. as the predominant group (55.40%) of fungi causing keratitis among 110 patients, followed by *C. albicans* (18.91%) and *Fusarium* sp. (10.81%). Agricultural activity-related ocular trauma was the principal cause of such keratitis.

4.3 Diagnosis of Fungal Infections of the Eye

Fungal infections of the cornea are frequently caused by species of *Fusarium*, *Aspergillus*, *Curvularia*, and *Candida*. Fungal keratitis usually manifests as a rapidly developing process, with fungi frequently found deep in the equine cornea. Fungi and bacteria are capable of promoting enzymatic degradation of the cornea. This process is aggravated by attracted neutrophils, which degranulate in the corneal stroma and release additional lytic enzymes. The diagnosis of keratomycosis is made by the finding of fungal elements during cytologic examination of corneal scrapings or mycologic culture, or during histopathologic examination of a keratectomy specimen (Richter et al. 2003).

Culture remains the cornerstone of diagnosis; direct microscopic detection of fungal structures in corneal scrapes or biopsies permits a rapid presumptive diagnosis. The most critical pieces of information regarding infections of the eye are the clinical history, clinical examination, and accurate identification of the causative micro-organisms. A good history and eye examination may provide sufficient information to suggest the pathogenesis of the disease and likely micro-organisms. For example, diminishing vision and pain in the eye of a patient wearing contact lenses in the presence of a corneal ulcer strongly suggest an infectious keratitis caused by bacteria, saprophytic fungi, or amoebae. The diagnosis of fungal infections requires the clinician to (1) establish the presence of ophthalmic pathology (which may require special instruments, such as a scanning slit confocal microscope; Florakis et al. 1997), (2) obtain tissue in which the fungus is visualized, and (3) isolate the responsible fungus. Fungal isolation by culture is particularly important since tissue strains frequently do not allow one to determine the identity of filamentous fungi or yeasts with any degree of certainty. Isolation allows one to perform both authoritative identification and antifungal testing when necessary. In most other circumstances the clinician will be obliged to establish the diagnosis by isolating the causative micro-organism directly from the eye or adnexal tissue.

Aspergillus, *Fusarium* spp. (Ando and Takatori 1982; Sehgal et al. 1981), and occasionally *C. albicans* (Schwab and Dawson 1995) are cultured from the conjunctiva of healthy and diseased eyes; therefore, it is difficult to establish saprophytic fungal isolates as pathogens of the conjunctiva unless a biopsy is performed, and this is rarely necessary. *Pneumocystis carinii* may cause conjunctivitis, but this organism cannot be cultured (Ruggli et al. 1997). Material may be expressed from an infected lacrimal duct or, if required, an incision can be made and tissue can be obtained for culture and appropriate stains. Not infrequently, invasive orbital disease arises from the paranasal sinuses, usually the ethmoid and sphenoid sinuses. A computerized tomography (CT) scan of the orbit and paranasal sinuses will establish the extent of disease, and biopsy, curettage, and drainage of the infected sinus can obtain adequate material. *Aspergillus* spp., zygomycetes, and other filamentous fungi are the usual pathogens. Rhinocerebral zygomycosis involving the orbit can often be diagnosed by biopsy of necrotic tissue from the hard palate or nose. The use of an exceedingly thin, round-ended platinum spatula or, alternatively, a scalpel blade or small needle allows for scrapings to be obtained from the corneal surface for stains and cultures (Wilhelmus et al. 1994). Ample tissue is needed, so multiple corneal scrapings are usually performed. Biopsy of the cornea or keratoplasty may be required to provide sufficient diagnostic material. Saprophytic filamentous fungi more often than not cause post-traumatic keratomycosis. Rarely are yeasts involved in post-trauma keratitis (Thomas 1994).

4.3.1 The Detection of Fungal Elements

In tissue or smears detection of fungal elements is enhanced and detected with significantly greater sensitivity using acridine orange (Kanungo et al. 1991), Calcofluor white (Chander et al. 1993), or lactophenol cotton blue (LCB; Thomas et al. 1991; Byrne et al. 1995) stains. The first two stains have the added advantage of demonstrating other pathogens, such as bacteria, amoebic exocysts, and microsporidial spores. This may be important, because traumatic injuries to the cornea may involve more than one pathogen. Similarly, the use of a battery of fluorescein-conjugated lectins has been shown to be useful in detection of ocular mycoses (Robin et al. 1986). The use of such tests as a chitin assay (Lamps et al. 1995) or polymerase chain reaction (PCR; Okhravi et al. 1998) may prove useful, but these assays currently suffer from a cumbersome technique in the former and lack of detection across fungal genera in the later.

4.3.2 Culture and Identification

Apart from the conventional culture techniques on SDA slants, and LCB preparation of the growth for distinguishing between yeasts and mycelia, and for the identification

of mycelia fungi, one can also opt for a slide culture technique (Rippon 1982) which visualizes aerial hyphae of molds, making the microscopic identification easier. Yeasts can be speciated by looking for chlamydospore formation on cornmeal agar and germ tube production, as well as by using various sugar fermentation and assimilation tests, urease test, and other biochemical tests (Rippon 1988).

4.3.3 Molecular Methods for the Diagnosis of Mycotic Keratitis

PCR assay, PCR-SSCP (single strand conformation polymorphism) and PCR-RFLP (restriction fragment length polymorphism) techniques have also been standardized for fungal identification (Chen et al. 2002). Of these, the PCR is universally accepted as the most popular technique as it can yield quick results, confirming the diagnosis of mycotic keratitis within a few hours, whereas culture takes at least 5–6 days for a positive detection (Ferrer et al 2002). Gaudio et al. (2002) developed a PCR-based assay to amplify a part of the fungal 18S r-RNA gene, which was used for detection of fungal DNA in corneal scrapings. PCR and fungal culture results matched in 74% of cases. Thus at present PCR assay seems quite promising for the diagnosis of fungal keratitis, offering a definite advantage over culture methods. However, its main drawback is its occasional false positivity that can be overcome by application of stringency in laboratory procedures and proper standardization of the techniques. Despite this, PCR remains an effective method for diagnosing keratomycosis. It is also a more sensitive and rapid method than the conventional mycologic procedures. In addition, PCR is of great benefit in rapidly detecting the presence of the organism difficult to culture. The sensitivity of PCR, taking culture as the gold standard, was quite high, between 89% and 94%, whereas specificity ranged from 50% to 88% (Ferrer et al. 2002; Gaudio et al. 2002). Some of the well-cited studies on molecular identification of ocular fungi are summarized here.

Kappe et al. (1998) used molecular probe for detection of pathogenic fungi in the presence of human tissue. Four primer systems, amplifying fragments of the gene coding for the small ribosomal subunit (18S r-RNA), were characterized with pure cultures of 65 medically relevant fungal species plus two mushrooms. A primer cocktail (TR1/CA1-TR2/AF2) amplified 59 of 67 fungal species; the universal fungal primer 1 (UF1), in combination with the eukaryotic primers S3 or EU1, amplified 64 and 65 of 67 fungal species respectively. The design of an additional primer (RZY1) enabled the amplification of the missing members of the zygomycetes. The primer systems amplified all the medically relevant fungi tested. These included eight *Candida* spp. and seven other yeast species, 13 dermatophytes, 32 molds (including six zygomycetes and five dimorphic fungi), and two mushrooms. Eleven controls including DNA from Schistosoma mansoni, *Escherichia coli*, *Mycobacterium tuberculosis* and human tissue were not amplified. The oligonucleotide CA hybridized with *C. albicans*, *Candida tropicalis*, and *Candida parapsilosis*; the oligonucleotide TR hybridized with the 13 dermatophytes; the

oligonucleotide AF hybridized with *A. fumigatus, A. flavus, Aspergillus terreus, Aspergillus nidulans, Aspergillus versicolor, Aspergillus tamarii, Aspergillus clavatus,* and *Aspergillus fischeri,* but not with *A. niger* or *A. versicolor*; and the oligonucleotide HC hybridized with three varieties of *Histoplasma capsulatum*. These oligonucleotides did not hybridize with the other fungi or the controls. The specificity of the designed primer systems was confirmed by selective amplification of fungal DNA from human lung tissue spiked with fungal biomass and from vitrectomy fluid of a patient with *Candida* endophthalmitis.

Okhravi et al. (1998) determined the usefulness of PCR and RFLP analysis in the identification and speciation of *Candida* spp. that causes ocular infection. Oligonucleotide primers based on the cytochrome P450 L1 A1 demethylase gene were used to successfully amplify by PCR a single 1.0 kb and a single 500 bp DNA fragment from *C. albicans, C. tropicalis, Candida krusei, C. glabrata, C. parapsilosis,* and *Candida pelliculosa* genomic DNA. RFLPs within the PCR product were identified after restriction enzyme digestion. The sensitivity of the amplification reaction after two rounds of PCR was 10 fg genomic *C. albicans* DNA or one copy of the gene. No amplification product was obtained when DNA from *C. guilliermondii, A. fumigatus, F. solani*, human leukocytes, or ten species of bacteria was used as a template. Experiments with spiked normal vitreous demonstrated equal sensitivity as long as the volume of vitreous did not exceed 20% of the total PCR volume. RFLP analysis of the PCR product generated from each species obtained from the first- and second-round amplification products enabled species identification after digestion with specific endonucleases. Application of the technique to four clinical samples was successful. PCR-RFLP analysis has great potential in the rapid detection and identification of *Candida* spp. and in the provision of a useful laboratory tool.

Ferrer et al. (2002) used molecular techniques to determine whether sequence analysis of internal transcribed spacer/5.8S ribosomal DNA (rDNA) can be used to detect fungal pathogens in patients with ocular infections (endophthalmitis and keratitis). Internal transcribed spacer 1 (ITS1) and ITS2 and 5.8S rDNA were amplified by PCR and seminested PCR to detect fungal DNA. Fifty strains of 12 fungal species (yeasts and molds) were used to test the selected primers and conditions of the PCR. PCR and seminested PCR of this region were carried out to evaluate the sensitivity and specificity of the method. It proved possible to amplify the ITS2/5.8S region of all the fungal strains by this PCR method. All negative controls (human and bacterial DNA) were PCR-negative. The sensitivity of the seminested PCR amplification reaction by DNA dilutions was one organism per PCR, and the sensitivity by cell dilutions was fewer than ten organisms per PCR. Intraocular sampling or corneal scraping was undertaken for all patients with suspected infectious endophthalmitis or keratitis (nonherpetic) respectively, between November 1999 and February 2001. PCRs were subsequently performed with 11 ocular samples. The amplified DNA was sequenced, and aligned against sequences in GenBank at the National Institutes of Health. The results were PCR-positive for fungal primers for three corneal scrapings, one aqueous sample, and one vitreous sample; one of them was negative by culture. Molecular fungal

identification was successful in all cases. Bacterial detection by PCR was positive for three aqueous samples and one vitreous sample; one of these was negative by culture. Amplification of ITS2/5.8S rDNA and molecular typing shows potential as a rapid technique for identifying fungi in ocular samples.

Various other workers have used molecular techniques for identification and diagnoses of pathogenic fungi from eye infection and other sources.

4.4 Soil and Other Environments as Reservoir of Ocular Fungi

Fungi are ubiquitous members of soil microbial communities, but constitute a varying proportion of the biomass in different systems. They tend to dominate in soils containing high proportions of organic matter and low pH, and generally constitute a smaller proportion in intensively managed mineral soils. They are involved in a plethora of functional roles in soil. The fungi are an immensely diverse group of organisms, encompassing a huge range of forms from microscopic single-celled yeasts to large macrofungi, as exemplified by the well-known mushrooms and toadstools and the largest of fruitbodies, the giant puffball (Bridge and Spooner 2001).

The great majority of the >80,000 fungal species so far named and described are likely to occur in the soil environment at some stage in their life-cycle. Fungi therefore have many different functions in soils, which include both active roles, such as the degradation of dead plant material, and inactive roles where propagules are present in the soil as resting states. Survey of the soil fungal diversity, which were popular during the 1960s and 1970s, have reappeared in the literature with the advent of DNA-based, culture-independent methods of analysis. The development of molecular techniques has provided a new range of tools that can provide clear insights into specific interactions and activities in soil environments. The combination of broad-spectrum PCR detection, coupled with SSCP or denaturing gradient gel electrophoresis, can give more accurate answers to fundamental questions on ecosystem diversity. This technique does not however distinguish between active and resting stages, and in order to interpret results accurately, some a priori knowledge is necessary (Bridge and Spooner 2001). Thus it is not surprising that opportunistic and pathogenic fungi are isolated from soil. It has long been known that soil is the main reservoir of ocular fungi. However, in recent years the number of fungal infections in human eyes has increased, which triggers an interest to examine the source and reservoir of such fungi and how they cause of various fungal infections in the eye.

Fungi are ubiquitous in the natural environment, appearing in air, water, and soil. The diversity of free living micro-soil fungi has been well-established and known for a very long time (Srivastava and Mishra 1972; Alexander 1985; Ahmad 1988; Paul 2007; Imran 2009). Occurrence of such fungi from environmental sources including air is expected. In poorly ventilated buildings with damaged and poor air-conditioning systems, there may be an increase in the concentration of

mycotoxicogenic molds, *Penicillium* and *Aspergillus* spp. (Garrison et al. 1993). Similarly, airborne microflora in hospital rooms were the subject of numerous studies as a potential cause of hospital infections (Herman 1980; Li and Hou; 2003; Arnow et al. 1991; Pini et al. 2004). A study from Tabbara and Al Jabarti (1998) reported an outbreak of *Aspergillus* endophthalmitis in five patients after cataract extraction during hospital construction in Jeddah, Saudi Arabia. Severe postoperative uveitis occurred in all five patients and failed to subside with topical steroid therapy. The causative organism was identified as *A. fumigatus* in each case.

In a well-designed study, air-borne fungi in the Departments of Dermatology, Venereology and Allergology of the Medical University in Wroclaw, Poland were studied by Lukaszuk et al. (2007). Thus, air is the common source of fungal spores and the fungal aerosols and can enter into the host body by direct contact or through inhalation. On the other hand, soil is considered as the major reservoir for almost all types of fungi — saprophytes, pathogenic, symbiotic, etc. These fungi are associated with plants and or soil have good opportunity to come in contact to human eye specially to agricultural workers, farmers, during sowing, plowing field, crop harvesting and processing of crops. Other reservoir of fungi which may come in contact to human is animals and organic matter including; animal wastes FYM may be potential source of ocular fungi. Fungi associated with normal eye may constitute potential ocular pathogenic or opportunistic fungi. In a separate study of soil fungi from the same geographical area, occurrence of the soil fungi isolated and identified includes species of *Aspergillus, Trichoderma, Geotrichum, Alternaria, Monilia,* and *Mycelia sterilia* (Zafar and Ahmad 2005).

A more extensive investigation of the soil fungi from agricultural soil of Aligarh showed 2.5×10^5 to 7.9×10^5 $CFUg^{-1}$ of filamentous soil fungi. Common members of the genera were *Aspergillus, Penicillium, Rhizopus, Curvularia, Trichoderma, Trichophyton, Mucor, Mycelia sterillia, Monotospora, Verticillium, Alternaria, Cladosporium, Hormodendrum, Trichothecium*; and *Fusarium* (Imran 2009).

A comparative study of the occurrence of fungi from diseased human eye (patients attended the Institute of Ophthalmology, JawaharLal Nehru Medical College and hospital for eye microbiological examination) during the year 1988 was analyzed by Ahmad (1988), and is presented in Table 4.5. In this comparative study occurrence of the filamentous fungi from diseased human eye, soil, plant material, and animal body surface indicated the common occurrence of various fungi both from diseased human eye and environmental sources. The *Aspergillus* was the most common fungi isolated both from environment and diseased eyes. Among *Aspergillus* isolates, *A. niger* (Fig. 4.3a), *A. glaucus, A. flavus* and *A. terreus* (Fig. 4.3b) were commonly isolated. Other fungi such as *Alternaria* (Fig. 4.3c) and *Microsporum* (Fig. 4.3d) were also isolated from soil and normal eye.

Occurrence of such fungi from the hospital environment, including air, was also reported by Lukaszuk et al. (2007) from Poland. *A. niger, Penicillium citrinum* and *C. albicans* were isolated more frequently in the air of the Department, of Dermatology, Venereology and Allergology of the Medical University in Wroclaw.

Table 4.5 Occurrence of fungi in human eyes and other sources

Source of isolation	No. of samples examined (% of positive sample)	Common fungi isolated
Diseased human eye (corneal ulcer, cataract, conjunctivitis)	90 (24.4)	*Aspergillus* spp., *Fusarium* spp., *Alternaria* spp., *Trichophyton* spp., *Microsporum* spp., *Curvularia* spp., *Rhizopus* spp.
Soil from agricultural fields	10 (100)	*Aspergillus* spp., *Alternaria* spp., *Curvularia* spp., *Trichophyton* spp., *Fusarium* spp., unidentified
Plant material (at wheat-thrashing site)	10 (100)	*Aspergillus* spp., *Rhizopus* spp., unidentified
Animal tail (buffalo)	10 (80)	*Aspergillus* spp., *Rhizopus* spp., *Mycelia sterilia,* unidetified

Values in *parenthesis* indicate number of positive samples

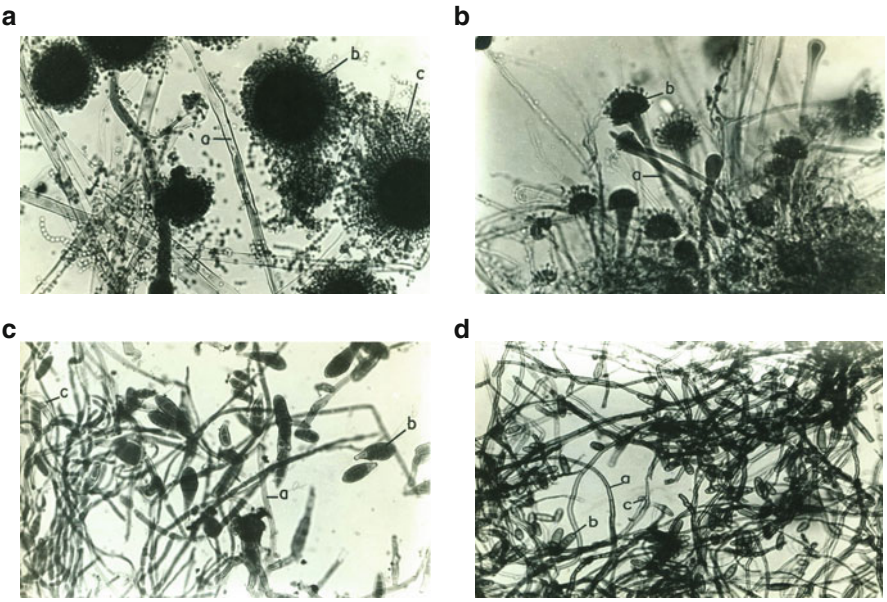

Fig. 4.3 **a** Microscopic photographs of *A. niger* isolated from diseased eye: (*a*) conidiophore, (*b*) radiate head, and (*c*) chain of conidia. **b** Microscopic photographs of *A. terreus* isolated from diseased eye: (*a*) conidiophore, and (*b*) vesicle having fertile sterigmate. **c** Microscopic photographs of *Alternaria* sp. isolated from diseased eye: (*a*) septate hyphae, (*b*) conidium showing longitudinal, and (*c*) transverse septations. **d** Microscopic photographs of *Microsporum* sp. isolated from diseased eye: (*a*) septate hyphae, (*b*) macroconidia showing septations, and (*c*) microconidium

Furthermore, some studies have highlighted animal as reservoir for these infections. Humans coming in contact with these infected animals by one or other means may spread fungal keratitis. In India, 50% of cases of keratomycosis in human beings are reported in association with injuries by paddy straw, stalk, sheath, and

thorns (Sansom et al. 2005). Among domestic animals, fungal keratitis is most common in the horse. One study of the conjunctival fungal flora of several domestic animal species, including horses and cattle, incorporated cows and horses without clinical evidence or recent history of keratitis or other ocular disease (Elligott et al. 2006). In a study investigating the fungal flora of the normal conjunctiva, fungi were isolated in 94% of the horses, in 100% of the cows, in 22% of the dogs and in 40% of the cats. Yeasts comprised 13% of the isolates of the fungal flora. The most common fungal species isolated from the normal equine eye are *Aspergillus*, *Fusarium*, *Penicillium, Alternaria* and *Cladosporium* spp. (Richter et al. 2003). Fungi were isolated from conjunctival swabs of 100% of the bovine and 95% of the equine subjects. Of a total of 95 bovine fungal isolates, 75 of which were identifiable, *Penicillium* sp. and *Cladosporium* sp. were most frequently isolated, accounting for 11 (12%) and 16 (16%) of the isolates respectively. Only three (3%) of the bovine fungal isolates were identified as *Aspergillus* sp. Of a total of 88 equine conjunctival fungal isolates, 81 of which were identifiable, 23 of the isolates (26%) were identified as *Aspergillus* sp. *Penicillium* sp. were the next most frequently isolated fungi among the horses, accounting for 19 (22%) of the total isolates (Elligott et al. 2006).

Thus based on the comparative occurrence of various fungal genera in various environmental sources (soil, air, plant and animals) and human eye, it can be concluded that soil is the main reservoir of the mycotic flora including ocular fungi. The possible mode of transmission of fungi from various sources to the eye is depicted in schematic diagram Fig. 4.4.

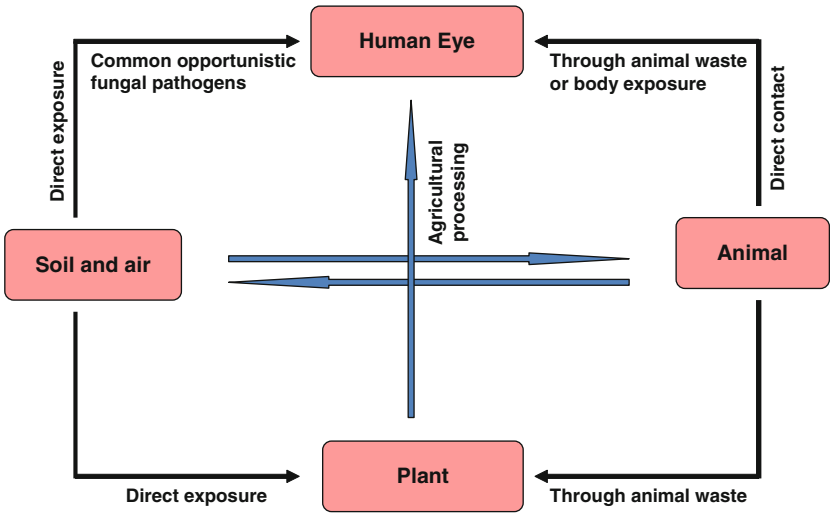

Fig. 4.4 Possible mode of transmission of fungi between eye and other reservoirs

4.5 Conclusion

Fungal infections of the cornea continue to be an important cause of ocular morbidity, particularly in the agricultural communities of the developing world. A proper understanding of agent and host factors involved in these infections will improve the outcome of this condition. Fungi isolated from normal eyes, diseased eyes and various reservoirs such as soil have great morphological and even taxonomic similarities. This indicates that fungi of the eye could be easily isolated from different sources which may act as reservoir for these fungi. Future research into ophthalmic mycoses reservoirs and their contribution to the increasing number of cases of fungal infection is needed. The use of improved diagnostic techniques and to distinguish between opportunistic, pathogenic, and nonpathogenic ocular fungi present in different reservoirs and the sources of contamination of such fungi in the eye needs to be extensively examined. Rapid and accurate identification of the fungal species which have potential to cause ocular infection will permit the immediate initiation of specific preventive, prophylactic, or antifungal therapy. A possible mode of transmission and factors encouraging fungi to reside in the normal eye need to be investigated.

References

Agrawal V, Biswas J, Madhavan HN, Mangat G, Reddy MK, Saini JS, Sharma S, Srinivasan M (1994) Current perspectives in infectious keratitis. Indian J Ophthalmol 42:171–192

Ahmad I (1988) A comparative study of the pathogenic and saprophytic fungi of human eye, soil, plant and animal sources. MSc (Microbiology) dissertation, Aligarh Muslim University, Aligarh

Ainbinder D, Parmley VC, Mader TH, Nelson ML (1998) Infectious crystalline keratopathy caused by *Candida guilliermondii*. Am J Ophthalmol 125:723–725

Alexander M (1985) Introduction to soil microbiology, 2nd edn. John Wiley, New York

Al-Rajhi A, Awad AH, Al-Hedaithy SS, Forster RK, Caldwell KC (1993) *Scytalidium dimidiatum* fungal endophthalmitis. Br J Ophthalmol 77:388–390

Ando N, Takatori K (1982) Fungal flora of the conjunctival sac. Am J Ophthalmol 94:67–74

Aristimuno B, Nirankari VS, Hemady RK, Rodrigues MM (1993) Spontaneous ulcerative keratitis in immunocompromised patients. Am J Ophthalmol 115:202–208

Arnow PM, Sadigh M, Costas C, Weil D, Chudy R (1991) Endemic and epidemic aspergillosis associated with in-hospital replication of *Aspergillus* organism. J Infect Dis 164:998–1002

Baradkar VP, De A, Mathur M, Lanjewar M, Kumar S (2008) Mycotic keratitis from Mumbai. Bombay Hosp J 50:200–204

Bartley GB (1995) Blastomycosis of the eyelid. Ophthalmology 102:2020–2023

Basak SK, Basak S, Mohanta A, Bhowmick A (2005) Epidemiological and microbiological diagnosis of suppurative keratitis in Gangetic West Bengal, Eastern India. Indian J Ophthalmol 53:17–22

Bharathi MJ, Ramakrishnan R, Vasu S, Meenakshi R, Palaniappan R (2003) Epidemiological characteristics and laboratory diagnosis of fungal keratitis: a three-year study. Indian J Ophthalmol 51:315–321

Borderie V, Bourcier TM, Poirot JL, Baudrimont M, Prudhomme de Saint-Maur P, Laroche L (1997) Endophthalmitis after *Lasiodiplodia theobromae* corneal abscess. Graefes Arch Clin Exp Ophthalmol 235:259–261

Bridge P, Spooner B (2001) Soil fungi: diversity and detection. Plant Soil 232:147–154

Buczek-Kalakucka S, Polz-Dacewicz M (2006) Asymptomatic and symptomatic ocular infections related to contact lens wearing. Ann Univ Mariae Curie 61:731–734

Burnier SV, Sant'Anna AE (1997) Palpebral paracoccidioidomycosis. Mycopathologia 140:29–33

Byrne KA, Burd EM, Tabbara KF, Hyndiuk RA (1995) Diagnostic microbiology and cytology of the eye. Butterworth-Heinemann, Boston, MA

Cameron J, Badr IA, Risco JM, Abboud E, Gaonnah S (1998) Endophthalmitis cluster from contaminated donor corneas following penetrating keratoplasty. Can J Ophthalmol 33:8–13

Carney MD, Tabassian A, Guerry RK (1996) Pseudo-*Allescheria boydii* endophthalmitis. Retina 16:263–264

Chander J, Chakrabarti A, Sharma A, Saini JS, Panigarhi D (1993) Evaluation of Calcofluor staining in the diagnosis of fungal corneal ulcer. Mycoses 36:243–245

Chander J, Singla N, Agnihotri N, Arya SK, Deep A (2008) Keratomycosis in and around Chandigarh: a five year study from a north Indian tertiary care hospital. Indian J Pathol Microbiol 51:304–306

Chen SC, Halliday CL, Meyer W (2002) A review of nucleic acid diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays. Med Mycol 40:333–357

Chowdhary A, Singh K (2005) Spectrum of fungal keratitis in North India. Cornea 24:8–15

Coccia L, Calista D, Boschini A (1999) Eyelid nodule: a sentinel lesion of disseminated cryptococcosis in a patient with acquired immunodeficiency syndrome. Arch Ophthalmol 117:271–272

Das T, Gopinathan U, Sharma S (1994) Exogenous *Helminthosporium* endophthalmitis. Br J Ophthalmol 78:492–493

David GJ, Stephen TJ, Andrew R, John DKG (2007) Fungal keratitis in London: microbiological and clinical evaluation. Cornea 26:1082–1086

Doczi I, Gyetvai T, Kredics L, Nagy E (2004) Involvement of Fusarium spp. in fungal keratitis. Clin Microbiol Infect 10(9):773–776

Elligott CR, Wilkie DA, Kuonen VJ, Bras ID, Neihaus A (2006) Primary *Aspergillus a*nd *Fusarium* keratitis in a Holstein cow. Vet Ophthalmol 9:175–178

Elmer TY (2009) Alternaria keratitis: clinical presentation and resolution with topical fluconazole or intrastromal voriconazole and topical caspofungin. Cornea 28:116–119

Eschete M, King JW, West BC, Oberle A (1981) *Penicillium chrysogenum* endophthalmitis. Mycopathologia 74:125–127

Ferrer C, Munoz G, Alio JL, Abad JL, Colon F (2002) Polymerase chain reaction diagnosis in fungal keratitis caused by *Alternaria alternata*. Am J Ophthalmol 33:398–399

Florakis G, Moazami G, Schubert H, Koester CJ, Auran JD (1997) Scanning slit confocal microscopy of fungal keratitis. Arch Ophthalmol 115:1461–1463

Font R, Parsons MA, Keener MJ, Shaver RP, Foos RY (1995) Involvement of anterior chamber angle structures in disseminated histoplasmosis: report of three cases. Ger J Ophthalmol 4:107–115

Fridkin S, Kremer FB, Bland LA, Padhye A, McNeil MM, Jarvis WR (1996) *Acremonium kiliense* endophthalmitis that occurred after cataract extraction in an ambulatory surgical center and was traced to an environmental reservoir. Clin Infect Dis 22:222–227

Garg P, Gopinathan U, Choudhary K, Rao GN (2000) Keratomycosis – clinical and microbiologic experience with dematiaceous fungi. Ophthalmology 107:574–580

Garrison RA, Robertson LD, Koehn RD, Wynn SR (1993) Effect of heating–ventilation–air conditioning system sanitation on air borne fungal populations in residential environments. Ann Allergy 71:548–556

Garrity JD, Herman C, Imes R, Fries P, Hughes CF, Campbell RJ (1993) Optic nerve sheath decompression for visual loss in patients with acquired immunodeficiency syndrome and cryptococcal meningitis with papilledema. Am J Ophthalmol 116:472–478

Gaudio PA, Gopinanthan U, Sangwan V, Hughes TE (2002) Polymerase chain reaction based detection of fungi in infected corneas. Br J Ophthalmol 86:755–760

Glasgow B, Engstrom RE Jr, Holland GN, Kreiger AE, Wool MG (1996) Bilateral endogenous *Fusarium* endophthalmitis associated with acquired immunodeficiency syndrome. Arch Ophthalmol 114:873–877

Gonzales CA, Scott IU, Chaudhry NA, Luu KM, Miller D, Murray TG, Davis JL (2000) Endogenous endophthalmitis caused by *Histoplasma capsulatum* var. *capsulatum*. A case report and literature review. Ophthalmology 107:725–729

Gopinathan U, Garg P, Fernandes M, Sharma S, Athamanathan S, Rao GN (2002) The epidemiological features and laboratory results of fungal keratitis: a 10 year review at a referral eye care center in South India. Cornea 21:555–559

Guarro J, Gams W, Pujol I, Gene J (1997) *Acremonium* species: new emerging fungal opportunists – *in vitro* antifungal susceptibilities and review. Clin Infect Dis 25:1222–1229

Guarro J, Akiti T, Almada-Horta R, Leite-Filho LAM, Gene J, Ferreira-Gomes S, Aguilar C, Ortoneda M (1999) Mycotic keratitis due to *Curvularia senegalensis* and *in vitro* antifungal susceptibilities of *Curvularia* spp. J Clin Microbiol 37:4170–4173

Gunawerdena SA, Ranasinghe KP, Arseceuleratne SN, Scimon CR, Ajello L (1994) Survey of mycotic and bacterial keratitis in Sri Lanka. Mycopathologia 127:77–81

Hagan M, Wright E, Newman M, Dolin P, Johnson G (1995) Causes of suppurative keratitis in Ghana. Br J Ophthalmol 79:1024–1028

Hammer M, Harding S, Wynn P (1983) Post-traumatic fungal endophthalmitis caused by *Exophiala jeanselmei*. Ann Ophthalmol 15:853–855

Herman LG (1980) *Aspergillus* in patient care areas. Ann N Y Acad Sci 353:140–146

Hoflin-Lima AL, Roizenblatt R (2002) Therapeutic contact lens-related bilateral fungal keratitis. CLAO J 28:149–150

Imran M (2009) Interaction of heavy metals with indigenous isolates of free living rhizospheric fungi and their plant growth promoting potentials. PhD thesis submitted to Aligarh Muslim University, Aligarh

Jager M, Chodosh J, Huang AJ, Alfonso EC, Culbertson WW, Forster RK (1994) *Aspergillus niger* as an unusual cause of scleritis and endophthalmitis. Br J Ophthalmol 78:584–586

Johnson TE, Casiano RR, Kronish JW, Tse DT, Meldrum M, Chang W (1999) Sino-orbital aspergillosis in acquired immunodeficiency syndrome. Arch Ophthalmol 117:57–64

Kanungo R, Srinivasan R, Rao RS (1991) Acridine orange staining in early diagnosis of mycotic keratitis. Acta Ophthalmol 69:750–753

Kappe R, Okeke CN, Fauser C, Maiwald M, Sonntag HG (1998) Molecular probes for the detection of pathogenic fungi in the presence of human tissue. J Med Microbiol 47:811–820

Khor WB, Aung T, Saw SM, Wong TY, Tambyah PA, Tan AL, Beuerman R, Lim L, Chan WK, Heng WJ, Lim J, Loh RSK, Lee SB, Tan DTH (2006) An outbreak of *Fusarium* keratitis associated with contact lens wear in Singapore. JAMA 295:2867–2873

Klotz SA, Penn CC, Negvesky GJ, Butrus SI (2000) Fungal and parasitic infections of the eye. Clin Microbiol Rev 13:662–685

Lamps C, Oeltmann TN, Collins MJ Jr, Robinson RD, Logan RA, Head WS, O'Day DM (1995) Development of a chitin assay for the quantification of fungus. Curr Eye Res 14:637–641

Lee BL, Holland GN, Glasgow BJ (1996) Chiasmal infarction and sudden blindness caused by mucormycosis in AIDS and diabetes mellitus. Am J Ophthalmol 122:895–896

Levin LA, Avery R, Shore JW, Woog JJ, Baker AS (1996) The spectrum of orbital aspergillosis: a clinicopathological review. Surv Ophthalmol 41:142–154

Li CS, Hou PA (2003) Bioaerosol characteristics in hospital clean rooms. Sci Total Environ 305:169–176

Liesegang TJ (1998) Fungal keratitis. In: Kaufman HE, Barron BA, McDonald MB (eds) The Cornea. Butterworth-Heinemann, Boston, MA, pp 219–245

Locher DH, Adesina A, Wolf TC, Imes CB, Chodosh J (1998) Post-operative *Rhizopus* scleritis in a diabetic man. J Cataract Refract Surg 24:562–565

Lukaszuk C, Krajewska-Kulak E, Boran E, Szepietowski J, Bialynicki-Birula R, Kulak W, Rolka H, Oksiejczuk E (2007) Analysis of the incidence of the fungal pathogens in air of the Department of Dermatology, Venereology and Allergology of Medical University in Wroclaw. Adv Med Sci 52:15–17

Marshall DH, Brownstein S, Jackson WB, Mintsioulis G, Gilberg SM, Al-Zeerah BF (1997) Post-traumatic corneal mucormycosis caused by *Absidia corymbifera*. Ophthalmology 104:1107–1111

McGinnis MR (1980) Laboratory Handbook of Medical Mycology. Academic, New York

Morinelli EN, Dugel PU, Riffenburgh R, Rao NA (1993) Infectious multifocal choroiditis in patients with acquired immune deficiency syndrome. Ophthalmology 100:1014–1021

Muccioli C, Belfort B Jr, Neves R, Rao N (1995) Limbal and choroidal *Cryptococcus* infection in the acquired immunodeficiency syndrome. Am J Ophthalmol 120:539–540

Nayak N (2008) Fungal infections of the eye — laboratory diagnosis and treatment. Nepal Med Coll J 10:48–63

Nitzulescu V, Niculescu M (1975) Ophthalmopathy determined by *Candida humicola*. Arch Roum Pathol Exp Microbiol 34:357–361

Nitzulescu V, Niculescu M (1976) Three cases of ocular candidiasis caused by *Candida lipolytica*. Arch Roum Pathol Exp Microbiol 35:269–272

Okhravi N, Dart JK, Towler HM, Lightman S (1997) *Paecilomyces lilacinus* endophthalmitis with secondary keratitis: a case report and literature review. Arch Ophthalmol 115:1320–1324

Okhravi N, Adamson P, Mant R, Matheson M, Midgley G, Towler H, Lightman S (1998) Polymerase chain reaction and restriction fragment length polymorphism mediated detection and speciation of *Candida* spp. causing intraocular infection. Invest Ophthalmol Vis Sci 39:859–866

Panda A, Sharma N, Das G, Kumar N, Satpathy G (1997) Mycotic keratitis in children: epidemiologic and microbiologic evaluation. Cornea 16:295–299

Paul EA (2007) Soil microbiology, ecology and biochemistry, 3rd edn. Academic, Canada

Pavan PR, Margo CE (1993) Endogenous endophthalmitis caused by *Bipolaris hawaiiensis* in a patient with acquired immunodeficiency syndrome. Am J Ophthalmol 116:644–645

Periman LM, Harrison DA, Kim J (2003) Fungal keratitis after photorefractive keratectomy: delayed diagnosis and treatment in a co-managed setting. J Refract Surg 19:364–366

Petersen M, Althaus C, Santen R, Gerhaz CD (1997) Endogenous *Aspergillus* endophthalmitis in AIDS. Klin Monbl Augenheilkd 211:400–402

Pflugfelder SC, Flynn HW, Zwickey TA, Forster RK, Tsiligianni A, Culbertson WW, Mandelbaum S (1988) Endogenous fungal endophthalmitis. Ophthalmology 95:19–30

Pini G, Donato R, Faggi E, Fanci R (2004) Two years of fungal aerobiocontamination survey in a Florentine haematology ward. Eur J Epidemiol 19:693–698

Rao N, Nerenberg AV, Forster DJ (1991) *Torulopsis candida* (*Candida famata*) endophthalmitis simulating *Propionibacterium* syndrome. Arch Ophthalmol 109:1718–1721

Read RW, Chuck RS, Rao NA, Smith RE (2000) Traumatic *Acremonium atrogriseum* keratitis following laser-assisted in situ keratomileusis. Arch Ophthalmol 118:418–421

Richter M, Hauser B, Kaps S, Spiess BM (2003) Keratitis due to *Histoplasma* spp. in a horse. Vet Ophthalmol 6:99–103

Rippon JW (1982) Laboratory Mycology Section II Culture methods, media, stains and serologic procedures. In: Rippon JW (ed) Medical Mycology: The pathogenic fungi and the pathogenic Actinomycetes, 2nd edn. WB Saunders, Philadelphia, pp 772–795

Rippon JW (1988) Candidiasis and the pathogenic yeasts. In: Rippon JW (ed) Medical Mycology: The pathogenic fungi and the pathogenic Actinomycetes, 2nd edn. WB Saunders, Philadelphia, pp 536–581

Robin J, Arffa RC, Avni I, Rao NA (1986) Rapid visualization of three common fungi using fluorescein-conjugated lectins. Invest Ophthalmol Vis Sci 27:500–506

Rodenbiker HT, Ganley JP (1980) Ocular coccidioidomycosis. Surv Ophthalmol 24:263–290

Rosa RH Jr, Miller D, Alfonso EC (1994) The changing spectrum of fungal keratitis in South Florida. Ophthalmology 101:1005–1013
Ruggli G, Weber R, Messmer EP, Font RL, Moll C, Bernauer W (1997) *Pneumocystis carinii* infection of the conjunctiva in a patient with acquired immune deficiency syndrome. Ophthalmology 104:1853–1856
Saha R, Das S (2006) Mycological profile of infectious keratitis from Delhi. Indian J Med Res 123:159–164
Saha S, Banerjee D, Khetan A, Sengupta J (2009) Epidemiological profile of fungal keratitis in urban population of West Bengal, India. Oman J Ophthalmol 2:114–118
Sandhu DK, Randhawa IS, Singh D (1981) The correlation between environmental and ocular fungi. Indian J Ophthalmol 29:177–182
Sansom J, Featherstone H, Barnett KC (2005) Keratomycosis in six horses in the United Kingdom. Vet Rec 156:13–17
Saran BR, Pomilla PV (1996) Retinal vascular nonperfusion and retinal neovascularization as a consequence of cytomegalovirus retinitis and cryptococcal choroiditis. Retina 16:510–512
Schwab IR, Dawson CR (1995) Conjunctiva. In: Vaughan DG, Asbury T, Riordan-Eva P (eds) General ophthalmology. Appleton & Lange, Norwalk, Conn., pp 95–122
Schwartz L, Loignon LM, Webster RG Jr (1978) Post traumatic phycomycosis of the anterior segment. Arch Ophthalmol 96:860–863
Seegal BC, Khorazo DL (1972) Microbiology of the eye. Mosby, St Louis, p 361
Sehgal S, Dhawan S, Chhiber S, Sharma M, Talwar P (1981) Frequency and significance of fungal isolations from conjunctival sac and their role in ocular infections. Mycopathologia 73:17–19
Shmookler MP, Kolsky BM (1996) Unilateral optic neuritis caused by *Histoplasma capsulatum* in a patient with the acquired immunodeficiency syndrome. Am J Ophthalmol 121:324–326
Shokohi T, Nowroozpoor-Dailami K, Moaddel-Haghighi T (2006) Fungal keratitis in patients with corneal ulcer in Sari, Northern Iran. Arch Iran Med 9:222–227
Slack JW, Hyndiuk RA, Harris GJ, Simons KB (1992) Blastomycosis of the eyelid and conjunctiva. Ophthal Plast Reconstr Surg 8:143–149
Specht C, Mitchell KT, Bauman AE, Gupta M (1991) Ocular histoplasmosis with retinitis in a patient with acquired immune deficiency syndrome. Ophthalmology 98:1356–1359
Srdic N, Radulovic S, Nonkovic Z, Velimirovic S, Cvetkovic L, Vico I (1993) Two cases of exogenous endophthalmitis due to *Fusarium moniliforme* and *Pseudomonas* species as associated aetiological agents. Mycoses 36:441–444
Srinivas RK, Philip LTH, Emmy LYM, Hunter YKL, Dennis LSC (2007) A case series of contact lens-associated Fusarium keratitis in Hong Kong. Cornea 26:1205–1209
Srinivasan M (2004) Fungal keratitis. Curr Opin Ophthalmol 15:321–327
Srinivasan R, Kanungo R, Goyal JL (1991) Spectrum of oculomycosis in South India. Acta Ophthalmol 69:744–749
Srinivasan M, Ganzales CA, George C (1997) Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, South India. Br J Ophthalmol 81:965–971
Srivastava SN, Mishra SR (1972) *Rhizopus* root rot of sugar beet — a new host record in India. Indian Phytopath 25:154–155
Stevens DA, Kan VL, Judson MA, Morrison VA, Dummer S, Denning DW, Bennett JE, Walsh TJ, Patterson TF, Pankey GA (2000) Practice guidelines for diseases caused by *Aspergillus*. Clin Infect Dis 30:696–709
Sudan R, Sharma YR (2003) Keratomycosis: clinical diagnosis, medical and surgical treatment. JK Sci 5:3–10
Tabbara KF, Al Jabarti A (1998) Hospital construction-associated outbreak of ocular aspergillosis after cataract surgery. Ophthalmology 105:522–526
Tanure MA, Cohen EJ, Grewal S, Rapuano CJ, Laibson PR (2000) Spectrum of fungal keratitis at Wills Eye Hospital, Philadelphia, Pennsylvania. Cornea 19:307–312
Thomas PA (1994) Mycotic keratitis — an underestimated mycosis. J Med Vet Mycol 32:235–256
Thomas PA (2003a) Current perspectives on ophthalmic mycoses. Clin Microbiol Rev 16:730–797

Thomas PA (2003b) Fungal infections of the cornea. Eye 17:852–862
Thomas P, Kuriakose T, Kirupashanker MP, Maharajan VS (1991) Use of lactophenol cotton blue mounts of corneal scrapings as an aid to the diagnosis of mycotic keratitis. Diagn Microbiol Infect Dis 14:219–224
Ula J, Irmgard B, Kathryn C (2009) Fungal keratitis: changing pathogens and risk factors. Cornea 6:638–643
Westenfeld F, Alston WK, Winn WC (1996) Complicated soft tissue infection with prepatellar bursitis caused by *Paecilomyces lilacinus* in an immunocompetent host: case report and review. J Clin Microbiol 34:1559–1562
Whitcher JP, Srinivasan M, Upadhyay MP (2001) Corneal blindness: a global perspective. Bull World Health Organ 79:214–221
Whitcup SM, Fenton RM, Pluda JM, De Smet MD, Nussenblatt RB, Chan CC (1992) *Pneumocystis carinii* and *Mycobacterium aviumintracellulare* infection of the choroid. Retina 12:331–335
Wilhelmus KR, Liesegang TJ, Osato MS, Jones DB (1994) Laboratory diagnosis of ocular infections. American Society for Microbiology, Washington, DC
Wilson LA, Sawant AD, Ahearn DG (1991) Comparative efficacies of soft contact lens disinfectant solutions against microbial films in lens cases. Arch Ophthalmol 109:1155–1157
Witherspoon C, Kuhn F, Owens SD, White MF, Kimble JA (1990) Endophthalmitis due to *Sporothrix schenckii* after penetrating ocular injury. Ann Ophthalmol 22:385–388
Yau TH, Rivera-Velazquez PM, Mark AS, Cytryn AS, Levy CS, Shmookler BM, Kolsky MP (1996) Unilateral optic neuritis caused by *Histoplasma capsulatum* in a patient with the acquired immunodeficiency syndrome. Am J Ophthalmol 121:324–326
Yohai RA, Bullock JD, Aziz AA, Markert RJ (1994) Survival factors in rhino-orbital-cerebral mucormycosis. Surv Ophthalmol 39:3–22
Zafar S, Ahmad I (2005) Fungal diversity of metal contaminated agricultural contaminated soils and *in vitro* fungi-toxicity of heavy metals. Poll Res 24:793–799

Chapter 5
Antifungal Drugs Mode of Action and Recent Development

Yoshikazu Sakagami

Abstract Mycosis is still one of the most problematic illness worldwide in hospitals and homes etc. Many antifungal drugs have been developed recently in the world. Voriconazole, posaconazole and ravuconazole (three new azoles), caspofungin, anidulafungin and micafungin (three new echinocandins), mycograb of monoclonal antifungal antibody, butenafin hydrochloride (one of the benzylamine compounds), terbinafine (an allylamine antifungal agent), and amphotericin B (one of the polyene groups) are representative antifungal drugs used worldwide. Recently, new antifungal drugs such as polyene SPE-843, acrylamide T-2307, ambruticin analog, isavuconazole, icofungipen, and pyridobenzimidazole 75-4590 have also been introduced. In this report, I discuss the introduction of several recently developed antifungal drugs, and also describe the mechanism of action of antifungal drugs and the combination effect between antifungal drugs. Finally, I emphasize in this report that both combination therapy and the development of new antifungal drugs are to be expected in the near future.

5.1 Antifungal Drugs, Recent Development

After 10 years absence (between 1990 and 1999) without any of new antifungal agents and intensive research being introduced into clinical practice, three new azoles (voriconazole — Pfizer, posaconazole — Schering-Plough, ravuconazole — Bristol–Myers Squibb) and three new echinocandins (caspofungin — MSD, anidulafungin — Astellas–Pfizer, micafungin — Fujisawa, now Astellas) were patented. Voriconazole (2000), posaconazole (2005), ravuconazole (2007) from the group of azoles, and

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caspofungin (2002), anidulafungin (2004) and micafungin (2006) from the group of echinocandins, with unique mode(s) of action (cell wall synthesis inhibition) different from that of polyenes, azoles, and antimetabolites, together with a new monoclonal antifungal antibody (Mycograb), have been approved and introduced to clinical practice (Vladimir 2007).

Echinocandins exhibit fungicidal activity against *Candida* species, including triazole-resistant isolates, and fungistatic activity against *Aspergillus* species. While fungistatic against mold, echinocandins may hold promise for the treatment of these pathogens when given in combination with amphotericin B or broad-spectrum triazoles, such as voriconazole. To date, resistance to echinocandins has been reported in only two patients. Echinocandins exhibit concentration-dependent activity against *Candida* species. In clinical trials, caspofungin has demonstrated efficacy in treating candidemia, esophageal candidiasis, and febrile neutropenia. Micafungin has demonstrated efficacy as antifungal prophylaxis in hematopoietic stem cell transplant recipients and in the treatment of esophageal candidiasis. Anidulafungin received approved labeling from the Food and Drug Administration in February 2006. Clinical efficacy data will be forthcoming. Echinocandins are fungicidal against yeast and fungistatic against mold. Their limited toxicity profile and minimal drug–drug interactions make them an attractive new option for the treatment of invasive fungal infections. Their cost may limit their use as initial therapy for patients with fungemia in medical centers or intensive care units with a high rate of triazole-resistant *Candida* infections (Michele 2006).

Butenafin hydrochloride [the trade names Mentax, Butop (India)] was produced from 1992, and terbinafine (commercially available, Lamisil, Novartis Pharma Co., Ltd.) were introduced from 1993 in Japan. Now they are popular antifungal drugs in Japan, and available as OTC (over the counter) drugs.

Terbinafine is an allylamine antifungal agent which has fungicidal activity against a wide variety of dermatophytes, molds and certain dimorphic fungi, and fungistatic activity against *Candida albicans*. Oral terbinafine is the treatment of choice for dermatophyte onychomycosis, as it achieves high rates of mycological and clinical cure, is generally well-tolerated, and has a relatively low potential for drug interactions. It must also be considered a first-line treatment option, along with itraconazole, in cutaneous mycoses which warrant systemic treatment; topical terbinafine is a treatment of choice in less extensive mycoses. The use of terbinafine in non-dermatophyte or mixed infections has not been fully defined (McClellan et al. 1999).

Terbinafine is also the drug of choice for dermatophyte onychomycosis. Adjunct therapies, such as topical agents or surgical approaches, may improve outcomes in patients who have risk factors for incomplete response or recurrence (Loo Daniel 2006).

Espinel-Ingroff (2009) reported new antifungal drugs such as such as polyene SPE-843, acrylamide T-2307, ambruticin analog, isavuconazole, icofungipen, and pyridobenzimidazole 75-4590.

5.2 Mode of Action

The mode of action of several antifungal agents against fungi is shown in Table 5.1. The mode of action of agents in the polyene group such as amphotericin B (commercially available, Fingizone etc.) and nystatine (Diflucan, Pfizer) is principally the inhibition of the plasma membrane enzymes. The mode of action of agents in the azole group such as clotrimazole (Empecid, Bayer Health Care Co., Ltd.), miconazole (Florid F, Mochida Pharm. Co., Ltd.), fluconazole (Diflucan or Trican, Pfizer), ketoconazole (Nizoral, by Johnson & Johnson et al.), itraconazole (Itrizole, Janssen Pharmaceutical K.K.), voriconazole (VFEND, Pfizer), posaconazole (Noxafil, Schering–Plough) and ravuconazole (Eisai Co., Ltd.) was inhibition of biosynthesis of ergosterol, the main component of the cytoplasmic membrane.

The principal mechanism of action of agents of the echinocandins group such as caspofungin (brand name Cancidas worldwide; not available in Japan), anidulafungin (Eraxis, Pfizer) and micafungin was noncompetitive inhibition of β-(1,3)-D-glucan synthase, an essential component of the cell wall of many fungi that is not present in mammalian cells (Michele 2006).

Table 5.1 Several groups of antifungal drugs focused to recent development

Group	General name	Mode of action (bactericidal mechanisms)
Polyenes	Amphotericin B	Inhibition of the plasma membrane enzymes
	Nystatine	
	Clotrimazole	
	Miconazole	
	Fluconazole	
Azoles	Ketoconazole	Inhibition of biosynthesis of ergosterol, main component of cytoplasmic membrane
	Itraconazole	
	Voriconazole	
	Posaconazole	
	Ravuconazole	
	Caspofungin	
Chandin (Echinocandins)	Anidulafungin	Noncompetitive inhibition of β-(1,3)-D-glucan synthase
	Micafungin	
Allylamine	Terbinafine hydrochloride	Inhibition of biosynthesis of ergosterol in cytoplasmic membrane constitution
Benzylamine	Butenafin hydrochloride	
Flucytocine	5-Fluorocytosine	Inhibition of nucleic acid biosynthesis
New monoclonal antifungal antibody	Mycograb	Target to HSP90 (heat shock protein)
Interference with the function of microtubules	Griseofulvin	Disruption of spindle and cytoplasmic microtubule function

The echinocandins inhibit fungal cell-wall synthesis, a novel therapeutic target; thus, they are effective against azole-resistant species. Their metabolism is independent of hepatic cytochrome P450 enzymes, minimizing drug interactions. They are available only as i.v. formulations (Loo Daniel 2006).

Terbinafine is an allylamine antifungal agent which has fungicidal activity against a wide variety of dermatophytes, molds and certain dimorphic fungi, and fungistatic activity against *C. albicans*. The mode of action of terbinafine was the inhibition of biosynthesis of ergosterol in cytoplasmic membrane constitution.

Inorganic salts could be used as potent transungual permeation enhancers on terbinafine. Terbinafine permeation (three- to fivefold) through the nail and drug load (four- to sevenfold) in the nail were enhanced significantly when salts were used at 0.5 M concentration. Increase in salt concentration up to 1 M increased the permeation, which decreased with further increase in salt concentration (>1 M) (Nair et al. 2009).

Butenafine hydrochloride is one of the benzylamine compounds. The mode of action of butenafine hydrochloride is also the inhibition of biosynthesis of ergosterol in cytoplasmic membrane constitution in the growth process of *Trichophyton ruburum* and *Trichophyton mentagrophytes* (Kaken Pharmaceutical Co., Ltd. 1999).

Butenafine hydrochloride exhibits potent fungicidal activity particularly against dermatophytes, aspergilli, dimorphic and dematiaceous fungi. The drug has excellent penetration into the epidermis and a prolonged retention time following topical application, conferring residual therapeutic activity after treatment cessation. Butenafine also possesses anti-inflammatory activity. Topical butenafine 1% cream has been reported to be efficacious for tinea pedis, tinea corporis and tinea cruris in many randomized clinical trials when used for shorter duration. Its efficacy against pityriasis versicolor, seborrheic dermatitis and as an anticandidal agent is not yet fully established (Singal 2008).

The mode of action of flucytosine (5-fluorocytosine: a common brand name is Ancobon) was the inhibition of nucleic acid biosynthesis. Flucytosine is a synthetic antimycotic compound, first synthesized in 1957. It has no intrinsic antifungal capacity, but after it has been taken up by susceptible fungal cells, it is converted into 5-fluorouracil, which is further converted to metabolites that inhibit fungal RNA and DNA synthesis (Vermes et al. 2000).

The target site of Mycograb, a new monoclonal antifungal antibody, was HSP90 (heat shock protein). It combines the site, and would take concerted effect. The first recombinant antibody fragment, Mycograb (NeuTec Pharma plc), against *Candida* HSP90 is now in clinical trials in patients with disseminated candidiasis in Europe and the US. Laboratory and early clinical data support the concept of synergy between Mycograb and amphotericin B. This should improve the outcome and diminish the risk of resistance occurring to either drug, without an increase in toxicity, as this should be minimal in a human antibody fragment representing the natural antibody that a patient produces on recovery (Burnie and Matthews 2004).

Burnie and Matthews (2003) reported that the development of antibody-based therapeutics, such as Mycograb, against novel fungal targets offers a new

approach to combating the spread of resistance and reducing mortality (Burnie and Matthews 2003).

The mode of action of other antifungal drugs such as griseofulvin reported by Develoux (2001) is as follows: griseofulvin is a metabolic product of *Penicillium* spp. It was the first available oral agent for the treatment of dermatophytoses and has now been used for more than 40 years. Griseofulvin is fungistatic; the exact mechanism by which it inhibits the growth of dermatophytes is uncertain. Several possibilities are invoked: inhibition of fungal cell mitosis and nuclear acid synthesis, and probable interference with the function of microtubules (Develoux 2001).

5.3 Combination Effect Between Antifungal Agents

There have been many reports concerning the combination effect between antifungal agents etc. Over this period, some representative reports concerning the combination effect of antifungal agents etc. were presented.

The synergism of polyene antibiotics with 5-fluorocytosine was reported by Polak (1978). The synergism of 5-fluorocytosine with four polyene antibiotics (amphotericin B, candicidin, trichomycin and nystatin) has been investigated in three *in vitro* models measuring the fungistatic and fungicidal activity as well as the development of resistant mutants. In these three models, candicidin and trichomycin exerted a higher synergistic effect than amphotericin B or nystatin. *In vivo* (systemic treatment of septicemic candidiasis of the mouse), the combination of 5-fluorocytosine with amphotericin B was, however, the most effective. Only with this combination a complete cure (culturally negative) was observed. For topical treatment of *Candida vaginitis* in rats, the combination of 5-fluorocytosine with candicidin proved the most active. As far as the biochemical basis of synergism is concerned in *C. albicans* in the presence of polyene antibiotics, the incorporation of fluorinated pyrimidines was increased, and the reduction of uptake of histidine by 5-fluorocytosine alone significantly enhanced. These effects could not be observed in *Cryptococcus neoformans*. The release of amino acids, phosphate and potassium caused by polyenes was significantly more pronounced in cells pretreated with 5-fluorocytosine. Thus, the interaction between 5-fluorocytosine and the polyenes may, in fact, be mutual.

Combined *in vitro* activity of amphotericin B and 5-fluorocytosine against *C. neoformans* and *C. albicans* was reported by Chen et al. (1982). The *in vitro* combined antifungal activity of amphotericin B and 5-fluorocytosine against ten strains of *C. albicans* and eight strains of *C. neoformans* was studied. Serial twofold dilutions of amphotericin B (0.1–12.8 μg/ml) were tested with serial twofold dilutions of 5-fluorocytosine (0.39–400 μg/ml) by using the broth dilution method. A synergistic effect was seen for 17 strains with 50 μg/ml of 5-fluorocytosine added to amphotericin B. Only one strain was killed by 0.8 μg/ml or less of amphotericin B alone, whereas all strains were killed by the addition of 50 μg/ml of 5-fluorocytosine.

Ketoconazole and flucytosine alone and in combination against *Candida* spp. in a neutropenic site in rabbits was reported by Hughes et al. (1986). Ketoconazole and flucytosine were administered alone and in combination for 10 days to rabbits, with four *Candida* isolates growing in subcutaneously implanted semipermeable chambers. The peak concentrations of ketoconazole in serum and in the chamber were 20.3 and 3.8 μg/ml respectively, and the concentrations of flucytosine were 47.7 and 37.3 μg/ml respectively. The two drugs combined resulted in better fungistatic activity than either drug alone against all four isolates. Correlation of efficacy in the rabbit model with *in vitro* MICs was good for flucytosine, but poor for ketoconazole.

In vitro evaluation of combination of fluconazole and flucytosine against *C. neoformans* var. *neoformans* was reported by Nguyen et al. (1995). The combination of fluconazole and flucytosine has yielded encouraging clinical results in human immunodeficiency virus patients with *Cryptococcal meningitis*. To investigate the biological basis of this finding, the authors performed *in vitro* combination testing of fluconazole and flucytosine against 50 clinical strains of *C. neoformans* var. *neoformans*. Synergy (fractional inhibitory concentration index of <1.0) was observed in 62% of cases, while antagonism (fractional inhibitory concentration index of >2.0) was not observed. For cases in which synergy was not achieved (autonomous or additive effects), the beneficial effect of the combination was still seen (i.e., there was still a decrease, although not as dramatic, in the MIC of one or both drugs when used in combination). The *in vitro* inhibitory action of flucytosine was greatly enhanced by the addition of fluconazole; the flucytosine MICs for Cryptococcus isolates were markedly decreased, to concentrations which were several-fold lower than the achievable cerebrospinal fluid flucytosine concentration. On the other hand, the addition of flucytosine did not greatly enhance the *in vitro* activity of fluconazole if the initial fluconazole MIC for the isolate was above or equal to 8 μg/ml. Controlled clinical studies are warranted to further elucidate the potential utility of fluconazole–flucytosine combination therapy.

In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera* was reported by Vital et al. (2003). The combined effects of antifungal and antibiotic drugs against *Exophiala spinifera* were evaluated *in vitro* by the checker-board method, calculated as a fractional inhibitory concentration index. Amphotericin B was combined with flucytosine and ciprofloxacin (one of the new quinolone compounds), whereas itraconazole was combined with ciprofloxacin, levofloxacin, lomefloxacin and sulfadiazine. Synergistic effects were observed for the combinations of itraconazole with ciprofloxacin and levofloxacin, and amphotericin B with ciprofloxacin and flucytosine. No antagonism was observed for any combination tested.

In vitro antifungal combination effects of micafungin with fluconazole, voriconazole, amphotericin B, and flucytosine against clinical isolates of *Candida* species were reported by Nishi et al. (2009). Micafungin is an echinocandin antifungal agent that exhibits potent activity against most species of *Candida* and *Aspergillus*. The authors investigated the *in vitro* antifungal combination effects of micafungin

with four other antifungal agents — fluconazole, voriconazole, amphotericin B, and flucytosine — against clinical isolates of 54 *Candida* spp. by checker-board analysis. The synergistic antifungal effects of micafungin–fluconazole and micafungin–voriconazole were 11% and 15% respectively, and the latter displayed a synergistic activity of 63% against *Candida glabrata*. Antagonism was not observed in any of the combinations tested.

Synergistic activity of lysozyme and antifungal agents against *C. albicans* biofilms on denture acrylic surfaces was reported by Samaranayake et al. (2009). Denture-related oral candidiasis is a recalcitrant fungal infection not easily resolved by topical antifungals. The antimycotic protein lysozyme in saliva is an important host defense mechanism, although its activity against *Candida* biofilms on denture acrylic has not been evaluated. Research to establish a clinically relevant denture acrylic assay model to develop standardized *C. albicans* biofilms, and to assess the inhibitory effects of lysozyme both alone and combined with antifungals (nystatin, amphotericin B, ketoconazole and 5-fluorocytosine) on sessile *Candida* cells was performed. Research to visualize the accompanying ultrastructural changes was also performed. The authors suggest that agents found in biological fluids such as lysozyme could be a safe adjunct to antifungals in future treatment strategies for recalcitrant candidal infections.

Fluconazole alone or combined with flucytosine for the treatment of AIDS-associated *cryptococcal meningitis* was reported by Milefchik et al. (2008). Both fluconazole and flucytosine are available in oral formulations, and have activity against *C. neoformans*. The authors conducted a prospective phase II dose escalation study employing doses of fluconazole ranging from 800 to 2,000 mg daily for 10 weeks, used alone or combined with flucytosine at 100 mg/kg per day for the first 4 weeks. They found that increasing doses of fluconazole were associated with an increase in survival and a decrease in the time to conversion of the cerebrospinal fluid from culture-positive to culture-negative. Addition of flucytosine to fluconazole improved outcomes in each dosing cohort. High doses of fluconazole alone or combined with flucytosine were well-tolerated.

Mycograb (NeuTec Pharma) is a human recombinant monoclonal antibody against HSP90 which, in laboratory studies, was revealed to have synergy with amphotericin B against a broad spectrum of *Candida* species. Mycograb plus lipid-associated amphotericin B produced significant clinical and culture-confirmed improvement in outcome for patients with invasive candidiasis (Pachl et al. 2006).

Espinel-Ingroff (2009) reviewed the synergistic effect of antifungal drugs as follows: synergistic effects of micafungin + caspofungin, micafungin + amphotericin B or + flucytosine, voriconazole + lovastatin, azoles + retigeric acid, caspofungin + calcinerium, and amphotericin + EDTA were found.

On the other hand, antagonistic effects of fluconazole and 5-fluorocytosine on candidacidal action of amphotericin B in human serum was reported by Martin et al. (1994). This study addressed the effects of fluconazole and 5-fluorocytosine on the candidacidal activity of amphotericin B in the presence of human serum. *C. albicans* isolate that was susceptible to all three agents according to standard testing procedures was employed. Fungicidal activity was estimated by using a flow

cytometric procedure that exploited the fact that yeast cells killed by amphotericin B diminish in size and take up propidium iodide. The following findings were made. (a) Fluconazole and 5-fluorocytosine each failed to inhibit pseudohyphal formation and cell aggregation, even when applied at 10 and 50 μg/ml respectively for up to 10 h. Hence, these agents were not fungistatic when tested in the presence of serum. (b) Simultaneous application of 5-fluorocytosine had neither enhancing nor inhibitory effects on the fungicidal activity of amphotericin B. However, yeasts that were preincubated for 20 h with 5-fluorocytosine became less susceptible to killing by amphotericin B. (c) Fluconazole exerted a frank antagonistic effect on the fungicidal activity of amphotericin B. Thus, under *in vitro* conditions, both fluconazole and 5-fluorocytosine can overtly antagonize the candidacidal action of amphotericin B.

5.4 Future Developments

Mori (2008) reported about the future aspects of antifungal therapy. Both further combination therapy and the development of new antifungal drugs might be expected. Because the main structural component of cells on eubacteria was the same as for human cells, the development of new antifungal drugs would be difficult. Then, only a few antifungal drugs were available to address systemic mycosis or deep-seated mycosis. The development of new antifungal drugs targeted on chitin in the cell wall of fungi might be expected in the near future.

Espinel-Ingroff (2009) reported that the incidence and prevalence of serious mycoses continues to be a public health problem. Despite aggressive treatment with new or more established licensed antifungal agents, these infections are an important cause of morbidity and mortality, especially in immunocompromised patients. Although the antifungal activity of numerous compounds has been examined, most of them are at the *in vitro* or animal models of efficacy stages. Therefore, further investigation should be carried out to realize the true clinical utility of these compounds.

References

Burnie J, Matthews R (2003) The role of antibodies against hsp 90 in the treatment of fungal infections. Drug News Perspect 16:205–210

Burnie J, Matthews R (2004) Genetically recombinant antibodies: new therapeutics against candidiasis. Expert Opin Biol Ther 4:233–241

Chen HH, Liushih RN, Hsieh WC (1982) Combined *in vitro* activity of amphotericin B and 5-fluorocytosine against *Cryptococcus neoformans* and *Candida albicans*. Chin J Microbiol Immunol 15:106–112

Develoux M (2001) Griseofulvin. Ann Dermatol Venereol 128:1317–1325

Espinel-Ingroff A (2009) Novel antifungal agents, targets or therapeutic strategies for the treatment of invasive fungal diseases: a review of the literature (2005–2009). Rev Iberoam Micol 26:15–22

Hughes CE, Peterson LR, Beggs WH, Gerding DN (1986) Ketoconazole and flucytosine alone and in combination against *Candida* spp. in a neutropenic site in rabbits. J Antimicrob Chemother 18:65–72

Kaken Pharmaceutical Co., Ltd. Press release, JAPAN 21 April, 1999

Loo Daniel S (2006) Systemic antifungal agents: an update of established and new therapies. Adv Dermatol 22:101–124

Martin E, Maier F, Bhakdi S (1994) Antagonistic effects of fluconazole and 5-fluorocytosine on candidacidal action of amphotericin B in human serum. Antimicrob Agents Chemother 38:1331–1338

McClellan KJ, Wiseman LR, Markham A (1999) Terbinafine. An update of its use in superficial mycoses. Drugs 58:179–202

Michele MI (2006) Echinocandins in the management of invasive fungal infections, part 1. Am J Health Syst Pharm 63:1693–1703

Milefchik E, Leal MA, Haubrich R, Bozzette SA, Tilles JG, Leedom JM, McCutchan JA, Larsen RA (2008) Fluconazole alone or combined with flucytosine for the treatment of AIDS-associated *cryptococcal meningitis*. Med Mycol 46:393–395

Mori T (2008) Medical history and progress in infectious diseases, especially systemic fungal infections in Japan (in Japanese). Jpn J Med Mycol 49:5–17

Nair AB, Sammeta SM, Vaka SR, Narasimha Murthy S (2009) A study on the effect of inorganic salts in transungual drug delivery of terbinafine. J Pharm Pharmacol 61:431–437

Nguyen MH, Barchiesi F, McGough DA, Yu VL, Rinaldi MG (1995) *In vitro* evaluation of combination of fluconazole and flucytosine against *Cryptococcus neoformans* var. *neoformans*. Antimicrob Agents Chemother 39:1691–1695

Nishi I, Sunada A, Toyokawa M, Asari S, Iwatani Y (2009) *In vitro* antifungal combination effects of micafungin with fluconazole, voriconazole, amphotericin B and flucytosine against clinical isolates of *Candida* species. J Infect Chemother 15:1–5

Pachl J, Svoboda P, Jacobs F, Vandewoude K, van der Hoven B, Spronk P, Masterson G (2006) A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in combination with an antibody-based inhibitor of Heat Shock Protein 90 in patients with invasive candidiasis. Clin Infect Dis 42:1404–1413

Polak A (1978) Synergism of polyene antibiotics with 5-fluorocytosine. Chemotherapy 24:2–16

Samaranayake YH, Cheung BP, Parahitiyawa N, Seneviratne CJ, Yau JY, Yeung KW, Samaranayake LP (2009) Synergistic activity of lysozyme and antifungal agents against *Candida albicans* biofilms on denture acrylic surfaces. Arch Oral Biol 54:115–126

Singal A (2008) Butenafine and superficial mycoses: current status. Expert Opin Drug Metab Toxicol 4:999–1005

Vermes A, Guchelaar HJ, Dankert J (2000) Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. J Antimicrob Chemother 46:171–179

Vital RG, Afeltra J, de Hoog GS, Rijs AJ, Verweij PE (2003) *In vitro* activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera*. J Antimicrob Chemother 51:1297–1300

Vladimir K (2007) Antifungal drug discovery, six new molecules patented after 10 years of feast: why do we need new patented drugs apart from new strategies? Recent Pat Antiinfect Drug Discov 2:182–187

Chapter 6
Antifungal-Induced Nephrotoxicity in Pediatrics: A State of the Art

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Abstract Fungal infections are common in children with defects in host defences and in preterm newborns are responsible for considerable morbility and mortality. Thus antifungins are important in the treatment of fungal infections, particularly in at risk babies. Therefore, antifungins may be responsible for important side effects such as nephrotoxicity. In particular, the nephrotoxicity is an important adverse effect of amphotericin B, which is higher in preterm babies than at-term neonates and infants. Therefore, Amphotericin B lipid formulations (AmBisome, AMLC, ABCD) showed less nephrotoxicity than Amphotericin B, in preterm babies, newborns and immunocompromized babies. Alternative antifungins (azoles, fluorinated pyrimidines and echinocardins) are currently available in children and newborns. Fluconazole is the most widely used and relatively well-tolerated azole. Unfortunately, clinical experience of treating children with the newer azoles (i.e., Variconazole) is more limited in newborns and children. Moreover, the treatment with Caspofungin (Echinocandin) could be considered a potential alternative to conventional antifungal therapy (Amphotericin B lipid formulations). Finally, other echinocandins are available but relatively few studies report safety data in newborns and children.

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6.1 Introduction

During recent decades, invasive fungal infections have become one of the major complications in children with defects in host defences: this derives from an increase in the number of children receiving antineoplastic therapies (Groll and Lehrnbecher 2005). *Candida* spp. and *Aspergillus* spp. are the most common fungal pathogens isolated, even if less common emerging pathogens such as *Fusarium* spp. and *Scedosporium* spp. are also reported as causes of life-threatening mycoses (Groll et al. 1999; Ponton et al. 2000). Mortality rates in children with candidemia range from 19% to 31% (Pappas et al. 2003), while invasive aspergillosis is associated with even greater mortality (68%–77%) (Lin et al. 2001).

Fungal infections are also common in the neonatal period, and are responsible for considerable morbidity and overall mortality in preterm newborns (Benjamin et al. 2006a, b). In particular, invasive fungal infection due to *Candida* species accounts for about 10% of all cases of nosocomial sepsis in very low birth weight (VLBW) infants and the risk is inversely related to gestational age and birth weight (Stoll et al. 2002; Farmaki et al. 2007). Therefore, the prevention and treatment of *Candida* infections is a significant issue in these infants.

6.2 Amphotericin B

Amphotericin B, a polyene macrolide antibiotic introduced in therapy in 1956, is still considered the gold standard for the treatment of invasive fungal infections in adults and children, including neonates (Khoory et al. 1999; Rao and Ali 2005). The molecule binds to ergosterol (a sterol component unique to fungal cell membranes) and to a lesser degree to cholesterol in mammalian cells, alters cell permeability, and induces pore formation, leading to the leakage of cytoplasmic contents and fungal death (Zaoutis and Walsh 2007).

Nephrotoxicity is an important adverse effect of amphotericin B that generally occurs within the first 2 weeks of treatment, is usually reversible (Goldman and Koren 2004), and seems to depend on the modality of administration: continuous infusions result in lower nephrotoxicity than for intermittent infusions (Eriksson et al. 2001; Peleg and Woods 2004). Numerous mechanisms have been proposed for amphotericin B nephrotoxicity (Goldman and Koren 2004). These include the vehicle (deoxycholate) in which amphotericin is administered (Zager et al. 1992), ischemic injury due to a reduction of renal blood flow and GFR (Tolins and Raij 1988; Sabra et al. 1990), increase in salt concentrations at the macula densa leading to enhanced stimulation of the tubuloglomerular feedback, and vasoconstriction (Gerkens and Branch 1980). Interaction of amphotericin B with cholesterol on the human tubular cell membrane has also been postulated (Sabra et al. 1990) and apoptosis in proximal tubular cells and medullary interstitial cells has been documented (Varlam et al. 2001). Acute renal failure, the most serious complication, is rare. More frequent is tubulotoxicity, which includes potassium and magnesium

Table 6.1 Nephrotoxicity of amphotericin B in newborns and children

Reference	Study population	No	Manifestations	No patients (%)
Baley et al. (1984)	ELBW infants	10	Elevated Cr/BUN, oliguria/ anuria, hypokalemia	7/10 (70%)
Turner et al. (1985)	Newborns	13	Elevated Cr/BUN, hypokalemia	6/13 (46%)
Baley et al. (1990)	VLBW infants	13	Hypokalemia	5/13 (38%)
Butler et al. (1990)	Newborns	36	Elevated Cr/BUN	4/36 (11%)
Kingo et al. (1997)	VLBW infants	18	Hypokalemia, decreased urine output	4/18 (22%)
Fernandez et al. (2000)	VLBW infants	23	Elevated Cr/BUN	7/23 (35%)
Holler et al. (2004)	ELBW infants	25	Elevated Cr, decreased urine output	11/25 (44%)
Turcu et al. (2009)	ELBW infants	37	Elevated Cr, decreased urine output	16/37 (43%)
Goldman et al. (2007)	Children	90	Elevated Cr	52/90 (58%)

loss in urine, renal tubular acidosis and loss of urinary concentrating ability. Hypokalemia is common (Fanos and Cataldi 2000).

A number of authors identified specific risk factors for amphotericin B-induced nephrotoxicity: cumulative dose, average daily dose, treatment duration (Wingard et al. 1999; Fisher et al. 1989; Luber et al. 1999), pre-existing renal impairment, hypovolemia, hyponatriemia, and concurrent use of nephrotoxic drugs such as cyclosporine and ciprofloxacin (Goldman et al. 2007). Renal complications can be minimized with careful management of fluid and electrolyte balance (Mayer et al. 2002).

In the literature, different authors reported cases of nephrotoxicity due to amphotericin B in children and newborn infants (Table 6.1).

Among a study population of 90 hospitalized pediatric patients (mean age 103 months) treated with amphotericin B (mean daily dose 0.85 ± 0.2 mg/kg/d, mean length of treatment 11 days), in 52 children (58%) the CrCl decreased 20% or more from the baseline despite efforts to prevent nephrotoxicity (large volume of fluids, NaCl supplementation). Concurrently, patients were treated with other 131 different medications and the association of amphotericin B with cyclosporine, nystatin and ciprofloxacin was related to nephrotoxicity (Goldman et al. 2007).

6.2.1 Preterm Babies

Among a population of 25 extremely low birth weight (ELBW) infants (mean GA 25 ± 1 weeks, mean BW 738 ± 37 g), renal problems (low urine output and high creatinine levels) occurred in 44% of newborns (Holler et al. 2004).

Recently, among a study population of 37 ELBW infants (mean GA 25 ± 2 weeks, mean BW 724 ± 158 g) treatment for amphotericin B-induced renal problems was successful in 16/37 patients (43%), but the number of neonates who developed nephrotoxicity was lower in the case of sodium intake (Turcu et al. 2009). On the other hand, other authors (Johnson et al. 1984; Glick et al. 1993) found the drug to be well tolerated in premature newborns.

6.2.2 *Newborns and Infants*

With regard to neonates, they seem to tolerate amphotericin B better than adults, with lower nephrotoxicity (Scarcella et al. 1998). Hypokalemia, however, may be severe and extended usage may lead to renal impairment (Zaoutis and Walsh 2007).

Several authors (Baley et al. 1984, 1990; Turner et al. 1985; Butler et al. 1990; Kingo et al. 1997; Fernandez et al. 2000) have reported the toxicity of amphotericin B in preterm and term neonates. Most authors noted changes in renal function (evidenced by changes in blood urea nitrogen, creatinine, and/or electrolytes), frequently reversible, in a percentage varying from 11% to 70%.

6.3 Amphotericin B Lipid Formulations

During recent decades, different efforts have been made in an attempt to prevent nephrotoxic effects of conventional amphotericin B and a revolution has occurred with the introduction of lipid formulations of the drug. The lipid–drug complexation appears to stabilize amphotericin B, so it cannot interact with cholesterol in human cellular membranes before reaching the fungi (Janoff et al. 1993). Three lipid formulations of amphotericin B were developed: (1) liposomal amphotericin B (AmBisome), with the drug incorporated in the membrane of small unilamellar vesicles composed of hydrogenated soy phosphatidyl choline, cholesterol and distearoyl phosphatidyl glycerol, (2) amphotericin B lipid complex (ABLC), where the drug is complexed with two lipids in a 1:1 drug–lipid molar ratio, and (3) amphotericin B colloidal dispersion (ABCD), with the drug complexed with sodium cholesteryl sulfate in a 1:1 molar ratio (Goldman and Koren 2004).

These three differ significantly in their pharmacokinetic properties, with lower amphotericin B formulations serum levels observed after ABLC and ABCD administration compared to AmBisome (Hiemenz and Walsh 1996). All three preparations demonstrate similar efficacy and share a considerable reduction in nephrotoxicity compared to conventional amphotericin B, as shown in three different meta-analyses (Johansen and Gotzsche 2000; Barrett et al. 2003; Girois et al. 2006) reporting a 49%–75% reduction in nephrotoxicity with lipid preparations. In every case, if nephrotoxicity secondary to a lipid formulation occurs, it occurs after a longer course of therapy (Prentice et al. 1997; White et al. 1998).

Different authors have studied premature infants with severe fungal infections treated with AmBisome (Scarcella et al. 1998; Weitkamp et al. 1998 Al Arishi et al. 1998; Walsh et al. 1999a). No acute adverse effects occurred, and renal parameters were all normal: the only reported side-effect was a transient hypokalemia observed in some patients (Scarcella et al. 1998; Weitkamp et al. 1998).

ABLC has been shown to be safe in one large open-label multicenter study in 111 children with invasive mycoses (mean age 9.3 years): the mean serum creatinine remained stable during up to 6 weeks of ABLC therapy (Walsh et al. 1999b). The safety and efficacy of ABLC were also assessed in 548 immunocompromized

children treated for invasive mycoses: there were few clinically significant deleterious effects on renal function, with elevations in serum creatinine observed in 33.6% of patients (Wiley et al. 2005). Other authors (Adler-Shohet et al. 2001) studied the safety and efficacy of ABLC in 11 neonates (eight preterm) with systemic *Candida* infections, previously treated with conventional amphotericin B that induced acute nephrotoxicity or was not able to eradicate infection. Renal function did not change appreciably in eight of the 11 infants.

6.4 Other Antifungals

Alternative antifungals are currently available for use in children and newborns: the azoles (itraconazole, fluconazole, and voriconazole), the fluorinated pyrimidines (flucytosine), and the echinocandins (caspofungin, micafungin, and anidulafungin).

6.4.1 Azoles

Triazole antifungal agents demonstrate a broad spectrum of activity against both filamentous and yeast organisms, a good safety profile, and an excellent oral bioavailability. They act by inhibiting ergosterol synthesis (Sheehan et al. 1999). Itraconazole was the first triazole approved by the Food and Drug Administration (FDA) for clinical use.

6.4.1.1 Fluconazole

Fluconazole is by far the most widely used azole and has been reported to be relatively well tolerated. Mild and transient increases of liver enzymes, without clinical complications, are the most common adverse effects described in infants (Kicklighter et al. 2001; Kaufman et al. 2001, 2005; Manzoni et al. 2007). With regard to renal tolerability, in an observational study of therapeutic fluconazole (6 mg/kg daily) given to treat *Candida* sepsis, two of 40 babies developed raised serum creatinine levels, but none needed to stop treatment (Huttova et al. 1998). Several studies of prophylactic fluconazole did not report clinically important nephrotoxicity (Kaufman et al. 2001; Manzoni et al. 2007; Clerihew et al. 2007; McCrossan et al. 2007).

6.4.1.2 Voriconazole

Clinical experience of treating children with the newer azoles is more limited. According to the manufacturer (Pfizer Inc 2004), intravenous voriconazole is not

recommended in patients with moderate to severe renal insufficiency due to the possible accumulation in the proximal tubule of the vehicle sulfobutyl ether β-cyclodextrin sodium observed in animals (Frank et al. 1976), even if modifications of the original β-cyclodextrin have diminished its renal toxicity (Uekama et al. 1993).

Data regarding safety of voriconazole in newborns and children are limited and mostly reported as case reports. A 14-day-old neonate born at 27 weeks' gestation developed a disseminated fluconazole-resistant *Candida albicans* infection and received a combination of iv voriconazole and AmBisome: this protocol resulted safe despite the developmentally limited renal function of the infant and the concurrent administration of other nephrotoxic drugs (Muldrew et al. 2005).

Two published case reports described voriconazole therapy in newborns (Maples et al. 2003; Kohli et al. 2008) and no renal side-effects were noted. Other authors (Jabado et al. 1998; van't Hek et al. 1998) reported a good tolerability of voriconazole administered to two children with chronic granulomatous disease developing invasive fungal infections.

Among 58 children (ages 9 months to 15 years) treated with voriconazole i.v. and orally for proven or probable fungal infection, two patients had elevated serum creatinine levels (Walsh et al. 2002). The same authors (Walsh et al. 2004) reported, among a population of 35 immunocompromised children, abnormal renal function and elevations in serum creatinine (resolved with a dose reduction) in one patient following multiple dosing of intravenous voriconazole.

6.4.2 Flucytosine

Flucytosine acts as an antimetabolite and is activated by deamination within the fungal cells to 5-fluorouracil, resulting in an inhibition of fungal DNA and protein synthesis. Its spectrum is restricted to *Candida* species and *Cryptococcus neoformans* with evidence of primary and acquired resistance in some strains, so it should not be used as monotherapy (Vermes et al. 2000).

In preterm infants, it is used in combination with amphotericin B for its synergism and high penetration in the central nervous system. However, being available only for oral administration, its use in newborns is limited (Almirante and Rodriguez 2007).

6.4.3 Echinocandins

Echinocandins form a new family of antifungal agents acting on a different pathway: they inhibit β(1,3)-D-glucan synthesis, an essential component of the fungal cell wall, with fungistatic as well as fungicidal effects (Letscher-Bru and

Herbrecht 2003). Due to this selectivity towards fungal cell wall enzymes, the side-effects of echinocandins are moderate in humans (Stone et al. 2002), and they could be considered a potential alternative to the highly nephrotoxic amphotericin B.

6.4.3.1 Caspofungin

Abnormalities in laboratory tests (serum creatinine and blood urea nitrogen elevations, and hypokalemia) are uncommon with all echinocandins, but rather more frequent with caspofungin, the first echinocandin approved in therapy (Vazquez and Sobel 2006). In every case, as assessed in different clinical trials, nephrotoxicity of caspofungin was less pronounced compared to amphotericin B and ranged from 0% to 1.4% (Villanueva et al. 2001; Arathoon et al. 2002). Nevertheless, in another trial the rate of nephrotoxicity was higher, and affected 8.4% of patients (Mora-Duarte et al. 2002). Some authors (Wegner et al. 2005) characterized the extent and nature of cell-damaging effects of caspofungin *in vitro* and demonstrated moderate and dose-dependent cytotoxic effects on both proximal and distal tubular cells, with the latter more vulnerable towards caspofungin.

Different studies examined the safety of echinocandins in pediatric patients and mostly concerned caspofungin, usually administered to patients unresponsive to conventional antifungal therapies. Hesseling et al. (2003) reported for the first time the use of caspofungin in an ELBW infant born at 24 weeks gestation who developed a systemic candidemia and was unresponsive to amphotericin B and flucytosine: although the antifungal failed in this end-stage therapy, no side-effects were observed.

An ELBW infant (GA 25 weeks, BW 810 g) with persistent candidemia, initially unsuccessfully treated with amphotericin B changed to ABLC (due to reduced urine output and elevated creatinine) and then with fluconazole, responded to therapy with caspofungin and improved renal function (Smith et al. 2007). Another ELBW infant (GA 27 weeks, BW 980 g), who developed *Candida parapsilosis* septicaemia and was resistant to both amphotericin B and fluconazole, was treated with caspofungin for 21 days: the infection was eradicated and renal function tests were normal (Yalaz et al. 2006).

Two term newborns with persistent candidemia, unresponsive to catheter removal and amphotericin B treatment, received in addition caspofungin that was well-tolerated (Belet et al. 2006). Odio et al. (2004) reported ten candidemic newborns (one term, nine preterm) treated with caspofungin after failure of therapy with amphotericin B: renal function tests did not show any value above normal.

Some authors (Natarajan et al. 2005) analyzed the use of caspofungin in 13 critically ill neonates (12 preterm and one term) with documented candidemia who failed to respond to conventional antifungal therapy with amphotericin B and/or fluconazole or flucytosine: transient hypokalemia was observed in two patients. Eighteen newborns and infants (<3 months of age), previously treated with intravenous amphotericin B for highly suspected or documented candidiasis, received

multiple once-daily doses of caspofungin as a 1-h infusion. Even if one or more clinical and laboratory adverse events were observed in 94.4% of patients, none resulted drug-related but rather were consistent with prematurity and/or other birth complications (Saez-Llorenz et al. 2009). Caspofungin was generally well-tolerated also in nine older infants and toddlers (mean age 13 months) with fever and neutropenia (Neely et al. 2009). Franklin et al. (2003) reported the safety of caspofungin in 25 immunocompromized children: no patient experienced a severe adverse drug-related event and had to discontinue the therapy.

One open-label study evaluated the tolerability of caspofungin in 39 children (ages 2–17 years) with neutropenia. The treatment was generally well tolerated and none of the patients developed serious drug-related adverse events: six children, receiving concomitantly an aminoglycoside, experienced a transient decrease in creatinine clearance to 75% of baseline value (Walsh et al. 2005).

In a large multicenter retrospective survey (Groll et al. 2006), 64 severely immunocompromised pediatric patients (mean age 11.5 years) received caspofungin as single agent or in combination: mild adverse events were observed in 53.1% of patients, but they were not related to renal function (serum creatinine was not different from baseline values).

Other authors (Merlin et al. 2006) analyzed the data of 20 children (mean age 12 years) treated with caspofungin for proven or probable invasive fungal infections: two patients had moderate hypokalemia, but kidney function of patients with renal insufficiency did not further deteriorate.

Among 49 pediatric patients treated with caspofungin for invasive aspergillosis or candidiasis, 13 (27%) had mild clinical adverse events possibly related to the therapy, but none related to renal function (Zaoutis et al. 2009).

6.4.3.2 Other Echinocandins

As regards the other echinocandins, relatively few studies report safety data in newborns and children. Among a total of 77 neutropenic pediatric patients (2–17 years of age) treated with micafungin, mean serum creatinine levels did not change from baseline to the end of therapy (Seibel et al. 2005). Among a substudy population of 52 pediatric patients with invasive candidiasis (comprising seven preterm infants and 14 subjects <2 years), three cases of hypokalemia were observed and two patients experienced drug discontinuation due to respectively an increase in serum creatinine levels and a worsening of renal failure (Queiroz-Telles et al. 2008).

A total of 18 premature infants (BW > 1,000 g) and five preterm weighing 500–1,000 g with infections caused by *Candida* spp. were treated with micafungin: a moderate but persistent hypokalemia possibly related to the study drug was observed in one patient (Heresi et al. 2006). Micafungin was added to other antifungal drugs for the treatment of cutaneous aspergillosis in a premature infant (GA 24 weeks, BW 651 g). The only adverse event possibly related to micafungin was hypokalemia (Santos et al. 2007). Recently, four premature infants (mean GA

24 weeks, mean BW 579 g), diagnosed with *Candida* infections and previously treated with fluconazole, showed no apparent drug-related side-effects after receiving micafungin (Kawaguchi et al. 2009).

Anidulafungin was generally well-tolerated in 25 neutropenic pediatric patients (2–16 years old) with invasive fungal infections: only one child experienced an elevation in serum blood urea nitrogen (Benjamin et al. 2006a, b).

6.5 Antifungal Drugs

6.5.1 Comparative Data of the Literature

Relatively few pediatric trials have been conducted to compare nephrotoxicity of different antifungals in pediatric patients (Table 6.2).

As reported in a recent systematic review (Blyth et al. 2007), among antifungals used in children and newborns, azoles, echinocandins, and amphotericin B lipid formulations all are or seem to be associated with a significantly lower nephrotoxicity than for amphotericin B deoxycholate.

In detail, some authors (Jeon et al. 2007) compared the efficacy and safety of AmBisome and amphotericin B deoxycholate given to 46 VLBW infants (mean GA 262 weeks, mean BW 856 ± 236 g) to treat systemic candidiasis. The incidence of

Table 6.2 Nephrotoxicity comparison between different antifungal drugs

Reference	Setting	No	Treatment	% (Significance)
Prentice et al. (1997)	Children with fever and neutropenia	202	amph B vs AmBisome (two doses)	21% vs 8%–11% (NS)
Walsh et al. (1999a, b)	Children with fever and neutropenia	687	amph B vs AmBisome	34% vs 19% ($p = 0.001$)
Jeon et al. (2007)	VLBW infants with systemic candidiasis	46	amph B vs AmBisome	55% vs 21% ($p = 0.029$)
White et al. (1998)	Children with fever and neutropenia	46	amph B vs ABCD	52% vs 12% ($p = 0.01$)
Sandler et al. (2000)	Children with fever and neutropenia	49	amph B vs ABCD	52% vs 12% ($p = 0.003$)
Lopez Sastre et al. (2003)	Newborns with invasive candidiasis	110	AmBisome vs ABLC	6% vs 3% (NS)
Linder et al. (2003)	Newborns with candidemia	56	amph B vs AmBisome vs ABCD	–
Driessen et al. (1996)	Newborns with fungal septicaemia	23	amph B vs fluconazole	9% vs 0% ( )
Queiroz-Telles et al. (2008)	Children with candidiasis	106	AmBisome vs micafungin	11% vs 5.8% ( )
Mondal et al. (2004)	Children with candidemia	42	Fluconazole vs itraconazole	17% vs 12% (NS)

renal toxicity, defined as a 50% increase in serum creatinine levels, was significantly lower in the AmBisome group (21% vs 55%, $p = 0.029$). In a randomized multicenter trial involving 687 children with persistent fever and neutropenia, nephrotoxic effects were significantly less frequent among patients treated with AmBisome compared to those receiving conventional amphotericin B (19% vs 34%, $p = 0.001$) (Walsh et al. 1999a, b). In two pediatric double-blind randomized trials involving respectively 46 and 49 children, a reduced rate of nephrotoxicity was observed with ABCD compared to amphotericin B deoxycholate (52% vs 12%) (White et al. 1998; Sandler et al. 2000). On the other hand, other authors did not observe any significant difference in nephrotoxicity between amphotericin B deoxycholate and AmBisome given at two different doses (21% vs 8%–11%) (Prentice et al. 1997) or ABLC (6% vs 3%) (Lopez Sastre et al. 2003). Finally, Linder et al. (2003) did not find any nephrotoxic effect among three groups of newborns with candidemia treated with conventional amphotericin B, AmBisome or ABCD.

Both azoles and echinocandins seem to cause less nephrotoxicity than amphotericin B and AmBisome (Driessen et al. 1996; Walsh et al. 2002; Queiroz-Telles et al. 2008), while no trials comparing ABLC or ABCD to azoles and echinocandins exist. Finally, Mondal et al. (2004) did not find any significant difference in nephrotoxicity between children treated with fluconazole or itraconazole (17% vs 12%).

6.6 Conclusions

Fungal infections are common in children with defects in host defences and are responsible for considerable morbility and mortality overall in preterm newborns. Antifungins are important means to treat fungal infections. Therefore, the nephrotoxicity is an important adverse effect of antifungal agents. Frequently, the choice of which antifungin could be used in at-risk babies depend upon its reduced renal risk profile. Summarizing, Amphotericin B lipid formulations showed less nephrotoxicity than Amphotericin B. Among alternative antifungals for children and newborns, Fluconazole is the most widely used and tolerated azole. Unfortunately, the clinical experience of treating children with other newer azoles is more limited. The treatment with Caspofungin (Echinocandins) could be considered a potential alternative to conventional antifungal therapy. Finally, other echinocandins are available but relatively few studies report safety data in newborns and children.

References

Adler-Shohet F, Waskin H, Lieberman JM (2001) Amphotericin B lipid complex for neonatal invasive candidiasis. Arch Dis Child Fetal Neonatal Ed 84:F131–F133

Al Arishi H, Frayha HH, Kalloghlian A, Al Alaiyan SA (1998) Liposomal amphotericin B in neonates with invasive candidiasis. Am J Perinatol 15:643–648

Almirante B, Rodriguez D (2007) Antifungal agents in neonates: issues and recommendations. Paediatr Drugs 9:311–321

Arathoon EG, Gotuzzo E, Noriega LM, Berman RS, DiNubile MJ, Sable CA (2002) Randomized, double-blind, multicenter study of caspofungin versus amphotericin B for treatment of oropharyngeal and esophageal candidiases. Antimicrob Agents Chemother 46:451–457

Baley JE, Kliegman RM, Fanaroff AA (1984) Disseminated fungal infections in very low-birthweight infants: therapeutic toxicity. Pediatrics 73:153–157

Baley JE, Meyers C, Kliegman RM, Jacobs MR, Blumer JL (1990) Pharmacokinetics, outcome of treatment and toxic effects of amphotericin B and 5-fluorocytosine in neonates. J Pediatr 116:791–797

Barrett JP, Vardulaki KA, Conlon C, Cooke J, Daza-Ramirez P, Evans EG, Hawkey PM, Herbrecht R, Marks DI, Moraleda JM, Park GR, Senn SJ, Viscoli C (2003) Amphotericin B systematic review study group. A systematic review of the antifungal effectiveness and tolerability of amphotericin B formulations. Clin Ther 25:1295–1320

Belet N, Çiftçi E, İnce E, Dalgiç N, Öncel S, Güriz H, Yağmurlu A, Dindar H, Doğru Ü (2006) Caspofungin treatment in two infants with persistent fungaemia due to *Candida lipolytica*. Scand J Infect Dis 38:559–562

Benjamin DK, Driscoll T, Seibel NL, Gonzalez CE, Roden MM, Kilaru R, Clark K, Dowell JA, Schranz J, Walsh TJ (2006a) Safety and pharmacokinetics of intravenous anidulafungin in children with neutropenia at high risk for invasive fungal infections. Antimicrob Agents Chemother 50:632–638

Benjamin DK, Stoll BJ, Fanaroff AA, McDonald SA, Oh W, Higgins RD, Duara S, Poole K, Laptook A, Goldberg R, National Institute of Child Health and Human Development Neonatal Research Network (2006b) Neonatal candidiasis among extremely low birth weight infants: risk factors, mortality rates and neurodevelopmental outcomes at 18 to 22 months. Pediatrics 117:84–92

Blyth CC, Palasanthiran P, O'Brien TA (2007) Antifungal therapy in children with invasive fungal infections: a systematic review. Pediatrics 119:772–784

Butler K, Rench M, Baker C (1990) Amphotericin B as a single agent in the treatment of systemic candidiasis in neonates. Pediatr Infect Dis J 9:51–56

Clerihew L, Austin N, McGuire W (2007) Systemic antifungal prophylaxis for very-low-birthweight infants: systematic review. Arch Dis Child Fetal Neonatal Ed 93:F198–F200

Driessen M, Ellis JB, Cooper PA, Wainer S, Muwazi F, Hahn D, Gous H, de Villiers FP (1996) Fluconazole vs amphotericin B for the treatment of neonatal fungal septicaemia: a prospective randomized trial. Pediatr Infect Dis J 15:1107–1112

Eriksson U, Seifert B, Schaffner A (2001) Comparison of effects of amphotericin B deoxycholate infused over 4 or 24 hours: randomised controlled trial. Br Med J 322:579–582

Fanos V, Cataldi L (2000) Amphotericin B-induced nephrotoxicity: a review. J Chemother 12:463–470

Farmaki E, Evdoridou J, Pouliou T, Bibashi E, Panagopoulou P, Filioti J, Benos A, Sofianou D, Kremenopoulos G, Roilides E (2007) Fungal colonization in the neonatal intensive care unit: risk factors, drug susceptibility and association with invasive fungal infections. Am J Perinatol 24:127–135

Fernandez M, Moylett EH, Noyola DE, Baker CJ (2000) Candidal meningitis in neonates: a 10-year review. Clin Infect Dis 31:458–463

Fisher MA, Talbot GH, Maislin G (1989) Risk factor for amphotericin B-associated nephrotoxicity. Am J Med 87:547–552

Frank DW, Gray JE, Weaver RN (1976) Cyclodextrin nephrosis in the rat. Am J Pathol 83:367–378

Franklin JA, McCormick J, Flynn PM (2003) Retrospective study of the safety of caspofungin in immunocompromised pediatric patients. Pediatr Infect Dis J 22:747–749

Gerkens JF, Branch RA (1980) The influence of sodium status and furosemide on canine acute amphotericin B nephrotoxicity. J Pharmacol Exp Ther 214:306–311

Girois SB, Chapuis F, Decullier E, Revol BGP (2006) Adverse effects of antifungal therapies in invasive fungal infections: review and meta-analysis. Eur J Clin Microbiol Infect Dis 5:138–149
Glick C, Graves GR, Feldman S (1993) Neonatal fungemia and amphotericin B. South Med J 86:1368–1371
Goldman RD, Koren G (2004) Amphotericin B nephrotoxicity in children. J Pediatr Hematol Oncol 26:421–426
Goldman RD, Ong M, Wolpin J, Doyle J, Parshuram C, Koren G (2007) Pharmacological risk factors for amphotericin B nephrotoxicity in children. J Clin Pharmacol 47:1049–1054
Groll AH, Lehrnbecher T (2005) New antifungal drugs and the pediatric cancer patient: current status of clinical development. Klin Pëdiatr 217:158–168
Groll AH, Kurz M, Schneider W, Witt V, Schmidt H, Schneider M, Schwabe D (1999) Five-year survey of invasive aspergillosis in a pediatric cancer centre: epidemiology, management and long-term survival. Mycoses 42:431–442
Groll AH, Attarbaschi A, Schuster FR, Herzog N, Grigull L, Dworzak MN, Beutel K, Laws HJ, Lehrnbecher T (2006) Treatment with caspofungin in immunocompromised paediatric patients: a multicentre survey. J Antimicrob Chemother 57:527–535
Heresi GP, Gertsmann DR, Reed MD, van den Anker JN, Blumer JL, Kovanda L, Keirns JJ, Buell DN, Kearns GL (2006) The pharmacokinetics and safety of micafungin, a novel echinocandin, in premature infants. Pediatr Infect Dis J 25:1110–1115
Hesseling M, Weindling M, Neal T (2003) First reported use of caspofungin in an extremely low-birth-weight neonate. J Matern Fetal Neonatal Med 14:31
Hiemenz JW, Walsh TJ (1996) Lipid formulations of amphotericin B: recent progress and future direction. Clin Infect Dis 22(Suppl 2):S133–S144
Holler B, Omar SA, Farid MD, Patterson MJ (2004) Effects of fluid and electrolyte management on amphotericin B-induced nephrotoxicity among extremely low birth weight infants. Pediatrics 113:e608–e616
Huttova M, Hartmanova I, Kralinsky K, Filka J, Uher J, Kurak J, Krizan S, Krcmery V (1998) *Candida* fungemia in neonates treated with fluconazole: report of forty cases, including eight with meningitis. Pediatr Infect Dis J 17:1012–1015
Jabado N, Casanova JL, Haddad E, Dulieu F, Fournet JC, Dupont B, Fischer A, Hennequin C, Blanche S (1998) Invasive pulmonary infection due to *Scedosporium apiospermum* in two children with chronic granulomatous disease. Clin Infect Dis 27:1437–1441
Janoff AS, Perkins WR, Saletan SL (1993) Amphotericin B lipid complex (ABLC): a molecular rationale for the attenuation of amphotericin B-related toxicities. J Liposome Res 3:451–471
Jeon GW, Koo SH, Lee JH, Hwang JH, Kim SS, Lee EK, Chang W, Chang YS, Park WS (2007) A comparison of AmBisome® to amphotericin B for treatment of systemic candidiasis in very low birth weight infants. Yonsei Med J 48:619–626
Johansen HK, Gotzsche PC (2000) Amphotericin B lipid soluble formulations vs amphotericin B in cancer patients with neutropenia. Cochrane Database Syst Rev 3:CD000969
Johnson DE, Thompson TR, Green TP, Ferrieri P (1984) Systemic candidiasis in very low-birth-weight infants (less than 1,500 grams). Pediatrics 73:138–143
Kaufman D, Boyle R, Hazen KC, Patrie JT, Robinson M, Donowitz LG (2001) Fluconazole prophylaxis against fungal colonization and infection in preterm infants. N Engl J Med 345:1660–1666
Kaufman D, Boyle R, Hazen KC, Patrie JT, Robinson M, Grossman LB (2005) Twice weekly fluconazole prophylaxis for prevention of invasive *Candida* infection in high-risk infants of $<$1000 grams birth weight. J Pediatr 147:172–179
Kawaguchi C, Arai I, Yasuhara H, Sano R, Nishikubo T, Takahashi Y (2009) Efficacy of micafungin in treating four premature infants with candidiasis. Pediatr Int 51:220–224
Khoory BJ, Vino L, Dall'Agnola A, Fanos V (1999) *Candida* infections in neonates: a review. J Chemother 11:367–378
Kicklighter SD, Springer SC, Cox T, Hulsey TC, Turner RB (2001) Fluconazole for prophylaxis against candidal rectal colonization in the very low birth weight infant. Pediatrics 107:293–298

Kingo AR, Smyth JA, Waisman D (1997) Lack of evidence of amphotericin B toxicity in very low birth weight infants treated for systemic candidiasis. Pediatr Infect Dis J 16:1002–1003

Kohli V, Taneja V, Sachdev P, Joshi R (2008) Voriconazole in newborns. Indian Pediatr 45:236–238

Letscher-Bru V, Herbrecht R (2003) Caspofungin: the first representative of a new antifungal class. J Antimicrob Chemother 51:513–521

Lin SJ, Schranz J, Teitsch SM (2001) Aspergillosis case-fatality rate: systematic review of the literature. Clin Infect Dis 32:358–366

Linder N, Klinger G, Shalit I, Levy I, Ashkenazi S, Haski G, Levit O, Sirota L (2003) Treatment of candidaemia in premature infants: comparison of three amphotericin B preparations. J Antimicrob Chemother 52:663–667

Lopez Sastre JB, Coto Cotallo GD, Fernandez Colomer B, Grupo de Hospitales Castrillo (2003) Neonatal invasive candidiasis: a prospective multicenter study of 118 cases. Am J Perinatol 20:153–163

Luber AD, Maa L, Lam M, Guglielmo BJ (1999) Risk factors for amphotericin B-induced nephrotoxicity. J Antimicrob Chemother 43:267–271

Manzoni P, Stolfi I, Pugni L, Decembrino L, Magnani C, Vetrano G, Tridapalli E, Corona G, Giovannozzi C, Farina D, Arisio R, Merletti F, Maule M, Mosca F, Pedicino R, Stronati M, Mostert M, Gomirato G (2007) Italian task force for the study and prevention of neonatal fungal infections; italian society of neonatology. A multicenter, randomized trial of prophylactic fluconazole in preterm neonates. N Engl J Med 356:2483–2495

Maples HD, Stowe CD, Saccente SL, Jacobs RF (2003) Voriconazole serum concentrations in an infant treated for *Trichosporon beigelii* infection. Pediatr Infect Dis J 11:1022–1024

Mayer J, Doubek M, Doubek J, Horky D, Scheer P, Stepanek M (2002) Reduced nephrotoxicity of conventional amphotericin B therapy after minimal nephroprotective measures: animal experiments and clinical study. J Infect Dis 186:379–388

McCrossan BA, McHenry E, O'Neill F, Onq G, Sweet DG (2007) Selective fluconazole prophylaxis in high-risk babies to reduce invasive fungal infection. Arch Dis Child Fetal Neonatal Ed 92:F454–F458

Merlin E, Galambrun C, Ribaud P, Blanc T, Michel G, Auvrignon A, Stephan JL (2006) Efficacy and safety of caspofungin therapy in children with invasive fungal infections. Pediatr Infect Dis J 25:1186–1188

Mondal RK, Singhi SC, Chakrabarti AMJ (2004) Randomised comparison between fluconazole and itraconazole for the treatment of candidaemia in a pediatric intensive care unit: a preliminary study. Pediatr Crit Care Med 5:561–565

Mora-Duarte J, Betts R, Rotstein C, Colombo AL, Thompson-Moya L, Smietana J, Lupinacci R, Sable C, Kartsonis N, Perfect J (2002) Comparison of caspofungin and amphotericin B for invasive candidiasis. N Engl J Med 347:2020–2029

Muldrew KM, Maples HD, Stowe CD, Jacobs RF (2005) Intravenous voriconazole therapy in a preterm infant. Pharmacotherapy 25:893–898

Natarajan G, Lulic-Botica M, Rongkavilit C, Pappas A, Bedard M (2005) Experience with caspofungin in the treatment of persistent fungemia in neonates. J Perinatol 25:770–777

Neely M, Jafri HS, Seibel N, Knapp K, Adamson PC, Bradshaw SK, Strohmaier KM, Sun P, Bi S, Dockendorf MF, Stone JA, Kartsonis NA (2009) Pharmacokinetics and safety of caspofungin in older infants and toddlers. Antimicrob Agents Chemother 53:1450–1456

Odio CM, Araya R, Pinto LE, Castro CI, Vasquez S, Alfaro B, Saenz A, Herrera ML, Walsh TJ (2004) Caspofungin therapy of neonates with invasive candidiasis. Pediatr Infect Dis J 23: 1093–1097

Pappas PG, Rex JH, Lee J, Hamill RJ, Larsen RA, Powderly W, Kauffman CA, Hyslop N, Mangino JE, Chapman S, Horowitz HW, Edwards JE, Dismukes WE (2003) NIAID Mycoses Study Group. A prospective observational study of candidemia: epidemiology, therapy and influences on mortality in hospitalized adult and pediatric patients. Clin Infect Dis 37:634–643

Peleg AY, Woods ML (2004) Continuous and 4 h infusion of amphotericin B: a comparative study involving high risk haematology patients. J Antimicrob Chemother 54:803–808

Pfizer Inc (2004) Vfend (voriconazole) package insert. Pfizer, New York, NY

Ponton J, Rüchel R, Clemons KV, Coleman DC, Grillot R, Guarro J, Aldebert D, Ambroise-Thomas P, Cano J, Carrillo-Muñoz AJ, Genè J, Pinel C, Stevens DA, Sullivan DJ (2000) Emerging pathogens. Med Mycol 38(Suppl 1):225–236

Prentice HG, Hann IM, Herbrecht R, Aoun M, Kvaloy S, Catovsky D, Pinkerton CR, Sa S, Jacobs F, Oakhill A, Stevens RF, Darbyshire PJ, Gibson BE (1997) A randomized comparison of liposomal versus conventional amphotericin B for the treatment of pyrexia of unknown origin in neutropenic patients. Br J Haematol 98:711–718

Queiroz-Telles F, Berezin E, Leverger G, Freire A, van der Vyver A, Chotpitayasunondh T, Konja J, Diekmann-Berndt H, Koblinger S, Groll AH, Arrieta A (2008) Micafungin versus liposomal amphotericin B for pediatric patients with invasive candidiasis. Pediatr Infect Dis J 27:820–826

Rao S, Ali U (2005) Systemic fungal infections in neonates. J Postgrad Med 51:S27–S29

Sabra R, Takahaski R, Branch RA, Badr KF (1990) Mechanism of amphotericin B-induced reduction of glomerular filtration rate. J Pharmacol Exp Ther 253:34–37

Saez-Llorenz X, Macias M, Maiya P, Pineros J, Jafri HS, Chatterjee A, Ruiz G, Raghavan J, Bradshaw SK, Kartsonis NA, Sun P, Strohmaier KM, Fallon M, Bi S, Stone JA, Chow JW (2009) Pharmacokinetics and safety of caspofungin in neonates and infants less than 3 months of age. Antimicrob Agents Chemother 53:869–875

Sandler ES, Mustafa MM, Tkaczewski I, Graham ML, Morrison VA, Green M, Trigg M, Abboud M, Aquino VM, Gurwith M, Pietrelli L (2000) Use of amphotericin B colloidal dispersion in children. J Pediatr Hematol Oncol 22:242–246

Santos RP, Sanchez PJ, Mejias A, Benjamin DK, Walsh TJ, Patel S, Jafri HS (2007) Successful medical treatment of cutaneous aspergillosis in a premature infant using liposomal amphotericin B, voriconazole and micafungin. Pediatr Infect Dis J 26:364–366

Scarcella A, Pasquariello MB, Giugliano B, Vendemmia M, De Lucia A (1998) Liposomal amphotericin B treatment for neonatal fungal infections. Pediatr Infect Dis J 17:146–148

Seibel NL, Schwartz C, Arrieta A, Flynn P, Shad A, Albano E, Keirns J, Lau WM, Facklam DP, Buell DN, Walsh TJ (2005) Safety, tolerability and pharmacokinetics of micafungin (FK463) in febrile neutropenic pediatric patients. Antimicrob Agents Chemother 49:3317–3324

Sheehan DJ, Hitchcock CA, Sibley CM (1999) Current and emerging azole antifungal agents. Clin Microbiol Rev 12:40–79

Smith PB, Steinbach WJ, Cotten CM, Schell WA, Perfect JR, Walsh TJ, Benjamin DK (2007) Caspofungin for the treatment of azole resistant candidemia in a premature infant. J Perinatol 27:127–129

Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran S, Laptook AR, Stevenson DK, Papile LA, Poole WK (2002) Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. Pediatrics 110:285–291

Stone EA, Fung HB, Kieschenbaum HL (2002) Caspofungin: an echinocandin antifungal agent. Clin Ther 24:351–377

Tolins JP, Raij L (1988) Adverse effect of amphotericin B administration on renal hemodynamics in the rat: neurohumoral mechanisms and influence of calcium channel blockade. J Pharmacol Exp Ther 245:594–599

Turcu R, Patterson MJ, Omar S (2009) Influence of sodium intake on amphotericin B-induced nephrotoxicity among extremely premature infants. Pediatr Nephrol 24:497–505

Turner RB, Donowitz LG, Hendley JO (1985) Consequences of candidemia for pediatric patients. Am J Dis Child 139:178–180

Uekama K, Shiotani K, Irie T, Ishimaru Y, Pitha J (1993) Protective effects of cyclodextrin sulphates against gentamicin-induced nephrotoxicity in the rat. J Pharm Pharmacol 45: 745–747

Uhlenbrock S, Zimmermann M, Fegeler W, Jürgens H, Ritter J (2001) Liposomal amphotericin B for prophylaxis of invasive fungal infections in high-risk paediatric patients with chemotherapy-related neutropenia: interim analysis of a prospective study. Mycoses 44:455–463

van't Hek LG, Verweij PE, Weemaes CM, van Dalen R, Yntema J, Meis JF (1998) Successful treatment with voriconazole of invasive aspergillosis in chronic granulomatous disease. Am J Respir Crit Care Med 157:1694–1696

Varlam DE, Siddiq MM, Parton LA, Russmann H (2001) Apoptosis contributes to amphotericin B-induced nephrotoxicity. Antimicrob Agents Chemother 45:679–685

Vazquez JA, Sobel JD (2006) Anidulafungin: a novel echinocandin. Clin Infect Dis 43: 215–222

Vermes A, Guchelaar HJ, Dankert J (2000) Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. J Antimicrob Chemother 46:171–179

Villanueva A, Arathoon EG, Gotuzzo E, Berman RS, DiNubile MJ, Sable CA (2001) A randomized double-blind study of caspofungin versus amphotericin for the treatment of candidal esophagitis. Clin Infect Dis 33:1529–1535

Walsh TJ, Finberg RW, Arndt C, Hiemenz J, Schwartz C, Bodensteiner D, Pappas P, Seibel N, Greenberg RN, Dummer S, Schuster M, Holcenberg JS (1999a) Liposomal amphotericin B for empirical therapy in patients with persistent fever and neutropenia. National Institute of Allergy and Infectious Diseases Mycoses Study group. N Engl J Med 340:764–771

Walsh TJ, Seibel NL, Arndt C, Harris RE, Dinubile MJ, Reboli A, Hiemenz J, Chanock SJ (1999b) Amphotericin B lipid complex in pediatric patients with invasive fungal infections. Pediatr Infect Dis J 18:702–708

Walsh TJ, Lutsar I, Driscoll T, Dupont B, Roden M, Ghahramani P, Hodges M, Groll AH, Perfect JR (2002) Voriconazole in the treatment of aspergillosis, scedosporiosis and other invasive fungal infections in children. Pediatr Infect Dis J 21:240–248

Walsh TJ, Karlsson MO, Driscoll T, Arguedas AG, Adamson P, Saez-Llorens X, Vora AJ, Arrieta AC, Blumer J, Lutsar I, Milligan P, Wood N (2004) Pharmacokinetics and safety of intravenous voriconazole in children after single- or multiple-dose administration. Antimicrob Agents Chemother 48:2166–2172

Walsh TJ, Adamson PC, Seibel NL, Flynn PM, Neely MN, Schwartz C, Shad A, Kaplan SL, Roden MM, Stone JA, Miller A, Bradshaw SK, Li SX, Sable CA, Kartsonis NA (2005) Pharmacokinetics, safety and tolerability of caspofungin in children and adolescents. Antimicrob Agents Chemother 49:4536–4545

Wegner B, Baer P, Gauer S, Oremek G, Hauser IA, Geiger H (2005) Caspofungin is less nephrotoxic than amphotericin B *in vitro* and predominantly damages distal renal tubular cells. Nephrol Dial Transplant 20:2071–2079

Weitkamp JH, Poets CF, Sievers R, Musswessel E, Groneck P, Thomas P, Bartmann P (1998) *Candida* infection in very low birth-weight infants: outcome and nephrotoxicity of treatment with liposomal amphotericin B (AmBisome). Infection 26:11–15

White MH, Bowden RA, Sandler ES, Graham ML, Noskin GA, Wingard JR, Goldman M, van Burik JA, McCabe A, Lin JS, Gurwith M, Miller CB (1998) Randomized, double-blind clinical trial of amphotericin B colloidal dispersion vs amphotericin B in the empirical treatment of fever and neutropenia. Clin Infect Dis 27:296–302

Wiley JM, Seibel NL, Walsh TJ (2005) Efficacy and safety of amphotericin B lipid complex in 548 children and adolescents with invasive fungal infections. Pediatr Infect Dis J 24: 167–174

Wingard JR, Kubilis P, Lee L, Yee G, White M, Walshe L, Bowden R, Anaissie E, Hiemenz J, Lister J (1999) Clinical significance of nephrotoxicity in patients treated with amphotericin B for suspected or proven aspergillosis. Clin Infect Dis 29:1402–1407

Yalaz M, Akisu M, Hilmioglu S, Calkavur S, Cakmak B, Kultursay N (2006) Successful caspofungin treatment of multidrug resistant *Candida parapsilosis* septicaemia in an extremely low birth weight neonate. Mycoses 49:242–245

Zager RA, Bredl CR, Schimpf BA (1992) Direct amphotericin B-mediated tubular toxicity: assessment of selected cytoprotective agents. Kidney Intern 41:1588–1594

Zaoutis T, Walsh TJ (2007) Antifungal therapy for neonatal candidiasis. Curr Opin Infect Dis 20:592–597

Zaoutis TE, Jafri HS, Huang L, Locatelli F, Barzilai A, Ebell W, Steinbach WJ, Bradley J, Lieberman JM, Hsiao C, Seibel N, Laws H, Gamba M, Petrecz M, Taylor AF, Strohmaier KM, Chow JW (2009) A prospective, multicenter study of caspofungin for the treatment of documented *Candida* or *Aspergillus* infections in pediatric patients. Pediatrics 123:877–884

Chapter 7
Antifungal Resistance: Cellular and Molecular Mechanisms

Ricardo Araujo and Ana Espinel-Ingroff

Abstract The incidence of life-threatening fungal infections, a critical public health threat, has dramatically increased, and infections due to resistant strains represent a therapeutic drug selection challenge. Although MICs are not the best predictors of patient response to therapy and clinical breakpoints are still not available for all licensed agent/species combinations, susceptibility testing remains the most practical tool for *in vitro* resistance detection. Some fungal species may be inherently resistant to an antifungal; however, the increasing use of antifungal prophylactic and empirical treatments has led to the emergence of resistance. The overexpression of genes encoding proteins of biosynthetic pathways or efflux pumps and modifications of target enzyme(s) represent the most relevant molecular mechanisms involved in fungal resistance. The cellular and molecular mechanisms responsible for antifungal and multidrug *in vitro* resistance will be reviewed in this chapter as well as their impact in patient care.

7.1 Introduction

Drug resistance has been known since the first antibiotics were introduced for bacterial infection treatment. There are two kinds of resistance: clinical and *in vitro* resistance. Clinical resistance concerns patient response to therapy, regardless of *in vitro* susceptibility; it can be affected by drug pharmacokinetics/pharmacodynamics (e.g. tissue availability), interaction with other drugs, host status and

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immune response, patient management, and the infecting isolate virulence factors (Pemán et al. 2009).

In vitro resistance can be primary/intrinsic for the resistance found in organisms naturally, or secondary/acquired for organisms that were susceptible but became resistant during therapy. Susceptibility testing remains the most practical tool for the detection of *in vitro* fungal resistance. Patients infected with fungal strains which are *in vitro* resistant to antifungal agents have shown a lower rate of treatment success (Kanafani and Perfect 2008; Lass-Flörl et al. 2005; Rodriguez-Tudela et al. 2008a). Resistance outbreaks originate from either the latter mechanism (Bulik et al. 2009; Howard et al. 2009; Nolte et al. 1997) or from the environment (Snelders et al. 2009). Today, there are several antifungal agents available for the treatment of fungal infections. The known resistance mechanisms for these agents will be described, but it is not within the scope of this chapter to cover clinical resistance.

7.2 Resistance to Polyenes

7.2.1 Polyenes for Antifungal Therapy

The polyenes were the first group of antifungal agents – available for over 40 years – for the systemic treatment of yeast and mould infections. Among the polyenes, amphotericin B formulations (conventional, liposomal and lipid complex) are usually administered to patients suffering from severe and distinct fungal infections. The polyenes act by forming pores in the fungal membrane that leads to transmembrane potential loss and affect fungal cell viability. The most commonly used polyenes are amphotericin B formulations, which are licensed as empirical therapy (liposomal formulation in neutropenic febrile patients), and as primary or salvage therapy against aspergillosis, cryptococcosis, coccidioidomycosis, histoplasmosis, zygomycosis, and several forms of systemic candidiasis including candidemia (Erjavec et al. 2009; Pappas et al. 2009). Most fungi are usually susceptible to amphotericin B, but a few species are intrinsically resistant such as some *Alternaria* spp. isolates (Pastor and Guarro 2008), *Aspergillus flavus* (Araujo et al. 2007; Chandrasekar 2009), *Aspergillus lentulus* (Balajee et al. 2005), *Aspergillus terreus* (Araujo et al. 2007; Lass-Flörl et al. 2009), *Aspergillus ustus* (Panackal et al. 2006), *Candida lusitaniae* (Pfaller et al. 1994), *Fusarium* spp. (Azor et al. 2008; Tortorano et al. 2008), *Paecilomyces lilacinus* (Castelli et al. 2008; Espinel-Ingroff 2008), *Penicillium marneffei* (Supparatpinyo et al. 1993), *Scedosporium apiospermum* (Alastruey-Izquierdo et al. 2007; Espinel-Ingroff 2008), *Scedosporium prolificans* (Espinel-Ingroff 2008), *Scopulariopsis brevicaulis* (Salmon et al. 2010), *Sporothrix schenckii* (Alvarado-Ramírez and Torres-Rodríguez 2007), and a few zygomycetes (Almyroudis et al. 2007; Espinel-Ingroff 2008). However, infections caused by isolates of these species have been occasionally treated with success by amphotericin B, despite high MICs (>2 μg/ml) (Nolte et al. 1997).

7.2.2 Yeast Resistance to Polyenes

Acquired resistance to amphotericin B has been described among species typically susceptible to this agent, such as *Candida albicans*, *Candida tropicalis* and *Candida glabrata* (MICs $\geq$ 16 μg/ml following treatment with this polyene) (Dick et al. 1980; Krogh-Madsen et al. 2006; Nolte et al. 1997). A decrease of membrane ergosterol content was found in these isolates, which indicated that resistance was caused by removal of the membrane antifungal target. The ergosterol deficiency can be due to defects in the *ERG3* gene, which leads to the accumulation of other sterols in the membrane (Dick et al. 1980). The role of *ERG3* and *ERG11* genes for addition of lanosterol and eburicol to the membrane of *C. albicans* mutants has been verified by Sanglard et al. (2003). Although Geber et al. (1995) did not find a decrease of amphotericin B susceptibility following *ERG3* deletion in *C. glabrata*, they reported that *ERG6* plays an important role in its resistance to amphotericin B. The function of *ERG6* was more recently confirmed; a unique missense mutation in *ERG6* of *C. glabrata* — causing a substitution of a cysteine by a phenylalanine in the protein at the position 198 (C198F) — resulted in both low polyene activity and final steps of ergosterol pathway defects (Vandeputte et al. 2007). Recently, another six genes (*YGR035C*, *YOR1*, *ICT1*, *GRE2*, *PDR16* and *YPLO88W*) were found to be overexpressed in *Saccharomyces cerevisae* populations exposed to increasing concentrations of amphotericin B (Anderson et al. 2009). The genes *ERG1*, *ERG25*, *SKN1* and *KRE1* were found upregulated in *C. albicans* biofilms resistant to amphotericin B (Khot et al. 2006). But the role of those genes in acquired amphotericin B-resistant clinical isolates needs to be determined.

7.2.3 Mould Resistance to Polyenes

Among common moulds associated with invasive infections, *Aspergillus fumigatus* is usually susceptible *in vitro* to amphotericin B, but some isolates have been reported with decreased susceptibility to amphotericin B (Messer et al. 2006). Resistance mechanisms in moulds are still unknown. *ERG3A* and *ERG3B* genes were described as not essential for *A. fumigatus* viability (one isolate) and did not play a relevant role in *in vitro* susceptibility of this species to amphotericin B (Alcazar-Fuoli et al. 2006); both *A. fumigatus* single- and double-mutants and wild-type strain showed similar susceptibility to amphotericin B.

In addition to the defects in membrane ergosterol content, another mechanism involved in resistance to amphotericin B is the reduction of fungal oxidative cellular damage (Sokol-Anderson et al. 1986). For example, *A. terreus,* traditionally resistant to amphotericin B, has produced a higher amount of catalase (an important enzyme that protects fungal cells against oxidative damage) than that in *A. fumigatus* susceptible isolates (Blum et al. 2008b). Although this mechanism was not investigated in other fungi, it may be assumed that strains producing more catalase would be less susceptible to the polyenes.

The susceptibility to the polyenes is dependent on the nitrogen source. In the presence of bovine serum albumin or casein, *C. albicans* was less susceptible to amphotericin B and nystatin than in the presence of ammonium sulphate (Oliver et al. 2008). *A. fumigatus* has also been previously described as less susceptible *in vitro* to amphotericin B in the presence of human albumin (Rodrigues et al. 2005). The mechanism that is associated with amphotericin B activity alteration has not been determined. The fact that distinct pathways are involved in the development of polyene resistance should be considered.

7.3 Resistance to Azoles

7.3.1 Azole Antifungal Therapy

Several azoles are widely used in the treatment of severe fungal infections. The azoles act by blocking the pathway of ergosterol biosynthesis, specifically the enzymes 14-α-lanosterol demethylase in yeasts or the 14-α-sterol demethylase in moulds. These cytochrome enzymes are encoded by the *ERG11* and *CYP51* (*A* and *B*) genes, respectively in yeasts and moulds (Pemán et al. 2009). Some of the first available azoles (e.g. ketoconazole, miconazole) and triazoles (fluconazole and itraconazole) are still used in patient care. Azoles have good *in vitro* activity against a variety of yeasts and some moulds. The newest triazoles, voriconazole and posaconazole, have a broader spectrum of activity against yeasts as well as moulds (Chandrasekar 2009; Groll and Kolve 2004). The ergosterol biosynthetic pathway is crucial for studying the activity of all azoles. Today, this pathway is well understood; the group of enzymes that are involved at any level in this pathway have also been identified (Alcazar-Fuoli et al. 2008; Veen et al. 2003).

The triazoles are widely used as therapeutic antifungal agents. Fluconazole is licensed for a variety of yeast infections, especially those caused by *Candida* [prevention (hematopoietic stem cell transplant patients) and primary treatment of candidiasis in non-neutropenic patients] and *C. neoformans* (Erjavec et al. 2009; Pappas et al. 2009). Itraconazole serves as prophylactic and primary treatment of aspergillosis and allergic bronchopulmonary aspergillosis; also, it can be useful in the treatment of coccidioidomycosis, histoplasmosis, blastomycosis and sporotrichosis (Hope et al. 2008). Voriconazole is indicated for candidemia in non-neutropenic patients and for infections caused by *Candida* isolates resistant to fluconazole; it is the first line treatment for invasive aspergillosis and for infections caused by *S. apiospermum* and *Fusarium* spp. (Erjavec et al. 2009; Pappas et al. 2009). Posaconazole has been cleared as a prophylactic agent in immunosuppressed patients for the prevention of *Candida* and *Aspergillus* infections, and as salvage therapy against invasive aspergillosis, fusariosis, chromoblastomycosis and coccidioidomycosis (Hope et al. 2008; Pappas et al. 2009). Despite the broad spectrum of azole activity, a few fungal species are intrinsically resistant to these agents

(e.g. *Candida krusei* versus fluconazole, certain *Aspergillus* spp., *Fusarium* spp., *Scedosporium*, and the zygomycetes versus fluconazole, itraconazole and voriconazole) (Chandrasekar 2009). In general, the triazoles have fungistatic activity against most *Candida* spp. and *Aspergillus* spp., but voriconazole and posaconazole have fungicidal activity against *Aspergillus* spp. (Groll and Kolve 2004).

7.3.2 Yeast Resistance to Azoles

The azoles and especially the triazoles have been increasingly used during the last two decades, and their excessive use, especially of fluconazole, has resulted in worldwide reports of the emergence of resistance following azole therapy (Bulik et al. 2009; Chakrabarti et al. 2009; Małafiej et al. 2009). Usually resistance to fluconazole means cross-resistance with itraconazole, voriconazole, and/or posaconazole and other azoles. However, extended exposure of *C. krusei* to fluconazole or voriconazole without changing its susceptibility profile has been documented (Hautala et al. 2009). It is easy to induce *in vitro* resistance to the azoles and triazoles in the laboratory and because of that a distinct dynamic pattern for acquiring resistance has been demonstrated. Fluconazole resistance seems to be induced more rapidly *in vitro* than resistance to either voriconazole or posaconazole, but cross-resistance to all three drugs is only observed with posaconazole (Silva et al. 2009). The following azole resistance mechanisms have been described: (1) genetic mutations that reduce the target affinity to the drug, (2) overexpression of the genes encoding the target enzyme, (3) activation of efflux pumps that removed the drug from the fungal cell, (4) occurrence of aneuploidy or increased genomic instability, and (5) intervention of stress-response functions that increased the survival of the fungus.

7.3.2.1 Gene Expression and Mutations

Several point mutations in *ERG11* (which encodes the enzyme 14-α-lanosterol demethylase) were described in *C. albicans* and *C. tropicalis* clinical isolates that were associated with triazole resistance (Dunkel et al. 2008b; Nolte et al. 1997; Sanglard et al. 1998; Vandeputte et al. 2005). Earlier *in vitro* studies have revealed that two hotspot regions in *C. albicans ERG11* were more prone to alterations and easily originated azole resistance (Marichal et al. 1999). Those mutations are responsible for the reduction of the affinity of azoles to 14-α-lanosterol demethylase by changing the enzyme structure and conformation. The substitution of G484S in the same enzyme of *Cryptococcus neoformans* azole-resistant isolate has been reported (Sheng et al. 2009). In vitro exposure of yeasts to azoles has resulted in the identification of several other mutations, but their clinical importance needs to be explored (Cheng et al. 2007; Marichal et al. 1999; Sanglard et al. 1998).

In addition to point mutations, the overexpression of *ERG11* might result in the decrease of fungal susceptibility to triazoles (e.g. *C. glabrata* versus fluconazole) (Redding et al. 2003). It has been demonstrated that the presence of an azole agent causes increased expression and induction of *ERG11* promoter and accumulation of lanosterol (and/or depletion of ergosterol) in *C. albicans* membrane (Song et al. 2004). The direct interaction between azole exposure, ergosterol membrane content and gene expression, particularly *ERG11*, has been clarified when studying the effect of histone deacetylase inhibitors, such as trichostatin A (TSA), on azole susceptibility (Smith and Edlind 2002). TSA reduced itraconazole MICs for *C. albicans* after 24 h by interfering with the expression of several genes at various levels — *ERG11* was one of the genes directly affected by TSA (Smith and Edlind 2002). *C. albicans* with null mutations of *ERG3* were fluconazole-resistant (Sanglard et al. 2003).

Transcription factors may also interfere with yeast susceptibility to azoles. The zinc cluster transcription factor UPC2p regulates the expression levels of *ERG11* in the presence of triazoles. A gain-of-function mutation of UPC2p may lead to the increased expression of *ERG11* and to fluconazole resistance in *C. albicans* clinical isolates (Dunkel et al. 2008b).

7.3.2.2 Efflux Pumps

Another relevant resistance mechanism is the overexpression of genes encoding membrane efflux pumps. Two classes of efflux pumps are directly involved in removing triazoles from the cytoplasm: (1) pumps belonging to the major facilitator superfamily (MFS), and (2) the ATP-binding cassette (ABC) superfamily of transporters. *MDR1* and *FLU1* genes encode MFS transporters specific for fluconazole. Several researchers have reported the overexpression of the *MDR1* gene in fluconazole-resistant *C. albicans* clinical isolates (Franz et al. 1998; Perea et al. 2001), *C. dubliniensis* (Pinjon et al. 2005; Wirsching et al. 2001) and, more recently, *C. albicans* petite mutants less susceptible to fluconazole and voriconazole (Cheng et al. 2007). *FLU1* encodes a multidrug efflux transporter in *C. albicans*; this mechanism was also responsible for fluconazole resistance in *S. cerevisiae* mutants (Calabrese et al. 2000). The *FLR1* transporter in *C. glabrata* (also MFS transporter) has not been associated with fluconazole resistance, but it has been described in resistance to benomyl (Chen et al. 2007).

CDR1 and *CDR2* genes encoded ABC transporters in *C. albicans* (Sanglard et al. 1995; Sanglard et al. 1997), *C. glabrata* (Bennett et al. 2004; Miyazaki et al. 1998) and *C. dubliniensis* (Pinjon et al. 2005). These transporters were repeatedly involved in yeast resistance to different triazoles and to the uptake of other oligopeptides (Wakiec et al. 2008). There are other genes encoding specific ABC transporters and some of them were responsible for fluconazole resistance in clinical isolates: (1) *SNQ2* in *C. glabrata* (Sanguinetti et al. 2005), (2) specific genes detected *in vitro* in *C. tropicalis* (similar to *CDR1* in other *Candida* species) (Barchiesi et al. 2000),

(3) *ABC1* and *ABC2* in *C. krusei* (Katiyar and Edlind 2001), and (4) *AFR1* in *C. neoformans* (Posteraro et al. 2003).

The regulation and expression of efflux pump genes can be changed by multiple mechanisms. As previously described for *ERG11*, point mutations in transcription factors may affect both transporter groups (Morschhäuser 2010). Transcription factors have been observed for *MDR1*, such as the activator MRR1p (Morschhäuser et al. 2007) and the repressor REP1p (Chen et al. 2009). A single gain-of-function mutation in *MRR1* was responsible for the overexpression of *MDR1* in *C. albicans* (Schubert et al. 2008), and a few comparable gain-of-function mutations in the *MRR1* that encoded a transcription factor in *C. dubliniensis* (Dunkel et al. 2008a) resulted in the development of fluconazole resistance; the same applied for the transcription factors encoded by *PDR1* and *TAC1* in *C. albicans* and *C. glabrata* (Coste et al. 2007; Ferrari et al. 2009; Tsai et al. 2006; Vermitsky and Edlind 2004; Vermitsky et al. 2006; Znaidi et al. 2007). The simple overexpression of the transcription factors encoded by *CAP1* and *NDT80* also led to *C. albicans* azole resistance (Sellam et al. 2009b; Znaidi et al. 2009); this mechanism was similar to that found in the AP1p in *C. glabrata* (Chen et al. 2007). The uptake function of efflux pumps and intracellular accumulation of triazoles can be altered when the lipid and sterol contents of plasma membranes are changed (e.g. in *C. albicans*) (Löffler et al. 2000). The localization of the efflux pumps along the membrane, particularly of ones codified by *CDR1*, is also largely affected (Pasrija et al. 2008).

7.3.2.3 Cellular Stress

Antifungal stress among others can induce the expression of a large set of genes responsible for cell protection. In humans, the environmental conditions fluctuate a lot and it is extremely important for the fungal invaders to adapt and defend themselves against these "exterior" aggressions. Fungal genomic instability tends to increase in such stressful conditions and drug resistance can occur more rapidly. This fact was observed in *C. albicans* when the function of proteins encoded by DNA repair genes was compromised (Legrand et al. 2007). In fact, *C. albicans* is a "surprising" yeast that constantly shows a high genomic plasticity, which allows the formation of isochromosomes and the observation of aneuploidy and a high level of heterozygosity (Selmecki et al. 2006). The formation of isochromosomes originated azole resistance in *C. albicans* clinical strains when the region of the chromosome 5 (*ERG11*, *TAC1* and genes encoding efflux pumps are located in this region) is affected by such genomic mechanisms (Selmecki et al. 2006). Azole resistance arose from the increased copy number of these genes and it was also associated with other resistance mechanisms (e.g. point mutations in *ERG* genes) (Coste et al. 2007). In addition to *C. albicans*, *C. glabrata* also presented a highly variable karyotype and genotype that perhaps originated either from chromosomal rearrangements (Shin et al. 2007) or from the formation of new chromosomes (Poláková et al. 2009). Another relevant contributor to fungal stress has been the chaperone HSP90p that enables considerable changes in cell membranes and walls through

the interaction with calcineurin (a key sensor of environmental stress) (Cowen and Lindquist 2005). In fact, *HSP90* has participated in the development of azole-resistant *C. albicans* (via calcineurin) and its inhibition increased fungal susceptibility to azoles (Cowen et al. 2006, 2009). A recent study has shown that the role of the calcineurin pathway in the susceptibility to azoles is crucial. The depletion of the gene *RTA2*, involved in the calcineurin pathway, facilitated azole antifungal activity against *C. albicans* by changing the cell membrane vulnerability to triazoles (Jia et al. 2009). The inhibition of other signalling pathways (e.g. *TOR*) completely abolished azole resistance (Robbins et al. 2010). It is possible that all global regulators of cellular signalling implicated in the cellular responses to the environment participate and influence key steps in the development of azole resistance. Other fungal proteins, such as DDR48p and ADA2p, have promoted cellular stress responses and have a role in triazole-resistant *C. albicans* (Dib et al. 2008; Sellam et al. 2009a). The induction of an alternative respiratory pathway and expression of *AOX* gene has reduced *C. albicans* susceptibility to fluconazole (Yan et al. 2009).

In conclusion, azole resistance mechanisms in yeasts are likely to be multifactorial, involving both cellular and molecular events. The latest studies have shown that by interfering with stress contributor responses, signalling regulators, proteins involved in membrane changes and modelling of respiratory functions, these mechanisms may also alter yeast susceptibility to azoles.

7.3.3 Mould Resistance to Azoles

Acquired azole resistance in moulds is less understood than previously described for yeasts as only a few mechanisms have been described for *A. fumigatus* and *A. flavus* as follows. Point mutations in the *CYP51A* gene (encode 14-α-sterol demethylase) can result in amino acid substitutions in the enzyme. Until now, multi-azole resistance in *A. fumigatus* was caused by substitution of the glicine in position 54 and of methionine in position 220 by different amino acids, but substitution of L98H and two copies of 34 bp in the *CYP51A* promoter also have been identified (Howard et al. 2009; Mellado et al. 2007; Rodriguez-Tudela et al. 2008a, b; Snelders et al. 2008; Trama et al. 2005; van der Linden et al. 2009). Krishnan-Natesan et al. (2008) also reported some mutations in the *CYP51A* of *A. flavus* which explained the multi-azole resistance following azole *in vitro* exposure. In addition, other rarer substitutions may originate multi-azole resistance in *A. fumigatus* clinical isolates, particularly G138C, G448S, and a 53 bp tandem repeat in the promoter of *CYP51A* (Bellete et al. 2009; Hodiamont et al. 2009; Howard et al. 2006). Recently, several mutations were associated with azole resistance among *A. fumigatus* recovered from patients failing triazole treatment (Howard et al. 2009). Thus, it is expected that new mutations will be identified as the investigation of mould azole resistance mechanisms continues.

A few efflux pumps have been described in *A. fumigatus* and *A. flavus* (ABC transporters). *MDR1*, *ATRf*, *MDR2* and *MDR4* encoded ABC efflux pumps in *A. fumigatus*, while *MDR3* was responsible for an MFS transporter in this species (Morschhäuser 2010; Nascimento et al. 2003; Slaven et al. 2002; Tobin et al. 1997). These transporters may explain itraconazole resistance in a few clinical isolates of *A. fumigatus* (Denning et al. 1997; Slaven et al. 2002). *MDR1* was also described in *A. flavus* (Tobin et al. 1997), while several *ATR* transporters were identified in *A. nidulans* (Andrade et al. 2000). The participation of such transporters in azole-resistant clinical strains needs clarification.

The regulators of the cellular stress, particularly involving signalling pathways, may also play a relevant role in mould azole resistance. The inhibition of *HSP90* appeared to enhance the activity of azoles against *A. fumigatus* (Cowen et al. 2009).

7.4 Resistance to Echinocandins

7.4.1 Echinocandins for Antifungal Therapy

The echinocandins represent a unique class of antifungal agents that act by blocking the activity of 1,3-β-D-glucan synthase (GS), an important enzyme for the formation of the cell wall component 1,3-β-D-glucan. Caspofungin was the first agent to be cleared for treatment of candidemia in neutropenic and non-neutropenic patients, as salvage therapy for invasive aspergillosis and empirical treatment in febrile neutropenia (Bennett 2006; Pappas et al. 2009). More recently, micafungin has been licensed for prophylaxis of *Candida* infections in high-risk patients (allotransplant and >10 days neutropenia) and as primary therapy for invasive candidiasis, as well as anidulafungin for primary therapy against candidemia and other *Candida* infections in non-neutropenic patients (Erjavec et al. 2009; Naeger-Murphy and Pile 2009; Pappas et al. 2009). In general, the echinocandins show good *in vitro* activity against most *Candida* spp. and *Aspergillus* spp., but lower activity against *Candida parapsilosis*, *Candida metapsilosis*, *Candida orthopsilosis* and *Candida guilliermondii* and most moulds (Garcia-Effron et al. 2008; Pemán et al. 2009; Silva et al. 2009). *C. neoformans*, *Fusarium* spp., *Scedosporium* spp. and zygomycetes are intrinsically resistant to echinocandins (Chandrasekar 2009; Espinel-Ingroff 1998).

7.4.2 Fungal Resistance: Yeast and Moulds

Acquired resistance to echinocandins in *Candida* spp. clinical isolates has been described as a result of point mutations in the *FKS1* gene (Perlin 2007) or the substitution of one or more amino acids in the structure of the GS enzyme. The most common alterations in the GS of *C. albicans* clinical isolates were in the codons 641

and 645, which resulted in caspofungin resistance (Arendrup et al. 2009; Baixench et al. 2007; Katiyar et al. 2006; Perlin 2007). Alterations in GS were also associated with echinocandin resistance in *C. glabrata*, particularly the substitution of P665C (Cleary et al. 2008), and in *C. krusei* the substitutions of P665C and R1361G (Kahn et al. 2007; Park et al. 2005). However, echinocandin resistance in some *C. krusei* has not been related to mutations in *FKS1* (Hakki et al. 2006). *FKS2* mutations (mainly the substitution of F659V) led to echinocandin resistance in *C. glabrata* clinical isolates (Katiyar et al. 2006; Thompson et al. 2008). The innate lower susceptibility of *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* to echinocandins was explained by a point mutation in *FKS1*, amino acid alteration of P660A (Garcia-Effron et al. 2008). In this last report, the transformation of *C. parapsilosis* with a "normal" *FKS1* isolated from a susceptible *C. albicans* strain produced a *C. parapsilosis* mutant that was highly susceptible to caspofungin. Other laboratory *C. albicans* mutants (GS alterations F641S, L644F and S645C) were described as resistant to caspofungin (Balashov et al. 2006). In moulds, a single mutation in *A. fumigatus FKS1* (amino acid alteration of S678P in the GS structure) was associated with echinocandin resistance (Rocha et al. 2007). In addition, the primary echinocandin resistance of *Fusarium solani* and *S. prolificans* was explained by a substitution in GS (F639Y) (Katiyar and Edlind 2009).

The echinocandins are not a substrate of *C. albicans* transporters encoded by *CDR1*, *CDR2* and *MDR1*. The overexpression of CDR1p and MDR1p efflux pumps showed no effect in the susceptibility of *C. albicans* to echinocandins, while CDR2p only showed a moderate increase of caspofungin resistance (Niimi et al. 2006). Thus, efflux transporters are not a significant mechanism participating in echinocandin resistance. Other genes, however, are relevant to echinocandin activity, particularly *CYP56*. This gene codifies the protein DIT2p, whose absence increased the susceptibility of *C. albicans* to caspofungin (Melo et al. 2008). As previously described for azoles, *HSP90* plays a relevant role in echinocandin resistance. The inhibition of *HSP90* blocks calcineurin activation and increases the susceptibility of fungal cells, both yeast and moulds, to caspofungin (Singh et al. 2009).

7.5 Resistance to Other Antifungals

7.5.1 Flucytosine

Flucytosine is rarely used for the systemic treatment of fungal infections; it is especially administered for the treatment of specific yeast (especially *C. neoformans*) or mould infections in combination with other antifungal agents (Espinel-Ingroff 2008). Resistance to this antifungal is frequently acquired during therapy. Flucytosine is a base pyrimidine analogue that acts by inhibiting the synthesis of DNA and RNA (Vermes et al. 2000). Resistance to flucytosine has been attributed to point

mutations (codons 9 and 168) in the genes *FCY1* and *FCY2*, encoding the enzymes purine-cytosine permease and cytosine deaminase (Chapeland-Leclerc et al. 2005; Florent et al. 2009). Changes in the enzyme uracil phosphoribosyltransferase (encoded by *FUR1*) may also result in resistance to flucytosine (Papon et al. 2007).

7.5.2 *Terbinafine*

Terbinafine belongs to the allylamines class and inhibits the activity of the enzyme squalene epoxidase that participates in the synthesis of ergosterol. Like flucytosine, terbinafine is usually administered in combination with other antifungal agents for the treatment of systemic infections. Resistance to this agent has been reported following point mutations in squalene epoxidase codifying gene. Amino acid substitutions of P391L in *A. nidulans* and of F389L in ERGAp of *A. fumigatus* have been reported (Rocha et al. 2006). Efflux pumps encoded by *CDR1*, *CDR2* and *MDR1*, particularly *CDR2*, may also transport terbinafine. Overexpression of *CDR2* transporters resulted in a decreased *C. albicans* susceptibility to terbinafine (Sanglard et al. 1997). Additionally, histidone deacetylase inhibitors, such as TSA, increased the susceptibility of *C. albicans* to terbinafine by changing the membrane ergosterol content (Smith and Edlind 2002).

7.6 Resistance in the Environmental Versus in Clinical Isolates

The classification of a species as "susceptible" or "resistant" to antifungal agents is difficult to achieve and is never definitive. In the environment, microorganisms suffer natural mutations, becoming more or less susceptible to any drug. Mutations and evolution are happening continuously and the first data from genetic diversity confirm that fungi are highly diverse in the environment and may have high mutation rates (in addition to the dispersion ability that most fungi present) (Blum et al. 2008a; Ezov et al. 2006). Thus, there is constantly a small chance that a fungal organism becomes resistant to an antifungal agent without any contact with this particular agent. When the fungus is challenged with an antifungal agent, selective pressure chooses the more adaptable fungal strains, and the potential for developing antifungal resistance increases (acquired resistance). This fact is supported by (1) culturing the fungal cells in a medium with an antifungal agent, and (2) resistant isolates following antifungal treatment.

The triazoles are a group of antifungal agents that are important in the clinical, as well as in veterinary and agriculture settings (Beernaert et al. 2009; Serfling et al. 2007). Cross-resistance is common among azoles; fungal exposure to agricultural azoles may lead to pathogens becoming less susceptible to the triazoles used in patient care (Serfling et al. 2007).

7.6.1 Clinical and Epidemiologic Cutoff Values

At present, it is difficult to evaluate and quantify the level of resistance among environmental isolates, but this knowledge may be the first step in understanding the level of resistance to be expected in clinical isolates. The available clinical breakpoints for yeasts define the susceptibility values that separate the treatable from the non-treatable organisms (Dalhoff et al. 2009; EUCAST 2009; CLSI 2008; Espinel-Ingroff 2008; Pfaller et al. 2008, 2010). Although clinical breakpoints are not available for mould testing, epidemiologic cutoff values (ECVs) have been proposed for *A. fumigatus* and other mould species; an ECV represents the endpoint that separates the drug-susceptible wild type fungal organisms from acquired or mutational resistance strains (Dalhoff et al. 2009; Espinel-Ingroff et al. 2007; Pfaller et al. 2009; Rodriguez-Tudela et al. 2008b). However, these values are not clinical breakpoints, but will certainly help to better characterize mould isolates. It remains to be determined how the use of antifungal drugs – particularly prophylactic or empiric use – can impact future drug resistance. Horizontal transfer of genes coding resistance, common in bacteria, is not found in fungi (Hof 2008), and this fact represents a favourable point in restricting the dissemination of resistance in both environment and clinics.

7.7 Future Perspectives and Conclusions

Although a great deal of progress has been accomplished toward a better understanding of the different molecular mechanisms of resistance, much is needed to be learned regarding the clinical impact of such information. In the meantime, the available clinical breakpoints and ECV values could be helpful in monitoring the development of resistance during therapy as well as the selection of antifungal therapy. Identification of resistant or less susceptible isolates by available standard methods is helpful because the probability of clinical response to therapy is much higher when the infecting isolate is not resistant to the therapeutic agent.

References

Alastruey-Izquierdo A, Cuenca-Estrella M, Monzón A, Rodriguez-Tudela JL (2007) Prevalence and susceptibility testing of new species of *Pseudallescheria* and *Scedosporium* in a collection of clinical mold isolates. Antimicrob Agents Chemother 51:748–751

Alcazar-Fuoli L, Mellado E, Garcia-Effron G, Buitrago MJ, Lopez JF, Grimalt JO, Cuenca-Estrella JM, Rodriguez-Tudela JL (2006) *Aspergillus fumigatus* C-5 sterol desaturases Erg3A and Erg3B: role in sterol biosynthesis and antifungal drug susceptibility. Antimicrob Agents Chemother 50:453–460

Alcazar-Fuoli L, Mellado E, Garcia-Effron G, Lopez JF, Grimalt JO, Cuenca-Estrella JM, Rodriguez-Tudela JL (2008) Ergosterol biosynthesis pathway in *Aspergillus fumigatus*. Steroids 73:339–347

Almyroudis NG, Sutton DA, Fothergill AW, Rinaldi MG, Kusne S (2007) In vitro susceptibilities of 217 clinical isolates of zygomycetes to conventional and new antifungal agents. Antimicrob Agents Chemother 51:2587–2590

Alvarado-Ramírez E, Torres-Rodríguez JM (2007) In vitro susceptibility of *Sporothrix schenckii* to six antifungal agents determined using three different methods. Antimicrob Agents Chemother 51:2420–2423

Anderson JB, Sirjusingh C, Syed N, Lafayette S (2009) Gene expression and evolution of antifungal drug resistance. Antimicrob Agents Chemother 53:1931–1936

Andrade AC, Van Nistelrooy JG, Peery RB, Skatrud PL, De Waard MA (2000) The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. Mol Gen Genet 263:966–977

Araujo R, Pina-Vaz C, Rodrigues AG (2007) Susceptibility of environmental versus clinical strains of pathogenic *Aspergillus*. Int J Antimicrob Agents 29:108–111

Arendrup MC, Garcia-Effron G, Buzina W, Mortensen KL, Reiter N, Lundin C, Jensen HE, Lass-Flörl C, Perlin DS, Bruun B (2009) Breakthrough *Aspergillus fumigatus* and *Candida albicans* double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. Antimicrob Agents Chemother 53:1185–1193

Azor M, Gené J, Cano J, Sutton DA, Fothergill AW, Rinaldi MG, Guarro J (2008) In vitro antifungal susceptibility and molecular characterization of clinical isolates of *Fusarium verticillioides* (*F. moniliforme*) and *Fusarium thapsinum*. Antimicrob Agents Chemother 52:2228–2231

Baixench MT, Aoun N, Desnos-Ollivier M, Garcia-Hermoso D, Bretagne S, Ramires S, Piketty C, Dannaoui E (2007) Acquired resistance to echinocandins in *Candida albicans*: case report and review. J Antimicrob Chemother 59:1076–1083

Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA (2005) *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot Cell 4:625–632

Balashov SV, Park S, Perlin DS (2006) Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in FKS1. Antimicrob Agents Chemother 50:2058–2063

Barchiesi F, Calabrese D, Sanglard D, Falconi Di Francesco L, Caselli F, Giannini D, Giacometti A, Gavaudan S, Scalise G (2000) Experimental induction of fluconazole resistance in *Candida tropicalis* ATCC 750. Antimicrob Agents Chemother 44:1578–1584

Beernaert LA, Pasmans F, Van Waeyenberghe L, Dorrestein GM, Verstappen F, Vercammen F, Haesebrouck F, Martel A (2009) Avian *Aspergillus fumigatus* strains resistant to both itraconazole and voriconazole. Antimicrob Agents Chemother 53:2199–2201

Bellete B, Raberin H, Morel J, Flori P, Hafid J, Sung RT (2009) Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. Med Mycol 19:1–4

Bennett JE (2006) Echinocandins for candidemia in adults without neutropenia. N Engl J Med 355:1154–1159

Bennett JE, Izumikawa K, Marr KA (2004) Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. Antimicrob Agents Chemother 48:1773–1777

Blum G, Perkhofer S, Grif K, Mayr A, Kropshofer G, Nachbaur D, Kafka-Ritsch R, Dierich MP, Lass-Flörl C (2008a) A 1-year *Aspergillus terreus* surveillance study at the University Hospital of Innsbruck: molecular typing of environmental and clinical isolates. Clin Microbiol Infect 14:1146–1151

Blum G, Perkhofer S, Haas H, Schrettl M, Würzner R, Dierich MP, Lass-Flörl C (2008b) Potential basis for amphotericin B resistance in *Aspergillus terreus*. Antimicrob Agents Chemother 52:1553–1555

Bulik CC, Sobel JD, Nailor MD (2009) Susceptibility profile of vaginal isolates of *Candida albicans* prior to and following fluconazole introduction — impact of two decades. Mycoses (in press). doi: 10.1111/j.1439-0507.2009.01752.x

Calabrese D, Bille J, Sanglard D (2000) A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (FLU1) conferring resistance to fluconazole. Microbiology 146:2743–2754

Castelli MV, Alastruey-Izquierdo A, Cuesta I, Monzon A, Mellado E, Rodriguez-Tudela JL, Cuenca-Estrella M (2008) Susceptibility testing and molecular classification of *Paecilomyces* spp. Antimicrob Agents Chemother 52:2926–2928

Chakrabarti A, Chatterjee SS, Rao KL, Zameer MM, Shivaprakash MR, Singhi S, Singh R, Varma SC (2009) Recent experience with fungaemia: change in species distribution and azole resistance. Scand J Infect Dis 41:275–284

Chandrasekar P (2009) Invasive mold infections: recent advances in management approaches. Leuk Lymphoma 50:703–715

Chapeland-Leclerc F, Bouchoux J, Goumar A, Chastin C, Villard J, Noël T (2005) Inactivation of the FCY2 gene encoding purine–cytosine permease promotes cross-resistance to flucytosine and fluconazole in *Candida lusitaniae*. Antimicrob Agents Chemother 49:3101–3108

Chen CG, Yang YL, Tseng KY, Shih HI, Liou CH, Lin CC, Lo HJ (2009) Rep1p negatively regulating MDR1 efflux pump involved in drug resistance in *Candida albicans*. Fungal Genet Biol 46:714–720

Chen KH, Miyazaki T, Tsai HF, Bennett JE (2007) The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in *Candida glabrata*. Gene 386:63–72

Cheng S, Clancy CJ, Nguyen KT, Clapp W, Nguyen MH (2007) A *Candida albicans* petite mutant strain with uncoupled oxidative phosphorylation overexpresses MDR1 and has diminished susceptibility to fluconazole and voriconazole. Antimicrob Agents Chemother 51:1855–1858

Cleary JD, Garcia-Effron G, Chapman SW, Perlin DS (2008) Reduced *Candida glabrata* susceptibility secondary to an FKS1 mutation developed during candidemia treatment. Antimicrob Agents Chemother 52:2263–2265

Clinical Laboratory Standards Institute (2008) Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved standard, 3rd edn, CLSI document M27-A3. CLSI, Wayne, PA

Coste A, Selmecki A, Forche A, Diogo D, Bougnoux ME, d'Enfert C, Berman J, Sanglard D (2007) Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. Eukaryot Cell 6:1889–1904

Cowen LE, Carpenter AE, Matangkasombut O, Fink GR, Lindquist S (2006) Genetic architecture of Hsp90-dependent drug resistance. Eukaryot Cell 5:2184–2188

Cowen LE, Lindquist S (2005) Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science 309:2185–2189

Cowen LE, Singh SD, Köhler JR, Collins C, Zaas AK, Schell WA, Aziz H, Mylonakis E, Perfect JR, Whitesell L, Lindquist S (2009) Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. Proc Natl Acad Sci USA 106: 2818–2823

Dalhoff A, Ambrose PG, Mouton JW (2009) A long journey from minimum inhibitory concentration testing to clinically predictive breakpoints: deterministic and probabilistic approaches in deriving breakpoints. Infection 37:296–305

Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA, Warnock DW, Kelly SL (1997) Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 41:1364–1368

Dib L, Hayek P, Sadek H, Beyrouthy B, Khalaf RA (2008) The *Candida albicans* Ddr48 protein is essential for filamentation, stress response, and confers partial antifungal drug resistance. Med Sci Monit 14:BR113–BR121

Dick JD, Merz WG, Saral R (1980) Incidence of polyene-resistant yeasts recovered from clinical specimens. Antimicrob Agents Chemother 18:158–163

Dunkel N, Blass J, Rogers PD, Morschhäuser J (2008a) Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant *Candida albicans* strains. Mol Microbiol 69:827–840

Dunkel N, Liu TT, Barker KS, Homayouni R, Morschhäuser J, Rogers PD (2008b) A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol

biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate. Eukaryot Cell 7:1180–1190

Erjavec Z, Kluin-Nelemans H, Verweij PE (2009) Trends in invasive fungal infections, with emphasis on invasive aspergillosis. Clin Microbiol Infect 15:625–633

Espinel-Ingroff A (1998) Comparison of *in vitro* activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743, 872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. J Clin Microbiol 36:2950–2956

Espinel-Ingroff A (2008) Mechanisms of resistance to antifungal agents: yeasts and filamentous fungi. Rev Iberoam Micol 25:101–106

Espinel-Ingroff A, Arthington-Skaggs B, Iqbal N, Ellis D, Pfaller MA, Messer S, Rinaldi M, Fothergill A, Gibbs DL, Wang A (2007) Multicenter evaluation of a new disk agar diffusion method for susceptibility testing of filamentous fungi with voriconazole, posaconazole, itraconazole, amphotericin B, and caspofungin. J Clin Microbiol 45:1811–1820

EUCAST (2009) The European Committee on Antimicrobial Susceptibility Testing. http://eucast.www137.server1.mensemedia.net/index.php?id=59. Cited 23 September 2009

Ezov TK, Boger-Nadjar E, Frenkel Z, Katsperovski I, Kemeny S, Nevo E, Korol A, Kashi Y (2006) Molecular-genetic biodiversity in a natural population of the yeast *Saccharomyces cerevisiae* from "Evolution Canyon": microsatellite polymorphism, ploidy and controversial sexual status. Genetics 174:1455–1468

Ferrari S, Ischer F, Calabrese D, Posteraro B, Sanguinetti M, Fadda G, Rohde B, Bauser C, Bader O, Sanglard D (2009) Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. PLoS Pathog 5:e1000268. doi:10.1371/journal.ppat.1000268

Florent M, Noël T, Ruprich-Robert G, Da Silva B, Fitton-Ouhabi V, Chastin C, Papon N, Chapeland-Leclerc F (2009) Nonsense and missense mutations in FCY2 and FCY1 genes are responsible for flucytosine resistance and flucytosine–fluconazole cross-resistance in clinical isolates of *Candida lusitaniae*. Antimicrob Agents Chemother 53:2982–2990

Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhnke M, Morschhäuser J (1998) Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob Agents Chemother 42:3065–3072

Garcia-Effron G, Katiyar SK, Park S, Edlind TD, Perlin DS (2008) A naturally occurring proline-to-alanine amino acid change in Fks1p in *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* accounts for reduced echinocandin susceptibility. Antimicrob Agents Chemother 52:2305–2312

Geber A, Hitchcock CA, Swartz JE, Pullen FS, Marsden KE, Kwon-Chung KJ, Bennett JE (1995) Deletion of the *Candida glabrata* ERG3 and ERG11 genes: effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. Antimicrob Agents Chemother 39:2708–2717

Groll AH, Kolve H (2004) Antifungal agents: *in vitro* susceptibility testing, pharmacodynamics, and prospects for combination therapy. Eur J Clin Microbiol Infect Dis 23:256–270

Hakki M, Staab JF, Marr KA (2006) Emergence of a *Candida krusei* isolate with reduced susceptibility to caspofungin during therapy. Antimicrob Agents Chemother 50:2522–2524

Hautala T, Kakko S, Siitonen T, Saily M, Koistinen P, Koskela M (2009) Clinical *Candida krusei* isolates remain susceptible during extensive exposure to antifungal drugs. Med Mycol 4:1–6

Hodiamont CJ, Dolman KM, Ten Berge IJ, Melchers WJ, Verweij PE, Pajkrt D (2009) Multiple-azole-resistant *Aspergillus fumigatus* osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. Med Mycol 47:217–220

Hof H (2008) Is there a serious risk of resistance development to azoles among fungi due to the widespread use and long-term application of azole antifungals in medicine? Drug Resist Updat 11:25–31

Hope WW, Billaud EM, Lestner J, Denning DW (2008) Therapeutic drug monitoring for triazoles. Curr Opin Infect Dis 21:580–586

Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, Laverdiere M, Arendrup MC, Perlin DS, Denning DW (2009) Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis 15:1068–1076

Howard SJ, Webster I, Moore CB, Gardiner RE, Park S, Perlin DS, Denning DW (2006) Multi-azole resistance in *Aspergillus fumigatus*. Int J Antimicrob Agents 28:450–453

Jia XM, Wang Y, Jia Y, Gao PH, Xu YG, Wang L, Cao YY, Cao YB, Zhang LX, Jiang YY (2009) RTA2 is involved in calcineurin-mediated azole resistance and sphingoid long-chain base release in *Candida albicans*. Cell Mol Life Sci 66:122–134

Kahn JN, Garcia-Effron G, Hsu MJ, Park S, Marr KA, Perlin DS (2007) Acquired echinocandin resistance in a *Candida krusei* isolate due to modification of glucan synthase. Antimicrob Agents Chemother 51:1876–1878

Kanafani ZA, Perfect JR (2008) Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis 46:120–128

Katiyar S, Pfaller M, Edlind T (2006) *Candida albicans* and *Candida glabrata* clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob Agents Chemother 50: 2892–2894

Katiyar SK, Edlind TD (2001) Identification and expression of multidrug resistance-related ABC transporter genes in *Candida krusei*. Med Mycol 39:109–116

Katiyar SK, Edlind TD (2009) Role for Fks1 in the intrinsic echinocandin resistance of *Fusarium solani* as evidenced by hybrid expression in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother 53:1772–1778

Khot PD, Suci PA, Miller RL, Nelson RD, Tyler BJ (2006) A small subpopulation of blastospores in Candida albicans biofilms exhibit resistance to amphotericin B associated with differential regulation of ergosterol and beta-1, 6-glucan pathway genes. Antimicrob Agents Chemother 50:3708–3716

Krishnan-Natesan S, Chandrasekar PH, Alangaden GJ, Manavathu EK (2008) Molecular characterisation of cyp51A and cyp51B genes coding for P450 14alpha-lanosterol demethylases A (CYP51Ap) and B (CYP51Bp) from voriconazole-resistant laboratory isolates of *Aspergillus flavus*. Int J Antimicrob Agents 32:519–524

Krogh-Madsen M, Arendrup MC, Heslet L, Knudsen JD (2006) Amphotericin B and caspofungin resistance in Candida glabrata isolates recovered from a critically ill patient. Clin Infect Dis 42:938–944

Lass-Flörl C, Alastruey-Izquierdo A, Cuenca-Estrella M, Perkhofer S, Rodriguez-Tudela JL (2009) In vitro activities of various antifungal drugs against *Aspergillus terreus*: Global assessment using the methodology of the European Committee on Antimicrobial Susceptibility Testing. Antimicrob Agents Chemother 53:794–795

Lass-Flörl C, Griff K, Mayr A, Petzer A, Gastl G, Bonatti H, Freund M, Kropshofer G, Dierich MP, Nachbaur D (2005) Epidemiology and outcome of infections due to *Aspergillus terreus*: 10-year single centre experience. Br J Haematol 131:201–207

Legrand M, Chan CL, Jauert PA, Kirkpatrick DT (2007) Role of DNA mismatch repair and double-strand break repair in genome stability and antifungal drug resistance in *Candida albicans*. Eukaryot Cell 6:2194–2205

Löffler J, Einsele H, Hebart H, Schumacher U, Hrastnik C, Daum G (2000) Phospholipid and sterol analysis of plasma membranes of azole-resistant *Candida albicans* strains. FEMS Microbiol Lett 185:59–63

Małafiej E, Adamiec AC, Tworzyańska U (2009) Microbial profile and drug resistance of *Candida* strains isolated from the blood of children: an 11-year study. Mycoses 52:149–153

Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FC, Odds FC, Bossche HV (1999) Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology 145:2701–2713

Mellado E, Garcia-Effron G, Alcázar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M, Rodríguez-Tudela JL (2007) A new *Aspergillus fumigatus* resistance mechanism conferring

in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. Antimicrob Agents Chemother 51:1897–1904

Melo NR, Moran GP, Warrilow AG, Dudley E, Smith SN, Sullivan DJ, Lamb DC, Kelly DE, Coleman DC, Kelly SL (2008) CYP56 (Dit2p) in *Candida albicans*: characterization and investigation of its role in growth and antifungal drug susceptibility. Antimicrob Agents Chemother 52:3718–3724

Messer SA, Jones RN, Fritsche TR (2006) International surveillance of *Candida* spp. and *Aspergillus* spp.: report from the SENTRY Antimicrobial Surveillance Program (2003). J Clin Microbiol 44:1782–1787

Miyazaki H, Miyazaki Y, Geber A, Parkinson T, Hitchcock C, Falconer DJ, Ward DJ, Marsden K, Bennett JE (1998) Fluconazole resistance associated with drug efflux and increased transcription of a drug transporter gene, PDH1, in *Candida glabrata*. Antimicrob Agents Chemother 42:1695–1701

Morschhäuser J (2010) Regulation of multidrug resistance in pathogenic fungi. Fungal Genet Biol 47(2):94–106. doi:10.1016/j.fgb.2009.08.002

Morschhäuser J, Barker KS, Liu TT, BlaB-Warmuth J, Homayouni R, Rogers PD (2007) The transcription factor Mrr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in *Candida albicans*. PLoS Pathog 3:e164. doi:10.1371/journal.ppat.0030164

Naeger-Murphy N, Pile JC (2009) Clinical indications for newer antifungal agents. J Hosp Med 4:102–111

Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS (2003) Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother 47:1719–1726

Niimi K, Maki K, Ikeda F, Holmes AR, Lamping E, Niimi M, Monk BC, Cannon RD (2006) Overexpression of *Candida albicans* CDR1, CDR2, or MDR1 does not produce significant changes in echinocandin susceptibility. Antimicrob Agents Chemother 50:1148–1155

Nolte FS, Parkinson T, Falconer DJ, Dix S, Williams J, Gilmore C, Geller R, Wingard JR (1997) Isolation and characterization of fluconazole- and amphotericin B-resistant *Candida albicans* from blood of two patients with leukemia. Antimicrob Agents Chemother 41:196–199

Oliver BG, Silver PM, White TC (2008) Polyene susceptibility is dependent on nitrogen source in the opportunistic pathogen *Candida albicans*. J Antimicrob Chemother 61:1302–1308

Panackal AA, Imhof A, Hanley EW, Marr KA (2006) *Aspergillus ustus* infections among transplant recipients. Emerg Infect Dis 12:403–408

Papon N, Noël T, Florent M, Gibot-Leclerc S, Jean D, Chastin C, Villard J, Chapeland-Leclerc F (2007) Molecular mechanism of flucytosine resistance in *Candida lusitaniae*: contribution of the FCY2, FCY1, and FUR1 genes to 5-fluorouracil and fluconazole cross-resistance. Antimicrob Agents Chemother 51:369–371

Pappas PG, Kauffman CA, Andes D, Benjamin DK Jr, Calandra TF, Edwards JE Jr, Filler SG, Fisher JF, Kullberg BJ, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD, Infectious Diseases Society of America (2009) Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis 48:503–535

Park S, Kelly R, Kahn JN, Robles J, Hsu MJ, Register E, Li W, Vyas V, Fan H, Abruzzo G, Flattery A, Gill C, Chrebet G, Parent SA, Kurtz M, Teppler H, Douglas CM, Perlin DS (2005) Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical *Candida* sp. isolates. Antimicrob Agents Chemother 49:3264–3273

Pasrija R, Panwar SL, Prasad R (2008) Multidrug transporters CaCdr1p and CaMdr1p of *Candida albicans* display different lipid specificities: both ergosterol and sphingolipids are essential for targeting of CaCdr1p to membrane rafts. Antimicrob Agents Chemother 52:694–704

Pastor FJ, Guarro J (2008) *Alternaria* infections: laboratory diagnosis and relevant clinical features. Clin Microbiol Infect 14:734–746

Pemán J, Cantón E, Espinel-Ingroff A (2009) Antifungal drug resistance mechanisms. Expert Rev Anti Infect Ther 7:453–460

Perea S, López-Ribot JL, Kirkpatrick WR, McAtee RK, Santillán RA, Martínez M, Calabrese D, Sanglard D, Patterson TF (2001) Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother 45:2676–2684

Perlin DS (2007) Resistance to echinocandin-class antifungal drugs. Drug Resist Updat 10:121–130

Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, Jones RN, Turnidge J, Diekema DJ (2010) Wild-type MIC distributions and epidemiological cutoff values for the echinocandins and Candida spp. J Clin Microbiol 48:52–56

Pfaller MA, Diekema DJ, Ghannoum A, Rex JH, Alexander BD, Andes D, Brown SD, Chaturvedi V, Espinel-Ingroff A, Fowler CL, Johnson EM, Knapp CC, Motyl MR, Ostrosky-Zeichner L, Sheehan DJ, Walsh TJ, for the Clinical and Laboratory Standards Institute Antifungal Testing Subcommittee (2009) Wild type MIC distribution and epidemiological cutoff values for *Aspergillus fumigatus* and three triazoles as determined by the Clinical and Laboratory Standards Institute Broth Microdilution Methods. J Clin Microbiol 47(10):3142–3146. doi:10.1128/JCM.00940-09

Pfaller MA, Diekema DJ, Ostrosky-Zeichner L, Rex JH, Alexander BD, Andes D, Brown SD, Chaturvedi V, Ghannoum MA, Knapp CC, Sheehan DJ, Walsh TJ (2008) Correlation of MIC with outcome for *Candida* species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints. J Clin Microbiol 46: 2620–2629

Pfaller MA, Messer SA, Hollis RJ (1994) Strain delineation and antifungal susceptibilities of epidemiologically related and unrelated isolates of *Candida lusitaniae*. Diagn Microbiol Infect Dis 20:127–133

Pinjon E, Moran GP, Coleman DC, Sullivan DJ (2005) Azole susceptibility and resistance in *Candida dubliniensis*. Biochem Soc Trans 33:1210–1214

Poláková S, Blume C, Zárate JA, Mentel M, Jørck-Ramberg D, Stenderup J, Piskur J (2009) Formation of new chromosomes as a virulence mechanism in yeast *Candida glabrata*. Proc Natl Acad Sci USA 106:2688–2693

Posteraro B, Sanguinetti M, Sanglard D, La Sorda M, Boccia S, Romano L, Morace G, Fadda G (2003) Identification and characterization of a *Cryptococcus neoformans* ATP binding cassette (ABC) transporter-encoding gene, CnAFR1, involved in the resistance to fluconazole. Mol Microbiol 47:357–371

Redding SW, Kirkpatrick WR, Saville S, Coco BJ, White W, Fothergill A, Rinaldi M, Eng T, Patterson TF, Lopez-Ribot J (2003) Multiple patterns of resistance to fluconazole in *Candida glabrata* isolates from a patient with oropharyngeal candidiasis receiving head and neck radiation. J Clin Microbiol 41:619–622

Robbins N, Collins C, Morhayim J, Cowen LE (2010) Metabolic control of antifungal drug resistance. Fungal Genet Biol 47(2):81–93. doi:10.1016/j.fgb.2009.07.004

Rocha EM, Garcia-Effron G, Park S, Perlin DS (2007) A Ser678Pro substitution in Fks1p confers resistance to echinocandin drugs in *Aspergillus fumigatus*. Antimicrob Agents Chemother 51:4174–4176

Rocha EM, Gardiner RE, Park S, Martinez-Rossi NM, Perlin DS (2006) A Phe389Leu substitution in ergA confers terbinafine resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 50:2533–2536

Rodrigues AG, Araujo R, Pina-Vaz C (2005) Human albumin promotes germination, hyphal growth and antifungal resistance by *Aspergillus fumigatus*. Med Mycol 43:711–717

Rodriguez-Tudela JL, Alcazar-Fuoli L, Cuesta I, Alastruey-Izquierdo A, Monzon A, Mellado E, Cuenca-Estrella M (2008a) Clinical relevance of resistance to antifungals. Int J Antimicrob Agents 32:S111–S113

Rodriguez-Tudela JL, Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Monzon A, Cuenca-Estrella M (2008b) Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. Antimicrob Agents Chemother 52:2468–2472

Salmon A, Debourgogne A, Vasbien M, Clément L, Collomb J, Plénat F, Bordigoni P, Machouart M (2010) Disseminated *Scopulariopsis brevicaulis* infection in an allogeneic stem cell recipient: case report and review of the literature. Clin Microbiol Infect 16:508–512. doi: 10.1111/j.1198-743X.2009.02878.x

Sanglard D, Ischer F, Koymans L, Bille J (1998) Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother 42:241–253

Sanglard D, Ischer F, Monod M, Bille J (1997) Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology 143:405–416

Sanglard D, Ischer F, Parkinson T, Falconer D, Bille J (2003) *Candida albicans* mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob Agents Chemother 47:2404–2412

Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M, Bille J (1995) Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob Agents Chemother 39:2378–2386

Sanguinetti M, Posteraro B, Fiori B, Ranno S, Torelli R, Fadda G (2005) Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. Antimicrob Agents Chemother 49:668–679

Schubert S, Rogers PD, Morschhäuser J (2008) Gain-of-function mutations in the transcription factor MRR1 are responsible for overexpression of the MDR1 efflux pump in fluconazole-resistant *Candida dubliniensis* strains. Antimicrob Agents Chemother 52:4274–4280

Sellam A, Askew C, Epp E, Lavoie H, Whiteway M, Nantel A (2009a) Genome-wide mapping of the coactivator Ada2p yields insight into the functional roles of SAGA/ADA complex in *Candida albicans*. Mol Biol Cell 20:2389–2400

Sellam A, Tebbji F, Nantel A (2009b) Role of Ndt80p in sterol metabolism regulation and azole resistance in *Candida albicans*. Eukaryot Cell 8:1174–1183

Selmecki A, Forche A, Berman J (2006) Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. Science 313:367–370

Serfling A, Wohlrab J, Deising HB (2007) Treatment of a clinically relevant plant-pathogenic fungus with an agricultural azole causes cross-resistance to medical azoles and potentiates caspofungin efficacy. Antimicrob Agents Chemother 51:3672–3676

Sheng C, Miao Z, Ji H, Yao J, Wang W, Che X, Dong G, Lü J, Guo W, Zhang W (2009) Three-dimensional model of lanosterol 14 alpha-demethylase from *Cryptococcus neoformans*: active-site characterization and insights into azole binding. Antimicrob Agents Chemother 53:3487–2495

Shin JH, Chae MJ, Song JW, Jung SI, Cho D, Kee SJ, Kim SH, Shin MG, Suh SP, Ryang DW (2007) Changes in karyotype and azole susceptibility of sequential bloodstream isolates from patients with *Candida glabrata* candidemia. J Clin Microbiol 45:2385–2391

Silva AP, Miranda IM, Lisboa C, Pina-Vaz C, Rodrigues AG (2009) Prevalence, distribution, and antifungal susceptibility profiles of *Candida parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* in a tertiary care hospital. J Clin Microbiol 47:2392–2397

Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE (2009) Hsp90 governs echinocandin resistance in the pathogenic yeast *Candida albicans* via calcineurin. PLoS Pathog 5:e1000532. doi:10.1371/journal.ppat.1000532

Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, Denning DW (2002) Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol 36:199–206

Smith WL, Edlind TD (2002) Histone deacetylase inhibitors enhance *Candida albicans* sensitivity to azoles and related antifungals: correlation with reduction in CDR and ERG upregulation. Antimicrob Agents Chemother 46:3532–3539

Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ, Verweij PE (2009) Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. Appl Environ Microbiol 75:4053–4057

Snelders E, van der Lee HA, Kuijpers J, Rijs AJ, Varga J, Samson RA, Mellado E, Donders AR, Melchers WJ, Verweij PE (2008) Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PLoS Med 5:e219. doi:10.1371/journal.pmed.0050219

Sokol-Anderson ML, Brajtburg J, Medoff G (1986) Amphotericin B-induced oxidative damage and killing of *Candida albicans*. J Infect Dis 154:76–83

Song JL, Harry JB, Eastman RT, Oliver BG, White TC (2004) The *Candida albicans* lanosterol 14-alpha-demethylase (ERG11) gene promoter is maximally induced after prolonged growth with antifungal drugs. Antimicrob Agents Chemother 48:1136–1144

Supparatpinyo K, Nelson KE, Merz WG, Breslin BJ, Cooper CR Jr, Kamwan C, Sirisanthana T (1993) Response to antifungal therapy by human immunodeficiency virus-infected patients with disseminated *Penicillium marneffei* infections and *in vitro* susceptibilities of isolates from clinical specimens. Antimicrob Agents Chemother 37:2407–2411

Thompson GR 3rd, Wiederhold NP, Vallor AC, Villareal NC, Lewis JS 2nd, Patterson TF (2008) Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to *Candida glabrata* infection. Antimicrob Agents Chemother 52:3783–3875

Tobin MB, Peery RB, Skatrud PL (1997) Genes encoding multiple drug resistance-like proteins in *Aspergillus fumigatus* and *Aspergillus flavus*. Gene 200:11–23

Tortorano AM, Prigitano A, Dho G, Esposto MC, Gianni C, Grancini A, Ossi C, Viviani MA (2008) Species distribution and *in vitro* antifungal susceptibility patterns of 75 clinical isolates of *Fusarium* spp. from northern Italy. Antimicrob Agents Chemother 52:2683–2685

Trama JP, Mordechai E, Adelson ME (2005) Detection of *Aspergillus fumigatus* and a mutation that confers reduced susceptibility to itraconazole and posaconazole by real-time PCR and pyrosequencing. J Clin Microbiol 43:906–908

Tsai HF, Krol AA, Sarti KE, Bennett JE (2006) *Candida glabrata* PDR1, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. Antimicrob Agents Chemother 50:1384–1392

van der Linden JW, Jansen RR, Bresters D, Visser CE, Geerlings SE, Kuijper EJ, Melchers WJ, Verweij PE (2009) Azole-resistant central nervous system aspergillosis. Clin Infect Dis 48:1111–1113

Vandeputte P, Larcher G, Bergès T, Renier G, Chabasse D, Bouchara JP (2005) Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. Antimicrob Agents Chemother 49:4608–4615

Vandeputte P, Tronchin G, Bergès T, Hennequin C, Chabasse D, Bouchara JP (2007) Reduced susceptibility to polyenes associated with a missense mutation in the ERG6 gene in a clinical isolate of *Candida glabrata* with pseudohyphal growth. Antimicrob Agents Chemother 51:982–990

Veen M, Stahl U, Lang C (2003) Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*. FEMS Yeast Res 4:87–95

Vermes A, Guchelaar HJ, Dankert J (2000) Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. J Antimicrob Chemother 46:171–179

Vermitsky JP, Earhart KD, Smith WL, Homayouni R, Edlind TD, Rogers PD (2006) Pdr1 regulates multidrug resistance in *Candida glabrata*: gene disruption and genome-wide expression studies. Mol Microbiol 61:704–722

Vermitsky JP, Edlind TD (2004) Azole resistance in *Candida glabrata*: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. Antimicrob Agents Chemother 48:3773–3781

Wakiec R, Gabriel I, Prasad R, Becker JM, Payne JW, Milewski S (2008) Enhanced susceptibility to antifungal oligopeptides in yeast strains overexpressing ABC multidrug efflux pumps. Antimicrob Agents Chemother 52:4057–4063

Wirsching S, Moran GP, Sullivan DJ, Coleman DC, Morschhäuser J (2001) MDR1-mediated drug resistance in *Candida dubliniensis*. Antimicrob Agents Chemother 45:3416–3421

Yan L, Li M, Cao Y, Gao P, Cao Y, Wang Y, Jiang Y (2009) The alternative oxidase of *Candida albicans* causes reduced fluconazole susceptibility. J Antimicrob Chemother 64:764–773

Znaidi S, Barker KS, Weber S, Alarco AM, Liu TT, Boucher G, Rogers PD, Raymond M (2009) Identification of the *Candida albicans* Cap1p regulon. Eukaryot Cell 8:806–820

Znaidi S, De Deken X, Weber S, Rigby T, Nantel A, Raymond M (2007) The zinc cluster transcription factor Tac1p regulates PDR16 expression in *Candida albicans*. Mol Microbiol 66:440–452

Chapter 8
Multidrug Resistance in Fungi: The Role of Pleiotropic Drug Resistance Genes

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Abstract Multidrug resistance of pathogenic fungi to antifungal agents is a major impediment in combating fungal infections. Clinically important resistance of fungal pathogens to azoles and other antifungal drugs is often caused by over-expression of energy-dependent drug efflux pumps able to facilitate the efflux of drugs from the cells. The efflux pump proteins which belong to the ATP-binding cassette and major facilitator superfamilies are the most prominent contributors to multidrug resistance (MDR). The abundance of the drug transporters and their wider specificity suggest that these transporters are not exclusively drug exporters but also have other cellular functions. In this chapter, we focus on the role played by pleiotropic drug resistance genes in MDR, and their physiological relevance in lipid homeostasis of nonpathogenic and pathogenic fungal species.

8.1 Introduction

Multidrug resistance (MDR) is a well-described biological phenomenon, causing serious problems in the treatment of human cancers and microbial infections. MDR in fungi shares several similarities with processes in bacteria and higher eukaryotes. The molecular mechanism that forms the core of MDR in human cancer cells subjected to chemotherapy involves the overexpression of an ATP-dependent extrusion pump (known as P-glycoprotein) which facilitates the efflux of toxic compounds from the cells (Gottesman and Pastan 1993). Remarkably, a number of membrane-embedded energy-consuming ATP-binding cassette (ABC) superfamily and major facilitators superfamily (MFS) transporters have been implicated in the development of MDR phenotypes in various pathogenic eukaryotes and yeasts in

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particular, similar to the MDR phenotype of mammalian cells (Cannon et al. 2009). The MDR phenotype in yeast is known as pleiotropic drug resistance (PDR) and is caused by the increased expression of genes that encode these nonspecific drug-efflux pumps belonging to the ABC and MFS families of transporter proteins. MDR transporters of the yeast are responsible for the resistance of the cells to a broad spectrum of diverse xenobiotics, thereby mediating clinical antifungal resistance in fungal pathogens. Yeast *Saccharomyces cerevisiae* cells lacking endogenous ABC pumps are hypersensitive to many antifungal drugs, and thereby facilitate functional studies of ABC transporters from fungal pathogens such as *Candida albicans* and other *Candida* species. The presence of large number of drug transporters and their broad specificity suggest that these transporters not only play a role as drug exporters in yeasts, but also have other cellular functions (Prasad et al. 2006). The ABC and MFS groups of transporters differ in the way they actively translocate compounds across cell membrane. The ABC transporters bind to ATP and hydrolyze it ,and use the energy generated to transport solutes across cell membrane. ABC transporters can also transport compounds against an electrochemical gradient, thus acting as a primary active transporter system. MFS transporters are considered as secondary active transport systems (Lewis 1994), as the MFS-mediated transport is driven by the electrochemical proton-motive force, which is composed of membrane electric potential and a chemical proton gradient ($\Delta pH = pH_{external} - pH_{internal}$), across the plasma membrane (PM). A large number of uni-, sym-, and antiporters of sugar, peptides, drugs, and organic and inorganic ions fall within this superfamily (Pao et al. 1998). Both types of transporters contain distinctive protein domains, i.e., nucleotide binding domain (NBDs) in ABC pumps and transmembrane domains (TMDs) in both ABC and MFS pumps that confer substrate specificity (Fig. 8.1).

8.2 ABC Transporters

Resistance of pathogenic fungi to antifungal drugs is of rising concern, particularly in infected immunocompromised patients. The model yeast *S. cerevisiae* and the most common pathogenic yeast *C. albicans*, have a multitude of permeases (belonging to the major facilitator superfamily) and pumps (belonging to the ATP-binding cassette superfamily) for the efflux of multiple drugs. Most ABC proteins are purely ATP-driven membrane translocators, although some of them act as ion channels, channel regulators, receptors, proteases, and even sensing proteins (Higgins 1995). ABC transporters mediate membrane translocation of ions, heavy metals, antifungals, anticancer drugs, antibiotics, amino acids, phospholipids, steroids, glucocorticoids, carbohydrates, bile acids, fluorescent dyes, small peptides, and many others across the plasma membrane or across intracellular membranes (Higgins 1992; Kuchler and Thorner 1992; Dean and Allikmets 1995; Sipos and Kuchler 2006)

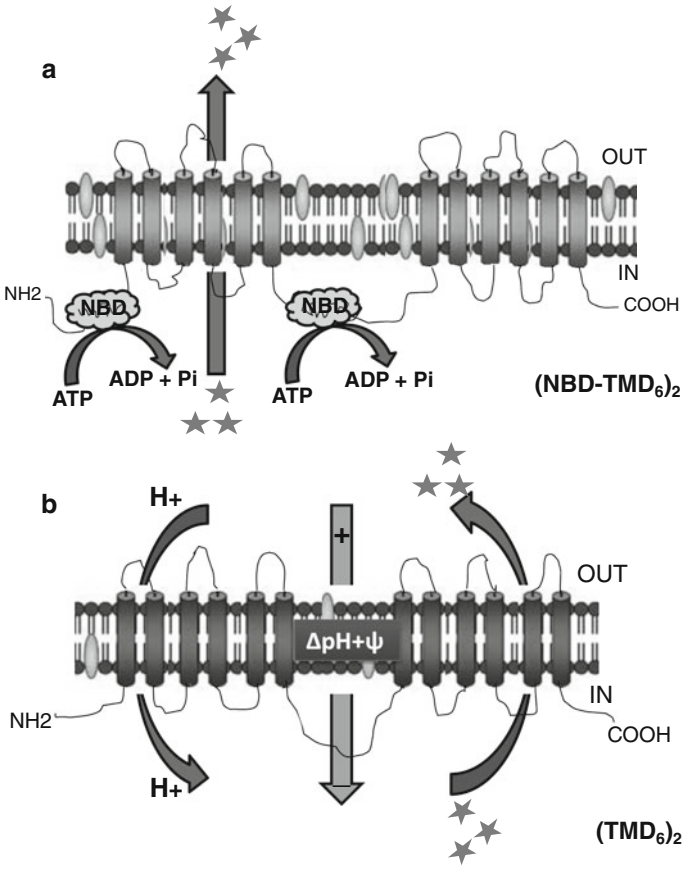

Fig. 8.1 Schematic representation of the topology of MDR transporters: the multidrug pump of the ABC (**a**) and the proton-motive-force dependent transporter of the MFS superfamily (**b**). The transmembrane electrochemical proton gradient is generated by the H+-translocating-ATPase present in the membrane

8.2.1 Structure and Function

Typical ABC transporters of fungi (Fig. 8.1) contain two intracytoplasmic regions, both of which harbour NBD for binding and hydrolysis of ATP, and two extensively hydrophobic regions, each containing six TMDs (TMD_6) (Del Sorbo et al. 2000). Based on gene homologies, ABC proteins in *S. cerevisiae* have been classified into three main subfamilies, the PDR, MDR, and MDR-associated protein (MRP) subfamilies (Decottignies and Goffeau 1997; Bauer et al. 1999; Sipos and Kuchler 2006). The domain arrangement in most MDR and MRP ABC proteins is $(TMD_6–NBD)_2$, and for most PDR pumps the arrangement is $(NBD–TMD_6)_2$ (Decottignies and Goffeau 1997). Other subfamilies of fungal ABC transporters have $NBD–TMD_6$ domain organization, and are described as

"half-sized" transporters. These are thought to be functional after assembly into homodimers or heterodimers (Del Sorbo et al. 2000). Like other ATPases, the NBD domains of ABC transporters contain the conserved Walker A and Walker B motifs (Walker et al. 1982) and a consensus sequence, (L,V)-SGG-(X) 3-R-hydrophobic residue-X-hydrophobic residue-A, known as ABC signature or C motif, preceding the Walker B motif. Unlike other ATPases, the Walker motifs of ABC transporters are separated by 90–120 amino acid residues (Hyde et al. 1990).

Yeast ABC pumps can transport a broad range of structurally and functionally unrelated substances. However, the mechanism behind the translocation of a wide variety of these xenobiotics by a single transporter is still unknown. Through photo-affinity labelling studies and genetic analysis, it has been demonstrated that both NBD and TMD make major contributions to substrate recognition and drug transport in mammalian transporters (Gottesman et al. 1995; Zhang et al. 1995). Several critical amino acid residues responsible for substrate recognition in Pdr5p and Cdr1p have been identified through transport inhibition studies and analysis of mutants (Kolaczkowski et al. 1996; Egner et al. 1998; Krishnamurthy et al. 1998a–c; Egner et al. 2000). Studies on inhibition of Pdr5p-mediated quenching of rhodamine 6G fluorescence have suggested that more than one drug-binding site is present in fungal ABC pumps (Kolaczkowski et al. 1996). The genetic separation of drug transport activity of Pdr5p from its inhibitor susceptibility indicated the presence of at least two distinct drug-binding sites in Pdr5p (Egner et al. 1998; Egner et al. 2000). The possible existence of multiple drug-binding sites and the broad substrate specificity indicates that a single fungal ABC transporter may utilize different mechanisms for drug transport. The actual mechanism of drug extrusion might depend on the location of the actual substrate, wherein drugs could either be "flopped" from the inner leaflet of the plasma membrane to the outside and removed by a "molecular vacuum cleaner" mechanism, or expelled by "classical" efflux from the aqueous cytoplasm through a pore formed by energized active transporter (Sipos and Kuchler 2006). Important information towards understanding the molecular structural mechanisms for drug transport across membranes comes from the high-resolution crystal structure of the entire *MsbA*, an essential bacterial ABC transporter functioning as a lipid flippase (Chang and Roth 2001). According to the hypothetical model proposed by Chang, the functional transporter forms a homodimer in the membrane that harbours a chamber confined with 12 trans-membrane α-helices. This chamber serves as a flipping environment where the lipid substrate flips from an energetically unfavourable situation into a more favourable position (Chang 2003). A recent study on structure of half size ABC transporter Sav1866 of *Staphylococcus aureus* suggests that the two TMDs in the homodimer provide inward-facing sites that bind drugs from the lipid bilayer or the cytoplasm and the NBDs and then form interfacial contacts mediated by the binding of two ATP molecules to conserved features on both NBDs. This induces the TMDs to undergo conformational change, and results in a cavity that is open extracellularly and closed intracellularly, thus enabling bound drug to be effluxed out from the cell (Dawson and Locher 2007). While similar structural information does not yet exist for ABC transporters of fungi, it is likely that these also function with such a mechanism.

8.3 PDR Genes and Role in Drug Resistance

The nonpathogenic yeast *S. cerevisiae* genome harbours about 30 genes encoding ABC transporter proteins, a large number of which are responsible for MDR (Sipos and Kuchler 2006). *S. cerevisiae* has served as a model organism for the genetic identification and characterization of MDR (Balzi and Goffeau 1995). ABC proteins in *S. cerevisiae* have been classified into five distinct subfamilies (Schüller et al. 2003), of which several members from two subfamilies (PDR and MRP/cystic fibrosis transmembrane conductance regulator (CFTR) are the best-characterized ABC transporters (Table 8.1). Numerous studies have unravelled the intricate and multifaceted nature of the yeast PDR network, which is composed of several genes that contribute to PDR phenotype and are tightly regulated in a complex regulatory circuit, involving transcription factors that control several target genes encoding ABC pumps (DeRisi et al. 2000; Wolfger et al. 2001), non-ABC genes such as membrane permeases of the MFS (Nourani et al. 1997), and others linked to stress response.

There are at least five plasma membrane-localized ABC transporters, which confer a PDR phenotype when overexpressed. These are Pdr5p, Snq2p, Pdr12p, Yor1p and Ycf1p. Pdr5p, Snq2p and Pdr12p have the $(NBD–TMD_6)_2$ topology and hence belong to the PDR subfamily, whereas Yor1p and Ycf1p are MRP-like transporters and have the $(TMD_6–NBD)_2$ domain organization. All localize to cell surface except Ycf1p (vacuolar pump), displaying remarkable broad substrate specificities. Induced expression of these ABC pumps leads to cellular efflux of a variety of drugs or cytotoxic compounds (Mahe et al. 1996; Egner et al. 1998; Kolaczkowski et al. 1998) and simultaneous resistance to a number of unrelated compounds. Certain PDR subfamily members such as Pdr10p and Pdr15p are connected to the general stress response machinery (Wolfger et al. 1997; Wolfger et al. 2004). Pdr12p plays an essential role in the response and adaptation to weak organic acid stress, and has specificity for transport of C1–C7 organic acids, such as sorbic, benzoic, and propionic acid (Piper et al. 1998; Kren et al. 2003). In addition to Yor1p and Ycf1p, Ybt1p and Bpt1p are representative of the MRP/CFTR subfamily of ABC genes. Null mutant strains lacking several ABC pumps exhibit complex susceptibility phenotypes. Cells lacking Pdr5p, Snq2p or Yor1p display pronounced hypersensitivities to xenobiotics, including antifungal and anticancer drugs (Mahe et al. 1996; Kolaczkowski et al. 1998; Wolfger et al. 2001). Null mutants for *yor1* or *pdr5* are sensitive to certain drugs but hyper-resistant to others. Also, *pdr5* cells are phytosphingosine-sensitive (PHS) but display increased efflux of long chain bases (LCBs), which is probably due to upregulation of *RSB1* encoding a putative LCB translocase (Kihara and Igarashi 2002; Kihara and Igarashi 2004). In *S. cerevisiae*, inactivation of a particular multidrug transporter triggers compensatory transcriptional stimulation of paralogous transporters. While resistance to specific Pdr5p substrates rose upon disruption of the *YOR1* or *SNQ2* gene, resistance to Yor1p and Snq2p-specific substrates increased upon deletion of *PDR5* (Kolaczkowska et al. 2008).

Table 8.1 Drug efflux pumps from nonpathogenic and pathogenic strains[a]

Protein	Substrate	Topology	Localization	Type
Saccharomyces cerevisiae				
Pdr5p	Drugs, phospholipids, steroids, antifungals	$(NBD–TMD_6)_2$	PM	ABC–PDR
Pdr10p	?	$(NBD–TMD_6)_2$	PM	ABC–PDR
Pdr15p	Herbicides, detergents	$(NBD–TMD_6)_2$	PM	ABC–PDR
Pdr11p	Sterol uptake?	$(NBD–TMD_6)_2$	PM	ABC–PDR
Aus1p	Sterols	$(NBD–TMD_6)_2$	PM	ABC–PDR
Pdr12p	Weak acids such as sorbic, benzoic, and propionic acid, fluoresceine	$(NBD–TMD_6)_2$	PM	ABC–PDR
Snq2p	Drugs, mutagens, steroids	$(NBD–TMD_6)_2$	PM	ABC–PDR
Ste6p	a-factor lipopeptide mating pheromone	$(TMD_6–NBD)_2$	PM	ABC–MRP
Yor1p	Phospholipids, oligomycin, reveromycine A	$(TMD_6–NBD)_2$	PM	ABC–MRP
Bpt1	GSH-conjugates, Cd2+, heavy metals	$NTE–NBD–TMD_6–NBD$	Vacuole	ABC–MRP
Vmr1p	Drugs?	$NTE–NBD–TMD_6–NBD$	Vacuole	ABC–MRP
Nft1p	Unknown	$NTE–NBD–TMD_6–NBD$	Vacuole	ABC–MRP
Ybt1p	Heavy metals, GSH conjugates, Bile acids	$NTE–NBD–TMD_6–NBD$	Vacuole	ABC–MRP
Ycf1p	GS-conjugates, Cd2+, diazaborine, bile acids, arsenite, arsenate and antimony	$(TMD_6–NBD)_2$	Vacuole	ABC–MRP
Flr1p	Fluconazole, benomyl, methotrexate	$(TMD)_6$	PM	MFS
Atr1p	4-Nitroquinoline *N*-oxide, aminotriazole	$(TMD)_7$	–	MFS
Sge1p	Crystal violet, Etbr, methylmethane sulfonate	$(TMD)_7$	PM	MFS
Azr1p	Ketoconazole, polymixinB, crystal violet, weak organic acids	$(TMD)_7$	PM	MFS
Aspergillus nidulans				
AtrA	?	$(NBD–TMD_6)_2$		ABC–PDR
AtrB	Cycloheximide, drugs, plant defense compounds	$(NBD–TMD_6)_2$		ABC–PDR
AtrC	?	$(TMD_6–NBD)_2$		ABC–MRP
AtrD	Drugs, antibiotics	$(TMD_6–NBD)_2$		ABC–MRP
Candida albicans				
Cdr1p	Antifungals, azoles, allylamine, rhodamine, drugs, dyes, phospholipids	$(NBD–TMD_6)_2$	PM	ABC–PDR
Cdr2p	Antifungals, azoles, allylamine, rhodamine, caspofungin, phospholipid	$(NBD–TMD_6)_2$	PM	ABC–PDR
Cdr3p	Phospholipid translocator	$(NBD–TMD_6)_2$		ABC–PDR

(*continued*)

Table 8.1 (continued)

Protein	Substrate	Topology	Localization	Type
Cdr4p	Phospholipid translocator	$(NBD–TMD_6)_2$		ABC–PDR
Cdr5p	Drugs?	$(NBD–TMD_6)_2$		ABC–PDR
Hst6p	a-factor export, drugs?	$(TMD_6–NBD)_2$		ABC–MRP
Yor1p	Aureobasidin A	$(TMD_6–NBD)_2$	PM	ABC–MRP
Ycf1p	Drugs	$(TMD_6–NBD)_2$	Vacuole	ABC–MRP
CaMdr1p	Fluconazole, ketoconazole, benomyl, methotrexate, azole, brefeldin A, cerulenin	$(TMD)_6$	PM	MFS
CaFlu1p	Mycophenolic acid	$(TMD)_6$		MFS
Candida dubliniensis				
CdCdr1p	Azoles	$(NBD–TMD_6)_2$		ABC–PDR
CdCdr2p	Azoles	$(NBD–TMD_6)_2$		ABC–PDR
CdMdr1p	Fluconazole, amorolfine, brefeldinA, cycloheximide, fluphenazine	$(TMD)_6$		MFS
Candida glabrata				
CgCdr1p	Azoles, R6G, cycloheximide, chloramphenicol, 5-FC, terbinafine, cerulenin	$(NBD–TMD_6)_2$		ABC–PDR
CgCdr2p/ Pdh1p	Azoles, R6G, cycloheximide, chloramphenicol, 5-FC, terbinafine, cerulenin	$(NBD–TMD_6)_2$		ABC–PDR
CgSnq2p	Azoles, 4-nitroquinoline-*N*-oxide	$(NBD–TMD_6)_2$		ABC–PDR
CgFlr1p	Fluconazole			MFS
Candida krusei				
CkAbc1p	Azoles, cycloheximide, cerulenin, rhodamine123	$(NBD–TMD_6)_2$		ABC–PDR
CkAbc2p	Fluconazole	$(NBD–TMD_6)_2$		ABC–PDR
Aspergillus fumigatus				
AfuMdr1p	Drugs	$(TMD_6–NBD)_2$		ABC–MDR
AfuMdr2p	–	$TMD_6–NBD$		ABC–MDR
AfuMdr3p	Itraconazole	$(TMD_7)_2$		MFS
AfuMdr4p	Itraconazole	$(TMD_6–NBD)_2$		ABC–MDR
Aspergillus flavus				
AflMdr1p	Drugs	$(TMD_6–NBD)_2$		ABC–MDR
Cryptococcus neoformans				
CneMdr1p	Azoles, R6G	$(TMD_6–NBD)_2$		ABC–MDR
CneAfr1p	Azoles, R6G	$(NBD–TMD_6)_2$		ABC–PDR

[a]Table compiled from data available in Bauer et al. (1999), Sipos and Kuchler (2006), Prasad et al. (2006) and Cannon et al. (2009)

ABC ATP-binding cassette, *TMD* transmembrane domain, *PM* plasma membrane, *Vac* vacuole, *GS* glutathione S, *UCB* unconjugated bilirubin, *NBD* nucleotide binding domain, *R6G* rhodamine 6 G, *5-FC* 5-flucocytosine, *Etbr* ethidium bromide, *NTE N*-terminal extension

The plasma membrane ABC transporter Pdr5p was cloned as a cycloheximide resistance gene (Balzi et al. 1994), as a gene mediating resistance to other mycotoxins such as sporidesmin (Bissinger and Kuchler 1994), and cross-resistant to cerulenin and cycloheximide (Hirata et al. 1994). Pdr5p, which is a central element of the PDR network in yeast *S. cerevisiae,* has functional homology with the human P-glycoprotein. It is the most abundant ABC transporter in *S. cerevisiae*, capable of extruding a large number of structurally unrelated hydrophobic compounds, including many classes of clinical antimycotics, across the plasma membrane in an ATP-dependent manner, hence providing resistance to these drugs upon overexpression (Kolaczkowski et al. 1996; Golin et al. 2003; Ernst et al. 2005). Pdr5p-specific ATPase activity shows complete, concentration-dependent inhibition by clotrimazole, which is also known to be a potent transport substrate. However, Pdr5p-mediated efflux of clotrimazole continues at intracellular concentrations of substrate that should eliminate all ATPase activity, and it has been proposed that GTPase and UTPase activities, which are resistant to clotrimazole, were responsible for this function (Golin et al. 2007). Mutational analysis of Pdr5p with altered drug specificity suggests that overall geometry of the Pdr5p contributes to acquiring the enormous range of drug specificity (Egner et al. 1998; Tutulan-Cunita et al. 2005). The Pdr5p mutant with a S1360F exchange in the predicted α-helical TMS10 causes limited substrate specificity for the antifungal ketoconazole (KTC), with poor resistance to itraconazole (ITC) and cycloheximide. Moreover, unlike in wild-type Pdr5p, inhibition of KTC resistance by the FK506 is completely absent in Pdr5p-S1360F (Egner et al. 1998).

Snq2p was identified as the PDR transporter conferring MDR, particularly resistance to mutagens such as 4-nitroquinoline-*N*-oxide (4-NQO) and methyl-nitro-nitrosoguanidine (Servos et al. 1993). Although cells lacking Snq2p are viable, they are hypersensitive to a vast number of xenobiotics. Snq2p also modulates resistance to metal ions such as Na+, Li+ and Mn2+. A *snq2pdr5* double-delete aggravates the effect on intracellular metal ion accumulation and metal sensitivity, implying some functional overlap between Snq2p and Pdr5p (Miyahara et al. 1996). Deletion of *PDR5* and *SNQ2* strongly increases pregnenolone and progesterone toxicity to yeast cells (Cauet et al. 1999), implying intracellular targets for these steroids.

Yor1p and Ycf1p are considered analogues of the human ABC transporters CFTR and MRP1 (MDR-related protein 1). They were identified for their ability to confer resistance to oligomycin (Katzmann et al. 1995), and cadmium (Wemmie and Moye-Rowley 1997) respectively. Deletion of Yor1p causes hypersensitivity to reveromycin A, oligomycin, as well as to various organic anions, and cells are also cadmium-sensitive (Katzmann et al. 1995; Cui et al. 1996). Ycf1p is a glutathione *S*-conjugate pump, and mediates vacuolar transport of oxidized glutathione and other glutathione *S*-conjugates from cytosol. Ycf1p also mediates resistance to arsenite, arsenate and antimony. Overexpression of YCF1 gene allows yeast cells to grow in the presence of a high concentration of cadmium, and its deletion renders the cells hypersensitive to cadmium (Wemmie et al. 1994; Ghosh et al. 1999). Two other ABC transporters, Bpt1p and Ybt1p, also localize to the vacuole, transporting

heavy metals and GSH conjugates from the cytosol (Rebbeor et al. 1998; Klein et al. 2002; Sharma et al. 2002).

Among various pathogenic fungi, most of the MDR-related studies have focussed on *Candida,* in particular *C. albicans*, as it accounts for the majority of systemic infections in immunocompromised individuals. Infections caused by nonalbicans species, such as *C. glabrata*, *C. parapsilosis, C. tropicalis,* and *C. krusei*, have also been increasing, especially in neutropenic patients and neonates (Orozco et al. 1998; Sanglard et al. 1998). In addition to C. *albicans,* clinical resistance to fluconazole (FLC) as a result of reduced intracellular accumulation has been reported for other pathogenic *Candida* species, including *C. tropicalis, C. glabrata, C. krusei*, and *C. dubliniensis*. The azole-resistant isolates of *Candida* cells mainly overexpress genes encoding multidrug efflux transporter proteins belonging to two superfamilies, the ABC transporters and MFS (Prasad et al. 2006).

Five major CDR genes (*CDR1, CDR2, CDR3, CDR4,* and *CDR5*) encoding ABC transporters with the $(NBD–TMD_6)_2$ topology have been identified and confirmed in *C. albicans* for exhibiting xenobiotic-transporting ATPase activity that could be associated with MDR (Arnaud et al. 2007). Others include *SNQ2* and *YOR1* (from the PDR family), *HST6* (from the MDR family), *YCF1* (from the CFTR family), and the *ELF1* (from the YEF family) (White 1997). Several of these genes have been shown to be upregulated in the presence of FLC, ITC, or fluphenazine (Rogers and Barker 2003; Karababa et al. 2004; Xu et al. 2006). Cdr1p was the first ABC transporter identified as a drug efflux pump of *C. albicans* (Prasad et al. 1995a, b) with PM localization (Hernaez et al. 1998). The gene encoding *CDR1* was cloned by functional complementation of the *S. cerevisiae PDR5* mutant, exhibiting hypersensitivity to cycloheximide and other drugs such as miconazole (MCZ) (Prasad et al. 1995a, b) or FLC/ketoconazole (KLC) (Sanglard et al. 1995). Cdr1p is a close homologue of the human MDR1/P-glycoprotein and *S. cerevisiae* Pdr5p. It plays a major role in resistance to FLC and MCZ, the two most common azole antimycotics used to treat candidiasis. In addition to its role in resistance to clinically relevant azoles (FLC, KTC, ITC), Cdr1p also confers resistance to allyamines (terbinafine), morpholines (amorolfine), and several other drugs (Prasad et al. 1995a, b; Sanglard et al. 1995; Sanglard et al. 1997). The expression of *CDR1* in different stress conditions has revealed a significant but transient enhancement of *CDR1* expression at elevated temperatures, and in presence of drugs (MCZ, nystatin, vinblastine) and human steroid hormones (b-estradiol, progesterone) (Krishnamurthy et al. 1998a–c).

A search for other homologues of *CDR1* in *C. albicans* led to the identification of *CDR2*. Cdr1p and Cdr2p pump proteins eliminate not only azoles and their derivatives, but also expel a variety of structurally unrelated compounds. Both Cdr1p and Cdr2p have relatively high level expression in *C. albicans* clinical isolates resistant to azoles (Sanglard et al. 1997). Upregulation of *CDR1* and *CDR2* was shown for four FLC-resistant isolates, relative to their susceptible parental strains (Liu et al. 2007). Like *CDR1*, *CDR2* is able to mediate resistance to azole and allylamine (terbinafine) antifungals, as well as to a variety of metabolic inhibitors including the fluorescent compound and PDR substrate rhodamine 6G when heterologously expressed in yeast, suggesting that they are ABC pump substrates

(Sanglard et al. 1997; Nakamura et al. 2001; Lamping et al. 2007). Azole-resistant clinical isolates with high levels of Cdr1p and Cdr2p also display high minimum inhibitory concentrations for caspofungin. Functional expression of both efflux pumps in *S. cerevisiae* reveals that Cdr2p mediates pronounced caspofungin resistance (Schuetzer-Muehlbauer et al. 2003). Deletion of *CDR1* gene renders the *C. albicans* strain highly susceptible to azole antifungals and many other drugs (Sanglard et al. 1996), and controlled overexpression of Cdr1p in a *C. albicans CDR1*-null mutant conferred resistance to FLC and other xenobiotics (Niimi et al. 2004). Overexpression strains have increased ability to efflux nystatin (Krishnamurthy et al. 1998a–c), but this does not lead to nystatin resistance (Niimi et al. 2004). Also, it has been demonstrated that disruption of *CDR2* did not increase drug sensitivity, most likely because of the low Cdr2p expression, and because Cdr1p alone mediates efficient antifungal efflux, thus compensating for *CDR2* disruption. Deletion of Cdr2p in *cdr1* background aggravated growth inhibition by various antifungals when compared to the single *cdr1* deletion mutant (Sanglard et al. 1997) suggesting that Cdr2p does play a role in antifungal resistance. While it is apparent that multiple mechanisms contribute to clinical *C. albicans* FLC resistance (White et al. 2002), high-level resistance in clinical isolates most often correlates with overexpression of *CDR1* and *CDR2*, and evidence is accumulating that *CDR1* expression is more critical than *CDR2* expression. A recent analysis of Cdr protein expression in *C. albicans* clinical isolates with decreased FLC susceptibilities revealed that Cdr1p was expressed in higher amounts than Cdr2p, and that most FLC efflux was mediated by Cdr1p rather than Cdr2p (Holmes et al. 2008).

C. glabrata has emerged as the second most common *Candida* species associated with fungemia and nosocomial infections (Pfaller et al. 2001). As with *C. albicans*, azole-resistance in *C. glabrata* clinical isolates is associated with increased expression of the ABC drug efflux pumps CgCdr1p and CgPdh1p (also called *Cg*Cdr2p) (Sanglard et al. 1999; Bennett et al. 2004). Cg*CDR1* overexpression confers resistance to azole antifungals, and deletion of the gene renders cells susceptible to azoles. A wide range of structurally and functionally diverse compounds are substrates of this efflux pump (Wada et al. 2002). The ABC transporter Cg*SNQ2* is highly similar to Sc*SNQ2*, and mediates resistance to azoles and a *SNQ2*-specific substrate, 4-nitroquinoline-*N*-oxide (Torelli et al. 2008). Expression of *C. glabrata* ABC transporters is controlled by the transcription factor Cg*PDR1*, an ortholog of Sc*PDR1*. Azole-resistant clinical isolates of *C. glabrata* showed higher expression of Cg*CDR1* and Cg*PDR1* than the susceptible parent strains, indicating the importance of the MDR network to azole resistance in clinical isolates (Ferrari et al. 2009). Disruption of Cg*PDR1* in a resistant clinical isolate led to increased susceptibility to azoles, which could be complemented by introduction of Cg*PDR1* under the control of the *ADH1* promoter to confer resistance (Tsai et al. 2006). Respiratory-deficient petite mutants of *C. glabrata* are highly multidrug-resistant (Sanglard et al. 2001). This mitochondrial control of MDR proceeds through the activation of CgPdr1p (Tsai et al. 2006). Single amino acid substitution mutant forms of Cg*PDR1* have been found that are linked with high-level transcription of both Cg*PDR1* and Cg*CDR1*, as well as robust MDR (Vermitsky and Edlind 2004).

Other ABC-transporters from *C. dubliensis* (Cd*CDR1* and Cd*CDR2*), *C. krusei* (ABC1 and 2), and *C. tropicalis* (*CDR1*-homologue), and from *Cryptococcus neoformans* (Cn*AFR1*, antifungal resistance1) were reported as upregulated in azole-resistant isolates (Moran et al. 1998; Barchiesi et al. 2000; Katiyar and Edlind 2001; Pinjon et al. 2003; Posteraro et al. 2003). ABC transporters Cd*CDR1* and Cd*CDR2* mediate FLC resistance in clinical isolates of *C. dubliensis* (Moran et al. 1998). These findings suggest that a similar resistance mechanism is responsible for acquired azole resistance in *C. dubliensis* and *C. albicans*. Ck*ABC1* and Ck*ABC2* are the only two putative ABC transporters reported for *C. krusei* with a role in drug resistance (Katiyar and Edlind 2001). Heterologous overexpression of CkAbc1p in *S. cerevisiae* showed that CkAbc1p is able to transport a large array of xenobiotics, including FLC, ITC, KTC, MCZ and voriconazole (VCZ) (Lamping et al. 2009).

The filamentous fungi *Aspergillus fumigatus* and *Aspergillus flavus* are two of the more widespread opportunistic species causing human aspergillosis. Most of the *in vitro* studies attempting to identify candidate efflux pumps that could contribute to azole resistance in *Aspergillus* have been performed in the model filamentous fungus *A. nidulans*. The ABC family transporters characterized in *A. nidulans* so far include AtrA, AtrB, AtrC, AtrC2 and AtrD (Del Sorbo et al. 1997; Angermayr et al. 1999; Andrade et al. 2000a, b). AtrA and AtrB encode PDR ABC pumps, and share the same topology $(NBD–TMD_6)_2$ with Pdr5p and Snq2p from *S. cerevisiae* and Cdr1p from *C. albicans*. AtrC, AtrC2, and AtrD belong to the MDR class of transporter, with a $(TMD_6–NBD)_2$ topology (Andrade et al. 2000a, b) sharing significant homology with human P-gp. AtrB and AtrD are the pumps most likely to contribute to MDR in *A. nidulans*. The role of *atrB* MDR was demonstrated by its ability to functionally complement a yeast *PDR5* null mutant for resistance to cycloheximide and other drugs. Expression of *atrB* was strongly enhanced after exposure of *A. nidulans* to several unrelated drugs, antibiotics, and plant defence compounds (Del Sorbo et al. 1997), suggesting a role in defence against natural toxic products. Functional analysis of these genes by gene disruption and overexpression revealed that *atrD* is involved in protection against a range of fungicides and/or xenobiotics (Andrade et al. 2000a, b). Two ABC transporter genes from *A. fumigatus*, Afu*MDR1* and Afu*MDR2*, and one ABC transporter gene from *A. flavus*, Afl*MDR1*, were also identified and cloned (Tobin et al. 1997). Afu*MDR1* and Afl*MDR1* are MDR-type transporters with $(TMD_6–NBD)_2$ topology. Afu*MDR1*- and Afl*MDR1*-encoded proteins displayed a high degree of similarity to human *MDR1*. Overexpression of AfuMdr1p in *S. cerevisiae* conferred increased resistance to cilofungin, an echinocandin B analogue (Tobin et al. 1997). AfuMdr2p is a half-size transporter with a TMD_6–NBD topology, and has been shown not to be involved in antifungal resistance. A third ABC gene from *A. fumigatus*, *ADR1*, has been discovered whose expression was found to be upregulated upon treatment with ITZ (Slaven et al. 1999), suggesting that *ADR1* is involved in modulating resistance to azole antimycotics. Apart from this, in another study that generated 26 ITC-resistant *A. fumigatus*, about half of the mutants displayed ITC resistance due to the overexpression of the efflux pump Afu*MDR3*, an MFS-type transporter of DHA2 family, and Afu*MDR4*, a typical MDR-type ABC transporter with the $(TMD_6–NBD)_2$ topology (Nascimento et al. 2003).

8.4 MFS Transporters

In addition to ABC transporters, proteins of the MFS also contribute to the PDR phenomenon displayed by yeast. The MFS superfamily consists of 61 families of which at least 20 different MFS proteins exhibit structural characteristics consistent with drug resistance, or have already been demonstrated to play a role in drug resistance. The MFS–MDR transporters are classified into two families according to the number of predicted transmembrane spans: (1) the Drug: H+ Antiporter-1 (12-TMS; DHA1) family, and (2) the Drug: H+ Antiporter-2 (14-TMS; DHA2) family (Gbelska et al. 2006; Gaur et al. 2008). *FLR1* of *S. cerevisiae* belonging to the DHA1 family has been shown to function in MDR, conferring resistance to FLC (Alarco et al. 1997), benomyl and methotrexate (Broco et al. 1999). Expression of *FLR1*- and *FLR1*-mediated resistance is regulated by the PDR transcription regulators *YAP1* and *PDR3*. Other MFS proteins involved in drug resistance include Qdr1p, Aqr1p (providing resistance to quinidine and KLC) and Tpo1-4p (providing resistance to polyamine toxicity) (Sa-Correia and Tenreiro 2002). MFS proteins of *S. cerevisiae* belonging to DHA2 family *ATR1, AZR1* and *SGE1* also function in MDR. Atr1p confers resistance to 4-nitroquinoline *N*-oxide and aminotriazole (Gompel-Klein and Brendel 1990), whereas Sge1p confers resistance to crystal violet, ethidium bromide, and methylmethane sulfonate (Ehrenhofer-Murray et al. 1998). Azr1p provides resistance to KLC, polymixinB, and crystal violet, and to the weak organic acids acetic acid and propionic acid (Sa-Correia and Tenreiro 2002).

The first MFS transporter gene to be characterized from *C. albicans* was Ca*MDR1* (Fling et al. 1991). CaMdr1p is a DHA1 MFS transporter, and was initially identified as a gene that conferred resistance to the tubulin-binding agent benomyl and tetrahydrofolate reductase inhibitor methotrexate (Ben-Yaacov et al. 1994). Heterologous expression of Ca*MDR1* in *S. cerevisiae* conferred resistance to FLC and KTC, but not to MCZ or ITC (Lamping et al. 2007; Pasrija et al. 2007), and its overexpression has been linked to azole, brefeldin A and cerulenin resistance in *C. albicans* (Hiller et al. 2006). Another *C. albicans* gene encoding DHA1 MFS transporter, *FLU1* (FLC resistance) (Calabrese et al. 2000) has been isolated by complementation in an azole hypersusceptible *S. cerevisiae PDR5* mutant. Disruption of *FLU1* in *C. albicans* had little effect on FLC susceptibility, but made cells sensitive to mycophenolic acid (Prasad et al. 2006).

MDR in *C. glabrata* is also influenced by transcriptional control of MFS protein expression. The Cg*FLR1* gene is induced by the *C. glabrata* Yap1p homologue (CgAP-1), and confers resistance to a range of agents, including FLC (Chen et al. 2007). *C. glabrata* Cg*ap-1* mutants were found to have normal FLC tolerance, while Cg*pdr1* mutants were hypersensitive to this drug. The homologue of Ca*MDR1* has been implicated in FLC resistance in *C. dubliensis* (Moran et al. 1998; Wirsching et al. 2001). Expression of Cd*MDR1* in a FLC-susceptible *pdr5* null mutant of *S. cerevisiae* conferred a FLC-resistant phenotype. Northern analysis of FLC-susceptible and FLC-resistant *C. dubliensis* isolates revealed that FLC resistance was associated with increased expression of Cd*MDR1* mRNA (Vanden Bossche et al.

1994). Regulation of PDR genes, which has been studied extensively in pathogenic fungi, is beyond the scope of this chapter. Interested readers may refer to a recent review (Morschhäuser 2009).

8.5 Efflux Pumps and Lipid Homeostasis

As discussed above, increased extrusion of antifungal compounds mediated by efflux pump proteins, belonging to either the ABC superfamily or MFS, mediate, MDR in fungi. Since these proteins are localized in PM, fluctuations in membrane lipid composition affect proper localization and functioning of the MDR efflux pump proteins. In particular, the efflux pumps of the ABC superfamily are susceptible to imbalances in membrane-raft lipid constituents. Hence, this part focuses on the significance of the membrane environment in functioning of the drug-efflux pumps, and explores the correlation between MDR and membrane lipid homoeostasis.

8.5.1 Phospholipid Homeostasis

The lipid composition of the cell membrane is the central determinant regulating intake of compounds from the external environment to the interior of the cell. The distribution of lipid components in the inner and outer leaflets of the PM is asymmetric, and is known to be controlled in part by the PDR pathway (Shahi and Moye-Rowley 2009). In the majority of cell types, phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer) are located in the inner leaflet, whereas phosphatidylcholine (PtdCho), sphingomyelin and glycolipids are located in the outer leaflet of the PM (Diaz and Schroit 1996). Loss of the specific asymmetrical distribution of membrane lipids has been linked to various physiological and pathological consequences (Herrmann and Devaux 1990; Diaz and Schroit 1996; Toti et al. 1997). In humans, MDR1/P-glycoprotein has been shown to be involved in maintaining the membrane lipid asymmetry, where it acts as a general phospholipid translocator for different phospholipids and sphingomyelins. A few ABC transporters of *S. cerevisiae* and *C. albicans* have also been shown to function as phospholipid translocators. Absence of ABC transporter *YOR1* or *PDR5* resulted in increased accumulation of a fluorescent PtdEtn, suggesting that Pdr5p and Yor1p are PtdEtn translocators. The accumulation of PtdEtn was even higher in *yor1 pdr5* double delete strain, indicating that the transporters act independently (Kean et al. 1997; Decottignies et al. 1998).

Cdr1p of *C. albicans* elicits energy-dependent in-to-out translocation (floppase) of phospholipids (Dogra et al. 1999). Involvement of *CDR1* in phospholipid translocation was confirmed by a decrease in the availability of PtdEtn in the exoplasmic leaflet of the PM of a homozygous *CDR1* disruptant. In addition, a

double disruption of *CDR1* and *CDR2* resulted in even lesser PtdEtn in the outer leaflet compared to the single *CDR1* disruption, thereby suggesting that *CDR2* could also contribute to phospholipid translocation. Drugs like FLC, cycloheximide and MCZ can affect transbilayer movement of phospholipids mediated by Cdr1p and Cdr2p, but have no effect on Cdr3p-mediated transbilayer exchange (Smriti et al. 2002). These studies suggest that Cdr1p and Cdr2p presumably have common binding sites for drugs and phospholipids, while the flippase activity of Cdr3p is independent of drug binding. The difference in the directionality of phospholipid transfer between Cdrps could be linked to their ability to efflux cytotoxic drugs.

Consistent with a function in membrane lipid transport, changes in membrane fluidity also appear to affect Cdr1p function (Smriti and Prasad 1999; Mukhopadhyay et al. 2002). The influence of membrane fluidity on the functions of Cdr1p was examined by studying expression of Cdr1p in different isogenic *S. cerevisiae erg* mutants, which accumulated various intermediates of the ergosterol biosynthesis thus altering the membrane fluidity. The accumulation of rhodamine 123, β-estradiol, FLC, and floppase (phosphatidyl-ethan-olamine translocase) activity associated with Cdr1p was measured to ascertain their responses to an altered membrane phase. The floppase activity appeared to be favoured by enhanced membrane fluidity, whereas there was significant reduction in effluxing of substrates and the Cdr1p's ability to confer multiple drug resistance (Smriti and Prasad 1999).

During the MDR response in *C. albicans* and *S. cerevisiae*, phosphatidylinositol transfer protein homologues designated Pdr16p and Pdr17p are also induced, which influence phospholipid levels and drug resistance. Loss of the *PDR16* gene from *S. cerevisiae* (van den Hazel et al. 1999), *C. albicans* (Saidane et al. 2006), or *C. glabrata* (Kaur et al. 2004) caused an increase in azole sensitivity, again supporting a common role for this factor in all these yeasts. Several eukaryotic ABC transporters are present in lipid rafts (Pohl et al. 2005), which are highly specialized lipid domains within biological membranes with a number of proposed biological functions (Simons and Vaz 2004). Several studies link the master PDR regulators Pdr1p and Pdr3p to asymmetric lipid distribution and phospholipid metabolism (Kean et al. 1997; Hallstrom et al. 2001; Hanson et al. 2002; Nichols 2002). It has been shown that the compound with potential membrane-damaging or -perturbing effects might function as an activating signal for Pdr1p and Pdr3p, suggesting a role for their target genes in membrane lipid organization or remodelling (Schuller et al. 2007).

8.5.2 Sphingolipid Homeostasis

Along with ABC transporters, another major category of PDR target genes are loci encoding proteins involved in biosynthesis of a major PM lipid called sphingolipids. Sphingolipids presumably associate with ergosterol to form detergent-resistant microdomains called lipid rafts that are concentrated in the outer leaflet of the PM (Gulshan and Moye-Rowley 2007). PDR-mediated transcriptional control has been demonstrated to impact sphingolipid biosynthesis. CaCdr1p has been shown

to be selectively recruited to lipid rafts, and it has been found that any imbalance in the raft lipid constituents due to compromised sphingolipid or ergosterol biosynthesis results in mislocalization of CaCdr1p, with severely impaired drug resistance (Mukhopadhyay et al. 2004; Pasrija et al. 2008). For example, Cdr1p is inappropriately targeted to intracellular cell compartments when sphingolipid biosynthesis is compromised in *sur4, fen1,* and *ipt1* mutants, or ergosterol biosynthesis in *erg24, erg6,* and *erg4* mutants.

Analysis of the expression of the Sc*IPT1* gene encoding inositol phosphotransferase, the last step in sphingolipid biosynthesis (Dickson et al. 1997), established a direct connection between the PDR pathway and biosynthesis of this class of membrane lipids. Sc*IPT1* has been demonstrated to be transcriptionally induced by ScPdr1p and ScPdr3p (Hallstrom et al. 2001), and responds to signals known to induce the *S. cerevisiae* PDR pathway. Loss of Sc*IPT1* altered drug resistance of the resulting mutants, and suggests that normal sphingolipid content is required for wild type levels of drug tolerance (Kolaczkowski et al. 2004).

Control of intracellular levels of sphingoid LCB is crucial, since inappropriate increase of these sphingolipid precursors leads to toxicity of the cell (Skrzypek et al. 1998; Chung et al. 2000). The expression of the putative LCB efflux transporter ScRsb1p is regulated by ScPdr1p and ScPdr3p (Kihara and Igarashi 2004; Panwar and Moye-Rowley 2006). Strains lacking ScPdr5p and ScYor1p ABC transporter proteins were observed to be highly resistant to PHS, and this phenotype was shown to be dependent on the presence of the *ScRSB1* and *ScPDR1* genes. It was argued that a regulatory signal may be transduced by *Sc*Pdr1p to *Sc*Rsb1p upon imbalance of phospholipid asymmetry in the PM. Sc*RSB1* homologues referred to as *RTA* genes have been identified in both *C. albicans* and *C. glabrata*. *CgRTA1* and *CaRTA3* are induced under conditions that lead to activation of other MDR genes (Karababa et al. 2004; Vermitsky et al. 2006). A close relative of Ca*RTA3* called Ca*RTA2* has been directly studied in *C. albicans*, and found to contribute to azole resistance in this pathogen (Jia et al. 2008). These observations reveal an interesting connection between function of PDR target genes and sphingolipid biosynthetic intermediates. Apart from these, ScYor1p was independently identified as a high-copy-number mediator of resistance to aureobasidin A, which is an inhibitor of sphingolipid biosynthesis (Cui et al. 1996). Intriguingly, ScYor1p was also identified as a mediator of resistance to the ceramide synthase inhibitor fumonisin B1 (Mao et al. 2000). These data suggest that ScYor1p may play a role in controlling levels of endogenous sphingolipid intermediates.

8.5.3 Sterol Homeostasis

Steroids are known to be substrates for Pdr5p and Snq2p (Kolaczkowski et al. 1996; Mahe et al. 1996). Mutations in Pdr5p that affect sterol homeostasis also alter FLC resistance. *Pdr1-100*, a gain of function allele of *PDR1* is known to upregulate

PDR5, thus leading to a high level of drug resistance. An *erg3* mutant impaired in ergosterol biosynthesis is also resistant to FLC. Interestingly, the resistance to FLC decreased in a *S. cerevisiae erg3 pdr1-100* double mutant strain, which was attributed to a competition between the endogenous sterols and azoles, both being substrates of Pdr5p (Kontoyiannis 2000). This was further supported by another observation that Pdr5p functions less efficiently in *erg* mutant strains of *S. cerevisiae*, suggesting that loss of ergosterol may enhance passive diffusion of compounds across the altered membrane (Kaur and Bachhawat 1999; Emter et al. 2002). Several studies have documented that Cdr1p can also specifically transport human steroid hormones, namely β-estradiol and corticosterone. The *CDR1*-mediated steroid transport activity was demonstrated by using a *pdr5* null mutant strain of *S. cerevisiae*. This *S. cerevisiae* transformant harbouring the *CDR1* gene accumulated less (two- to threefold) β-estradiol and corticosterone than the non-transformed counterpart (Krishnamurthy et al. 1998a–c). Any fluctuation in sterol composition, which in turn affects membrane fluidity, also alters functioning of ABC drug transporters of yeasts (Kaur and Bachhawat 1999; Emter et al. 2002; Mukhopadhyay et al. 2002). In order to ascertain the functioning of the drug extrusion pumps Cdr1p (ABC family) and Mdr1p (major facilitator superfamily) of *C. albicans* in different lipid environments, they were independently expressed in *S. cerevisiae erg* mutant background. While the fold change in drug resistance mediated by Mdr1p remained the same or increased in *erg* mutants (*erg2, erg3, erg4,* and *erg6*), resistance to FLC and cycloheximide mediated by Cdr1p decreased, indicating that Cdr1p function depends on lipid composition.

In summary, among other physiological function(s), a major role of ABC pumps is the dynamic regulation of membrane lipid composition and bilayer asymmetry. This is in agreement with the fact that many hydrophobic or amphipathic lipid-like molecules are "drug-like" substrates for the major yeast PDR transporters. Taken together, it appears that the functioning of the yeast ABC pump is closely linked to the status of membrane lipids, wherein the overall drug susceptibility phenotype of a cell appears to be interplay of drug diffusion, efflux pumps and membrane lipid environment.

8.6 Strategies to Combat Efflux-Mediated Antifungal Resistance

As discussed above, efflux pumps contribute in a major way to MDR, hindering effective treatment of fungal infections. The detailed mechanistic understanding of a fungal nuclear receptor-like gene regulatory pathway has revealed novel therapeutic targets for developing inhibitors to minimize MDR (Thakur et al. 2008). The inhibition of multidrug transporters or their regulators could in principle thwart the fungal pathogens from effluxing out antifungal drugs, and also affect their capacity to respond transiently to drug stress. Few approaches can be envisaged to negate the impact of efflux, all of which depend on maintaining a high concentration of the

antifungal agent at its site of action (Cannon et al. 2009). One among them would be to use antifungals that are probably not the substrates of fungal efflux, for example, polyene and echinocandin antifungals are not efflux pump substrates at their therapeutic concentrations. Secondly, pump inhibitors can be developed that in combination with existing effective drug could chemosensitize cells. A wide range of compounds that are pseudosubstrates of efflux pumps or that can modify pump activity by other mechanisms have been identified for human drug efflux pump, such as P-glycoprotein (Ganapathi and Grabowski 1983; Tsujimura et al. 2008), and a similar strategy could be used for development of fungal ABC transporter inhibitors. Other than this, inhibitors could be designed that act indirectly on efflux, e.g., de-energizing the transport by lowering the cytoplasmic ATP concentrations (for ABC pumps) or depleting the electrochemical potential of PM (for MFS transporters). It has been demonstrated that inhibitors of C. albicans Pma1p (PM H^+ ATPase) also inhibit azole resistance at a concentration below the MIC (Monk et al. 2005).

The ability to functionally express individual fungal transporters in model organisms such as S. *cerevisiae* has enabled the analysis of pump functions and screening for pump inhibitors. Some of the identified pump inhibitors may be useful in pump protein crystallization and structural resolution. The structures of fungal transporters will enable in silico modelling and structure directed development of more effective pump inhibitors. In *S. cerevisiae* and *C. glabrata*, xenobiotic substrates of ABC transporters have recently been shown to directly bind to the Pdr1p family of transcription regulator proteins via a nuclear-receptor-like pathway. The authors suggest that development of small-molecule antagonists that will bind Pdr1p and its orthologs could prevent activation of efflux pump genes (Thakur et al. 2008). Finally, another approach to combating antifungal resistance due to efflux upregulation could be to use calcineurin (mediates stress tolerance) inhibitors such as FK506 which render normally fungistatic azole FLZ fungicidal (Uppuluri et al. 2008), or to increase uptake rather than decrease efflux of drugs, a strategy that is currently being used for combating human ABC transporter-mediated drug resistance (Dubikovskaya et al. 2008). Insights into the structural element in drug molecules and transport proteins that are required for substrate–transporter interaction has enabled further research to shed light on the molecular mechanism behind antifungal drug resistance (Golin et al. 2003; Hanson et al. 2005). Detailed analysis of structures of fungal efflux pumps and their inhibitors will make a major contribution to designing more potent inhibitors of efflux pumps.

References

Alarco AM, Balan I, Talibi D, Mainville N, Raymond M (1997) AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires FLR1 encoding a transporter of the major facilitator superfamily. J Biol Chem 272:19304–19313

Andrade AC, Del Sorbo G, Van Nistelrooy JG, Waard MA (2000a) The ABC transporter AtrB from *Aspergillus nidulans* mediates resistance to all major classes of fungicides and some natural toxic compounds. Microbiology 146:1987–1997

Andrade AC, Van Nistelrooy JG, Peery RB, Skatrud PL, De Waard MA (2000b) The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. Mol Gen Genet 263:966–977

Angermayr K, Parson W, Stoffler G, Haas H (1999) Expression of atrC — encoding a novel member of the ATP binding cassette transporter family in *Aspergillus nidulans* — is sensitive to cycloheximide. Biochim Biophys Acta 1453:304–310

Arnaud MB, Costanzo MC, Skrzypek MS et al (2007) Sequence resources at the Candida genome database. Nucleic Acids Res 35:D452–D456

Balzi E, Goffeau A (1995) Yeast multidrug resistance: the PDR network. J Bioenerg Biomembr 27:71–76

Balzi E, Wang M, Leterme S, Van Dyck L, Goffeau A (1994) PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. J Biol Chem 269:2206–2214

Barchiesi F, Calabrese D, Sanglard D et al (2000) Experimental induction of fluconazole resistance in *Candida tropicalis* ATCC 750. Antimicrob Agents Chemother 44:1578–1584

Bauer BE, Wolfger H, Kuchler K (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochim Biophys Acta 1461:217–236

Ben-Yaacov R, Knoller S, Caldwell GA, Becker JM, Koltin Y (1994) *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. Antimicrob Agents Chemother 38:648–652

Bennett JE, Izumikawa K, Marr KA (2004) Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. Antimicrob Agents Chemother 48:1773–1777

Bissinger PH, Kuchler K (1994) Molecular cloning and expression of the *Saccharomyces cerevisiae* STS1 gene product. A yeast ABC transporter conferring mycotoxin resistance. J Biol Chem 269:4180–4186

Broco N, Tenreiro S, Viegas CA, Sa-Correia I (1999) FLR1 gene (ORF YBR008c) is required for benomyl and methotrexate resistance in *Saccharomyces cerevisiae* and its benomyl-induced expression is dependent on pdr3 transcriptional regulator. Yeast 15:1595–1608

Calabrese D, Bille J, Sanglard D (2000) A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (FLU1) conferring resistance to fluconazole. Microbiology 146:2743–2754

Cannon RD, Lamping E, Holmes AR et al (2009) Efflux-mediated antifungal drug resistance. Clin Microbiol Rev 22:291–321

Cauet G, Degryse E, Ledoux C, Spagnoli R, Achstetter T (1999) Pregnenolone esterification in *Saccharomyces cerevisiae*. A potential detoxification mechanism. Eur J Biochem 261: 317–324

Chang G (2003) Multidrug resistance ABC transporters. FEBS Lett 555:102–105

Chang G, Roth CB (2001) Structure of MsbA from E. coli: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. Science 293:1793–1800

Chen KH, Miyazaki T, Tsai HF, Bennett JE (2007) The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in *Candida glabrata*. Gene 386:63–72

Chung N, Jenkins G, Hannun YA, Heitman J, Obeid LM (2000) Sphingolipids signal heat stress-induced ubiquitin-dependent proteolysis. J Biol Chem 275:17229–17232

Cui Z, Hirata D, Tsuchiya E, Osada H, Miyakawa T (1996) The multidrug resistance-associated protein (MRP) subfamily (Yrs1/Yor1) of *Saccharomyces cerevisiae* is important for the tolerance to a broad range of organic anions. J Biol Chem 271:14712–14716

Dawson RJ, Locher KP (2007) Structure of the multidrug ABC transporter Sav1866 from *Staphylococcus aureus* in complex with AMP-PNP. FEBS Lett 581:935–938

Dean M, Allikmets R (1995) Evolution of ATP-binding cassette transporter genes. Curr Opin Genet Dev 5:779–785

Decottignies A, Goffeau A (1997) Complete inventory of the yeast ABC proteins. Nat Genet 15:137–145

Decottignies A, Grant AM, Nichols JW, de Wet H, McIntosh DB, Goffeau A (1998) ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J Biol Chem 273:12612–12622

Del Sorbo G, Schoonbeek H, De Waard MA (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet Biol 30:1–15

Del Sorbo G, Andrade AC, Van Nistelrooy JG, Van Kan JA, Balzi E, De Waard MA (1997) Multidrug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters. Mol Gen Genet 254:417–426

DeRisi J, van den Hazel B, Marc P, Balzi E, Brown P, Jacq C, Goffeau A (2000) Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. FEBS Lett 470:156–160

Diaz C, Schroit AJ (1996) Role of translocases in the generation of phosphatidylserine asymmetry. J Membr Biol 151:1–9

Dickson RC, Nagiec EE, Wells GB, Nagiec MM, Lester RL (1997) Synthesis of mannose-(inositol-P)2-ceramide, the major sphingolipid in *Saccharomyces cerevisiae*, requires the IPT1 (YDR072c) gene. J Biol Chem 272:29620–29625

Dogra S, Krishnamurthy S, Gupta V, Dixit BL, Gupta CM, Sanglard D, Prasad R (1999) Asymmetric distribution of phosphatidylethanolamine in *C. albicans*: possible mediation by CDR1, a multidrug transporter belonging to ATP binding cassette (ABC) superfamily. Yeast 15:111–121

Dubikovskaya EA, Thorne SH, Pillow TH, Contag CH, Wender PA (2008) Overcoming multidrug resistance of small-molecule therapeutics through conjugation with releasable octaarginine transporters. Proc Natl Acad Sci U S A 105:12128–12133

Egner R, Bauer BE, Kuchler K (2000) The transmembrane domain 10 of the yeast Pdr5p ABC antifungal efflux pump determines both substrate specificity and inhibitor susceptibility. Mol Microbiol 35:1255–1263

Egner R, Rosenthal FE, Kralli A, Sanglard D, Kuchler K (1998) Genetic separation of FK506 susceptibility and drug transport in the yeast Pdr5 ATP-binding cassette multidrug resistance transporter. Mol Biol Cell 9:523–543

Ehrenhofer-Murray AE, Seitz MU, Sengstag C (1998) The Sge1 protein of *Saccharomyces cerevisiae* is a membrane-associated multidrug transporter. Yeast 14:49–65

Emter R, Heese-Peck A, Kralli A (2002) ERG6 and PDR5 regulate small lipophilic drug accumulation in yeast cells via distinct mechanisms. FEBS Lett 521:57–61

Ernst R, Klemm R, Schmitt L, Kuchler K (2005) Yeast ATP-binding cassette transporters: cellular cleaning pumps. Methods Enzymol 400:460–484

Ferrari S, Ischer F, Calabrese D et al (2009) Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. PLoS Pathog 5:e1000268

Fling ME, Kopf J, Tamarkin A, Gorman JA, Smith HA, Koltin Y (1991) Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. Mol Gen Genet 227:318–329

Ganapathi R, Grabowski D (1983) Enhancement of sensitivity to adriamycin in resistant P388 leukemia by the calmodulin inhibitor trifluoperazine. Cancer Res 43:3696–3699

Gaur M, Puri N, Manoharlal R, Rai V, Mukhopadhayay G, Choudhury D, Prasad R (2008) MFS transportome of the human pathogenic yeast *Candida albicans*. BMC Genomics 9:579

Gbelska Y, Krijger JJ, Breunig KD (2006) Evolution of gene families: the multidrug resistance transporter genes in five related yeast species. FEMS Yeast Res 6:345–355

Ghosh M, Shen J, Rosen BP (1999) Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 96:5001–5006

Golin J, Ambudkar SV, Gottesman MM, Habib AD, Sczepanski J, Ziccardi W, May L (2003) Studies with novel Pdr5p substrates demonstrate a strong size dependence for xenobiotic efflux. J Biol Chem 278:5963–5969

Golin J, Kon ZN, Wu CP et al (2007) Complete inhibition of the Pdr5p multidrug efflux pump ATPase activity by its transport substrate clotrimazole suggests that GTP as well as ATP may be used as an energy source. Biochemistry 46:13109–13119

Gompel-Klein P, Brendel M (1990) Allelism of SNQ1 and ATR1, genes of the yeast *Saccharomyces cerevisiae* required for controlling sensitivity to 4-nitroquinoline-N-oxide and aminotriazole. Curr Genet 18:93–96

Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62:385–427

Gottesman MM, Hrycyna CA, Schoenlein PV, Germann UA, Pastan I (1995) Genetic analysis of the multidrug transporter. Annu Rev Genet 29:607–649

Gulshan K, Moye-Rowley WS (2007) Multidrug resistance in fungi. Eukaryot Cell 6:1933–1942

Hallstrom TC, Lambert L, Schorling S, Balzi E, Goffeau A, Moye-Rowley WS (2001) Coordinate control of sphingolipid biosynthesis and multidrug resistance in *Saccharomyces cerevisiae*. J Biol Chem 276:23674–23680

Hanson L, May L, Tuma P et al (2005) The role of hydrogen bond acceptor groups in the interaction of substrates with Pdr5p, a major yeast drug transporter. Biochemistry 44:9703–9713

Hanson PK, Grant AM, Nichols JW (2002) NBD-labeled phosphatidylcholine enters the yeast vacuole via the pre-vacuolar compartment. J Cell Sci 115:2725–2733

Hernaez ML, Gil C, Pla J, Nombela C (1998) Induced expression of the *Candida albicans* multidrug resistance gene CDR1 in response to fluconazole and other antifungals. Yeast 14:517–526

Herrmann A, Devaux PF (1990) Alteration of the aminophospholipid translocase activity during *in vivo* and artificial aging of human erythrocytes. Biochim Biophys Acta 1027:41–46

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8:67–113

Higgins CF (1995) The ABC of channel regulation. Cell 82:693–696

Hiller D, Sanglard D, Morschhauser J (2006) Overexpression of the MDR1 gene is sufficient to confer increased resistance to toxic compounds in *Candida albicans*. Antimicrob Agents Chemother 50:1365–1371

Hirata D, Yano K, Miyahara K, Miyakawa T (1994) *Saccharomyces cerevisiae* YDR1, which encodes a member of the ATP-binding cassette (ABC) superfamily, is required for multidrug resistance. Curr Genet 26:285–294

Holmes AR, Lin YH, Niimi K et al (2008) ABC transporter Cdr1p contributes more than Cdr2p does to fluconazole efflux in fluconazole-resistant *Candida albicans* clinical isolates. Antimicrob Agents Chemother 52:3851–3862

Hyde SC, Emsley P, Hartshorn MJ et al (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 346:362–365

Jia XM, Ma ZP, Jia Y et al (2008) RTA2, a novel gene involved in azole resistance in *Candida albicans*. Biochem Biophys Res Commun 373:631–636

Karababa M, Coste AT, Rognon B, Bille J, Sanglard D (2004) Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. Antimicrob Agents Chemother 48:3064–3079

Katiyar SK, Edlind TD (2001) Identification and expression of multidrug resistance-related ABC transporter genes in *Candida krusei*. Med Mycol 39:109–116

Katzmann DJ, Hallstrom TC, Voet M, Wysock W, Golin J, Volckaert G, Moye-Rowley WS (1995) Expression of an ATP-binding cassette transporter-encoding gene (YOR1) is required for oligomycin resistance in *Saccharomyces cerevisiae*. Mol Cell Biol 15:6875–6883

Kaur R, Bachhawat AK (1999) The yeast multidrug resistance pump, Pdr5p, confers reduced drug resistance in erg mutants of *Saccharomyces cerevisiae*. Microbiology 145:809–818

Kaur R, Castano I, Cormack BP (2004) Functional genomic analysis of fluconazole susceptibility in the pathogenic yeast *Candida glabrata*: roles of calcium signaling and mitochondria. Antimicrob Agents Chemother 48:1600–1613

Kean LS, Grant AM, Angeletti C, Mahe Y, Kuchler K, Fuller RS, Nichols JW (1997) Plasma membrane translocation of fluorescent-labeled phosphatidylethanolamine is controlled by transcription regulators, PDR1 and PDR3. J Cell Biol 138:255–270

Kihara A, Igarashi Y (2002) Identification and characterization of a *Saccharomyces cerevisiae* gene, RSB1, involved in sphingoid long-chain base release. J Biol Chem 277:30048–30054

Kihara A, Igarashi Y (2004) Cross talk between sphingolipids and glycerophospholipids in the establishment of plasma membrane asymmetry. Mol Biol Cell 15:4949–4959

Klein M, Mamnun YM, Eggmann T, Schuller C, Wolfger H, Martinoia E, Kuchler K (2002) The ATP-binding cassette (ABC) transporter Bpt1p mediates vacuolar sequestration of glutathione conjugates in yeast. FEBS Lett 520:63–67

Kolaczkowska A, Kolaczkowski M, Goffeau A, Moye-Rowley WS (2008) Compensatory activation of the multidrug transporters Pdr5p, Snq2p, and Yor1p by Pdr1p in *Saccharomyces cerevisiae*. FEBS Lett 582:977–983

Kolaczkowski M, Kolaczowska A, Luczynski J, Witek S, Goffeau A (1998) *In vivo* characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. Microb Drug Resist 4:143–158

Kolaczkowski M, Kolaczkowska A, Gaigg B, Schneiter R, Moye-Rowley WS (2004) Differential regulation of ceramide synthase components LAC1 and LAG1 in *Saccharomyces cerevisiae*. Eukaryot Cell 3:880–892

Kolaczkowski M, van der Rest M, Cybularz-Kolaczkowska A, Soumillion JP, Konings WN, Goffeau A (1996) Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. J Biol Chem 271:31543–31548

Kontoyiannis DP (2000) Efflux-mediated resistance to fluconazole could be modulated by sterol homeostasis in *Saccharomyces cerevisiae*. J Antimicrob Chemother 46:199–203

Kren A, Mamnun YM, Bauer BE et al (2003) War1p, a novel transcription factor controlling weak acid stress response in yeast. Mol Cell Biol 23:1775–1785

Krishnamurthy S, Gupta V, Snehlata P, Prasad R (1998a) Characterisation of human steroid hormone transport mediated by Cdr1p, a multidrug transporter of *Candida albicans*, belonging to the ATP binding cassette super family. FEMS Microbiol Lett 158:69–74

Krishnamurthy S, Gupta V, Prasad R, Panwar SL (1998b) Expression of CDR1, a multidrug resistance gene of *Candida albicans*: transcriptional activation by heat shock, drugs and human steroid hormones. FEMS Microbiol Lett 160:191–197

Krishnamurthy S, Chatterjee U, Gupta V, Prasad R, Das P, Snehlata P, Hasnain SE (1998c) Deletion of transmembrane domain 12 of CDR1, a multidrug transporter from Candida albicans, leads to altered drug specificity: expression of a yeast multidrug transporter in baculovirus expression system. Yeast 14:535–550

Kuchler K, Thorner J (1992) Secretion of peptides and proteins lacking hydrophobic signal sequences: the role of adenosine triphosphate-driven membrane translocators. Endocr Rev 13:499–514

Lamping E, Ranchod A, Nakamura K et al (2009) Abc1p is a multidrug efflux transporter that tips the balance in favor of innate azole resistance in *Candida krusei*. Antimicrob Agents Chemother 53:354–369

Lamping E, Monk BC, Niimi K et al (2007) Characterization of three classes of membrane proteins involved in fungal azole resistance by functional hyperexpression in *Saccharomyces cerevisiae*. Eukaryot Cell 6:1150–1165

Lewis K (1994) Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem Sci 19:119–123

Liu TT, Znaidi S, Barker KS et al (2007) Genome-wide expression, location analyses of the *Candida albicans* Tac1p regulon. Eukaryot Cell 6:2122–2138

Mahe Y, Lemoine Y, Kuchler K (1996) The ATP binding cassette transporters Pdr5 and Snq2 of *Saccharomyces cerevisiae* can mediate transport of steroids *in vivo*. J Biol Chem 271:25167–25172

Mao C, Xu R, Bielawska A, Obeid LM (2000) Cloning of an alkaline ceramidase from *Saccharomyces cerevisiae*. An enzyme with reverse (CoA-independent) ceramide synthase activity. J Biol Chem 275:6876–6884

Miyahara K, Mizunuma M, Hirata D, Tsuchiya E, Miyakawa T (1996) The involvement of the *Saccharomyces cerevisiae* multidrug resistance transporters Pdr5p and Snq2p in cation resistance. FEBS Lett 399:317–320

Monk BC, Niimi K, Lin S et al (2005) Surface-active fungicidal D-peptide inhibitors of the plasma membrane proton pump that block azole resistance. Antimicrob Agents Chemother 49:57–70

Moran GP, Sanglard D, Donnelly SM, Shanley DB, Sullivan DJ, Coleman DC (1998) Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. Antimicrob Agents Chemother 42:1819–1830

Morschhäuser J (2009) Regulation of multidrug resistance in pathogenic fungi. Fungal Genet Biol. doi:doi: 10.1016/ j.fgb.2009.1008.1002

Mukhopadhyay K, Kohli A, Prasad R (2002) Drug susceptibilities of yeast cells are affected by membrane lipid composition. Antimicrob Agents Chemother 46:3695–3705

Mukhopadhyay K, Prasad T, Saini P, Pucadyil TJ, Chattopadhyay A, Prasad R (2004) Membrane sphingolipid–ergosterol interactions are important determinants of multidrug resistance in *Candida albicans*. Antimicrob Agents Chemother 48:1778–1787

Nakamura K, Niimi M, Niimi K et al (2001) Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. Antimicrob Agents Chemother 45:3366–3374

Nascimento AM, Goldman GH, Park S et al (2003) Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother 47:1719–1726

Nichols JW (2002) Internalization and trafficking of fluorescent-labeled phospholipids in yeast. Semin Cell Dev Biol 13:179–184

Niimi M, Niimi K, Takano Y, Holmes AR, Fischer FJ, Uehara Y, Cannon RD (2004) Regulated overexpression of CDR1 in *Candida albicans* confers multidrug resistance. J Antimicrob Chemother 54:999–1006

Nourani A, Wesolowski-Louvel M, Delaveau T, Jacq C, Delahodde A (1997) Multiple-drug-resistance phenomenon in the yeast *Saccharomyces cerevisiae*: involvement of two hexose transporters. Mol Cell Biol 17:5453–5460

Orozco AS, Higginbotham LM, Hitchcock CA et al (1998) Mechanism of fluconazole resistance in *Candida krusei*. Antimicrob Agents Chemother 42:2645–2649

Panwar SL, Moye-Rowley WS (2006) Long chain base tolerance in *Saccharomyces cerevisiae* is induced by retrograde signals from the mitochondria. J Biol Chem 281:6376–6384

Pao SS, Paulsen IT, Saier MH Jr (1998) Major facilitator superfamily. Microbiol Mol Biol Rev 62:1–34

Pasrija R, Banerjee D, Prasad R (2007) Structure and function analysis of CaMdr1p, a major facilitator superfamily antifungal efflux transporter protein of *Candida albicans*: identification of amino acid residues critical for drug/H+ transport. Eukaryot Cell 6:443–453

Pasrija R, Panwar SL, Prasad R (2008) Multidrug transporters CaCdr1p and CaMdr1p of *Candida albicans* display different lipid specificities: both ergosterol and sphingolipids are essential for targeting of CaCdr1p to membrane rafts. Antimicrob Agents Chemother 52:694–704

Pfaller MA, Diekema DJ, Jones RN, Sader HS, Fluit AC, Hollis RJ, Messer SA (2001) International surveillance of bloodstream infections due to Candida species: frequency of occurrence and *in vitro* susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY antimicrobial surveillance program. J Clin Microbiol 39:3254–3259

Pinjon E, Moran GP, Jackson CJ, Kelly SL, Sanglard D, Coleman DC, Sullivan DJ (2003) Molecular mechanisms of itraconazole resistance in *Candida dubliniensis*. Antimicrob Agents Chemother 47:2424–2437

Piper P, Mahe Y, Thompson S et al (1998) The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J 17:4257–4265

Pohl A, Devaux PF, Herrmann A (2005) Function of prokaryotic and eukaryotic ABC proteins in lipid transport. Biochim Biophys Acta 1733:29–52

Posteraro B, Sanguinetti M, Sanglard D et al (2003) Identification and characterization of a *Cryptococcus neoformans* ATP binding cassette (ABC) transporter-encoding gene, CnAFR1, involved in the resistance to fluconazole. Mol Microbiol 47:357–371

Prasad R, Murthy SK, Gupta V (1995a) Multiple drug resistance in *Candida albicans*. Acta Biochim Pol 42:497–504

Prasad R, De Wergifosse P, Goffeau A, Balzi E (1995b) Molecular cloning and characterization of a novel gene of *Candida albicans*, CDR1, conferring multiple resistance to drugs and antifungals. Curr Genet 27:320–329

Prasad R, Gaur NA, Gaur M, Komath SS (2006) Efflux pumps in drug resistance of Candida. Infect Disord Drug Targets 6:69–83

Rebbeor JF, Connolly GC, Dumont ME, Ballatori N (1998) ATP-dependent transport of reduced glutathione on YCF1, the yeast orthologue of mammalian multidrug resistance associated proteins. J Biol Chem 273:33449–33454

Rogers PD, Barker KS (2003) Genome-wide expression profile analysis reveals coordinately regulated genes associated with stepwise acquisition of azole resistance in *Candida albicans* clinical isolates. Antimicrob Agents Chemother 47:1220–1227

Sa-Correia I, Tenreiro S (2002) The multidrug resistance transporters of the major facilitator superfamily, 6 years after disclosure of *Saccharomyces cerevisiae* genome sequence. J Biotechnol 98:215–226

Saidane S, Weber S, De Deken X, St-Germain G, Raymond M (2006) PDR16-mediated azole resistance in *Candida albicans*. Mol Microbiol 60:1546–1562

Sanglard D, Ischer F, Bille J (2001) Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. Antimicrob Agents Chemother 45:1174–1183

Sanglard D, Ischer F, Monod M, Bille J (1996) Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob Agents Chemother 40:2300–2305

Sanglard D, Ischer F, Monod M, Bille J (1997) Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology 143:405–416

Sanglard D, Ischer F, Calabrese D, Micheli M, Bille J (1998) Multiple resistance mechanisms to azole antifungals in yeast clinical isolates. Drug Resist Updat 1:255–265

Sanglard D, Ischer F, Calabrese D, Majcherczyk PA, Bille J (1999) The ATP binding cassette transporter gene CgCDR1 from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. Antimicrob Agents Chemother 43:2753–2765

Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M, Bille J (1995) Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob Agents Chemother 39:2378–2386

Schuetzer-Muehlbauer M, Willinger B, Krapf G, Enzinger S, Presterl E, Kuchler K (2003) The *Candida albicans* Cdr2p ATP-binding cassette (ABC) transporter confers resistance to caspofungin. Mol Microbiol 48:225–235

Schuller C, Mamnun YM, Wolfger H, Rockwell N, Thorner J, Kuchler K (2007) Membrane-active compounds activate the transcription factors Pdr1 and Pdr3 connecting pleiotropic drug resistance and membrane lipid homeostasis in *Saccharomyces cerevisiae*. Mol Biol Cell 18:4932–4944

Schüller C, Bauer BE, Kuchler K (2003) Inventory and evolution of fungal ABC protein genes. In: Holland IB, Cole SP, Kuchler K, Higgins CF (eds) ABC proteins: from Bacteria to Man. Academic, Amsterdam, pp 279–293

Servos J, Haase E, Brendel M (1993) Gene SNQ2 of *Saccharomyces cerevisiae*, which confers resistance to 4-nitroquinoline-N-oxide and other chemicals, encodes a 169 kDa protein homologous to ATP-dependent permeases. Mol Gen Genet 236:214–218

Shahi P, Moye-Rowley WS (2009) Coordinate control of lipid composition and drug transport activities is required for normal multidrug resistance in fungi. Biochim Biophys Acta 1794:852–859

Sharma KG, Mason DL, Liu G, Rea PA, Bachhawat AK, Michaelis S (2002) Localization, regulation, and substrate transport properties of Bpt1p, a *Saccharomyces cerevisiae* MRP-type ABC transporter. Eukaryot Cell 1:391–400

Simons K, Vaz WL (2004) Model systems, lipid rafts, and cell membranes. Annu Rev Biophys Biomol Struct 33:269–295

Sipos G, Kuchler K (2006) Fungal ATP-binding cassette (ABC) transporters in drug resistance and detoxification. Curr Drug Targets 7:471–481

Skrzypek MS, Nagiec MM, Lester RL, Dickson RC (1998) Inhibition of amino acid transport by sphingoid long chain bases in *Saccharomyces cerevisiae*. J Biol Chem 273:2829–2834

Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IA, Denning DW (1999) Induced expression of a novel *Aspergillus fumigatus* putative drug efflux gene in response to itraconazole. Fungal Genet Newsl 46:64

Smriti KSS, Prasad R (1999) Membrane fluidity affects functions of Cdr1p, a multidrug ABC transporter of *Candida albicans*. FEMS Microbiol Lett 173:475–481

Smriti KS, Dixit BL, Gupta CM, Milewski S, Prasad R (2002) ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. Yeast 19:303–318

Thakur JK, Arthanari H, Yang F et al (2008) A nuclear receptor-like pathway regulating multidrug resistance in fungi. Nature 452:604–609

Tobin MB, Peery RB, Skatrud PL (1997) Genes encoding multiple drug resistance-like proteins in *Aspergillus fumigatus* and *Aspergillus flavus*. Gene 200:11–23

Torelli R, Posteraro B, Ferrari S, La Sorda M, Fadda G, Sanglard D, Sanguinetti M (2008) The ATP-binding cassette transporter-encoding gene CgSNQ2 is contributing to the CgPDR1-dependent azole resistance of *Candida glabrata*. Mol Microbiol 68:186–201

Toti F, Schindler V, Riou JF et al (1997) Another link between phospholipid transmembrane migration and ABC transporter gene family, inferred from a rare inherited disorder of phosphatidylserine externalization. Biochem Biophys Res Commun 241:548–552

Tsai HF, Krol AA, Sarti KE, Bennett JE (2006) *Candida glabrata* PDR1, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. Antimicrob Agents Chemother 50:1384–1392

Tsujimura S, Saito K, Nawata M, Nakayamada S, Tanaka Y (2008) Overcoming drug resistance induced by P-glycoprotein on lymphocytes in patients with refractory rheumatoid arthritis. Ann Rheum Dis 67:380–388

Tutulan-Cunita AC, Mikoshi M, Mizunuma M, Hirata D, Miyakawa T (2005) Mutational analysis of the yeast multidrug resistance ABC transporter Pdr5p with altered drug specificity. Genes Cells 10:409–420

Uppuluri P, Nett J, Heitman J, Andes D (2008) Synergistic effect of calcineurin inhibitors and fluconazole against *Candida albicans* biofilms. Antimicrob Agents Chemother 52: 1127–1132

van den Hazel HB, Pichler H, do Valle Matta MA, Leitner E, Goffeau A, Daum G (1999) PDR16 and PDR17, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs. J Biol Chem 274:1934–1941

Vanden Bossche H, Marichal P, Odds FC (1994) Molecular mechanisms of drug resistance in fungi. Trends Microbiol 2:393–400

Vermitsky JP, Edlind TD (2004) Azole resistance in *Candida glabrata*: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. Antimicrob Agents Chemother 48:3773–3781

Vermitsky JP, Earhart KD, Smith WL, Homayouni R, Edlind TD, Rogers PD (2006) Pdr1 regulates multidrug resistance in *Candida glabrata*: gene disruption and genome-wide expression studies. Mol Microbiol 61:704–722

Wada S, Niimi M, Niimi K, Holmes AR, Monk BC, Cannon RD, Uehara Y (2002) *Candida glabrata* ATP-binding cassette transporters Cdr1p and Pdh1p expressed in a *Saccharomyces cerevisiae* strain deficient in membrane transporters show phosphorylation-dependent pumping properties. J Biol Chem 277:46809–46821

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J 1: 945–951

Wemmie JA, Moye-Rowley WS (1997) Mutational analysis of the *Saccharomyces cerevisiae* ATP-binding cassette transporter protein Ycf1p. Mol Microbiol 25:683–694

Wemmie JA, Szczypka MS, Thiele DJ, Moye-Rowley WS (1994) Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1. J Biol Chem 269:32592–32597

White TC (1997) Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother 41:1482–1487

White TC, Holleman S, Dy F, Mirels LF, Stevens DA (2002) Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob Agents Chemother 46:1704–1713

Wirsching S, Moran GP, Sullivan DJ, Coleman DC, Morschhauser J (2001) MDR1-mediated drug resistance in *Candida dubliniensis*. Antimicrob Agents Chemother 45:3416–3421

Wolfger H, Mamnun YM, Kuchler K (2001) Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. Res Microbiol 152:375–389

Wolfger H, Mamnun YM, Kuchler K (2004) The yeast Pdr15p ATP-binding cassette (ABC) protein is a general stress response factor implicated in cellular detoxification. J Biol Chem 279:11593–11599

Wolfger H, Mahe Y, Parle-McDermott A, Delahodde A, Kuchler K (1997) The yeast ATP binding cassette (ABC) protein genes PDR10 and PDR15 are novel targets for the Pdr1 and Pdr3 transcriptional regulators. FEBS Lett 418:269–274

Xu Z, Zhang LX, Zhang JD et al (2006) cDNA microarray analysis of differential gene expression and regulation in clinically drug-resistant isolates of *Candida albicans* from bone marrow transplanted patients. Int J Med Microbiol 296:421–434

Zhang X, Collins KI, Greenberger LM (1995) Functional evidence that transmembrane 12 and the loop between transmembrane 11 and 12 form part of the drug-binding domain in P-glycoprotein encoded by MDR1. J Biol Chem 270:5441–5448

Chapter 9
Laboratory Diagnosis of Fungal Infections: An Overview

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Abstract With the increasing rate of fungal infection, it is important for clinical mycologists to know the details of classical and newer molecular approaches in diagnosis of fungal infection. The classical approach includes appropriate specimen collection and transport, as fungus isolation is not so easy as for bacteriological samples. Direct microscopic examination with 10% KOH and Calcofluor white staining plays a very important role, as it helps to provide a presumptive diagnosis to the physician and also permits the selection of appropriate culture media. The fungal culture media used are always two, one being a non-selective medium and the other with addition of antibiotics and cycloheximide, thus reducing bacterial and saprobic fungal contamination. To obtain a better fungal morphology, slide culture is usually done. There are certain differential test media to identify the different species of fungi. As the media for culture are available in plates or tubes, with their respective advantages and disadvantages, their use depends on the preference of the laboratory concerned. As we all know that fungi causing disease are either yeasts, molds or dimorphic, hence the culture media is incubated at two different temperatures of 30°C (for the mold form of all fungi) and 35°C (for yeast forms of dimorphic fungi). As it takes a long time for fungi to grow, they should be

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incubated for at least 30 days before giving a negative culture report. Fungal serology is currently performed only in selected reference laboratories. The test is rapid, and aids in diagnosis of systemic fungal infection, and also helps in the study of epidemiology of the fungal infection. The commonly used serological tests are ELISA, EIA, immunodiffusion and latex agglutination test, with commercially available kits. Now with the advent of molecular techniques including PCR and DNA probes, identification of fungal infection has become even more sensitive and specific. It is rapid, and can detect fungi even in culture plates contaminated with bacteria or other saprobic fungi. In this review, we will describe in detail the important clinical aspects in the laboratory diagnosis of fungal infections, and hope that it will be a reference chapter for beginners as well as for established researchers working in the current field.

9.1 Introduction

Many general microbiology laboratories are inefficient in diagnosing fungal infections, though it requires relatively simple technology and equipment. Medical microbiologists need to correct this situation, as there is a significant increase in recent years in the incidence of serious invasive fungal infections. The increasing incidence of invasive fungal infection originates from the increase in the number of patients who are at high risk, which can be attributed to the factors such as

- The use of potent chemotherapeutic agents that alter the host's immune response
- The increased number of AIDS cases with longer survival
- Individuals who are immunosuppressed or who have reduced numbers or compromised function of the circulating polymorphonuclear leukocytes
- The success of intensive care units in prolonging the survival of highly compromised patients
- Intravenous drug abuse
- More aggressive treatment of cancer patients, particularly those with leukemia and lymphoma
- The increased number of bone marrow transplant and solid organ transplant procedures
- Widespread use of broad-spectrum antibiotics
- New and more widely used prosthetic devices
- Catheter-borne infections among the patients

The laboratory diagnosis of fungal infections relies largely on direct as opposed to indirect methods, i.e., while serology often contributes to the definitive diagnosis of a bacterial or viral infections, it has little importance in diagnosing fungal infection, and is performed only by certain reference laboratories only. It is important that the laboratory receives a correct type of specimen, with adequate clinical data, so that the appropriate investigation can be carried out. Information on factors such as travel or residence in any particular geographical area, animal contacts and

the occupation of the patient enables the mycologist to direct or modify the diagnostic procedures towards a particular fungus or group of fungal agents.

Methods used for the identification of the fungi are a mixture of classical and newer commercially available systems. Therefore, in this chapter we will describe in detail the important clinical aspects in the laboratory diagnosis of fungal infections

9.2 The Classical Approaches Used in the Laboratory Diagnosis of Fungal Infections

9.2.1 Specimen Selection

The laboratory diagnosis of a fungal infection starts with careful collection of the appropriately selected clinical specimen. Table 9.1 shows the organisms likely to be isolated from selected specimens. The specimen must contain the viable etiologic agent for its proper recovery and identification. The anatomic site in which the organism is present must be carefully selected, and the specimen collected in such a manner that it will allow the fungus to remain viable in its "natural" state, without contamination (Merz and Roberts 2003a).

When the appropriate site is selected, the collected specimen should be placed in a suitable container and sent to the laboratory without delay.

9.2.2 Specimen Collection

Specimens should be appropriately collected, stored and processed on time, as faults in any of these steps may cause a wrong diagnosis or a delay in appropriate therapy to the patient. The guidelines for collection of specimens — such as respiratory tract, sterile body fluids [cerebrospinal fluid (CSF), peritoneal, pericardial, synovial fluid and vitreous humor, urine and blood], skin, hair, nails, tissue biopsies and bone marrow, exudate/pus, external eye, prostatic secretions, vaginal secretions, and stool (Milne and Barnetson 1974; Jones et al. 1981; Bille et al. 1983, 1984;

Table 9.1 Organisms likely to be isolated from clinical specimens

Specimen	Organism likely to be isolated
Sputum, bronchoalveolar lavage	*Aspergillus* species
Cerebrospinal fluid	*Cryptococcus neoformans*
Skin, hair & nails	Dermatophytes
Blood, urine and other sterile body fluids	Dimorphic fungi and other pathogenic fungi causing systemic mycoses
Vaginal secretions	*Candida* species

Telenti and Roberts 1989; Geha and Roberts 1994; Waite and Woods 1998; Vetter et al. 2001; Fuller et al. 2001) — are as follows:

1. Collect the specimen from an active lesion; old "burned-out" lesions often do not contain viable organisms.
2. Collect the specimen under aseptic conditions.
3. Collect a sufficient amount of the specimen.
4. Use sterile collection devices and containers.
5. Label the specimens appropriately; consider all clinical specimens should be considered as potential biohazards and handle with care using universal precautions.

9.2.3 Specimen Transport and Storage

For best results, all clinical specimens should be microscopically examined and cultured as soon as possible. Except for blood and corneal scrapings, it is not necessary to transport the specimen in a transport medium. Blood should be placed in the preferred culture system or the lysis-centrifugation system (an isolator). Corneal scrapings should be inoculated onto the medium when collected. Specimens should not be frozen before culture, and those not likely to contain contaminating microorganisms (e.g., CSF) and those that contain dermatophytes (e.g., skin, nails and hair) should not be refrigerated. Transported specimens should never be allowed to dry out. Sputum specimens may be transported without preservative, if only dimorphic fungi are to be recovered (Hariri et al. 1982).

9.2.4 Specimen Processing

When specimens reach the laboratory, they must be appropriately processed to ensure viability of the etiologic agent and to minimize the chance of contamination. Specimens from normally sterile sites, e.g., CSF and peritoneal fluid, need no special processing, but may be cultured directly onto primary recovery media. If the volume is adequate, specimens should be concentrated by centrifugation membrane filtration, and the centrifugated specimen aseptically transferred to primary recovery media for culture and on to a clean microscope slide for direct microscopic examination. Aseptically collected tissue should be homogenized in a tissue grinder, mincer or stomacher. A highly viscous specimen, such as sputum, should be liquefied before culture, and should be concentrated by centrifugation if it is too dilute (Sarosi et al. 1985).

Specimens that are not processed immediately should be held at room temperature, as *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* do not survive well in frozen or iced specimens. Several fungal species

can be recovered from samples that have been in transit for as long as 16 days, if not allowed to dry out; therefore, an attempt should always be made to recover fungi even if processing is delayed (Sarosi et al. 1985).

9.2.5 *Fungal Culture*

The recovery of fungi from a clinical specimen begins with the selection of appropriate primary culture media. Two types of culture media are possible: (a) a non-selective medium, which supports the growth of any potentially pathogenic fungus in the specimen, for example brain–heart infusion agar (BHI agar), and (b) a selective medium, which selectively inhibits contaminating microorganisms and is more selective for the recovery of pathogenic fungi. The selectivity is achieved by the addition of one or more antibiotics, including penicillin (20 U/ml), streptomycin (40 U/ml), gentamicin (5 μg/ml), or chloramphenicol (16 μg/ml). Ciprofloxacin in a concentration of 5 μg/ml also inhibits the growth of contaminating bacteria. It is not necessary to use these on specimens from sterile sites. It is usually used for specimens that are likely to contain contaminating bacteria (e.g., urine, skin, etc). Cycloheximide (Acti-Dione) in a concentration of 0.5 μg/ml may be added to prevent the overgrowth of certain rapidly growing environmental molds. Certain pathogenic fungi such as *C. neoformans*, *Candida krusei* (other *Candida sps*), *Trichosporon cutaneum*, *Pseudallescheria boydii*, and *Aspergillus sps* may be partially or totally inhibited by cycloheximide; therefore, a non-selective medium must always be used in parallel.

9.2.5.1 Media Used for Culture

The media used for fungal cultures are usually solid, except when blood is cultured, for which biphasic brain–heart infusion agar broth is used. The media are usually provided in two forms, as agar plates or agar tubes (screw-capped tubes are used, as cotton-plugged tubes are unsatisfactory for fungal cultures). The choice between the two is optional, taking into account of their respective advantages and disadvantages (kindly refer to Tables 9.2 and 9.3). The culture media used for recovering fungi

Table 9.2 Advantages and disadvantages of agar tubes used for fungal culture

No.	Advantages	Disadvantages
1.	More easily stored, more easily handled, require less space for incubation and easy to transport	Relatively poor isolation of colonies, a reduced surface area for culturing and a tendency to promote anaerobiosis if capped tightly
2.	Lower dehydration rate and less hazardous to handle	Difficulty in preparing stained mounts for microscopic examinations

Table 9.3 Advantages and disadvantages of agar plates used for fungal culture

No.	Advantages	Disadvantages
1.	Provide better aeration of cultures	Agar tends to dehydrate during the extended incubation period
2.	Large surface area for better isolation of colonies	Many laboratories discourage the use of culture plates because of safety considerations
3.	Greater ease of handling and easier to examine and subculture	If *Coccidioides* is suspected in a clinical specimen, plates should never be used
4.	Fungal colonies in mixed cultures are easier to separate	–
5.	Tease mounts or transparency tape preparations are more effectively made from plate cultures	–

from clinical specimens have been grouped as primary recovery medium and differential test medium, described in detail in Tables 9.4 and 9.5 respectively.

9.2.5.2 Method of Identification of Growth on Culture Media

When a fungus is recovered from a clinical specimen, a decision must be made as to its importance as a cause of disease, as this is very helpful in knowing whether the isolate is a "classic pathogen" or one of the opportunistic fungi associated with infection of immunocompromised hosts (Kwon-Chung and Bennett 1992).

9.2.6 Microscopic Examination

9.2.6.1 Direct Method

It is highly recommended that a direct microscopic examination be made on most specimens submitted for fungal cultures; however, its usefulness should be reemphasized (Merz and Roberts 1995). This is the most rapid method for laboratory diagnosis currently available, even in small setups. The direct microscopic examination of clinical specimens depends on its type, i.e., if it is transparent, it may be examined without treatment, if it is opaque, it must be cleared to reveal fungal elements, or the elements must differentially stained within the specimen.

(a) Wet mount (KOH mount): Most of the specimens (skin, hair and nail) can be satisfactorily examined in wet mounts after partial digestion with 10–20% KOH. The specimens should be mounted under coverslip in KOH on a slide. This clears the material within 5–20 min depending on its thickness. Warming over low flame hastens the digestion of the keratin, but care must be taken to avoid overheating and therefore crystallization. KOH may be supplemented with dimethyl sulphoxide to hasten clearing, especially in skin scrapings and

Table 9.4 The primary recovery media used for fungal culture

No.	Media	Indications for use
1.	Brain–heart Infusion agar (BHI)	Primary recovery of saprophytic and dimorphic fungi
2.	Brain–heart infusion agar with antibiotics	Cycloheximide and chloramphenicol may be added for selective recovery of dimorphic molds such as *Blastomyces dermatitidis* and *Histoplasma capsulatum*
3.	Brain–heart infusion biphasic blood culture bottles	Recovery of fungi from blood
4.	Brain–heart Infusion agar + 5–10% sheep blood	For the recovery of fastidious strains, particularly *H. capsulatum*
5.	SABHI agar (Sabouraud's brain–heart infusion agar)	Primary recovery of saprophytic and dimorphic fungi, particularly fastidious strains
6.	Inhibitory mold agar (IMA)	Primary recovery of dimorphic pathogenic fungi. Saprophytic fungi and dermatophytes will not be recovered
7.	Mycosel/mycobiotic agar	Primary recovery of dermatophytes
8.	Sabouraud's dextrose agar (SDA)	This is the most commonly used medium in the diagnostic mycology laboratory. Its final pH is acidic (5.5–5.6), and contains relatively high concentration of sugar such as glucose or dextrose. Its use as primary recovery medium is discouraged because sometimes saprobic fungi grow rapidly on this medium and often overgrow, obscuring the true pathogen, and also it is insufficiently rich to recover most of the dimorphic fungi. It is best used for secondary workup of cultures
9.	Emmon's modified Sabouraud's dextrose agar	It differs from original Sabouraud's formulation in having lower concentration of glucose and a neutral pH. It is the preferred medium for the isolation of *B. dermatitidis*
10.	Sabouraud's dextrose agar with antibiotics	The following antibiotics are added to make the medium selective for the fungal pathogen: cycloheximide (0.5 μg/ml), chloramphenicol (16 μg/ml) and gentamicin (5 μg/ml)
11.	Potato flake agar	Primary recovery of saprobic and pathogenic fungi
12.	Dermatophyte test medium (DTM)	Primary recovery of dermatophytes, recommended as screening medium only
13.	Malt peptone agar	For primary recovery of saprobic and pathogenic fungi
14.	Dermatophyte identification medium (DIM)	Used for presumptive identification of dermatophytes

nail clippings (Roberts 1975). In wet mounts under low- and high-power microscope, yeast cells with or without pseudohyphae, septate hyphae, non-septate hyphae, spherules, arthrospores, or any of the fungal forms are visible. With hair, mosaic arrangement of spores may be seen on the surface of the shaft (ectothrix infection), or hyphal fragments and arthroconidia may be seen internally (endothrix infection).

(b) Calcofluor white stain (CFW): is superior to KOH preparation. Slides prepared by this method may be observed using fluorescent or brightfield microscopy, in which the fungal cells will fluoresce and hence be easily detectable. The fluorochrome optical brighteners bind to polysaccharides with β-glucose

Table 9.5 The differential test media used for fungal culture

No.	Media	Indications for use
1.	Cornmeal agar/cornmeal Tween 80 agar (CMA)	Identification of *Candida albicans* by its production of chlamydospores in this medium
2.	Cottonseed conversion agar	Conversion of dimorphic fungus *Blastomyces dermatitidis* from mold to yeast form
3.	Czapek's agar	For identification of *Aspergillus* species
4.	Niger seed agar/bird seed agar	Identification of *Cryptococcus neoformans*
5.	Ascospore agar	Detection of Ascospores in ascosporogenous yeasts such as *Saccharomyces* species
6.	Potato dextrose agar	Demonstration of pigment production by *Trichophyton rubrum*; preparation of microslide culture and sporulation of dermatophytes
7.	Rice medium	Identification of *Microsporum audouinii*
8.	*Trichophyton* agars 1–7	Identification of members of *Trichophyton* genus
9.	CHROMagar	Provides rapid presumptive identification of various *Candida* species (also *Trichosporon* & *Prototheca* species) on the basis of colony color and morphology produced on this medium
10.	Yeast extract agar	For the isolation and recognition of *Trichophyton verrucosum*

linkages such as cellulose as well as glucans and chitins. They bind non-specifically to the fungal cell wall polysaccharides (rich in glucans and chitin), such that the walls fluoresce brightly under ultraviolet illumination. The CFW-supplemented KOH, especially in corneal scrapings, can detect even a scanty amount of fungal elements (Hageage and Harrington 1984).

9.2.6.2 Other Staining Methods

(a) Gram's stain: fungi are Gram-positive, and seen as violet-colored budding yeast forms in stained smears, as the hyphae are distorted during preparation of smear and staining and do not take the stain.
(b) Giemsa stain: stained smears of sputum, pus and biopsies are advised in patients suspected of histoplasmosis.
(c) Wright's stain: used for the examination of bone marrow and peripheral blood smears and the detection of *H. capsulatum* and *C. neoformans*.
(d) Papanicolaou stain: used for examination of secretions for the presence of malignant cells, but also detects fungal elements which stain pink to blue.
(e) India ink (or nigrosin) mount: may be prepared from CSF specimens when *C. neoformans* is suspected, and shows the presence of encapsulated budding yeast forms in positive specimens.

These methods have shown satisfactory results in the detection of fungal elements in various clinical specimens (Chandler et al. 1989; Conner et al. 1997).

9.3 Role of Serological Tests in Laboratory Diagnosis of Fungal Infections

Serological procedures, together with clinical findings and patient's history, are often useful in obtaining an early diagnosis of the life-threatening systemic fungal diseases (Kaufman et al. 1997). The use of serological tests to measure antibodies is most useful in the diagnosis of acute, systemic, fungal diseases. Agglutination (latex agglutination), precipitation (immunodiffusion), complement fixation test (CFT), and enzyme immunosorbent assays (EIA) are widely available commercially for use in the diagnosis of aspergillosis, blastomycosis, coccidioidomycosis, histoplasmosis, and paracoccidioidomycosis. Serological procedure detect antibodies to specified fungi; they are therefore of limited value in diagnosing disease in immunocompromised patients. To overcome this drawback, considerable effort is being directed towards developing tests to detect fungal antigens, in the serum and other body fluids, as a means of diagnosing the increasing number of opportunistic fungal diseases in immunocompromised patients (Yeo and Wong 2002).

9.4 Molecular Approaches in the Laboratory Diagnosis of Fungal Infections

The limitations of antibody detection and the problems of sensitivity associated with antigen detection have prompted the evaluation of polymerase chain reaction (PCR) for the diagnosis of invasive aspergillosis (Alexander 2002; Kawazu et al. 2003). The main advantages of PCR appear to be that it detects low burden of fungal genetic material and warns of the presence of possible invasive aspergillosis (IA). Early reports described methods to amplify the gene for *Aspergillus fumigatus* 18 kDa ribonucleotoxin (Reddy et al. 1993). Subsequently, a PCR, based on universally conserved sequences within fungal large rDNA, including that of *A. fumigatus*, was described (Haynes et al. 1995). Amplification assays using PCR have been developed to detect DNA of *Aspergillus* species (Lass-Florl et al. 2000; Buchheidt et al. 2001, 2002; Ferns et al. 2002). While most PCR systems for the detection of aspergillosis use whole blood or bronchoalveolar lavage (BAL) as a source, the possibility of using serum was investigated by Yamakami et al. (1996). Walsh et al. (1995) reported the use of single-strand conformational polymorphism (SSCP) as a technique for detecting and delineating differences between fungal species and genera.

Light cycler technique combines rapid *in vitro* amplification of DNA in glass capillaries with real-time species determination and quantification of DNA load. Results are available within 1 hour of testing, and sensitivity is exquisite. Loeffler et al. (2000a) has used this technology to develop assays for *Aspergillus* species. One of the simplest approaches used has been in situ hybridization using specific nucleic acid probes for the identification of organisms in patient specimens.

Amplification assays using PCR allow for detection of small amounts of target DNA in clinical specimens. Specific primers with or without specific probes have been used with some success. Recently, Yeo and Wong (2002) and Chen et al. (2002) have summarized specific targets used and detection methods. Nucleic acid sequencing has been used with great success for the identification of fungi in culture (Turenne et al. 1999).

A comprehensive database of fungal sequences is available in MicroSeq 500 microbial identification system (Applied Biosystems, Foster City, CA, USA). This database is not complete, but when used with GenBank and other public databases, many fungi may be more easily identified than using traditional identification methods. The use of molecular methods is important to investigate the epidemiology and environmental sources of fungi that infect immunocompromised patients and, in some instances, immunocompetent patients. Epidemiologic typing can determine whether or not organisms share the same DNA profile, and this can be related to environmental isolates to determine the point source. The use of molecular tools has permitted the reduction of hospital-acquired infections and their spread.

9.5 The Laboratory Identification of Fungal Isolates

The identification of the isolates has been divided into four sections as shown below covering (1) yeasts and yeast-like organisms, (2) dermophytes, (3) dimorphic fungi, and (4) *Aspergillus* species.

9.5.1 Laboratory Identification of Yeasts and Yeast-Like Organisms

Yeasts can be identified by morphological and biochemical methods (Fig. 9.1).

9.5.1.1 Media Used for Identification

Cornmeal Agar

The commonly used differential medium both for genus identification as well as speciation is the cornmeal agar plate (Dalmau plate) supplemented with Tween 80 or rice agar (de Hoog et al. 2000). Production of large thick-walled, terminal, chlamydospores is diagnostic of *Candida albicans* and *C. dubliniensis* (kindly refer to Table 9.5).

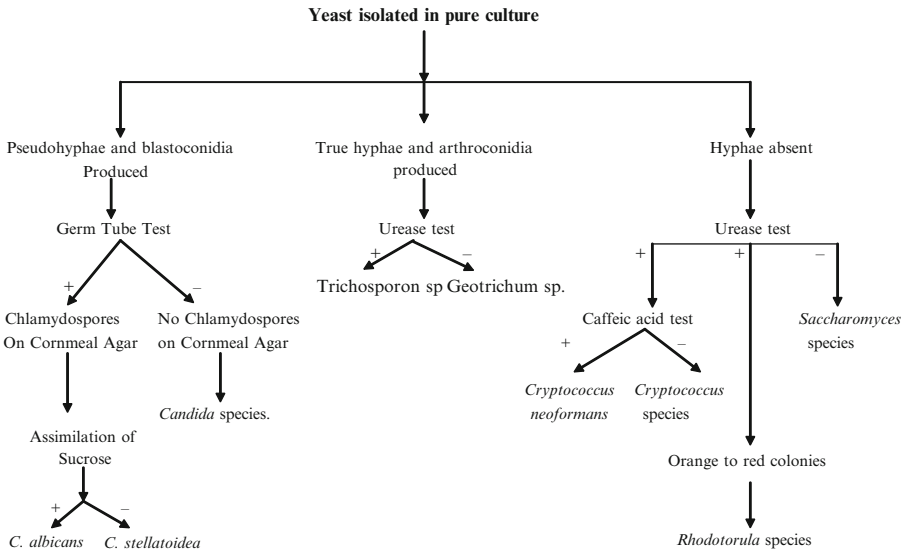

Fig. 9.1 The diagrammatic representation of steps of identification of an isolate of yeasts in pure culture

CHROMagar

An important development was introduced by Odds and Bernaerts (1994) consisting of the CHROMagar system, which uses chromogenic substances (kindly refer to Tables 9.5 and 9.6).

Bird Seed Agar/Niger Seed Agar

An isolate suspicious for *C. neoformans* can be confirmed by demonstrating the production of caffeic acid, either through the production of a maroon-red pigment on bird seed agar or directly by inoculating a reagent-impregnated filter paper strip with a portion of the unknown colony and observing for the development of a black pigment (Table 9.5).

Germ Tube Test

The principle of this test is the ability of *C. albicans* or *C. dubliniensis* blastospores to produce true germ tubes (long tube-like projections extending from the yeast cells, with no constriction at the point of origin from the yeast cell) under defined conditions, i.e., a colony emulsified in 0.5 ml serum (sheep or human) and incubated at 37°C for 2–4 h. *C. tropicalis* produces "pseudo-germ tubes" or pseudohyphae, which are constricted at the base or point of germ tube origin from yeast cell.

Table 9.6 The CHROMagar medium shows the following colors of the colonies when incubated at 30°C for 48–72 h

Species of *Candida*	Colony color
Candida albicans	Light-green
Candida parapsilosis	Cream-colored
Candida tropicalis	Blue with pink halo
Candida krusei	Pink
Candida glabrata	Purple
Candida dubliniensis	Dark-green

The demonstration of germ tube is also known as the Reynolds–Braude phenomenon (Merz and Roberts 2003b).

9.5.1.2 Biochemical Tests for Identification

Rapid Urease Test

The rapid urease test is a most useful tool for screening for urease-producing yeasts recovered from respiratory secretions and other clinical specimens. This test is a presumptive means for identifying the yeasts as *C. neoformans*. As crytococci have large amounts of urease, positive results using such tests can be seen within 15 min (Zimmer and Roberts 1979).

Carbohydrate Utilization Tests

Candida species can utilize carbohydrates both oxidatively (assimilation) and anaerobically (fermentation). Various techniques have been developed for assessing the assimilation patterns. The classical method assessed assimilation by determining the ability of a given yeast isolate to grow in a set of defined minimal liquid media supplemented with different carbohydrates. The other medium used for identification of yeasts by determining carbohydrate assimilation is the yeast nitrogen base agar (Wickerham and Burton 1948).

This method was later on replaced by the auxanographic technique (Hazen and Howell 2003), which employs minimal media agar plates on which paper disks impregnated with different carbohydrates are placed, and the growth ability of the yeast around a specific disk is an indication of the yeast's ability to assimilate that carbohydrate. This method is simpler and quicker, and therefore more adapted for routine use. Modifications of this principle are used by various commercial kits, such as the API 20C, API ID32C, Vitek, Minitek, Uni-Yeast-Tek, and others.

Other methods such as the microscan yeast identification method have been described (Land et al. 1991; St Germain and Beauchesne 1991). RapID yeast plus system (Innovative Diagnostic Systems, Norcross, GA, USA) is a qualitative micro-method using conventional and chromogenic substrates to identify the medically important yeasts. Kitch et al. (1996) showed that the system correctly

identified more than 90% of 286 strains tested within 5 h, without the need of additional tests. Fermentation tests are more difficult to perform. The classical tests involved liquid media supplemented with different carbohydrates, a color indicator to assess pH changes to measure acid formation, and a tool to assess gas production (inverted Durham tube). The medium used is yeast fermentation broth.

9.5.1.3 Serodiagnosis for Identification

Detection of Antigen

Detection of *Candida* antigens in the body fluids is an important diagnostic tool, particularly in immunocompromised patients, who have difficulties in mounting antibodies at a detectable level (Walsh and Chanock 1997). Antigen is generally detected in serum; however, it can be found in other body fluids, such as urine (Ferreira et al. 1990). Although several techniques have been described (De Bernardis et al. 1993; Reboli 1993), currently the most widely used tests include latex agglutination (LA) and ELISA (Fujita and Hashimoto 1992). Some of these are commercially available, such as the Cand-Tec or Pastorex LA systems or the lately introduced Platelia system, a modified ELISA method. The antigens detected by different systems include mannan (Pastorex LA), an undefined glycoprotein (Cand-Tec LA), a 47 kDa protein, enolase, and specific mannosides (Walsh and Chanock 1997; Sendid et al. 2002).

Detection of Antibodies

Tests aimed at detecting antibodies to *Candida* antigens released and/or expressed during deep-seated infections include the gel immunodiffusion (ID) or counter-immunoelectrophoresis (CIE) techniques to detect antibodies to *Candida* cytoplasmic antigens, which are standardized by their protein content (Reiss et al. 2002). Other systems used include the ELISA or LA tests. Detection of antibodies to defined antigens believed to circulate in body fluids during infection, such as enolase (Mitsutake et al. 1994), heat shock proteins (47-kDa breakdown of HSP90) (Walsh and Chanock 1997), and specific mannosides (Poulain et al. 2002), although not widely used has been evaluated. It has been suggested that concurrent determination of both antigens and antibodies in clinical samples may increase the sensitivity of the diagnosis of systemic Candidiasis (Sendid et al. 2002), in both immunocompetent and immunocompromised patients.

9.5.1.4 Detection of Fungal Metabolites for Identification

Detection of arabinitol, a candidal metabolite which is released during infection into the patient's body fluids, possesses a diagnostic value (Reiss and Morrison 1993).

Arabinotol as a fungal metabolite was first described by Kiehn and Bernard (1979). Serum is generally used for the detection of arabinotol, although it is also found in urine. Arabinitol is released by most of the pathogenic *Candida* species except *C. krusei* and *C. glabrata*. The techniques to detect arabinitol include gas-liquid chromatography (GLC) and enzymatic fluorometric measurements (Reiss and Morrison 1993; Switchenko et al. 1994). Another Candidal metabolite is the cell wall component (1→3)-beta-D-glucan (Miyazaki et al. 1995) which is used for diagnosis of fungal infections. These metabolite-identification tests are known as G-test and commercial kits for this test are available (Hossain et al. 1997). However, it should be remembered that glucan is found in other fungi as well; therefore its value as a diagnostic tool is debatable (Verweij et al. 1998; Chryssanthou et al. 1999).

9.5.1.5 Molecular Techniques for Identification

These techniques are based on detection of Candidal DNA in patient's body fluids, involving the use of specific DNA probes. This approach became more promising as a possible diagnostic tool with the development of DNA-amplification techniques, particularly PCR. Several possible probes have been described, such as the actin gene, the gene encoding cytochrome P450 14-lanosterol demethylase (Burgener-Kairuz et al. 1994), the chitin synthetase gene (Jordan 1994), SAP genes (Flahaut et al. 1998), mitochondrial DNA or the candidal DNA repetitive elements (CARE). A widely used probe consists of the rRNA gene complex (Maiwald et al. 1994; Kappe et al. 1998). Some of these are species-specific, while others have wider specificity (Niesters et al. 1993). DNA probes can be used for species identification after these have been isolated in cultures. An example of such an approach is a study by Weissman et al. (1995) which used the benomyl resistance gene for *C. albicans* molecular identification. PCR has been developed to detect DNA of *Candida* (Chryssanthou et al. 1999; Loeffler et al. 2000b; Wahyuningsih et al. 2000; Ahmad et al. 2002). Yeasts in blood cultures have been identified by using PCR, and most were species of *Candida* (Chang et al. 2001).

9.5.2 Laboratory Identification of Dermatophytes

Any mold recovered in culture from specimens labeled skin, nail or hair should be evaluated for the presence of dermatophytes (or ringworm fungi), which are the commonest cause of superficial mycoses. However, not all superficial mycoses are caused by dermatophytes; the yeasts give rise to intertrigos and infect moist skin folds. Yeast infections are often associated with predisposing factors such as diabetes, obesity, or constant immersion of hands in water (causing paronychia).

The identification of these molds is made using a combination such as (a) growth rate of the colony, (b) colony morphology, and (c) microscopic features. The detailed characteristics of the most frequently encountered dermatophytes in the diagnostic mycology laboratory are shown in Tables 9.7 and 9.8 (Rebell and Taplin 1974; Rippon 1988; Suarez et al. 1991; Weitzman et al. 1995; Larone 1995; St-Germain and Summerbell 1996).

9.5.2.1 Primary Media for Identification of Dermatophytes

A primary medium for the isolation of dermatophytes should be selective against bacteria and non-dermatophyte saprobic molds such as:

Mycosel/Mycobiotic Agar

This medium contains phytone peptone, dextrose, agar, cycloheximide, and chloramphenicol, and is used for primary recovery of dermatophytes (Table 9.4).

Sabouraud's Dextrose Agar with Antibiotics

SDA with chloramphenicol and cycloheximide is commonly used for primary isolation of dermatophytes (Table 9.4).

Dermatophyte Test Medium (DTM)

DTM incorporates cycloheximide to inhibit saprobic contaminating molds, and is used for primary isolation of dermatophytes. DTM incorporates gentamicin and chloramphenicol to inhibit bacteria, and a phenol red indicator that changes color from yellow to red when the medium becomes alkaline as the result of growth of dermatophytes, usually within 2 weeks (Rebell and Taplin 1974). However, this medium gives certain false-positive results i.e., a nonpathogenic fungi can turn this medium red (Merz et al. 1970). Some systemically infecting fungi such as *Coccidioides immitis* (Salkin 1973) can resemble a dermatophyte on this medium. In addition, some dermatophytes, for example *M. canis*, may give a false-negative reaction (Moriello and Deboer 1991). This has the disadvantage of not allowing visualization of pigmentation on the reverse of the colony — a characteristic used in identification. Therefore, DTM is a good screening medium for the isolation of dermatophytes, but not a specific indicator of dermatophytes (Table 9.4).

Table 9.7 The characteristics of the most frequently encountered dermatophytes in the diagnostic mycology laboratory are

Dermatophyte	Epidermophyton flocossum	Microsporon audouinii	Microsporum canis	Microsporum gypseum
Rate of growth & pathogenicity:	Within 10 days	7–10 days	6–10 days	Within 6 days
	Infects skin of groin and feet	Causes Ringworm of scalp	Infects scalp and skin	Infects scalp and skin
Grossly:	Colony khaki-colored	Velvety brownish to salmon pink	Cottony colony	Colony cinnamon colored
		Reverse tan to salmon pink	Reverse has lemon yellow pigment	Reverse is light tan
Microscopically:	Hyphae is septate	Hyphae is septate	Hyphae is septate	Hyphae is septate
Macroconidia:	Macroconidia are large club-shaped, thin-walled, smooth and multicelled (1–9 celled), and are arranged in clusters	Sterile hyphae, thick-walled chlamydospores are present, Conidia rare and irregular	Abundant macroconidia which are thick walled, rough, multicelled (15 septa present), spindle-shaped, with pointed curved tip	Abundant macroconidia which are thin-walled, multicelled (4–6 septa) broad, club-shaped with rounded tips
Microconidia:	Microconidia absent	Scanty microconidia	Rare microconidia	Scanty microconidia
Special aspects:	–	No growth on rice medium	–	–

Table 9.8 The characteristics of most frequently encountered dermatophytes in the diagnostic mycology laboratory are

Dermatophyte	*Trichophyton rubrum*	*Trichophyton mentagrophytes*	*Trichophyton tonsurans*	*Trichophyton verrucosum*	*Trichophyton schoenleinii*	*Trichophyton violaceum*
Rate of growth & 14–21 days	pathogenicity:	Within 14 days	7–10 days	12 days	14–21 days	15 days
	Infects skin and nail	Infects skin, hair, & nail. Cause of athlete's foot	Causes scalp ringworm	Infects scalp, beard & skin. Acquired from cattle	Causes chronic scarring scalp infection leading to permanent hair loss	Infects scalp, skin, hair, and nails
Grossly:	Velvety, white downy to pink granular	Granular to powdery, white to tan in color	Powdery, cream or yellow with central furrows	Slow growing, glabrous to velvety white colonies, colony smooth on surface partially submerged in medium	Smooth waxy, brownish colony	Very slow-growing, waxy, violet to purple in color
	Wine-red pigment on reverse	Buff to reddish brown pigment on reverse	Reverse is yellow to tan		Reverse is white	
Microscopically:	Hyphae is septate	Hyphae is septate	Hyphae is septate	Hyphae is septate	Hyphae is septate and usually sterile. Many antler-type hyphae seen (Favic chandelier)	Branched tortuous septate hyphae that are sterile
Macroconidia:	Usually absent, or few long pencil shaped macroconidia	Cigar shaped macroconidia with terminal rat-tail filaments	Rare, macroconidia. Balloon forms found when present	Macroconidia rare, but form characteristic rat-tail type when present		

(*continued*)

Table 9.8 (continued)

Dermatophyte	*Trichophyton rubrum*	*Trichophyton mentagrophytes*	*Trichophyton tonsurans*	*Trichophyton verrucosum*	*Trichophyton schoenleinii*	*Trichophyton violaceum*
				Antler hyphae and chlamydospores arranged in chains are observed with septa appearing like fission planes		
Microconidia:	Abundant microconidia, teardrop shaped, borne laterally from hyphae	Cluster of spherical microconidia present	Abundant microconidia, irregular in size	Microconidia rare	Absent	Absent
Special aspects:	Urea agar ↓ Negative in 2 days	Urea agar ↓ Positive in 2 days	Urea agar ↓ Negative in 2 days It does not grow on *Trichophyton* no. 1 agar	Increased growth at 37°C and on *Trichophyton* no. 4 agar	–	Increased growth on *Trichophyton* no. 4 agar

Dermatophyte Identification Medium (DIM)

DIM is used for presumptive identification of dermatophytes. If there is growth of dermatophytes, the color of the medium changes from greenish blue to purple within 24–48 h after growth. It gives fewer false-positive results than DTM (Table 9.4).

9.5.2.2 Specific Media for Identification of Dermatophytes

Potato Dextrose Agar

This is a reference medium, especially for the production of pigment (Table 9.5).

Rice Medium

Rice grains are used to separate *Microsporum audouinii* and *M. canis*, as the former grows very poorly on this substrate (Table 9.5).

Casamino Acids/Erythritol/Albumin Medium

This medium is used for the isolation of dermatophytes from lesions heavily contaminated with bacteria or with cycloheximide-tolerant *C. albicans* (Fischer and Kane 1974).

Bromocresol Purple (BCP)/Casein Yeast Extract Agar

This medium is designed for the isolation and recognition of *Trichophyton verrucosum* in specimens (Kane and Smitka 1978). The addition of 0.1% yeast extract or thiamine to Sabouraud's agar may increase the isolation rate of dermatophytes, especially of *T. verrucosum* (Aly 1994).

Bromocresol Milk Solids Glucose Agar

Depending on the type of growth and change of indicator color occurring on this medium, one can easily identify the several dermatophytes (Weitzman et al. 1995).

Trichophyton Agars 1–7

A series of agar media (*Trichophyton* agars, Difco) (Table 9.9) incorporating growth factors can be helpful in grouping species of *Trichophyton* by demonstrating their requirements for special growth factors (Clayton and Midgley 1989).

Table 9.9 Shows *Trichophyton* agar medium used for dermatophytes

Trichophyton agar no.	Contents	*Trichophytons* which require these nutrients
1	Vitamin-free casein basal medium	*Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton schoenleinii*
2	Casien basal medium + inositol	Other *Trichophyton* species
3	Casein basal medium + inositol + thiamine	*Trichophyton verrucosum* (16% isolates)
4	Casein basal medium + thiamine	*T. verrucosum, Trichophyton tonsurans, Trichophyton violaceum*
5	Casein basal medium + nicotinic acid	Other *Trichophyton* species
6	Ammonium nitrate basal medium	Other *Trichophyton* species
7	Ammonium nitrate basal medium + histidine	Other *Trichophyton* species

Sporulation-Stimulating Media

Cornmeal, potato flake, potato glucose, Sabouraud's glucose + 3–5% NaCl (Kane and Fischer 1975), lactrimel (Kaminski 1985), and autoclaved polished rice grains (McGinnis 1980).

9.5.2.3 Tests for Identification of Dermatophytes

In Vitro Hair Perforation Test

This test was originally devised to distinguish atypical isolates of *T. mentagrophytes* and *T. rubrum*. It may also be used to distinguish *M. equinum* from *M. canis*. The test is taken as positive when the dermatophyte species show wedge-shaped perforations in the hair. It is positive in *T. mentagrophytes* and *M. canis*, and negative in *T. rubrum* and *M. equinum*. This test is helpful in identifying other dermatophyte species (Clayton and Midgley 1989).

Urease Test

The ability to hydrolyse urea (Clayton and Midgley 1989), either in Christensen's urea agar or a broth medium, aids in the distinction of *T. rubrum* (urease-negative) and *T. mentagrophytes* (urease-positive). The dermatophyte *T. raubitschekii*, considered to be a variant of *T. rubrum* (Kwon-Chung and Bennett 1992; Graser et al. 2000a, b) or as a distinct species by others, is urease-positive (Kane et al. 1981). A broth medium is preferred over agar medium because it is more sensitive (Kane and Fischer 1971). The test is considered negative if there is no color change from straw to reddish purple within 7 days at 23–30°C. Cultures to be tested must be

pure, because bacterial contamination can produce a false-positive reaction. Hence, when in doubt about the purity of the culture, incubate the test culture on a blood agar medium to detect presence of bacteria, before carrying out the urease test.

Test for Temperature Tolerance and High-Temperature Enhancement of Growth

If *T. verrucosum* is suspected, two culture media enriched with thiamine are inoculated and incubated at 37°C, as this is the only dermatophyte whose growth is enhanced at this high temperature (Clayton and Midgley 1989; Weitzman et al. 1995).

9.5.2.4 Trichophytin Skin Test (Immunodiagnosis) for Dermatophytes

Skin test with dermatophytic antigen is important for the diagnosis of dermatophytosis. Trichophytin is a crude extract from dermatophytes, producing positive delayed-type hypersensitivity (DTH), tuberculin-like response in most adults. DTH responses to intradermal injections of trichophytin are commonly observed in the normal population (Grossman et al. 1975). A galactomannan peptide is the reactive component of the antigen. Patients without the delayed-type or with an immediate-type reaction are more susceptible to chronic dermatophytosis. Similarly, experimental dermatophyte infections in animal models appear to result in CMI to the antigens of the infecting dermatophyte (Green and Balish 1980).

9.5.2.5 Molecular Techniques for Dermatophytes

The conventional laboratory procedures for the identification of dermatophytes are either slow or lack specificity; the application of nucleic-acid amplification technology has made rapid and precise identification of dermatophytes possible. Recently, Liu et al. (2000) have shown that when one of the four random primers (OPAA11, OPD18, OPAA17, and OPU15) was used in an arbitrarily primed polymerase chain reaction (AP-PCR), up to 20 of the 25 dermatophyte species or subspecies under investigation could be distinguished on the basis of characteristic band patterns detected in agarose gel electrophoresis. Turin et al. (2000) have developed new assays to detect DNA of dermatophytes.

9.5.3 Laboratory Identification of the Dimorphic Fungi

The dimorphic fungi are causative agents of "deep-seated" mycoses in humans. Whenever a dimorphic fungus is suspected in a clinical specimen, it should be

inoculated in two media and kept at 25–30°C and 35–37°C to obtain its mold form and yeast form respectively. The mold form is the infective form but never found in tissues; however, they are recovered on culture media. Only the yeast form (parasitic form) is seen in the tissues. The definitive identification of the dimorphic fungi is traditionally made on the basis of the microscopic morphology of both mold and parasitic (yeast or spherule) forms. The characteristic features of medically important dimorphic fungi are shown in Table 9.10.

9.5.3.1 Microscopic Examination of Dimorphic Fungi

The quickest means of diagnosing most mycoses is by immediate microscopic examination of infected host material. This approach is usually futile with sporotrichosis, because the cells of *S. schenckii* normally occur very sparsely within infected material. However, staining the histological tissues with fluorescent antibody can quickly provide its specific identification. In the wet mount made from clinical specimens, *B. dermititidis* appears as a round yeast cell with double wall and often with a single broad-based bud, and all cells are of uniform size. Round-to-oval yeast cells measuring 2–4 mm in diameter, characteristic of *H. capsulatum*, may be seen in clinical specimens stained with fungal stains. Microscopic demonstration of *C. immitis*, particularly the endosporulating spherules, provides the most direct and secure method of diagnosis. The direct microscopic observation of multiple-budding yeast forms in 10% KOH preparations of clinical specimens is the most recommended way for diagnosing the infection by *P. braseliensis* (Lacaz et al. 2002). Rapid bedside diagnosis of *Penicilliosis marneffei* can be made by microscopic examination of Wright's stained bone marrow aspirates and/or touch smear of skin biopsy or lymph node biopsy specimens (Supparatpinyo et al. 1994). The yeast cells of *P. marneffei* can also be seen in peripheral blood smear of patient with fulminant disease (Supparatpinyo and Sirisanthana 1994).

9.5.3.2 Media Used for Identification of Dimorphic Fungi

The dimorphic fungi have the capability of growing in culture media containing cycloheximide (except *P. marneffei*, whose growth is inhibited by cycloheximide); this is a valuable property in separating out the saprophytic filamentous molds, particularly their environmental look-alike, which grow poorly or not at all. Thus media used for primary isolation of dimorphic fungi include brain-heart infusion (BHI) agar with or without antibiotics, brain–heart Infusion (BHI) agar + 5–10% sheep red blood cells (especially for the recovery of *H. capsulatum*), inhibitory mold agar (IMA), Sabouraud's brain–heart infusion agar (SABHI), and Sabouraud's dextrose agar (SDA). The preferred medium for isolation of *B. dermatitidis* is Emmon's modified Sabouraud's dextrose agar, as the decreased sugar content of this medium favors its recovery. Colonies grow slowly; usually 7–14 days are required for primary isolation. In cases in which spore concentration in a clinical

Table 9.10 Characteristic features of medically important dimorphic fungi

Dimorphic fungi:	*Blastomyces dermatitidis*	*Coccidioides immitis*	*Histoplasma capsulatum*	*Sporothrix schenckii*	*Paracoccidioides brasiliensis*	*Penicillium marneffei*
Rate of growth & pathogenicity:	Slow, within 14 days Causes Blastomycosis; chronic infection, characterized by suppurative & granulomatous lesions in any part of body	Moderate, within 10 days Causes Coccidioidomycosis; which is highly infectious disease. Cultures should be dealt only in Biological Safety Cabinet	Slow, within 15–20 days Causes Histoplasmosis; which may be acute benign pulmonary disease or chronic fatal disease. It may be localized or disseminated (primarily to reticuloendotheliai system)	Rapid, matures in 4 days Causes Sporotrichosis; a chronic infection beginning as skin infection and then involving lymphatics	Very slow, within 21 days Causes Paracoccidioidomycosis; a chronic granulomatous disease of skin, mucous membrane and internal organs	Rapid within 3–5 days Causes *Penicilliosis marneffei*;Infects reticuloendothelial system. Emerging pathogen, infects immunocompromised paients (AIDS patients)
Colony morphology:	At first yeast like, then "prickly", finally cottony with white aerial mycelium	Colony is at first moist, grayish, membranous, and soon develops a white, cottony aerial mycelium	White to brown or pinkish, with a fine dense cottony texture	At first small and white with no cottony aerial hyphae. Later on becomes moist, wrinkled, leathery, or velvety, & darkens to brown or black	Colony white, heaped compact, usually folded, almost glabrous with short nap of white aerial mycelium, that turns brown with age	Grayish-white colonies initially, lateron become wooly to granular in texture, yellow-orange, or green center, periphery is white with radial folds
At 25°C on SDA		Reverse is white	Reverse is white			Reverse is bright rose color, due to water soluble red pigment, that diffuses in medium
At 37°C on BHI agar	Cream to tan in color, heaped or wrinkled & waxy	-do-	Moist, white, yeast like colonies. Yeast phase may be	Cream or tan, smooth, yeast-like	Colony heaped, cream to tan, becoming waxy, yeast-like	Yeast-like cerebriform, pinkish-white convoluted colonies

(*continued*)

Table 9.10 (continued)

Dimorphic fungi:	*Blastomyces dermatitidis*	*Coccidioides immitis*	*Histoplasma capsulatum*	*Sporothrix schenckii*	*Paracoccidioides brasiliensis*	*Penicillium marneffei*
	in appearance. Yeast phase may be inhibited by Chloramphenicol & Cycloheximide		inhibited by Chloramphenicol & Cycloheximide			
						Red pigment poorly produced or not at all
Microscopic morphology:						
Mold form (at 25°C):	Septate hyphae with, club-shaped conidia having thin-smooth walls, borne at the tips of long straight Conidiophores (Lollipop)	Septate hyphae which segment into barrel shaped arthroconidia, that alternate with empty cells (giving rise to alternate staining)	Septate hyphae with tiny microconidia produced early on. Later, large, spiked spherical macroconidia are diagnostic	Septate hyphae with branching and bearing small pear-shaped conidia, produced radially at the tip of straight conidiophores, in a "rosette-like" cluster	Septate branched hyphae with some intercalary and terminal chlamydospores. Few microconidia seen occasionally	Septate branched hyphae with chains of elliptical or lemon-shaped, smooth-walled phialoconidia arising basipetally from ends of tapered phialides (giving sausage-like appearance) which have prominent cross-walls (characteristic)
Yeast form (at 37°C):	Spherical yeast cells, 10–15 μm in diameter, with single broad-based bud and appearing to be double contoured	Large spherules, 75–200 μm in diameter, non-budding, thick-walled, containing 3–5 μm endospores. Empty & collapsed "ghost" spherules may be seen	Small, round or oval budding cells and occasional abortive hyphae seen. Seen intracellularly in bone marrow stained with Wright or Giemsa stain	Round, oval & fusiform budding cells, commonly called "cigar bodies"	Large, spherical, smooth, thick-walled mother cell with single and multiple buddings resembling "mariner's wheel" or "steering wheel"	Small 2–6 μm, yeast-like cells that have single transverse septum but no budding cells are produced

specimen is particularly high in sputum, growth may be observed within 4–7 days. Colony surface is gray-white and cob-web in consistency. The hyphae of dimorphic fungi are delicate, septate and hyaline. Their arrangement in parallel bundles may be seen. Species identification of the dimorphic fungi is based on the size, shape, morphology, and arrangement of conidia. Species confirmation is done by yeast conversion (or nucleic acid probe). The species of medical importance include *B. dermatitidis, Penicillium marneffei, Paracoccidioides brasiliensis, Sporothrix schenckii, H. capsulatum,* and *C. immitis.*

9.5.3.3 Tests for Identification of Dimorphic Fungi

Exoantigen Test

The exoantigen test was considered for many years as the method of choice for definitive identification of dimorphic fungi (Kaufman and Standard 1978). This test identifies the mycelia form of dimorphic fungi (Kaufman et al. 1983). This method relied on the principle that soluble antigens are produced by fungi when grown in a broth medium, which can be extracted and concentrated and subsequently reacted with standardized antiserum. This is a double immunodiffusion reaction in agar. This test with a proper antiserum is 100% specific for *B. dermatitidis*, and can be applied for the identification of other dimorphic fungi. *B. dermatitidis* can be specifically identified by this method, even when the extracted culture is contaminated (Di Salvo et al. 1981). However, the exoantigen test has been replaced by specific nucleic acid probes that provide a more rapid and specific identification.

Direct Fluorescent Antibody Test (DFA)

The direct fluorescent antibody test for *B. dermatitidis* was developed by Kaplan and Kaufman (1963). These fluorescent antibody reagents are, however, available only at certain reference laboratories, and are not commercially offered.

Indirect Fluorescent Antibody Test

A specific indirect fluorescent antibody reagent was developed to facilitate the rapid detection and identification of *P. marneffei* in histological sections (Kaufman et al. 1995). This test is specific, and shows no cross reaction with *H. capsulatum*.

Skin Tests

Individuals who become infected with *Histoplasma* or *Coccidioides* develop a DTH reaction within 1–14 days which may persist for many years. This sensitization can

be shown by skin tests in which induration and erythema occur 24–72 h following intradermal inoculation of the appropriate fungal antigen (Histoplasmin and Coccidioidin). However, this is usually not employed for diagnosis due to false-positive reactions in endemic areas and cross-reactions that occur because of common antigens shared by the major fungal pathogens (Edwards et al. 1969). The skin tests may be falsely negative in disseminated disease, and skin tests boost the levels of antibodies, thus causing confusion in the interpretation of serological tests (Campbell and Hill 1964). The skin test is however useful for determining the prevalence of infection in epidemiological studies.

9.5.3.4 Serodiagnosis for Dimorphic Fungi

Blastomyces Dermatitidis

The two serological tests in general use which detect antibodies to *B. dermatitidis* and are useful for the diagnosis of blastomycosis are (a) the complement fixation test (CFT), and (b) the immunodiffusion (ID) precipitin test. The EIA test is a more recent development in the diagnosis of blastomycosis. A non-commercial radioimmunoassay has been reported that incorporated BAD1, which is a target of both antibody and T-cell responses (Klein and Jones 1990).

Histoplasma Capsulatum

For *H. capsulatum*, ID and CFT tests are used for the detection of antibodies. The precipitin bands (H and M) are demonstrated by ID test using histoplasmin as the antigen. H precipitin bands can be demonstrated in less than 25% of patients, and are clear during the first 6 months after infection, whereas M bands appear in about 75% of individuals and may persist for several years after the resolution of infection (Wheat et al. 1982; Williams et al. 1994).The complement fixation test is performed with both histoplasmin and whole yeast cell antigens. Titers of 1:8–1:16 are suggestive of active infection, and titers of 1:32 or higher are more suggestive of active disease.

The most widely used is an immunoassay for the detection of *Histoplasma* antigen (Durkin et al. 1997). This test has proven useful for the detection of antigenemia in patients having disseminated disease (Wheat et al. 1986a), particularly those with AIDS (Wheat et al. 1992). *Histoplasma* polysaccharide antigen is found in blood, urine, CSF and bronchoalveolar lavage fluid of more than 90% of individuals with disseminated histoplasmosis capsulate, and in urine of 75% of those with extensive pneumonitis after acute exposure (Wheat et al. 1986b; Wheat and Kaufman 2003).

Coccidioidis Immitis

For *C. immitis* also, two assays, i.e., CFT and ID, are employed for diagnosis. The precipitin antibody which is IgM in nature was detected by the tube precipitin (TP) test (Smith et al. 1956). It can now be detected by immunodiffusion test corresponding to tube precipitin test (IDTP). This antibody can be detected by antigen-coated latex particles and by EIA. The antigen with which it reacts is mainly polysaccharide, but it can also be a 120 kDa glucosidase (Kruse and Cole 1992) or a 21 kDa proteinase (Resnick et al. 1987) that can be released into the culture medium (Zimmer and Pappagianis 1989). The antibodies detected by CFT appear late and IgG in nature, and their titer clearly correlates with the extent and severity of the disease. The reacting antigen is a chitinase (Johnson and Pappagianis 1992) which is released by the maturing, then rupturing spherules. This antibody can be detected by EIA (Gade et al. 1992). CFT titers as low as 1:2–1:4 suggest infection and titers greater than 1:16 are indicative of active disease with metapulmonary dissemination, e.g., to skin, bone or even the meninges (Smith et al. 1956).

Paracoccidioides Brasiliensis

For paracoccidioidomycosis, several serological tests have been developed and extensively studied. Agar gel immunodiffusion (ID), CFT, and CIE tests have been employed extensively (Mendes-Giannini et al. 1994). Several other tests are also available, such as indirect immunofluorescence, indirect hemagglutination, ELISA, and dot blot immunobinding and Western blotting (Mendes-Giannini et al. 1984; Taborda and Camargo 1993, 1994; Martins et al. 1997).

Penicillium Marneffei

A microimmunodiffusion test using mycelia culture filtrate antigen of *P. marneffei* (Sekhon et al. 1982) has been used to monitor the serological response of an AIDS patient with *P. marneffei* (Viviani et al. 1993). An indirect fluorescent antibody test for detecting IgG antibody, using germinating conidia and yeast cells as antigens, was able to detect the IgG titers of 160 or more in patients infected with *P. marneffei* (Yuen et al. 1994). In addition, a gene (MP1) encoding a highly antigenic cell wall mannoprotein (Mp1p) has been cloned, and purified recombinant antigenic mannoprotein produced and tested in an ELISA for antibody detection (Cao et al. 1998). In order to assess the presence of antigen in the body fluids of the patients affected with *P. marneffei*, rabbit polyclonal antibodies against arthroconidial filtrate (or yeast phase) were used in an immunodiffusion and latex agglutination test, with a specificity of 100% (Kaufman et al. 1996). Encouraging results were obtained with antibody reagent against the recombinant mannoprotein Mp1p that was used to test sera from AIDS and non-AIDS patients with

penicilliosis in an ELISA assay with specificity of 100% and sensitivity of 65% (Cao et al. 1999).

9.5.3.5 Molecular Techniques for Dimorphic Fungi

Fungal cells are lysed, and the ribosomal RNA is hybridized to a DNA probe which has been labeled with acridinium ester (Stockman et al. 1993). In short, the hybrids are detected by the chemiluminescence of the attached probe. Amplification assays using PCR have been developed to detect DNA of *Histoplasma* (Bialek et al. 2002; Rickerts et al. 2002), *Blastomyces* (Sandhu et al. 1995; Bialek et al. 2003), *Coccidioides* (Sandhu et al. 1995), *Paracoccidioides* (Gomes et al. 2000), *P. marneffei* (Vanittanakom et al. 2002). Recently, real-time PCR has been used for the identification of culture isolates of *H. capsulatum* (Martagon-Villamil et al. 2003), and the detection of *H. capsulatum* DNA has also been reported in a few clinical specimens, but these are preliminary data and clinical trials will be needed to confirm the utility of the test as a diagnostic tool. Oligonucleotide primers for selective amplification of *P. marneffei* DNA were designed from the nuclear rDNA internal transcribed spacer region (LoBuglio and Taylor 1995). Using nested PCR, the test was 100% successful in amplifying *P. marneffei* DNA. It was possible to use the PCR-hybridization technique to detect *P. marneffei* DNA in EDTA blood samples collected from AIDS patients with penicilliosis. Recently, new PCR primers were designed based on the 18SrDNA sequence, and were used in a single and nested PCR method for the rapid identification of *P. marneffei* (Vanittanakom et al. 2002). It was possible to perform a very young culture of *P. marneffei* (2-day-old filamentous colony, 2 mm in diameter) by this assay. Another method which was developed to provide rapid identification of dimorphic fungi including *P. marneffei* was a PCR–EIA method (Lindsley et al. 2001). The DNA was amplified by using universal fungal primers and was directed to the conserved regions of rDNA. The PCR amplicons were then detected colorimetrically in an enzyme immunoassay format for specific fungal species.

9.5.4 *Laboratory Identification of* Aspergillus *Species*

Direct microscopic examination of sputum preparations is often helpful in the diagnosis of allergic aspergillosis. Microscopic examination of sputum is seldom helpful in patients with suspected IA, but examination of bronchoalveolar lavage (BAL) specimens is often rewarding (Delvenne et al. 1993). Typical mycelium may be detected in wet preparations of necrotic material from cutaneous lesions or sinus washings, but isolation of the etiologic agent in culture is essential to confirm diagnosis.

The definitive diagnosis of aspergillosis depends upon the isolation of the etiologic agent in culture. Moreover, because *Aspergillus* species are commonly found in the air, their isolation must be interpreted with caution (Nalesnik et al. 1980).

Their isolation from sputum is more convincing if multiple colonies are obtained on a plate, or the same fungus is recovered on more than one occasion. A positive culture is indicative of infection, but may also merely represent colonization (Delvenne et al. 1993). However, the isolation of *A. fumigatus* from the respiratory tract specimens in heart transplant recipients is highly predictive of IA (Munoz et al. 2003). Colony morphology and microscopic features of medically important *Aspergillus* species are well-described in Table 9.11.

9.5.4.1 Media Used for Identification of *Aspergillus* Species

Czapek Agar

Subculture of an isolate to Czapek agar with incubation at 25–30°C allows identification of *Aspergillus* species (Table 9.5).

Potato Dextrose Agar

Potato dextrose agar is particularly useful for inducing sporulation so that the identification of the isolate becomes easier (Table 9.5).

9.5.4.2 Skin Test for Identification of *Aspergillus* Species

Skin tests with *A. fumigatus* antigen are useful in the diagnosis of allergic aspergillosis. Patients with uncomplicated extrinsic asthma caused by *Aspergillus* species give an immediate type I reaction. Those with allergic bronchopulmonary aspergillosis (ABPA) give an immediate type I reaction, and may also give a delayed type IV reaction.

9.5.4.3 Serodiagnosis of *Aspergillus* Species

Many potential systems for the immunodiagnosis of aspergillosis have been described (Barnes 1993; Kappe and Seeliger 1993). Those based on detection of antibody to the organism have been very successful in allergic aspergillosis and aspergilloma, and those used for the detection of fungal antigen have great potential for the diagnosis of IA (Manso et al. 1994).

Tests for Antibodies

Detection of specific precipitating antibodies to *Aspergillus* species by double diffusion, CIE or ELISA has provided the basis for the most frequently used

Table 9.11 Characteristic features of medically important *Aspergillus* species

Aspergillus species	*Aspergillus fumigatus*	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus terreus*
Pathogenicity	Most pathogenic species, causing aspergillosis commonly in the lungs	Usually considered a contaminant, but also known to cause disease in debilitated patients (opportunistic invaders)	Usually considered a contaminant, but also known to cause disease in immunosuppressed patients (opportunistic invaders)	Commonly considered a contaminant, but known to cause infection
Gross morphology	Colonies are velvety or powdery at first, later turning to smoky green	Colonies are velvety, yellow to green or brown	Colonies are wooly at first, then turning dark brown to black (characteristic peppered effect)	Colonies are granular, radially rugose and cinnamon buff, brown or orange brown
	Reverse is white to tan	Reverse is golden to reddish brown	Reverse is buff or yellow-gray	Reverse is white to brown
Microscopic morphology	Septate hyphae with smooth conidiophores of variable length	Septate hyphae with conidiophores which are relatively long, rough, pitted and spiny	Septate hyphae with smooth conidiophores of variable length	Septate hyphae with smooth, short (<250 μm) conidiophores
	Phialides single, usually cover upper half vesicle, parallel to the axis of stalk	Phialides are single and/or double, cover the entire vesicle, and point out in all directions (daisy-petal-like arrangement)	Phialides are double (biseriate) covering the entire vesicle, "form radiate head"	Phialides are double (biseriate) compactly columnar and cover the top half of club-shaped small vesicle

serological tests for the diagnosis of aspergillosis (Hopwood and Evans 1991; Barnes 1993). The presence of one or more weak precipitin bands is one of the diagnostic criteria accepted for the diagnosis of ABPA. It has been reported that all serum specimens from patients with IA contain antibodies to a 58 kDa conconavalin A-binding antigen identified by SDS-PAGE and immunoblotting. However, the specificity of this antigen has not been well-documented (Fratamico and Buckley 1991).

Tests for Antigens

Methods have been sought for diagnosing IA infection in immunocompromised hosts, which would rely on the measurement of a cell component (antigen) of fungal origin and thereby be independent of the host's ability to respond (Barnes 1993). Aspergillus galactomannan circulates during infection and appears in the urine, presumably after clearance by receptor-mediated process by Kupffer's cell in the liver. In addition to galactomannan, at least seven other aspergillus-related antigens have been detected by immunoblotting, in the urine of patients with invasive disease (Ansborg et al. 1994). The Platelia *Aspergillus* test system is a Sandwich ELISA kit that is currently available in many reference laboratories, providing for means for detection of *Aspergillus* circulating galactomannan early in the course of progressive disease. Complement fixation test using aspergillin as antigen is also used as a serological test, and titer $\geq$1:32 is suggestive of infection. The galactomannan EIA (GM-EIA) assay offers promise of being an important test for IA (Wheat 2003).

The value of granulocyte–macrophage (GM) detection by the Pastorex *Aspergillus* LA test (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) has been evaluated by a number of groups (Hopwood et al. 1995; Verweij et al. 1995a, b, c), and the test showed sensitivities up to 95% with serum samples from patients with a high index of suspicion for IA. ELISA is also used for detection of GM.

The detection of circulating β-1,3-D-glucan is another investigative strategy for diagnosis of IA. The plasma concentration of β-1,3-D-glucan has been measured at the time of routine cultures in febrile episodes (Obayashi et al. 1995). More recent studies have confirmed the usefulness of this approach (Hossain et al. 1997).

9.6 Laboratory Safety Considerations

Biosafety in the clinical laboratory is of the utmost importance to the laboratory worker and to those who work in adjacent areas. The fungi produce conidia that are easily aerosolized and may contaminate the laboratory, air-handling specimens and in some instance other cultures and specimens. Comprehensive safety guidelines should be available to laboratory workers to minimize the risk of acquiring infection in this setting.

The following are the guiding principles which should be remembered and practiced by laboratory personnel:

(a) All clinical specimens should be considered as infectious, and universal precautions must be used to protect laboratory personnel.
(b) All mold cultures and clinical specimens must be handled in a class II biological safety cabinet (BSC), with no exceptions, to prevent aerosolization of their conidia within the laboratory.
(c) An electric incinerator or a gas flame should be used to decontaminate the loop used for transfer of yeast cultures. Flaming should be done on the entire wire or loop and not only the tip.
(d) Cultures of organisms suspected of being pathogens should be sealed with tape to prevent laboratory contamination, and should be autoclaved as soon as the definitive identification is made.

References

Ahmad S, Khan Z, Mustafa AS, Khan ZU (2002) Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection and biochemical methods for species identification. J Clin Microbiol 40:2483–2489

Alexander BD (2002) Diagnosis of fungal infection: new technologies for the mycology laboratory. Transpl Infect Dis 4(Suppl 3):32–37

Aly R (1994) Culture media for growing dermatophytes. J Am Acad Dermatol 31:S107–S108

Ansborg R, vov Heinegg EH, Rath PM (1994) Aspergillus antigenuria compared to antigenemia in bone marrow transplant recipients. Eur J Clin Microbiol Infect Dis 13:582–589

Barnes AJ (1993) Aspergillus infection: does serodiagnosis work? Serodiagn Immunother Infect Dis 5:135–138

Bialek R, Feucht A, Aepinus C, Just-Nubling G, Robertson VJ, Knobloch J, Hohle R (2002) Evaluation of two nested PCR assays for detection of Histoplasma capsulatum DNA in human tissue. J Clin Microbiol 40:1644–1647

Bialek R, Cirera AC, Herrmann T, Aepinus C, Shearn-Bochsler VI, Legendre AM (2003) Nested PCR assays for detection of Blastomyces dermatitidis DNA in paraffin-embedded canine tissue. J Clin Microbiol 41:205–208

Bille J, Stockman L, Roberts GD, Horstmeier CD, Ilstrup DM (1983) Evaluation of a lysis-centrifugation system for recovery of yeasts and filamentous fungi from blood. J Clin Microbiol 18:469–471

Bille J, Edson RS, Roberts GD (1984) Clinical evaluation of the lysis-centrifugation blood culture system for the detection of fungemia and comparison with a conventional biphasic broth blood culture system. J Clin Microbiol 19:126

Buchheidt D, Spiess B, Hehlmann R (2001) Systemic infections with Aspergillus species in patients with hematological malignancies: current serologic and molecular diagnostic approaches. Onkologie 24:531–536

Buchheidt D, Baust C, Skladny H, Baldus M, Brauninger S, Hehlmann R (2002) Clinical evaluation of a polymerase chain reaction assay to detect Aspergillus. Br J Haematol 116:803–811

Burgener-Kairuz P, Zuber JP, Jaunin P, Buchman TG, Bille J, Rossier M (1994) Rapid detection and identification of Candida albicans and Torulopsis (Candida) glabrata in clinical specimens by species–species nested PCR amplification of a cytochrome P-450 Lanosterol-alpha-demethylase (L1A1) gene fragment. J Clin Microbiol 32:1902–1907

Campbell CC, Hill GB (1964) Further studies on the development of complement-fixing antibodies and precipitins in healthy histoplasmin-sensitive persons following a single histoplasmin skin test. Am Rev Respir Dis 90:927–934

Cao L, Chen DL, Lee C, Chan CM, Chan KM, Vanittanakom N, Tsang DN, Yuen KY (1998) Detection of specific antibodies to an antigenic mannoprotein for diagnosis of Penicillium marneffei penicilliosis patients. J Clin Microbiol 36:3028–3031

Cao L, Chan KM, Chen D, Vanittanakom N, Lee C, Cham CM, Sirisanthana T, Tsang DN, Yuen KY (1999) Detection of cell wall mannoprotein Mp1p in culture supernatants of Penicillium marneffei and in sera of penicilliosis patients. J Clin Microbiol 37:981–986

Chandler FW, Kaplan W, Ajello L (1989) A colour atlas and textbook of the histopathology of mycotic diseases. Wolfe Medical, London

Chang HC, Leaw SN, Huang AH, Wu TL, Chang TC (2001) Rapid identification of yeast in positive blood cultures by a Multiplex PCR method. J Clin Microbiol 39:3466–3471

Chen SCA, Halliday CL, Meyer W (2002) A review of nucleic acid-based diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays. Med Mycol 40:333–357

Chryssanthou E, Klingspor L, Tollemar J, Petrini B, Larsson L, Christensson B, Ringden O (1999) PCR and other non-culture methods for diagnosis of invasive candida infections in allogenic bone marrow and solid organ transplant recipients. Mycoses 42:239–247

Clayton YM, Midgley G (1989) Identification of agents of superficial mycoses. In: Evans EGV, Richardson MD (eds) Medical Mycology-a practical approach. IRL at Oxford University Press, Oxford, pp 65–95

Conner DH, Chandler FW, Schwartz DA, Manz HJ, Lack EE (1997) Pathology of infectious diseases. Appleton and Lange, Stamford, CT

De Bernardis F, Girmenia C, Boccanera M, Adriani D, Martino P, Cassone A (1993) Use of a monoclonal antibody in a dot immunobinding assay for detection of a circulating mannoprotein of Candida spp., in neutropenic patients with invasive candidiasis. J Clin Microbiol 31:3142–3146

de Hoog GS, Guarro J, Gene J, Figueras MJ (eds) (2000) Ascomycetous yeasts genus: Candida. In: Atlas of Clinical Fungi, 2nd edn. Centtraalbureau voor Schimmelcultures, Delft, The Netherlands, pp 180–226

Delvenne P, Arrese JE, Thiry A, Borlee-Hermans G, Pierard GE, Boniver J (1993) Detection of cytomegalovirus, Pneumocystis carinii and Aspergillus species in bronchoalveolar fluid — a comparison of techniques. Am J Clin Pathol 100:414–418

Di Salvo AF, Terreni AA, Wooten AK (1981) Use of the exoantigen test to identify Blastomyces dermitiidis, Coccidioides immitis and Histoplasma capsulatum in mixed cultures. Am J Clin Pathol 75:825–826

Durkin MM, Connolly PA, Wheat LJ (1997) Comparison of radioimmunoassay and enzyme-linked immunoassay methods for detection of Histoplasma capsulatum antigen. J Clin Microbiol 35:2252–2255

Edwards LB, Acquaviva FA, Livesay VT, Cross FW, Palmer CE (1969) An atlas of sensitivity to tuberculin, PPD-B and histoplasmin in the United States. Am Rev Respir Dis 99:1–132

Ferns RB, Fletcher H, Bradley S, Mackinnon S, Hunt C, Tedder RS (2002) The prospective evaluation of a nested polymerase chain reaction assay for the early diagnosis of Aspergillus infection in patients with leukemia or undergoing allograft treatment. Br J Haematol 119:720–725

Ferreira RP, Yu B, Niki Y, Armstrong D (1990) Detection of Candida antigenuria in disseminated candidiasis by immunoblotting. J Clin Microbiol 28:1075–1078

Fischer JB, Kane J (1974) The laboratory diagnosis of dermatophytosis complicated by Candida albicans. Can J Microbiol 20:167–182

Flahaut M, Sanglard D, Monod M, Bille J, Rossier M (1998) Rapid detection of Candida albicans in clinical samples by DNA amplification of common regions from C. albicans-secreted aspartic proteinase genes. J Clin Microbiol 36:395–401

Fratamico PM, Buckley HR (1991) Identification and characterization of an immunodominant 58-kilodalton antigen of Aspergillus fumigatus recognized by sera of patients with invasive aspergillosis. Infect Immun 59:309–315

Fujita S, Hashimoto T (1992) Detection of serum Candida antigens by enzyme-linked immunosorbent assay and a latex agglutination test with anti-Candida albicans and anti-Candida krusei antibodies. J Clin Microbiol 30:3132–3137

Fuller DD, Davis TE Jr, Denys GA, York MK (2001) Evaluation of BACTEC MYCO/F Lytic medium for recovery of mycobacteria, fungi, and bacteria from blood. J Clin Microbiol 39:2933–2936

Gade W, Ledman DW, Wethington R, Yi A (1992) Serologic responses to various Coccidioides antigen preparations in a new enzyme immunoassay. J Clin Microbiol 30:1907–1912

Geha DJ, Roberts GD (1994) Laboratory detection of fungemia. Clin Lab Med 14:83–97

Gomes GM, Cisalpino PS, Taborda CP, de Camargo ZP (2000) PCR for diagnosis of paracoccidioidomycosis. J Clin Microbiol 38:3478–3480

Graser Y, de Hoog GS, Kuijpers AFA (2000a) Recent advances in the molecular taxonomy of dermatophytes. In: Kushwaha RKS, Guarro J (eds) Biology of dermatophytes and other keratinophilic fungi. Revista Iberoamericana de Micologia, Bilbao, Spain, pp 17–21

Graser Y, Kuijpers AFA, Presber W, de Hoog GS (2000b) Molecular taxonomy of Trichophyton rubrum complex. J Clin Microbiol 38:3329–3336

Green F, Balish E (1980) Trichophyton mentagrophytes dermatophytosis in germfree guinea pigs. J Invest Dermatol 75:476–480

Grossman J, Baum J, Gluckman J, Fusner J, Condemi JJ (1975) The effect of ageing and acute illness on delayed hypersensitivity. J Allergy Clin Immunol 55:268–275

Hageage G, Harrington B (1984) Use of calcofluor white in clinical mycology. Lab Med 15:109

Hariri ALR, Hemple HO, Kimberlin CL, Goodman NL (1982) Effects of time elapse between sputum collection and culturing on isolation of clinically significant fungi. J Clin Microbiol 15:425–428

Haynes KA, Westerneng TJ, Fell JW, Moens W (1995) Rapid detection and identification of pathogenic fungi by polymerase chain reaction amplification of large subunit ribosomal DNA. J Med Vet Mycol 33:319–325

Hazen KC, Howell SA (2003) Candida, Cryptococcus, and other yeasts of medical importance. In: Murray PR, Baron EJ et al (eds) Manual of Clinical Microbiology, 8th edn. ASM, Washington DC, pp 1693–1711

Hopwood V, Evans EGV (1991) Serological tests in the diagnosis and prognosis of fungal infection in the compromised patient. In: Warnock DW, Richardson MD (eds) Fungal infection in the compromised patient, 2nd edn. Wiley, Chichester, pp 311–353

Hopwood V, Johnson EM, Cornish JM, Foot AB, Evans EG, Warnock DW (1995) Use of Pastorex Aspergillus antigen latex agglutination test for the diagnosis of invasive aspergillosis. J Clin Pathol 48:210–213

Hossain MA, Miyazaki T, Mitsutake K, Kakeya H, Yamamoto Y, Yanagihara K, Kawamura S, Otsubo T, Hirakata Y, Tashiro T, Kohno S (1997) Comparison between Wako-WB003 and Fungitech G tests for detection of (1, 3)-beta-D-glucan in systemic mycoses. J Clin Lab Anal 11:73–77

Johnson SM, Pappagianis D (1992) The coccidioidal complement fixation and immunodiffusion-complement fixation antigen is a chitinase. Infect Immun 60:2588–2592

Jones DB, Liesegang TJ, Robinson NM (1981) Cumitech 13. American Society for Microbiology, Washington, DC

Jordan JA (1994) PCR identification of four medically important Candida species by using a single primer pair. J Clin Microbiol 32:2962–2967

Kaminski GW (1985) The routine use of modified Borelli's lactritmel (MBLA). Mycopathologia 91:57–59

Kane J, Fischer JB (1971) The differentiation of Trichophyton rubrum and T. mentagrophytes by use of Christensen's urea broth. Can J Microbiol 17:911–913

Kane J, Fischer JB (1975) The influence of sodium chloride on the growth and morphology of dermatophytes and some other keratinophilic fungi. Can J Microbiol 21:742–749
Kane J, Smitka CM (1978) Early detection and identification of Trichophyton verrucosum. J Clin Microbiol 8:740–747
Kane J, Salkin IF, Weitzman I, Smitka C (1981) Trichophyton raubitschekii sp. nov. Mycotaxon 13:259–266
Kaplan W, Kaufman L (1963) Specific fluorescent antiglobulins for the detection and identification of Blastomyces dermititidis yeast-phase cells. Mycopathologia 19:173–180
Kappe R, Seeliger HPR (1993) Serodiagnosis of deep-seated fungal infection. Curr Top Med Mycol 5:247–280
Kappe R, Okeke CN, Fauser C, Maiwald M, Sonntag HG (1998) Molecular probes for the detection of pathogenic fungi in the presence of human tissue. J Med Microbiol 47:811–820
Kaufman L, Standard P (1978) Improved version of the exoantigen test for identification of Histoplasma capsulatum. J Clin Microbiol 8:42–45
Kaufman L, Kovacs JA, Reiss E (1997) Clinical Immunomycology. American Society for Microbiology, Washington, DC
Kaufman L, Standard PG, Weeks RJ, Padhye AA (1983) Detection of two Blastomyces dermititidis serotypes by exoantigen analysis. J Clin Microbiol 18:110–114
Kaufman L, Standard PG, Anderson SA, Jalbert M, Swisher BL (1995) Development of specific fluorescent-antibody test for tissue form of Penicillium marneffei. J Clin Microbiol 33:2136–2138
Kaufman L, Standard PG, Jalbert M, Kantipong P, Limpakarnjanarat K, Mastro TD (1996) Diagnostic antigenemia tests for penicilliosis marneffei. J Clin Microbiol 34:2503–2505
Kawazu M, Kanda Y, Goyama S, Takeshita M, Nannya Y, Niino M, Komeno Y, Nakamoto T, Kurokawa M, Tsujino S, Ogawa S, Aoki K, Chiba S, Motokura T, Ohishi N, Hirai H (2003) Rapid diagnosis of invasive pulmonary aspergillosis by quantitative polymerase chain reaction using bronchial lavage fluid. Am J Hematol 72:27–30
Kiehn TE, Bernard EM (1979) Candidiasis: detection by gas liquid chromatography of D-arabinitol, a fungal metabolite, in human serum. Science 206:577–580
Kitch TT, Jacobs MR, McGinnis MR, Appelbaum PC (1996) Ability of Rapid Yeast Plus system to identify 304 clinically significant yeasts within 5 hours. J Clin Microbiol 34:1069–1071
Klein BS, Jones JM (1990) Isolation, purification, and radiolabeling of a novel 120-kD surface protein on Blastomyces dermititidis yeasts to detect antibody in infected patients. J Clin Invest 85:152–161
Kruse D, Cole GT (1992) A seroactive 120-kilodalton β-1, 3-glucanase of Coccidioides immitis which may precipitate in spherule-morphogenesis. Infect Immun 60:4350–4363
Kwon-Chung J, Bennett J (1992) Medical Mycology. Lea and Febiger, Philadelphia, PA
Lacaz CS, Porto E, Martins JEC, Heins-Vaccari EM, Melo NT (2002) Paracoccidioidomicose. In: Lacaz CS, Porto E et al (eds) Micologia medica, 9th edn. Sarvier, Sao Paulo, Brasil, pp 629–729
Land GA, Salkin IF, el-Zaatari M, McGinnis MR, Hashem G (1991) Evaluation of Baxter-Microscan 4-hour enzyme-based yeast identification system. J Clin Microbiol 29:718–722
Larone DH (1995) Medically Important Fungi, 3rd edn. American Society of Microbiology, Washington, DC
Lass-Florl C, Rath PM, Niederwieser D, Kofler G, Wurzner R, Krezy A, Dierich MP (2000) Aspergillus terreus infections in hematological malignancies: molecular epidemiology suggest association with in-hospital patients. J Hosp Infect 46:31–35
Lindsley MD, Hurst SF, Iqbal NJ, Morrison CJ (2001) Rapid identification of dimorphic and yeast-like fungal pathogens using specific DNA probes. J Clin Microbiol 39:3505–3511
Liu D, Coloe S, Baird R, Pedersen J (2000) Application of PCR to the identification of dermatophyte fungi. J Med Microbiol 49:493–497
LoBuglio KF, Taylor JW (1995) Phylogeny and PCR identification of the human pathogenic fungus Penicillium marneffei. J Clin Microbiol 33:85–89

Loeffler J, Hagmeyer L, Hebart H, Henke N, Schumacher U, Einsele H (2000a) Rapid detection of point mutations by fluorescence resonance energy transfer and probe melting curves in Candida species. Clin Chem 46:631–635

Loeffler J, Henke N, Hebart H, Schmidt D, Hagmeyer L, Schumacher U, Einsele H (2000b) Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. J Clin Microbiol 38:586–590

Maiwald M, Kappe R, Sonntag HG (1994) Rapid presumptive identification of medically relevant yeasts to the species level b polymerase chain reaction and restriction enzyme analysis. J Med Vet Mycol 32:115–122

Manso E, Montillo M, De Sio G, D'Amico S, Discepoli G, Leoni P (1994) Value of antigen and antibody detection in the serological diagnosis of invasive aspergillosis in patients with hematological malignancies. Eur J Clin Microbiol Infect Dis 13:756–760

Martagon-Villamil J, Shreshtha N, Sholtis M, Isada CM, Hall GS, Bryne T, Lodge BA, Reller LB, Procop GW (2003) Identification of Histoplasma capsulatum from culture extracts by real-time PCR. J Clin Microbiol 41:1295–1298

Martins R, Marques S, Alves M, Fecchio D, deFranco MF (1997) Serological follow-up of patients with paracoccidioidomycosis treated with itraconazole using Dot-blot, ELISA and western-blot. Rev Inst Med Trop Sao Paulo 39:261–269

McGinnis MR (1980) Laboratory Handbook of Medical Mycology. Academic, New York

Mendes-Giannini MJ, Camargo ME, Lacaz CS, Ferreira AW (1984) Immunoenzymatic absorption test for serodiagnosis of paracoccidioidomycosis. J Clin Microbiol 20:103–108

Mendes-Giannini MJS, Del Negro GB, Siqueira AM (1994) Serodiagnosis. In: Franco M, Lacaz CS et al (eds) Paracoccidioidomycosis. CRC, Boca Raton, FL, pp 345–363

Merz W, Roberts G (1995) Detection and recovery of fungi from clinical specimens. In: Murray P et al (eds) Manual of Clinical Microbiology. ASM, Washington, DC

Merz WG, Roberts GD (2003a) Algorithms for detection and identification of fungi. In: Murray PR, Baron EJ et al (eds) Manual of Clinical Microbiology, 8th edn. ASM, Washington, DC, pp 668–685

Merz WG, Roberts GD (2003b) Detection and recovery of fungi from clinical specimens. American Society for Microbiology, Washington, DC

Merz WG, Berger CL, Silva-Hutner M (1970) Media with pH indicators for the isolation of dermatophytes. Arch Dermatol 102:545–547

Milne LJR, Barnetson RStC (1974) Diagnosis of dermatophytoses using vinyl adhesive tape. Sabouraudia 12:162–165

Mitsutake K, Kohno S, Miyazaki T, Miyazaki H, Maesaki S, Koga H (1994) Detection of Candida enolase antibody in patients with candidiasis. J Clin Lab Anal 8:207–210

Miyazaki T, Kohno S, Mitsutake K, Maesaki S, Tanaka K, Ishikawa N, Hara K (1995) Plasma (1→3)-beta-D-glucan and fungal antigenemia in patients with candidemia, aspergillosis, and cptococcosis. J Clin Microbiol 33:3115–3118

Moriello KA, Deboer DJ (1991) Fungal flora of haircoat of cats with and without dermatophytosis. J Med Vet Mycol 29:285–292

Munoz P, Alcala L, Sanchez Conde M, Palomo J, Yanez J, Pelaez T, Bouza E (2003) The isolation of Aspergillus fumigatus from respiratory tract specimens in heart transplant recipients is highly predictive of invasive aspergillosis. Transplantation 75:326–329

Nalesnik MA, Myerowitz RL, Jenkins R, Lenkey J, Herbert D (1980) Significance of Aspergillus species isolated from respiratory secretions in the diagnosis of invasive pulmonary aspergillosis. J Clin Microbiol 11:370–376

Niesters HG, Goessens WH, Meis JF, Quint WG (1993) Rapid, polymerase chain reaction based identification assays for Candida species. J Clin Microbiol 31:904–910

Obayashi T, Yoshida M, Mori T, Goto H, Yasuoka A, Iwasaki H, Teshima H, Kohno S, Horiuchi A, Ito A, Yamaguchi H, Shimada K, Kawai T (1995) Plasma (1-3)-β-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet 345:17–20

Odds FC, Bernaerts RIA (1994) CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important Candida species. J Clin Microbiol 32:1923–1929

Poulain D, Slomianny C, Jouault T, Gomez JM, Trinel PA (2002) Contribution of phopholipomannan to the surface expression of beta-1, 2-oligimannosides in Candida albicans and its presence in cell wall extracts. Infect Immun 70:4323–4328

Rebell G, Taplin D (1974) Dermatophytes, their recognition identification. University of Miami Press, Coral Gables, FL

Reboli AC (1993) Diagnosis of invasive candidiasis by a dot immunobinding assay for Candida antigen detection. J Clin Microbiol 31:518–523

Reddy LV, Kumar A, Kurup VP (1993) Specific amplification of Aspergillus fumigatus DNA by polymerase chain reaction. Mol Cell Probes 7:121–126

Reiss E, Morrison CJ (1993) Nonculture methods for diagnosis of disseminated candidiasis. Clin Microbiol Rev 6:311–323

Reiss E, Kaufman L, Kovacs JA, Lindsley MD (2002) Clinical immunomycology. In: Rose NR, Hamilton RG, Detrick B (eds) Manual of Clinical Laboratory Immunology. ASM, Washington, DC, pp 559–583

Resnick S, Pappagianis D, McKerrow JH (1987) Proteinase production by the parasitic cycle of the pathogenic fungus Coccidioides immitis. Infect Immun 55:2807–2815

Rickerts V, Bialek R, Tintelnot K, Jacobi V, Just-Nubling G (2002) Rapid PCR-based diagnosis of disseminated histoplasmosis in an AIDS patient. Eur J Clin Microbiol Infect Dis 21:821–823

Rippon JW (1988) Medical Mycology, the Pathogenic fungi and Pathogenic Actionmycetes, 3rd edn., WB Saunders, Philadelphia

Roberts GD (1975) Detection of fungi in clinical specimens by phase-contrast microscopy. J Clin Microbiol 2:261

Salkin IF (1973) Dermatophyte test medium: evaluation with non-dermatophyte pathogens. Appl Microbiol 26:134–137

Sandhu GS, Kline BC, Stockman L, Roberts GD (1995) Molecular probes for diagnosis of fungal infections. J Clin Microbiol 33:2913–2919

Sarosi GA, Armstrong D, Davies SF, George RB, Graybill JR, Pennington JE et al (1985) Laboratory diagnosis of mycotic and specific fungal infections. Am Rev Respir Dis 132:1373–1379

Sekhon AS, Li JSK, Gag AK (1982) Penicillosis marneffei: serological and exoantigen studies. Mycopathologia 77:51–57

Sendid B, Poirot JL, Tabouret M, Bonnin A, Caillot D, Camus D, Poulain D (2002) Combined detection of mannanaemia and anti mannan antibodies as a strategy for the diagnosis of systemic infection caused by pathogenic Candida species. J Med Microbiol 51:433–442

Smith CE, Saito MT, Simons SA (1956) Pattern of 39, 500 serological tests in coccidioidomycosis. J Am Med Assoc 160:546–552

St Germain G, Beauchesne D (1991) Evaluation of the MicroScan Rapid Yeast Identification panel. J Clin Microbiol 29:2296–2299

St-Germain G, Summerbell RC (1996) Identifying filamentous fungi: a clinical laboratory handbook. Star Publishing Co., Belmont CA

Stockman L, Clark KA, Hunt JM, Roberts JD (1993) Evaluation of commercially available acridinium ester-labeled chemiluminescent DNA probes for culture identification of Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans and Histoplasma capsulatum. J Clin Microbiol 31:845–850

Suarez SM, Silvers DN, Scher RK, Pearlstein HH, Auerbach R (1991) Histologic evaluation of nail clippings for diagnosing onychomycosis. Arch Dermatol 127:1517–1519

Supparatpinyo K, Sirisanthana T (1994) Disseminated Penicillium marneffei infection diagnosed on examination of a peripheral blood smear of a patient with human immunodeficiency virus infection. Clin Infect Dis 18:246–247

Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE, Sirisanthana T (1994) Disseminated Penicillium marneffei infection in Southeast Asia. Lancet 344:110–113

Switchenko AC, Miyada CG, Goodman TC, Walsh TJ, Wong B, Becker MJ, Ullman EF (1994) An automated enzymatic method for measurement of D-arabinitol, a metabolite of pathogenic Candida species. J Clin Microbiol 32:92–97

Taborda CP, Camargo ZP (1993) Diagnosis of paracoccidioidomycosis by passive haemagglutination assay of antibody using a purified and specific antigen-gp43. J Med Vet Mycol 31:155–160

Taborda CP, Camargo ZP (1994) Diagnosis of paracoccidioidomycosis by dot immunobinding assay for antibody detection using the purified and specific antigen-gp43. J Clin Microbiol 32:554–556

Telenti A, Roberts GD (1989) Laboratory detection of fungemia. J Clin Microbiol 8:825–831

Turenne CY, Sanche SE, Hoban DJ, Karlowsky JA, Kabani AM (1999) Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. J Clin Microbiol 37:1846–1851

Turin L, Riva F, Galbiati G, Cainelli T (2000) Fast, simple and highly sensitive double-rounded polymerase chain reaction assay to detect medically relevant fungi in dermatological specimens. Eur J Clin Invest 30:511–518

Vanittanakom N, Vanittanakom P, Hay RJ (2002) Rapid identification of Penicillium marneffei by PCR-based detection of specific sequence on the rRNA gene. J Clin Microbiol 40:1739–1742

Verweij PE, Latge JP, Rijs AJ, Melchers WJ, BEDe P, Hoogkamp-Korstanje JA, Meis JF (1995a) Comparison of antigen detection and PCR assay using bronchoalveolar lavage fluid for diagnosing invasive pulmonary aspergillosis in patients receiving treatment for haematological malignancies. J Clin Microbiol 33:3150–3153

Verweij PE, Poulain D, Obayashi T, Patterson TF, Denning DW, Ponton J (1998) Current trends in detection of antigenemia, metabolites and cell wall markers for the diagnosis and therapeutic monitoring of fungal infections. Med Mycol 36(Suppl 1):146–155

Verweij PE, Rijs AJ, BEDe P, Horrevorts AM, Hoogkamp-Korstange JA, Meis JF (1995b) Clinical evaluation and reproducibility of the Pastorex Aspergillus antigen latex agglutination test for diagnosing invasive aspergillosis. J Clin Pathol 48:474–476

Verweij PE, Stynen D, Rijs AJ, BEDe P, Hoogkamp-Korstanje JA, Meis JF (1995c) Sandwich enzyme-linked immunosorbent assay compared with Pastorex latex agglutination test for diagnosing invasive aspergillosis in immunocompromised patients. J Clin Microbiol 33:1912–1914

Vetter E, Torgerson C, Feuker A, Hughes J, Harmsen S, Schleck C, Horstmeier C, Roberts G, Cockerill F III (2001) Comparison of the BACTEC MYCO/F Lytic bottle to the isolator tube. BACTEC Plus Aerobic F/bottle, and BACTEC Anaerobic Lytic/10 bottle and comparison of the BACTEC Plus Aerobic F/bottle to the Isolator tube for recovery of bacteria, mycobacteria, and fungi from blood. J Clin Microbiol 39:4380–4386

Viviani MA, Tortorano AM, Rizzardini G, Quirino T, Kaufman L, Padhye AA, Ajello L (1993) Treatment and serological studies of an Italian case of penicilliosis marneffei contracted in Thailand by a drug addict infected with the human immunodeficiency virus. Eur J Epidemiol 9:79–85

Wahyuningsih R, Freisleben HJ, Sonntag HG, Schnitzler P (2000) Simple and rapid detection of Candida albicans DNA in serum by PCR for diagnosis of invasive candidiasis. J Clin Microbiol 38:3016–3021

Waite TR, Woods GL (1998) Evaluation of Myco Flytic medium for recovery of mycobacteria and fungi from blood. J Clin Microbiol 36:1176–1179

Walsh TJ, Chanock SJ (1997) Laboratory diagnosis of invasive candidiasis: a rationale for complementary use of culture and nonculture-based detection systems. Int J Infect Dis 1 (Suppl 1):S11–S19

Walsh TJ, Francesconi A, Kasai M, Chanock SJ (1995) PCR and single-stranded conformational polymorphism for recognition of medically important fungi. J Clin Microbiol 33:3216–3220

Weissman Z, Berdicevsky I, Cavari B (1995) Molecular identification of Candida albicans. J Med Vet Mycol 33:205–207

Weitzman I, Kane J, Summerbell RC (1995) Trichophyton, microsporum, epidermophyton & agents of superficial mycoses. In: Murray PR, Baron EJ et al (eds) Manual of Clinical Microbiology, 6th edn. American Society for Microbiology, Washington, DC, pp 791–808

Wheat LJ (2003) Rapid diagnosis of invasive aspergillosis by antigen detection. Transpl Infect Dis 5:158–166

Wheat LJ, Kaufman CA (2003) Histoplasmosis. Infect Dis Clin North Am 17:1–19, vii

Wheat LJ, Connolly-Stringfield P, Williams B, Connolly K, Blair R, Bartlett M, Durkin M (1992) Diagnosis of histoplasmosis in patients with the acquired immunodeficiency syndrome by detection of Histoplasma capsulatum polysaccharide antigen in bronchoalveolar lavage fluid. Am Rev Respir Dis 145:1421–1424

Wheat J, French ML, Kohler RB, Zimmerman SE, Smith WR, Norton JA, Eitzen HE, Smith CD, Slama TG (1982) The diagnostic laboratory tests for histoplasmosis: analysis of experience in a large urban outbreak. Ann Intern Med 97:680–685

Wheat J, French ML, Kamel S, Tewari RP (1986a) Evaluation of cross-reactions in Histoplasma capsulatum serologic tests. J Clin Microbiol 23:493–499

Wheat LJ, Kohler RB, Tewari RP (1986b) Diagnosis of disseminated histoplasmosis by detection of Histoplasma capsulatum antigen in serum and urine specimens. N Engl J Med 314:83–88

Wickerham LJ, Burton KA (1948) Carbon assimilation tests for the classification of yeasts. J Bacteriol 56:363–371

Williams B, Fojtasek M, Connolly-Stringfield P, Wheat J (1994) Diagnosis of histoplasmosis by antigen detection during an outbreak in Indianapolis, IN. Arch Pathol Lab Med 118:1205–1208

Yamakami Y, Hashimoto A, Tokimatsu I, Nasu M (1996) PCR detection of DNA specific for Aspergillus species in serum of patients with invasive aspergillosis. J Clin Microbiol 34:2464–2468

Yeo SF, Wong B (2002) Current status of nonculture methods for diagnosis of invasive fungal infections. Clin Microbiol Rev 15:465–484

Yuen KY, Wong SS, Tsang DN, Chau PY (1994) Serodiagnosis of Penicillium marneffei infection. Lancet 344:44–45

Zimmer BL, Pappagianis D (1989) Immunoaffinity isolation and partial characterization of the Coccidioides immitis antigen detected by tube precipitin and immunodiffusion-tube presipitin tests. J Clin Microbiol 27:1759–1766

Zimmer BL, Roberts GD (1979) Rapid selective urease test for presumptive identification of Cryptococcus neoformans. J Clin Microbiol 10:380–381

Chapter 10
Combinational Antifungal Therapy and Recent Trends in Drug Discovery

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Abstract Invasive fungal infections are a major problem in immunocompromised patients. This has necessitated an increased interest in the development of new antifungals to treat these life-threatening infections. However, our means of combating fungal infections are still lagging behind those for bacterial infections, due to toxicity and the lower clinical efficacy of available antifungals against some invasive fungal infection. Thus, more efforts are needed in antifungal drug discovery, as well as in developing effective ways of minimizing toxicity and improving delivery of available antifungal drugs. One approach is the effective use of newer antifungal agents in combination therapy against invasive aspergillosis, cryptococcosis and candidiasis. On the other hand, identifying and validating new antifungal drug targets is a prerequisite for new antifungal drug discovery. These new targets might be discovered by both conventional but improved assays and genomic approaches. Also, targeting virulence is expected to be a new paradigm for antifungals. In this chapter, an attempt is made to describe recent progress in combinational therapy and new approaches to antifungal drug discovery.

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10.1 Introduction

Fungal diseases in humans can be classified as (a) allergic reactions to fungal proteins, (b) toxic reactions to toxins present in certain fungi, and (c) infection (mycoses). Mycoses are of different types based on the site of infection and the nature of causative agents. In healthy persons, common forms of mycoses are superficial, cutaneous, or subcutaneous and in certain instances are systemic, causing a variety of conditions ranging from athlete's foot and nail infections to severe life-threatening disseminated diseases. On the other hand, immunocompromised individuals are susceptible to a large number of opportunistic fungal pathogens which cause systemic mycoses. Indeed, the last two decades have witnessed a remarkable increase in the incidence of deep-seated disseminated mycoses (Barrett 2002). The reason for this increase is mainly due to the advent of aggressive cancer chemotherapy, use of immunosuppressant for organ transplants, excessive and indiscriminate use of broad-spectrum antibacterial agents, and the rise in the number of cases of human immunodeficiency virus (HIV) infections. Candidiasis, aspergillosis, and cryptococcosis are the leading invasive fungal infections that cause substantial morbidity and mortality in nosocomial settings and among immunocompromised patients (Truan et al. 1994). *Candida* spp. are considered to be the fourth most common blood stream isolates in the United States (Magill et al. 2006). *Aspergillus* spp. are the leading cause of pneumonic mortality in patients with acute leukemia or bone marrow/hematopoietic stem cell transplants, and those who have surpassed cytomegalovirus infections in this setting. These infections are difficult to diagnose during lifetime, and, when diagnosed, often refractory to treatment with existing therapy (Walsh et al. 2000). Furthermore, ongoing demographic trends would tend to strongly suggest that the number of fungal infections will continue to increase due to the aging of the population in the developed countries (Kauffman 2001). For many years, amphotericin B and fluconazole have been the standard therapy for treatment of severe fungal infection (Dismukes 2000). Unfortunately, these established agents suffer from a number of limitations such as nephrotoxicity associated with amphotericin B, limited spectrum activity of fluconazole, and development of resistance among *Candida* spp. (Dismukes 2000; Cha and Sobel 2004).

Various antifungal drugs currently available for the treatment of systemic fungal infections are amphotericin B and lipid formulations of amphotericin B, 5-fluorocytosine, and the azoles (miconazole, ketoconazole, fluconazole, and itraconazole). Currently the optimal properties being sought in new antifungal drug candidates include inhibition of fungal cell wall biosynthesis, potency comparable with that of amphotericin B, safety comparable with fluconazole, and fungicidal activity both *in vitro* and *in vivo* (Andriole 1999). Interestingly, several new drugs have recently been approved by the US Food and Drug Administration, including voriconazole, caspofungin, and micafungin. Like two other triazoles (posaconazole, ravuconazole), caspofungin and micafungin represent the first new class (the echinocandin lipopeptides) of antifungal agents approved after several decades of

intensive research (Zaas and Steinbach 2005). In spite of these recent advances, the currently marketed antifungals represent only three major chemical groups, i.e., polyenes, azoles, and echinocandins, and target only two cellular structures, namely cell membrane and cell wall. However, to preclude wide-spectrum drug resistance and to provide safe and more efficacious treatment of fungal infections, a wider repertoire of antifungal drugs and new approaches for their identification and development are still required.

Considering the complex nature of human fungal infection and host–fungi interactions, multidimensional approaches are needed to develop ways and means for effective management/treatment of fungal infections. Both classical (with improved test methods), modern genomics, and proteomics-based approaches are to be explored to identify new drug targets and develop simple assays for screening natural products or transforming existing safe natural antifungals to enhance their efficacy, as well as exploring combinational drug therapy against drug resistant fungi. Recently, some progress has been made in using classical approaches to discovering antifungal drugs from natural products, including microbial sources (Marinelli 2009) which indicated that new antifungals could be developed if systemic and improved strategies are used.

In this article, we review the recent progress made in combinational antifungal therapy and current approaches such as genomic-based drug discovery. In order to develop new antifungal drugs new targets and innovative delivery systems for existing drugs are needed.

10.2 Combinational Antifungal Therapy

Recent advances in antifungal development have made available more pharmacologic compounds to choose from for the management of these fungal infections. The major challenges in management of invasive fungal infections should be kept in mind before adopting any strategy to combat such refractory fungal infection. These challenges, as described by Johnson and Perfect (2007), may include:

(a) Rapid and accurate diagnosis of infections
(b) Manipulating immune reconstitution, as too much or too little of a host immune response can be problematic
(c) Optimizing pharmacokinetics/pharmacodynamics of drugs; poor drug bioavailability, drug interaction, and toxicities can contribute to clinical failure
(d) Empiric/pre-emptive prevention strategies
(e) Surgical intervention and deciding between old and new antifungals

The role of combination antifungal therapy has been well-established for fungal infections such as cryptococcal meningitis. The availability of new antifungals and the increased incidence of mould infections such as hematopoiesis in stem cell transplant recipients have stimulated interest in the clinical use of combination antifungal therapy. In recent years, several authors have written excellent reviews

covering both *in vitro* and *in vivo* antifungal combinations and the progress made in the treatment of various fungal infections (Cuenca-Estrella 2004; Johnson and Perfect 2007; Baddley and Pappas 2007; Espinel-Ingroff 2009). Here, we provide a brief account of the concept and applications of combinational antifungal therapy in combating fungal infection.

10.2.1 Principle of Combinational Therapy

A possible approach to overcoming antifungal drug resistance and high mortality rates due to severe fungal infections is to combine two or three classes of antifungals, especially drugs having different mechanisms of action. The overall goal of combination antifungal therapy is to achieve increased clinical efficacy through drug synergy and to avoid or minimize toxicity to the patient. Combination of drugs may result in different outcomes defined as synergy, antagonism, additivity, and indifference. When the combinational activity of drugs is greater than that of an individual drug, it is defined as synergy. If it is lower, it is defined as antagonism. Additivity is observed when efficacy in combination is no higher than the sum of individual drugs, whereas indifference indicates no enhancement in efficacy at all. There are several advantages and points to be considered in combination therapy, as described by Baddley and Pappas (2005).

(a) Increased potency and extent of fungal killing
(b) Broader spectrum of activity targeting potentially resistant pathogens
(c) Counter-emergence of drug resistance
(d) Reduction of individual drug dosages to minimize their toxicities

In addition to the above advantages, there are several important strategies for combinational antifungal therapy, each of which depends on a complex interaction between antifungal agent, the host and the pathogens, such as the pharmacokinetics and pharmacodynamics of the drugs (Medoff 1983; Lewis and Kontoyiannis 2001). The disposition of the drug in the body in terms of absorption, distribution and elimination should be considered when a combinational approach is required. For example, use of an agent in combination that reduces the metabolic activity of a drug would result in increased concentration of the drug, leading to enhanced efficacy. Also, the use in combination of two antifungals with different target sites may result in the active concentration of one being affected by the activity of the other. However, it is generally assumed with combinational therapy that two drugs should be administered at the same time. It is important to note that peak tissue concentrations and optimal killing may vary between two antifungal drugs of different classes depending on the antimicrobial activity pattern. For example, amphotericin B and echinocandins exhibits their concentration-dependent killing, whereas azole and flucytosine have a time-dependent pattern of killing (Andes 2003).

Drug combinations may be based on the different modes of action of two antifungal drugs. Most of the antifungal drugs can be grouped into three major

classes: (1) inhibitors of cell wall synthesis (echinocandin, nikomycin) (2) inhibitors of cell membrane (azoles, polyenes), and (3) inhibitors of protein/DNA synthesis (flucytosine, sordarin). Using a combination of drugs that act at different sites may theoretically result in synergistic killing of the fungal pathogen, and several examples are based on this principle (Eliopoulos and Moellering 1991; Lewis and Kontoyiannis 2001; Barchiesi et al. 1998; Johnson et al. 2004). Combinations of new agents together with more traditional antifungals have been shown to possess some synergistic or at least additive activity against many pathogenic fungi *in vitro* and *in vivo*. But caution is still needed since some antifungal combinations have also demonstrated antagonistic activity. Well-controlled clinical trials are required to define the most efficacious antifungal regimen. Furthermore, these trials should evaluate the side-effect potential of combination therapy and the pharmaco economic impact (Vazquez 2007).

10.2.2 In Vitro Antifungal Drug Interactions

Various methods are described in the scientific literature to determine the synergistic interactions between two compounds *in vitro* and *in vivo* (Arroyo et al. 1997). The most commonly used In vitro methods include the checkerboard method (Eliopoulos and Moellering 1991), where a fractional inhibitory concentration index (FICI) is calculated to determine combination efficiencies. Other methods are time-kill assay, E-test, and the response surface model (Eliopoulos and Moellering 1991; Sugar 1995; Lewis and Kontoyiannis 2001). Each method has its own advantages and limitations. However, checkerboard and time-kill assays are more popularly used *in vitro* methods because of their ease of use.

The checkerboard method employs the testing of two antifungal agents in microtitre plates, several dilutions (twofold) above and below their MICs, against a given fungi being tested. The results are interpreted by the pattern obtained on an isobologram which displays fractional inhibitory concentration indices (FICI) on an arithmetic scale. FICI is calculated as the sum of FICs of individual drugs.

$$\mathrm{FICI} = (\mathrm{FIC_A} + \mathrm{FIC_B}) = (A/A_c) + (B/B_c),$$

where (A) is the minimum inhibitory concentration of drug A alone, (A_c) is the MIC of drug A in combination, (B) is the MIC of drug B alone, and (B_c) is the MIC of drug B in combination. The restriction with this method is that it can measure only one-dimensional interaction, resulting in variation in data analysis, and thus may not give actual FICs in certain cases (Meletiadis et al. 2003). It is generally agreed by many experts in this field that an FICI value slightly lower or above the theoretical cut-off value of 1.0 indicates no interaction (Odds 2003b), an FICI value $\leq$0.5 indicates synergism, an FICI value $>$4 indicates antagonism, and an FICI value $>$0.5 but $\leq$4 indicate no interactions.

A more complex alternative to the checkerboard method is response surface models (Te Dorsthorst et al. 2002; Meletiadis et al. 2003) that rely on data generated by the three-dimensional nature of antifungal interactions, incorporating interaction parameters and uncertainty of the estimates.

In vitro studies of combination antifungal therapy have been reported by various workers, mainly against candidiasis, cryptococcosis and aspergillosis (Keele et al. 2001; Johnson et al. 2004; Cuenca-Estrella et al. 2005; Baddley and Pappas 2007). The data obtained indicated variable results depending on the experimental conditions, and nature and types of the fungal isolates. The interactions may be synergistic, antagonistic, additive or no interaction. Some workers reported a reduction in the emergence of flucytosine resistance in *Candida* spp. when used in combination with amphotericine B (Polak 1978). The same combination has also been reported to be synergistic or indifferent against *Aspergillus* spp. and *Cryptococcus* spp. (Denning et al. 1992; Keele et al. 2001). Combinations of terbinafine and azoles have also produced synergistic interactions against *Candida* spp. (Barchiesi et al. 1998). But azoles in combination with amphotericin B have mostly shown indifference or antagonistic behavior against *Aspergillus* spp. (Denning et al. 1992), whereas caspofungin in combination with amphotericin B produced a synergistic-to-indifferent effect (Arikan et al. 2002). Dannaoui et al 2004 reported synergistic interaction of caspofungin with flucytosine and plus amphotericin B or voriconazole in triple combination against *Aspergillus* spp. In a study by Cuenca-Estrella et al. (2005), synergistic interaction between amphotericin B and itraconazole. and between voriconazole and caspofungin, was observed against itraconazole-resistant isolates of *Aspergillus fumigatus*, Voriconazole also exhibited synergistic interaction with micafungin against *Aspergillus* isolates but was indifferent for *Candida albicans* (Heyn et al. 2005). In a triple combination, voriconazole, caspofungin, and amphotericin B showed synergy against *Aspergillus* isolates (O'Shaughnessy et al. 2006). A report showed that inactivity of posaconazole against zygomycetes was overcome by combining it with caspofungin, highlighting effective use of combinational therapy in treating newly emerging pathogens in immunocompromised patients (Guembe et al. 2007). Also, concentration-dependent synergism and antagonism were observed for triple drug combination against *Aspergillus* spp. (Meletiadis et al. 2007). Lower concentrations of voriconazole and amphotericin B and intermediate concentration of caspofungin showed synergy, but a higher concentration produced an antagonistic effect. A multi laboratory study from Chaturvedi et al. (2008) found reproducible results showing synergistic interaction of amphotericin B, posaconazole, caspofungin, and voriconazole against *candida krusei*, indicating successful application of combinational therapy. A recent study has highlighted the synergistic interaction of micafungin with fluconazole, voriconazole, amphotericin B, and flucytosine against *Candida* sp (Nishi et al. 2009). We have listed here some selected interaction studies as an example in Table 10.1.

In addition to various fungal agents from different classes being tested in combination, combinations of antifungals and antibacterials have recently been exploited for effective therapy. For example, rifampicin, an antibacterial agent

Table 10.1 List of various antifungal combinations tested *in vitro* against pathogenic fungi

Combinations	Organisms		
	Candida spp.	*C. neoformans*	*Aspergillus* spp.
AMB + azoles	A, I	I	S, A, I
AMB + echinocandins	S, I	S, I	S, I
AMB + 5-FC	S, I	S, A, I	S, A, I
AMB + TBF	S, I	NA	S, A, I
Azoles + echinocandins	S, I	S, I	S, I
Azoles + TBF	S, I	NA	S
Azoles + 5-FC	S, A, I	S, I	I
TBF + Cyclosporine A	S	NA	NA
Azoles + Cyclosporine A	S, I	NA	NA
Azoles + Amiodarone	S	NA	NA
AMB + Ciprofloxacin	S	NA	S
Echino + Nikkomycin Z	S	NA	S

Source: Cuenca-Estrella (2004), Lewis and Kontoyiannis (2005), Oliveira et al. (2005), Fohrer et al. (2006), Brun et al. (2007), Baddley and Pappas (2007), Guo et al. (2008), Li et al. (2008), Sandovsky-Losica et al. (2008), Stergiopoulou et al. (2008)
AMB amphotericin B, *FC* flucytocine, *TBF* terbinafine, *MCF* micafungin, *S* synergy, *A* antagonism, *I* indifferent (includes both additive and no interaction), *NA* not available

acting through blocking of DNA synthesis, exhibits no antifungal activity, but in combination with amphotericin B it enters the fungal cell to inhibit DNA transcription. Synergy for this combination has been reported against isolates of *Candida* spp., *Aspergillus* spp., *Fusarium* spp., Mucorales, and *C. neoformans*, but no antagonism is observed (Dannaoui et al. 2002). Likewise, some other studies have also shown synergy between antifungal agents and the fluoroquinolones such as ciprofloxacin, levofloxacin, and ofloxacin, and the macrolides against some fungal isolates (Vitale et al. 2003). Recently, ciprofloxacin was shown to be interacting synergistically with amphotericin B against *C. albicans* and *A. fumigatus* (Stergiopoulou et al. 2008). Also, several nonantimicrobials have been evaluated in combination with antifungals, such as cyclosporin and tacrolimus, the known calcineurin inhibitors, which have increased the *in vitro* efficacy of both fluconazole and caspofungin against *Candida* spp., *Aspergillus* spp., and *C. neoformans* (Kontoyiannis et al. 2003; Onyewu et al. 2003; Sun et al. 2008). An *in vitro* study for *A. fumigatus* has also indicated synergy between itraconazole and amiodarone, and between lansoprazole and nifedipine. In vitro combination of calcium pump blockers with itraconazole has even shown synergy against resistant strains (Afeltra et al. 2004). Recently, a report highlighted effective uses of amiodarone and cyclosporine A in separate combination with azoles against drug-resistant *C. albicans* (Guo et al. 2008; Li et al. 2008). Nikkomycin Z and retigeric acid had showed synergy with voriconazole, caspofungin, and azoles respectively against *C. albicans* isolate (Sandovsky-Losica et al. 2008; Sun et al. 2009). Anticarcinoma compound methotrexate has also been found interacting differently with antifungals against *Aspergillus* sp. It has shown synergy with terbinafine, no interaction with itraconazole and amphotericin B, and indifferent action with voriconazole (Yang et al. 2009).

10.2.3 *In Vivo* Antifungal Drug Interactions and Clinical Trials

In vitro testing of drugs exhibits inherent susceptibility to pathogens, but *in vivo* testing shows the efficacy with which this combination could be exploited in clinical use, considering pharmacodynamic and kinetic parameters. However, additional factors also have an influence on clinical efficacy, such as the difficulty in diagnosing deep mycosis, the heterogeneity of the patient affected, and the host immunity, etc. Several animal models have been implicated for assessment of synergy between antifungal compounds. However, the results obtained are variable because of the divergence of methodologies adopted and differences in the animal species involved; also, infection site and immune status have an effect on the real infection situation. For example, many animal models rely on intravenous inoculation of yeasts or conidia; this does not mimic the initiation and progress of the majority of fungal infections in humans. In addition, some animal models raise doubts about the validity of results because they employ lower doses of antifungal agents than are recommended for clinical use. Therefore, numbers of reports on *in vivo* and clinical trials are still limited, but some of the individual case reports are available (Johnson et al. 2004; Baddley and Pappas 2007). Some reports state that, similarly to *in vitro* studies of a combination of flucytosine and amphotericin B, synergistic or indifferent results were obtained *in vivo* against *Cryptococcus* spp. and *Aspergillus* spp. (Bava and Negroni 1992; Arroyo et al. 1997). Also, the *in vivo* combination of azoles with amphotericin B against *Aspergillus* spp. has shown similar to *in vitro* outcomes (Polak 1988). However, some *in vitro* combinations have not resulted in consistency *in vivo*, as is the case with azoles and amphotericin B against *Candida* spp. (Sugar et al. 1995). In general, combinations of echinocandin with other antifungls have produced favorable results. Some of the *in vivo* combination experimental data have been summarized in Table 10.2.

In addition, several clinical trials need to be conducted to evaluate the proposed formulations. Various clinical studies and case studies have been conducted with

Table 10.2 List of various antifungal combinations tested *in vivo* against pathogenic fungi

Combinations	Organisms		
	Candida spp.	*Cryptococcus* spp.	*Aspergillus* spp.
AMB + FC	S, I	S, I	S, I
AMB + ICZ	A, I	S, I	A
AMB + FCZ	A, I	I	I
AMB + TBF	NA	NA	I
AMB + echinocandins	I	NA	S, I
FCZ + echinocandins	NA	NA	NA
FC + FCZ	S, I	S, I	NA
PSZ + echinocandins	NA	NA	S

Source: Johnson et al. (2004), Cacciapuoti et al. (2006), Fohrer et al (2006), Baddley and Pappas (2007)

AMB amphotericin B, *FC* flucytocine, *TBF* terbinafine, *PSZ* posaconazole, *S* synergy, *A* antagonism, *I* indifferent (includes both additive and no interaction)

different combinations of antifungal drugs similar to those reported as being successful *in vivo*. Clinical data for cryptococcosis have confirmed that amphotericin B plus flucytosine is the most active combination for patients with cryptococcal meningitis. Clinical trials in candidaemia also suggested improved outcomes among patients receiving amphotericin B plus fluconazole versus fluconazole alone. In aspergillosis, several experimental models suggest the benefit of a variety of antifungal combinations (Baddley and Pappas 2005). Some of the *in vivo* studies are summarized here. The first clinical application of combination therapy was conducted for invasive candidiasis by oral administration of amphotericin B and flucytosine, and found effective over fluconazole in a randomized study (Smego et al. 1984). A double-blind study by Rex et al. (2003) on 219 patients for 14 days demonstrated 69% successful outcome for combination therapy using amphotericin B (0.6–0.7 mg Kg^{-1}) and fluconazole (800 mg) compared to 56% for monotherapy using fluconazole (800 mg) in treating non-neutropenic patients with candidemia ($p = 0.043$). A first large-scale clinical trial, in the pre-AIDS era, involving 51 patients over 4 years, was conducted for treating cryptococcal meningitis using a combination of amphotericin B (0.3 mg Kg^{-1}) and flucytosine (150 mg Kg^{-1}) versus amphotericin B alone (0.4 mg Kg^{-1}) for 70 days, and showed marked improvement for combinational therapy over monotherapy ($p < 0.05$). For the same disease, with the advent of the AIDS pandemic, several pilot-scale or large-scale studies comprising a combination of amphotericin B and flucytosine over amphotericin B alone were conducted. Combination therapy was found more effective in clearing CSF infections (Larsen et al. 1990). On the other hand, a study from Saag et al. (1992) reported effective use of fluconazole alone over amphotericin B used either alone or in combination with flucytosine in HIV patients with acute cryptococcal meningitis. However, Brouwer et al. (2004) demonstrated effective use of combinations therapy (amphotericin B with flucytosine, amphotericin B with fluconazole, or amphotericin B with flucytosine and fluconazole) over monotherapy (amphotericin B alone) for AIDS-associated cryptococcal meningitis. Azoles have been exploited in combination with flucytosine to cure mainly such infections (Mayanja-Kizza et al. 1998). However, some reports describe effective use of fluconazole in combination with flucytosine in treating candidemia due to fluconazole-resistant isolates (Girmenia et al. 2003). A double-blind randomized study by Barbaro et al. (1996) involving treatment of esophageal candidiasis revealed better efficacy of itraconazole along with flucytosine over monotherapy. The combination of amphotericin B and azoles has also been evaluated successfully against candidal endocarditis (Thakur et al. 1994) and invasive aspergillosis (Duchini et al. 2002). Invasive aspergillosis has responded well to the use of caspofungin in combination with either itraconazole or lipid formulation of amphotericin B (Rubin et al. 2002). The combination of amphotericin B with itraconazole against aspergillosis is the most studied, appears to be safe, and is recommended for use (Denning et al. 2003). A prospective, multicenter study by Singh et al. (2006) on 40 patients with solid organ transplants suffering from invasive aspergillosis highlighted 67.5% survival success using a combination of voriconazole with caspofungin, compared with 51% for the control treated with lipid formulation of

amphotericin B. Recently, a retrospective study carried out on haematological cancer patients for the period of 2001–2007 revealed successful implication of liposomal amphotericin B with caspofungin in treating invasive fungal infections (Rieger et al. 2008). Some of the selected clinical studies are listed in Table 10.3.

However, prior to recommendation of drugs after clinical trials, some issues need to be considered, such as rapid and accurate diagnosis of mycoses, understanding or manipulation of the patient's immunity, optimization of drug pharmacokinetics and dynamics in host tissue, empiric or pre-emptive prevention strategies adopted against infections, surgical intervention, and activity of antifungals, whether newer or old class drugs.

10.3 New Strategy for Antifungal Drug Discovery

In order to develop new approaches to antifungal therapy, new targets, novel compounds and innovative delivery systems are required. In this regard, the cell membrane, cell wall and associated virulence factors, and putative genes have been targeted. Novel delivery systems consisting of cyclodextrins, cochleates, nanoparticles/nanosphere and long-circulating (stealth) liposome substantially modulate the pharmacokinetics of existing compounds and may also be useful in enhancing the efficacy of antifungal agents (Walsh et al. 2000). In addition, some new targets for antifungal agents may be identified involving fungal cell wall, a structure essential to fungi but lacking in mammalian cells. Some of such major macromolecular targets are chitin β-glucan and mannoproteins (Cabib et al. 1982; Cabib et al. 1988; Klis 1994; Oehischiager and Czyrewska 1992). Similarly, plasma membrane and targets based on synthesis of ergosterol, phospholipids, sphingolipid and proton ATPase, efflux pump, protein synthesis, intermediary metabolism of nucleic acid, signal transduction, and cell cycle, etc. can also offer new antifungal drug targets (Sangamwar et al. 2008). In this chapter, we have focused on progress made in antifungal drug discovery targeting virulence factors and genomics-based approaches.

10.3.1 Targeting Virulence and Pathogenicity

The common themes of microbial pathogenicity, as described by Jones and Falkow (1996), indicate that pathogenicity is the ability of a pathogen to cause disease, and the degree of pathogenicity is called virulence. The term 'virulence factors' refers to a set of genes that augment the ability of the parasite to survive and to grow within the host and that are essential to the parasite's ability to produce disease. In a review article, Perfect (1996) emphasized the need for a broad definition of virulence gene to encompass molecular antifungal targets. Various virulence

Table 10.3 List of clinical trials findings conducted for antifungals in combination therapy against invasive fungal infections

Target disease	Treatment combination	Trial population	Experiment design	Results
Cryptococcal meningitis	AMB + 5FC AMB	Non AIDS patients	Prospective, random trial	Cure: 23/34 (68%) vs 15/32 (47%)
	AMB + 5FC AMB	AIDS patients	Prospective, double-blinded, multicenter, random trial	CSF sterilization: 122/200 (60%) vs 91/179 (51%) ($P = 0.06$)
	AMB + 5FC/FLZ AMB	AIDS patients	Prospective, random, open-label trial	Fungicidal effect within 14 days: −0.54 vs −0.31 log cfu/day
Candidiasis	AMB + FLZ FLZ	Non-neutropenic patients	Prospective, random trial	Success at 20 days: 69% vs 56% ($P = 0.043$)
	AMB + 5FC FLZ	Non-neutropenic surgical patients	Prospective, random trial	Time to elimination: 5.5 vs 8.5 days ($P = 0.03$)
	AMB + Mycograb AMB	Invasive candidiasis	Multicenter, blinded, randomized trial	Mortality reduced: 18% vs 4% ($P < 0.001$)
Aspergillosis	VCZ + CAS AMB	Solid organ transplant patients	Prospective, multicenter, cohort study	90 day survival: 67% (27/40) vs 51% (24/47) ($P = 0.11$)
	MICA + other antifungals MICA	Immunocompromised patients	Prospective, open-label study, primary or salvage	Primary therapy response: 29.4% vs 50% Salvage therapy response: 34.55 vs 40.9%

Source: Johnson and Perfect (2007), Wirk and Wingard (2008)
AMB amphotericinB, *5FC* flucytosine, *FLZ* fluconazole, *VCZ* voriconazole, *CAS* Caspofungin, *MICA* micafungin

factors, which include structure products of the microbial cell, play a significant role in determining whether the microbial strain is virulent or avirulent. Various virulence factors exist; interestingly, some of them are of obvious importance. Therefore, virulence factors worth considering as an antimicrobial target should fulfill Koch's postulates (Falkow 1988), in so far as genetic disruption is linked to attenuation.

In the recent past, effective antifungal therapy has become particularly important as the number of immunocompromised patients with life-threatening fungal infections has increased. Further, due to the emergence of multi drug resistance, it has been realized that it is not adequate only to target the essential processes of fungal physiology, but also at least address the virulence process. The simple idea behind this is no longer to try to kill the microorganisms acting as a pathogen by all means, but rather to hinder them from causing any harm to the host. This in fact may offer a kinder and gentler approach to antimicrobial chemotherapy (Alksne and Projan 2000). There has been a recent interest and enthusiasm for virulence factors as antifungal targets among scientific workers (De Backer and Van Dijck 2003; Niimi et al. 1999; Perfect 1996; Selitrennikoff et al. 2001). Various virulence factors of pathogenic fungi have been known and characterized at molecular level (See Chapter 2). For fungal virulence factors to be established, the corresponding knockout strain should be shown to be a virulent one when assayed in an appropriate animal model; reintroduction of the wild-type alleles should restore virulence. An advantage of using virulence factors as drug targets is that they are usually pathogen-specific and not found in the human host. However, a disadvantage is that the chance of finding a virulence factor that is conserved among fungal pathogens is difficult or low. On the other hand, virulence and pathogenicity of a pathogen is a multifactorial phenomenon and targeting only single major virulence factors may not be highly effective. Some workers believe that virulence factors are conceptually more likely to be targets for prophylactic rather than therapeutic agents and may be highly specific to a single species or strain within a species.

Fortunately, during the last few years, virulence factors of fungi and their inhibitors have, at least to some extent, been discovered and characterized. This should provide new options for the development of potential antifungal therapeutics, as described by Gauwerky et al. 2009. However, like many other antimicrobial treatment strategies, the concept of virulence factor-oriented antimicrobial chemotherapy has its limitations, one being the fact that the inherent specificity makes people expect a relatively narrow spectrum of action (Projan and Youngman 2002). However, there are possible ways and means of overcoming such problems. Some other studies have indicated that virulence factors of the fungal pathogen can be altered. For example, aspartic proteinase (Sap), a prime virulence factor of *C. albicans,* is upregulated in HIV patients treated with fluconazole at sub-MIC concentration (Wu et al. 2000). Further, Navarathna et al. (2005) were able to show in animal experiment that the virulence of *C. albicans* strains is increased upon exposure to sub-MICs of fluconazole. A variety of virulence factors have been observed in fungal pathogenesis (for detail see Chapter 2) and reported to be targeted *in vitro* and *in vivo* for a possible new approach for describing new

antifungals. Some of them are secretory aspartic proteinases (SAPs), the major virulence factors of *C. albicans*. These are hydrolytic enzymes and encoded by a gene family with ten members (Sap1–Sap10). Other virulence factors include phospholipases (Mirbod et al. 1995; Birch et al. 1996; Ghannoum 2000), catalases (Latge 2001; Paris et al. 2003), melanin (Brush and Money 1999; Huffnagle et al. 1995; Langfelder et al. 2003), calcineurin (Blankenship et al. 2003; Cramer et al. 2008), lipid signaling molecules (Sugimoto et al. 2004; Mare et al. 2005), and several other putative virulence factors in one or other pathogenic fungi candidate are in the development of new antifungal drugs targeting virulence. Several inhibitors of these factors have been reported by various workers. Pepstatin A is a specific aspartic proteinase inhibitor and reduces *Candida* adherence *in vitro*, but its clinical application showed no value (Ollert et al. 1995). Didanosine (an antiretroviral agent) and squinavir (HIV proteinase inhibitors) were found effective in clearance of oral candidiasis in HIV patients. Squinavir, retnovir and indinavir, being HIV proteinase inhibitors, also inhibited candidal SAPs *in vitro* (Zingman 1996; Cauda et al. 1999). Among the SAPs, sap2 plays a greater role in pathogenesis, and was found to be inhibited by a congenitor of pesptatin named A-70450 (Ascencio et al. 2005). Some compounds, like alexidine hydrochloride, have been found to inhibit phospholipases in *C. albicans*, *C. neoformans*, and *A. flavus*, as they share structural similarity to phospholipid substrates (Ganendren et al. 2004). In addition, some natural products such as tacrolimus, cyclosporine A inhibit calcineurin in *C. albicans* and *C. neoformans* (Liu et al. 1991; Huai et al. 2002). In addition, olive extracts and saponins are reported to be inhibitors of elastases and hyphal formation respectively in *Candida* infections (Battinelli et al. 2006; Zhang et al. 2006). Furthermore, Aureobasdin A and Khafrefungin have been shown to inhibit inositol phosphoryl ceramide synthase in *Candida* spp., *C. neoformans*, and *Aspergillus* spp. (Gauwerky et al. 2009).

10.3.2 Use of Genomic Approaches in Antifungal Drug Discovery

Antifungal drug discovery is starting to benefit from the enormous advances in the genomic field which have occurred in recent decades. The decline in the success of the more classical drug-screening strategies in identifying potent, specific and nontoxic antifungal therapeutics has highlighted the need of alternative approaches (De Backer et al. 2002). One such approach using novel genetics and genomic-based strategies will aid in the discovery of novel drug targets. The current paradigm in the discovery of antifungal drug targets focuses on basically two classes of targets to evaluate: genes essential for viability, and those for virulence or pathogenicity. These genomic-based approaches, however, are in the stage of infancy for antifungal drugs, but it is expected that recent advances in genetics and genome-based technologies will allow us not only to identify and validate novel fungal drug targets but hopefully in the longer run also to discover potent therapeutic antifungal agents (De Backer and Van Dijck 2003).

10.3.2.1 Principle of Genomic-Based Antifungal Drug Discovery

The application of fungal genomics offers an unparalleled opportunity to develop novel antifungals. The release of the genome sequence of *C. albicans* in 2000 started a post-genomic era. The subsequent release of the sequence of many fungal pathogens such as *A. fumigatus* and *Cryptococcus neoformans* and the emergence of genome-wide characterization technologies have further improved its application. At present more than ten fungal genomes have been sequenced including those of *Saccharomyces cerevisiae, C. albicans, Candida glabrata, A. fumigatus, Cryptococcus neoformans, debaryomyces hansenii, Kluyveromyces lactis, Schizosaccharomyces pombe, Yarrwia lipolitica*, and *Eremothecium gossypii* (Liu et al. 2006). Publicly available, there are complete fungal genome sequences, namely for *A. fumigatus, Cryptococcus neoformans, C. albicans, Saccharomyces cerevisae, Neurospora carassa, Schizosaccharomyces pombe,* and *Magnaporthe grisea* (Jiang et al. 2002). These covers fungi from ascomycetes as well as basidiomycetes, providing a wealth of comparative information. The concept and principle of genomic-based antifungal drug discovery is similar to antibacterial or antiviral drug discovery as described by Odds (2005). Some of the key features are determination of the complete DNA sequence of a pathogen followed by designation of the open reading frames (ORFs) that encode functional products. However, it is a difficult and time-consumingg process, and requires a combination of bioinformatics and gene-expression technology. To define potential antimicrobial targets, the sequence of microbial genes are compared with that of the human genome, and microbial genes with a low degree of amino acid sequence homology to the human gene are listed as potentially microbe-specific genes of the target. The next most important task is the reduction of ORFs found by whole genome sequencing to a short list of genes specific to the microorganisms. The assumption underlying the genome approach is that an inhibitor of the products of microbial genes will not have an equivalent target in human host and will therefore work as a specific antimicrobial agent. Approaches to determining the functions of the unknown genes include: (a) specific mutagenesis to determine phenotypic consequences or changes in gene expression profile (De Backer and Van Dijck 2003), (b) transposon mutagenesis to reveal gene functions (Firon et al. 2003), and (c) two-hybrid screening to find sequences that interact with the gene of interest and post-transcriptional gene silencing with antisense RNA (De Backer et al. 2001b, Gorlach et al. 2002; Liu et al. 2002) and over expression of the gene in a foreign host.

Once a list of genes of interest has been compiled, the list is further reduced by consideration of issues such as target function, patentability, and the ease with which a high-throughput screen can be devised for inhibitors of the gene products (Spaltmann et al. 1999). Different approaches for large-scale identification of essential genes in *C. albicans* are used by Roemer et al. (2003). The applications of comparative genomics for identification of antifungal targets are used by different workers (Haselbeck et al. 2002; Willins et al. 2002). The next step is for the investigator to design novel chemical compounds or classes that can be predicted to inhibit the newly selected target (Anderson 2003) or screen existing compound

libraries for the target inhibitor. The Hits (the first inhibitor not necessarily the optimum molecules) can be optimized to leads (more potent inhibitor) by computer analysis of drug target interaction and of predicted pharmacokinetics (adsorption, distribution, metabolism excretion, etc).

10.3.2.2 Identification and Validation of New Antifungal Targets

A considerable amount of work is in progress in this direction especially in the identification of essential genes as potential drug targets from key fungal pathogens including *C. albicans* (De Backer et al. 2001a; Roemer et al. 2003; Sambrook et al. 1989). It is believed that a more difficult task is target prioritization in terms of, for example, degree of essentiality, broad spectrum potential, drug target potential, fungal specificity, availability of functional assays, and amenability to high-throughput screening (HTS). This would probably present a greater challenge to the research community (Bruccoleri et al. 1998). Recently, excellent review articles have been published covering recent progress made in this direction, and the types of obstacles faced by a scientist when a fungal pathogen is subjected to genomic strategies and target validation (Bays and Margolis 2004; Baddley and Pappas 2005; Agarwal et al. 2008). There are several molecular genetic technologies being used to identify and validate antifungal drug targets, such as (a) gene expression profiling approaches, (b) alternative methods to DNA microarray such as serial analysis of gene expression (SAGE), (c) RNA mediated gene silencing approaches, and (d) insertional mutagenesis-based approaches, as described by De Backer and Van Dijck (2003).

The availability of genome sequence data for some fungi has made it possible to identify new potential antifungal targets by using bio-information. These targets may be identified on the basis that these functions are essential for viability, they are conserved amongst fungal pathogens, and they have diverged significantly or are absent in humans. Using a genome comparison (or concordance) tool, Liu et al. (2006) identified 240 conserved genes as candidates for potential antifungal targets in ten fungal genomes. To facilitate the identification of essential genes in *C. albicans*, they developed a repressible *C. albicans MET3* (Ca*MET3*) promoter system capable of evaluating gene essentiality on a genome-wide scale. The Ca*MET3* promoter was found to be highly amenable to controlled gene expression, a prerequisite for use in target-based whole-cell screening. Furthermore, Potential targets involved in the process such as cell-wall biosynthesis and ergosterol biosynthesis are attractive partly because they are fungus-specific. Cell-wall modules have been characterized which are involved in many fungus-specific physiological processes, environmental protection, morphogenesis, and virulence (Hoyer 2001; De Groot et al. 2005; Pardini et al. 2006). Many putative antifungal targets have been reported by various workers; some of them are listed in Table 10.4.

Other types of drug target have been suggested on the basis that the target molecule should not be conserved in humans. These include the translation initiation factor and even central metabolic enzymes (Dominguez and Martin 1998; Rodaki et al. 2006). A good example is heat shock protein 90 (HSp-90).

Table 10.4 Examples of some known putative genes exploited as targets for discovery of new antifungal agents

Fungus	Genes	Product/function	Inhibitors known
A. fumigatus, A. nidulans, C. neoformans, H. capsulatum	PRP8	Prp8 protein	No
Candida spp., *Aspergillus* spp.		Inositol phosphoryl-ceramide synthase	Yes (natural products)
A. fumigatus, C. neoformans *C. albicans*	PMA1	Plasma membrane H^+-ATPase	Yes (ebselen)
A. fumigatus	ERG11	Phenotypic	No
C. albicans	CDC68	Transcript elongation factor	No
C. albicans	VRG4	Golgi apparatus GDP-mannose transporter	No
C. albicans	CHS1	Chitin synthase 1	Yes (HWY-289)
C. albicans	NMT	*N*-myristoyl transferase	Yes (RO-09-4609)
C. neoformans	RAM1	Protein-farnesyltransferase β-subunit homologue	Yes (FPT inhibitor 3, calbiochem)
C. albicans, C. neoformans	CNA1/CNB1	Calcineurin A or B	Yes (cyclosporine and PK506)
C. albicans	FBA1	Fructose-1,6-bisphosphate aldolase enzyme	No

Source: Odds (2005), Rodaki et al. (2006), Hu et al. (2007), Billack et al. (2009)

This conserved molecule is the target of the therapeutic antibody, Mycograb. However, a putative drug target identified by a bioinformatics approach might not yet have yielded clinically useful antifungal drugs. Therefore, functional genomics is proving useful in helping to define the mode of action of drugs (Weig and Brown 2007). Global effects of a drug upon gene expression are analyzed in this regard. In *C. albicans*, this has been addressed at the level of transcriptome and the proteome (Liu et al. 2005; Bruneau et al. 2003). For example, transcript profiling showed that ketoconazole affects the expression of *C. albicans* genes involved in lipid, fatty acid and sterol metabolism, and that caspofungin, a β-1,3-glucan synthase inhibitor, influences genes involved in cell wall biosynthesis. These genomic datasets have largely reinforced previous assumptions about the mode of action of these well-characterized drugs. More to the point, these experiments have confirmed that genomic approaches can provide valuable information about the mode of action of a new antifungal by revealing which genes are regulated in response to the drug.

10.3.3 Progress in the Genomic Approach to Antifungal Drug Discovery

The genomic approach to antifungal target discovery is not without pitfall. There are many problems in identifying and validating an eligible gene target. There are many uncertainties in gene annotation and functional assignment of genes

(Stahura and Bajorath 2001). Genes of unknown function present a curious difficulty when taking the rational approach to target discovery. Fungal pathogens typically present significant obstacles when subjected to molecular genetics or genomics strategies, as described by De Backer and Van Dijck (2003).

- Diploidy or polyploidy of strain requires multiple rounds of gene inactivation to achieve a knockout mutant.
- Resistance to transformation in the fungal cell may require many independent transformations to obtain mutant cells for screening.
- Use of different codon leads to incorrect expression of heterologous genes in pathogens, and of genes from pathogens in hosts.
- Non-availability of functional mutagenesis vehicles, e.g., transposons force the development of novel alternative means of mutagenesis
- Non-availability or incomplete genome sequence prevents efficient use of comparative genomics and genome expression profiling strategies

Good antifungal drug targets are typically considered to be either gene products essential for viability or so-called virulence factors. Essential genes can be identified by creating a conditional gene-knockout strain in which one copy of a gene is disrupted or deleted and other alleles are placed under the control of an inducible promoter, so that the corresponding gene product is not made under repressed conditions, and is subsequently monitored for viability and or growth. Various molecular techniques are now available to identify and validate the novel antifungal gene target. Various essential genes have been identified and validated as drug targets; some of them are listed in Table 10.5.

Table 10.5 Examples of some essential virulence genes in pathogenic fungi identified as targets for antifungal drug discovery

Organism targeted	Virulence genes	Factors
Candida sp	PHR1,2	pH-dependent morphology
	SAP1-7	Proteinases
	PLB1	Phospholipases
	Heme3	Porphyrin biosynthesis
Cryptococcus sp	CnCal-A	Calcineurin
	Cap59	Capsule
	NI	Mannitol
	CLC1	Melanin production
	LAC1	Iron homeostasis
	ENA1	ATPase
Histoplasma sp	YP3	Transition phase
	NI	Heat shock
Aspergillus sp	AFA/P	Elastase
	HYP1	Hydrophobin
	LAE1	Hyphal formation
	GLI	Gliotoxin
	ALB1	Conidial morphology

Source: Walsh et al. (2000), Idnurm et al. (2004), Bok et al. (2005, 2006), Sugui et al. (2007), Idnurm et al. (2009)

Three main molecular genetics technologies used to identify and validate antifungal drug targets are based on gene expression profiling, RNA-mediated gene silencing, and insertional mutagenesis. There are excellent review articles available describing details of these techniques; our aim is to introduce the concept of genomics in antifungal drug discovery. However, gene expression profiling approaches as described by De Backer and Van Dijck (2003) are illustrated here as an example.

A major tool used to identify novel drug targets is genome-wide transcriptome analysis (expression profiling at the RNA level using DNA microarrays) or proteome analysis (expression profiling at the protein level using two-dimensional gel electrophoresis). Several papers have described the application of these technologies for the model yeast *Saccharomyces cerevisiae* and some of the reported approaches are also very useful in the field of antifungal drug research (De Backer et al. 2002). Gene expression profiling studies in pathogenic organisms are limited but expanding rapidly on *C. albicans* (Enjalbert et al. 2003). Earlier work used a subset of annotated *C. albicans* genes, but now DNA microarrays containing most or all of the *C. albicans* genes are available. Gene expression profiling studies in *C. albicans* or other pathogens are classified in four categories on the basis of experimental set- up (Conway and Schoolnik 2003).

In a first category, cells are treated with a drug or other stresses and the gene expression profile is compared with nontreated control cells (De Backer et al. 2002). When drugs are used, the expression profile is referred to as a drug-specific gene expression signature, and studies have shown that DNA microarray analysis of a mutant in which the target of the drug has been deleted results in a similar signature. In a study by De Backer et al. (2001b), 296 genes differentially expressing after treatment of *C. albicans* with itraconazole were identified, indicating that the mechanism of action of a drug, or its molecular point of intervention, can be identified through the use of gene expression profiling experiments with DNA microarrays. Many genes of the ergosterol biosynthesis pathway, targeted by itraconazole, were upregulated in the drug-treated cells. To identify alternative molecular targets for the drug, it would be interesting to determine the expression profile of erg11 or conditional erg5 deletion mutants (two direct targets of azole drugs) after treatment.

In a second category, gene expression profiling of transcription factor mutants is used to identify cellular targets that play a central role in the control of metabolism and virulence, for example, the yeast-to-hyphae transition (Nantel et al. 2002). Disruption of many transcription factors results in strains that are affected in yeast-to-hyphae transition. For example, disruption of TUP1 and NRG1 results in constitutive pseudo-hyphal and hyphal growth, whereas disruption of EFG1, CPH1 and CPH2 results in strains that are defective in yeast-to-hyphae induction. Reduction in virulence from these mutants confirms involvement of those genes in pathogenesis. DNA microarray analysis of these deletion mutants allows the identification of many targets regulated by the corresponding transcription factors.

A third category involves the comparison of global expression profiles of two phenotypic variants of the same strain. For example, the comparison of expression profiles of white (W) and opaque (O) switch phenotypes of the *C. albicans* WO-1 strain revealed that these phenotypes differ in their ability to form hyphae, in their surface properties, and in their drug susceptibility, and showed a completely different metabolic gene expression pattern (Lan et al. 2002). The W cells are more fermentative, whereas the O cells seem to be more respirative. This high-frequency switching (white-to-opaque cells at 10^{-4}–10^{-2} and opaque-to-white at 10^{-2}–10^{-1}) demonstrates that combined therapy with drugs that prevent expression of genes important for virulence in both cell types as opposed to one cell type would be the better option. The observation that the virulence of W and O cell types is also dependent on the infection site in the host (Kvaal et al. 1999) supports this theory. In addition, the infection site itself could result in differential expression, thus illustrating the interplay between pathogen and host (Mukherjee et al. 2003).

In a fourth category, RNA is isolated directly from the pathogen within the host (Kato-Maeda et al. 2001). In most cases, there is a correlation between the ability to change morphology *in vitro* and virulence *in vivo*, but genes have been identified that are either nonessential or have no effect on yeast-to-hyphae induction *in vitro*, but which when deleted reduce virulence (Kato-Maeda et al. 2001). Experiments using implanted dialysis chambers of cell cultures that mimic the host environment are being conducted. The interaction between macrophages and *S. cerevisiae* cells has been used to study gene expression following ingestion of the yeast cells by macrophages (Lorenz and Fink 2001). Genes of the glyoxylate pathway, such as ICL1, MLS1, MDH2 and CIT2, were found to be strongly upregulated. *C. albicans* cells, which are not killed by the macrophage, react similarly to such ingestion by strongly inducing genes of the glyoxylate pathway, permitting the use of C2 compounds. The *C. albicans* icl1 D/icl1D strain has no apparent phenotype under laboratory conditions, but it is strongly affected in virulence in a mouse model for systemic candidiasis. This finding, together with the fact that these genes are not essential during normal growth and that the glyoxylate pathway does not occur in human cells, makes these enzymes prime targets for specific antifungal and antibacterial drugs.

Recently, an approach for screening of *Candida* genome was adapted by Xu et al. (2007), which is a fitness test using reverse genetic assay, and genes related to the mechanism of action of the probe compounds were clearly identified, supporting their functional roles and genetic interactions. In this report, chemical–genetic relationships were provided for multiple FDA-approved antifungal drugs (fluconazole, voriconazole, caspofungin, 5-fluorocytosine, and amphotericin B) as well as additional compounds targeting ergosterol, fatty acid and sphingolipid biosynthesis, microtubules, actin, secretion, rRNA processing, translation, glycosylation, and protein-folding mechanisms. They also demonstrated how chemically induced haploinsufficiency profiles could be used to identify the mechanism of action of novel antifungal agents, thereby illustrating the potential utility of this approach to antifungal drug discovery.

10.4 Conclusion

In recent years, the data generated on antifungal combination therapy, in both *in vitro* and *in vivo* models, have been encouraging in many cases. However, many *in vitro* studies are not useful in animal models and many animal studies are not effective in clinical trials. In vitro data provide a base line for drug combination, but they can only be useful if such interactions are demonstrated under animal and clinical evaluation. Since the nature of fungal infection and its treatment require a multi-dimensional approach, combination therapy seems to be an attractive option for specific cases. It should not be generalized, as an injudicious use may create a serious problem of multi-drug resistance in fungi. On the other hand, there is a need to generate data on possible synergy interaction of antifungal with other natural and safe compounds *in vitro* and evaluate them in animal models for their efficacy and safety in combating fungal infections.

Furthermore, there is a need for new antifungal drugs to be addressed in a more holistic and productive way. This requires a continuous and concerted effort to develop more productive, traditional, hit-and-miss whole microbe-screening approaches for identifying new compounds, and the molecular mechanism of action of the compound could be explored through molecular tools and techniques (Odds 2003a). The assumption by certain groups of workers that classical approaches for drug discovery have become unproductive is not correct. There is a need to redesign strategies for more productive outcomes. The genomic approach at present is enriching much-needed basic molecular information which will hopefully become very useful in drug development in the future. Odds (2005) pointed out that the refined, rational, scientific approaches do not yet necessarily accelerate the process of antifungal discovery, since compounds with high activity against molecular targets would need to be designed to work against intact cells. Their molecular activities commonly fail to extrapolate to intact fungi, often because they are unable to gain access to an intracellular target. And, of course, not all compounds that work against intact fungi show activity in animal models of fungal infection. Clearly a combination of technologies, old and new, provides the optimum platform for antifungal discovery. So far, molecular approaches to target identification and the input of genomic information have successfully validated many targets for new inhibitory drugs. No antifungal drugs that are in clinical use have been developed through genomics, since several constraints are being faced in identifying and validating targets for drug development or heading to clinical development. The genomic era is still in its infancy with regad to its role in drug discovery. It takes many years to develop, validate and test a new antifungal drug. Hence, it is too early to decide the impact of genomics on antifungal drug development. Furthermore, it is clear that genomics is proving useful by defining the global effects of antifungal drugs upon the fungal cell, and the mechanism of cellular adaptation to antifungals. Genomic approaches are also helping to define potentially useful tools for the diagnosis and prognosis of systemic fungal infection (Weig and Brown 2007).

We therefore suggest that our efforts should not be directed only to one approach for combating fungal infection. A holistic and multidimensional approach is required to provide new treatment strategies and novel drugs and drug combinations. Whatever be the future of the genomics-based approach for antifungal drug, one thing is very clear; that it would be a very powerful tool for studying the molecular mechanism of drug action and gaining in-depth understanding of gene expression and functions, both in host and pathogen, under disease conditions, which may open new dimensions in combating fungal infection in the future.

References

Afeltra J, Vitale RG, Mouton JW, Verweij PE (2004) Potent synergistic *in vitro* interaction between nonantimicrobial membraneactive compounds and itraconazole against clinical isolates of Aspergillus fumigatus resistant to itraconazole. Antimicrob Agents Chemother 48: 1335–1343

Agarwal AK, Xu T, Jacob MR, Feng Q, Li XC, Walker LA, Clark AM (2008) Genomic and genetic approaches for the identification of antifungal drug targets. Infect Disord Drug Targets 8:2–15

Alksne LE, Projan SJ (2000) Bacterial virulence as a target for antimicrobial chemotherapy. Curr Opin Biotechnol 11:625–636

Anderson AC (2003) The process of structure based drug design. Chem Biol 10:787–797

Andes DA (2003) In vivo pharmacodynamic of antifungal drugs in treatment of candidiasis. Antimicrob Agents Chemother 47:1179–1186

Andriole VT (1999) Current and future antifungal therapy: new targets for antifungal agents. J Antimicrob Chemother 44:151–162

Arikan S, Lozano-Chiu M, Paetznick V, Rex JH (2002) In vitro synergy of caspofungin and amphotericin B against *Aspergillus* and *Fusarium* spp. Antimicrob Agents Chemother 46:245–247

Arroyo J, Medoff G, Kobayashi GS (1997) Therapy of murine aspergillosis with amphotericin B in combination with rifampin or 5-fluorocytosine. Antimicrob Agents Chemother 11:21–25

Ascencio MA, Gardunco E, Perez-Giraldo C, Blanco MT, Hurtado C, Gomez-Garcia AC (2005) Exposure to therapeutic concentrations of ritonavir, but not saquinavir, reduces secreted aspartyl proteinase of *Candida parapsilosis*. Chemotherapy 51:252–255

Baddley JW, Pappas PG (2005) Antifungal combination therapy: clinical potential. Drugs 65:1461–1480

Baddley JW, Pappas PG (2007) Combination antifungal therapy for the treatment of invasive yeast and mold infections. Curr Infect Dis Rep 9:448–456

Barbaro G, Barbarini G, Di Lorenzo G (1996) Fluconazole vs itraconazole–flucytosine association in the treatment of esophageal candidiasis in AIDS patients. A double-blind, multicenter placebo-controlled study. The *Candida esophagitis* Multicenter Italian Study (CEMIS) Group. Chest 110:1507–1514

Barchiesi F, Di Francesco LF, Compagnucci P, Arzeni D, Giacometti A, Scalise G (1998) In vitro interaction of terbinafine with amphotericin B, fluconazole, and itraconazole against clinical isolates of *Candida albicans*. J Antimicrob Chemother 41:59–65

Barrett D (2002) From natural products to clinically useful antifungals. Biochimica et Biophysica Acta 1587:224–233

Battinelli L, Canielea C, Cristianib M, Bisiqnanob G, Sajjab A, Mazzantia G (2006) In vitro antifungal and anti-elastase activity of some aliphatic aldehydes from *Olea europaea* L. fruit. Phytomedicine 13:558–563

Bava AJ, Negroni R (1992) Comparative study of six antifungal treatments in an experimental model of murine cryptococcosis. Eur J Epidemiol 8:422–426

Bays N, Margolis J (2004) Yeast as a budding technology in target validation. Drug Discov Today Tech 1:157–162

Billack B, Santoro M, Lau-Cam C (2009) Growth inhibitory action of ebselen on fluconazole-resistant *Candida albicans*: role of the plasma membrane H+-ATPase. Microb Drug Resist 15:77–83

Birch M, Robson G, Law D, Denning DW (1996) Evidence of multiple extracellular phospholipase activities of *Aspergillus fumigatus*. Infect Immun 64:751–755

Blankenship JR, Wormley FL, Boyce MK, Schell WA, Filler SG, Perfect JR, Heitman J (2003) Calcineurin is essential for *Candida albicans* survival in serum and virulence. Eukaryot Cell 2:422–430

Bok JW, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Keller NP (2005) LaeA, a regulator of morphogenetic fungal virulence factors. Eukaryot Cell 4:1574–1582

Bok JW, Chung DW, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Kirby KA, Keller NP (2006) GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to *Aspergillus fumigatus* virulence. Infect Immun 74:6761–6768

Brouwer AE, Rajanuwong A, Chierakul W, Griffin GE, Larsen RA, White NJ, Harrison TS (2004) Combination therapies for HIV associated cryptococcal meningitis: a randomized trial. Lancet 363:1764–1767

Bruccoleri R, Dougherty T, Davison D (1998) Concordance analysis of microbial genomes. Nucleic Acids Res 26:4482–4486

Brun YF, Dennis CG, Greco WR, Bernacki RJ, Pera PJ, Bushey JJ, Youn RC, White DB, Segal BH (2007) Modeling the combination of amphotericin B, micafungin, and nikkomycin Z against *Aspergillus fumigatus in vitro* using a novel response surface paradigm. Antimicrob Agents Chemother 51:1804–1812

Bruneau JM, Isabelle M, Eric T, Raymond L, Francoise S, Claude F, Jean-Pierre LC, Valerie L, Dominique L, John H (2003) Drug induced proteome changes in *Candida albicans*: comparison of the effect of β-(1, 3) glucan synthase inhibitors and two triazoles, fluconazole and itraconazole. Proteomics 3:325–336

Brush L, Money NP (1999) Invasive hyphal growth in Wangiella dermatitidis is induced by stab inoculation and shows dependence upon melanin biosynthesis. Fungal Genet Biol 28:190–200

Cabib E, Roberts RL, Bowers B (1982) Synthesis of the yeast cell wall and its regulation. Annu Rev Biochem 51:763–793

Cabib E, Bowers B, Sburlati A, Silverman SJ (1988) Fungal cell wall synthesis: the construction of biological structure. Microbial Sci 5:370–375

Cacciapuoti A, Halpern J, Mendrick C, Norris C, Patel R, Loebenberg D (2006) Interaction between posaconazole and caspofungin in concomitant treatment of mice with systemic *Aspergillus* infection. Antimicrob Agents Chemother 50:2587–2590

Cauda R, Tacconelli E, Tumbarello M, Morace G, De Bernardis F, Torosantucci A, Cassone A (1999) Role of protease inhibitors in preventing recurrent oral candidosis in patients with HIV infection: a prospective case-control study. J Aquir Immune Def Syndr 21:20–25

Cha R, Sobel JD (2004) Fluconazole for the treatment of candidiasis: 15 years experience. Expert Rev Anti Infect Ther 2:357–366

Chaturvedi V, Ramani R, Ghannoum MA, Killian SB, Holliday N, Knapp C, Ostrosky-Zeichner L, Messer SA, Pfaller MA, Iqbal NJ, Arthington-Skaggs BA, Vazquez JA, Sein T, Rex JH, Thomas J, Walsh TJ (2008) Multilaboratory testing of antifungal combinations against a quality control isolate of *Candida krusei*. Antimicrob Agents Chemother 52:1500–1502

Conway T, Schoolnik GK (2003) Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. Mol Microbiol 47:879–889

Cramer RA Jr, Perfect BZ, Pinchai N, Park S, Perlin DS, Asfaw YG, Heitman J, Perfect JR, Steinbach WJ (2008) Calcineurin target CrzA regulates conidial germination, hyphal growth, and pathogenesis of *Aspergillus fumigatus*. Eukaryot Cell 7:1085–1097

Cuenca-Estrella M (2004) Combinations of antifungal agents in therapy — what value are they? J Antimicrob Chemother 54:854–869

Cuenca-Estrella M, Gomez-Lopez A, Garcia-Effron G, Alcazar-Fuoli L, Mellado E, Buitrago MJ, Rodriguez-Tudela JL (2005) Combined activity *in vitro* of caspofungin, amphotericin azole agents against Itraconazole-resistant clinical isolates of *Aspergillus fumigatus*. Antimicrob Agents Chemother 49:1232–1235

Dannaoui E, Afeltra J, Meis JFGM, Verweij PE, Network E (2002) In vitro susceptibilities of zygomycetes to combinations of antimicrobial agents. Antimicrob Agents Chemother 46: 2708–2711

Dannaoui E, Lortholary O, Dromer F (2004) In vitro evaluation of double and triple combinations of antifungal drugs against *Aspergillus fumigatus* and *Aspergillus terreus*. Antimicrob Agents Chemother 48:970–978

de Backer MD, Van Dijck P (2003) Progress in functional genomics approaches to antifungal drug target discovery. Trends Microbiol 11:470–478

de Backer MD, Itliyana T, Ma XJ, Vandononick S, Luyten WHML, Bossche V (2001a) Genomic profiling of the response of *Candida albicans* to itraconazole treatment using a DNA microarray. Antimicrob Agents Chemother 45:1660–1670

de Backer MD, Nelissen B, Logghe M, Viaene J, Loonen I, Vandoninck S, de Hoogt R, Dewaele S, Simons FA, Verhasselt P, Vanhoof G, Contreras F, Luyten WHML (2001b) An antisense based functional genomics approach for identification of genes critical for growth of *Candida albicans*. Nat Biotechnol 19:235–241

de Backer MD, Van Dijck P, Luyten WH (2002) Functional genomics approaches for the identification and validation of antifungal drug targets. Am J Pharmacogenomics 2:113–127

de Groot PW, Tam AF, Klis FM (2005) Features and functions of covalently linked proteins in fungal cell walls. Fungal Genet Biol 42:657–675

Denning DW, Hanson LH, Perlman AM, Stevens DA (1992) In vitro susceptibility and synergy studies of *Aspergillus* species to conventional and new agents. Diagn Microbial Infect Dis 15:21–34

Denning DW, Kibbler CC, Barnes RA (2003) British Society for Medical Mycology proposed standards of care for patients with invasive fungal infections. Lancet Infect Dis 3:230–240

Dismukes WE (2000) Introduction to antifungal drugs. Clin Infect Dis 30:653–657

Dominguez JM, Martin JJ (1998) Identification of elongation factor 2 as the essential protein targeted by sordarins in *Candida albicans*. Antimicrob Agents Chemother 42:2279–2283

Duchini A, Redfield DC, McHutchison JG, Brunson ME, Pockros PJ (2002) Aspergillosis in liver transplant recipients: successful treatment and improved survival using a multistep approach. South Med J 95:897–899

Eliopoulos GM, Moellering RC (1991) Antimicrobial combinations. In: Lorian V (ed) Antibiotics in laboratory medicine, 3rd edn. Williams & Wilkins, Baltimore, MD, pp 432–492

Enjalbert B, Nantel A, Whiteway M (2003) Stress-induced gene expression in *Candida albicans*: absence of a general stress response. Mol Biol Cell 14:1460–1467

Espinel-Ingroff A (2009) Novel antifungal agents, targets or therapeutic strategies for the treatment of invasive fungal diseases: a review of the literature (2005-2009). Rev Iberoam Micol 26:15–22

Falkow S (1988) Molecular Koch's postulates applied to microbial pathogenicity. Rev Infect Dis 10:S274–S276

Firon A, Villalba F, Beffa R, d'Enfert C (2003) Identification of essential genes in the human fungal pathogen *Aspergillus fumigatus* by transposon mutagenesis. Eukaryot Cell 2:247–255

Fohrer C, Fornecker L, Nivoix Y, Cornila C, Marinescu C, Herbrecht R (2006) Antifungal combination treatment: a future perspective. Int J Antimicrob Agents 27S:S25–S30

Ganendren R, Widmer F, Singhal V, Wilson C, Sorrell T, Wright L (2004) In vitro antifungal activities of inhibitors of phospholipases from the fungal pathogen *Cryptococcus neoformans*. Antimicrob Agents Chemother 48:1561–1569

Gauwerky K, Boreli C, Korting HC (2009) Targeting virulence: a new paradigm for antifungals. Drug Discov Today 14:214–222

Ghannoum MA (2000) Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 12:122–143

Girmenia C, Venditti M, Martino P (2003) Fluconazole in combination with flucytosine in the treatment of fluconazole-resistant *Candida* infections. Diagn Microbiol Infect Dis 46:227–231

Gorlach JM, McDade HC, Perfect JR, Cox GM (2002) Antisense repression in *Cryptococcus neoformans* as a laboratory tool and potential antifungal strategy. Microbiology 148:213–219

Guembe M, Guinea J, Pelaz T, Torres-Narbona M, Bouza E (2007) Synergistic effect of posaconazole and caspofungin against clinical zygomycetes. Antimicrob Agents Chemother 51:3457–3458

Guo Q, Sun S, Yu J, Li Y, Cao L (2008) Synergistic activity of azoles with amiodarone against clinically resistant *Candida albicans* tested by chequerboard and time-kill methods. J Med Microbiol 57:457–462

Haselbeck R, Wall D, Jiang B, Ketela T, Zyskind J, Bussey H, Foulkes JG, Roemer T (2002) Comprehensive essential gene identification as a platform for novel anti-infective drug discovery. Curr Pharm Des 8:1155–1172

Heyn K, Tredup A, Salvenmoser S, Muller FMC (2005) Effect of voriconazole combined with micafungin against *Candida*, *Aspergillus*, and *Scedosporium* spp. and *Fusarium solani*. Antimicrob Agents Chemother 49:5157–5159

Hoyer LL (2001) The ALS gene family of *Candida albicans*. Trends Microbiol 9:176–180

Hu W, Sillaots S, Lemieux S, Davison J, Kauffman S, Breton A, Linteau A, Xin C, Bowman J, Becker J, Jiang B, Roemer T (2007) Essential gene identification and drug target prioritization in *Aspergillus fumigatus*. PLoS Pathog 3:0001–0015

Huai Q, Kim HY, Liu Y, Zhao Y, Mondragon A, Liu JO, Ke H (2002) Crystal structure of calcineurin–cyclophilin–cyclosporin shows common but distinct recognition of immunophilin-drug complexes. Proc Natl Acad Sci USA 99:12037–12042

Huffnagle GB, Chen GH, Curtis JL, McDonald RA, Strieter RM, Toews GB (1995) Down regulation of the afferent phase of T cell mediated pulmonary inflammation and immunity by a high melanin producing strain of *Cryptococcus neoformans*. J Immunol 155:3507–3516

Idnurm A, Reedy JL, Nussbaum JC, Heitman J (2004) *Cryptococcus neoformans* virulence gene discovery through insertional mutagenesis. Eukaryotic Cell 3:420–429

Idnurm A, Walton FJ, Floyd A, Reedy JL, Heitman J (2009) Identification of *ENA1* as a virulence gene of the human pathogenic fungus *Cryptococcus neoformans* through signature-tagged insertional mutagenesis. Eukaryotic Cell 8:315–326

Jiang B, Bussey H, Roemer T (2002) Novel strategies in antifungal lead discovery. Curr Opin Microbiol 5:466–471

Johnson MD, Perfect JR (2007) Combination antifungal therapy: what can and should we expect? Bone Marrow Transplant 40:297–306

Johnson MD, MacDougall C, Ostrosky-Zeichner L, Perfect JR, Rex JH (2004) Combination antifungal therapy. Antimicrob Agents Chemother 48:693–715

Jones BD, Falkow S (1996) Salmonellosis: Host immune responses and bacterial virulence determinants. Annu Rev Immunol 14:533–561

Kato-Maeda M, Gao Q, Small PM (2001) Microarray analysis of pathogens and their interaction with hosts. Cell Microbiol 3:713–719

Kauffman CA (2001) Fungal infections in older adults. Clin Infect Dis 33:550–555

Keele DJ, DeLallo VC, Lewis RE, Ernst EJ, Klepser ME (2001) Evaluation of amphotericinn B and flucytosine in combination against *Candida albicans* and *Cryptococcus neoformans* using time kill methodology. Diagn Microbiol Infect Dis 14:121–126

Klis FM (1994) Cell wall assembly in yeast. Yeast 10:851–869

Kontoyiannis DP, Lewis RE, Osherov N, Albert ND, May GS (2003) Combination of caspofungin with inhibitors of the calcineurin pathway attenuates growth *in vitro* in *Aspergillus* species. J Antimicrob Chemother 51:313–316

Kvaal C, Lachke SA, Srikantha T, Daniels K, McCoy J, Soll DR (1999) Misexpression of the opaque-phase-specific gene PEP1 (SAP1) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. Infect Immun 67: 6652–6662

Lan CY, Newport G, Murillo LA, Jones T, Scherer S, Davis RW, Agabian N (2002) Metabolic specialization associated with phenotypic switching in *Candida albicans*. Proc Natl Acad Sci USA 99:14907–14912

Langfelder K, Streibel M, Jahn B, Haase G, Brakhage AA (2003) Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet Biol 38:143–158

Larsen RA, Leal ME, Chan LS (1990) Fluconazole compared with amphotericin B plus flucytosine for cryptococcal meningitis in AIDS: a randomized trial. Ann Intern Med 113:183–187

Latge JP (2001) The pathobiology of *Aspergillus fumigatus*. Trends Microbiol 9:382–389

Lewis RE, Kontoyiannis DP (2001) Rationale for combination antifungal therapy. Pharmacotherapy 21:S149–S164

Lewis RE, Kontoyiannis DP (2005) Micafungin in combination with voriconazole in Aspergillus species: a pharmacodynamic approach for detection of combined antifungal activity *in vitro*. J Antimicrob Chemother 56:887–892

Li Y, Sun S, Guo Q, Ma L, Shi C, Su L, Li H (2008) In vitro interaction between azoles and cyclosporin A against clinical isolates of *Candida albicans* determined by the chequerboard method and time-kill curves. J Antimicrob Chemother 61:577–585

Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL (1991) Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP-FK506 complexes. Cell 66: 807–815

Liu H, Cottrell TR, Pierini LM, Goldman WE, Doering TL (2002) RNA interference in the pathogenic fungus *Cryptococcus neoformans*. Genetics 160:463–470

Liu TT, Lee REB, Barker KS, Lee RE, Wei L, Homayouni R, Rogers PD (2005) Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. Antimicrob Agents Chemother 49:2226–2236

Liu M, Healy MD, Dougherty BA, Esposito KM, Maurice TC, Mazzucco CE, Bruccoleri RE, Davison DB, Frosco M, Barrett JF, Wang YK (2006) Conserved fungal genes as potential targets for broad-spectrum antifungal drug discovery. Eukaryotic Cell 5:638–649

Lorenz MC, Fink GR (2001) The glyoxylate cycle is required for fungal virulence. Nature 412:83–86

Magill SS, Shields C, Sears CL, Choti M, Merz WG (2006) Triazole cross resistance among *Candida* spp.: case report, occurrence among bloodstream isolates, and implications for antifungal therapy. J Clin Microbiol 44:529–535

Mare L, Iatta R, Montagna MT, Luberto C, Poeta MD (2005) APP1 transcription is regulated by IPC1-DAG pathway and is controlled by ATF2 transcription factor in *Cryptococcus neoformans*. J Biol Chem 280:36055–36064

Marinelli F (2009) Chapter 2. From microbial products to novel drugs that target a multitude of disease indications. Methods Enzymol 458:29–58

Mayanja-Kizza H, Oishi K, Mitarai S, Yamashita H, Nalongo K, Watanabe K, Izumi T, Ococi-Jungala AK, Mugerwa R, Nagatake T, Matsumoto K (1998) Combination therapy with fluconazole and flucytosine for cryptococcal meningitis in Ugandan patients with AIDS. Clin Infect Dis 26:1362–1366

Medoff G (1983) Antifungal action of rifampin. Rev Infect Dis 5(Suppl 3):S614–S619

Meletiadis J, Mouton JW, Meis JFGM, Verweij PE (2003) In vitro drug interaction modelling of combinations of azoles with terbinafine against clinical *Scedosporium prolificans* isolates. Antimicrob Agents Chemother 47:106–117

Meletiadis J, Stergiopoulou T, O'Shaughnessy EM, Peter J, Walsh TJ (2007) Concentration-dependent synergy and antagonism within a triple antifungal drug combination against *Aspergillus* species: analysis by a new response surface model. Antimicrob Agents Chemother 51:2053–2064

Mirbod F, Banno Y, Ghannoum MA, Ibrahim AS, Nakashima S, Kitajima Y, Cole GT, Nozawa Y (1995) Purification and characterization of lysophospholipasetransacylase (h-LPTA) from a highly virulent strain of *Candida albicans*. Biochim Biophys Acta 1257:181–188

Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA (2003) Differential expression of *Candida albicans* phospholipase B (PLB1) under various environmental and physiological conditions. Microbiology 149:261–267

Nantel A, Dignard D, Bachewich C, Harcus D, Marcil A, Bouin AP, Sensen CW, Hogues H, Hoog MVH, Gordon P, Rigby T, Benoit F, Tessier DC, Thomas DY, Whiteway M (2002) Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. Mol Cell Biol 13:3452–3455

Navarathna DHMLP, Hornby JM, Hoerrmann N, Parkhurst AM, Duhamel GE, Nickerson KW (2005) Enhanced pathogenicity of *Candida albicans* pretreated with sub inhibitory concentrations of fluconazole in a mouse model of disseminated candidiasis. J Antimicrob Chemother 56:1156–1159

Niimi M, Cannon RD, Monk BC (1999) *Candida albicans* pathogenicity: a proteomic perspective. Electrophoresis 20:2299–2308

Nishi I, Sunada A, Toyokawa M, Asari S, Iwatani Y (2009) In vitro antifungal combination effects of micafungin with fluconazole, voriconazole, amphotericin B, and flucytosine against clinical isolates of *Candida* species. J Infect Chemother 15:1–5

O'Shaughnessy EM, Meletiadis J, Stergiopoulou T, Demchok JP, Walsh TJ (2006) Antifungal interactions within the triple combination of amphotericin B, caspofungin and voriconazole against *Aspergillus* species. J Antimicrob Chemother 58:1168–1176

Odds FC (2003a) Reflections on the question: what does molecular mycology have to do with the clinician treating the patient? Med Mycol 41:1–6

Odds FC (2003b) Synergy, antagonism and what the chequerboard puts between them. J Antimicrob Chemother 52:1

Odds FC (2005) Genomics, molecular targets and the discovery of antifungal drugs. Rev Iberoam Micol 22:229–237

Oehlschlager AC, Czyzewska E (1992) Rationally designed inhibitors for sterol biosynthesis. In: Sutcliffe J, Georgopapadakao N (eds) Emerging Targets in Antibacterial and Antifungal Chemotherapy. Chapman and Hall, New York, pp 437–475

Oliveira ER, Fothergill AW, Kirkpatrick WR, Coco BJ, Patterson TF, Redding SW (2005) In vitro interaction of posaconazole and caspofungin against clinical isolates of *Candida glabrata*. Antimicrob Agents Chemother 49:3544–3545

Ollert MW, Wende C, Gorlich M, McMullan-Vogel CG, Zepelin MB, Vogel CW, Korting HC (1995) Increased expression of *Candida albicans* secretory proteinase, a virulence factor, in isolates from human immunodeficiency virus positive patients. J Clin Microbiol 33:2543–2549

Onyewu C, Blankenship JR, Poeta DM, Heitman J (2003) Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. Antimicrob Agents Chemother 47:956–964

Pardini G, De Groot PWJ, Coste AT, Karababa M, Klis FM, de Koster CG, Sanglard D (2006) The CRH family coding for cell wall GPI proteins with a predicted transglycosidase domain affects cell wall organization and virulence of *Candida albicans*. J Biol Chem 281:40399–40411

Paris S, Wysong D, Debeaupuis JP, Shibuya K, Philippe B, Diamond RD, Latge JP (2003) Catalases of *Aspergillus fumigatus*. Infect Immun 71:3551–3562

Perfect JR (1996) Fungal virulence genes as targets for antifungal chemotherapy. Antimicrob Agents Chemother 40:1577–1583

Polak A (1978) Synergism of polyene antibiotics with 5-fluorocytosine. Chemotherapy 24:2–16

Polak A (1988) Combination therapy with antifungal drugs. Mycoses 31(Suppl 2):S45–S53

Projan SJ, Youngman PJ (2002) Antimicrobials: new solutions badly needed. Curr Opin Microbiol 5:463–465

Rex JH, Pappas PG, Karchmer AW, Sobel J, Edwards JE, Hadley S, Brass C, Vazquez JA, Chapman SW, Horowitz HW, Zervos M, McKinsey D, Lee J, Babinchak T, Bradsher RW,

Cleary JD, Cohen DM, Danziger L, Goldman M, Goodman J, Hilton E, Hyslop NE, Kett DH, Lutz J, Rubin RH, Scheld WM, Schuster M, Simmons B, Stein DK, Washburn RG, Mautner L, Chu TC, Panzer H, Rosenstein RB, Booth J, National Institute of Allergy and Infectious Diseases Mycoses Study Group (2003) A randomized and blinded multicenter trial of high dose fluconazole plus placebo versus fluconazole plus amphotericin B as therapy for candidemia and its consequences in non neutropenic subjects. Clin Infect Dis 36:1221–1228

Rieger CT, Ostermann H, Kolb HJ, Fiegl M, Huppmann S, Morgenstern N, Tischer J (2008) A clinical cohort trial of antifungal combination therapy: efficacy and toxicity in haematological cancer patients. Ann Hematol 87:915–922

Rodaki A, Young T, Brown AJP (2006) Effects of depleting the essential central metabolic enzyme, fructose-1, 6-bisphosphate aldolase, upon the growth and viability of *Candida albicans*: implications for antifungal drug target discovery. Eukaryot Cell 5:1371–1377

Roemer T, Jiang B, Davison J, Ketela T, Veillette K, Breton A, Tandia F, Linteau A, Sillaots S, Marta C, Martel N, Veronneau S, Lemieux S, Kauffman S, Becker J, Storms R, Boone C, Bussey H (2003) Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. Mol Microbiol 50:167–181

Rubin MA, Carroll KC, Cahill BC (2002) Caspofungin in combination with itraconazole for the treatment of invasive aspergillosis in humans. Clin Infect Dis 34:1160–1161

Saag MS, Powderly WG, Cloud GA, Robinson P, Grieco MH, Sharkey PK, Thompson SE, Sugar AM, Tuazon CU, Fisher JF et al (1992) Comparison of amphotericin B with fluconazole in the treatment of acute AIDS-associated cryptococcal meningitis. N Engl J Med 326:83–89

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sandovsky-Losica H, Shwartzman R, Lahat Y, Segal E (2008) Antifungal activity against *Candida albicans* of nikkomycin Z in combination with caspofungin, voriconazole or amphotericin B. J Antimicrob Chemother 62:635–637

Sangamwar AT, Deshpande UD, Pekamwar SS (2008) Antifungals: need to search for a new molecular target. Indian J Pharmaceut Sci 70:423–430

Selitrennikoff CP, Alex L, Miller TK, Clemons KV, Simon MI, Stevens DA (2001) *COS-I*, a putative two component histidine kinase of *Candida albicans*, is an *in vivo* virulence factor. Med Mycol 39:69–74

Singh N, Limaye AP, Forrest G, Safdar N, Muñoz P, Pursell K, Houston S, Rosso F, Montoya JG, Patton P, Del Busto R, Aguado JM, Fisher RA, Klintmalm GB, Miller R, Wagener MM, Lewis RE, Kontoyiannis DP, Husain S (2006) Combination of voriconazole and caspofungin as primary therapy for invasive aspergillosis in solid organ transplant recipients: a prospective, multicenter, observational study. Transplantation 81:320–326

Smego RA, Perfect JR, Durack DT (1984) Combined therapy with amphotericin B and 5-fluorocytosine for *Candida meningitis*. Rev Infect Dis 6:791–801

Spaltmann F, Blunck M, Ziegelbauer K (1999) Computer-aided target selection — prioritizing targets for antifungal drug discovery. Drug Discov Today 4:17–26

Stahura FL, Bajorath J (2001) Bio and chemo informatics beyond data management: crucial challenges and future opportunities. Drug Discov Today 7:S41–S47

Stergiopoulou T, Meletiadis J, Sein T, Papaioannidou P, Tsiouris I, Roilides E, Walsh JT (2008) Isobolographic analysis of pharmacodynamic interactions between antifungal agents and ciprofloxacin against *Candida albicans* and *Aspergillus fumigatus*. Antimicrob Agents Chemother 52:2196–2204

Sugar AM (1995) Use of amphotericin B with azole antifungal drugs: what are we doing? Antimicrob Agent Chemother 39:1907–1912

Sugar AM, Hitchcock CA, Troke PF, Picard M (1995) Combination therapy of murine invasive candidiasis with fluconazole and amphotericin B. Antimicrob Agents Chemother 39: 598–601

Sugimoto Y, Sakoh H, Yamada K (2004) IPC synthase as a useful target for antifungal drugs. Curr Drug Targets Infect Disord 4:311–322

Sugui JA, Pardo J, Chang YC, Mullbacher A, Zarember KA, Galvez EM, Brinster L, Zerfas P, Gallin JI, Simon MM, Kwon-Chung KJ (2007) Role of *laeA* in the regulation of *alb1*, *gliP*, conidial morphology, and virulence in *Aspergillus fumigatus*. Eukaryot Cell 6:1552–1561

Sun S, Li Y, Guo Q, Shi C, Yu J, Ma L (2008) In vitro interactions between tacrolimus and azoles against *Candida albicans* determined by different methods. Antimicrob Agents Chemother 52:409–417

Sun L, Sun S, Cheng A, Wu X, Zhang Y, Lou H (2009) In vitro activities of retigeric acid B alone and in combination with azole antifungal agents against *Candida albicans*. Antimicrob Agents Chemother 53:1586–1591

Te Dorsthorst DTA, Verweij PE, Meletiadis J, Bergervoet M, Punt NC, Meis JFGM, Mouton JW (2002) In vitro interaction of flucytosine combined with amphotericin B or fluconazole against thirty five yeast isolates determined by both the fractional inhibitory concentration index and the response surface approach. Antimicrob Agents Chemother 46:2982–2989

Thakur RK, Skelcy KM, Kahn RN, Cannon L, Cherukuri R (1994) Successful treatment of Candida prosthetic valve endocarditis with a combination of fluconazole and amphotericin B. Crit Care Med 22:712–714

Truan G, Epinat JC, Rougeulle C, Cullin C, Pompon D (1994) Cloning and characterization of a yeast cytochrome b5 encoding gene which suppresses ketoconazole hypersensitivity in a NADPH P-450 reductase deficient strain. Gene 142:123–127

Vazquez JA (2007) Combination antifungal therapy: the new frontier. Future Microbiol 2:115–139

Vitale RG, Afeltra J, De Hoog GS, Rijs AJ, Verweij PE (2003) In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against Exophiala spinifera. J Antimicrob Chemother 51:1297–1300

Walsh TJ, Viviani MA, Arathoon E, Chiou C, Ghannoum M, Groll AH, Odds FC (2000) New targets and delivery systems for antifungal therapy. Med Mycol 38:335–347

Weig M, Brown AJP (2007) Genomics and the development of new diagnostics and anti-Candida drugs. Trends Microbiol 15:310–317

Willins DA, Kessler M, Walker SS, Reyes GR, Cottarel G (2002) Genomics strategies for antifungal drug discovery — from gene discovery to compound screening. Curr Pharm Des 8:1137–1154

Wirk B, Wingard JR (2008) Combination antifungal therapy: from bench to bed side. Curr Fungal infect Rep 2:43–48

Wu T, Wright K, Hurst SF, Morrison CJ (2000) Enhanced extracellular production of aspartyl proteinase, a virulence factor, by C*andida albicans* isolates following growth in sub inhibitory concentrations of fluconazole. Antimicrob Agents Chemother 4:1200–1208

Xu D, Jiang B, Ketela T, Lemieux S, Veillette K, Martel N, Davison J, Sillaots S, Trosok S, Bachewich C, Bussey H, Youngman P, Roemer T (2007) Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans*. PLoS Pathog 3:835–848

Yang J, Wan Z, Wang X, Liu W, Li R (2009) In vitro Interactions between antifungals and methotrexate against *Aspergillus* spp. Mycopathologia 168:237–242

Zaas AK, Steinbach WJ (2005) Micafungin: The US perspective. Exp Rev Anti Infect Ther 3:183–190

Zhang JD, Xu Z, Cao YB, Chen HS, Yan L, An MM, Gao PH, Wang Y, Jia XM, Jiang YY et al (2006) Antifungal activities and action mechanisms of compounds from *Tribulus terrestris* L. J Ethnopharmacol 103:76–84

Zingman BS (1996) Resolution of refractory AIDS-related mucosal candidiasis after initiation of didanosine plus saquinavir. New Engl J Med 334:1674–1675

Chapter 11
Role of De-Escalation and Combination Therapy Strategies in the Management of Invasive Fungal Infection: A Multidisciplinary Point of View

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Abstract The high morbidity, mortality, and healthcare costs associated with invasive fungal infections, especially in the critical care setting and immunocompromised host, have made them an excellent target for prophylactic, empiric, and preemptive therapy interventions, principally based on early identification of risk factors. Early diagnosis and treatment are associated with a better prognosis. In recent years there have been important developments in antifungal pharmacotherapy. An approach to the new diagnosis tools in the clinical mycology laboratory and an analysis of the use of new antifungal agents and their application in different clinical situations has been made. Furthermore, an attempt at developing a state of the art in each clinical scenario (ICU, hematological and solid organ transplant patients) has been performed, with the aim of choosing the best strategy for each clinical situation (prophylaxis, preemptive, empirical or targeted therapy). The high

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mortality rates in these settings make mandatory the application of early de-escalation therapy in critically ill patients with fungal infection. In addition, the possibility of antifungal combination therapy might be considered in solid organ transplant and hematological patients.

11.1 Introduction

Hospital medicine has advanced greatly in the past few decades. Patients with complex medical and surgical disorders are surviving longer due to equally complex medical and surgical interventions, which often involve the "collateral damage" of avoiding normal body defensive mechanisms.

Invasive fungal infections (IFIs), especially in the critical care setting, and solid organ transplant (SOT) and hematological patients have become an excellent target for prophylactic, empiric, and pre-emptive therapy interventions due to their increasing incidence, high morbidity and mortality rates, and associated healthcare costs. Early diagnosis and treatment are associated with a better prognosis. Although at present the number of systemic antifungal agents has increased significantly, the choice of antifungal drug must be based on the individual characteristics of the patient, clinical scenario and the presence of hemodynamic instability. A tailored therapy (de-escalation) must also be considered in some clinical situations.

The aim of this multidisciplinary review was to analyze the best option for treating this special population patients, describing the antifungal armamentary, and developing an state of the art in each clinical scenario (ICU, hematological and transplant patients) in an attempt to choose the best strategy for each clinical situation (de-escalation and/or combination therapy).

11.2 Systemic Antifungal Agents Today

According to their mechanism of action, antifungal agents can be classified as:

- Antifungals targeting fungal cell membrane
  - Polyenes: amphotericin B, nystatin
  - Azoles:
    Imidazoles: miconazole, ketoconazole
    Triazoles: fluconazole, itraconazole, voriconazole, posaconazole, ravuconazole
  - Alilamines: terbinafine
- Antifungals targeting fungal cell wall:
  - Echinocandins: caspofungin, micafungin, anidulafungin
  - Nikkomycin Z (peptide-nucleoside antibiotic)
- Antifungals targeting DNA/RNA and antimetabolites
  - Flucytosine

- Antifungals targeting protein synthesis:
  - Flucytosine
  - Sordarins

The main characteristics of the antifungals most used for IFIs are shown in Tables 11.1 and 11.2 (DRUGDEX® System 2008; Mensa et al. 2007; Richardson et al. 2007).

11.3 Antifungal Administration Routes

The existing systemic antifungals permit mainly intravenous (IV) administration, but only fluconazole, itraconazole, and voriconazole have oral formulation, allowing sequential administration. However, the IV route remains the main administration route for treatment of most IFIs. Over the last few years, inhalers have been added as an innovative administration route for some antifungal drugs.

11.3.1 Intravenous (IV) Route

General recommendations for preparation and administration of IV solutions are indicated in Table 11.3, as well as the usual dose interval.

Although intermittent perfusions are recommended, continuous infusion administration has been proposed over the past few years, with claims it could reduce the risk of nephrotoxicity associated with amphotericin B (AmB). The preliminary results of an open study, including 80 neutropenic patients with randomized hematologic neoplasias during 4 h infusion versus continuous infusion, suggest more advantages for continuous infusion. The maximum average daily dose was 0.95 mg/kg and 0.96 mg/kg respectively. A significantly lower incidence of reactions related to the infusion was found (fever, shivers, migraine, etc.) in the case of continuous administration, which also favored the creatinine clearance values (Eriksson et al. 2001).

Another cohort study on 81 febrile neutropenic hematological patients, including those with bone marrow transplant with a higher IFI risk, evaluated the administration of AmB in intermittent infusion during 4 h, (average daily dose of 0.69 ± 0.2 mg/kg), compared to a continuous infusion (dose 0.79 ± 0.2 mg/kg/day). Kidney failure, defined as the duplication of baseline creatinine, was seen in 45% and 10% of the patients respectively (OR 0.14; IC 95% 0.04–0.5; $p < 0.001$). The result was similar in patients with allogenic transplant and in those who were given other nephrotoxic drugs. A multivariate logistic regression showed that continuous infusion was the only variable significantly associated with kidney failure, with a protective effect (Peleg and Woods 2004).

The first experiences with lung transplant and hematological patients with IFI also evidenced that continuous infusion is well-tolerated, safe, and efficient in these

Table 11.1 Most important pharmacological characteristics of antifungal agents

	Susceptibility patterns	Adverse reactions	Interactions	Pregnancy	Breast feeding
Polyenes					
AMB-d	*Aspergillus* spp (except *A. terreus* and some *A. flavus* strains), *Blastomyces dermatitidis*, *Candida* spp (except *C. lusitaniae*), *Coccidioides immitis*, *Cryptococcus neoformans*, *Fusarium* spp, *Sporothrix schenckii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Scedosporium* spp, *Trichosporon* spp, *Zygomycetes*	Shivers, fever, vomits Anaphylaxis, hot flushes, muscular pain Renal deterioration (34–60%) dose-dependent and cumulative Hypokalemia, hypomagnesemia Normocytic–normochromic progressive anemia	–Nephrotoxic drugs –Antineoplastics (doxorubicin, carmustine, cyclophosphamide, 5-FU) –Hypokaliemic drugs (corticoids, digitalis, neuromuscular blockers)	Crosses placenta No teratogenic effects on animals (B category) No effects on humans (little experience)	No data Avoid exposure
AMB-l	*Aspergillus* spp (except *A. terreus* and some *A. flavus* strains), *B. dermatitidis, Candida* spp (except *C. lusitaniae), C. immitis, C. neoformans, Fusarium* spp, *S. schenckii, H. capsulatum, P. brasiliensis, Scedosporium* spp, *Trichosporon* spp, *Zygomycetes*	Shivers, fever Hypokalemia Hepatic alterations (alkaline phosphatase, bilirubin, transaminase) Renal function deterioration (10–20%)	Less potential of interaction with nephrotoxic medications than AMB-d	Crosses placenta No teratogenic effects on animals (category B) No effects on humans (little experience)	No data Avoid exposure
AMB-cl	*Aspergillus* spp (except *A. terreus* and some *A. flavus* strains), *B. dermatitidis, Candida*	Shivers, fever Hypokalemia Liver alterations (alkaline phosphatase, bilirubin)	Less potential of interaction with nephrotoxic medications than AMB-d	Crosses placenta No teratogenic effects on animals (category B)	No data Avoid exposure

	spp (except *C. lusitaniae), C. immitis, C. neoformans, Fusarium* spp, *S. schenckii, H. capsulatum, P. brasiliensis, Scedosporium* spp, *Trichosporon* spp, *Zygomycetes*	Renal function deterioration (<25%) Less incidence of anemia than AMB-d		No effects on humans (little experience)	
FCZ	*Candida* spp (except *C. krusei, C. norvegensis, C. ciferrii, C. inconspicua), C. neoformans, Trichosporon* spp, dermatophytes, *B. dermatitidis, H. capsulatum, C. immitis, P. brasiliensis,*	Safe and well tolerated (1–2% withdrawal due to ADR) GI discomfort (nausea, vomiting, diarrhea, abdominal pain) migraine, cutaneous exanthema Transitory increase of hepatic enzymes (hepatitis exceptionally) Alopecia (prolonged treatments and high doses)	Inhibition of liver metabolism of other drugs (anticoagulants, tricyclic antidepressants, oral antidiabetics, cyclosporin, fenitoine, terfenadine, astemizole, benzodiazepines, Celecoxib, losartan, tacrolimus, teofiline, zidovudine) –Rifampcine → FCZ metabolism induction → CpFCZ reduction (25%)	Unknown if it crosses placenta Teratogenic effects in animals (category C) and humans (little experience)	Concentration in mother's milk similar to Cp Risk not evaluated → evaluate risk/benefit
ICZ	*Aspergillus* spp, *B. dermatitidis, Candida* spp, *C. immitis, C. neoformans, H. capsulatum, P. marneffei, P. brasiliensis, S. apiospermum, S. schenckii, Malassezia* spp, dermatophytes	Safe (<5% withdrawal due to ADR) Gastrointestinal discomfort, migraine, pruritus, vertigo Higher indicence with oral solution (osmotic cyclodextrin effect) Transitory increase of liver	High potential of interactions –Inhibition of liver metabolism of other drugs (anticoagulants, oral antidiabetics, cisapride, cyclosporin, terfenadine, astemizole, benzodiazepines, calcioantagonists,	Unknown if it crosses placenta No teratogenic effects on animals (category C) No experience in humans	No data Avoid exposure

(*continued*)

Table 11.1 (continued)

	Susceptibility patterns	Adverse reactions	Interactions	Pregnancy	Breast feeding
		enzymes (hepatitis exceptionally)	digoxin, quinadin, tacrolimus, protease inhibitors, vinca alkaloids, statins) –Rifampicin metabolism induction –Rifampicin, rifabutin, isoniazid, fenitoin, carbamazepine, fenobarbital → ICZ metabolism induction –Antiacids, antiulcerous → decrease ICZ capsule absorption		
PCZ	*Candida* spp, *Aspergillus* spp, *C. neoformans, Trichosporon* spp, *P. boydii, P.aecilomyces spp,* dermatophytes, *Malassezia furfur, Fusarium* spp, *C. immitis, H. capsulatum, B. dermatitidis, Scedosporium* spp, *S. schenckii, Zygomycetes*	Gastrointestinal discomfort (vomiting, nausea, abdominal pain, diarrhea, dyspepsia, dry mouth, flatulence), migraine, dizziness, drowsiness, test alterations of liver function (ALT, AST, bilirubin, alkaline phosphatase, GGT), neutropenia, electrolytic imbalance (hypokalemia), anorexia, asthenia, fatigue, parestesia, fever, rash, QT interval	Inhibition of liver metabolism of other drugs (anticoagulants, oral antidiabetics, cisapride, cyclosporin, terfenadine, astemizole, benzodiazepines, calcioantagonists, quinadin, tacrolimus, sirolimus, vinca alkaloids, ergometrine alkaloids, statins) –Rifampicin, rifabutin, fenitoin, carbamazepine, fenobarbital → PCZ metabolism induction	Animal studies (skeletal anomalies in rats, embriotoxicity in rabbits). Category C No studies in humans	No studies in humans Excretion through maternal milk in animals studies (rats) Avoid exposure

		prolongation, liver damage, adrenal insufficiency	–Cimetidine → decreases PCZ absorption		
VCZ	*Candida* spp, *Aspergillus* spp, *C. neoformans, Rhodotorula spp, Trichosporon spp, dermitáceos, Paecilomyces spp,* dermatophytes, *P. marneffei, M. furfur, Fusarium spp, C. immitis, H. capsulatum, B. dermatitidis, Scedosporium* spp	Fever, migraine, abdominal pain, nausea, vomiting, diarrhea, peripheral edema, eyesight alterations (20–45%), increase of liver function test (10–20%), toxicodermia, photosensitivity	–Inhibition of liver metabolism of other drugs (anticoagulants, oral antidiabetics, cisapride, cyclosporin, terfenadine, astemizole, benzodiazepines, calcioantagonists, quinadin, tacrolimus, sirolimus, vinca alkaloids, ergometrine alkaloids, statins, omeprazole, fenitoine, protease inhibitors) –Rifampicin, rifabutin, fenitoin, carbamazepine, fenobarbital, ritonavir → VCZ metabolism induction –Cimetidine, erythromicine → VCZ metabolism inhibition	Unknown if it crosses placenta Teratogenic and embiotoxic (in animals) Lengthens duration of pregnancy and birth (animals) Teratogenic effects in humans (Category D) Evaluate risk/benefit	No data Avoid exposure
Echinocandins					
ANI	*Candida* spp, *Aspergillus* spp, *H. capsulatum, B. dermatitidis, C. immitis, S. schenckii, dermatiaceous, (Alternaria, Curvularia, Exophiala, Fonsecaea),*	Fever, migraine, abdominal discomfort, nausea, vomiting, diarrea, phlebitis, pruritos, bronchospasm, erythema, transitory	Cyclosporin increases the AUC of ANI by 22% Dosage adjustment not required when administered with cyclosporine, tacrolimus,	No data in humans Slight effect on development in rabbits Avoid use if there are alternatives	No data in humans Excreted through milk (animals) Avoid exposure

(*continued*)

Table 11.1 (continued)

	Susceptibility patterns	Adverse reactions	Interactions	Pregnancy	Breast feeding
	P. boydii, Paecilomyces variotii, Acremonium spp	increase of liver enzymes, hypomagnesiemia, hypokaliemia	rifampicine, voriconazole or AmB		
CAS	*Candida* spp, *Aspergillus* spp, *Histoplasma capsulatum, Blastomyces dermatitidis, Coccidioides immitis, Sporothrix schenckii, dermatiaceous (Alternaria, Curvularia, Exophiala, Fonsecaea), Pseudallescheria boydii, Paecilomyces variotii, Acremonium* spp	Safe and well tolerated (3–4% withdrawal due to ADR) Fever, migraine, abdominal discomfort, nausea, vomiting, diarrhea, phlebitis, hot flushes, shivers, tachycardia, dyspnea, anemia, ADR related with infusion (pain, pruritus, erythema), increase in liver enzymes (specially at high doses associated with cyclosporin), electrolytic imbalance (hypomagnesaemia, hypocalcemia, hypokalemia, hyponatremia)	CAS reduces tracolimus AUC by 20% Cyclosporin increases the AUC of CAS by 35% Enzymatic inducers (efavirenz, nevirapin, dexametasone, fenitoin, carbamenzapine...) can increase the AUC of CAS Rifampicin → inicial increase of AUC (60%) and Cmin (170%) of CAS → gradual decrease → Cmin decrease of CAS (30%) after 2 weeks	No data in humans Fetal toxicity (low weight, imcomplete ossification...) in animals (Category C) Crosses placenta (in animals)	No data in humans Excreted through milk (animals) Avoid exposure

AMB-d amphotericin B deoxycholate, *AMB-l* amphotericin B Liposome, *AMB-cl* amphotericin B lipid complex, *FCZ* fluconazole, *ICZ* itraconazole, *PCZ* posaconazole, *VCZ* voriconazole, *ANI* anidulafungin, *CAS* caspofungin, *ADR* adverse drug reaction, *AUC* area under curve, *Cmin* minimum plasma concentration

Table 11.2 Pharmakocinetic properties of antifungal agents

	Absorption	Distribution	Metabolism	Excretion	Dosage adjustment in RF	Dosage adjustment in HI
Polyenes						
AMB-d	No oral absorption	Vd: 4 l/kg PPB >90% Tissue distribution: Wide in liver, spleen, lung, kidney Low in CSF (2–4%)	No/unknown	Renal (40% dose in urine, 2–5% in 24 h → tissue accumulation) *t*½: 24 h Can appear in urine up to 8 weeks after treatment. Not removed by hemodialysis or peritoneal dialysis (<5%) → supplementary doses not required	Yes Evaluate interval increase (36 h) in severe RF (Cl-Cr<10 ml/min)	Dose adjustment not required
AMB-l	No oral absorption	Vd: 0.1–0.44 l/kg PPB >90% Tissue distribution: wide in liver, spleen, lung Low in kidney, heart, brain, lymph nodes Low in CSF	No/unknown	Renal excretion up to 10% *t*½: 7–153 h Not removed by hemodialysis or peritoneal dialysis (<5%) → supplementary doses not required	No data Evaluate interval increase (36 h) in severe RF (Cl-Cr<10 ml/min)	Dose adjustment not required
AMB-cl	No oral absorption	Vd: 2.3 l PPB >90% Tissue distribution: wide in liver, spleen. Low in kidney, lung (variable). Low in CSF	No/unknown	1% dose in urine after 7 days *t*½: 173–235 h Dose-dependent not linear kinetics Not removed by hemodialysis or peritoneal dialysis (<5%) → supplementary doses not required	No data Evaluate interval increase (36 h) in severe RF (Cl-Cr<10 ml/min)	Dose adjustment not required

(*continued*)

Table 11.2 (continued)

	Absorption	Distribution	Metabolism	Excretion	Dosage adjustment in RF	Dosage adjustment in HI
Triazoles						
FCZ	F: ~90% t_{max}: 0.5–6 h Food does not influence	Vd: 0.56–0.82 l/kg PPB: 11–12% t_{SS}: 7 days Tissue distribution: Wide (nails, skin, lung, pericardia, vagina, saliva, sputum, blisters). C_{CSF}: 50–88% of C_p	Low liver metabolism (~10%)	Renal: 61–88% (11% as metabolites) Feces: 2% not metabolized $t½$: 20–30 h Approx. 50% is removed by hemodialysis, → administer total dose after hemodialysis	Moderate RF (Cl-Cr: 20–50 ml/min) (therapeutic interval increase (48 h) Severe RF (Cl-Cr<20 ml/min) → half the dose and therapeutic interval increase (48 h)	Dose adjustment not required
ICZ	F: 20–100% F oral sol. > F caps (30–60%) t_{max}: 4 h (2.2–5 h) pH influence (caps) –Acidic drinks → absorption increase –Antiacids, anticancerous → absorption decrease Food influence: –Caps → F increase → take with food	Vd: 11 l/kg PPB >99% t_{SS}: 48 h (IV) Tissue distribution: Very Wide (kidney, liver, spleen, bone, stomach, muscle, skin, nails, lung, endometrium, cervical mucus, vagina, adipose tissue). Low in CSF (<5-%)	Wide liver metabolism (mainly by CYP3A4) Metabolism saturation Active metabolite: Hydroxi-ICZ ($Cp_{Hidroxi-ICZ} > Cp_{ICZ}$)	1% unaltered by urine (40% inactive metabolites) Feces: 55% (3–18% not metabolized) $t½$: 20–30 h Not removed by hemodialysis or peritoneal dialysis (<5%) → supplementary doses not require	Oral dose adjustment not required. IV administration should not be used in severe RF because of accumulation of hydroxypropyl-beta-cyclodextrin (toxic)	Dose adjustment not required

	–Oral sol. → F decrease → take without food					
PCZ	Saturation at dose >800 mg (If high Cp is necessary, shorten interval without increasing doseage) Administer with food or nutritional supplements (absorption increase)	Vd: 4.9–18.8 l/kg PPB >98% t_{ss} = 7–10 days Tissue distribution: very wide	Glucuronidation (UDP-Glucuronosyl-transferase) Inactive metabolites Not metabolized by CYP450	High fecal excretion as unaltered drug (∼66%) Low renal excretion (14% of the dose; <1% as unaltered drug) $t½$: 25–31 h Not removed by hemodialysis → supplementary doses not required	Dose adjustment not required	Dose adjustment not required (Consider in severe HI)
VCZ	Rapid (t_{max}: 1–2 h) and complete (F: 96%) absorption High fat meals decrease C_{max} (34%) and AUC (24%)	Vd: 4.6 l/kg PPB: 58% t_{SS} = 24 h with shock dosage/5–6 days without shock dosage Tissue distribution: Wide (brain, liver, kidney, heart, lung, spleen) C_{CSF}: 20–67% of Cp	Wide liver metabolism by citP450 (CYP2C19, CYP2C9 and CYP3A4) Genetic polymorphism (oriental people are slow acetylators) Main metabolite (N-oxyde) inactive	<2% elimination unaltered by urine (80% metabolites in urine) $t½$ dose-dependent (non-linear pharmakocinetic) → 6 h (with 200 mg oral administration) Low elimination by dialysis → supplementary doses not required. Possible utility of dialysis in case of intoxication by SBECD accumulation (sulfobutylether-β-cyclodextrin)	Oral dose adjustment unnecessary IV administration not recommended in moderate-severe RF by SBECD accumulation (nephrotoxic effect)	–Acute: Dose adjustment not required (monitor liver function) –Mild-Moderate. Reduce dose (same charge dose; half of the maintenance dose) –Severe → no data

(*continued*)

Table 11.2 (continued)

	Absorption	Distribution	Metabolism	Excretion	Dosage adjustment in RF	Dosage adjustment in HI
Echinocandins						
ANI	No oral absorption	Vd: 0.56 l/kg PPB: 99% t_{SS} = 24 h with shock dosage Tissue distribution: no studies	Spontaneous chemical degradation No liver metabolism	Fecal (>90%) and liver (<1%) as inactive metabolites t½: ~24 h Not removed by hemodialysis → supplementary doses not required	Dose adjustment not required	Dose adjustment not required
CAS	No oral absorption	Vd: 0.3 l/kg PPB: ~97% t_{SS} = 24 h with shock dosage Wide tissue distribution (liver, kidney, large intestine > spleen, small intestine, lung > heart, brain). No distribution in CSF	Spontaneous peptide hydrolysis and N-acetylation. Does not use citP450. Inactive metabolites	1.4% elimination unaltered by urine (41% metabolites in urine) Slow elimination (Cl: 10–12 ml/min) Polyphasic elimination (alpha, beta and gamma phases) Initial t½: 9–15 h t½: 40–50 h Not removed by hemodialysis → supplementary doses not required	Dose adjustment not required	–Mild: dose adjustment not required –Moderate: Reduce dose (70 mg day 1; then; 35 mg) –Severe. No experience

AMB-d amphotericin B deoxycholate, *AMB-l* amphotericin B Liposome, *AMB-cl* amphotericin B lipid complex, *FCZ* fluconazole, *ICZ* itraconazole, *PCZ* posaconazole, *VCZ* voriconazole, *ANI* anidulafungin, *CAS* caspofungin, *RF* renal failure, *HI* hepatic insufficiency, *F* bioavailability, C_{max} maximum plasma concentration, t_{max} time in which maximum plasma concentration is reached, *Vd* distribution volume, *PPB* plasma protein binding, t_{ss} time in which stationary state is reached, *CSF* cerebrospinal fluid, C_{LCR} concentration in cerebrospinal fluid, *Cp* plasma concentration, t½ elimination half-life, *Cl* clearance, *Cl-Cr* creatinin clearance

Table 11.3 Doses and administration routes of systemic antifungal drugs

	Presentation	Reconstitution volume	Diluent and concentration	Administration	Usual dose[c]
AmB[a]	Lyophilized 50 mg vial	10 ml API 5 mg/ml	Glucose 5% ≤0,1 mg/ml	Infusion time 2–6 h	Adults and children: 0.25–1 mg/kg/day Maximum dose: 1.5 mg/kg/day
AmB-LC[a]	50–100 mg vial, suspension for infusion (5 mg/ml in 10 and 20 ml)	–	Glucose 5% 1 mg/ml[b] 5 μ filter to add diluent	Dose test 1 mg/20ml × 15–20 min Unique infusion speed at 2.5 mg/kg/h	Adults and children: 3–5 mg/kg/day
AmB-L	Lyophilized 50 mg vial	12 ml API	Glucose 5% (10, 20%) No sodium chloride 0.9% 0.20–2 mg/ml	Infusion time 2 h (30–60 min if well tolerated)	Adults and children: 1–3 mg/kg/day Maximum dose: Up to 5 mg/kg/day
Anidulafungin	Lyophilized 100 mg vial	30 ml diluent from medication	0.9% Sodium chloride and 5% glucose Disolution 0.36 mg/ml	250 ml volume Infusion speed 3 ml/min	Adults: Dose charge 200 mg/day + 100 mg/day
Caspofungin	Lyophilized 50 and 70 mg vial	10.5 ml API 5 mg/ml	0.9% Sodium chloride and lactated ringer 100–250 ml		Dose charge 70 mg + 50 mg/day Weight > 80 kg: 70 mg/day
Fluconazole IV	2 mg/ml vial solution for 50, 100 and 200 ml perfusion	–	20% glucose, Ringer solution, Hartman solution, 4.2% sodium bicarbonate, 0.9% Sodium chloride.	Infusion speed ≤10 ml/min Administer 60 ml of the solution (200 mg)	Adults: 200–400 mg/day IV 100–400 mg/day oral Children: 3–12 mg/kg/day
Fluconazole oral	50, 100, 150 and 200 mg capsules 10 mg/ml (35 ml) Oral suspension	–	–	- Bioavailability ≈ 90% - Food does not affect	

(*continued*)

Table 11.3 (continued)

	Presentation	Reconstitution volume	Diluent and concentration	Administration	Usual dose[c]
Itraconazole IV	25 ml (250 mg) ampoules	100 ml bag, 0.9% Sodium chloride	Diluent common for the administration If another is used, it can precipitate	Infusion speed 1 ml/min	Adults: 200 mg/24 h IV 100–200 mg/12 h oral
Itraconazole oral	100 mg Capsules 200 mg/5 ml (150 ml) Oral solution	–	–	Bioavailability ≈ 55% → maximum when administered alter a full meal	
Posaconazole oral	40 mg/ml (200 mg/5 ml–105 ml) Oral suspension	–	–	– Low absorption and major Bioavailability with food rich in fat – Should be administered with food or food supplement	Adults: With food: 400 mg/12 h Without food: 400 mg/6 h
Voriconazole IV	Lyophilized 200 mg vial	19 ml API 10 mg/ml	No solutions with 4.2% sodium bicarbonate	Infusion time 1–2 h Maximum speed 3 mg/kg/h	Adults and children ≥12 years: 6 mg/kg/12 h the first day + 3–4 mg/kg/12 h following days If not tolerated: 3 mg/kg/12 h
Voriconazole oral	50 and 200 mg tablets Oral suspension powder 40 mg/ml	–	–	–Bioavailability ≈ 96% Administer 1 h before or after food	>40 kg: 200 mg/12 h ≤40 kg: 100 mg/12 h Maximum dose: 400 mg/12 h 200 mg/12 h When administered with fenitoin

AmB amphotericin B desoxycolate, *AmB-LC* amphotericin B lipid complex, *AmB-L* lipid Amphotericin B, *API* water for injectables

[a]It is recommended to administer test dose of 1 mg in 10–30 min in order to test tolerance. [b]In patients with cardiovascular illness, dilute in less volume until there is a 2 mg/ml concentration. [c]Patients with normal renal function

patients (Schulenburg et al. 2005; Speich et al. 2002). A recent publication analyzing the use of continuous infusion in cases of IFI affecting central nervous system, showed that administration of higher doses of AmB has an acceptable safety profile (Redmond et al. 2007).

Nevertheless, the use of continuous infusion is still controversial. Whilst some authors defend this strategy before opting for lipid or liposomic formulations (Johnson 2004; Schneemann and Bachli 2004), others do not consider it totally acceptable because of limited experience of its application. Moreover, the pharmakodynamics pattern of AmB (depending on concentration) favors the use of fractionated higher doses (Lewis and Wiederhold 2003). In the same way, Altmannsberger et al. (2007), when repeating the experience provided by Peleg and Woods (2004), found no significant advantages between intermittent and continuous infusion in patients with a high IFI risk.

11.3.2 Oral Route

The main characteristic data about antifungal oral administration are summarized in Table 11.3.

11.3.3 Inhalation Route

To date, the use of inhaled administration of antifungal drugs is limited. The scarce clinical experience with this route, and the complexity of establishing both the correct dose and ideal formulation makes it difficult to reach firm conclusions for their indications in patients at risk. Nevertheless, this route has the advantage of reaching high concentrations of the antifungal in the site of infection (lung), reducing systemic adverse reactions (Clemente et al. 2007). AmB has been used for more than a decade in the IFI prophylaxis of immunocompromised patients (neutropenic, TPH especially allogenic, lung transplant and other transplant patients at high risk) (Sole and Nieto 2007). Published results for the use of inhalated AmB for prophylaxis of IFI in lung transplant patients have demonstrated a reduction of episodes of IFI and a safety profile in this population (Alexander et al. 2006; Clemente et al. 2007; Drew 2006; Drew et al. 2004; Lowry et al. 2007; Mohammad and Klein 2006; Palmer et al. 2001)

Recently, the *in vitro* suitability of caspofungin for aerosol administration has been characterized (Wong-Beringer et al. 2005). Caspofungin solution with adjustments appears to have physicochemical and aerodynamic characteristics suitable for aerosolization. However, further *in vivo* testing is warranted.

In conclusion, aerosolized antifungal therapy is a promising route of drug delivery for pulmonary aspergillosis, due to attainment of high localized concentrations (Mohammad and Klein 2006; Sole and Nieto 2007).

11.4 Treatment of Invasive Candidiasis in ICU Patients and De-Escalation Therapy

A 2006 publication has shown a shift toward the use of antifungal drugs other than fluconazole due to the increasing number of non-*C.albicans* (NCA) isolates (Sendid et al. 2006). Consequently, the application of an early de-escalation therapy in critically ill patients with fungal infection should be recommended (Zaragoza and Pemán 2006). For this reason, the increasing prevalence of NCA isolates voriconazole (due to its broad spectrum and good profile in the ICU setting), caspofungin, anidulafungin, and micafungin (particularly in renal dysfunction) could be attractive options in critically ill patients. Finally, the choice of antifungal drug must be based on the individual characteristics of the patient, and particularly focus on the presence of renal or hepatic failure, hemodynamic instability, and possible interactions with other drugs. The presence of hemodynamic instability is a major factor for choosing empirical therapy. This fact has been considered by guidelines published recently enhancing a tailored therapy (de-escalation), especially with severe sepsis or septic shock (Fluckiger et al. 2006; Pappas et al. 2004). All these guidelines, including the last ongoing IDSA guidelines, heavily recommend the use of echinocandins in noneutropenic patients with IC when hemodynamic instability is present. The high rate of clinical success of these agents in candidemia, their low toxicity, their excellent safety profile and their broad spectrum against non-*albicans* species make this recommendation feasible. A recent publication has corroborated the use of caspofungin in critically ill patients (DiNubile et al. 2007).

In conclusion, IFIs, especially in the critical care setting, have become an excellent target for prophylactic, empiric, and pre-emptive therapy interventions due to high morbidity and mortality rates, an increasing incidence, and associated healthcare costs. Early diagnosis and treatment are associated with a better prognosis in IC. Invasive candidiasis in critically ill patients can be treated with fluconazole, voriconazole, AmB, or echinocandins. Finally, the choice of antifungal drug must be based on the individual characteristics of the patient, and particularly focus on the presence of renal or hepatic failure and possible interactions with other drugs. A strategy for the de-escalation of antifungal drugs, and the diagnosis status–treatment protocol, are proposed in Fig. 11.1.

11.5 Combination Therapy

11.5.1 ICU Patients

The availability of new antifungal agents with single mechanisms of action and improved tolerability has widened the possibility for the use of combination antifungal therapy (i.e., a combination of two antifungal drugs) for difficult-to-treat,

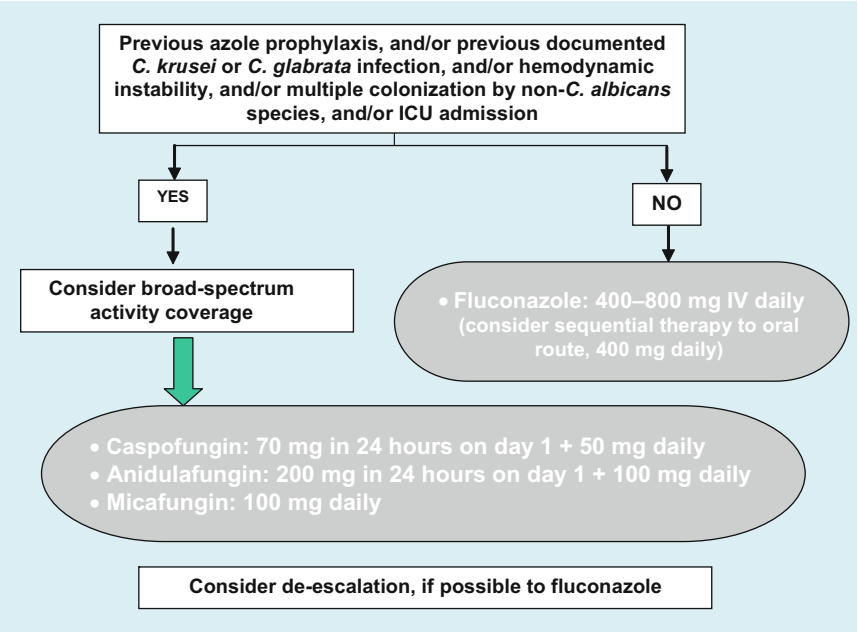

Fig. 11.1 Hospital algorithm for antifungal drug use for suspected or proven invasive candidiasis, based on a de-escalation strategy. *ABLC*: amphotericin B lipid complex; *BID*: twice daily; *ICU*: intensive care unit; *IV*: intravenous

opportunistic mycoses. Furthermore, additive *in vitro* interactions of voriconazole and the echinocandins have been observed. Few randomized clinical trials have examined the role of this type of therapy for invasive mycoses, and no prospective randomized trial of antifungal combinations has been completed for invasive mold infections. The results of *in vitro* studies and animal models suggest that combination therapy with azoles and echinocandins may have additive activity against *Aspergillus* species and suggest a great potential for combination therapy, confirming the need for further investigation (Marr 2004). However, the possible benefit of combination therapy with voriconazole for disseminated cryptococcosis and invasive candidiasis (or other emerging yeasts) needs to be elucidated. At present, a combination of Amb plus flucytosine or monotherapy with fluconazole, caspofungin, voriconazole, or Amb may be more desirable in these settings.

We must also consider the association of other drugs with antifungal agents as combination therapy. For instance, Mycograb (NeuTec Pharma, Manchester, UK), a human recombinant monoclonal antibody against heat-shock protein 90, has been shown to act synergistically with Amb *in vitro* against a broad spectrum of *Candida* species (Matthews et al. 2003). Subsequently, a double-blind, randomized study was conducted to determine whether lipid-associated Amb plus Mycograb was superior to Amb plus placebo in patients with culture-confirmed invasive candidiasis (Pachl et al. 2006). Patients received a lipid-associated formulation of Amb

plus a 5-day course of Mycograb or placebo, having been stratified on the basis of Candida species (*C. albicans* vs NCA). A favorable overall response was defined as a complete clinical and mycologic response, with resolution of all signs and symptoms of candidiasis and culture-confirmed eradication of the pathogen.

At day 10, a favorable response was obtained for 29 of 61 patients (48%) in the Amb-alone group, compared with 47 of 56 patients (84%) in the Mycograb combination-therapy group [odds ratio (OR) 5.8, 95% confidence interval 2.41–13.79; $P < 0.001$]. A greater percentage of patients in the combination-therapy group, compared with the Amb-alone group, met individual efficacy criteria, including clinical response (86% vs 52%, OR 5.4, 95% confidence interval 2.21–13.39; $P < 0.001$), mycologic response (89% vs 54%, OR 7.1, 95% confidence interval 2.64–18.94; $P < 0.001$), *Candida*-attributable mortality rate (4% vs 18%, OR 0.2, 95% confidence interval 0.04–0.80; $P = 0.025$), and rate of culture-confirmed clearance of the infection (hazard ratio 2.3, 95% confidence interval 1.4–3.8; $P = 0.001$). These results underscore the potential of combination therapy in critically ill patients. Nevertheless, further clinical trials are required to clarify this issue in the ICU setting.

11.5.2 Hematological Patients

It is difficult to say something specific of hematological patients because most of our current knowledge on IFI comes from patients with hematological malignancies treated with myelotoxic chemotherapy followed in most instances by an allogeneic hematopoietic stem cell transplant (HSCT). However, from a hematological point of view, several considerations can be made. Firstly, it should be remembered that hematological patients are prone to IFI since most of them receive myelotoxic chemotherapy and usually have more than one of the well-known risk factors for IFI (e.g., long-lasting neutropenia, older age, active cancer, corticosteroid therapy, administration of broad spectrum antibiotics, allogeneic HSCT, central venous catheter, or organ dysfunction). Secondly, hematological patients are usually polymedicated and are thus exposed to harmful drug interactions. Thirdly, it is important to emphasize that the same individual patient will be at risk at several time-points through the entire treatment plan for his/her underlying disease. Thus, the planned antineoplastic treatment should be kept in mind when designing antifungal strategies for hematological patients. Historically, outcomes for IFI have been disappointing and associated with a high mortality rate.

Poor response rates for primary and salvage monotherapy therapy for IFI and the availability of increasingly safer agents with differing mechanisms of action have prompted hematologists to use early combination therapy. Arguments for considering combination therapy include enhanced fungal killing (synergy), an enhanced spectrum of activity, prevention of development of resistance, and reduction of drug-related toxicities (Kontoyiannis and Lewis 2004). Favorable responses were observed among HSCT patients failing polyene-based therapy for IA with a

combination of voriconazole and caspofungin compared to voriconazole monotherapy (Marr et al. 2004). Recently, a French multicenter randomized study comparing combination therapy with AmB-L (3 mg/kg/d) plus caspofungin (70 mg/day and 50 mg/d thereafter) versus high-dose AmB-L monotherapy (10 mg/kg/d) for primary treatment of IA was published (Caillot et al. 2007). A favorable overall response was observed in 67% of combination therapy recipients compared to 27% of high-dose therapy recipients ($P = 0.028$). The results of this small pilot, representing the first prospective study of combination therapy in IA, are encouraging but need confirmation. Combination antifungal therapies are expensive and potentially toxic and, despite some recent encouraging reports, there are limited well-designed randomized-controlled trials to guide the practicing clinician faced with managing these problems.

Because echinocandins and triazoles are different classes of antifungal drugs with different modes of action, the use of this two-drug combination has recently received much attention in medical mycology. Several *in vivo* and *in vitro* studies with echinocandins and voriconazole against aspergilli have been evaluated and all show a lack of antagonism. Considering the advent of antifungal combination in hematological patients, it will be highly interesting to investigate the use of voriconazole with anidulafungin. At present, only a few data are available on the use of this combination therapy in IFI. In 5 years, we will have a better understanding of the *in vitro–in vivo* correlation of anidulafungin against infections due to *Aspergillus* spp. Further clinical trials are warranted to define whether anidulafungin is a useful candidate for combination with voriconazole for antifungal therapy. Therefore, well-defined clinical trials are necessary to define the most efficacious antifungal combination. In these clinical trials, the evaluation of an adverse event profile, as well as the pharmaco-economic impact, should also be taken into account. The answers to these and other questions will help define the future directions in antifungal therapy in leukemia and HSCT patients.

11.5.3 Solid Organ Transplant Recipients

Renal, liver, heart, and lung transplantations are now considered to be the standard therapeutic interventions in patients with end-stage organ failure. Infectious complications following solid organ transplantation are relatively common due to the transplant recipients' overall immunosuppressed status. The incidence of invasive mycoses following solid organ transplant ranges from 5 to 60% depending on the organ transplanted. Moreover, invasive fungal infection accounts for significant morbidity and mortality in solid organ transplantation, ranging between 25 and 95% depending on the type of fungus and its organ localization. For diverse reasons, it is therefore better to consider in the following section differentiating between patients with lung transplant (LT) and the recipients of other types of non-pulmonary solid organ transplantation.

11.5.3.1 Lung Transplantation

IFI occur in 15–35% of patients after lung transplant (LT) and over 80% are caused by *Candida* spp. and *Aspergillus* spp., with an overall mortality rate of nearly 60% (Alexander and Tapson 2001; Kotloff et al. 2004; Marik 2006; Segal and Walsh 2006; Singh and Husain 2003; Sole et al. 2005). However, the overall incidence of invasive mycoses in LT has declined over the past decade. This may be related to improved surgical techniques, decreases in the length of operations, units of blood transfused, more effective prophylactic strategies, and refinements in immunosuppressive regimens.

Data from the compilation and synthesis of existing studies give a variable incidence of *Aspergillus* infection of 6% in the published series (ranging from 2.2 to 30%). These wide ranges translate the differences in definition criteria for IA, immunosuppressive therapy, and antifungal prophylaxis existing in each lung transplant program. It is known that infection by *Aspergillus* may manifest in lung transplant recipients in three different forms: colonization, tracheobronchitis/anastomotic infections, or invasive pulmonary/disseminated aspergillosis (Nicod et al. 2001). Although cases of allergic bronchopulmonary aspergillosis (ABPA) have been reported, this is a rare eventy that only occurs in transplant patients with CF (Helmi et al. 2003).

To prevent invasive pulmonary aspergillosis, multiple strategies and antifungal drugs have been utilized such as oral itraconazole, voriconazole, or aerosolized AmB used alone or in combination. Aerosolized-medication regimens are an attractive option, as drug interactions and systemic toxicities are likely to be limited (Drew 2006). Several centers have reported on the safety of aerosolized AmB with a variety of dosing regimens (Calvo et al. 1999; Monforte et al. 2003; Reichenspurner et al. 1997), and others with aerosolized Amb lipid formulations (Drew et al. 2004; Lowry et al. 2007; Palmer et al. 2001). Our institution has used aerosolized AmB as part of the post-lung transplant protocol since 1994 (Calvo et al. 1999). For the last 3 years we have also been using Amb lipid complex, with the same respiratory tolerability and safety as aerosolized AmB, but the Amb lipid complex results in patients being more comfortable for long periods of time (50 mg inhaled/weekly), and they are more likely to adhere to the treatment. With regard to oral prophylaxis, a study (Husain et al. 2006) was recently published which examined the efficacy and toxicity of a strategy of universal *de novo* antifungal prophylaxis with voriconazole compared to targeted antifungal prophylaxis. The main finding of this study was that the overall rate of IA at 1 year decreased to 1.5% with universal voriconazole prophylaxis as compared to 23.5% with a targeted prophylaxis strategy. Interestingly, the rate of *Candida* colonization, particularly non-*albicans* species, in the voriconazole group was significantly higher. In the voriconazole prophylaxis cohort, 27% of the lung transplant recipients had normal liver enzymes throughout the course of the study. The main handicap of this azole therapy is the strong interaction with immunosuppressors which makes it necessary to monitor calcineurin inhibitors to avoid toxicity or rejection. Another interesting finding was that universal voriconazole prophylaxis did not increase the rate of non-*Aspergillus* fungal infections (especially zygomycosis).

Newer azoles (voriconazole, posaconazole) with predictable bioavailability should be preferred over the azole (itraconazole) with erratic bioavailability. Available echinocandins (caspofungin, micafungin, and anidulafungin) may have an important role in antifungal prophylaxis because of their antifungal profile, pharmacokinetics and security; however, they are expensive and need intravenous administration. Lipid preparations of Amb appear to be ideal for inhalational administration; however, there are not rigorous pharmacokinetic studies in lung transplant recipients to determine the appropriate dose and schedule of their administration. Monforte and colleagues have demonstrated that aerosolized AmB and lipid preparations of Amb are safe and achieve high concentrations in BAL fluid for the first 24 h and 14 days respectively, following nebulisation (Monforte et al. 2003, 2005). These lipid formulations permit delayed administration (every 7–14 days), which results in a bête acceptance by the patient. Although the incidence of IFI seems to be reduced with aerosolized AmB prophylaxis, the efficacy of this approach has not been determined in a large prospective clinical trial. Furthermore, without detectable levels of AmB in the circulation, extrapulmonary fungal infections may not be prevented by this strategy. In addition, it is important to take into consideration the type of delivery systems used for inhaled drugs (Corcoran et al. 2006; Hagerman et al. 2006). Contamination of the nebulization systems used in the prophylaxis with AmB nebulized in LT has also been described (Monforte et al. 2003). Contamination of the nebulizing systems may be the origin of respiratory infections, and occurs frequently when no strict cleaning and disinfection protocol is followed.

Another question is how long prophylaxis should be maintained. The majority of centers agree to apply universal prophylaxis during the first period post transplant (3 months); after this time, each center uses a tailored prophylaxis. It is also recommended to use nebulized antifungal prophylaxis and/or pre-emptive therapy with antifungal agents (voriconazole) in patients with chronic rejection and respiratory samples positive for *Aspergillus*, even without clinical or radiological signs, mainly in single lung transplant patients due to the high risk of IA (Sole et al. 2005). This pre-emptive treatment should last for at least 6 months, the time-period over which colonization has been shown to precede disseminated infection (Singh and Husain 2003), and in some cases for life.

With regard to the treatment, voriconazole, an extended-spectrum highly lipophilic triazole with 98% oral bioavailability, is actually the first choice for initial therapy of IA in LT patients and other immunosuppressed hosts. Other potentially effective therapies include lipid formulations of Amb and echinocandins. Combination therapy using a triazole and an echinocandin has been evaluated in SOT, with a significant reduction in mortality in those patients with renal failure and infected by *A. fumigatus* (Singh et al. 2006).

11.5.3.2 Non-Pulmonary Solid Organ Transplantation

Frequency, incidence, and clinical and epidemiological characteristics of IFIs in recipients of non-pulmonary solid organ transplantation (NP-SOT) are very

different from those which occur in patients with lung transplantation (LT) and hematopoietic stem cells transplantation (HSCT). The incidence of fungal infection varies with type of SOT Table 11.4, though *Candida* spp. and *Aspergillus* spp. account for most IFI in SOT recipients (Silveira and Husain 2007). Liver transplant recipients have the highest reported incidence of *Candida* infections, while lung transplant recipients have the highest rate of *Aspergillus* infections (Singh 2005;

Table 11.4 Etiological and clinical characteristics of IFI according to the type of SOT

Organ	Incidence of IFI (%)	Usual etiologic agent (s)	Variables portending higher risk of IA	Mortality (%) of IA
Renal	0–20	76–95% *Candida* (mainly urinary tract infections), 0–25% *Aspergillus*	Graft failure requiring hemodialysis, high level and prolonged duration of corticosteroids	77
Heart	5–20	70–90% *Aspergillus*, 8–25% *Candida*	Isolation of *Aspergillus* species in respiratory tract cultures, reoperation, post-transplant hemodialysis, CMV disease	78
Liver	5–40	35–90% *Candida*, 9–45% *Aspergillus*	Retransplantation, renal failure (particularly requiring renal replacement therapy), fulminant hepatic failure as an indication for transplantation, primary allograft failure, high transfusion requirements, use of monoclonal antibodies,	87
Lung/Heart-Lung	10–45	43–72% *Candida*, 25–50% *Aspergillus*	Single lung transplant, CMV infection, rejection and augmented immunosuppression, obliterative bronchitis, *Aspergillus* colonization, acquired hypogammaglobulinemia, presence of bronchial stents, reperfusion injury, airway ischemia	68
Pancreas (+ kidney)	10–40	>90% *Candida*, 0–3% *Aspergillus*	Similar factors to the liver and kidney transplant, graft lost (vascular graft thrombosis, post-reperfusion pancreatitis), enteric drainage, alemtuzumab-containing immunosupressive regimen	100
Small bowel	30–60	80–100% *Candida*, 0–5% *Aspergillus*	Not clearly determined, similar factors to others intra-abdominal SOT recipients; Graft rejection/ dysfunction, enhanced immunosuppression , anastomotic disruption, multi-visceral transplant	66

SOT solid organ transplantation/solid organ transplant recipients, *IFI* invasive fungal infection, *IA* invasive aspergillosis, *CMV* cytomegalovirus

Singh and Paterson 2005; Sole et al. 2005; Sole and Salavert 2008). Recent epidemiological studies suggest the emergence of resistant strains of *Candida* as well as mycelial fungi other than *Aspergillus* in these patients. Moreover, significant percentages of fungal infections are occurring late in the course of transplantation. SOT recipients are also at risk for *Cryptococcus* infections (Singh et al. 2007) and reactivation of endemic mycoses such as histoplasmosis and coccidiomycosis. Recent changes in the epidemiology of fungal infections in SOT recipients have occurred and newer data on the diagnosis, prophylaxis, and treatment of fungal infections in these patients have appeared in the last years. Emergence of newer and more potent antifungal agents with lower toxicity potentially changes the concept of antifungal prophylaxis (Metcalf and Dockrell 2007; van Burik 2005).

Targeted prophylaxis against *Candida* and *Aspergillus* species is recommended in all SOT, with the exception of LT, where universal prophylaxis against *Aspergillus* species is recommended owing to the lack of prospective data on the risk factor analysis. Fluconazole should be used for prophylaxis against *Candida* species unless the institution has a high rate of non-*albicans* infections. In this case, Amb preparations, echinocandins, or newer azoles should be used. Voriconazole can be used for prophylaxis against *Aspergillus* species; alternatives include posaconazole, echinocandins, and Amb preparations. Inhaled Amb can also be used in NP-SOT, as is the case in LT, but there are not sufficient data to justify this type of prophylaxis. In any case, the accurate duration of prophylaxis still remains poorly defined owing to the lack of randomized clinical trials.

Therefore, the recommendations for prophylaxis against *Aspergillus* in NP-SOT (especially in liver transplant recipients) are a lipid formulation of Amb (at doses of 2.5–5 mg/kg/day), voriconazole, or caspofungin, with a duration of 3–4 weeks or until resolution of risk factors. Antifungal prophylaxis may be warranted whenever more than one risk factor is present. Routine antifungal prophylaxis is not warranted in heart transplant recipients, although in patients deemed to be at high risk (Table 11.4), itraconazole may be an option (Munoz et al. 2004). Oral itraconazole at 400 mg daily from day 5 after transplantation for 3–6 months has been associated with a significantly lower incidence of IA than in an earlier cohort that did not receive antifungal prophylaxis (2 vs 9.6%; $P < 0.05$).

The availability of new antifungal agents with unique mechanisms of action and improved tolerability has widened the possibilities for the use of combination antifungal therapy for difficult-to-treat opportunistic mycoses (Chamilos and Kontoyiannis 2006), especially against serious and disseminated forms of invasive aspergillosis.

An important advance in the antifungal armamentarium is the availability of the newer broad-spectrum azoles voriconazole and posaconazole, which have been studied for IA (Raad et al. 2007; Walsh et al. 2007). Researchers have studied the echinocandin class as a salvage therapy in IA, and results have been encouraging, making potential combination therapy with these drugs attractive (Denning et al. 2006; Maertens et al. 2004, 2006). Generally, for *in vitro* studies, combinations of AmB and azoles against *Aspergillus* spp show indifference or antagonism. Studies of AmB in combination with echinocandins have ranged from indifference to

synergy (Arikan et al. 2002). Combinations of the echinocandins with triazoles range from synergistic activity to indifference, but, importantly, no antagonism has been reported (Perea et al. 2002). Animal models of IA generally confirm results predicted by *in vitro* combination tests. Antagonism is often seen in the combination of AmB plus azoles (Clemons et al. 2005; Polak et al. 1982). Recently, several important *in vivo* studies evaluating newer azoles and echinocandin combinations have suggested that combination therapy is superior to single therapy by clinical parameters such as survival, galactomannan antigenemia, and reduced colony counts in tissues (Kirkpatrick et al. 2002; Petraitis et al. 2003). Importantly, no antagonism was demonstrated in these studies. Other studies have evaluated AmB and echinocandin combinations with favorable results, showing that combination therapy resulted in reduced kidney burden of organisms, increased survival, and improved histopathologic findings with combination therapy. However, a study by Petraitis et al. (1999) found neither synergy nor antagonism with the combination of micafungin and AmB. These *in vivo* studies demonstrated that combinations of expanded-spectrum triazoles plus an echinocandin or AmB plus an echinocandin have clinical potential and warrant further clinical investigation.

Antifungal combinations are increasingly used in clinical practice to improve outcomes for refractory mycoses. However, the use of this therapy is largely governed by empiricism (Kontoyiannis and Lewis 2004). Although important clinical trials have used monotherapy for the treatment of aspergillosis, data from clinical studies on the use of combination antifungal therapy for aspergillosis are lacking. Most information on combination therapy for the treatment of aspergillosis is derived from retrospective case series and reviews, especially in hematological patients (Aliff et al. 2003; Kontoyiannis et al. 2003) or HSCT recipients, which do not allow for appropriate critical analyses. Only a small number of clinical studies report on the use of combination therapy of expanded-spectrum triazoles plus echinocandins or AmB preparations plus echinocandins for IA (Marr et al. 2004). Most studies are limited by retrospective evaluation, use of historical controls, and use of combination therapy as salvage treatment. Nevertheless, these studies, reviewed below, bring attention to toxicity concerns and important design issues that are necessary for future successful combination therapy clinical trials.

No single randomized study on antifungal combination therapy in SOT patients has been performed (Baddley and Pappas 2005). Existing information does not support the use of combination therapy in invasive candidiasis in SOT patients. Indeed, initial combination therapy with Amb and flucytosine is recommended for SOT patients with central nervous system cryptococcosis. The combination of voriconazole and terbinafine may be an attractive option for *S. prolificans* infections. The aggregate of current data does not support the primary use of combination antifungal therapy for IA infections. However, for patients who develop breakthrough infections while receiving first-line monotherapy or have more resistant molds, combination antifungal therapy may provide additional antifungal activity and may be warranted (Wirk and Wingard 2008). A prospective study of voriconazole plus caspofungin as initial therapy for IA in SOT patients found that combination therapy was independently associated with reduced mortality in patients with renal failure

Table 11.5 Indications of combined antifungal therapy in IFI[a,b,c].(Almyroudis and Segal 2009; Baddley et al. 2008; Baddley and Pappas 2005; Bohme et al. 2009; Falcone et al. 2009; Gadea and Mensa 2008; Kontoyiannis et al. 2009; Mensa et al. 2009; Mukherjee et al. 2005; Munoz et al. 2006; Pachl et al. 2006; Pappas et al. 2009a,b; Perkhofer and Lass-Florl 2009; Reed et al. 2008; Salavert and Zaragoza 2009; Segal 2009; Sloan et al. 2008; Spellberg et al. 2009; Steinbach et al. 2005; Walsh et al. 2008; Wirk and Wingard 2008)

Fungal infection (evidence degree[d])	Indications	Scheme of combined treatment	
		Primary	Alternative
Candidiasis (A-II, B-I, B-III)	–Salvage treatment after monotherapy failure in severe ill (APACHE > 20) or immunocompromised patients –Candidemia or invasive candidiasis in patients with prolonged neutropenia (>2 weeks) and metastasic lesions due to haematogenous dissemination –Non surgical native valve endocarditis or after monotherapy failure –Prosthetic valve endocarditis with septic metastasis –CNS ventriculitis –Osteomyelitis –Foreign bodies infected with difficult replacement or withdraw	Lipid formulation of Amb + fluconazole [both at candidemia or invasive candidiasis doses]	Echinocandin + 1st or 2nd generation azole [standardized doses] Lipid formulations of Amb + efungumab (Mycograb™) [1 mg/kg/12 h, iv, 5 days]
Aspergillosis (B-II, C-II, C-III)	–Salvage treatment after monotherapy failure –CNS infection –Severe disseminated infection (sepsis or organ dysfunction) in immunocompromised patients (SOT, HSCT, HIV with CD4+ <100/mm^3) –Severe pulmonary infection –Transplant recipients with risk factors: renal failure, GVHD, high doses of steroids, anti-TNF	Voriconazole + echinocandin [standardized doses]	Echinocandin [standardized doses] + Lipid formulation of Amb [5–7.5 mg/kg/day]
Cryptococosis (A-I, B-II)	–Meningitis (induction therapy in HIV+) –Severe disseminated infection in patients non-HIV+ –Prolonged immunosuppression –Immune reconstitution failure –Therapeutic failure or salvage therapy	–Induction therapy: Liposomal Amb [4 mg/kg/day] + flucytosine [25 mg/kg/6 h, po], 2 weeks –No in consolidation therapy	Fluconazole [400–800 mg/day, iv/po] + flucytosine [25 mg/kg/6 h], 2 weeks Liposomal Amb [4 mg/kg/day, iv] + fluconazole [400–800 mg/day, iv/po], or voriconazole [standardized doses], or posaconazole [200 mg/6 h, po]

(*continued*)

Table 11.5 (continued)

Fungal infection (evidence degree[d])	Indications	Scheme of combined treatment	
		Primary	Alternative
Zygomycosis (A-III, B-II)	–Salvage treatment after monotherapy failure –Rhinocerebral or severe pulmonary infection –Severe disseminated infection in immunocompromised patients	Liposomal Amb [5–7.5 mg/kg/day, iv] + caspofungin [standardized dose]	Lipid formulation of Amb [5–7.5 mg/kg/day] + other echinocandin [standardized doses] Posaconazole [200 mg/6 h, po] + caspofungin [standardized doses] Lipid formulation of Amb [5–7.5 mg/kg/day] [#] + posaconazole [200 mg/6 h, po]
Other mycoses (B-III, C-III)	–*Scedosporiosis* –*Fusariosis* –*Blastoschizomycosis* –*Trichosporonosis*	Voriconazole + terbinafine [500 mg/day, po] Voriconazole + Lipid formulation of Amb [standardized dose] 1st or 2nd generation azole [high doses] + Lipid formulation of Amb Voriconazole + Lipid formulation of Amb [standardized dose]	1st or 2nd generation azole + terbinafine ± echinocandin Voriconazole or lipid formulation of Amb + caspofungin, Posaconazole + Lipid formulation of Amb Lipid formulation of Amb + flucytosine [100 mg/kg/day, po] or echinocandin [standardized dose] Posaconazole + Lipid formulation of Amb [standardized dose]

1st generation azoles: ketoconazole, fluconazole, itraconazole; 2nd generation azoles: voriconazole, posaconazole; Echinocandins: caspofungin, anidulafungin and micafungin

po per os (by mouth), *iv* intravenous, *CNS* central nervous system, *SOT* solid organ transplant, *HSCT* haematopoietic stem cell transplantation, *HIV* human immunodeficiency virus, *GVHD* graft-versus-host disease, *TNF* tumor necrosis factor

[a]It is desirable that diagnosis and treatment of these mycoses be guided by a mycologist and an infectious diseases consultant

[b]Some of these mycoses could need instrumental (catheter, prosthetic devices or foreign bodies withdraw) or surgical handling

[c]Length of treatment is difficult to determine in these infections and must be individualized

[d]The evidence degree is based in the IDSA (*Infectious Diseases Society of America*) categorizations

and in those with *A. fumigatus* infection, even when adjusted for other predictive factors of mortality in the study population (Singh et al. 2006). Investigators treated 40 IA patients with voriconazole plus caspofungin and compared them to 47 historical controls treated with a lipid formulation of AmB. Survival at 90 days was 67.5% (27/40) in the voriconazole and caspofungin group and 51% (24/47) in the control group ($P = 0.117$). Successful outcome was greater in the voriconazole and caspofungin group compared to historical controls (70% vs 51%; $P = 0.08$), but the difference was not statistically significant. In a multivariate analysis of factors associated with survival, in patients with renal failure (HR 0.32, 95% CI, 0.12–0.85, $P = 0.02$), or *A.fumigatus* infection (HR 0.37, 95% CI, 0.16–0.84, $P = 0.019$), combination therapy was independently associated with improved survival. This study is important because it included many solid organ transplant recipients, but it is limited by the use of a historical control group.

Because of the previously mentioned limitations, responses with combination therapy must be interpreted with caution. Importantly, these studies suggest that combination therapies are relatively safe and lack antagonism, but the superiority of combination therapy has not yet been determined. Moreover, drug interactions and health care costs associated with combination antifungal therapy have not been fully elucidated. Considering the *in vitro*, *in vivo*, and available clinical data for AmB plus echinocandins and triazoles plus echinocandins, these important clinical research questions require a randomized clinical trial.

In summary, combination therapy, sometimes including surgical resection, should be considered for severe forms of IFI and IA in SOT patients (Table 11.5); however, multicenter studies of such patients are urgently needed. A large clinical trial studying combination therapy for IA is feasible but would require a collaborative international effort and substantial support from both the pharmaceutical industry and clinicians.

11.6 Conclusion

In summary, combination therapy, sometimes including surgical resection, should be considered for severe forms of IFI in critically ill, hematological, and SOT patients (Table 11.5) according to the type of invasive fungal infection, especially when *Aspergillus* spp is involved; however, multicenter studies of such patients are urgently needed. A large clinical trial studying combination therapy for IA is feasible but would require a collaborative international effort and substantial support from both the pharmaceutical industry and clinicians in order to clarify the potential benefits of this strategy and more specifically the role of azoles and echinocandins combination.

The application of this approach should always be considered in the severe forms of these infections. De-escalation strategy offers a complementary role to combination therapy, allowing the narrowing of the spectrum of the antifungal therapy when the clinical situation of the patient has been improved.

References

Alexander BD, Tapson VF (2001) Infectious complications of lung transplantation. Transpl Infect Dis 3:128–137

Alexander BD, Dodds Ashley ES, Addison RM, Alspaugh JA, Chao NJ, Perfect JR (2006) Non-comparative evaluation of the safety of aerosolized amphotericin B lipid complex in patients undergoing allogeneic hematopoietic stem cell transplantation. Transpl Infect Dis 8:13–20

Aliff TB, Maslak PG, Jurcic JG, Heaney ML, Cathcart KN, Sepkowitz KA, Weiss MA (2003) Refractory Aspergillus pneumonia in patients with acute leukemia: successful therapy with combination caspofungin and liposomal amphotericin. Cancer 97:1025–1032

Almyroudis NG, Segal BH (2009) Prevention and treatment of invasive fungal diseases in neutropenic patients. Curr Opin Infect Dis 22:385–393

Altmannsberger P, Holler E, Andreesen R, Krause SW (2007) Amphotericin B deoxycholate: no significant advantage of a 24 h over a 6 h infusion schedule. J Antimicrob Chemother 60:180–182

Arikan S, Lozano-Chiu M, Paetznick V, Rex JH (2002) In vitro synergy of caspofungin and amphotericin B against *Aspergillus* and *Fusarium* spp. Antimicrob Agents Chemother 46:245–247

Baddley JW, Pappas PG (2005) Antifungal combination therapy: clinical potential. Drugs 65:1461–1480

Baddley JW, Benjamin DK Jr, Patel M, Miro J, Athan E, Barsic B, Bouza E, Clara L, Elliott T, Kanafani Z, Klein J, Lerakis S, Levine D, Spelman D, Rubinstein E, Tornos P, Morris AJ, Pappas P, Fowler VG Jr, Chu VH, Cabell C (2008) *Candida* infective endocarditis. Eur J Clin Microbiol Infect Dis 27:519–529

Bohme A, Ruhnke M, Buchheidt D, Cornely OA, Einsele H, Enzensberger R, Hebart H, Heinz W, Junghanss C, Karthaus M, Kruger W, Krug U, Kubin T, Penack O, Reichert D, Reuter S, Silling G, Sudhoff T, Ullmann AJ, Maschmeyer G (2009) Treatment of invasive fungal infections in cancer patients – recommendations of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). Ann Hematol 88:97–110

Caillot D, Thiebaut A, Herbrecht R, de Botton S, Pigneux A, Bernard F, Larche J, Monchecourt F, Alfandari S, Mahi L (2007) Liposomal amphotericin B in combination with caspofungin for invasive aspergillosis in patients with hematologic malignancies: a randomized pilot study (Combistrat trial). Cancer 110:2740–2746

Calvo V, Borro JM, Morales P, Morcillo A, Vicente R, Tarrazona V, Paris F (1999) Antifungal prophylaxis during the early postoperative period of lung transplantation. Valencia lung transplant group. Chest 115:1301–1304

Chamilos G, Kontoyiannis DP (2006) The rationale of combination antifungal therapy in severely immunocompromised patients: empiricism versus evidence-based medicine. Curr Opin Infect Dis 19:380–385

Clemente BS, Fernandez PA, Gil LG, Cabanas Poy MJ, Oliveras AM, Hidalgo AE (2007) Administration of anti-infective agents through the inhaled route. Farm Hosp 31:112–119

Clemons KV, Espiritu M, Parmar R, Stevens DA (2005) Comparative efficacies of conventional amphotericin b, liposomal amphotericin B (AmBisome), caspofungin, micafungin, and voriconazole alone and in combination against experimental murine central nervous system aspergillosis. Antimicrob Agents Chemother 49:4867–4875

Corcoran TE, Venkataramanan R, Mihelc KM, Marcinkowski AL, Ou J, McCook BM, Weber L, Carey ME, Paterson DL, Pilewski JM, McCurry KR, Husain S (2006) Aerosol deposition of lipid complex amphotericin-B (Abelcet) in lung transplant recipients. Am J Transplant 6:2765–2773

Denning DW, Marr KA, Lau WM, Facklam DP, Ratanatharathorn V, Becker C, Ullmann AJ, Seibel NL, Flynn PM, van Burik JA, Buell DN, Patterson TF (2006) Micafungin (FK463),

alone or in combination with other systemic antifungal agents, for the treatment of acute invasive aspergillosis. J Infect 53:337–349
DiNubile MJ, Lupinacci RJ, Strohmaier KM, Sable CA, Kartsonis NA (2007) Invasive candidiasis treated in the intensive care unit: observations from a randomized clinical trial. J Crit Care 22:237–244
Drew R (2006) Potential role of aerosolized amphotericin B formulations in the prevention and adjunctive treatment of invasive fungal infections. Int J Antimicrob Agents 27(Suppl 1):36–44
Drew RH, Dodds AE, Benjamin DK Jr, Duane DR, Palmer SM, Perfect JR (2004) Comparative safety of amphotericin B lipid complex and amphotericin B deoxycholate as aerosolized antifungal prophylaxis in lung-transplant recipients. Transplantation 77:232–237
DRUGDEX® System (2008) Greenwood Village, CO, USA. http://www.thomsonhc.com. Thomson Healthcare
Eriksson U, Seifert B, Schaffner A (2001) Comparison of effects of amphotericin B deoxycholate infused over 4 or 24 hours: randomised controlled trial. Br Med J 322:579–582
Falcone M, Barzaghi N, Carosi G, Grossi P, Minoli L, Ravasio V, Rizzi M, Suter F, Utili R, Viscoli C, Venditti M (2009) *Candida* infective endocarditis: report of 15 cases from a prospective multicenter study. Medicine (Baltimore) 88:160–168
Fluckiger U, Marchetti O, Bille J, Eggimann P, Zimmerli S, Imhof A, Garbino J, Ruef C, Pittet D, Tauber M, Glauser M, Calandra T (2006) Treatment options of invasive fungal infections in adults. Swiss Med Wkly 136:447–463
Gadea I, Mensa J (2008) Potential of anidulafungin in combined therapy. Enferm Infecc Microbiol Clin 26(Suppl 14):51–55
Hagerman JK, Hancock KE, Klepser ME (2006) Aerosolised antibiotics: a critical appraisal of their use. Expert Opin Drug Deliv 3:71–86
Helmi M, Love RB, Welter D, Cornwell RD, Meyer KC (2003) *Aspergillus* infection in lung transplant recipients with cystic fibrosis: risk factors and outcomes comparison to other types of transplant recipients. Chest 123:800–808
Husain S, Paterson DL, Studer S, Pilewski J, Crespo M, Zaldonis D, Shutt K, Pakstis DL, Zeevi A, Johnson B, Kwak EJ, McCurry KR (2006) Voriconazole prophylaxis in lung transplant recipients. Am J Transplant 6:3008–3016
Johnson JR (2004) Reduction of nephrotoxicity associated with amphotericin B deoxycholate. Clin Infect Dis 38:303–307
Kirkpatrick WR, Perea S, Coco BJ, Patterson TF (2002) Efficacy of caspofungin alone and in combination with voriconazole in a guinea pig model of invasive aspergillosis. Antimicrob Agents Chemother 46:2564–2568
Kontoyiannis DP, Lewis RE (2004) Toward more effective antifungal therapy: the prospects of combination therapy. Br J Haematol 126:165–175
Kontoyiannis DP, Hachem R, Lewis RE, Rivero GA, Torres HA, Thornby J, Champlin R, Kantarjian H, Bodey GP, Raad II (2003) Efficacy and toxicity of caspofungin in combination with liposomal amphotericin B as primary or salvage treatment of invasive aspergillosis in patients with hematologic malignancies. Cancer 98:292–299
Kontoyiannis DP, Ratanatharathorn V, Young JA, Raymond J, Laverdiere M, Denning DW, Patterson TF, Facklam D, Kovanda L, Arnold L, Lau W, Buell D, Marr KA (2009) Micafungin alone or in combination with other systemic antifungal therapies in hematopoietic stem cell transplant recipients with invasive aspergillosis. Transpl Infect Dis 11:89–93
Kotloff RM, Ahya VN, Crawford SW (2004) Pulmonary complications of solid organ and hematopoietic stem cell transplantation. Am J Respir Crit Care Med 170:22–48
Lewis RE, Wiederhold NP (2003) The solubility ceiling: a rationale for continuous infusion amphotericin B therapy? Clin Infect Dis 37:871–872
Lowry CM, Marty FM, Vargas SO, Lee JT, Fiumara K, Deykin A, Baden LR (2007) Safety of aerosolized liposomal versus deoxycholate amphotericin B formulations for prevention of invasive fungal infections following lung transplantation: a retrospective study. Transpl Infect Dis 9:121–125

Maertens J, Raad I, Petrikkos G, Boogaerts M, Selleslag D, Petersen FB, Sable CA, Kartsonis NA, Ngai A, Taylor A, Patterson TF, Denning DW, Walsh TJ (2004) Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. Clin Infect Dis 39:1563–1571

Maertens J, Glasmacher A, Herbrecht R, Thiebaut A, Cordonnier C, Segal BH, Killar J, Taylor A, Kartsonis N, Patterson TF, Aoun M, Caillot D, Sable C (2006) Multicenter, noncomparative study of caspofungin in combination with other antifungals as salvage therapy in adults with invasive aspergillosis. Cancer 107:2888–2897

Marik PE (2006) Fungal infections in solid organ transplantation. Expert Opin Pharmacother 7:297–305

Marr K (2004) Combination antifungal therapy: where are we now, and where are we going? Oncology 18:24–29

Marr KA, Boeckh M, Carter RA, Kim HW, Corey L (2004) Combination antifungal therapy for invasive aspergillosis. Clin Infect Dis 39:797–802

Matthews RC, Rigg G, Hodgetts S, Carter T, Chapman C, Gregory C, Illidge C, Burnie J (2003) Preclinical assessment of the efficacy of Mycograb, a human recombinant antibody against fungal HSP90. Antimicrob Agents Chemother 47:2208–2216

Mensa J, Gatell JM, Azanza JR (2007) Guía de terapéutica antimicrobiana, 17th edn. Elsevier Doyma S.L., Barcelona

Mensa J, de la CR, Carreras E, Cuenca EM, Garcia Rodriguez JA, Gobernado M, Picazo J, Aguado JM, Sanz MA (2009) Treatment of fungal infections in patients with hematologic neoplasia. Med Clin (Barc) 132:507–521

Metcalf SC, Dockrell DH (2007) Improved outcomes associated with advances in therapy for invasive fungal infections in immunocompromised hosts. J Infect 55:287–299

Mohammad RA, Klein KC (2006) Inhaled amphotericin B for prophylaxis against invasive *Aspergillus* infections. Ann Pharmacother 40:2148–2154

Monforte V, Roman A, Gavalda J, Lopez R, Pou L, Simo M, Aguade S, Soriano B, Bravo C, Morell F (2003) Nebulized amphotericin B concentration and distribution in the respiratory tract of lung-transplanted patients. Transplantation 75:1571–1574

Monforte V, Roman A, Gavalda J et al (2005) Prophylaxis for *Aspergillus* infection in lung transplantation: pharmacokinetics, safety and efficacy. J Heart Lung Transplant 24: S108–S109, Abstract

Mukherjee PK, Sheehan DJ, Hitchcock CA, Ghannoum MA (2005) Combination treatment of invasive fungal infections. Clin Microbiol Rev 18:163–194

Munoz P, Rodriguez C, Bouza E, Palomo J, Yanez JF, Dominguez MJ, Desco M (2004) Risk factors of invasive aspergillosis after heart transplantation: protective role of oral itraconazole prophylaxis. Am J Transplant 4:636–643

Munoz P, Singh N, Bouza E (2006) Treatment of solid organ transplant patients with invasive fungal infections: should a combination of antifungal drugs be used? Curr Opin Infect Dis 19:365–370

Nicod LP, Pache JC, Howarth N (2001) Fungal infections in transplant recipients. Eur Respir J 17:133–140

Pachl J, Svoboda P, Jacobs F, Vandewoude K, der HB Van, Spronk P, Masterson G, Malbrain M, Aoun M, Garbino J, Takala J, Drgona L, Burnie J, Matthews R (2006) A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. Clin Infect Dis 42:1404–1413

Palmer SM, Drew RH, Whitehouse JD, Tapson VF, Davis RD, McConnell RR, Kanj SS, Perfect JR (2001) Safety of aerosolized amphotericin B lipid complex in lung transplant recipients. Transplantation 72:545–548

Pappas PG, Rex JH, Sobel JD, Filler SG, Dismukes WE, Walsh TJ, Edwards JE (2004) Guidelines for treatment of candidiasis. Clin Infect Dis 38:161–189

Pappas PG, Chetchotisakd P, Larsen RA, Manosuthi W, Morris MI, Anekthananon T, Sungkanuparph S, Supparatpinyo K, Nolen TL, Zimmer LO, Kendrick AS, Johnson P, Sobel

JD, Filler SG (2009a) A phase II randomized trial of amphotericin B alone or combined with fluconazole in the treatment of HIV-associated cryptococcal meningitis. Clin Infect Dis 48:1775–1783

Pappas PG, Kauffman CA, Andes D, Benjamin DK Jr, Calandra TF, Edwards JE Jr, Filler SG, Fisher JF, Kullberg BJ, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD (2009b) Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis 48:503–535

Peleg AY, Woods ML (2004) Continuous and 4 h infusion of amphotericin B: a comparative study involving high-risk haematology patients. J Antimicrob Chemother 54:803–808

Perea S, Gonzalez G, Fothergill AW, Kirkpatrick WR, Rinaldi MG, Patterson TF (2002) In vitro interaction of caspofungin acetate with voriconazole against clinical isolates of *Aspergillus* spp. Antimicrob Agents Chemother 46:3039–3041

Perkhofer S, Lass-Florl C (2009) Anidulafungin and voriconazole in invasive fungal disease: pharmacological data and their use in combination. Expert Opin Investig Drugs 18: 1393–1404

Petraitis V, Petraitiene R, Legit RJ (1999) Combination antifungal therapy with FK463 plus amphotericin B in treatment of experimental pulmonary aspergillosis. 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco (Abstract 2003)

Petraitis V, Petraitiene R, Sarafandi AA, Kelaher AM, Lyman CA, Casler HE, Sein T, Groll AH, Bacher J, Avila NA, Walsh TJ (2003) Combination therapy in treatment of experimental pulmonary aspergillosis: synergistic interaction between an antifungal triazole and an echinocandin. J Infect Dis 187:1834–1843

Polak A, Scholer HJ, Wall M (1982) Combination therapy of experimental candidiasis, cryptococcosis and aspergillosis in mice. Chemother 28:461–479

Raad II, Hanna HA, Boktour M, Jiang Y, Torres H, Afif C, Kontoyiannis DP, Hachem RY (2007) Novel antifungal agents as salvage therapy for invasive aspergillosis in patients with hematologic malignancies: posaconazole compared with high-dose lipid formulations of amphotericin B alone or in combination with caspofungin. Leukemia 22:496–503

Redmond A, Dancer C, Woods ML (2007) Fungal infections of the central nervous system: a review of fungal pathogens and treatment. Neurol India 55:251–259

Reed C, Bryant R, Ibrahim AS, Edwards J Jr, Filler SG, Goldberg R, Spellberg B (2008) Combination polyene–caspofungin treatment of rhino-orbital-cerebral mucormycosis. Clin Infect Dis 47:364–371

Reichenspurner H, Gamberg P, Nitschke M, Valantine H, Hunt S, Oyer PE, Reitz BA (1997) Significant reduction in the number of fungal infections after lung-, heart–lung, and heart transplantation using aerosolized amphotericin B prophylaxis. Transplant Proc 29:627–628

Richardson M, Jones B, Rautema R (2007) Therapeutic guidelines in systemic fungal infections, 4th edn. Remedica, London

Salavert M, Zaragoza R (2009) Future role of micafungin in the treatment of invasive mycoses caused by filamentous fungi. Rev Iberoam Micol 26:81–89

Schneemann M, Bachli EB (2004) Continuous infusion of amphotericin B deoxycholate: a cost-effective gold standard for therapy of invasive fungal infections? Clin Infect Dis 38:303–304

Schulenburg A, Sperr W, Rabitsch W, Knobl P, Thalhammer F (2005) Brief report: practicability and safety of amphotericin B deoxycholate as continuous infusion in neutropenic patients with hematological malignancies. Leuk Lymphoma 46:1163–1167

Segal BH (2009) Aspergillosis. N Engl J Med 360:1870–1884

Segal BH, Walsh TJ (2006) Current approaches to diagnosis and treatment of invasive aspergillosis. Am J Respir Crit Care Med 173:707–717

Sendid B, Cotteau A, Francois N, D'Haveloose A, Standaert A, Camus D, Poulain D (2006) Candidaemia and antifungal therapy in a French University Hospital: rough trends over a decade and possible links. BMC Infect Dis 6:80

Silveira FP, Husain S (2007) Fungal infections in solid organ transplantation. Med Mycol 45:305–320

Singh N (2005) Invasive aspergillosis in organ transplant recipients: new issues in epidemiologic characteristics, diagnosis, and management. Med Mycol 43(Suppl 1):S267–S270

Singh N, Husain S (2003) *Aspergillus* infections after lung transplantation: clinical differences in type of transplant and implications for management. J Heart Lung Transplant 22:258–266

Singh N, Paterson DL (2005) *Aspergillus* infections in transplant recipients. Clin Microbiol Rev 18:44–69

Singh N, Limaye AP, Forrest G, Safdar N, Munoz P, Pursell K, Houston S, Rosso F, Montoya JG, Patton P, del Busto R, Aguado JM, Fisher RA, Klintmalm GB, Miller R, Wagener MM, Lewis RE, Kontoyiannis DP, Husain S (2006) Combination of voriconazole and caspofungin as primary therapy for invasive aspergillosis in solid organ transplant recipients: a prospective, multicenter, observational study. Transplantation 81:320–326

Singh N, Alexander BD, Lortholary O, Dromer F, Gupta KL, John GT, del Busto R, Klintmalm GB, Somani J, Lyon GM, Pursell K, Stosor V, Munoz P, Limaye AP, Kalil AC, Pruett TL, Garcia-Diaz J, Humar A, Houston S, House AA, Wray D, Orloff S, Dowdy LA, Fisher RA, Heitman J, Wagener MM, Husain S (2007) Cryptococcus neoformans in organ transplant recipients: impact of calcineurin-inhibitor agents on mortality. J Infect Dis 195:756–764

Sloan D, Dlamini S, Paul N, Dedicoat M (2008) Treatment of acute cryptococcal meningitis in HIV infected adults, with an emphasis on resource-limited settings. Cochrane Database Syst Rev CD005647

Sole A, Nieto M (2007) Nebulized antifungal prophylaxis: role of amphotericin B lipid complex. Med Intensiva 5:64–70

Sole A, Salavert M (2008) Fungal infections after lung transplantation. Transplantation Rev 22:89–104

Sole A, Morant P, Salavert M, Pemán J, Morales P (2005) *Aspergillus* infections in lung transplant recipients: risk factors and outcome. Clin Microbiol Infect 11:359–365

Speich R, Dutly A, Naef R, Russi EW, Weder W, Boehler A (2002) Tolerability, safety and efficacy of conventional amphotericin B administered by 24-hour infusion to lung transplant recipients. Swiss Med Wkly 132:455–458

Spellberg B, Walsh TJ, Kontoyiannis DP, Edwards J Jr, Ibrahim AS (2009) Recent advances in the management of mucormycosis: from bench to bedside. Clin Infect Dis 48:1743–1751

Steinbach WJ, Perfect JR, Cabell CH, Fowler VG, Corey GR, Li JS, Zaas AK, Benjamin DK Jr (2005) A meta-analysis of medical versus surgical therapy for Candida endocarditis. J Infect 51:230–247

van Burik JA (2005) Role of new antifungal agents in prophylaxis of mycoses in high risk patients. Curr Opin Infect Dis 18:479–483

Walsh TJ, Raad I, Patterson TF, Chandrasekar P, Donowitz GR, Graybill R, Greene RE, Hachem R, Hadley S, Herbrecht R, Langston A, Louie A, Ribaud P, Segal BH, Stevens DA, van Burik JA, White CS, Corcoran G, Gogate J, Krishna G, Pedicone L, Hardalo C, Perfect JR (2007) Treatment of invasive aspergillosis with posaconazole in patients who are refractory to or intolerant of conventional therapy: an externally controlled trial. Clin Infect Dis 44:2–12

Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens DA, van Burik JA, Wingard JR, Patterson TF (2008) Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327–360

Wirk B, Wingard JR (2008) Combination antifungal therapy: from bench to bedside. Curr Infect Dis Rep 10:466–472

Wong-Beringer A, Lambros MP, Beringer PM, Johnson DL (2005) Suitability of caspofungin for aerosol delivery: physicochemical profiling and nebulizer choice. Chest 128:3711–3716

Zaragoza R, Pemán J (2006) Invasive fungal infections in critically ill patients: different therapeutic options and a uniform strategy. Rev Iberoam Micol 23:59–63

Chapter 12
Challenges to the Management of Pulmonary Mycoses in Allogeneic Hematopoietic Stem Cell Transplantation

Michael Koldehoff

Abstract Pulmonary mycoses are counted among the most dangerous complications in allogeneic hematopoietic stem cell transplantation. Despite the establishment of antifungal chemoprophylaxis and empirical antifungal treatment, they frequently leads to respiratory failure and are still associated with an extraordinarily poor prognosis. However, the emergence of new antimycotics with alternative mechanisms of action and decreased toxicity in combination with the development of new non-culture-based diagnostic techniques may allow earlier, more aggressive, and more effective antifungal treatment approaches. In addition, the optimized use of new technologies designed to augment spontaneous breathing efforts by patients, mechanical ventilation, as well as the advantages of early tracheostomy, contributes to the hope for a considerable improvement in the outcome of respiratory failure due to pulmonary mycoses following allogeneic hematopoietic stem cell transplantation.

12.1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) has been established as a curative treatment for a multitude of malignant and nonmalignant disorders. However, this procedure may cause life-threatening complications, especially in the early post-transplant phase. Depending on risk factors, the incidence of invasive fungal infections (IFI) in allogeneic HSCT recipients ranges from 14% to 25% (Martino et al. 2002; Cornely et al. 2003). Pulmonary complications are one of the major causes of respiratory failure in this context (Cooke and Yanik 2004; Hamza et al. 2004; Kojima et al. 2005). Despite recent published guidelines for treatment

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of IFI based on etiology, these infections retain a high morbidity and mortality in the HSCT setting (Barnes and Stallard 2001; Bach et al. 2001; CDCP 2000; Huaringa et al. 2000; Cornely et al. 2009). Based on the risk factors, physicians have attempted to predict IFI and focus prophylaxis on high-risk recipients. The introduction of a variety of new antifungal compounds over the past decade has focused attention on strategies as a means to decrease the threat of IFI. Weaning of allogeneic HSCT recipients off artificial ventilation (AV) is a clinical challenge, particularly in the light of IFI, duration of neutropenia, conditioning regimen-related toxicity, graft-versus-host disease (GVHD), immunosuppressive therapy, and steroid-induced myopathy. The rising incidence of IFI, especially invasive aspergillosis, compromises therapeutic outcomes in hematologic cancer patients and in transplant recipients. Additionally, early diagnosis of IFIs is critical (Greene et al. 2007), but usually diagnosis is delayed, which hampers the outcome of further treatment.

12.1.1 Spectrum of Fungal Pathogens

The most common pathogens responsible for pulmonary mycoses in recipients of allogeneic HSCT are *Candida* and *Aspergillus* species (Wald et al. 1997; Chamilos et al. 2006). The most prevalent *Candida* species are *albicans* (accounting for 50%–60% of *Candida* infections), *krusei*, *glabrata*, *tropicalis, lusitaniae*, and *parapsilosis*. Among the *Aspergillus* species *fumigatus* is the most prevalent, followed by *flavus, terreus*, and *niger*. However, the emergence of less common but medically important fungal pathogens has contributed to the rate of morbidity and mortality in allogeneic HSCT recipients. These pathogens include septate filamentous fungi (e.g., *Fusarium* spp., *Acremonium* spp., *Scedosporium* spp., dark-walled molds) and aseptate or hyposeptate *zygomycetes* spp. (Walsh et al. 2004a; Jahagirdar and Morrison 2002). Other comparatively rare fungal pathogens that may cause invasive infections include *Cryptococcus* spp., *Histoplasma* spp., *Coccidioides* spp., *Trichosporon* spp., and *Pseudallescheria* spp. (Wingard 1999; Marr and Bowden 1999; Marr et al. 2002b).

12.1.2 Pathogenesis and Risk Factors of Pulmonary Mycoses Following Allogeneic HSCT

The occurrence of IFI is limited to immunocompromised individuals, indicating that an impaired host defense is a prerequisite for the development of pulmonary mycoses. These infections develop either from endogenous organisms colonizing mucosal surfaces such as *Candida* spp., whose proliferation is facilitated by antibiotic suppression of the bacterial flora, or through exposure to an environmental

source and subsequent acquisition by respiratory inhalation (e.g., in pulmonary aspergillosis). Successful competition with other microbial pathogens enables fungal organisms to grow in sufficient numbers to overcome phagocytes. Mucosal injury due to conditioning-related mucositis additionally facilitates the breakdown of the mucosal barrier. Having overcome the mucosal barrier, various virulence properties of the respective fungal organisms allow these pathogens to invade and cause tissue damage in the immunocompromised host (Wald et al. 1997; Huaringa et al. 2000; Chamilos et al. 2006; Walsh et al. 2004a). Invasive pulmonary infections with *Candida* spp. present frequently in the context of a disseminated disease, often with positive blood cultures. In contrast, pulmonary aspergillosis may cause isolated nodular lesions, multiple lesions, or diffuse pulmonary infiltrates (Wald et al. 1997; Chamilos et al. 2006). The time-distribution of the incidence of pulmonary mycoses following allogeneic HSCT is characterized by an early peak as a consequence of neutropenia (increase starts in the second week of neutropenia) and a later peak which is explained by the development of acute GVHD and steroid treatment, resulting in a suppression of cell-mediated immunity and phagocytosis (Nichols 2003). Recent trends show an increase in the late IFI, which now accounts for the majority of cases of IFI (Marr et al. 2002a) (Table 12.1).

12.1.3 Prevention of IFI Following Allogeneic HSCT

A high standard of hygienic precautions and infection control measures is crucial in allogeneic HSCT. The nosocomial acquisition of *Aspergillus* spores may be prevented by the use of high-efficiency particulate air filters or laminar airflow (CDCP 2000). Since *Candida* infections usually develop as endogenous infections, prevention of environmental exposure does not reduce the risk of candidiasis. To prevent invasive candidal disease, the CDC guidelines and the Infectious Diseases Working Party of the German Society for Hematology and Oncology recommend the use of fluconazole 400 mg day^{-1} orally or intravenously for fungal prophylaxis of fluconazole-susceptible *Candida* spp. (CDCP 2000; Cornely et al. 2009; Hughes et al. 2002; Pappas et al. 2004). This approach has been supported by two randomized trials which showed a decrease in IFIs after HSCT subsequent to fluconazole prophylaxis. Fluconazole 400 mg d^{-1} was significantly superior to placebo in both the reduction of breakthrough IFI and the decrease of IFI-attributable mortality

Table 12.1 Risk factors for pulmonary mycoses following allogeneic HSCT

Prior to engraftment	Post-engraftment	General risk factors
Prolonged neutropenia	GVHD	Advanced age
Conditioning regimen related mucosal damage	Steroid therapy	Unrelated or mismatched donors
		IFI in the past medical history
Fungal overgrowth		Concomitant respiratory infections
		Concomitant CMV infections

(Goodman et al. 1992; Slavin et al. 1995). In a longitudinal observation survival benefit extended beyond the period of fluconazole treatment (75 days) and was accompanied by a lower incidence of intestinal graft-versus-host disease (Marr et al. 2002c). Moreover, fluconazole has been reported to protect from cyclophosphamide toxicity (Upton et al. 2007). On the other hand, since the introduction of fluconazole prophylaxis a shift to azole-resistant *Candida* species in post-transplant IFI has been observed (Fukuda et al. 2003). A recent study comparing fluconazole with itraconazole for the prevention of fungal infections in allogeneic HSCT recipients indicated that itraconazole provided a better protection against molds, but toxicities and intolerance limited its use (Marr et al. 2004).

Aspergillus prophylaxis is reasonable in certain high-risk groups such as patients with severe GVHD who require prolonged immunosuppressive therapy or patients with a previous history of invasive aspergillosis. Although the prophylactic use of amphotericin B has been evaluated in various studies, data are limited and the CDC guidelines do not recommend its routine use in the prophylaxis of aspergillosis. Amphotericin B inhalation in the deoxycholate formulation was considered active in reducing invasive pulmonary aspergillosis on the basis of noncomparative studies (Hertenstein et al. 1994). The only large multicenter trial did not confirm these results (Schwartz et al. 1999). In a noncomparative evaluation of inhalational amphotericin B lipid complex (ABLC) 50 mg d^{-1} and concomitant fluconazole 400 mg d^{-1} were found to be safe in allogeneic stem cell recipients (Alexander et al. 2006). A placebo-controlled trial on aerosolized liposomal amphotericin B (LAmB) resulted in a significant reduction of invasive pulmonary aspergillosis, but did not improve survival. All patients received fluconazole at an undisclosed dose and route (Rijnders et al. 2008). Aerosolized LAmB appears to be effective, but does not prevent IFI other than pulmonary. Adverse events in these trials included coughing, bad taste and nausea.

Prophylactic use of LAmB remains attractive, due to its lower toxicity. Recently a randomized clinical trial compared LAmB 50 mg q48h with no prophylaxis in a population with hematologic malignancies. In this reasonably sized and dosage study on the prophylactic properties of the drug, the investigators observed a significant reduction in the rates for proven and probable IFI as well as IFI-attributable mortality rates (Penack et al. 2006). However, neither approach of amphotericin B formulations has been sufficiently evaluated and cannot be recommended at present. A recent study conducted in allogeneic HSCT recipients compared itraconazole versus fluconazole from day +1 to +100 post-transplant. Proven IFI occurred in 9% treated with itraconazole versus 25% in recipients treated with fluconazole during the first 6 months post-transplant ($p < 0.01$) and translated for lower fungal-related mortality (Winston et al. 2003). Posaconazole has been compared to fluconazole or itraconazole in a randomized, open-label clinical trial in patients undergoing induction chemotherapy for acute myelogenous leukemia or myelodysplastic syndrome. At a dose of 600 mg d^{-1} posaconazole resulted in a significant reduction in proven and probable IFI, mainly by reducing the incidence rate of aspergillosis. Attributable and overall mortality were significantly reduced as well. Safety, including the overall rate of patients with serious adverse events,

was comparable between the three drugs. The only difference was a higher rate of patients on posaconazole experiencing possibly or probably related serious adverse events than patients on fluconazole or itraconazole prophylaxis. However, these events did not translate into a higher rate of study-drug discontinuation (Cornely et al. 2007). In another trial, allogeneic HSCT recipients with severe graft-versus-host disease were randomly allocated to receive posaconazole or fluconazole in a double-blinded fashion. Recipients receiving posaconazole prophylaxis had reduced rates of proven and probable IFI and attributable mortality. Posaconazole was found to be as safe and tolerated as fluconazole in this trial (Ullmann et al. 2007). These studies offer the first hope that the broad-spectrum azoles may indeed reduce the risk of serious mold infections in high-risk patient groups. Prophylaxis with posaconazole 600 mg d^{-1} is recommended during induction chemotherapy induced neutropenia in patients with AML or MDS due to its effects on the rate of reduction of IFI and death. Posaconazole 600 mg d^{-1} is recommended in HSCT recipients with GVHD because of the reduction in the rates of IFI and attributable mortality. Several immunomodulatory strategies to enhance host defense mechanisms against IFI have been investigated in recent years including hematopoietic growth factors, granulocyte infusions, gamma interferon, and keratinocyte growth factor (Walsh et al. 2004a). The possible contribution of these new treatment approaches to an improved control of IFI remains to be evaluated by clinical studies.

12.2 Diagnosis of Pulmonary Mycosis

The first clinical sign of IFI is persistent fever refractory to broad-spectrum antibiotic treatment. A consensus definition for possible, probable and proven IFI was published by Ascioglu et al. in Ascioglu et al. (2002). The proposed criteria are somewhat complex, but the criteria that will certainly apply to most patients are summarized in Table 12.2.

Table 12.2 Main diagnostic criteria for IFI used in the EORTC/MSG diagnostic criteria

Category, type of IFI	Description
Proven IFI	Positive histopathologic examination of infected tissues
	±
	Positive microbiologic culture of the same specimen (this criteria is required for the genus and species-specific definition of the IFI)
Probable IFI	Clinical criteria for having an IFI
	+
	Radiological findings suggestive of an IFI
	+
	Positive microbiological sample obtained from the site of disease
Possible IFI	Clinical criteria for having an IFI
	+
	Radiological findings suggestive of an IFI

(For full details see Ascioglu et al. 2002)

Diagnosis of fungal pneumonia includes radiological imaging (X-ray, thoracic CT scans including high-resolution or spiral CT scans), microscopy and microbiologic cultures (blood, throat swabs, washes from mouth and nose, sputum, salvia and bronchial secretions, and tissue biopsy obtained by BAL). However, the culture-based diagnostic tools are characterized by a low sensitivity and specificity and often, if at all, only become positive at a late stage of infection (Verweij and Meis 2000). In recent years, new molecular non-culture-based diagnostic techniques have been established in order to provide a definitive diagnosis of IFI at an earlier stage of infection. These new methods include PCR detection of fungal DNA and a variety of serologic tests such as detection of circulating fungal antigens or antifungal antibodies. For instance, the fungal cell-wall component galactomannan has been shown to be a sensitive marker for invasive aspergillosis (Kontoyiannis et al. 2002; Becker et al. 2003; Spiess et al. 2003; McLintock and Jones 2004). The accuracy of the β-glucan assay (a cell-wall component of many fungi) for diagnosis of aspergillosis and candidiasis and its role in IFI diagnosis remain unclear, in part because the few published studies report widely varying specificity (Wheat 2006). These new diagnostic tests may not only be of considerable benefit for high risk patients who should be screened for fungal infections at regular intervals, but they have also proven to be useful in monitoring the response to antifungal therapy (Verweij and Meis 2000; McLintock and Jones 2004). However, more prospective studies are required to establish the utility and accuracy of non-culture-based tests for early diagnosis and improved outcome in allogeneic HSCT recipients suffering from IFI.

12.3 Antifungal Agents Used in the Treatment of IFI in Allogeneic HSCT

Conventional amphotericin B (amphotericin B deoxycholate) has long been the standard treatment for pulmonary mycoses, although responses are suboptimal in severely immunosuppressed patients and toxic effects often limit its use. The development of lipid-based delivery technologies offered the possibility of reduced toxicity and resulted in the birth of three lipid formulations of amphotericin (LFAB): ABLC, amphotericin B colloidal dispersion, and LAmB (Herbrecht et al. 2003; Ostrosky-Zeichner et al. 2003). These agents have the same antifungal spectrum and activity as amphotericin B deoxycholate. The pharmacokinetic properties differ between these formulations, with remarkable differences in the tissue distribution. To date, the clinical significance of these observations has not been fully elucidated. Preclinical data suggest that, compared to conventional amphotericin, higher concentrations of lipid formulations are needed to exert similar antifungal effects. However, since the antifungal activity of all LFAB is equivalent to conventional amphotericin B and the drug-related toxicity is reduced significantly,

lipid formulations should generally be preferred to conventional amphotericin B in allogeneic HSCT patients. Interestingly, a recent trial showed that initial use of higher doses of LAmB at 10 mg kg^{-1} b.w day^{-1} did not improve efficacy and was associated with more toxicity (Muñoz et al. 2008).

Itraconazole has a wide spectrum of activity, including *Aspergillus* spp., *Candida albicans*, and non-*albicans* species, but cross-resistance to fluconazole-resistant *Candida* spp. has been shown (Bagg et al. 2003). Two large studies have recently been reported comparing the use of itraconazole with fluconazole for primary prophylaxis in high-risk patients who were recipients of allogeneic HSCT. These have confirmed that itraconazole is effective in this setting in reducing the rate of IFI. However, there are concerns with regard to increased toxicity and the potential for drug interaction with itraconazole compared with fluconazole (Winston et al. 2003; Marr et al. 2004).

Additionally, bioavailability differs among patients. The shift to fluconazole-resistant non-*albicans Candida* infections increased research efforts into newer antifungal agents. In recent years three new antimycotics, voriconazole, posaconazole and caspofungin, have been established and are nowadays used frequently in allogeneic HSCT recipients with IFI. Voriconazole or posaconazole are members of the triazole family of antifungal agents and interact with ergosterol synthesis. Voriconazole has the broadest antifungal spectrum of clinically used azoles. It is available in oral and injectable solution. Posaconazole is a second-generation azole that has a similar constitution to that of itraconazole. Caspofungin belongs to the echinocandin class of antimycotics which inhibit β-1,3-D-glucan synthesis in the fungal cell wall. Both azole (voriconazole or posaconazole) and caspofungin are effective in the therapy of *Aspergillus* and *Candida* (including non-*albicans Candida* species) infections (Chiou et al. 2000; Groll and Walsh 2001; Walsh et al. 2002; Herbrecht et al. 2002; Pfaller et al. 2001; Pfaller et al. 2002). In examination using clinical isolates, posaconazole had more potent fungicidal activity than amphotericin B, itraconazole, or voriconazole, and its characteristics include its effectiveness against Zygomycetes and *Rhizopus* (Van Burik et al. 2006).

In a randomized, multicenter trial comparing LAmB with voriconazole for empirical antimycotic therapy in patients with neutropenia and persistent fever, voriconazole was as effective as, and better tolerated than, LAmB (Walsh et al. 2002). Another recent randomized study compared voriconazole with amphotericin B in immunocompromised patients with proven or probable invasive aspergillosis, and demonstrated the superiority of voriconazole as initial therapy in terms of response rate, survival rate, and safety (Herbrecht et al. 2002). Voriconazole is a substrate and inhibitor of hepatic cytochrome P450 isoenzymes, with a stronger drug interaction than fluconazole or itraconazole. Possible side-effects include hallucinations, visual disturbances, and liver toxicity, which is obviously related to increased voriconazole blood levels. Severe nephrotoxicity has not been reported so far, but dose adjustments have to be undertaken in patients with preterminal renal failure.

A multicenter, randomized trial evaluating posaconazole versus fluconazole for the treatment of oropharyngeal candidiasis in subjects with HIV/AIDS demonstrated that posaconazol was as effective as fluconazole in producing a successful clinical outcome. Adverse events were similar between treatment arms. On day 14, mycological success was 68% in both arms, but by day 42 significantly more posaconazole recipients than fluconazole recipients continued to have mycological success (40.6% vs. 26.4%; $p < 0.04$). Fewer posaconazole recipients than fluconazole recipients experienced clinical relapse (31.5% vs. 38.2%). The observation reported here indicated that oral posaconazole has a tolerability profile and may offer a beneficial alternative to other antifungal agents currently in clinical use for treating oropharngeal candidiasis (Vazquez et al. 2006). Posaconazole inhibits cytochrome P450 isoenzyme 3A4 and is highly interactive with other drugs. Concomitant use of phenytoin, among others, is contraindicated. Transfer to the cerebrospinal fluid is low and the rate of protein binding is high. In an open-label, multicenter study in patients with invasive aspergillus and other mycoses, who were refractory to or intolerant of conventional antifungal therapy, posaconazole demonstrated an overall success rate of 42% vs. 26% for the control subjects ($p < 0.06$). These findings indicate that posaconazole is an alternative to salvage therapy for patients with invasive aspergillosis who are refractory to or intolerant of previous antifungal therapy (Walsh et al. 2007).

In studies comparing caspofungin with LAmB for empirical antimycotic therapy in patients with persistent febrile neutropenia, the overall response was similar. However, the subgroup with proven IFIs responded significantly better to caspofungin, resulting in an improved overall survival rate (Walsh et al. 2004b). In patients with invasive candidiasis, caspofungin was superior to conventional amphotericin B in terms of all drug-related adverse effects such as nephrotoxicity, hypokalemia, infusion-related events, and the need for drug-related discontinuation in general (Mora-Duarte et al. 2002). The most clinically relevant adverse event of caspofungin is its interaction with calcineurin inhibitors: though caspofungin has no effect on the pharmacokinetics of cyclosporin, their concomitant use was associated with an increased risk for hepatic dysfunction. These data imply a need for reduction of caspofungin doses in HSCT recipients with elevated liver function tests. In contrast to this, caspofungin reduces tacrolimus blood levels by up to 20% so that upward tacrolimus dose adjustments may be necessary (Sable et al. 2002). Nephrotoxicity related to caspofungin has not been reported so far. A reliable method for the measurement of caspofungin blood levels has not been published so far. Micafungin at a dose of 50 mg d^{-1} has been compared to fluconazole in a large double-blind trial undergoing autologous or allogeneic HSCT. Invasive candidiasis was effectively prevented by both regimes, and the rate of aspergillosis was lower in the micafungin group but did not reach significance in those subgroups despite the fact of successful prophylaxis in the primary composite endpoint. No significant reduction of the overall and attributable fungal mortality was detected (Van Burik et al. 2004).

12.4 Respiratory Support

In HSCT recipients with pulmonary infections, the early initiation of augmented spontaneous breathing with continuous positive airway pressure (CPAP) can delay the development of acute respiratory failure (Ghosh et al. 1999). However, artificial respiration may be necessary with further respiratory deterioration. The need for AV in patients with hematologic malignancy significantly worsens prognosis (Peters et al. 1998). In recent years, however, survival has improved and AV is no longer considered futile therapy (Ferrà et al. 2007). Weaning off AV can be difficult in patients with hematologic malignancies and HSCT recipients because of a variety of factors including regimen-related pulmonary toxicity and steroid-induced critical illness myopathy (Koldehoff and Zakrzewski 2005). HSCT recipients should undergo tracheostomy within 10 days after initiation of AV in all cases requiring prolonged ventilatory assistance (MacIntyre et al. 2001). The advantages of early tracheostomy include improved weaning off AV and reduced sedation, especially in patients suffering from steroid induced myopathy, the possibility of supportive intermittent ventilator assistance [biphasic positive airway pressure (BIPAP)/CPAP], less exhaustion due to decreased airway resistance; enhanced patient mobility, facilitated recovery of the patient's physical condition, and a reduced incidence of ventilator associated pneumonias (Heffner 2001; Jaeger et al. 2002). We reported on four cases of pulmonary IFI in the early posttransplant HSCT recipients showing various risk factors associated with a poor prognosis (pretransplant fungal infections, myeloablative chemotherapy, hepatic dysfunction, renal failure, vasopressor support, and AV). Through early tracheotomy in combination with optimized anti-infective regimens including new antimycotic, all patients recovered from respiratory failure and were brought back to spontaneous breathing. Even though our observations are based on only four cases and have therefore to be interpreted with caution, their potential clinical importance is evident (Koldehoff et al. 2005).

12.5 Conclusions

The extraordinarily high morbidity and mortality associated with pulmonary mycoses and subsequent respiratory failure is still one of the major issues in our effort to improve the outcome of allogeneic HSCT. However, with the substantial progress that has been made in recent years, including the development of new antimycotics, we now have an increased opportunity to control these infections and stabilize the pulmonary condition of these high-risk patients. The choice of the appropriate antifungal agent will always depend on efficacy, the toxicity profile, and economic considerations (see Table 12.3). Yet current experience with new antimycotics suggests that their use should be seriously considered in post-transplant IFIs such as pulmonary mycoses.

Table 12.3 Efficacy, toxicity and costs of conventional amphotericin B (cAmB), lipid formulations of amphotericin B (LFAB), fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin

	Efficacy		Overall toxicity	Costs
	Candida	*Aspergillus*		
cAmB	High	High	High	Low
LFAB	High	High	Moderate	Very high
Fluconazole	High[a]	None	Very low	Moderate
Itraconazole	High[a]	High	Low	High
Voriconazole	High[a]	High	Low	High
Posaconazole	High	High	Low	High
Caspofungin	High	High	Low	Very high

[a]May not be effective in *Candida krusei* and *Candida glabrata*

Table 12.4 Therapy of proven invasive fungal infections

	Invasive candidiasis	Invasive aspergillosis
1. Line	Fluconazole 400–800 mg day^{-1} Caspofungin 50 mg day^{-1} (70 mg day^{-1})	Voriconazole 3–4 mg kg^{-1} b.w. $q12h^{-1}$ (6 mg kg^{-1} b.w. q12h day^{-1}) LFAB 1–6 mg kg^{-1} b.w. day^{-1}
2. Line	LFAB 1–6 mg kg^{-1} b.w. day^{-1} Posaconazole 400 mg $q12h^{-1}$	Caspofungin 50 mg day^{-1} (70 mg day^{-1}) Posaconazole 400 mg $q12h^{-1}$

In allogeneic HSCT patients with proven invasive candidiasis, the first-line therapeutic options are fluconazole (restricted to patients who did not receive prophylactic fluconazole) and caspofungin. Second-line treatment consists of LFAB and posaconazole. Central lines should be removed whenever possible. In allogeneic HSCT patients with proven invasive aspergillosis the first therapeutic choices are voriconazole or LFAB with posaconazole or caspofungin being the second-line treatment (Michallet and Ito 2009). The establishment of non-culture-based molecular diagnostic techniques, and the availability of new antimycotics with alternative mechanisms of action and decreased toxicity, allow for earlier, more aggressive and thus potentially more effective antifungal treatment approaches (Table 12.4).

In nonresponders to first-line and second-line single-agent antimycotic therapy, combination therapy involving these agents can still be effective. Reasonable combinations include caspofungin/LFAB, caspofungin/azoles, and azoles/LFAB (Kontoyiannis et al. 2003; Rubin et al. 2002; Damaj et al. 2004). Empirical antifungal therapy covering azole-resistant *Candida* species and molds should be initiated immediately in allogeneic HSCT recipients with suspected IFI since delayed treatment is associated with an increased mortality. Patients should be evaluated extensively for fungal infections including early thoracic CT. Voriconazole is still often used as the first-line agent in these high-risk settings for IFI, but LFAB is an adequate alternative (Wingard and Leather 2004; Walsh et al. 1999). Otherwise, caspofungin or posaconazole can be used as an alternative.

In combination with the optimized use of augmented spontaneous breathing and mechanical ventilation, including early tracheostomy, the above-described diagnostic and therapeutic advances contribute to a considerable improvement in management, and will subsequently lead to a better outcome of respiratory failure due to pulmonary mycoses following allogeneic HSCT. In order to precisely demonstrate the effect of the new antimycotics on the outcome of pulmonary mycoses during allogeneic HSCT, further prospective studies and systematic analyses are needed.

References

Alexander BD, Dodds Ashley ES, Addison RM, Alspaugh JA, Chao NJ, Perfect JR (2006) Noncomparative evaluation of the safety of aerosolized amphotericin B lipid complex in patients undergoing allogeneic hematopoietic stem cell transplantation. Transpl Infect Dis 8:13–20

Ascioglu S, Rex JH, de Pauw B, Bennett JE, Bille J, Crokaert F, Denning DW, Donnelly JP, Edwards JE, Erjavec Z, Fiere D, Lortholary O, Maertens J, Meis JF, Patterson TF, Ritter J, Selleslag D, Shah PM, Stevens DA, Walsh TJ, Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer; Mycoses Study Group of the National Institute of Allergy and Infectious Diseases (2002) Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 34:7–14

Bach PB, Schrag D, Nierman DM, Horak D, White P, Young JW, Groeger JS (2001) Identification of poor prognostic features among patients requiring mechanical ventilation after hematopoietic stem cell transplantation. Blood 98:3234–3240

Bagg J, Sweeney MP, Lewis MA, Jackson MS, Coleman D, Al MA, Baxter W, McEndrick S, McHugh S (2003) High prevalence of non-albicans yeasts and detection of anti-fungal resistance in the oral flora of patients with advanced cancer. Palliat Med 17:477–481

Barnes RA, Stallard N (2001) Severe infections after bone marrow transplantation. Curr Opin Crit Care 7:362–366

Becker MJ, Lugtenburg EJ, Cornelissen JJ, van der Schee C, Hoogsteden HC, De Marie S (2003) Galactomannan detection in computerized tomography-based broncho-alveolar lavage fluid and serum in haematological patients at risk for invasive pulmonary aspergillosis. Br J Haematol 121:448–457

Centers for Disease Control and Prevention, Infectious Diseases Society of America, American Society of Blood and Marrow Transplantation (2000) Guidelines for preventing opportunistic infections among hematopoietic stem cell transplant recipients. Biol Blood Marrow Transplant 6:659–713

Chamilos G, Luna M, Lewis RE, Bodey GP, Chemaly R, Tarrand JJ, Safdar A, Raad II, Kontoyiannis DP (2006) Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989-2003). Haematologica 91:986–989

Chiou CC, Groll AH, Walsh TJ (2000) New drugs and novel targets for treatment of invasive fungal infections in patients with cancer. Oncologist 5:120–135

Cooke KR, Yanik G (2004) Acute lung injury after allogeneic stem cell transplantation: is the lung a target of acute graft-versus-host disease? Bone Marrow Transplant 34:753–765

Cornely OA, Böhme A, Buchheidt D, Einsele H, Heinz WJ, Karthaus M, Krause SW, Krüger W, Maschmeyer G, Penack O, Ritter J, Ruhnke M, Sandherr M, Sieniawski M, Vehreschild JJ, Wolf HH, Ullmann AJ (2009) Primary prophylaxis of invasive fungal infections in patients

with hematologic malignancies. Recommendations of the Infectious Diseases Working Party of the German Society for Haematology and Oncology. Haematologica 94:113–122
Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ, Helfgott D, Holowiecki J, Stockelberg D, Goh YT, Petrini M, Hardalo C, Suresh R, Angulo-Gonzalez D (2007) Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N Engl J Med 356:348–359
Cornely OA, Ullmann AJ, Karthaus M (2003) Evidence-based assessment of primary antifungal prophylaxis in patients with hematologic malignancies. Blood 101:3365–3372
Damaj G, Ivanov V, le Brigand B, D'incan E, Doglio MF, Bilger K, Faucher C, Vey N, Gastaut JA (2004) Rapid improvement of disseminated aspergillosis with caspofungin/voriconazole combination in an adult leukemic patient. Ann Hematol 83:390–393
Ferrà C, Marcos P, Misis M, Morgades M, Bordejé ML, Oriol A, Lloveras N, Sancho JM, Xicoy B, Batlle M, Klamburg J, Feliu E, Ribera JM (2007) Outcome and prognostic factors in patients with hematologic malignancies admitted to the intensive care unit: a single-center experience. Int J Hematol 85:195–202
Fukuda T, Boeckh M, Carter RA, Sandmaier BM, Maris MB, Maloney DG, Martin PJ, Storb RF, Marr KA (2003) Risks and outcomes of invasive fungal infections in recipients of allogeneic hematopoietic stem cell transplants after nonmyeloablative conditioning. Blood 102:827–833
Ghosh K, Rafique B, Tirkey J, Benjamin E, Jacob S, Goes J (1999) Successful non-invasive ventilatory support in a patient with regimen-related toxicity during allogeneic bone marrow transplantation. Bone Marrow Transplant 23:833–834
Goodman JL, Winston DJ, Greenfield RA, Chandrasekar PH, Fox B, Kaizer H, Shadduck RK, Shea TC, Stiff P, Friedman DJ et al (1992) Controlled trial of fluconazole to prevent fungal infection in patients undergoing bone marrow transplantation. N Engl J Med 326:845–851
Greene RE, Schlamm HT, Oestmann JW, Stark P, Durand C, Lortholary O, Wingard JR, Herbrecht R, Ribaud P, Patterson TF, Troke PF, Denning DW, Bennett JE, de Pauw BE, Rubin RH (2007) Imaging findings in acute invasive pulmonary aspergillosis: clinical significance of the halo sign. Clin Infect Dis 44:373–379
Groll AH, Walsh TJ (2001) Caspofungin: pharmacology, safety and therapeutic potential in superficial and invasive fungal infections. Expert Opin Investig Drugs 10:1545–1558
Hamza NS, Ghannoum MA, Lazarus HM (2004) Choices aplenty: antifungal prophylaxis in hematopoietic stem cell transplant recipients. Bone Marrow Transplant 34:377–389
Heffner JE (2001) The role of tracheotomy in weaning. Chest 120:477S–481S
Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, Kern WV, Marr KA, Ribaud P, Lortholary O, Sylvester R, Rubin RH, Wingard JR, Stark P, Durand C, Caillot D, Thiel E, Chandrasekar PH, Hodges MR, Schlamm HT, Troke PF, de Pauw B (2002) Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 347:408–415
Herbrecht R, Natarajan-Ame S, Nivoix Y, Letscher-Btu V (2003) The lipid formulation of amphotericin B. Expert Opin Pharmacother 4:1277–1287
Hertenstein B, Kern WV, Schmeiser T, Stefanic M, Bunjes D, Wiesneth M, Novotny J, Heimpel H, Arnold R (1994) Low incidence of invasive fungal infections after bone marrow transplantation in patients receiving amphotericin B inhalations during neutropenia. Ann Hematol 68:21–26
Huaringa AJ, Leyva FJ, Giralt SA, Blanco J, Signes-Costa J, Velarde H, Champlin RE (2000) Outcome of bone marrow transplantation patients requiring mechanical ventilation. Crit Care Med 28:1014–1017
Hughes WT, Armstrong D, Bodey GP, Bow EJ, Brown AE, Calandra T, Feld R, Pizzo PA, Rolston KVI, Shenep JL, Young LS (2002) 2002 Guidelines for the use of antimicrobial agents in neutropenic patients with cancer. Clin Infect Dis 34:730–751
Jaeger JM, Littlewood KA, Durbin CG Jr (2002) The role of tracheostomy in weaning from mechanical ventilation. Respir Care 47:469–480

Jahagirdar BN, Morrison VA (2002) Emergine fungal pathogens in patients with hematologic malignancies and marrow/stem-cell transplant recipients. Semin Respir Infect 17:113–120

Kojima R, Tateishi U, Kami M, Murashige N, Nannya Y, Kusumi E, Sakai M, Tanaka Y, Kanda Y, Mori S, Chiba S, Kusumoto M, Miyakoshi S, Hirai H, Taniguchi S, Sakamaki H, Takaue Y (2005) Chest computer tomography of late invasive aspergillosis after allogeneic hematopoietic stem cell transplantation. Biol Blood Marrow Transplant 11:506–511

Koldehoff M, Elmaagacli AH, Steckel NK, Trenschel R, Hlinka M, Ditschkowski M, Beelen DW (2005) Successful treatment of patients with respiratory failure due to fungal infection after allogeneic hematopoietic stem cell transplantation. Transpl Infect Dis 7:137–145

Koldehoff M, Zakrzewski JL (2005) Modern management of respiratory failure due to pulmonary mycoses following allogeneic hematopoietic stem-cell transplantation. Am J Hematol 79:158–163

Kontoyiannis DP, Hachem R, Lewis RE, Rivero GA, Torres HA, Thornby J, Champlin R, Kantarjian H, Bodey GP, Raad II (2003) Efficacy and toxicity of caspofungin in combination with liposomal amphotericin B as primary or salvage treatment of invasive aspergillosis in patients with hematologic malignancies. Cancer 98:292–299

Kontoyiannis DP, Reddy BT, Torres HA, Luna M, Lewis RE, Tarrand J, Bodey GP, Raad II (2002) Pulmonary candidiasis in patients with cancer: an autopsy study. Clin Infect Dis 34:400–403

MacIntyre NR, Cook DJ, Ely EW Jr, Epstein SK, Fink JB, Heffner JE, Hess D, Hubmayer RD, Scheinhorn DJ (2001) Evidence-based guidelines for weaning and discontinuing ventilatory support: a collective task force facilitated by the American College of Chest Physicians; the American Association for Respiratory Care; and the American College of Critical Care Medicine. Chest 120:375S–395S

Marr KA, Bowden RA (1999) Fungal infections in patients undergoing blood and marrow transplantation. Transpl Infect Dis 1:237–246

Marr KA, Carter RA, Boeckh M, Martin P, Corey L (2002a) Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. Blood 100: 4358–4366

Marr KA, Carter RA, Crippa F, Wald A, Corey L (2002b) Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin Infect Dis 34:909–917

Marr KA, Crippa F, Leisenring W, Hoyle M, Boeckh M, Balajee SA, Nichols WG, Musher B, Corey L (2004) Itraconazole versus fluconazole for prevention of fungal infections in patients receiving allogeneic stem cell transplants. Blood 103:1527–1533

Marr KA, Seidel K, Slavin MA, Bowden RA, Schoch HG, Flowers ME, Corey L, Boeckh M (2002c) Prolonged fluconazole prophylaxis is associated with persistent protection against candidiasis-related death in allogeneic marrow transplant recipients: long-term follow-up of a randomized, placebo-controlled trial. Blood 96:2055–2061

Martino R, Subira M, Rovira M, Solano C, Vazquez L, Sanz GF, Urbano-Ispizua A, Brunet S, la Camara D (2002) Invasive fungal infections after allogeneic peripheral blood stem cell transplantation: incidence and risk factors in 395 patients. Br J Haematol 116:475–482

McLintock LA, Jones BL (2004) Advances in molecular and serological diagnosis of invasive fungal infection in haemato-oncology patients. Br J Haematol 126:289–297

Michallet M, Ito JI (2009) Approaches to the management of invasive fungal infections in hematologic malignancy and hematopoietic cell transplantation. J Clin Oncol 27:3398–3409

Mora-Duarte J, Betts R, Rotstein C, Colombo AL, Thompson-Moya L, Smietana J, Lupinacci R, Sable C, Kartsonis N, Perfect J (2002) Comparison of caspofungin and amphotericin B for invasive candidiasis. N Engl J Med 347:2020–2029

Muñoz P, Guinea J, Narbona MT, Bouza E (2008) Treatment of invasive fungal infections in immunocompromised and transplant patients: AmBiLoad trial and other new data. Int J Antimicrob Agents 32:S125–S131

Nichols WG (2003) Management of infectious complication in the hematopoietic stem cell transplant recipient. J Intensive Care Med 18:295–312

Ostrosky-Zeichner L, Marr KA, Rex JH, Cohen SH (2003) Amphotericin B: time for a new "gold standard". Clin Infect Dis 37:415–425

Pappas PG, Rex JH, Sobel JD, Filler SG, Dismukes WE, Walsh TJ, Edwards JE (2004) Guidelines for treatment of candidiasis. Clin Infect Dis 38:161–189

Penack O, Schwartz S, Martus P, Reinwald M, Schmidt-Hieber M, Thiel E, Blau IW (2006) Low-dose liposomal amphotericin B in the prevention of invasive fungal infections in patients with prolonged neutropenia: results from a randomized, single-center trial. Ann Oncol 17: 1306–1312

Peters SG, Meadows JA III, Gracey DR (1998) Outcome of respiratory failure in hematologic malignancy. Chest 94:99–102

Pfaller MA, Messer SA, Hollis RJ et al (2001) In vitro activities of posaconazole (SCH 56592) compared with those of itraconazole and fluconazole against 3685 clinical isolates of *Candida* spp. and *Cryptococcus neoformans*. Antimicrob Agents Chemother 45:2862–2864

Pfaller MA, Messer SA, Hollis RJ et al (2002) SENTRY Participans Group. Antifungal activities of posaconazole, ravuconazole, and voriconazole compared to those of itraconazole and amphotericine B against 239 clinical isolates of *Aspergillus* spp. And other filamentous fungi: report from SENTRY Antimicrobial Surveillance Program, 2000. Antimicrob Agents Chemother 46:1032–1037

Rijnders BJ, Cornelissen JJ, Slobbe L, Becker MJ, Doorduijn JK, Hop WC, Ruijgrok EJ, Löwenberg B, Vulto A, Lugtenburg PJ, de Marie S (2008) Aerosolized liposomal amphotericin B for the prevention of invasive pulmonary aspergillosis during prolonged neutropenia: a randomized, placebo-controlled trial. Clin Infect Dis 46:1401–1408

Rubin MA, Carroll KC, Cahill BC (2002) Caspofungin in combination with itraconazole for the treatment of invasive aspergillosis in humans. Clin Infect Dis 34:1160–1161

Sable CA, Nguyen BY, Chodakewitz JA, DiNubile MJ (2002) Safety and tolerability of caspofungin acetate in the treatment of fungal infections. Transpl Infect Dis 4:25–30

Schwartz S, Behre G, Heinemann V, Wandt H, Schilling E, Arning M, Trittin A, Kern WV, Boenisch O, Bosse D, Lenz K, Ludwig WD, Hiddemann W, Siegert W, Beyer J (1999) Aerosolized amphotericin B inhalations as prophylaxis of invasive aspergillus infections during prolonged neutropenia: results of a prospective randomized multicenter trial. Blood 93:3654–3661

Slavin MA, Osborne B, Adams R, Levenstein MJ, Schoch HG, Feldman AR, Meyers JD, Bowden RA (1995) Efficacy and safety of fluconazole prophylaxis for fungal infections after marrow transplantation-a prospective, randomized, double-blind study. J Infect Dis 171:1545–1552

Spiess B, Buchheidt D, Baust C, Skladny H, Seifarth W, Zeilfelder U, Leib-Mosch C, Morz H, Hehlmann R (2003) Development of a LightCycler PCR assay for detection and quantification of *Aspergillus fumigatus* DNA in clinical samples from neutropenic patients. J Clin Microbiol 41:1811–1818

Ullmann AJ, Lipton JH, Vesole DH, Chandrasekar P, Langston A, Tarantolo SR, Greinix H, Morais de Azevedo W, Reddy V, Boparai N, Pedicone L, Patino H, Durrant S (2007) Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. N Engl J Med 356:335–347

Upton A, McCune JS, Kirby KA, Leisenring W, McDonald G, Batchelder A, Marr KA (2007) Fluconazole coadministration concurrent with cyclophosphamide conditioning may reduce regimen-related toxicity postmyeloablative hematopoietic cell transplantation. Biol Blood Marrow Transplant 13:760–764

Van Burik JA, Hare RS, Solomon HF et al (2006) Psoaconazole is effective as salvage therapy in zygomycosis: a retrospective summary of 91 cases. Clin Infect Dis 42:e61–e65

Van Burik JA, Ratanatharathorn V, Stepan DE, Miller CB, Lipton JH, Vesole DH, Bunin N, Wall DA, Hiemenz JW, Satoi Y, Lee JM, Walsh TJ (2004) Micafungin versus fluconazole for prophylaxis against invasive fungal infections during neutropenia in patients undergoing hematopoietic stem cell transplantation. Clin Infect Dis 39:1407–1416

Vazquez JA, Skiest DJ, Nieto L et al (2006) A multicenter randomized trial evaluating posaconazole versus fluconazole for the treatment of oropharyngeal candidiasis in subjects with HIV/AIDS. Clin Infect Dis 42:1179–1186

Verweij PE, Meis JF (2000) Microbiological diagnosis of invasive fungal infections in transplant recipients. Transpl Infect Dis 2:80–87

Wald A, Leidenring W, van Burik JA, Bowden RA (1997) Epidemiology of aspergillus infection in a large cohort of patients undergoing bone marrow transplantation. J Infect Dis 175: 1459–1466

Walsh TJ, Finberg RW, Arndt C et al (1999) Liposomal amphotericin B for empirical therapy in patients with persistent fever and neutropenia. N Engl J Med 340:764–771

Walsh TJ, Groll A, Hiemenz J, Fleming R, Roilides E, Anaissie E (2004a) Infections due to emerging and uncommon medically important fungal pathogens. Clin Microbiol Infect 10:48–66

Walsh TJ, Pappas P, Winston DJ, Lazarus HM, Petersen F, Raffalli J, Yanovich S, Stiff P, Greenberg R, Donowitz G, Schuster M, Reboli A, Wingard J, Arndt C, Reinhardt J, Hadley S, Finberg R, Laverdiere M, Perfect J, Garber G, Fioritoni G, Anaissie E, Lee J (2002) Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. N Engl J Med 346:225–234

Walsh TJ, Raad I, Patterson TF et al (2007) Treatment of invasive aspergillosis with posaconazole in patients who are refractory to or intolerant of conventional therapy: an externally controlled trial. Clin Infect Dis 44:2–12

Walsh TJ, Teppler H, Donowitz GR, Maertens JA, Baden LR, Dmoszynska A, Cornely OA, Bourque MR, Lupinacci RJ, Sable CA, dePauw BE (2004b) Caspofungin versus liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia. N Engl J Med 351:1391–1402

Wheat LJ (2006) Antigen detection, serology, and molecular diagnosis of invasive mycoses in the immunocompromised host. Transpl Infect Dis 8:128–139

Wingard JR, Leather H (2004) A new era of antifungal therapy. Biol Blood Marrow Transplant 10:73–90

Wingard JR (1999) Fungal infections after bone marrow transplant. Biol Blood Marrow Transplant 5:55–68

Winston DJ, Maziarz RT, Chandrasekar PH, Lazarus HM, Goldman M, Blumer JL, Leitz GJ, Territo MC (2003) Intravenous and oral itraconazole versus intravenous and oral fluconazole for long-term antifungal prophylaxis in allogeneic hematopoietic stem-cell transplant recipients. A multicenter, randomized trial. Ann Intern Med 138:705–713

Chapter 13
Aspartic Peptidase Inhibitors as Potential Bioactive Pharmacological Compounds Against Human Fungal Pathogens

André Luis Souza dos Santos

Abstract The development of novel antifungal drugs is becoming more demanding every day since existing drugs either have too many side-effects or they tend to lose effectiveness due to resistant fungal strains. In view of this, a number of new strategies to obstruct fungal biological processes have emerged; one of them is focused on peptidase inhibition. This particular class of hydrolytic enzymes cleaves peptide bond in proteinaceous substrates, a reaction extremely important in maintaining the physiology of all living cells. Interestingly, peptidases are also essential virulence factors for prokaryotic and eukaryote micro-organisms, including fungi, during all stages of the infection process. Consequently, peptidases are potential targets for the development of future antifungal drugs. This chapter will focus on the potential use of aspartic peptidase inhibitors against human fungal pathogens, showing the capability of these bioactive pharmacological compounds to arrest vital fungal processes such as growth, differentiation, nutrition, and interaction with host components.

13.1 Introduction

Recent decades have seen a significant increase in the incidence of invasive fungal infections. Fungi can cause life-threatening diseases, particularly in patients with weakened immunological systems. Although treatment options are available for these individuals, dose-limiting toxicity and the appearance of drug-resistant

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organisms are growing problems. Fungi are eukaryote cells with conserved gene sequences closely related to metazoans, including humans, which reflects in similarities at both biochemical and immunological levels. For these reasons, the generation of specific, effective, and nontoxic drugs against human fungal pathogens is extremely complicated. Up to now, the antifungal armamentarium for the treatment of serious fungal infections remains limited. In this context, the need for the discovery of novel fungal targets as well as new bioactive compounds is extremely urgent. With this task in mind, extensive studies have revealed a globally conserved role for proteolytic enzymes in crucial biological processes during different steps of the fungi cell cycle, such as growth, proliferation, differentiation and nutrition. Similarly, a conserved and essential role for peptidases in fungal virulence has been established, for instance in helping the fungal cells in the interaction with key host components and in the evasion of host immune response. Taken together, this fungal target provides a robust platform from which novel antimicrobial compounds should be developed for therapeutic approaches in a variety of clinical settings. In this scenario, aspartic peptidases are already the targets of some clinically useful drugs (peptidase inhibitors), and a variety of factors make these enzymes appealing to those seeking novel antifungal therapies. An example that highlights the recent success of the application of aspartic peptidase inhibitors has been the use of highly active antiretroviral therapy (HAART) in individuals with the acquired immunodeficiency syndrome (AIDS), which has drastically reduced the prevalence of fungal infections, especially candidiasis and cryptococcosis. This result seems to be due to aspartic peptidase inhibitors of the human immunodeficiency virus (HIV) included in HAART, which directly bind to essential aspartic peptidases produced by some fungi, interfering with vital metabolic processes of these fungal cells. This chapter describes the knowledge in the area of aspartic peptidase inhibitors, with special emphasis on their role in fungal development and virulence.

13.2 Proteolytic Enzymes and Proteolytic Inhibitors

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteolytic enzymes are classified in subgroup 4 of group 3 (hydrolases) (EC 3.4.). A redundant set of terms is used by the scientific community to refer to proteolytic enzymes, including the terms protease, peptide hydrolase, and peptidase. Of these, peptidase is the most recent proposed terminology (Barrett et al. 2001). Peptidases are degradative enzymes that catalyze the cleavage of peptide bonds in macromolecular proteins and oligomeric peptides. All peptidases bind their substrates in a groove or cleft, where amide bond hydrolysis occurs. Amino acid side chains of substrates occupy proteolytic enzyme subsites in the groove, designated as S_3, S_2, S_1, S_1', S_2', S_3', that bind to corresponding substrate/inhibitor residues P_3, P_2, P_1, P_1', P_2', P_3' with respect to the cleavable amide bond (Fig. 13.1).

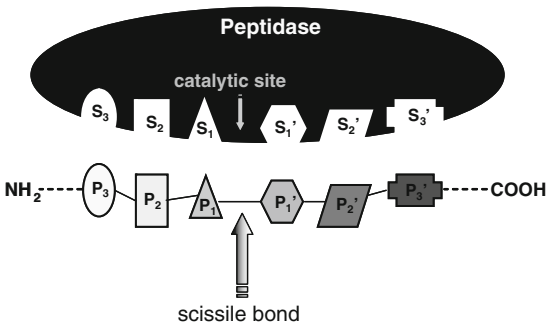

Fig. 13.1 Schematic representation of binding region and catalytic site of a hypothetical peptidase with six subsites (S_1–S_3 and S_1'–S_3'), recognizing a sequence of six amino acids (P_1–P_3 and P_1'–P_3') in the substrate

Peptidases are classified on the basis of three major criteria: (1) type of reaction catalyzed, (2) chemical nature of the catalytic site, and (3) evolutionary relationship with reference to amino acid sequence and protein structure (Rao et al. 1998; Barrett et al. 2001, 2003; Rawlings et al. 2004a). Peptidases are broadly subdivided into two major groups depending on their site of action: exopeptidases and endopeptidades. Exopeptidases cleave the peptide bond proximal to the amino (NH_2) or carboxy (COOH) termini of the proteinaceous substrate, whereas endopeptidases cleave peptide bonds within a polypeptide chain. Based on their site of action at the NH_2, the exopeptidases are classified as aminopeptidases, dipeptidyl peptidases, or tripeptidyl peptidases which act at a free NH_2 terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide or a tripeptide respectively. Carboxypeptidases or peptidyl peptidases act at the COOH terminal of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be further divided into three major groups: serine, metallo, and cysteine carboxypeptidases, based on the functional group present at the active site of the enzymes. Similarly, endopeptidases (also known as proteinases) are classified according to essential catalytic residues at their active sites in: serine, metallo, glutamic, threonine, cysteine, and aspartic endopeptidases (Fig. 13.2). Conversely, there are a few miscellaneous peptidases that do not precisely fit into the standard classification (Rao et al. 1998; Barrett et al. 2001).

In terms of enzyme specificity, the cleavage of peptide bonds by peptidases may be specific, e.g., limited proteolysis, which depends on the amino acid sequence surrounding the hydrolyzed site, or unspecific, which results in complete degradation of proteins to oligopeptides and/or amino acids. By cleaving peptide bonds, peptidases may change the biological properties of polypeptide chains. Specific proteolysis often results in protein and peptide activation or inactivation, and unspecific proteolysis in their degradation, and thus these enzymes are potentially dangerous for cells, and may alter their environment. The activity of a peptidase is regulated at multiple levels including the level of production, the activation of the peptidase generally synthesized in an inactive proform, and the production of specific inhibitors (Rao et al. 1998; Barrett et al. 2001; Rawlings et al. 2004b).

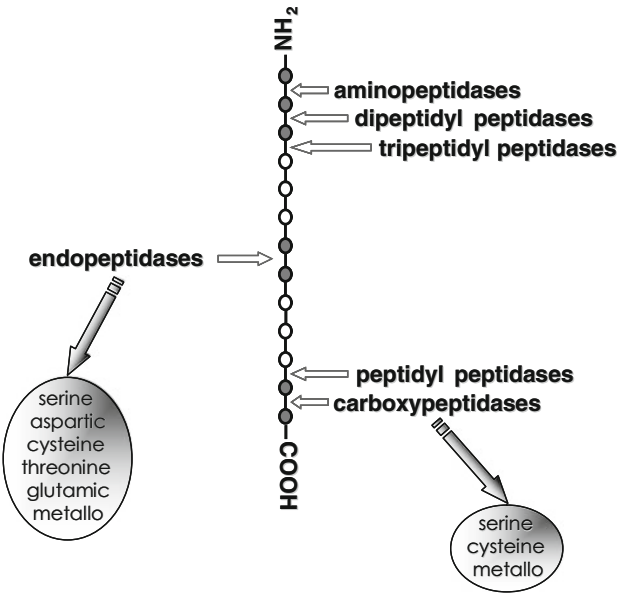

Fig. 13.2 Classification of peptidases. *Open circles* represent amino acids and *gray circles* indicate the amino acid sequence that is bond to the peptidase. *White arrows* point to the site of cleavage. The *gray arrows* indicate the classes of endopeptidases and carboxypeptidases according to the chemical group present in their catalytic sites

Recently, the MEROPS database (http://www.merops.sanger.ac.uk) has provided a catalog and structure-based classification of proteolytic enzymes and their inhibitors. In this novel classification system, the peptidases are grouped into families on the basis of statistically significant similarities between the protein sequences in the part termed "peptidase unit" that is most directly responsible for hydrolytic activity. Families that are thought to have common evolutionary origins and are known or expected to have similar tertiary folds are grouped into clans (Barrett et al. 2001; Rawlings et al. 2004a, b).

The class of a peptidase is routinely determined according to the effects of proteolytic inhibitors on the enzymatic activity. The proteolytic inhibitors can be divided into two functional classes on the basis of their interaction with the target peptidase: (1) irreversible trapping reactions and (2) reversible tight-binding reactions (Fig. 13.3). Inhibitors which bind through a trapping mechanism change conformation after cleaving an internal peptide bond and "trap" the enzyme molecule covalently; neither the inhibitor nor peptidase can participate in further reactions. In tight-binding reactions, the inhibitor binds directly to the active site of the peptidases; these reactions are reversible and the inhibitor can dissociate from the proteolytic enzyme in either the virgin state, or after modification by the peptidase. Based on their structural dichotomy, the proteolytic inhibitors can be generally classified in two large groups: low molecular weight peptidomimetic inhibitors and protein peptidase inhibitors composed of one or more peptide chains. Proteolytic

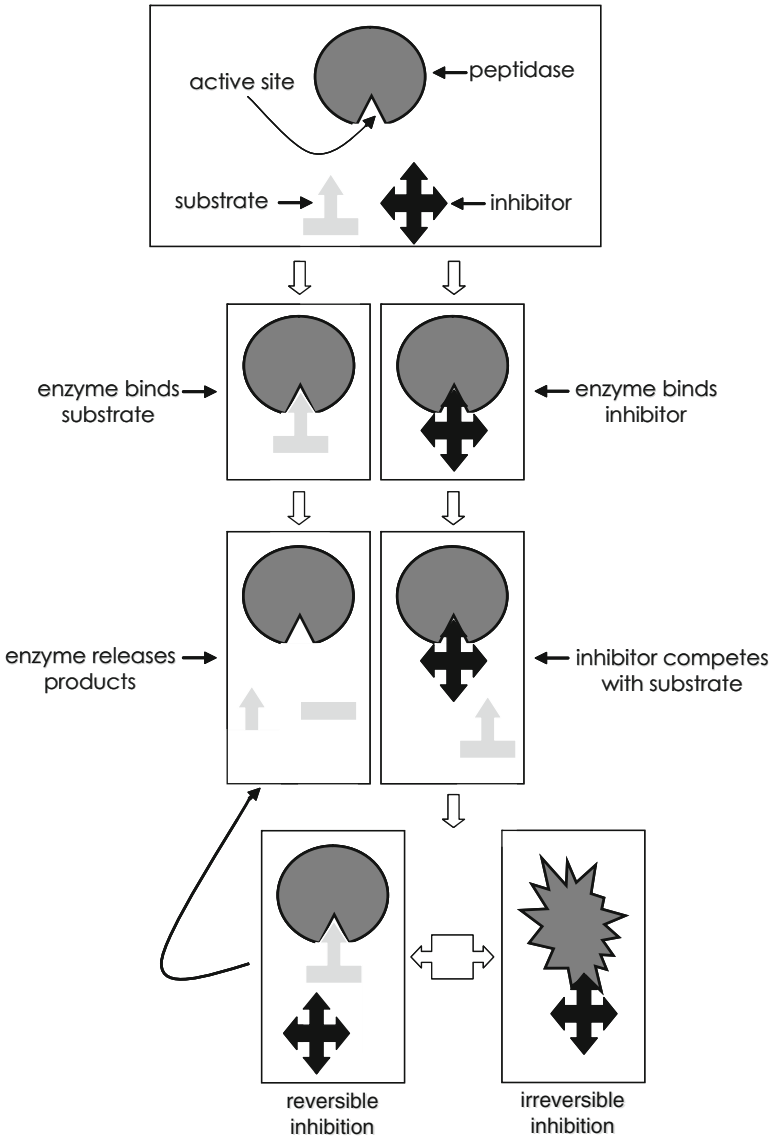

Fig. 13.3 Mechanisms of peptidase inhibition. The peptidase inhibitor competes with the substrate to bind to the active site of a peptidase, and two distinct possibilities arise: (1) substrate binds to the catalytic site and then is cleaved by the peptidase, which releases the products, or (2) inhibitor binds to the active site and by steric hindrance blocks the substrate attachment. In this second case, the inhibitor can promote an irreversible (the conformational structure of the peptidase is completely lost) or reversible inhibition (in this second case, for example, when the inhibitor disconnects to the enzyme, the substrate can bind to it)

inhibitors can be further classified into five groups (metallo, serine, threonine, cysteine, and aspartic peptidase inhibitors) according to the mechanism employed at the active site of peptidases they inhibit. Some proteolytic inhibitors interfere with more than one type of peptidase (Rawlings et al. 2004b).

Peptidases are the single class of enzymes that occupy a pivotal position with respect to their applications in both physiological and commercial fields (Rao et al. 1998). Additionally, peptidases play several physiological roles and are essential factors for homeostatic control in both prokaryotes and eukaryotes. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions, accounting for ~2% of the genes in humans, infectious organisms, and other forms of life (Barrett et al. 2001). Since peptidases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of biological systems, including fungi. The fungal life cycle requires the successful execution of several difficult tasks, including invasion across the host integuments, procurement of nutrients for growth and development, and evasion of host immune defenses (van Burik and Magee 2001). In this context, both surface and secreted peptidases produced by fungal cells could facilitate the colonization and infection of the host and contribute to the production of disease (Fig. 13.4). So, fungal peptidases can play a variety of roles in establishing, maintaining, and exacerbating an infection.

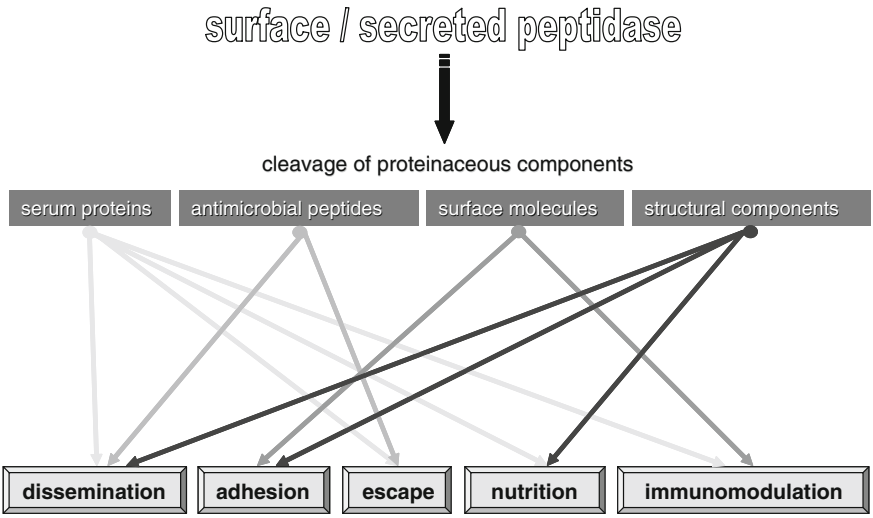

Fig. 13.4 Microbial peptidases (surface and/or secreted forms) are able to cleave different host components such as serum proteins, antimicrobial peptides, surface molecules, and structural proteinaceous compounds. The degradation of host proteins can help the micro-organisms in several steps of their life cycle and pathogenesis, including dissemination, adhesion, escape, nutrition, and immunomodulation of the host immune response

13.2.1 Aspartic-Type Peptidases

Aspartic peptidases (EC 3.4.23.X) are a family of proteolytic enzymes characterized by two essential catalytic aspartic acid residues at the active site, which are located in an extended cleft that can accommodate at least seven amino acids of a substrate molecule that have high-sequence homology, three-dimensional structure similarity, low optimal pH value, and a scission preference between large and hydrophobic amino acids (Davies 1990; Blundell and Johnson 1993; Coates et al. 2006). While most aspartic peptidases conform to these characteristics, substantial differences exist in terms of catalytic properties, cellular localization, and biological functions (Koelsch et al. 1994). In general, the most widely accepted catalytic mechanism is an acid-based system involving two active aspartic acid residues in the active site and a water molecule that resides between them (Fig. 13.5). These two aspartic acid residues act as a proton donor and acceptor respectively, to catalyze the hydrolysis of peptide bonds in substrates. The water molecule is partly activated by an aspartate and makes a nucleophilic attack at a specific carbonyl carbon in the substrate. The carbonyl oxygen, in turn, captures a proton from another aspartic acid in the active site, resulting in a tetrahedral intermediate. This intermediate is the crucial transition state. Restabilizing from the transition state, the amino moiety from the substrate becomes a better leaving group, and the substrate is cleaved into two peptide fragments (Nguyen et al. 2008) (Fig. 13.5).

All aspartic peptidases are produced as inactive precursors (zymogens) which require activation either by autolysis caused by a change in pH upon secretion or by the action of other proteolytic enzymes. Aspartic peptidases are characteristically inhibited by the microbial peptide pepstatin A (Fig. 13.6), which contains the

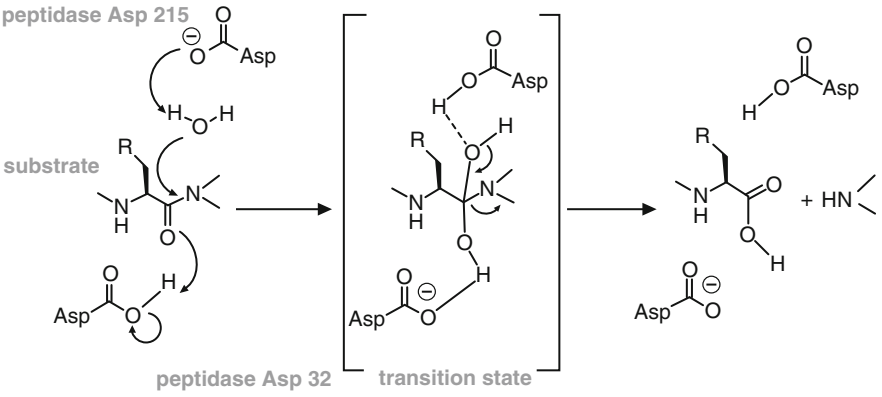

Fig. 13.5 Catalytic mechanism of aspartic-type peptidases proposed by Nguyen et al. (2008). A water molecule tightly bond to the aspartates in the native enzyme is proposed to nucleophilically attack the scissile bond carbonyl. The resulting tetrahedral intermediate is stabilized by hydrogen bonds to the negatively charged carboxyl of aspartate 32. Fission of the scissile C–N bond is accompanied by transfer of a proton to the leaving amino group either from Asp 215. *Dashed lines* indicate hydrogen bonds

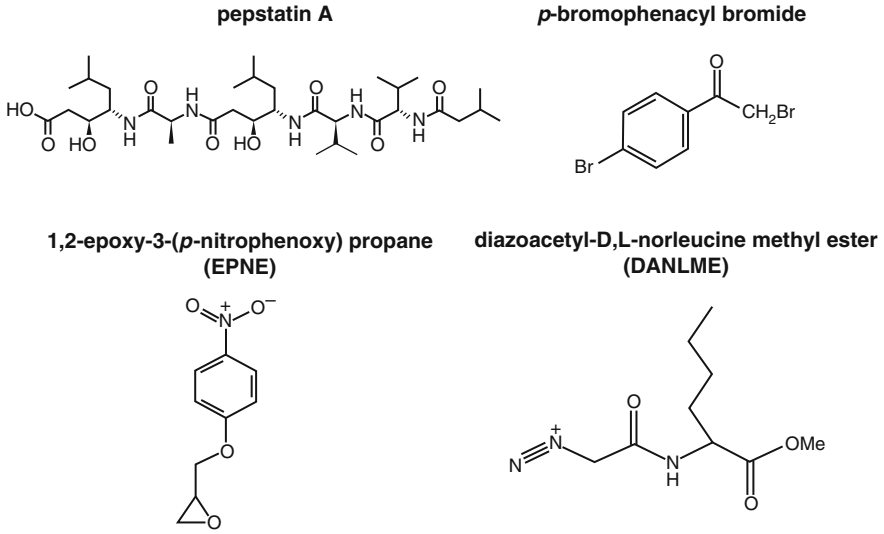

Fig. 13.6 Archetypal aspartic peptidase inhibitors

unusual amino acid statine. Statine is an analog of L-leucine, differing from this amino acid by the insertion of a –CHOH–CH_2– group between the Cα and the main chain carbonyl group. Diazoacetylnorleucine methyl ester, 1,2-epox-3-(*p*-nitrophenoxy) propane, and *p*-bromophenacyl bromide have also been used as diagnostic reagents for aspartic endopeptidases (Fig. 13.6).

The aspartic peptidases are classified in clan A in the MEROPS database, which classifies peptidases and their inhibitors based on similarities at the tertiary and primary structural levels (Rawlings et al. 2006). There are 15 families and 17 subfamilies of aspartic peptidases that are widely distributed in vertebrates, plants, fungi, protozoa, bacteria, and viruses. Most eukaryotic aspartic peptidases are monomeric and consist of a single chain of around 330 amino acids that forms two similar domains with the active site located in between. In contrast, retroviral aspartic peptidases are dimeric, consisting of two identical subunits, each roughly equivalent to one domain of a eukaryotic aspartic peptidase (Davies 1990; Takahashi 1995; Hill and Phylip 1997; James 1998; Rao et al. 1998; Cooper 2002; Fruton 2002; Dash et al. 2003). Mammalian aspartic peptidases are classified into two clans, AA and AD, based on differences in tertiary structure. While clan AA contains the classical aspartic peptidases, clan AD contains peptidases that hydrolyze peptide bonds within biological membranes. Clan AA is further divided into the two families A1 and A2. Family A1 includes pepsin-like enzymes such as pepsin, gastricin, rennin, cathepsin D and E, plasmepsins, and histo-aspartic peptidase. The A2 family, also termed the retropepsin family, includes HIV retropepsin (Rawlings et al. 2006). Most fungal aspartic peptidases are classified into clan AA and family A1 (Rawlings et al. 2006), as summarized in Table 13.1.

Table 13.1 Fungal aspartic peptidases deposited in the MEROPS database

Fungal species	MEROPS database		
	Peptidase name	Clan	Family (subfamily, identifier)
Penicillium janthinellum	Penicillopepsin	AA	A1 (A, A01.011)
Rhizopus microsporus	Rhizopuspepsin	AA	A1 (A, A01.012)
Rhizomucor miehei	Mucorpepsin	AA	A1 (A, A01.013)
Candida albicans	Candidapepsin SAP1	AA	A1 (A, A01.014)
Saccharomyces cerevisiae	Barrierpepsin	AA	A1 (A, A01.015)
Aspergillus saitoi	Aspergillopepsin I	AA	A1 (A, A01.016)
Cryphonectria parasitica	Endothiapepsin	AA	A1 (A, A01.017)
Saccharomyces cerevisiae	Saccharopepsin	AA	A1 (A, A01.018)
Aspergillus fumigatus	Peptidase F	AA	A1 (A, A01.026)
Hypocrea lixii	Trichodermapepsin	AA	A1 (A, A01.027)
Saccharomyces cerevisiae	Yapsin-1	AA	A1 (A, A01.030)
Saccharomyces cerevisiae	Yapsin-2	AA	A1 (A, A01.031)
Saccharomyces cerevisiae	Yapsin-3	AA	A1 (A, A01.035)
Yarrowia lipolytica	Axp peptidase	AA	A1 (A, A01.036)
Candida tropicalis	Canditropsin	AA	A1 (A, A01.037)
Candida parapsilosis	Candiparapsin	AA	A1 (A, A01.038)
Syncephalastrum racemosum	Syncephapepsin	AA	A1 (A, A01.042)
Podospora anserina	Podosporapepsin	AA	A1 (A, A01.044)
Schizosaccharomyces pombe	Yps1 protein	AA	A1 (A, A01.056)
Candida albicans	Candidapepsin SAP2	AA	A1 (A, A01.060)
Candida albicans	Candidapepsin SAP3	AA	A1 (A, A01.061)
Candida albicans	Candidapepsin SAP4	AA	A1 (A, A01.062)
Candida albicans	Candidapepsin SAP5	AA	A1 (A, A01.063)
Candida albicans	Candidapepsin SAP6	AA	A1 (A, A01.064)
Candida albicans	Candidapepsin SAP7	AA	A1 (A, A01.065)
Candida albicans	Candidapepsin SAP8	AA	A1 (A, A01.066)
Candida albicans	Candidapepsin SAP9	AA	A1 (A, A01.067)
Aspergillus oryzae	Oryzepsin	AA	A1 (A, A01.072)
Candida parapsilosis	Sapp2p peptidase	AA	A1 (A, A01.076)
Aspergillus niger	CtsD peptidase	AA	A1 (A, A01.077)
Filobasidiella neoformans	CnAP1 peptidase	AA	A1 (A, A01.078)
Aspergillus niger	PepAa peptidase	AA	A1 (A, A01.079)
Aspergillus niger	PepAb peptidase	AA	A1 (A, A01.080)
Aspergillus niger	PepAc peptidase	AA	A1 (A, A01.081)
Hypocrea lixii	SA76 peptidase	AA	A1 (A, A01.082)
Candida albicans	Candidapepsin SAP10	AA	A1 (A, A01.085)
Saccharomyces cerevisiae	GPI-anchored aspartic peptidase	AA	A1 (A, A01.A61)
Saccharomyces cerevisiae	Yapsin-6	AA	A1 (A, A01.A62)

Based on their importance in health and disease, peptidase inhibitors have already been developed into blockbuster drugs and diagnostics, many others are in clinical trials, and some peptidases are themselves being trialed as vaccines or diagnostics (reviewed by Abbenante and Fairlie 2005). In this context, several positive reasons are proposed for the use of aspartic peptidases as validated drug targets: (1) good experimental tools including recombinant enzymes and inhibitors are already available, (2) there is a large body of data on host and other aspartic

peptidases, and (3) relevant expertise exists in academia and industry in order to synthesize potential aspartic inhibitors; some of them are actually employed in the medical arena.

13.3 Aspartic Peptidases of Human Pathogenic Fungi: A New Target for Fungal Drug Development

All pathogenic micro-organisms have developed mechanisms that allow successful colonization or infection of the host (Finlay and Falkow 1989; van Burik and Magee 2001). Consequently, most pathogens have developed an effective battery of putative virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease, and overcome host defenses. Aspartic peptidases are involved in a myriad of biochemical processes in a variety of fungal cells (Stewart and Abad-Zapatero 2001; Monod et al. 2002; Naglik et al. 2003a). A number of fungi are able to produce both surface and/or secreted aspartic-type peptidases that facilitate penetration into the host organism and counteract its defense systems. Thus there is interest in the design of therapeutic inhibitors for controlling such pathogens. A considerable amount of information is now available on the aspartic extracellular peptidases of human fungal pathogens, especially those produced by the genus *Candida*.

13.3.1 Candida spp

The production and secretion of aspartic peptidases, generally designated as Saps, have been demonstrated or suggested as virulence attributes in opportunistic pathogens of the genus *Candida* (De Bernardis et al. 2001; Hube and Naglik 2001; Naglik et al. 2003a, 2004) Recently, genome mining and phylogenetic analyses revealed the presence of new members of the *SAP* superfamily in *Candida tropicalis* (*SAPT*, $n = 8$), *Candida guilliermondii* (*SAPGU*, $n = 8$), *Candida parapsilosis* (*SAPP*, $n = 11$) and *Candida lusitaniae* (*SAPLU*, $n = 3$) in comparison to *Candida albicans* (*SAP*, $n = 10$) and *Candida dubliniensis* (*SAPDU*, $n = 8$) (Parra-Ortega et al. 2009). Interestingly, construction of a phylogenetic tree of the Sap superfamily generated 12 families of genes from six *Candida* species: *SAP1-3*/*SAPDU1-3* and *SAPT4; SAP4-6; SAPP1-3; SAPT1* and *SAP8*/*SAPDU8; SAPT2; SAPGU* and *SAPLU; SAPT3; SAPP* (two distinct families); *SAP7*/*SAPDU7; SAP10*/*SAPDU10;* and *SAP9*/*SAPDU9* (Parra-Ortega et al. 2009). No typical *SAP* genes have been described in *Candida glabrata*, a species more phylogenetically related to *Saccharomyces cerevisiae* than other pathogenic *Candida* species. However, both *C. glabrata* and *S. cerevisiae* possess several yapsins, which are nonsecreted, glycosylphosphatidylinositol (GPI)-anchored aspartic peptidases involved in cell wall

integrity and cell–cell interactions (Krysan et al. 2005). Among the *Candida* species that produce Sap, a huge volume of experiments have certainly been conducted cincerning *C. albicans* which generated a tremendous knowledge about Sap production and function.

Saps are differentially secreted, depending on the *C. albicans* strain and environmental conditions (White and Agabian 1995; Costa et al. 2003; Santos et al. 2006) (Fig. 13.7). Studies of the biochemical properties of Sap isoenzymes have been performed by using the purified protein from culture supernatants (Fig. 13.7)

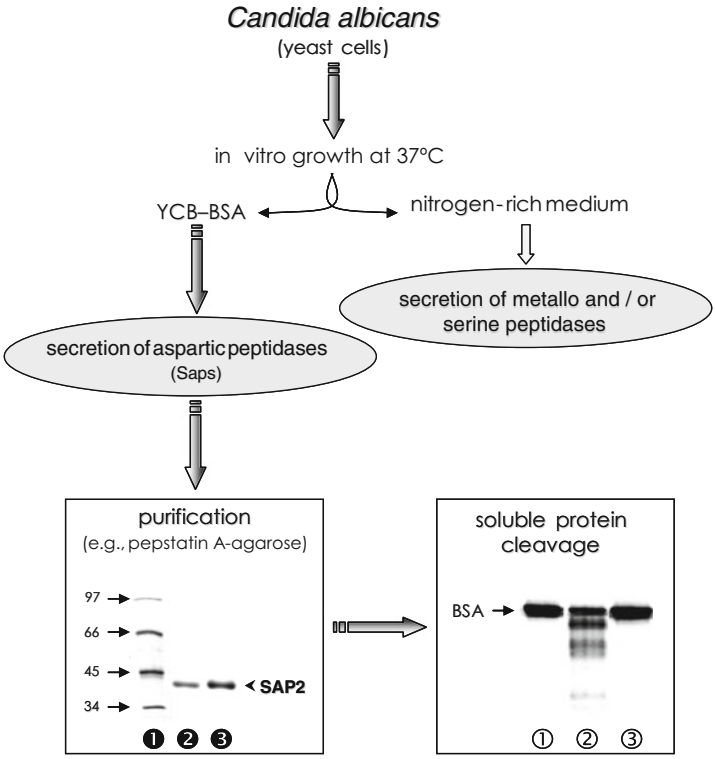

Fig. 13.7 Modulation of peptidase expression in *Candida albicans* according to the culture growth conditions. When cultured in nitrogen-rich condition, such as brain–heart infusion medium, yeast cells secreted metallo and/or serine peptidases, but no aspartic ones (for review see Costa et al. 2003 and Santos et al. 2006). Conversely, yeast cells grown in yeast carbon base supplemented with bovine serum albumin (YCB-BSA), as the sole source of nitrogen, extracellularly released aspartic peptidases, especially Sap2 isoenzyme, which can be purified by several chromatographic procedures such as pepstatin A-agarose. After SDS-PAGE, a predictable polypeptide with 43 kDa (White and Agabian 1995) was evidenced by silver staining (❷) or by western blotting using anti-Sap1-3 antibody (❸). Line ❶ shows the molecular masses of the protein standards expressed in kilodaltons. Sap2 is capable in hydrolyzing different protein substrates, like BSA, in acidic pH conditions. This fragmentation generates low molecular mass peptides (②). However, pepstatin A at micromolar range fully inhibited the BSA cleavage (③). Line ① represents the intact BSA molecule incubated only with buffer

or by recombinant expression in heterologous systems. Eight of these peptidases (Saps1–8) are secreted into the extracellular environment, while Sap9 and Sap10 are membrane GPI-anchored proteins (Hube and Naglik 2001). Saps1–3 are expressed predominantly in yeast cells, whereas Saps4–6 are expressed in hyphal forms. Saps1–3 have optimal activity in a pH range of 2.5–5.0, in contrast to Saps4–6 that have greater activity at a higher pH range (Hube et al. 1994). Another difference is the net charge: while Saps1–3 are negatively charged on the whole, Sap5 is positive (Borelli et al. 2008). Enzymatic studies of some isoenzymes (Sap1–3 and Sap6) have also revealed significant differences in substrate specificities (Fusek et al. 1997; Smolenski et al. 1997; Koelsch et al. 2000), suggesting that the large set of isoenzymes provides *C. albicans* with a capacity to hydrolyze a variety of substrates under a wide range of conditions. Furthermore, detailed analysis of the *in vitro* expression of *SAP* genes revealed that the major peptidase gene expressed in *C. albicans* yeast forming at 30°C–37°C in medium-containing proteins such as BSA or hemoglobin, as the sole nitrogen source, is *SAP2* (Hube et al. 1994). However, at 25°C, levels of Sap8 protein and *SAP8* transcripts increase, suggesting that the expression of this gene is temperature-regulated (Monod et al. 1998). Expression of *SAP9* and *SAP10* appears to be independent of environmental conditions, since both genes are constitutively expressed in all growth forms (Felk et al. 2002; Schaller et al. 2003). Expression of *SAP1* (Morrow et al. 1992) and *SAP3* (White et al. 1993) is regulated during phenotypic switching; the isoenzymes are expressed in the "opaque" but not in the "white" form of strain WO-1. By modulating the expression of several molecules, including Saps, *Candida* possesses the remarkable ability to survive and proliferate in a radically changing environment, adapting their growth to physiological extremes of pH, osmolarity, availability of nutrients, and temperature (Naglik et al. 2003a).

Sap2 is the most abundant virulence factor expressed during *C. albicans* infection, and the principal protein known to induce antibody response during *Candida* infection in humans. Individuals with candidiasis have high titers of antibodies to Sap2, with soluble antigens present in their serum (Na et al. 1999). However, no evidence was found to demonstrate that specific or nonspecific antibodies in serum or saliva could inhibit *C. albicans* Sap2 activity using a BSA hydrolysis assay (Naglik et al. 2005). Evidence was also found of the presence of Sap antigen in all organs of immunocompromised patients who had died of systemic *C. albicans* infections, such as the mucosa, central nervous system, lung, heart, liver, pancreas, and kidney (Rüchel et al. 1991). Mice immunized with *C. albicans* extracts enriched with Sap2 have shown a decrease in mucosal tissue fungal burden (Na et al. 1999). Intradermal administration of purified Sap2 with alum to BALB/c mice, which have been infected intraperitoneally with *C. albicans*, has resulted in a 20-fold decrease in kidney colonization (Vilanova et al. 2004). Therefore, Sap2 is considered to be a candidate for a vaccine against systemic candidiasis.

Sap2 has broad substrate specificity (Hube 1996). Extracellular matrix and host surface proteins such as keratin, human stratum corneum, collagen, laminin, fibronectin, vimentin, and mucin are efficiently degraded by this Sap isoenzyme. Several host defense proteins, such as salivary lactoferrin, the peptidase inhibitor

α_2-macroglobulin (a natural peptidase inhibitor in human plasma), cystatin A (a cysteine peptidase inhibitor found in human epidermal tissue and fluids), human endothelin-1 precursor (a vasoconstrictive peptide that alters vascular homeostasis), enzymes of the respiratory burst of macrophages, C3 complement protein, and almost all immunoglobulins, including secretory IgA (which is normally resistant to most bacterial peptidases) can also be efficiently hydrolyzed by Sap2 (reviewed by Hube 1996; Naglik et al. 2003a). In addition, Villar et al. (2007) demonstrated that *C. albicans* is able to degrade E-cadherin, the major protein in the epithelial cell junction, and that the degradation was mediated by Sap5. Sap9 and Sap10 play a role in adhesion, cell separation during budding, and cell surface integrity (Albrecht et al. 2006). Moreover, deletion of *SAP9* and *SAP10* modified the adhesion properties of *C. albicans* to epithelial cells and caused attenuated epithelial cell damage during experimental oral infection, suggesting a unique role for these peptidases in both cellular processes and host–pathogen interactions. A recent study has shown that Sap9 produced by *C. albicans* cells efficiently and rapidly degrades histatin-5, a salivary antimicrobial peptide, resulting in loss of its anticandidal potency (Meiller et al. 2009).

The importance of specific Sap isoenzymes for the pathogenicity of *C. albicans* has been investigated by comparing the virulence of mutants deleted for individual or multiple *SAP* genes with that of a wild-type control strain in different infection models. For instance, *sap1*Δ, *sap2*Δ and *sap3*Δ single mutants as well as triple mutants lacking the highly homologous *SAP4–SAP6* genes exhibited attenuated virulence after intravenous infection of mice, indicating that all these genes have important roles for the normal progression of a systemic infection (Hube et al. 1997; Sanglard et al. 1997). Mutants deleted for either *SAP1*, *SAP2* or *SAP3* were also found to be less virulent in a rat model of *Candida* vaginitis, whereas mutants lacking *SAP4–SAP6* did not exhibit a detectable virulence defect under these conditions (De Bernardis et al. 1999a). On the other hand, only the latter mutants showed reduced virulence in a murine model of *Candida* peritonitis, while deletion of *SAP1*, *SAP2* or *SAP3* had no significant effect in this infection model (Kretschmar et al. 1999). The importance of Saps for tissue invasion and damage seems to depend on the infection model used. For example, when the interaction of mutants lacking one of the *SAP1–SAP3* genes with endothelial cells was investigated, only Sap2, but not Sap1 or Sap3, was found to contribute to the ability of *C. albicans* to damage endothelial cells (Ibrahim et al. 1998). Furthermore, *Candida* peptidases have been shown to activate the pro-inflammatory cytokine interleukin-1β from its precursor, suggesting a role for Saps in the activation and maintenance of the inflammatory response at epithelial surfaces *in vivo* (Beausejour et al. 1998). Saps may also contribute to the pathogenesis of inflammatory mucosal lesions in an *in vitro* model of vaginal candidiasis based on reconstituted human vaginal epithelium (RHVE) by inducing the upregulation of epithelial proinflammatory cytokines including interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, gamma interferon (IFN-γ), and tumor necrosis factor-alpha (TNF-α) responses in comparison with cytokine expression in noninfected tissue (Schaller et al. 2005). Addition of the aspartic peptidase inhibitor pepstatin A,

a hexapeptide from *Streptomyces*, strongly reduced the cytokine response of RHVE. Also, *SAP*-null mutants lacking either *SAP1* or *SAP2* caused reduced tissue damage and had a significantly reduced potential to stimulate cytokine expression. These data show that the potential of specific Saps to cause tissue damage correlates with an epithelium-induced proinflammatory cytokine response, which may be crucial in controlling and managing *Candida* infections at the vaginal mucosa *in vivo* (Schaller et al. 2005). In another work, *SAP7* gene transcript was detected in 60% of oral candidiasis patients as opposed to 25% of *Candida* carriers by means of RT-PCR (Naglik et al. 1999). Felk et al. (2002) found that *SAP7* transcript was not induced in response to an intraperitoneal model of *C. albicans* infection that examined invasion of parenchymal organs. Vaginal samples examined for the presence of *SAP7* transcript showed that strains collected from patients with vaginal candidiasis induced *SAP7* significantly compared to samples collected from carriers (Naglik et al. 2003b). Furthermore, a study examining infection of RHVE found induction of *SAP7* transcript after 24 h of infection (Schaller et al. 2003). Taylor et al. (2005) reported that *SAP7* of *C. albicans* was induced after vaginal infection of mice. Conversely, virulence during vaginal infection was not affected in a *Δsap7/Δsap7* mutant strain; only a partial virulence phenotype was detectable after intravenous injection.

There are several reports indicating that invasive strains of *C. albicans* produced significantly more extracellular proteolytic activity than the commensal strains did (Chakrabarti et al. 1991; De Bernardis et al. 2001; Yücel and Kantarcıoglu 2001). The mean secretory peptidase activity of *C. albicans* isolates from the oropharyngeal candidiasis in an HIV-positive group was significantly higher when compared with the isolates from the control group ($P < 0.05$). Peptidase activity within the population of *C. albicans* isolates from HIV-positive individuals was independent of the patient's clinical disease stage and no correlation of the peptidase activities with the *C. albicans* serotype was found. Because *C. albicans* isolates from HIV-positive subjects invariably have elevated Sap production, it appears that HIV, or a factor related to its infection, exerts a positive selective pressure for high Sap production by *C. albicans*. In this way, HIV-1 envelope proteins (gp160 and gp41) enhance the virulence of *C. albicans* by increasing the secretion and activity of Sap and induce a dysfunction of phagocytic cells (Gruber et al. 1998). Additionally, Gokce et al. (2007) detected higher levels of extracellular peptidase activity in *C. albicans* strains than non-*albicans Candida* strains (89.7% vs. 25.8%, $P < 0.05$), which is consistent with the findings (40% vs. 10%, $P < 0.05$) of Ozkan et al. (2005). De Bernardis et al. (1999b) found that cutaneous isolates of *C. parapsilosis* had uniformly elevated Sap activity, more than four times higher than the enzyme activity of the blood isolates. Similarly, Cassone et al. (1995) detected a higher proteolytic activity in vaginal *C. parapsilosis* isolates when compared with blood isolates. Fotedar and Al-Hedaithy (2005) demonstrated that only 32% of the *C. dubliniensis* isolates ($n = 87$) exhibited moderate activity of secretory aspartic peptidase whereas a vast majority (68%) of them were nonproteolytic. Conversely, a strong peptidase activity was observed for 79% of *C. albicans* strains ($n = 52$) while the remaining 21% isolates showed moderate peptidase activity.

Interestingly, a positive correlation of peptidase activity to antifungal susceptibility was evident. The *C. albicans* isolates from the HIV-positive group that were characterized by higher levels of peptidase activity were also less susceptible to the widely used azole antifungal agents ketoconazole and fluconazole (Ollert et al. 1995). *C. albicans* strains exposed to subinhibitory concentrations of antifungals belonging to the azole, echinocandin, and pyrimidine analog classes showed an elevated activity of Sap2 in the culture filtrates (Wu et al. 2000; Ripeau et al. 2002). In addition, the expression of *SAP2* and activity of the secreted Sap2 gene product was upregulated in fluconazole-exposed yeast cells (Copping et al. 2005). In fact, Navarathna et al. (2005) showed in animal experiments that the virulence of *C. albicans* strains is increased upon exposure to subinhibitory concentrations of fluconazole.

The ability of *Candida* species to form biofilms has important clinical repercussions due to their increased resistance to antifungal therapy and the ability of yeast cells within the biofilms to withstand host immune defenses (Silva et al. 2009). Mendes et al. (2007) showed that biofilms of *C. albicans* consistently secrete more Saps than their planktonic counterparts, suggesting that increased Sap production is yet another mechanism by which yeasts persist on host surfaces, in addition to the expression of the foregoing virulence attributes. Another intriguing observation is that the Sap-encoding genes *SAP1-10* were differentially expressed in *C. albicans* oral isolates that possess distinct genome backgrounds, referred to as *b* and *c* karyotype profiles, in which the *c* karyotype express a higher number of *SAP1-10* genes than *b*-type strains during their *in vitro* propagation (Tavanti et al. 2004). The host immune system is the major factor determining whether this opportunistic yeast behaves as a commensal or as a pathogen. In this sense, Tavanti et al. (2006) demonstrated that all *c*-type *C. albicans* strains were able to develop hyphal forms, resist intracellular killing, replicate and escape from macrophage cells. Conversely, the *b*-type isolates, which were shown to be more efficiently ingested by macrophages than the *c*-type strains, were susceptible to intracellular killing and predominantly found as blastoconidia inside macrophages, suggesting that the *c* type strains are better suited to behave as a more virulent strain cluster.

Most of the clinical isolates of *C. parapsilosis*, *C. tropicalis*, *C. dubliniensis*, and *C. lusitaniae* are peptidase producers and usually have more than one Sap isoenzyme (Monod et al. 1994; Dostál et al. 2003; Naglik et al. 2003a). However, the role of Saps in the virulence of non-*albicans Candida* has not been fully clarified. For instance, *C. tropicalis* secretes dominantly Sapt1p *in vitro* in a medium containing BSA as the sole source of nitrogen. Sapt1p was isolated from the culture medium and its three-dimensional structure was determined (Symersky et al. 1997). Fusek et al. (1993) isolated two aspartic peptidases secreted by *C. parapsilosis* that have similar molecular masses, though they vary in their N-terminal sequences and isoelectric points. The substrate specificities detected with a synthetic peptide also varied. Weak proteolytic activities of aspartic peptidase secreted by two *C. lusitaniae* strains were detected in culture media using fluorogenic substrate (Capobianco et al. 1992). As previously mentioned, *C. glabrata* does not produce any aspartic peptidase belonging to the Sap superfamily; however, it possesses

several yapsin genes (named *YPS*). The *S. cerevisiae* yapsins are a family of five GPI-linked aspartic peptidases (Yps1–3, Yps6 and Yps7) that have been shown to cleave peptides C-terminal to basic residues both *in vitro* and *in vivo* (Krysan et al. 2005). Transcript profiling of *C. glabrata* yeast cells ingested by macrophages reveals global changes in metabolism as well as increased expression of *YPS* gene family (Kaur et al. 2007). Genetic analysis evidenced that the *C. glabrata YPS* genes were required for survival during the stationary phase or under conditions of cell wall stress, for adherence to mammalian cells, for survival in macrophages, and for virulence in a murine model of disseminated candidiasis. By monitoring the processing of a cell wall adhesin named Epa1, the authors showed that Yps peptidases play an important role in cell wall remodeling by removal and release of GPI-anchored cell wall proteins in response to different host environments. Alternatively, the Yps peptidases might protect *C. glabrata* from immune recognition by acting to remove GPI-cell-wall protein targets of the innate or adaptive immune responses (Kaur et al. 2007).

13.3.2 Other Pathogenic Fungi

Although a significant amount of information is known about the aspartic peptidases produced by *C. albicans*, knowledge of the aspartic-type proteolytic system of most other human pathogenic fungi is still in its early stages. In this area, most studies focus on the detection, identification, and biochemical/molecular characterization of both cellular and extracellular aspartic-type peptidases; however, there is a paucity of information regarding the biological role of this class of hydrolytic enzyme. Table 13.2 summarizes the published data about the aspartic peptidase produced by human pathogenic/opportunistic fungi, including species belonging to the following genera: *Aspergillus*, *Coccidioides*, *Cryptococcus*, *Fonsecaea*, *Histoplasma*, *Paracoccidioides*, and *Sporothrix*.

13.4 Aspartic Peptidase Inhibitors: A Novel Alternative for Fungal Treatment?

The last decade has witnessed an effervescence of research interest in the development of potent inhibitors of various aspartic peptidases (reviewed by Dash et al. 2003; Horimoto et al. 2009). As an enzyme family, aspartic peptidases are a relatively small group that has received enormous interest because of their significant roles in human diseases, such as involvement of renin in hypertension (Scott et al. 2006), cathepsin D in metastasis of breast cancer (Benes et al. 2008), cathepsin E in the immune system (Zaidi and Kalbacher 2008), BACE (β-site amyloid precursor protein cleaving enzyme) in Alzheimer's disease (Vassar 2002), pepsin

Table 13.2 Aspartic peptidases produced by human pathogenic fungi

Fungal species	Morphological form	Aspartic peptidases: biochemical and immunological properties		
		Localization (name)	MM (p*I*)	Main findings
Aspergillus fumigatus	Mycelia/ conidiophores	Extracellular (PEP1)		Immunogenic molecule; possible allergen associated with allergic bronchopulmonary aspergillosis; plays a lesser role in tissue invasion (Reichard et al. 1994), detected in human lung infected by *A. fumigatus* (Reichard et al. 1996); *pep*-mutant revealed no significant difference in virulence between the wild-type strain and the mutant in an intravenous model of aspergillosis (Reichard et al. 1997)
Aspergillus fumigatus	Mycelia	Cell wall (PEP2)	39 kDa	Possible participation in the weakening of the cell wall to allow fungal cell growth (Reichard et al. 1997)
Aspergillus fumigatus	Mycelia	Extracellular (CtsD)		Detected in *Galleria mellonella* insect larvae inoculated with *A. fumigatus*; anti-CtsD antibody exhibited a protective effect against fungal infection in this insect model system (Vickers et al. 2007)
Aspergillus niger	Mycelia	Extracellular (PEPA and PEPB) and cellular (PEPE)		Unknown (Van den Hombergh et al. 1997a, b)
Candida spp.	Yeasts/mycelia	Extracellular (Saps)		Well-known virulence factors that participate in numerous aspects of *Candida* biology and pathogenesis (see details in the Sect. 13.1)
Coccidioides immitis	Spherules	Cellular	43 kDa	Immunogenic molecule; stimulates immune T cells *in vitro*; protects mice against pulmonary infection with *C. immitis*; reduces fungal burden in *C. immitis*-challenged mice (Tarcha et al. 2006)
Cryptococcus neoformans	Yeasts	Extracellular (CnAP1)	51.6 kDa	Strongly inhibited by pepstatin A, but insensitive to indinavir, lopinavir and ritonavir (Pinti et al. 2007); transcriptional level of *cnap1* is constant in different media (with simple or complex nitrogenous source); *cnap1* expression depends on the fungal growth phase, with higher expression in stationary phase than in exponential one

(*continued*)

Table 13.2 (continued)

Fungal species	Morphological form	Aspartic peptidases: biochemical and immunological properties		
		Localization (name)	MM (p*I*)	Main findings
Cryptococcus neoformans	Yeasts	Several GPI-anchored to cell wall		Found that some of them are postulated to be functionally similar to yapsins (Levitz and Specht 2006; Eigenheer et al. 2007)
Fonsecaea pedrosoi	Conidia/ Mycelia	Extracellular		Inducible peptidases (just produced in chemically defined medium) (Palmeira et al. 2006a), cleavage of numerous substrates like human serum proteins, extracellular matrix components, and syalilated compounds; possible participation in conidia into mycelia transformation (Palmeira et al. 2006a,b, 2008)
Histoplasma capsulatum	Yeasts/ mycelia	Extracellular		Unknown, but activity was more pronounced in the yeast form than in the mycelial one (Muotoe-Okafor et al. 1996)
Paracoccidioidis brasiliensis	Yeasts	Cell wall/ extracellular	44 kDa (5.27)	Unknown (Tacco et al. 2009)
Sporothrix schenckii	Yeasts	Extracellular	39 kDa (3.80)	Immunogenic molecule (Yoshiike et al. 1993); hydrolyzes natural host protein substrates including stratum corneum, type I collagen, hemoglobin, BSA, α-casein and elastin (Tsuboi et al. 1987), as well as IgG_1 and IgG_2 (Da Rosa et al. 2009)

in various gastric disorders such as peptic ulcer disease (Cooper 2002), plasmepsins in malaria (Coombs et al. 2001), HIV-1 peptidase in AIDS (Cooper 2002), and Saps in candidal infections (Stewart and Abad-Zapatero 2001). Some of these diseases are major causes of death in both the developed and developing countries. As cited above, aspartic peptidases play pivotal roles in many diseases and the development of inhibitors to this class of hydrolytic enzymes raises many prospects for the treatment of these pathological conditions.

The advent of HIV peptidase inhibitors in late 1995 was hailed as a major step forward in the battle against HIV/AIDS (Flexner 1998). The drug combination in HAART involving HIV peptidase inhibitors has revolutionized the treatment of AIDS (Sepkowitz 2001). There are at present ten peptidase inhibitors licensed for clinical use in the treatment of HIV infections: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir (fosamprenavir), lopinavir, atazanavir, brecanavir, tipranavir, and darunavir (Fig. 13.8). The HIV peptidase inhibitors were the first success of structure-based drug design (Wlodawer and Erickson 1993). All of the clinically successful ones are competitive active site inhibitors. They interact with the active site in such a way as to keep the flaps of the enzyme tightly closed over the active

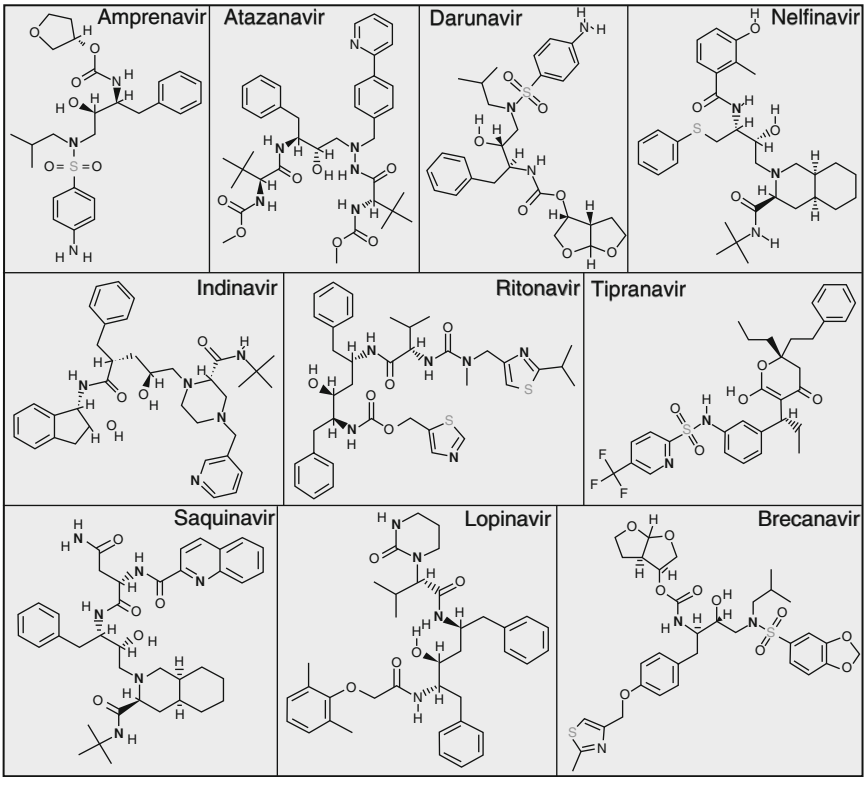

Fig. 13.8 Aspartic peptidase inhibitors used in the anti-HIV chemotherapy

site, thus mimicking the transition state and thereby effectively inactivating the enzyme. With the exception of tipranavir, which is based on a coumarin scaffold, all the HIV peptidase inhibitors are based on the "peptidomimetic" principle, that is, they contain a hydroxyethylene scaffold which mimics the normal peptide linkage (cleaved by the HIV peptidase) but which itself cannot be cleaved. Chemically, these inhibitors have generally hydrophobic moieties that interact with the mainly hydrophobic S_2–S_2' pockets in the active site. HIV peptidase inhibitors prevent cleavage of gag and gag–pol protein precursors in acutely and chronically infected cells, arresting maturation and thereby blocking the infectivity of nascent virions (Roberts et al. 1990; Flexner 1998). The main antiviral action of HIV peptidase inhibitors is thus to prevent subsequent waves of infection. Therapeutically useful HIV peptidase inhibitors bind to the active site with an affinity several orders of magnitude greater than peptide substrates. These agents are active against clinical isolates of HIV types 1 and 2, with the *in vitro* concentration of drug required to reduce viral production by 50% (IC_{50}) ranging from 2 nM to 60 nM (Roberts et al. 1990; Flexner 1998).

Inhibitors of HIV-encoded peptidase, combined with nucleoside analogs with antiretroviral activity, caused profound and sustained suppression of viral replication, increased $CD4^+$ T lymphocyte cell counts, reduced morbidity and mortality, promoted an improvement in the quality and prolongation of life, arresting the progression of the HIV/AIDS condition, with a dramatic fall in HIV-related opportunistic infections (UNAIDS/WHO 2005). These breakthroughs were seen not only in clinical trials but in the clinical setting and provided new hope for those with HIV. There was a shift from HIV representing an acute, rapidly fatal infectious disease to a chronic manageable illness (Palella et al. 1998; Randolph and DeGoey 2004; Crum et al. 2006; Mastrolorenzo et al. 2007; Walmsley 2007). In addition to direct antiviral effects, the HIV peptidase inhibitors also modulate apoptosis. A growing body of work demonstrates the antiapoptotic effects of HIV peptidase inhibitors on $CD4^+$ and $CD8^+$ T cells during HIV infection (Vlahakis et al. 2007). Some of the HIV peptidase inhibitors, such as ritonavir, have also been reported to have effects in non-HIV-related systems, showing inhibition of TNF-α-mediated cell death in Jurkat cells (Wolf et al. 2003), antiatherogenic properties on vascular smooth muscle cells (Kappert et al. 2004), and inhibition of brush-border microvilli assembly in *Escherichia coli* (Potter et al. 2003), as well as having antitumorigenic properties (Gaedicke et al. 2002; Sgadari et al. 2002). Since these drugs have already been shown to be safe for humans, the potential for using them in the treatment of conditions other than HIV infection has led to these effects being further studied. In this context, since the introduction of HAART in 1996, the mortality and morbidity for a wide variety of opportunistic viral, bacterial, parasitic, and fungal infections have decreased dramatically among HIV-infected individuals in economically developed countries (Palella et al. 1998; Centers for Disease Control and Prevention 2001). In a multinational cohort study involving 6941 HIV-positive individuals from Australia and ten European countries, when comparing the periods 1997–2001 and 1994–1996, there was a significant HAART-induced decrease in progression to all AIDS-defining diseases (e.g., candidiasis

from 17.0% to 5.7%, cryptosporidiosis from 3.1% to 0.2%, cytomegalovirus from 5.9% to 0.6%, *Pneumocystis carinii* pneumonia from 17.0% to 4.4%, toxoplasmosis from 3.4% to 1.4%, and tuberculosis from 6.4% to 2.6%) (Babiker et al. 2002; Pozio and Morales 2005).

13.4.1 Candida albicans

The fact that *C. albicans* is capable of adhering to plastic surfaces and to cells in various target tissues has widely been accepted as a first step in the pathogenesis of candidiasis and can be regarded as an important virulence factor of this fungus. Experimental studies suggested that elevated Sap production is an important aspect of *C. albicans* interaction with host tissue and strain virulence (Ghannoum and Abu Elteen 1986; Cassone et al. 1987; Kobayashi et al. 1989; Louie et al. 1994; De Bernardis et al. 2001). For example, *C. albicans* yeast cells efficiently adhered to primarily cultured human keratinocytes. In parallel, more than 95% of all yeast cells displayed germ tubes after a 60-min exposure to keratinocytes in adherence assays. However, *C. albicans* adherence was blocked by the acid peptidase inhibitor pepstatin A at 1 μM (45% inhibition) (Ollert et al. 1993), as was already shown for attachment and invasion to oral mucosa epithelium (Borg and Ruchel 1988) and exfoliated corneocytes (El-Maghrabi et al. 1990). Pepstatin A also reduced the adherence of *C. albicans* to vaginal mucosa epithelial cells (53.1%, 48.7% and 59.9% respectively to isolates from asymptomatic, vulvovaginal candidiasis, and recurrent vulvovaginal candidiasis patients) (Consolaro et al. 2006). Borg and Ruchel (1988) reported the capability of pepstatin A to block invasion of host tissue by hyphal cells at neutral pH. Interestingly, inhibition of Saps with pepstatin A prevents the initial penetration of *C. albicans* through mucosal surfaces, but not the dissemination of the fungus once the cells have already reached the blood vessels (Rüchel et al. 1990; Fallon et al. 1997; Naglik et al. 2004). These results suggest that the Saps are important in the early stages of infection but might not play such a significant role in the progression and dissemination of infection from the bloodstream to deeper host tissues. However, the lack of complete protection by pepstatin A might also be caused by its rapid clearance from the bloodstream (Rüchel et al. 1990; Dash et al. 2003). Additionally, the route of *Candida* challenge rather than the route of pepstatin A administration might be more relevant for observing the protective effects of this inhibitor. Another intriguing study showed that the pretreatment of *C. albicans* yeasts with pepstatin A also led to a significant decrease in the number of cells adhering to poly-L-lysine-coated slides, which represent a homogeneous and positively charged surface, and to Matrigel (which is a solubilized extract of basement membrane from Engel-Horm Swarm transplantable mouse tumor and therefore contains a variety of important components of endothelial cell layers), but did not affect the adherence to human buccal epithelial cells (Watts et al. 1998). All these results suggest a nonenzymatic role for the Sap in the adherence to both biotic and abiotic surfaces.

Pepstatin-like drugs, however, are not used clinically because of their metabolism in the liver and rapid clearance from blood. Since the introduction of HAART, researchers have noticed a concurrent reduction in the incidence of oral candidiasis (Zingman 1996). The reduction in candidal infections in HIV-infected patients in the HAART era might not have resulted solely from improved immunological status, such as increased $CD4^+$ T-cell count or decreased viral load, but could also be as a result of direct inhibition of *Candida* Saps, which belongs to the same family as HIV peptidase, by the HIV peptidase inhibitors in HAART. In this context, Hoegl et al. (1998) published a case of a patient receiving HAART who recovered from a *Candida* infection but maintained a low CD4+ T cell count, a finding that corroborates the hypothesis that HAART might have a direct effect on *C. albicans*. These findings led to studies showing that HIV aspartic peptidase inhibitors indeed led to a direct attenuating effect on *C. albicans* Saps *in vitro* and *in vivo*.

Korting et al. (1999) showed that saquinavir and indinavir were able to inhibit the Sap activity in a dose-dependent manner, when BSA, hemoglobin, or human stratum corneum were used as protein substrates. These inhibitory effects were observed in a therapeutically relevant dose range. Evidence for a direct protective role of saquinavir during experimental oral candidiasis was also demonstrated by a strong attenuated histological phenotype. The authors showed that without saquinavir, at 12 h after infection with *C. albicans*, a powerful epithelial lesion was observed with vacuolation, edema, and detachment of all epithelial layers; in addition, *C. albicans* was able to invade the epithelium. All the morphological alterations were robustly reduced when saquinavir was added at a concentration of 0.3 μM (Korting et al. 1999). In parallel, Cassone et al. (1999) demonstrated that indinavir and ritonavir exerted a marked dose-dependent inhibition of Sap production and activity when evaluated both antigenically and as direct enzyme activity on BSA as substrate. Also, both peptidase inhibitors noticeably inhibited growth of *C. albicans* in a medium with BSA as the sole nitrogen source, at acidic pH. As expected, in nitrogen-unlimited complex media, where *SAP* expression is not required for growth, the inhibitors did not arrest *C. albicans* growth. Of more importance, indinavir and ritonavir exerted a therapeutic effect in an experimental model of vaginal candidiasis (estrogen-dependent rat vaginitis), with an efficacy comparable to that of fluconazole, a well-known anticandidal agent (Cassone et al. 1999). In the same year, Borg-von Zepelin et al. (1999) showed that purified Sap1, Sap2 and Sap3 were inhibited by four distinct HIV aspartic peptidase inhibitors (ritonavir, saquinavir, indinavir, and nelfinavir) in a concentration-dependent manner, in which ritonavir was by far the most effective inhibitor of these Sap isoenzymes. In contrast, only a slight inhibition of activity was detected for purified Sap4, Sap5 and Sap6. These antiretroviral agents were able to inhibit *C. albicans* Saps1–3, which are involved in *Candida* adherence. In this study, the authors testified that ritonavir and saquinavir inhibited the adhesiveness of yeasts to Vero cells, whereas indinavir had no effect (Borg-von Zepelin et al. 1999). Corroborating these findings, ritonavir, indinavir, and saquinavir attenuated the adherence of *C. albicans* to human vaginal epithelial cancer cell line HeLa S3; however, no

effect in the phagocytosis by polymorphonuclear leukocytes (PMNLs) was observed (Bektić et al. 2001). Interestingly, the adhesion process between *C. albicans* and human umbilical vein endothelial cells (HUVEC) was not reduced by ritonavir, indinavir, or saquinavir (Falkensammer et al. 2007), suggesting that inhibition of *C. albicans* adhesion by HIV peptidase inhibitors is clearly epithelial cell-specific. Compounding this group of experimental tests, Gruber et al. (1999a, b) described that indinavir promoted a dose-dependent decrease of cell wall-bound Sap antigen by flow cytometry analysis. In addition, while *C. albicans* yeast cells treated with or without indinavir retained their ability to form hyphae, hyphal elongation of drug-treated cells was delayed after incubation with indinavir.

It is also important to note that the inhibitory effects of HIV aspartic peptidase inhibitors in both *in vitro* and *in vivo* experiment models were observed at substantial inhibitor concentrations (of the μM order), much higher than those needed for HIV peptidase inhibition (of the nM order). This probably reflects a much lower affinity of these drugs for Sap compared with their high affinity for HIV peptidase (Flexner 1998). Another explanation is that in contrast to the very small and structurally simplified HIV peptidase, Saps are larger and more complex (Kato et al. 1987; Abad-Zapatero et al. 1996). They possess a relatively large active site which might be responsible for the broader substrate specificity and also their susceptibility to distinct aspartic peptidase inhibitors (Abad-Zapatero et al. 1996). Nevertheless, the above concentrations may be achieved under current HAART regimens both in the blood (Flexner 1998) and (at least for indinavir) in human saliva, as shown by Hughens et al. (1998). In future, derivatives of HIV aspartic peptidase inhibitors, being more specific for the fungal Saps, may form an alternative in the treatment of mucosal candidiasis insensitive to currently available antimycotics.

Tsang and Hong (2010) have investigated whether HIV peptidase inhibitors affect *C. albicans* adhesion to acrylic substances. In this study, *C. albicans* suspensions were pretreated with different concentrations of saquinavir, ritonavir, or indinavir for 1 h and allowed to adhere on acrylic strips that had been pretreated with pooled human saliva for 30 min, plus another hour in the presence of each drug. The test groups showed a significantly lower degree of adhesion than the controls. Adhesion was reduced by 50% at drug concentrations of 100 μM, 100 μM and 20 μM for saquinavir, ritonavir, and indinavir respectively.

13.4.2 Cryptococcus neoformans

C. neoformans is ubiquitous encapsulated yeast that is responsible for morbidity and mortality in patients with impaired cell-mediated immunity, especially in those with AIDS. Human infection begins with inhalation of desiccated yeasts or basidiospores followed by a local pulmonary infection. In immunocompetent individuals, primary lung infections with *C. neoformans* usually resolve without therapy, but in immunocompromised individuals, *C. neoformans* can disseminate to the

central nervous system, causing cryptococcal meningitis, which if untreated is always fatal (reviewed by Lin 2009). As previously mentioned in this review, HAART has beneficial effects on some opportunistic infections by fungal pathogens, known to be major causes of morbidity and mortality in AIDS patients, including *C. neoformans*.

In respect of *C. neoformans*, Mussini et al. (2004) showed that HIV-infected patients who experienced a cryptococcal meningitis taking HAART have a lower risk of relapse when the $CD4^+$ cell count increases to >100 cells/μl, even in the absence of antifungal maintenance therapy. As reported for *C. albicans*, the peptidase inhibitor present in the HAART directly inhibits the aspartic-type peptidase produced by *C. neoformans*. To test this hypothesis, the researchers evaluated the effect of HIV peptidase inhibitors on the secretory aspartic peptidase as well as on several parameters, such as fungal viability, growth, and susceptibility to immune effector cells. For this purpose, indinavir is the main peptidase inhibitor studied against *C. neoformans*, especially due to its diffusion through the blood–brain barrier (Haas et al. 2000).

The cultivation of *C. neoformans* cells for 48–72 h in the presence of 10 μM indinavir or 100 μg/ml pepstatin A considerably diminished the extracellular peptidase activity measured in the cell-free culture supernatant in comparison with yeast grown in the absence of these peptidase inhibitors. These data imply a direct inhibitory effect of indinavir on *C. neoformans* secreted peptidase(s). Indinavir did not inhibit *C. neoformans* growth until 48 h of incubation, but a significant decrease was observed after this time. In addition, this inhibition is a strictly dose-dependent phenomenon, reaching about 75% growth reduction at 50 μM dose (Blasi et al. 2004). These authors also evaluated the effects of indinavir on cryptococcal susceptibility to murine microglial cell line BV2, which is a prototype of immune effector cell. Nontreated yeast cells were phagocytosed by approximately 6% of the microglial cells, while *C. neoformans* cells pre-exposed to indinavir (10 μM) or pepstatin A (100 μg/ml) were ingested to a higher extent. In addition, the pretreatment of *C. neoformans* cells with indinavir enhanced the microglia-mediated anticryptococcal activity, resulting in damaging of ingested yeasts. In contrast, control yeasts exposed to BV2 cells, though being ingested, still retained a well-conserved morphology (Blasi et al. 2004).

Indinavir also selectively inhibited the production of some virulence factors of *C. neoformans,* including: urease, which promotes survival of the fungus within mammalian hosts, and peptidase activities, but not melanin and phospholipase synthesis. Moreover, indinavir interfered with capsule formation. As is well-known, the polysaccharide capsular size is important in influencing the anticryptococcal function of phagocytic cells; therefore, impairment of capsule formation could contribute to increased susceptibility to killing (Vecchiarelli et al. 1994). This effect could be particularly relevant *in vivo* because, apart from its antiphagocytic activity, the *C. neoformans* capsule is also endowed with immunosuppressive properties (Vecchiarelli 2000). Cooperatively, these effects led to increased susceptibility of *C. neoformans* to intracellular killing by PMNLs and peripheral blood mononuclear cells (PBMCs), which are natural effector cells. This phenomenon

was also correlated with the increase of respiratory burst, which enhanced the production of superoxide anion (O_2^-) in both cell types. Additionally, the antimicrobial capacity of granule extracts from PMNLs killed the indinavir-treated *C. neoformans* more efficiently than nontreated ones (Monari et al. 2005). In parallel, Pericolini et al. (2006, 2008) demonstrated that the inoculation of immunosuppressed mice with *C. neoformans* previously exposed to indinavir, in comparison to untreated *C. neoformans*, resulted in several beneficial effects, such as: (1) a more pronounced secretion of IL-12 by splenic dendritic cells, suggesting a more efficient Th1 protective response, (2) reduction of CD14 and FcγRs expression on splenic dendritic cells, and upregulation of CD86 and CD80 molecules, suggesting the maturation and activation of this ultimate antigen-presenting cell, (3) enhancement of IFN-γ and IL-2 production by splenic T cells and increase in their proliferation in response to fungal antigens, (4) survival from an otherwise lethal challenge, correlated with a drastic decrease in colony-forming units from two target organs, brain and liver, and (5) induced an expansion of dendritic cell with CD8α phenotype in spleens of infected hosts, resulting in an efficient T-cell-protective response. Collectively, these results showed that *C. neoformans* exposed to indinavir can somehow bypass or attenuate some immunosuppressive effects normally induced by the unexposed fungus.

Tipranavir, a nonpeptidic peptidase inhibitor that exhibits antiviral activity against many peptidase inhibitor-resistant HIV-1 isolates, induced in *C. neoformans* and *C. albicans* a significant reduction of both peptidase and phospholipase production. However, this inhibitor showed an opposite effect on the major virulence factors of *C. neoformans* and *C. albicans*: in the former, it inhibited the expression of the polysaccharide capsular, while in *C. albicans* it promoted the mycelial transition. Curiously, tipranavir impaired *in vitro* growth of *C. neoformans*, but not of *C. albicans*. Moreover, tipranavir-treated *C. neoformans*, but not *C. albicans*, proved more susceptible to killing by human neutrophils. Finally, tipranavir showed a therapeutic effect in experimental systemic cryptococcosis, as evaluated by reduced fungal burden in brain and liver of immunocompetent and immunodepressed mice. These data indicate that tipranavir could act in multiple ways by diversifying its effects on various opportunistic pathogenic fungi (Cenci et al. 2008).

Combined therapy has developed to diminish the fungal resistance phenomenon. The combination of peptidase inhibitors and antimycotic drugs could help in enhancing the therapeutic treatment against mycoses. In this context, the combination of antimycotic agents and saquinavir both at subinhibitory concentrations was effectively demonstrated against strains of *C. albicans* and *C. neoformans* isolated from HIV-seropositive patients. The results described by Casolari et al. (2004) showed that the interaction between saquinavir and different antimycotic drugs (amphotericin B, 5-fluorocytosine, miconazole. and fluconazole) did not result in antagonism, and fluconazole acts in a more synergistic way. The advantage of the synergic effect of the combination is to attenuate the resistance and accompanying toxic phenomena, above all in the event of long-term therapy, thanks to the use of low dosages of both antiviral and antimycotic drugs.

13.4.3 *Fonsecaea pedrosoi*

F. pedrosoi is the principal etiologic agent of chromoblastomycosis, a fungal disease whose pathogenic events are poorly understood. Chromoblastomycosis is a chronic, suppurative, and progressive mycosis of the skin and subcutaneous tissues. Current therapy for chromoblastomycosis is suboptimal due to the toxicity of the available therapeutic agents and the emergence of drug resistance. Compounding these problems is the fact that endemic countries and regions are economically poor (reviewed by Santos et al. 2007). The first report on peptidase production by *F. pedrosoi* was described by Palmeira et al. (2006a), who demonstrated that the pattern of peptidase secretion by *F. pedrosoi* conidial cells was highly dependent on the culture medium composition. In this way, conidia cultured on Kauffman complex medium secreted a peptidase, whose activity was restrained by some metallopeptidase inhibitors and which functioned over a large pH range encompassing both acidic and alkaline values. Conversely, in culture supernatant derived from chemically defined medium an extracellular aspartic peptidase was identified, which was active at very low pH and totally sensitive to pepstatin A. Moreover, mycelial forms of *F. pedrosoi* also released aspartic peptidase extracellularly (Palmeira et al. 2006b). Both conidial and mycelial aspartic peptidases were able to hydrolyze important serum proteins as well as extracellular matrix components. Therefore, the extracellular proteolytic enzymes produced by *F. pedrosoi* cells could support the initial development of this fungus inside the host, and the existence of two biochemically distinct secretory peptidases makes it possible to cover a wide range of host conditions. The effect of saquinavir, ritonavir, indinavir, and nelfinavir on the secreted proteolytic activity of *F. pedrosoi* cells was recently evaluated (Palmeira et al. 2006b, 2008). These compounds inhibited the extracellular aspartic proteolytic activity produced by both conidial and mycelial forms in a dose-dependent manner. Interestingly, recent isolated strains of *F. pedrosoi* produced higher levels of extracellular peptidase activity when compared with a laboratory-adapted strain (Palmeira et al. 2006b, 2008), suggesting that the production of secreted aspartic-type peptidases may be stimulated by interaction with the host.

Aspartic peptidases are apparently key regulators of the *in vitro* growth in *F. pedrosoi* (Palmeira et al. 2006b, 2008). Pepstatin A demonstrated a fungicidal activity against conidia in a cell-number and dose-dependent fashion. Accordingly, the HIV aspartic peptidase inhibitors mentioned above also abrogated the growth of conidial forms as well as their transformation into mycelial cells (Palmeira et al. 2008), an essential step during the *F. pedrosoi* life cycle and virulence (Santos et al. 2007). Probably the aspartic peptidase may be involved in the early transformation or remodeling of the conidia after it enters the host cells. Conidia-into-mycelia transformation in *F. pedrosoi* is accompanied by dramatic morphologic changes that could require peptidase-mediated degradation of several proteins, including conidia cytoskeleton components. The synergistic action on growth ability between nelfinavir and amphotericin B, when both were used at subinhibitory concentrations, was also observed. Interestingly, HIV peptidase inhibitor-treated conidial

cells presented irreversible ultrastructural alterations, as shown by transmission electron microscopy. After 1 h of treatment, both nelfinavir and saquinavir induced several morphological alterations, including: invaginations in the cytoplasmic membrane and withdrawal of the cytoplasmic membrane from within the cell wall, disorder and detachment of the cell wall, rupture of internal organelles, and the presence of large and irregular cytoplasmic vacuoles, some of them containing small vesicles. In some cells, the enlargement of intracellular vacuoles was so intense that they occupied almost the entire cytoplasm area. Furthermore, abnormal division disfiguring regular conidia morphology could be observed in cells treated with nelfinavir, while the saquinavir treatment seemed to induce breakage of the cell wall starting from extracellular environmental. Ritonavir and indinavir also promoted some of these aberrant cellular alterations, but in a lower proportion when compared to nelfinavir and saquinavir treatment. Furthermore, the aspartic peptidase inhibitors drastically reduced the adhesion and endocytic indexes during the interaction between *F. pedrosoi* conidia and epithelial cells (Chinese hamster ovary), fibroblasts, or macrophages, in a cell type-dependent manner. Moreover, these peptidase inhibitors arrested the conidia-into-mycelia transformation when in contact with CHO cells and enhanced the susceptibility killing by macrophage cells, promoting a significant reduction in the number of viable intracellular conidia (Palmeira et al. 2008).

13.4.4 Sporothrix schenckii

S. schenckii yeast cells, the morphological stage that is found in affected host tissues, are able to produce two distinct extracellular peptidases when grown in liquid medium containing albumin or collagen as a unique nitrogen source: a serine-type peptidase inhibited by chymostatin and an aspartic-type peptidase strongly blocked by pepstatin A (Tsuboi et al. 1987, 1988). The addition of pepstatin or chymostatin to the culture medium did not inhibit the cell growth of *S. schenckii*, but the addition of both inhibitors at a concentration of 10 μg/ml robustly inhibited yeast growth (Tsuboi et al. 1988). Thus, these enzymes could act synergistically, degrading collagen of the dermis and consequently helping the fungus to obtain nitrogen source for its *in vivo* proliferation. In order to corroborate this speculation using a sporotrichosis model, Lei et al. (1993) examined the effects of peptidase inhibitors on the cutaneous lesions of mice. Ointments containing 0.1% chymostatin, 0.1% pepstatin A, and 0.1% chymostatin plus 0.1% pepstatin A were applied twice daily on the inoculation site of hairless mouse skin, and the time courses of the lesions were examined. The inhibitory effect *in vivo* on *S. schenckii* was similar to that demonstrated in an *in vitro* study. Compared to the control, the time-course curve of the number of nodules present after the application of either pepstatin A or chymostatin was slightly suppressed. However, the application of both pepstatin A and chymostatin powerfully suppressed nodule formation. The infection of mice with

S. schenckii resulted in high titers of antibodies against both peptidases, showing that both enzymes were antigenic and produced *in vivo*. The time course of the antibody titers to both peptidases was paralleled to those macroscopic and microscopic observations in an experimental mouse sporotrichosis model (Yoshiike et al. 1993). Taken together, these results pointed out to the direct action of peptidase inhibitors on the *S. schenkii* and may lead to their use as possible topical therapeutic agents.

13.4.5 Histoplasma capsulatum

Before the era of HAART, the prevalence of *H. capsulatum* infections reached up to 30% of HIV-infected patients in hyperendemic areas of the southeastern part of the United States (McKinsey et al. 1997). The infection occurs more often in patients with a CD4 count <50 cells/mm^3 and is usually disseminated (Carme et al. 1990). An extremely interesting study showed that the treatment of a patient with AIDS and disseminated histoplasmosis with a combined therapy of itraconazole and HIV peptidase inhibitors (atazanavir, 300 mg once daily, and ritonavir, 100 mg once daily) promoted an improvement in the desired urinary *Histoplasma* antigen level and plasma itraconazole concentration, leading to a mycological cure (Koo et al. 2007).

13.5 Conclusions

To date, antifungal drug design has predominantly been based on targeting a singular cellular metabolic or biosynthetic process. However, because of frequent relapses and the ever-rising emergence of drug resistance, this strategy appears to be inadequate and the pressing demand for new approaches to drug discovery can no longer be neglected. In this sense, there have been substantive advances in our understanding of the use of peptidase inhibitors as therapeutic agents. Exploration of natural inhibitors and synthesis of peptidomimetic molecules have provided many promising compounds performing successfully in animal studies. Several peptidase inhibitors are undergoing further evaluation in human clinical trials. By combining new technology in molecular biology, X-ray crystallography, computer graphics, and biochemistry, structure-based drug design provides a parallel and cost-effective strategy for identification of new antifungal chemotherapy. In this context, aspartic peptidase-inhibitor crystal structures are currently available on the Protein Data Bank (PDB) database (http://www.rcsb.org/pdb/home/home.do) for some fungal peptidases, including penicillopepsin (*Penicillium janthinellum*), aspergillopepsin (*Aspergillus phoenicis* and *A. niger*), mucorpepsin (*Rhizomucor miehei*), saccharopepsin (*S. cerevisiae*), rhizopuspepsin (*Rhizopus chinensis*), and Saps (*C. albicans*, *C. parapsilosis*, and *C. tropicalis*), and these data could help in

the development of novel and potent antifungal compounds. Furthermore, future research into the synergistic capabilities of inhibitors will help elucidate the most effective combination therapies. Perhaps we can learn from the treatment of HIV where combination therapies targeting more than one essential factor have been successful. Combination therapies that target not only essential genes but also important virulence factors that are essential for certain steps in infection could be attractive in the treatment of fungal infections. Finally, peptidase inhibitor research should be viewed as a promising field in which medical advances are likely to be realized.

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References

Abad-Zapatero C, Goldman R, Muchmore SW, Hutchins C, Stewart K, Navaza J, Payne CD, Ray TL (1996) Structure of a secreted aspartic protease from *C. albicans* complexed with a potent inhibitor: implications for the design of antifungal agents. Protein Sci 5:640–652

Abbenante G, Fairlie DP (2005) Protease inhibitors in the clinic. Med Chem 1:71–104

Albrecht A, Felk A, Pichova I, Naglik JR, Schaller M, de Groot P, Maccallum D, Odds FC, Schafer W, Klis F, Monod M, Hube B (2006) Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host–pathogen interactions. J Biol Chem 281:688–694

Babiker A, Darbyshire J, Pezzotti P, Porter K, Rezza G, Walker SA, Beral V, Coutinho R, del Amo J, Gill N, Lee C, Meyer L, Tyrer F, Dabis F, Thiebaut R, Lawson-Aye S, Boufassa F, Hamouda O, Fischer K, Rezza G, Touloumi G, Hatzakis A, Karafoulidou A, Katsarou O, Brettle R, del Romero J, Prins M, van Benthem B, Kirk O, Pederson C, Hernández AI, Pérez-Hoyos S, Eskild A, Bruun JN, Sannes M, Sabin C, Lee C, Johnson AM, Phillips AN, Francioli P, Vanhems P, Egger M, Rickenbach M, Cooper D, Kaldor J, Ashton L, Vizzard J, Muga R, Day NE (2002) Changes over calendar time in the risk of specific first AIDS-defining events following HIV seroconversion, adjusting for competing risks. Int J Epidemiol 31:951–958

Barrett AJ, Rawlings ND, O'Brien EA (2001) The MEROPS database as a protease information system. J Struct Biol 134:95–102

Barrett AJ, Tolle DP, Rawlings ND (2003) Managing peptidases in the genomic era. Biol Chem 384:873–882

Beausejour A, Grenier D, Goulet JP, Deslauriers N (1998) Proteolytic activation of the interleukin-1beta precursor by *Candida albicans*. Infect Immun 66:676–681

Bektić J, Lell CP, Fuchs A, Stoiber H, Speth C, Lass-Flörl C, Borg-von Zepelin M, Dierich MP, Würzner R (2001) HIV protease inhibitors attenuate adherence of *Candida albicans* to epithelial cells *in vitro*. FEMS Immunol Med Microbiol 31:65–71

Benes P, Vetvicka V, Funsek M (2008) Cathepsin D — many functions of aspartic protease. Crit Rev Oncol Hematol 68:12–28

Blasi E, Colombari B, Orsi CF, Pinti M, Troiano L, Cossarizza A, Esposito R, Peppoloni S, Mussini C, Neglia R (2004) The human immunodeficiency virus (HIV) protease inhibitor

indinavir directly affects the opportunistic fungal pathogen *Cryptococcus neoformans*. FEMS Immunol Med Microbiol 42:187–195

Blundell TL, Johnson MS (1993) Catching a common fold. Protein Sci 2:877–883

Borelli C, Ruge E, Lee JH, Schaller M, Vogelsang A, Monod M, Korting HC, Huber R, Maskos K (2008) X-ray structures of Sap1 and Sap5: structural comparison of the secreted aspartic proteinases from *Candida albicans*. Proteins 72:1308–1319

Borg M, Ruchel R (1988) Expression of extracellular acid proteinase by proteolytic *Candida* spp. during experimental infection of oral mucosa. Infect Immun 56:626–631

Borg-von Zepelin M, Meyer I, Thomssen R, Würzner R, Sanglard D, Telenti A, Monod M (1999) HIV-protease inhibitors reduce cell adherence of *Candida albicans* strains by inhibition of yeast secreted aspartic proteases. J Invest Dermatol 113:747–751

Capobianco JO, Lerner CG, Goldman RC (1992) Application of a fluorogenic substrate in the assay of proteolytic activity and in the discovery of a potent inhibitor of *Candida albicans* aspartic proteinase. Anal Biochem 204:96–102

Carme B, Ngolet A, Ebikili B, Ngaporo AI (1990) Is African histoplasmosis an opportunistic fungal infection in AIDS? Trans R Soc Trop Med Hyg 84:293

Casolari C, Rossi T, Baggio G, Coppi A, Zandomeneghi G, Ruberto AI, Farina C, Fabio G, Zanca A, Castelli M (2004) Interaction between saquinavir and antimycotic drugs on *C. albicans* and *C. neoformans* strains. Pharmacol Res 50:605–610

Cassone A, de Bernardis F, Mondello F, Ceddia T, Agatensi L (1987) Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. J Infect Dis 156:777–783

Cassone A, de Bernardis F, Pontieri E, Carruba G, Girmenia C, Martino P, Fernández-Rodriguez M, Quindós G, Pontón J (1995) Biotype diversity of *C. parapsilosis* and its relationship to the clinical source and experimental pathogenicity. J Infect Dis 171:967–975

Cassone A, de Bernardis F, Torosantucci A, Tacconelli E, Tumbarello M, Cauda R (1999) *In vitro* and *in vivo* anticandidal activity of human immunodeficiency virus protease inhibitors. J Infect Dis 180:448–453

Cenci E, Francisci D, Belfiori B, Pierucci S, Baldelli F, Bistoni F, Vecchiarelli A (2008) Tipranavir exhibits different effects on opportunistic pathogenic fungi. J Infect 56:58–64

Centers for Disease Control and Prevention (2001) HIV/AIDS surveillance report. US Department of Health and Human Services Public Health Service, Atlanta, GA

Chakrabarti A, Nayak N, Talwar P (1991) *In vitro* proteinase production by *Candida* species. Mycopathologia 114:163–168

Coates L, Erskine PT, Mall S, Gill R, Wood SP, Myles DA, Cooper JB (2006) X-ray, neutron and NMR studies of the catalytic mechanism of aspartic proteinases. Eur Biophys J 35: 559–566

Consolaro ME, Gasparetto A, Svidzinski TI, Peralta RM (2006) Effect of pepstatin A on the virulence factors of *Candida albicans* strains isolated from vaginal environment of patients in three different clinical conditions. Mycopathologia 162:75–82

Coombs GH, Goldberg DE, Klemba M, Berry C, Kay J, Mottram JC (2001) Aspartic proteases of *Plasmodium falciparum* and other parasitic protozoa as drug targets. Trends Parasitol 17:532–537

Cooper JB (2002) Aspartic proteinases in disease: a structural perspective. Curr Drug Targets 3:155–174

Copping VM, Barelle CJ, Hube B, Gow NA, Brown AJ, Odds FC (2005) Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted proteinase genes. J Antimicrob Chemother 55:645–654

Costa EMMB, Santos ALS, Cardoso AS, Portela MB, Abreu CM, Alviano CS, Hagler NA, Soares RMA (2003) Heterogeneity of metallo and serine extracellular proteinases in oral clinical isolates of *Candida albicans* in HIV-positive and healthy children from Rio de Janeiro, Brazil. FEMS Immunol Med Microbiol 38:173–180

Crum NF, Riffenburgh RH, Wegner S, Agan BK, Tasker SA, Spooner KM, Armstrong AW, Fraser S, Wallace MR, Triservice AIDS Clinical Consortium (2006) Comparisons of causes of death

and mortality rates among HIV-infected persons: analysis of the pre-, early, and late HAART (highly active antiretroviral therapy) eras. J Acquir Immune Defic Syndr 41:194–200

Da Rosa D, Gezuele E, Calegari L, Goñi F (2009) Excretion–secretion products and proteases from live *Sporothrix shenckii* yeast phase: immunological detection and cleavage of human IgG. Rev Inst Med Trop São Paulo 51:1–7

Dash C, Kulkarni A, Dunn B, Rao M (2003) Aspartic peptidase inhibitors: implications in drug development. Crit Rev Biochem Mol Biol 38:89–119

Davies DR (1990) The structure and function of the aspartic proteinases. Ann Rev Biophys Chem 19:189–215

De Bernardis F, Arancia S, Morelli L, Hube B, Sanglard D, Schafer W, Cassone A (1999a) Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for *Candida* vaginitis. J Infect Dis 179:201–208

De Bernardis F, Mondello F, San Millan R, Ponton J, Cassone A (1999b) Biotyping and virulence properties of skin isolates of *C. parapsilosis*. J Clin Microbiol 37:3481–3486

De Bernardis F, Sullivan PA, Cassone A (2001) Aspartyl proteinases of *Candida albicans* and their role in pathogenicity. Med Mycol 39:303–313

Dostál J, Hamal P, Pavlícková L, Soucek M, Ruml T, Pichová I, Hrusková-Heidingsfeldová O (2003) Simple method for screening *Candida* species isolates for the presence of secreted proteinases: a tool for the prediction of successful inhibitory treatment. J Clin Microbiol 41:712–716

Eigenheer RA, Jin Lee Y, Blumwald E, Phinney BS, Gelli A (2007) Extracellular glycosylphosphatidylinositol-anchored mannoproteins and proteases of *Cryptococcus neoformans*. FEMS Yeast Res 7:499–510

El-Maghrabi EA, Dixon DM, Burnett JW (1990) Characterization of *Candida albicans* epidermolytic proteases and their role in yeast-cell adherence to keratinocytes. Clin Exp Dermatol 15:183–191

Falkensammer B, Pilz G, Bektic J, Imwidthaya P, Jöhrer K, Speth C, Lass-Flörl C, Dierich MP, Würzner R (2007) Absent reduction by HIV protease inhibitors of *Candida albicans* adhesion to endothelial cells. Mycoses 50:172–177

Fallon K, Bausch K, Noonan J, Huguenel E, Tamburini P (1997) Role of aspartic proteases in disseminated *Candida albicans* infection in mice. Infect Immun 65:551–556

Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, Sanglard D, Korting HC, Schafer W, Hube B (2002) *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. Infect Immun 70:3689–3700

Finlay BB, Falkow S (1989) Common themes in microbial pathogenicity. Microbiol Rev 53:210–230

Flexner C (1998) HIV-protease inhibitors. N Engl J Med 338:1281–1292

Fotedar R, Al-Hedaithy SS (2005) Comparison of phospholipase and proteinase activity in *Candida albicans* and *C. dubliniensis*. Mycoses 48:62–67

Fruton JS (2002) A history of pepsin and related enzymes. Quart Rev Biol 77:127–147

Fusek M, Smith EA, Monod M, Foundling SI (1993) *Candida parapsilosis* expresses and secretes two aspartic proteinases. FEBS Lett 327:108–112

Fusek M, Smith EA, Monod M, Dunn BM, Foundling SI (1997) Extracellular aspartic proteinases from *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* yeasts differ substantially in their specificities. Biochemistry 33:9791–9799

Gaedicke S, Firat-Geier E, Constantiniu O, Lucchiari-Hartz M, Freudenberg M, Galanos C, Niedermann G (2002) Antitumor effect of the human immunodeficiency virus protease inhibitor ritonavir: induction of tumor-cell apoptosis associated with perturbation of proteasomal proteolysis. Cancer Res 62:6901–6908

Ghannoum M, Abu Elteen K (1986) Correlative relationship between proteinase production adherence and pathogenicity of various strains of *Candida albicans*. J Med Vet Mycol 24:407–413

Gokce G, Cerikcioglu N, Yagci A (2007) Acid proteinase, phospholipase, and biofilm production of *Candida* species isolated from blood cultures. Mycopathologia 164:265–269

Gruber A, Lukasser-Vogl E, Borg-von Zepelin M, Dierich MP, Würzner R (1998) Human immunodeficiency virus type 1 gp160 and gp41 binding to *Candida albicans* selectively enhances candidal virulence *in vitro*. J Infect Dis 177:1057–1063

Gruber A, Lukasser-Vogl E, Zepelin MB, Dierich MP, Würzner R (1999a) Human immunodeficiency virus type 1 gp160 and gp41 binding to *Candida albicans* selectively enhances candidal virulence *in vitro*. J Infect Dis 177:1057–1063

Gruber A, Speth C, Lukasser-Vogl E, Borg-von Zepelin M, Dierich MP, Würzner R (1999b) Human immunodeficiency virus type 1 protease inhibitor attenuates *Candida albicans* virulence properties *in vitro*. Immunopharmacology 41:227–234

Haas DW, Stone J, Clough LA, Johnson B, Spearman P, Harris VL, Nicotera J, Johnson RH, Raffanti S, Zhong L, Bergqwuist P, Chamberlin S, Hoagland V, Ju WD (2000) Steady-state pharmacokinetics of indinavir in cerebrospinal fluid and plasma among adults with human immunodeficiency virus type1 infection. Clin Pharmacol Ther 68:367–374

Hill J, Phylip L (1997) Bacterial aspartic proteinases. FEBS Lett 409:357–360

Hoegl L, Thoma-Greber E, Röcken M, Korting HC (1998) HIV protease inhibitors influence the prevalence of oral candidosis in HIV-infected patients: a 2-year study. Mycoses 41:321–325

Horimoto Y, Dee DR, Yada RY (2009) Multifunctional aspartic peptidase prosegments. New Biotechnol 25:318–324

Hube B (1996) *Candida albicans* secreted aspartyl proteinases. Curr Top Med Mycol 7:55–69

Hube B, Naglik J (2001) *Candida albicans* proteinases: resolving the mystery of a gene family. Microbiology 147:1997–2005

Hube B, Monod M, Schofield DA, Brown AJ, Gow NA (1994) Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. Mol Microbiol 14:87–99

Hube B, Sanglard D, Odds FC, Hess D, Monod M, Schafer W, Brown AJ, Gow NA (1997) Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. Infect Immun 65:3529–3538

Hughens PW, Burger DM, de Graaff M, ter Hofstede HJ, Hoetelmans RM, Brinkman K, Meenhorst PL, Mulder JW, Koopmans PP, Hekster YA (1998) Saliva as a possible specimen for monitoring compliance and plasma levels in patients treated with indinavir. In: Program and abstracts 12th World AIDS Conference, Stockholm: International AIDS Society, Geneva, abstract 32330

James MNG (1998) Structure and function of aspartic protease: retroviral and cellular enzymes. Plenum, New York, pp 1–481

Kappert K, Caglayan E, Baumer AT, Sudkamp M, Fatkenheuer G, Rosenkranz S (2004) Ritonavir exhibits anti-atherogenic properties on vascular smooth muscle cells. AIDS 18:403–411

Kato I, Yasunuga T, Ikawa Y, Yoshinaka Y (1987) Inhibition of retroviral protease activity by an aspartyl proteinase inhibitor. Nature 329:654–656

Kaur R, Ma B, Cormack BP (2007) A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. PNAS 104:7628–7633

Kobayashi I, Kondoh Y, Shimizu K, Tanaka K (1989) A role of secreted proteinase of *Candida albicans* for the invasion of chick chorioallantoic membrane. Microbiol Immunol 33:709–719

Koelsch G, Mares M, Metcalf P, Funsek M (1994) Multiple functions of pro-parts of aspartic proteinase zymogen. FEBS Lett 343:6–10

Koelsch G, Tang J, Loy JA, Monod M, Jackson K, Foundling SI, Lin X (2000) Enzymic characteristics of secreted aspartic proteases of *Candida albicans*. Biochim Biophys Acta 1480:117–131

Koo HL, Hamill RJ, Andrade RA (2007) Drug–drug interaction between itraconazole and efavirenz in a patient with AIDS and disseminated histoplasmosis. Clin Infect Dis 45:e77–e79

Korting HC, Schaller M, Eder G, Hamm G, Böhmer U, Hube B (1999) Effects of the human immunodeficiency virus (HIV) proteinase inhibitors saquinavir and indinavir on *in vitro*

activities of secreted aspartyl proteinases of *Candida albicans* isolates from HIV-infected patients. Antimicrob Agents Chemother 43:2038–2042

Kretschmar M, Hube B, Bertsch T, Sanglard D, Merker R, Schroder M, Hof H, Nichterlein T (1999) Germ tubes and proteinase activity contribute to virulence of *Candida albicans* in murine peritonitis. Infect Immun 67:6637–6642

Krysan DJ, Ting EL, Abeijon C, Kroos L, Fuller RS (2005) Yapsins are a family of aspartyl protease required for cell wall integrity in *Saccharomyces cerevisiae*. Eukaryot Cell 4:1364–1374

Lei PC, Yoshiike T, Ogawa H (1993) Effects of proteinase inhibitors on the cutaneous lesion of *Sporothrix schenckii* inoculated hairless mice. Mycopathologia 123:81–85

Levitz SM, Specht CA (2006) The molecular basis for the immunogenicity of *Cryptococcus neoformans* mannoproteins. FEMS Yeast Res 6:513–524

Lin X (2009) *Cryptococcus neoformans*: morphogenesis, infection, and evolution. Infect Genet Evol 9:401–416

Louie A, Dixon DM, El-Maghrabi EA, Burnett JW, Baltch AL, Smith RP (1994) Relationship between *Candida albicans* epidermolytic proteinase activity and virulence in mice. J Med Vet Mycol 32:59–64

Mastrolorenzo A, Rusconi S, Scozzafava A, Barbaro G, Supuran CT (2007) Inhibitors of HIV-1 protease: current state of the art 10 years after their introduction. From antiretroviral drugs to antifungal, antibacterial and antitumor agents based on aspartic protease inhibitors. Curr Med Chem 14:2734–2748

McKinsey DS, Spiegel RA, Hutwagner L, Stanford J, Driks MR, Brewer J, Gupta MR, Smith DL, O'Connor MC, Dall L (1997) Prospective study of histoplasmosis in patients infected with human immunodeficiency virus: incidence, risk factors, and pathophysiology. Clin Infect Dis 24:1195–1203

Meiller TF, Hube B, Schild L, Shirtliff ME, Scheper MA, Winkler R, Ton A, Jabra-Rizk MA (2009) A novel immune evasion strategy of *Candida albicans*: proteolytic cleavage of a salivary antimicrobial peptide. PLoS ONE 4:e5039

Mendes A, Moraes AU, Carvalho AP, Rosa RT, Samaranayake LP, Rosa EA (2007) *Candida albicans* biofilms produce more secreted aspartyl protease than the planktonic cells. Biol Pharm Bull 30:1813–1815

Monari C, Pericolini E, Bistoni G, Cenci E, Bistoni F, Vecchiarelli A (2005) Influence of indinavir on virulence and growth of *Cryptococcus neoformans*. J Infect Dis 191:307–311

Monod M, Togni G, Hube B, Sanglard D (1994) Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. Mol Microbiol 13:357–368

Monod M, Hube B, Hess D, Sanglard D (1998) Differential regulation of *SAP8* and *SAP9*, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*. Microbiology 144:2731–2737

Monod M, Capoccia S, Lechenne B, Zaugg C, Holdom M, Jousson O (2002) Secreted proteases from pathogenic fungi. Int J Med Microbiol 292:405–419

Morrow B, Srikantha T, Soll DR (1992) Transcription of the gene for a pepsinogen, *PEP1*, is regulated by white-opaque switching in *Candida albicans*. Mol Cell Biol 12:2997–3005

Muotoe-Okafor FA, Gugnani HC, Obidoa OO (1996) Extracellular proteolytic enzyme activity of *Histoplasma capsulatum* var. *duboisii*. Mycopathologia 133:129–133

Mussini C, Pezzotti P, Miro JM, Martinez E, de Quiros JC, Cinque P, Borghi V, Bedini A, Domingo P, Cahn P, Bossi P, de Luca A, d'Arminio Monforte A, Nelson M, Nwokolo N, Helou S, Negroni R, Jacchetti G, Antinori S, Lazzarin A, Cossarizza A, Esposito R, Antinori A, Aberg JA (2004) Discontinuation of maintenance therapy for cryptococcal meningitis in patients with AIDS treated with highly active antiretroviral therapy: an international observational study. Clin Infect Dis 38:565–571

Na BK, Chung GT, Song CY (1999) Production, characterization and epitope mapping of a monoclonal antibody against aspartic proteinase of *Candida albicans*. Clin Diagn Lab Immunol 6:429–433

Naglik JR, Newport G, White TC, Fernandes-Naglik LL, Greenspan JS, Greenspan D, Sweet SP, Challacombe SJ, Agabian N (1999) *In vivo* analysis of secreted aspartyl proteinase expression in human oral candidiasis. Infect Immun 67:2482–2490

Naglik JR, Challacombe SJ, Hube B (2003a) *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 67:400–428

Naglik JR, Rodgers CA, Shirlaw PJ, Dobbie JL, Fernandes-Naglik LL, Greenspan D, Agabian N, Challacombe SJ (2003b) Differential expression of *Candida albicans* secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. J Infect Dis 188:469–479

Naglik JR, Albrecht A, Bader O, Hube B (2004) *Candida albicans* proteinases and host/pathogen interactions. Cell Microbiol 6:915–926

Naglik JR, Scott J, Rahman D, Mistry M, Challacombe SJ (2005) Serum and saliva antibodies do not inhibit *Candida albicans* Sap2 proteinase activity using a BSA hydrolysis assay. Med Mycol 43:73–77

Navarathna DH, Hornby JM, Hoerrmann N, Parkhurst AM, Duhamel GE, Nickerson KW (2005) Enhanced pathogenicity of *Candida albicans* pre-treated with subinhibitory concentrations of fluconazole in a mouse model of disseminated candidiasis. J Antimicrob Chemother 56:1156–1159

Nguyen JT, Hamada Y, Kimura T, Kiso Y (2008) Design of potent aspartic protease inhibitors to treat various diseases. Arch Pharm Chem Life Sci 341:523–535

Ollert MW, Sohnchen R, Korting HC, Ollert U, Brautigam S, Brautigam W (1993) Mechanisms of adherence of *Candida albicans* to cultured human epidermal keratinocytes. Infect Immun 61:4560–4568

Ollert MW, Wende C, Gorlich M, McMullan-Vogel CG, Borg-von Zepelin M, Vogel CW, Korting HC (1995) Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus-positive patients. J Clin Microbiol 33:2543–2549

Ozkan S, Kaynak F, Kalkancı A, Abbasoglu U, Kustimur S (2005) Slime production and proteinase activity of *Candida* species isolated from blood samples and the comparison of these activities with minimum inhibitory concentration values of antifungal agents. Mem Inst Oswaldo Cruz 100:319–323

Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N Engl J Med 338:853–860

Palmeira VF, Kneipp LF, Alviano CS, Santos ALS (2006a) The major chromoblastomycosis fungal pathogen *Fonsecaea pedrosoi* extracellularly releases proteolytic enzymes whose expression is modulated by culture medium composition: implications on the fungal development and cleavage of key's host structures. FEMS Immunol Med Microbiol 46: 21–29

Palmeira VF, Kneipp LF, Alviano CS, Santos ALS (2006b) Secretory aspartyl peptidase from mycelia of *Fonsecaea pedrosoi*: effect of HIV peptidase inhibitors. Res Microbiol 157:819–826

Palmeira VF, Kneipp LF, Rozental S, Alviano CS, Santos ALS (2008) Beneficial effects of HIV aspartyl peptidase inhibitors on the human pathogen *Fonsecaea pedrosoi*: promising compounds to arrest key fungal biological process and virulence. PLoS ONE 3:e3382

Parra-Ortega B, Cruz-Torres H, Villa-Tanaca L, Hernández-Rodríguez C (2009) Phylogeny and evolution of the aspartyl protease family from clinically relevant *Candida* species. Mem Inst Oswaldo Cruz 104:505–512

Pericolini E, Cenci E, Monari C, Perito S, Mosci P, Bistoni G, Vecchiarelli A (2006) Indinavir-treated *Cryptococcus neoformans* promotes an efficient antifungal immune response in immunosuppressed hosts. Med Mycol 44:119–126

Pericolini E, Cenci E, Gabrielli E, Perito S, Mosci P, Bistoni F, Vecchiarelli A (2008) Indinavir influences biological function of dendritic cells and stimulates antifungal immunity. J Leukoc Biol 83:1286–1294

Pinti M, Orsi CF, Gibellini L, Esposito R, Cossarizza A, Blasi E, Peppoloni S, Mussini C (2007) Identification and characterization of an aspartyl protease from *Cryptococcus neoformans*. FEBS Lett 581:3882–3886

Potter DA, Srirangam A, Fiacco KA, Brocks D, Hawes J, Herndon C, Maki M, Acheson D, Herman IM (2003) Calpain regulates enterocyte brush border actin assembly and pathogenic *Escherichia coli*-mediated effacement. J Biol Chem 278:30403–30412

Pozio E, Morales MAG (2005) The impact of HIV-protease inhibitors on opportunistic parasites. Trends Parasitol 21:58–63

Randolph JT, DeGoey DA (2004) Peptidomimetic inhibitors of HIV protease. Curr Top Med Chem 4:671–686

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635

Rawlings ND, Tolle DP, Barrett AJ (2004a) MEROPS: the peptidase database. Nucleic Acids Res 32:D160–D164

Rawlings ND, Tolle DP, Barrett AJ (2004b) Evolutionary families of peptidase inhibitors. Biochem J 378:705–716

Rawlings ND, Morton FR, Barrett AJ (2006) MEROPS: the peptidase database. Nucleic Acid Res 34:D270–D272

Reichard U, Eiffert H, Rüchel R (1994) Purification and characterization of an extracellular aspartic proteinase from *Aspergillus fumigatus*. J Med Vet Mycol 32:427–436

Reichard U, Monod M, Rfichel R (1996) Expression pattern of aspartic proteinase antigens in aspergilli. Mycoses 39:99–101

Reichard U, Monod M, Odds F, Rüchel R (1997) Virulence of an aspergillopepsin-deficient mutant of *Aspergillus fumigatus* and evidence for another aspartic proteinase linked to the fungal cell wall. J Med Vet Mycol 35:189–196

Ripeau JS, Aumont F, Belhumeur P, Ostrosky-Zeichner L, Rex JH, de Repentigny L (2002) Effect of the echinocandin caspofungin on expression of *Candida albicans* secretory aspartyl proteinases and phospholipase *in vitro*. Antimicrob Agents Chemother 46:3096–3100

Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Krohn A (1990) Rational design of peptide-based HIV proteinase inhibitors. Science 248:358–361

Rüchel R, Ritter B, Schaffrinski M (1990) Modulation of experimental systemic murine candidosis by intravenous pepstatin. Zentralbl Bakteriol 273:391–403

Rüchel R, Zimmermann F, Boning-Stutzer B, Helmchen U (1991) Candidiasis visualised by proteinase-directed immunofluorescence. Virchows Arch A Pathol Anat Histopathol 419:199–202

Sanglard D, Hube B, Monod M, Odds FC, Gow NA (1997) A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* causes attenuated virulence. Infect Immun 65:3539–3546

Santos ALS, Carvalho IM, Silva BA, Portela MB, Alviano CS, Soares RMA (2006) Secretion of serine peptidase by a clinical strain of *Candida albicans*: influence of growth conditions and cleavage of human serum proteins and extracellular matrix components. FEMS Immunol Med Microbiol 46:209–220

Santos ALS, Palmeira VF, Rozental S, Kneipp LF, Nimrichter L, Alviano DS, Rodrigues ML, Alviano CS (2007) Biology and pathogenesis of *Fonsecaea pedrosoi*, the major etiologic agent of chromoblastomycosis. FEMS Microbiol Rev 31:570–591

Schaller M, Bein M, Korting HC, Baur S, Hamm G, Monod M, Beinhauer S, Hube B (2003) The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an *in vitro* model of vaginal candidiasis based on reconstituted human vaginal epithelium. Infect Immun 71:3227–3234

Schaller M, Korting HC, Borelli C, Hamm G, Hube B (2005) *Candida albicans* secreted aspartic proteinases modify the epithelial cytokine response in an *in vitro* model of vaginal candidiasis. Infect Immun 73:2758–2765

Scott BB, McGeehan GM, Harrison RK (2006) Development of inhibitors of the aspartyl protease renin for the treatment of hypertension. Curr Protein Pept Sci 7:241–254

Sepkowitz KA (2001) AIDS — the first 20 years. N Engl J Med 344:1764–1772

Sgadari C, Monini P, Barillari G, Ensoli B (2002) Use of HIV protease inhibitors to block Kaposi's sarcoma and tumour growth. Lancet Oncol 4:537–547

Silva S, Henriques M, Martins A, Oliveira R, Williams D, Azeredo J (2009) Biofilms of non-*Candida albicans* species: quantification, structure and matrix composition. Med Mycol 20:1–9

Stewart K, Abad-Zapatero C (2001) *Candida* proteases and their inhibition: prospects for antifungal therapy. Curr Med Chem 8:941–948

Smolenski G, Sullivan PA, Cutfield SM, Cutfield JF (1997) Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, and Sap3 isoenzymes. Microbiology 143:349–356

Symersky J, Monod M, Foundling SI (1997) High-resolution structure of the extracellular aspartic proteinase from *Candida tropicalis* yeast. Biochemistry 36:12700–12710

Tacco BA, Parente JA, Barbosa MS, Bao SN, Goes TD, Pereira M, Soares CM (2009) Characterization of a secreted aspartyl protease of the fungal pathogen *Paracoccidioides brasiliensis*. Med Mycol 47:845–854

Takahashi K (1995) Aspartic peptidases: structure, function, biology and biomedical implications. Plenum, New York

Tarcha EJ, Basrur V, Hung CY, Gardner MJ, Cole GT (2006) A recombinant aspartyl protease of *Coccidioides posadasii* induces protection against pulmonary coccidioidomycosis in mice. Infect Immun 74:516–527

Tavanti A, Pardini G, Campa D, Davini P, Lupetti A, Senesi S (2004) Differential expression of secretory aspartyl proteinase genes (*SAP1-10*) in oral *Candida albicans* isolates with distinct karyotypes. J Clin Microbiol 42:4726–4734

Tavanti A, Campa D, Bertozzi A, Pardini G, Naglik JR, Barale R, Senesi S (2006) *Candida albicans* isolates with different genomic backgrounds display a differential response to macrophage infection. Microb Infect 8:791–800

Taylor BN, Hannemann H, Sehnal M, Biesemeier A, Schweizer A, Röllinghoff M, Schröppel K (2005) Induction of *SAP7* correlates with virulence in an intravenous infection model of candidiasis but not in a vaginal infection model in mice. Infect Immun 73:7061–7063

Tsang CS, Hong I (2010) HIV protease inhibitors differentially inhibit adhesion of *Candida albicans* to acrylic surfaces. Mycoses Jun 15 [Epub ahead of print]

Tsuboi R, Sanada T, Takamori K, Ogawa H (1987) Isolation and properties of extracellular proteinases from *Sporothrix schenckii*. J Bacteriol 169:4104–4109

Tsuboi R, Sanada T, Ogawa H (1988) Influence of culture medium pH and proteinase inhibitors on extracellular proteinase activity and cell growth of *Sporothrix schenckii*. J Clin Microbiol 26:1431–1433

UNAIDS/WHO (2005) Report on annual AIDS epidemic update. United Nations Publications, New York http://www.unaids.org/epi/2005/

van Burik JA, Magee PT (2001) Aspects of fungal pathogenesis in humans. Annu Rev Microbiol 55:743–772

van den Hombergh JP, Sollewijn Gelpke MD, van de Vondervoort PJ, Buxton FP, Visser J (1997a) Disruption of three acid proteases in *Aspergillus niger*-effect on protease spectrum, intracellular proteolysis, and degradation of target proteins. Eur J Biochem 247:605–613

van den Hombergh JP, van de Vondervoort PJ, Fraissinet-Tachet L, Visser J (1997b) *Aspergillus* as a host for heterologous protein production: the problem of proteases. Trends Biotechnol 15:256–263

Vassar R (2002) β-Secretase (BACE) as a drug target for Alzheimer's disease. Adv Drug Deliv Rev 54:1589–1602

Vecchiarelli A (2000) Immunoregulation by capsular components of *Cryptococcus neoformans*. Med Mycol 38:407–417

Vecchiarelli A, Pietrella D, Dottorini M, Monari C, Retini C, Todisco T, Bistoni F (1994) Encapsulation of *Cryptococcus neoformans* regulates fungicidal activity and the antigen presentation process in human alveolar macrophages. Exp Immunol 98:217–223

Vickers I, Reeves EP, Kavanagh KA, Doyle S (2007) Isolation, activity and immunological characterisation of a secreted aspartic protease, CtsD, from *Aspergillus fumigatus*. Protein Expr Purif 53:216–224

Vilanova M, Teixeira L, Caramalho I, Torrado E, Marques A, Madureira P, Ribeiro A, Ferreira P, Gama M, Demengeot J (2004) Protection against systemic candidiasis in mice immunized with secreted aspartic proteinase 2. Immunol 111:334–342

Villar CC, Kashleva H, Nobile CJ, Mitchell AP, Dongari-Bagtzoglou A (2007) Mucosal tissue invasion by *Candida albicans* is associated with E-cadherin degradation, mediated by transcription factor Rim 101p and protease Sap5p. Infect Immun 75:2126–2135

Vlahakis SR, Bennett SAL, Whitehead SN, Badley AD (2007) HIV protease inhibitors modulate apoptosis signaling *in vitro* and *in vivo*. Apoptosis 12:969–977

Walmsley S (2007) Protease inhibitor-based regimens for HIV therapy safety and efficacy. J Acquir Immune Defic Syndr 45:S5–S13

Watts HJ, Cheah FS, Hube B, Sanglard D, Gow NA (1998) Altered adherence in strains of *Candida albicans* harbouring null mutations in secreted aspartic proteinase genes. FEMS Microbiol Lett 159:129–135

White TC, Agabian N (1995) *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. J Bacteriol 177:5215–5221

White TC, Miyasaki SH, Agabian N (1993) Three distinct secreted aspartyl proteinases in *Candida albicans*. J Bacteriol 175:6126–6133

Wlodawer A, Erickson J (1993) Structure-based inhibitors of HIV-1 protease. Annu Rev Biochem 62:543–585

Wolf T, Findhammer S, Nolte B, Helm EB, Brodt HR (2003) Inhibition of TNF-alpha mediated cell death by HIV-1 specific protease inhibitors. Eur J Med Res 8:17–24

Wu T, Wright K, Hurst SF, Morrison CJ (2000) Enhanced extracellular production of aspartyl proteinase, a virulence factor, by *Candida albicans* isolates following growth in subinhibitory concentrations of fluconazole. Antimicrob Agents Chemother 44:1200–1208

Yoshiike T, Lei PC, Komatsuzaki H, Ogawa H (1993) Antibody raised against extracellular proteinases of *Sporothrix schenckii* in *S. schenckii* inoculated hairless mice. Mycopathologia 123:69–73

Yücel A, Kantarcıoglu AS (2001) The determination of some virulence factors (phospholipase, protease, germ tube formation and adherence) of *C. albicans* and the correlative relationship of these factors. Turk J Infect 15:517–525

Zaidi N, Kalbacher H (2008) Cathepsin E: a mini review. Biochem Biophys Res Commun 367:517–522

Zingman BS (1996) Resolution of refractory AIDS-related mucosal candidiasis after initiation of didanosine plus saquinavir. N Engl J Med 334:1674–1675

Chapter 14
Metabolic Pathways as Drug Targets: Targeting the Sulphur Assimilatory Pathways of Yeast and Fungi for Novel Drug Discovery

Anand Kumar Bachhawat and Amit Kumar Yadav

Abstract The incidence of fungal infections is increasing worldwide, and the management and treatment of fungal infections has become increasingly important. The antifungals which are currently available target only a few pathways, and their persistent use has resulted not only in increased drug resistance but also in the emergence of newer fungal pathogens with intrinsic resistance. There is thus an urgent need for novel antifungals. Among the metabolic pathways, the sulphur assimilatory pathways of the pathogenic yeasts and fungi appear to be suitable for antifungal development, due to the essential requirement of sulphur to the organism and the significant differences from the corresponding pathways in humans. In this chapter, we present the current understanding of the sulphur assimilatory pathways in different pathogenic yeast and fungi, and discuss several targets that are currently being investigated or have the potential for antifungal development.

14.1 Introduction

Among the 1.5 million yeast and fungi estimated to be in existence, only a small fraction (0.1%) cause disease in mammals, and among these only a few are frequently encountered as pathogens (Hawksworth 2001; Kwon-Chung and Bennett 1992). Many yeast and fungi, however, are commensals, in that they inhabit the human host without causing pathogenesis. Under certain conditions or in immunocompromised hosts, these can become pathogenic, and thus they are called opportunistic pathogens. With an increasing incidence of HIV, these opportunistic pathogens have become a major cause of morbidity and mortality.

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Fungal infections can be superficial, subcutaneous or invasive. Superficial infections (or cutaneous infections) affect hair, nails, and skin, and are caused by dermatophytes. *Trichophyton rubrum, Epidermophyton floccosum, Trichophyton mentagrophytes* and *Microsporum gypseum* are examples of common dermatophytes infecting humans (White et al. 2008, Venkatesan et al. 2007). Subcutaneous infections are caused by the infection of the dermis, for example the disease sporotrichosis caused by *Sporothrix schenckii*. The disseminated fungal infections, endemic and opportunistic are the most life-threatening. Invasive fungal infections are increasingly being caused by opportunistic pathogens in immunocompromised hosts. Among the most common opportunistic pathogens in this category are the commensals *Aspergillus*, *Candida,* and *Cryptococccus* (Pfaller and Diekema 2004).

Currently there are only a limited number of antifungal agents (Table 14.1), and persistent use of these has led to an increased resistance to antifungals and also the emergence of several new fungal pathogens that have intrinsic resistance to these antifungals. *Candida krusei* and *Candida glabrata,* for example, are intrinsically resistant to fluconazole, and an increased mortality rate has been reported with these species compared to *Candida albicans* (Cheson 1995).

There is therefore, an urgent need to discover newer and better antifungals. An ideal antifungal should not only have broad antifungal activity, and be fungicidal rather than fungistatic, but it should also be non-toxic, have low frequency of either acquired or intrinsic resistance, have minimal drug interactions, and also be inexpensive (Chapman et al. 2008).

Two different approaches are being taken towards discovering new antifungals. In the first approach, chemical libraries and natural product libraries are directly screened against different pathogenic yeast and fungi to identify potential antifungals. In a second approach, potential target proteins are first identified and validated *in vivo*, and then high-throughput screens based on the target protein are set up to screen different compound libraries to identify lead compounds which are then further developed.

Metabolic pathways are important targets of antifungals, and in fact most of the current antifungals in use target two key metabolic pathways of fungi — the

Table 14.1 Pathways targeted by currently used antifungals in humans

Pathway	Target enzyme	Drug class	Example
Ergesterol biosynthesis pathway	14α-Demethylase	Azoles (imidazole and triazoles)	Ketoconazole, fluconazole
	Squalene epoxidase	Allylamine	Terbinafine
	Δ^{14} Reductase and $\Delta^{7,8}$ isomerase	Phenylmorpholine	Amorolfine
	Ergesterol	Polyene	Amphotericin B
Cell wall biosynthesis	β-1, 3 Glucan polysaccharides	Echinocandins	Micafungin, caspofungin, anidulafungin
Nucleic acid biosynthesis	Nucleic acid biosynthesis	–	Flucytosine
Mitosis	Microtubule assembly	–	Griseofulvin

ergosterol biosynthetic pathway and the cell-wall biosynthesis pathway (Table 14.1). However, not all metabolic pathways can be exploited for antifungal development. Only those targets or pathways which are essential for the pathogen's survival *in vivo*, and where the mammalian equivalents are not present or are significantly different, are likely to be useful targets. Thus, the cell-wall biosynthetic pathway which is essential for fungal survival is a pathway absent in mammalian cells, while the ergosterol pathway has been the major target for antifungals because fungi make ergosterol (essential component of fungal membranes), as opposed to cholesterol in humans. However, owing to the similarity in the enzymes of ergosterol and cholesterol pathways, these antifungals also have a significant level of toxicity.

Surprisingly, very few other pathways have been targeted for antifungal development till recently. The explosion in whole genome sequences, including genome sequences of several pathogenic yeast and fungi (Jung et al. 2008), has made the target-based approach for antifungal development very attractive (Willins et al. 2002). Comparative genomics at the whole genome level has made it possible to identify potential targets in silico. These can then be validated *in vivo*, saving significant time and effort. This has led to many more enzymes being considered as potential drug targets (Wang and Shuman 2005; Rodaki et al. 2006), but they need careful validation.

14.2 The Sulphur Assimilatory Pathway of Humans is Distinct from Fungi: A Potential for Drug Discovery

The sulphur metabolic pathway of humans is significantly different from the pathway in yeasts and fungi. The salient features of the sulphur assimilatory pathway of humans and selected fungi/yeast are schematically shown in Fig. 14.1. The two major differences that can be immediately discerned are firstly the ability of yeast and fungi to reduce and incorporate inorganic sulphates, and secondly the reversibility of the transsulphuration pathways from methionine to cysteine that is seen in most fungi. This gives these organisms far greater

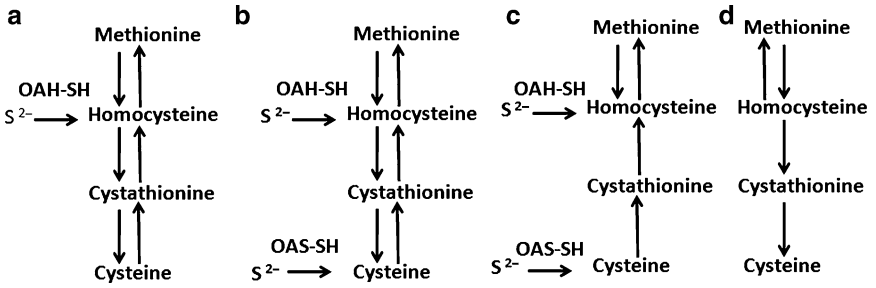

Fig. 14.1 Schematic representation of key features of sulphur assimilatory pathway in different organisms: *S. cerevisiae* (**a**), *N. crassa* and *A. nidulans* (**b**), *S. pombe* (**c**), and humans (**d**)

flexibility in the utilization of sulphur compounds, and thus, not only do they utilize inorganic sulphates, but they can also use different organic sulphur compounds such as cysteine, methionine, and glutathione as sources of sulphur. This is in contrast to humans, where methionine becomes an essential amino acid and has to be provided to meet the sulphur requirements in humans. Humans are thus not only unable to use inorganic sulphates, but are also unable to use organic sulphur sources such as cysteine and glutathione.

As sulphur is also an essential nutrient requirement of all living cells, being a component of the two sulphur-containing amino acids cysteine and methionine, as well as of several other sulphur compounds, such as glutathione and enzyme CoA, the sulphur pathway is essential for survival, and has the potential to be exploited for the development of new antifungals. In addition to yeast and fungi, parasitic protozoans (Nozaki et al. 2005; Walker and Barrett 1997) and pathogenic bacteria (Senaratne et al. 2006; Ejim et al. 2004) also have sulphur pathways that are more complex than in humans, and they also have the potential, and are currently being explored, for discovery of newer antifungals; but these will not be discussed here.

14.3 The Sulphur Assimilatory Pathways of the Yeast *Saccharomyces cerevisiae* and Other Model Yeast and Fungi

Detailed investigations on the sulphur assimilatory pathways have been carried out over the years in the yeast *S. cerevisiae* (reviewed in Thomas and Surdin-Kerjan 1997), and to a lesser extent in the fission yeast *Schizosaccharomyces pombe* and the fungi *Neurospora crassa* and *Aspergillus nidulans*.

In *S. cerevisiae*, transport of sulphate from the extracellular environment is mediated through two high-affinity sulphate transporters SUL1 and SUL2 (Cherest et al. 1997). The sulphate is acted upon by ATP sulphurylase (MET3) to form adenosine phosphosulphate (APS), followed by APS kinase (MET14) that acts on APS to form phosphoadenosine phosphosulphate (PAPS). PAPS reductase (MET16) reduces PAPS to form sulphite. Sulphite reductase (MET 1,5,8,10) reduces sulphite to sulphide, followed by incorporation of the sulphide into *O*-acetylhomoserine (formed by the esterification of homoserine with the *O*-acetyl group from acetyl-CoA using MET2 gene product) by the enzyme *O*-acetylhomoserine sulphydrylase (MET15) to generate the four-carbon chain of homocysteine (Fig. 14.2). Although sulphide is only incorporated into homocysteine in *S. cerevisiae*, many yeasts and fungi can also incorporate sulphide directly into cysteine by the action of *O*-acetyl serine-sulphydrylase (OAS-SH) or cysteine synthase using *O*-acetyl serine (OAS). *N. crassa* and *A. nidulans* preferentially use the OAS pathway, although both pathways exist in these fungi.

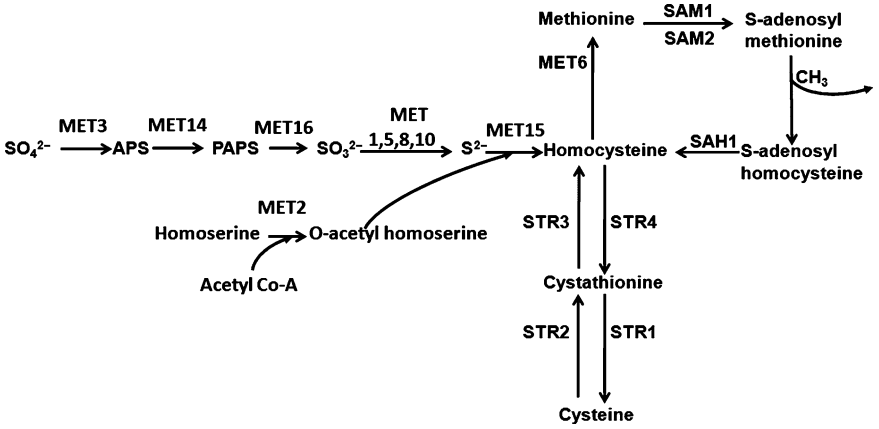

Fig. 14.2 Sulphur assimilatory pathways in *S. cerevisiae*

Humans also have sulphate transporters and the enzymes for sulphate activation. Interestingly, in humans the enzyme PAPS synthatase contains both APS kinase and ATP sulphurylase activity in a single polypeptide chain (Venkatachalam 2003). Humans however, lack the enzymes PAPS reductase and sulphite reductase, thus preventing them from reducing inorganic sulphur compounds.

Cysteine synthesis from homocysteine requires two enzymatic steps carried out by cystathionine beta synthase (STR4; Cherest and Surdin-Kerjan 1992; Ono et al. 1992) and cystathionine gamma lyase, (STR1; Cherest and Surdin-Kerjan 1992). The pathway from homocysteine to cysteine also exists in humans. Methionine is formed from homocysteine by the action of homocysteine methyl transferase or methionine synthase encoded by MET6 (Csaikl and Csaikl 1986). *S. cerevisiae* methionine synthase is cobalamine-independent (Burton et al. 1969). In contrast, methionine synthase in mammals is cobalamine-dependent (Chen et al. 1995). Methionine can also be converted to homocysteine by the action of S-adenosyl methione synthase (SAM1/SAM2) and the enzyme S-adenosyl homocysteine hydrolase (SAH1).

Cysteine can be converted to homocysteine, (and subsequently to methionine) by the action of two enzymes, cystathione gamma synthase (STR2) and cystathionine beta lyase (STR3) in *S. cerevisiae*. Most yeasts have this pathway. Interestingly, *S. pombe* lacks the reverse transsulphuration pathway for the conversion of homocysteine to cysteine (lacks STR4, STR1). *S. pombe* incorporates sulphide directly into cysteine and homocysteine, and has thus both the OAS-SH and OAH-SH pathways (Brzywczy and Paszewski 1994; Fujita and Takegawa 2004; Fig. 14.1).

The presence of the forward and reverse transsulphuration pathways in most yeasts and fungi, and the consequent interconvertibility of methionine and cysteine, allows not only sulphate, but any of the other sulphur compounds, such as cysteine, methionine, homocysteine or glutathione to be also used as a sole source of sulphur

in these yeasts. Studies with *S. cerevisiae*, have revealed that indeed each of these sulphur compounds have specific transporters for their uptake. Thus, methionine is preferentially taken up by MUP1 and MUP3 (Isnard et al. 1996), cysteine and homocysteine preferably by YCT1 (Kaur and Bachhawat 2007), and glutathione by HGT1 (Bourbouloux et al. 2000). Once transported, they can then be converted into the other sulphur compounds by the transsulphuration pathways. In the case of glutathione, it has been shown that the tripeptide is broken down into cysteine by the action of the DUG1, DUG2 and DUG3 proteins functioning as a DUG complex (Ganguli et al. 2007).

N. crassa has been shown to have the capability of utilizing several other sulphur compounds such as choline sulphates and aromatic sulphates (as well as other sulphate and sulphonate esters) as sources of sulphate. These are first transported, and then by the action of specific sulphatases such as arylsulphatases release the sulphate for subsequent assimilation. Furthermore, both *N. crassa* and *A. nidulans* can also obtain sulphur from the extracellular medium by secreting an alkaline protease into the growth medium to hydrolyse extracellular proteins and then transporting the peptides to fulfil their sulphur requirement (Hanson and Marzluf 1973, 1975).

The regulation of sulphur assimilation in *S. cerevisiae* occurs through the sulphur regulatory network involving the transcriptional activator, MET4 which is a basic leucine zipper protein. MET4 lacks DNA binding ability, and requires either CBF1 or one of the two transcription factors MET31/MET32 for promoter association. Thus MET4 forms an activation complex either with CBF1 (MET4–CBF1–MET28) or with MET31/MET32 (MET4–MET28–MET31/MET32). These complexes are activated during sulphur limitation, leading to their binding to the recognition sequences upstream of the sulphur assimilatory genes, and thus leading to their transcriptional activation. An inhibitory protein, MET30 (Kuras et al. 1996), is the substrate recognition subunit of the ubiquitin ligase complex SCF^{MET30} which causes ubiquitination of the transcriptional activator MET4. Transcriptional activation in *N. crassa* and *A. nidulans* occurs via CYS3 (Paietta et al. 1987) and METR (Natorff et al. 2003) respectively, which are the equivalents of *S. cerevisiae* MET4.

14.4 Studies on the Sulphur Assimilatory Pathways in the Pathogenic Yeasts/Fungi

As described in the earlier sections, studies with model yeasts and fungi have revealed that, unlike humans, these organisms have the ability to use a wide variety of sulphur sources. It is possible that some pathways might be important at some stages of infection, while others may be important at other stages of infection. This is especially so since the availability of the sulphur sources changes in the different tissues. The concentrations of sulphur amino acids in the plasma, for example, are

Table 14.2 Concentration of sulphur compounds in mammals (Markovich 2001; Jones et al. 2000; Guttormsen et al. 2004; Triguero et al. 1997)

Sulphur compound	Plasma conc.[a] (μM)	Intracellular conc.[b,c]
Inorganic sulphate	300	–
Cystine	40–50	~20 μM
Cysteine	8–10	~200 μM
Methionine	22	~100 μM
Homocysteine	7	~100 μM
Cystathionine	0.125	~60
Glutathione	1–3	10 mM

[a]Human
[b]Rat
[c]Intracellular concentrations of cysteine, cystine, methionine, homocysteine and cystathionine converted to μM conc. from nmol/g, taking intracellular conc. of glutathione in mM as standard

quite different from the intracellular concentrations within the cell (Table 14.2). Sulphur assimilation is also the route to the biosynthesis of glutathione that plays an important role in the oxidative stress response. As most pathogens face an oxidative stress *in vivo*, the sulphur pathway for this reason has also become important for the pathogens survival *in vivo*.

The importance of the sulphur assimilatory pathways *in vivo* is revealed from the observation that the sulphur pathways are up-regulated in conditions that mimic the *in vivo* conditions. During biofilm formation in *C. albicans* (Murillo et al. 2005; Garcia-Sanchez et al. 2004), many enzymes of the sulphur pathway were found to be up-regulated. Several genome-wide expression studies have been carried out in different *C. albicans* infection models showing the induction of sulphur pathway genes. These include studies with macrophages, neutrophils, and blood. It was observed that in neutrophils and in macrophages *C. albicans* up-regulates some sulphur and glutathione metabolism genes (Rubin-Bejerano et al. 2003; Lorenz et al. 2004; Enjalbert et al. 2003; Hromatka et al. 2005; Rogers and Barker 2003). In a study involving different components of blood, genes involved in the methionine biosynthesis were found to be up-regulated in the blood, in the mononuclear fraction (lymphocytes and monocyte) and in polymorphonuclear cells (eosinophils, basophils and neutrophils), but not in the plasma of blood (Fradin et al. 2005).

In genome-wide studies with another fungal pathogen prevalent in South America *Paracoccidiodes brasiliensis*, it was observed that in the yeast form and in cells undergoing transition from mycelium to the yeast form several genes of the inorganic pathway were induced (Felipe et al. 2005; Andrade et al. 2006; Bastos et al. 2007), while in a similar study during the mycelium-to-yeast transition and during the yeast phase of growth, both the pathways of inorganic and organic sulphur assimilation were found to be induced (Ferreira et al. 2006).

In the intracellular pathogen *H. capsulatum*, the yeast form showed up-regulation of sulphur assimilatory and glutathione metabolism genes (Hwang et al. 2003). Furthermore, under nitrosative stress and under iron limitation, enzymes of the sulphur assimilation were induced (Winters et al. 2008; Nittler et al. 2005).

Similarly, in *Cryptococcus neoformans* during heat shock or nitrosative stress or during phagocytosis by macrophage, genes of sulphur pathway and glutathione metabolism were found to be induced (Chow et al. 2007; Missall et al. 2006; Fan et al. 2005).

These studies highlight the importance of these pathways *in vivo*, the need to investigate the pathogenic microbes for their ability to assimilate sulphur, and also the need to evaluate the relative importance of the existing pathways inside the host. However, very limited studies have been carried out on pathogenic organisms. A brief outline of what we know in respect to the sulphur pathways of a few fungal pathogens is given below.

14.4.1 Candida glabrata *and* Candida albicans

C. albicans and *C. glabrata* are the most frequently isolated *Candida* species from patients suffering from candidiasis. They are frequently found as a commensal inside the human host, being found on the mucosal surface of the gastrointestinal and urogenital tract, along with other microorganisms. However, when there is intestinal tissue damage caused by antibiotic treatment or some other medication like chemotherapy, they escape into the blood of the host. Once inside the blood, they go to all the vital organs of human and colonize them, causing severe case of bloodstream infection.

The genome sequence of *C. albicans* suggests that the sulphur assimilatory pathways of *C. albicans* are quite similar to *S. cerevisiae*. However, when the gene for the enzyme OAH-SH (CaMET15) was disrupted in *C. albicans*, although it led to a severe defect of growth on sulphate it did not lead to complete organic sulphur auxotrophy, as seen in *S. cerevisiae met15Δ* strains (Viaene et al. 2000; Ganguli et al. unpublished data). This suggests that a second pathway for the generation of cysteine (such as OAS-SH, CysK) might exist in *C. albicans*, but this has not yet been investigated.

Comparative analysis of the sulphur assimilatory pathways of *C. glabrata* with *S. cerevisiae* reveals that *C. glabrata* follows the sulphur assimilatory pathways of *S. cerevisiae*. Similar to *S. cerevisiae* (but unlike *C. albicans)*, deletion of OAH-SH (CgMET15) led to strict organic sulphur auxotrophy in *C. glabrata*, and it was not able to grow on inorganic sulphate (Yadav and Bachhawat unpublished data).

Studies with the *met15Δ* strains of both *C. glabrata* and *C. albicans* have revealed that both yeasts can utilize methionine and cysteine, suggesting the presence of both forward and reverse transsulphuration pathways in these yeasts.

Both yeasts can utilize cystine as a sulphur source, although *S. cerevisiae* fails to utilize cystine (Yadav and Bachhawat unpublished data). Interestingly, only *C. albicans* but not *C. glabrata* utilizes glutathione as a sulphur source. The inability to utilize glutathione is due to the absence of a glutathione transporter in *C. glabrata* (Yadav and Bachhawat, unpublished data).

14.4.2 Histoplasma capsulatum

H. capsulatum causes the systemic fungal disease, histoplasmosis. The pathogenic fungi *H. capsulatum* occurs in two distinct morphological forms, the saprophytic mycelial form, and the pathogenic yeast form. At 23−25°C, *H. capsulatum* grows as a mycelium, while inside the human host at 37°C the mycelium gets converted into the yeast form. This dimorphism is caused by increase in temperature as well as the presence of compounds having sulphydryl groups (Scherr 1957). It has been shown that the mycelial form is prototrophic for cysteine, while the yeast form is auxotrophic for cysteine, and the maintenance of the yeast phase requires cysteine or cystine (Salvin 1949). The requirement for organic sulphur forms was found to be because of sulphite reductase, which was constitutive in the mycelial phase but was repressed in the yeast phase (Stetler and Boguslawski 1979).The lack of sulphite reductase activity in the yeast phase was subsequently shown to be due to its temperature sensitivity (Howard et al. 1980).

Histoplasma, was also shown to be able to take up cysteine and cystine, but mutants defective in the uptake of cysteine and cystine had no effect on the morphogenesis (Maresca et al. 1978; Jacobson and Harrell 1981). The presence of a cystine reductase for the utilization of cystine has also been proposed, but the gene has not been identified (Maresca et al. 1978).

14.4.3 Paracocciodiodes brasiliensis

P. brasiliensis is another dimorphic fungal pathogen which is the causative agent for the human systemic disease paracocidioidomycosis, which is most prevalent in South America. Infection occurs through the inhalation of conidia, which then transforms itself into the yeast form inside the human host.

The yeast form of *P. braseliensis* is unable to utilize the inorganic form of sulphur, while the hyphal form can (Paris et al. 1985). It was thus expected that the genes for the inorganic sulphur assimilation would be repressed, in the yeast-phase. However, genome-wide expression profiling revealed that in *P. braseliensis* during the mycelium-to-yeast transition and also during the yeast phase of growth, the pathways, both inorganic and organic, are up-regulated (Ferreira et al. 2006). It has therefore been suggested that the yeast form might not be able to transport the inorganic form of sulphur in the yeast form, thereby showing an organic sulphur requirement.

14.4.4 *Dermatophytes (*Trichophyton, Microsporum, *and* Epidermophton*)*

Dermatophytes cause fungal infection of skin, hair and nails, and these groups of diseases are called Tinea. Tinea infections are usually localized to the surface, and

are rarely systemic or disseminated. Early studies with these organisms have revealed that *M. gypseum* can utilize cysteine, cystine, methionine, and inorganic sulphate for its growth (Stahl et al. 1949). Among different inorganic sulphur forms, *M. gypseum* seems to utilize sulphate and sulphite preferentially (Kunert 1981). In the ecological niche of dermatophytes, keratin, which is rich in cystine, is present in abundance. Dermatophytes secrete sulphite during keratin degradation, and the secreted sulphite provides the reducing environment for the reduction of cystine in keratin to cysteine and S-sulphocysteine (Kunert 1972, 1976). The reduced form of keratin is then acted upon by the secreted exopeptidases and endopeptidases to release peptides and amino acids for their subsequent absorption.

14.4.5 Cryptococcus neoformans

C. neoformans is a saprophytic, dimorphic fungus which in its natural habitat is found in pigeon droppings and in contaminated soil. It is the causative agent of cryptococcosis. Cryptococcal infection begins with the inhalation of the yeast cells into lungs. Primary infections in lungs are asymptomatic in immunocompetent hosts. *C. neoformans* normally affects immunocompromised patients, causing pulmonary infections in them, and when the infection is disseminated it goes to the central nervous system and causes meningoencephalitis.

Some studies have been done to characterize the sulphur assimilatory pathways in this pathogen. A few genes of sulphur assimilation that include MET2, MET3 and MET6 have been targeted in this pathogen to attenuate its virulence. Interestingly, MET2, MET3 and MET6 gene deletions have provided some insights about the status of sulphur pathways in this yeast.

The *met3Δ* deletion of *C. neoformans* has been observed to grow well on cysteine and methionine, indicating that *C. neoformans* contains both forward and reverse transsulphuration pathways (Yang et al. 2002). However, the *C. neoformans met3Δ* did not grow as well as *S. cerevisiae met3Δ* in the presence of exogenous methionine, suggesting poor transport for methionine in this yeast as compared to *S. cerevisiae*. The m*et6Δ* mutant of *C. neoformans*, like *S. cerevisiae*, required methionine for its growth, which was not replaced by homocysteine or cysteine. In contrast, the *met3Δ* mutant of *C. neoformans* was able to satisfy its nutritional requirement by homocysteine, methionine, and cysteine, and it showed better growth when proline was used as a nitrogen source, suggesting that the uptake of these amino acids is under nitrogen repression. The expression of MET3 was not transcriptionally regulated by methionine; however, unlike MET3, MET6 expression was found to increase seven-fold in the presence of methionine (Pascon et al. 2004).

The *met2Δ* strain of *C. neoformans*, like the *met6Δ* and *met3Δ* strains, was a methionine auxotroph, and as with the *met3Δ* strain its growth on methionine was better when proline was used as a nitrogen source instead of ammonium sulphate (Nazi et al. 2007).

14.5 Metabolic Targets in the Sulphur Assimilatory Pathway

Although only a limited number of studies have been carried out on sulphur homeostasis with pathogenic yeasts and fungi, many steps in sulphur assimilation have the potential for targeting by antifungals. Listed below are some of the enzymes, transporters, and transcription factors that are being, and could possibly be, targeted for antifungal development.

14.5.1 Homoserine O*-Acetyl Transacetylase (MET2)*

MET2 encodes homoserine *O*-acetyl transacetylase (HAT) which is the first enzyme in the biosynthesis of methionine. HAT activates homoserine to form *O*-acetylhomoserine through esterification. In *C. neoformans, met2Δ* (like *met6Δ*) is a methionine auxotroph, and has been found to be avirulent in the mice inhalation infection model (Nazi et al. 2007), suggesting that MET2 could be an attractive antifungal target.

To identify inhibitors against MET2, compounds from a protein kinase inhibitor library were screened. Protein kinase inhibitors are built around a scaffold which mimics nucleotide substrates. HAT has a nucleotide recognition region for its substrate CoA. Out of 1,000 compounds screened, 40 were pursued further, based upon the inhibition they caused against HAT (50% inhibition). These compounds were further screened for their ability to compete for the substrate acetyl-CoA. Four compounds were selected as competitive inhibitors for acetyl-CoA. Among these, CTCQC (6-carbamoyl-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-4-carboxylic acid) appeared to be the most promising, as the remaining compounds posed solubility problems. CTCQC was found to be a competitive inhibitor of the substrate acetyl-CoA and non-competitive inhibitor of L-homoserine for the enzyme HAT. CTCQC had no effect on *C. neoformans* growth in minimal medium up to 128 μg/ml; however, this could be due to poor bioavailability of the compound, and thus the lead compound would need further modifications to increase its bioavailability and potency as an antifungal.

14.5.2 Methionine Synthase (MET6)

MET6 encodes methionine synthase which converts methionine to homocysteine. The MET6 encoded protein of fungi is a cobalamine-independent protein, in contrast to the human enzyme, which is cobalamine-dependent. Furthermore, the human and yeast enzymes have no sequence similarity, and have different evolutionary origins. The *met6Δ* of *C. neoformans* was found to be avirulent in the murine inhalation infection model compared to the wild-type (Pascon et al. 2004). The growth rate of *met6Δ* was also found to be much slower than that of *met3Δ*.

When *met3Δ* and *met6Δ* mutants were starved of methionine, *met3Δ* mutants remained viable up to 96 h, while CFU of *met6Δ* mutant declined after 96 h to 58% of the initial CFU. This slow growth rate caused delay in the production of melanin. However, unlike *met3Δ*, *met6Δ* deletion caused substantial reduction in capsule formation. The thick capsule of *C. neoformans* made of polysaccharides prevents phagocytosis of *C. neoformans* by macrophages and neutrophils, and thus *met6Δ* strains could have a secondary phenotype affecting virulence, further strengthening its potential use as a target.

As methionine synthase converts homocysteine to methionine, *met6Δ* mutants would have elevated levels of homocysteine. Homocysteine accumulation is known to inhibit sterol biosynthesis (Hatanaka et al. 1974; McCammon and Parks 1981; Parks and Casey 1995). The antifungal drug flucanozole inhibits sterol biosynthesis, and examining the effects of flucanozole on *met6Δ* mutants revealed that *met6Δ* mutants were two to four times more sensitive to the drug flucanozole. Calcineurin inhibitors act synergistically with flucanozole (Del Poeta et al. 2000). As *met6Δ* would also affect the sterol biosynthesis, they could be used in the combinatorial therapy with calcineurin inhibitors. This was indeed found to be the case, as the *met6Δ* mutants were four times more sensitive to FK506 (a calcineurin inhibitor).

Interestingly, attempts to make a strain of *C. albicans* deleted in both alleles of MET6 were not successful, even in the presence of exogenously added methionine (Suliman et al. 2007). This suggested that MET6 might be an essential gene in *C. albicans*. This observation was in contrast to what was seen in *C. neoformans*, where *met6Δ* could be rescued by exogenously added methionine. To verify this, the first allele was disrupted and the second allele was placed under a regulatable promoter (Gal promoter). The conditional mutant grew well under inducing conditions, even in the absence of methionine. However, under repressing conditions the conditional mutant could only grow in presence of methionine. It thus appears that the gene is essential in *C. albicans*, and that a minimal level of methionine synthase expression is required for growth, which would probably limit homocysteine accumulation and homocysteine induced toxicity.

MET6 is thus an attractive antifungal drug target owing to its dual effect of not only causing methionine auxotropy but also homocysteine accumulation, which is harmful as it causes aberrations in sterol biosynthesis. Furthermore, the human analogue is significantly different.

14.5.3 ATP Sulphurylase (MET3)

MET3 encodes ATP sulphurylase, which converts sulphate to APS in the inorganic sulphur assimilation in yeasts. This enzyme was evaluated in *C. neoformans* for its role in virulence (Yang et al. 2002). The *met3Δ* of *C. neoformans* showed a slow-growth phenotype which caused a defect in a known virulence factor, melanin (Kwon-chung and Rhodes 1986). Melanin is a hydrophobic compound that protects the pathogen against the harsh host environment. Melanin protects against

hypochlorite and hydrogen peroxide, which are products of the innate immunity (Wang *et al.* 1995). The *met3Δ* mutant in the murine inhalation infection model showed avirulence, and was deficient in survival in mice. The wild-type strain caused mortality in mice in 28 days, in contrast to the *met3Δ* strain, in which the mice survived up to 60 days post infection. This indicated that Met3p is required for both virulence and survival of *C. neoformans* in the murine infection model. It was thus concluded that the defect in virulence is because of lack of formation of cysteine and methionine in the *met3Δ* strain of *C. neoformans*. Thus, MET3 could be an attractive drug target. However, MET3-like activity exists in humans as part of the PAPS synthase enzyme. The effectiveness of MET3 targeting antifungals would therefore need to be carefully evaluated in terms of their effect on the human host.

14.5.4 Homoserine Dehydrogenase HOM6

HOM6 encodes homoserine dehydrogenase (HSD), which catalyzes the conversion of L-aspartate-semialdehyde (ASA) to L-homoserine. HSD is a part of the aspartate pathway that synthesizes the essential amino acids methionine, threonine, and isoleucine in fungi. A drug, 2-amino-4-oxo-5hydropentanoic acid (HON), which targets HSD enzyme has been used against fungal pathogens (Jacques et al. 2003). This drug was isolated from *streptomyces* species over 40 years ago in an anti-mycobacterial screen. HON has been shown to be effective against the human fungal pathogen *C. neoformans* (Yamaguchi et al. 1988), the plant pathogen *Cladosporium fulvus* (Yamaki et al. 1990), and *Candida albicans*. HON forms an adduct with the cofactor $NADP^+$ in the active site of HSD. The HON•$NADP^-$ adduct remains effectively irreversibly bound to the HSD active site, thus causing its inhibition. The aspartate pathway in which HSD participates is absent in mammals, making it an excellent drug target.

14.5.5 Transcription Factor Protein (MET4)

MET4 is a transcriptional activator for the sulphur assimilatory genes under sulphur starvation conditions. The antifungal drug azoxybacilin was found to target the inorganic sulphur assimilation pathway of fungi (Aoki et al. 1996) and cause the down-regulation of MET4 mRNA. This resulted in repression of genes involved in the inorganic sulphur assimilation (MET3, MET14, MET16, MET10, and MET25), which otherwise would have been up-regulated in sulphur starvation conditions. Interestingly, azoxybacilin caused moderate decrease in the mRNA level of these enzymes, with a maximal decrease seen for MET10 mRNA under sulphur starvation conditions. It was thus concluded that azoxybacilin, apart from acting at the transcriptional level of MET10 through MET4, is also acting post-transcriptionally on MET10 to cause its inhibition. As the inorganic sulphur assimilatory pathway is absent in humans, it could be an attractive target for drug designing.

14.5.6 Sulphite Transporter

As described in an earlier section, growth of dermatophytes on skin, hair and nails requires the secretion of sulphite. *S. cerevisiae* has a sulphite efflux pump SSU1 (Avram and Bakalinsky 1997). Its orthologue, AfuSSU1 was identified as the sulphite efflux pump in *Aspergillus fumigatus* (Lechenne *et al.* 2007). Subsequently, sulphite efflux pumps were found in the dermatophytes *T. rubrum* (TruSSU1) and *Arthroderma benhamiae* (AbeSSU1). It was also observed that the expression of TruSSU1 and AbeSSU1 (transporters in *T. rubrum* and *A. benhamiae*) was quite high compared to that of AfuSSU1 (transporter in *A. fuigatus)*, which probably renders these fungi (*T. rubrum* and *A. benhamiae*) more pathogenic then *Aspergillus*. However, this sulphite transporter was surprisingly not picked up in a microarray study done with the cells of *T. rubrum* grown on soy and keratin protein (Zaugg et al. 2009). Despite this, it seems likely that these transporters would be good antifungal targets, since inhibition of these transporters would prevent dermatophytes from hydrolysing keratin. Moreover, these sulphite transporters belong to the tellurite-resistance/dicarboxylate transporter (TDT) family, which is absent in humans.

14.5.7 Cystine Transporter

Cystine is one of the prominent sulphur compounds in blood plasma (Table 14.2). *C. albicans, C. glabrata, H. capsulatum, P. braseliensis* and dermatophytes such as *T. rubrum* and *A. benhamiae* all have the ability to utilize cystine. Other fungi may also have pathways for cystine utilization but have not been investigated. It thus appears that intervention in the cystine utilization pathway could be a possible antifungal drug target. This could be either in the recently identified cystine transporter found in *C. glabrata* and *C. albicans*, which lacks homologues in humans (Yadav and Bachhawat, unpublished data), or in the proposed cystine reductase. However, the cystine reductase may not be a specific reductase, and thus needs to await more definitive identification.

14.5.8 Glutathione Utilization Pathway and Glutathione Biosynthesis Pathway

Glutathione utilization in yeast and fungi requires the Dug complex (Dug1p, Dug2p, and Dug3p) and the yeast glutathione transporter Hgt1p. As glutathione is an abundant intracellular sulphur source, pathogens which have an intracellular location are likely to depend on glutathione as a source of sulphur. As both the

transporter and the Dug2p, Dug3p proteins lack homologues in humans, they could potentially be good drug targets, but that needs to be evaluated. In contrast to the intracellular concentrations, glutathione levels are low in plasma (Table 14.2). In such a case, GSH biosynthesis can possibly become essential for survival. Although yeast gamma glutamyl cysteine ligase, the first enzyme for the biosynthesis of glutathione, is in the same lineage as that of the humans, it is significantly different. Thus this enzyme could also be a potential drug target, but this needs validation. Interestingly, glutathione reductase (GLR1), which reduces the oxidized form of glutathione to the reduced form, has been shown to be important for virulence in *C. neoformans*, as *glr1* deletion was found to be avirulent, along with being sensitive to nitrosative stress and macrophage killing (Missall et al. 2006). This further highlights the importance of glutathione biosynthesis and utilization.

14.6 Concluding Remarks

Major differences between the sulphur assimilatory pathways of humans and human pathogens which include not only yeast and fungi but also parasitic protozoas and bacteria, makes the sulphur assimilatory pathway enzymes very attractive targets for antifungal (and also antibacterial and antiparasitic) drug development. As fungi have multiple pathways for assimilating sulphur, it is possible that different pathways become important at different stages of infection — especially since fungal infections occur at different levels from superficial to systemic. However, surprisingly, very little work has been done to determine which sulphur sources are important for a particular pathogen. In many cases, if multiple options exist for the pathogen, drugs should target multiple pathways to be effective *in vivo*. Although many pathways such as the cystine utilization pathway or the GSH utilization and even GSH biosynthesis pathway appear potentially good targets, these targets have to be evaluated *in vivo* before they can be conclusively considered as good targets for drug development. Finally, it must be mentioned that much of the work being done in the development of the sulphur pathway as an antifungal target has been based upon initial work done with model yeasts and fungi. However, there are bound to be significant differences and variations with the pathogenic fungi. The MET6 gene, which is essential in *C. albicans* but does not appears to be essential in either *S. cerevisiae* or the pathogenic *C. neoformans*, is a clear example. Therefore, care needs to be taken in extrapolating results from the model yeast and fungi. Nevertheless, it is clear that the sulphur pathways are bound to yield many targets for antifungal and antimicrobial activity. While in many cases the target may be very specific for an organism or a group of organisms, in other cases they may be able to act in a broader manner.

References

Andrade RV, Paes HC, Nicola AM, de Carvalho MJ, Fachin AL, Cardoso RS, Silva SS, Fernandes L, Silva SP, Donadi EA, Sakamoto-Hojo ET, Passos GA, Soares CM, Brigido MM, Felipe MS (2006) Cell organisation, sulphur metabolism and ion transport-related genes are differentially expressed in Paracoccidioides brasiliensis mycelium and yeast cells. BMC Genomics 7:208

Aoki Y, Yamamoto M, Hosseini-Mazinani SM, Koshikawa N, Sugimoto K, Arisawa M (1996) Antifungal azoxybacilin exhibits activity by inhibiting gene expression of sulfite reductase. Antimicrob Agents Chemother 40:127–132

Avram D, Bakalinsky AT (1997) SSU1 encodes a plasma membrane protein with a central role in a network of proteins conferring sulfite tolerance in Saccharomyces cerevisiae. J Bacteriol 179:5971–5974

Bastos KP, Bailao AM, Borges CL, Faria FP, Felipe MS, Silva MG, Martins WS, Fiuza RB, Pereira M, Soares CM (2007) The transcriptome analysis of early morphogenesis in Paracoccidioides brasiliensis mycelium reveals novel and induced genes potentially associated to the dimorphic process. BMC Microbiol 7:29

Brzywczy J, Paszewski A (1994) Sulfur amino acid metabolism in Schizosaccharomyces pombe: occurrence of two O-acetylhomoserine sulfhydrylases and the lack of the reverse transsulfuration pathway. FEMS Microbiol Lett 121:171–174

Bourbouloux A, Shahi P, Chakladar A, Delrot S, Bachhawat AK (2000) Hgt1p, a high affinity glutathione transporter from the yeast Saccharomyces cerevisiae. J Biol Chem 275: 13259–13265

Burton E, Selhub J, Sakami W (1969) The substrate specificity of 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase. Biochem J 111:793–795

Chapman SW, Sullivan DC, Cleary JD (2008) In search of the holy grail of antifungal therapy. Trans Am Clin Climatol Assoc 119:197–215, discussion 215–216

Cheson BD (1995) Infectious and immunosuppressive complications of purine analog therapy. J Clin Oncol 13:2431–2448

Chen Z, Chakraborty S, Banerjee R (1995) Demonstration that mammalian methionine synthases are predominantly cobalamin-loaded. J Biol Chem 270:19246–19249

Cherest H, Davidian JC, Thomas D, Benes V, Ansorge W, Surdin-Kerjan Y (1997) Molecular characterization of two high affinity sulphate transporters in *Saccharomyces cerevisiae*. Genetics 145:627–635

Cherest H, Surdin-Kerjan Y (1992) Genetic analysis of a new mutation conferring cysteine auxotrophy in Saccharomyces cerevisiae: updating of the sulfur metabolism pathway. Genetics 130:51–58

Chow ED, Liu OW, O'Brien S, Madhani HD (2007) Exploration of whole-genome responses of the human AIDS-associated yeast pathogen Cryptococcus neoformans var grubii: nitric oxide stress and body temperature. Curr Genet 52:137–148

Csaikl U, Csaikl F (1986) Molecular cloning and characterization of the MET6 gene of Saccharomyces cerevisiae. Gene 46:207–214

Del Poeta M, Cruz MC, Cardenas ME, Perfect JR, Heitman J (2000) Synergistic antifungal activities of bafilomycin A(1), fluconazole, and the pneumocandin MK-0991/caspofungin acetate (L-743, 873) with calcineurin inhibitors FK506 and L-685, 818 against Cryptococcus neoformans. Antimicrob Agents Chemother 44:739–746

Enjalbert B, Nantel A, Whiteway M (2003) Stress-induced gene expression in Candida albicans: absence of a general stress response. Mol Biol Cell 14:1460–1467

Ejim LJ, D'Costa VM, Elowe NH, Loredo-Osti JC, Malo D, Wright GD (2004) Cystathionine beta-lyase is important for virulence of Salmonella enterica serovar Typhimurium. Infect Immun 72:3310–3314

Fan W, Kraus PR, Boily MJ, Heitman J (2005) Cryptococcus neoformans gene expression during murine macrophage infection. Eukaryot Cell 4:1420–1433

Felipe MS, Andrade RV, Arraes FB, Nicola AM, Maranhao AQ, Torres FA, Silva-Pereira I, Pocas-Fonseca MJ, Campos EG, Moraes LM, Andrade PA, Tavares AH, Silva SS, Kyaw CM, Souza DP, Pereira M, Jesuino RS, Andrade EV, Parente JA, Oliveira GS, Barbosa MS, Martins NF, Fachin AL, Cardoso RS, Passos GA, Almeida NF, Walter ME, Soares CM, Carvalho MJ, Brigido MM (2005) Transcriptional profiles of the human pathogenic fungus Paracoccidioides brasiliensis in mycelium and yeast cells. J Biol Chem 280:24706–24714

Ferreira ME, Marques Edos R, Malavazi I, Torres I, Restrepo A, Nunes LR, de Oliveira RC, Goldman MH, Goldman GH (2006) Transcriptome analysis and molecular studies on sulfur metabolism in the human pathogenic fungus Paracoccidioides brasiliensis. Mol Genet Genomics 276:450–463

Fradin C, De Groot P, MacCallum D, Schaller M, Klis F, Odds FC, Hube B (2005) Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood. Mol Microbiol 56:397–415

Fujita Y, Takegawa K (2004) Characterization of two genes encoding putative cysteine synthase required for cysteine biosynthesis in Schizosaccharomyces pombe. Biosci Biotechnol Biochem 68:306–311

Ganguli D, Kumar C, Bachhawat AK (2007) The alternative pathway of glutathione degradation is mediated by a novel protein complex involving three new genes in Saccharomyces cerevisiae. Genetics 175:1137–1151

Garcia-Sanchez S, Aubert S, Iraqui I, Janbon G, Ghigo JM, d'Enfert C (2004) Candida albicans biofilms: a developmental state associated with specific and stable gene expression patterns. Eukaryot Cell 3:536–545

Guttormsen AB, Solheim E, Refsum H (2004) Variation in plasma cystathionine and its relation to changes in plasma concentrations of homocysteine and methionine in healthy subjects during a 24-h observation period. Am J Clin Nutr 79:76–79

Hanson MA, Marzluf GA (1973) Regulation of a sulfur-controlled protease in Neurospora crassa. J Bacteriol 116:785–789

Hanson MA, Marzluf GA (1975) Control of the synthesis of a single enzyme by multiple regulatory circuits in Neurospora crassa. Proc Natl Acad Sci USA 72:1240–1244

Hatanaka H, Ariga N, Nagai J, Katsuki H (1974) Accumulation of a sterol intermediate during reaction in the presence of homocysteine with cell-free extract of yeast. Biochem Biophys Res Commun 60:787–793

Hawksworth DL (2001) The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol Res 105:1422–1432

Howard DH, Dabrowa N, Otto V, Rhodes J (1980) Cysteine transport and sulfite reductase activity in a germination-defective mutant of Histoplasma capsulatum. J Bacteriol 141:417–421

Hromatka BS, Noble SM, Johnson AD (2005) Transcriptional response of Candida albicans to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. Mol Biol Cell 16:4814–4826

Hwang L, Hocking-Murray D, Bahrami AK, Andersson M, Rine J, Sil A (2003) Identifying phase-specific genes in the fungal pathogen Histoplasma capsulatum using a genomic shotgun microarray. Mol Biol Cell 14:2314–2326

Isnard AD, Thomas D, Surdin-Kerjan Y (1996) The study of methionine uptake in Saccharomyces cerevisiae reveals a new family of amino acid permeases. J Mol Biol 262:473–484

Jacques SL, Mirza IA, Ejim L, Koteva K, Hughes DW, Green K, Kinach R, Honek JF, Lai HK, Berghuis AM, Wright GD (2003) Enzyme-assisted suicide: molecular basis for the antifungal activity of 5-hydroxy-4-oxonorvaline by potent inhibition of homoserine dehydrogenase. Chem Biol 10:989–995

Jones DP, Carlson JL, Mody VC, Cai J, Lynn MJ, Sternberg P (2000) Redox state of glutathione in human plasma. Free Radic Biol Med 28:625–635

Jacobson ES, Harrell AC (1981) Selenocystine-resistant mutants of Histoplasma capsulatum. Mycopathologia 73:177–181

Jung K, Park J, Choi J, Park B, Kim S, Ahn K, Choi D, Kang S, Lee YH (2008) SNUGB: a versatile genome browser supporting comparative and functional fungal genomics. BMC Genomics 9:586

Kaur J, Bachhawat AK (2007) Yct1p, a novel, high-affinity, cysteine-specific transporter from the yeast Saccharomyces cerevisiae. Genetics 176:877–890

Kunert J (1972) Keratin decomposition by dermatophytes: evidence of the sulphitolysis of the protein. Experientia 28:1025–1026

Kunert J (1976) Keratin decomposition by dermatophytes. II. Presence of s- sulfocysteine and cysteic acid in soluble decomposition products. Z Allg Mikrobiol 16:97–105

Kunert J (1981) Inorganic sulphur sources for the growth of the dermatophyte Microsporum gypseum. Folia Microbiol (Praha) 26:196–200

Kuras L, Cherest H, Surdin-Kerjan Y, Thomas D (1996) A heteromeric complex containing the centromere binding factor 1 and two basic leucine zipper factors, Met4 and Met28, mediates the transcription activation of yeast sulfur metabolism. EMBO J 15:2519–2529

Kwon-Chung KJ, Bennett JE (1992) Medical mycology. Lea & Febiger, Philadelphia

Kwon-Chung KJ, Rhodes JC (1986) Encapsulation and melanin formation as indicators of virulence in Cryptococcus neoformans. Infect Immun 51:218–223

Lechenne B, Reichard U, Zaugg C, Fratti M, Kunert J, Boulat O, Monod M (2007) Sulphite efflux pumps in Aspergillus fumigatus and dermatophytes. Microbiology 153:905–913

Lorenz MC, Bender JA, Fink GR (2004) Transcriptional response of Candida albicans upon internalization by macrophages. Eukaryot Cell 3:1076–1087

Maresca B, Jacobson E, Medoff G, Kobayashi G (1978) Cystine reductase in the dimorphic fungus Histoplasma capsulatum. J Bacteriol 135:987–992

Markovich D (2001) Physiological roles and regulation of mammalian sulfate transporters. Physiol Rev 81:1499–1533

McCammon MT, Parks LW (1981) Inhibition of sterol transmethylation by S-adenosylhomocysteine analogs. J Bacteriol 145:106–112

Missall TA, Pusateri ME, Donlin MJ, Chambers KT, Corbett JA, Lodge JK (2006) Posttranslational, translational, and transcriptional responses to nitric oxide stress in Cryptococcus neoformans: implications for virulence. Eukaryot Cell 5:518–529

Murillo LA, Newport G, Lan CY, Habelitz S, Dungan J, Agabian NM (2005) Genome-wide transcription profiling of the early phase of biofilm formation by Candida albicans. Eukaryot Cell 4:1562–1573

Natorff R, Sienko M, Brzywczy J, Paszewski A (2003) The Aspergillus nidulans metR gene encodes a bZIP protein which activates transcription of sulphur metabolism genes. Mol Microbiol 49:1081–1094

Nazi I, Scott A, Sham A, Rossi L, Williamson PR, Kronstad JW, Wright GD (2007) Role of homoserine transacetylase as a new target for antifungal agents. Antimicrob Agents Chemother 51:1731–1746

Nittler MP, Hocking-Murray D, Foo CK, Sil A (2005) Identification of Histoplasma capsulatum transcripts induced in response to reactive nitrogen species. Mol Biol Cell 16:4792–4813

Nozaki T, Ali V, Tokoro M (2005) Sulfur-containing amino acid metabolism in parasitic protozoa. Adv Parasitol 60:1–99

Odds FC, Brown AJ, Gow NA (2003) Antifungal agents: mechanisms of action. Trends Microbiol 11:272–279

Ono B, Heike C, Yano Y, Inoue T, Naito K, Nakagami S, Yamane A (1992) Cloning and mapping of the CYS4 gene of Saccharomyces cerevisiae. Curr Genet 21:285–289

Paietta JV, Akins RA, Lambowitz AM, Marzluf GA (1987) Molecular cloning and characterization of the cys-3 regulatory gene of Neurospora crassa. Mol Cell Biol 7:2506–2511

Paris S, Duran-Gonzalez S, Mariat F (1985) Nutritional studies on Paracoccidioides brasiliensis: the role of organic sulfur in dimorphism. Sabouraudia 23:85–92

Parks LW, Casey WM (1995) Physiological implications of sterol biosynthesis in yeast. Annu Rev Microbiol 49:95–116

Pascon RC, Ganous TM, Kingsbury JM, Cox GM, McCusker JH (2004) Cryptococcus neoformans methionine synthase: expression analysis and requirement for virulence. Microbiology 150: 3013–3023

Pfaller MA, Diekema DJ (2004) Rare and emerging opportunistic fungal pathogens: concern for resistance beyond Candida albicans and Aspergillus fumigatus. J Clin Microbiol 42:4419–4431

Rodaki A, Young T, Brown AJ (2006) Effects of depleting the essential central metabolic enzyme fructose-1, 6-bisphosphate aldolase on the growth and viability of Candida albicans: implications for antifungal drug target discovery. Eukaryot Cell 5:1371–1377

Rogers PD, Barker KS (2003) Genome-wide expression profile analysis reveals coordinately regulated genes associated with stepwise acquisition of azole resistance in Candida albicans clinical isolates. Antimicrob Agents Chemother 47:1220–1227

Rubin-Bejerano I, Fraser I, Grisafi P, Fink GR (2003) Phagocytosis by neutrophils induces an amino acid deprivation response in Saccharomyces cerevisiae and Candida albicans. Proc Natl Acad Sci USA 100:11007–11012

Salvin SB (1949) Cysteine and related compounds in the growth of the yeast like phase of Histoplasma capsulatum. J Infect Dis 84:275–283

Scherr GH (1957) Studies on the dimorphism of Histoplasma capsulatum. I. The roles of -SH groups and incubation temperature. Exp Cell Res 12:92–107

Senaratne RH, De Silva AD, Williams SJ, Mougous JD, Reader JR, Zhang T, Chan S, Sidders B, Lee DH, Chan J, Bertozzi CR, Riley LW (2006) 5'-Adenosinephosphosulphate reductase (CysH) protects Mycobacterium tuberculosis against free radicals during chronic infection phase in mice. Mol Microbiol 59:1744–1753

Stahl WH, Mc QB et al (1949) Studies on the microbiological degradation of wool; sulfur metabolism. Arch Biochem 20:422–432

Stetler DA, Boguslawski G (1979) Cysteine biosynthesis in a fungus, Histoplasma capsulatum. Sabouraudia 17:23–34

Suliman HS, Appling DR, Robertus JD (2007) The gene for cobalamin-independent methionine synthase is essential in Candida albicans: a potential antifungal target. Arch Biochem Biophys 467:218–226

Thomas D, Surdin-Kerjan Y (1997) Metabolism of sulfur amino acids in Saccharomyces cerevisiae. Microbiol Mol Biol Rev 61:503–532

Triguero A, Barber T, Garcia C, Puertes IR, Sastre J, Vina JR (1997) Liver intracellular L-cysteine concentration is maintained after inhibition of the trans-sulfuration pathway by propargylglycine in rats. Br J Nutr 78:823–831

Venkatachalam KV (2003) Human 3'-phosphoadenosine 5′-phosphosulfate (PAPS) synthase: biochemistry, molecular biology and genetic deficiency. IUBMB Life 55:1–11

Venkatesan G, Singh AJA, Murugesan AG, Janaki C, Shanker SG (2007) *Trichophyton rubrum*-the predominant etiological agent in human dermatophytes in chennai, INDIA. African Journal of Microbiology Research 1:9–12

Viaene J, Tiels P, Logghe M, Dewaele S, Martinet W, Contreras R (2000) MET15 as a visual selection marker for Candida albicans. Yeast 16:1205–1215

Walker J, Barrett J (1997) Parasite sulphur amino acid metabolism. Int J Parasitol 27:883–897

Wang Y, Aisen P, Casadevall A (1995) Cryptococcus neoformans melanin and virulence: mechanism of action. Infect Immun 63:3131–3136

Wang LK, Shuman S (2005) Structure–function analysis of yeast tRNA ligase. RNA 11:966–975

White TC, Oliver BG, Graser Y, Henn MR (2008) Generating and testing molecular hypotheses in the dermatophytes. Eukaryot Cell 7:1238–1245

Willins DA, Kessler M, Walker SS, Reyes GR, Cottarel G (2002) Genomics strategies for antifungal drug discovery — from gene discovery to compound screening. Curr Pharm Des 8:1137–1154

Winters MS, Spellman DS, Chan Q, Gomez FJ, Hernandez M, Catron B, Smulian AG, Neubert TA, Deepe GS Jr (2008) Histoplasma capsulatum proteome response to decreased iron availability. Proteome Sci 6:36

Yamaguchi H, Uchida K, Hiratani T, Nagate T, Watanabe N, Omura S (1988) RI-331, a new antifungal antibiotic. Ann N Y Acad Sci 544:188–190

Yamaki H, Yamaguchi M, Imamura H, Suzuki H, Nishimura T, Saito H, Yamaguchi H (1990) The mechanism of antifungal action of (S)-2-amino-4-oxo-5-hydroxypentanoic acid, RI-331: the inhibition of homoserine dehydrogenase in Saccharomyces cerevisiae. Biochem Biophys Res Commun 168:837–843

Yang Z, Pascon RC, Alspaugh A, Cox GM, McCusker JH (2002) Molecular and genetic analysis of the Cryptococcus neoformans MET3 gene and a met3 mutant. Microbiology 148: 2617–2625

Zaugg C, Monod M, Weber J, Harshman K, Pradervand S, Thomas J, Bueno M, Giddey K, Staib P (2009) Gene expression profiling in the human pathogenic dermatophyte Trichophyton rubrum during growth on proteins. Eukaryot Cell 8:241–250

Chapter 15
Innate Immunity in Pathogenesis and Treatment of Dermatomycosis

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Abstract Innate and cell-mediated immunity are considered as the principal defense lines against fungal infections in humans. Most opportunistic mycoses occur in individuals with defective innate and/or adaptive cellular immunity. Skin and nail infections caused by dermatophyte fungi have become more common in recent years. The capacity of the skin (mainly stratum corneum) to resist infection depends on the innate, cutaneous production of molecules known as antimicrobial peptides (AMPs), and expression of some AMPs further increases in response to microbial invasion. Emerging evidence suggests that some of these peptides are important to immune defense by acting not only as natural antibiotics but also as cell-signaling molecules. Cathelicidins are unique AMPs that protect the skin. Therapies targeting control of cathelicidin and other AMPs might provide new approaches in the management of infectious skin diseases. A better understanding of reciprocal regulation between innate, humoral, and adaptive immune responses in the development of an optimal antifungal immunity may lead to a clarification of the mechanisms involved in host immunity to fungal infections. In this chapter, we review some of the dermatomycosis diseases, their casual agents, the role of innate immunity in pathogenesis, and treatment.

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15.1 Introduction

Infection caused by dermatophytic fungi in man and animals is common throughout the world, and causes a wide spectrum of diseases in humans. The majority of these are caused by a closely related group of keratinophilic fungi called dermatophytes, which cause ringworm infection or Tinea infection. Tinea infections are superficial fungal infections caused by the three genera of dermatophytes, *Trichophyton*, *Microsporum*, and *Epidermophyton* (Weitzman and Summerbell 1995). Dermatophytes are highly specialized pathogenic fungi, which primarily affect the stratum corneum (SC). Infection is generally cutaneous, and restricted to the nonliving cornified layers because of the inability of the fungi to penetrate the deeper tissues or organs of immunocompetent hosts (Dei Cas and Vernes 1986; King et al. 1975). Reactions to a dermatophyte infection may range from mild to severe as a consequence of the host's reactions to the metabolic products of the fungus, the virulence of the infecting strain or species, the anatomic location of the infection, and local environmental factors. Clinically, superficial dermatomycosis causes erythema and scaling of the skin. In the United States, dermatophytosis is second only to acne as the most frequently reported skin disease (Stern 1999). Indeed, in some areas of the world, *Trichophyton* infection is now considered a major public health problem (Fuller et al. 2003; Ghannoum et al. 2003; Gupta and Summerbell 1998; Hay et al. 2001).

The skin's first line of defense against invasion by microbial agents is the SC, a nonviable, desiccated layer of the epidermis (Jackson and Elias 1993). However, this physical barrier is susceptible to injuries that allow the entry of opportunistic microbial agents into the skin. Microbes have evolved a wide variety of mechanisms to breach the epidermis and induce disease. The innate immune system can immediately respond to this intrusion by helping to prevent further invasion. This immune response includes phagocytosis by neutrophils and macrophages, and their production of reactive oxygen intermediates that kill microbial agents (Fearon and Locksley 1996). A number of endogenous antimicrobial peptides (AMPs) have been shown to play an integral part in innate immunity (Lehrer et al. 1993). Two major classes of peptides in mammalian skin, β-defensins (Harder et al. 1997; Stolzenberg et al. 1997) and cathelicidins (Gallo et al. 1994; Frohm et al. 1997), have antimicrobial activity against bacterial, fungal, and viral pathogens (Harder et al. 1997; Frohm et al. 1997; Gropp et al. 1999). These peptides, which are produced by keratinocytes in the skin (Harder et al. 1997; Frohm et al. 1997), disrupt the membrane of the target microbe or penetrate the microbial membrane, interfering with intracellular functions. Similarly, the recruitment of circulating immune effector cells is also an absolute requirement for adequate protection against infection, but many pathogens have evolved systems to evade the cellular immune system (Gallo and Huttner 1998). Neutrophils play an important role in host defense by killing ingested microorganisms, and this mechanism includes the action of AMPs. Various neutrophil-derived AMPs have been identified, including the human cathelicidin cationic antimicrobial protein

(hCAP)3-18 and the neutrophil defensins. Neutrophils that have been stimulated to degranulate may release these peptides and other granule components into the extracellular environment. Here we discuss various types of infections caused by dermatophytes, how suppressed innate immunity helps in spreading of fungus, and the role of innate immunity including TLRs and AMPs in the prevention of the disease.

15.2 Dermatomycosis: An Increasing Problem

Dermatomycosis is a superficial infection or colonization of the keratinized tissue skin, hair, nails and furs in animal and man by a group of closely related fungi termed dermatophytes. Dermatophytes include three genera, *Epidermophyton*, *Microsporum*, and *Trichophyton* (Weitzman and Summerbell 1995). The genera and their descriptions are as follows.

15.2.1 Epidermophyton spp.

The type species is *Epidermophyton floccosum*. The macroconidia are broadly clavate, with typically smooth, thin to moderately thick walls with one to nine septa, 20–60 by 4–13 mm in size. They are usually abundant and borne singly or in clusters. Microconidia are absent. Only two species of this genus have been reported, and only *E. floccosum* is pathogenic.

15.2.2 Microsporum spp.

The type species is *Microsporum audouinii*. Macroconidia are having rough walls which may be asperulate, echinulate, or verrucose. Originally, the macroconidia were described by Emmons as spindle-shaped or fusiform, but the discovery of new species extended the range from obovate (egg-shaped) as in *Microsporum nanum* (Fuentes 1956) to cylindrofusiform as in *Microsporum vanbreuseghemii* (Georg et al. 1962). The macroconidia may have thin, moderately thick to thick walls and 1–15 septa, and range in size from 6–60 to 6–25 mm (Borelli 1965).

15.2.3 Trichophyton spp.

The type species is *Trichophyton tonsurans*. Macroconidia, when present, have smooth, usually thin walls and 1–12 septa, are borne singly or in clusters, and may

be elongate and pencil shaped, clavate, fusiform, or cylindrical. They range in size from 8–86 to 4–14 mm. Microconidia, usually more abundant than macroconidia, may be globose, pyriform or clavate, or sessile or stalked, and are borne singly along the sides of the hyphae or in grape-like clusters (Rippon 1988).

15.2.4 Infections

Tinea is the name given to a fungal skin infection (Fig. 15.1). Tinea is a fungus that can grow on skin, hair or nails. As it grows, it spreads out in a circle, leaving normal-looking skin in the middle. This makes it look like a ring. At the edge of the ring, the skin is lifted up by the irritation and looks red and scaly. Because of the way it looks, tinea infection is often called "ringworm" (the infection looks like a worm under the skin). Fig. 15.2 depicts pictures showing the types and distribution of infection on different part of body.

15.2.4.1 Tinea barbae

Tinea barbae, an infection of the bearded area, may be mild and superficial or a severe inflammatory pustular folliculitis, the latter form more commonly caused by

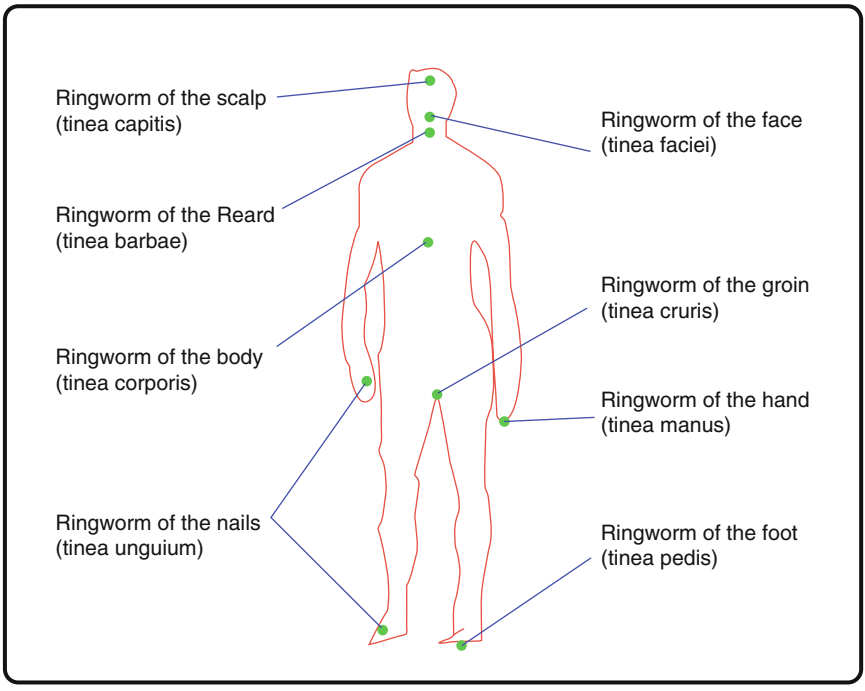

Fig. 15.1 A diagrammatic representation of the distribution of different dermatophytes

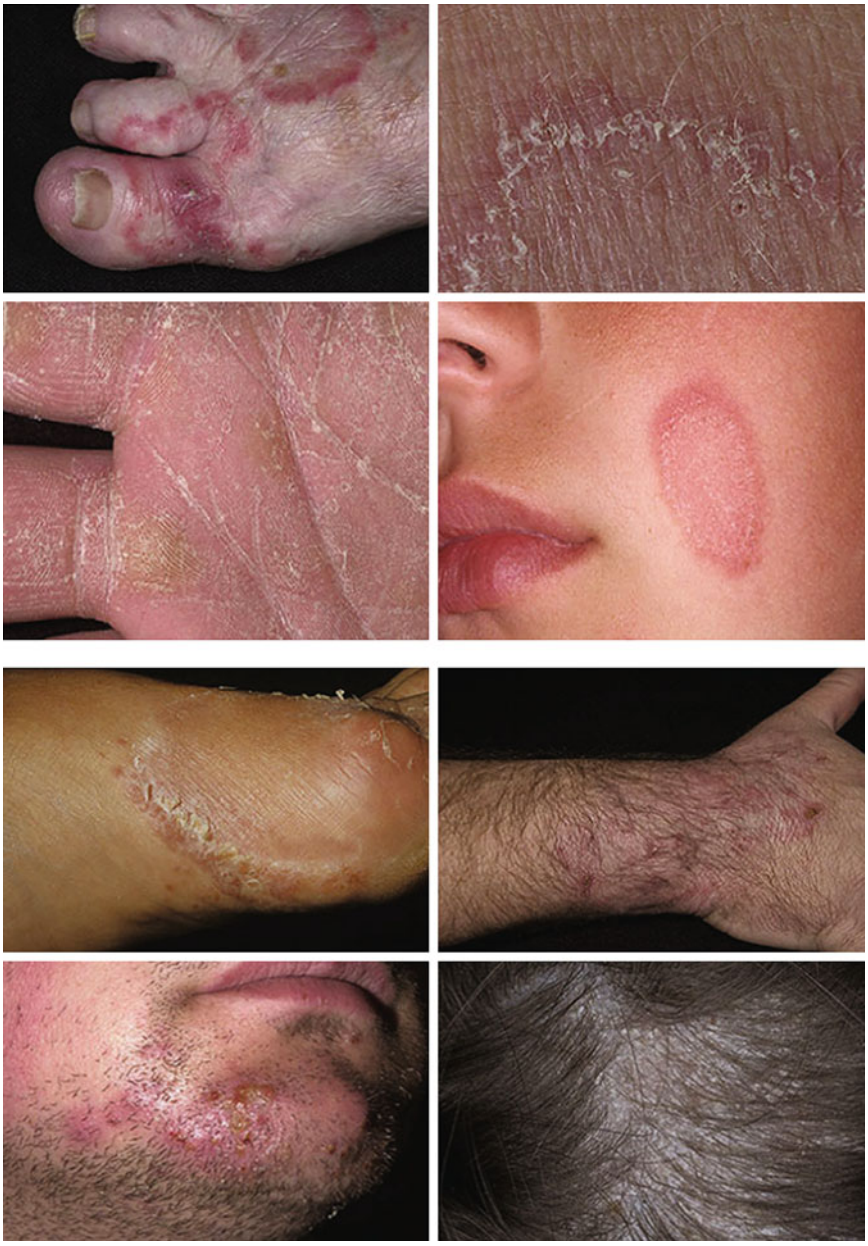

Fig. 15.2 Pictures showing the types and distribution of infection on different part of body. Clockwise from above, the feet (*Tinea pedis*), face area (*Tinea faciei*), scalp (*Tinea capitis*), ringworm of the body (*Tinea manuum*), invasion of the nail plate (*Tinea unguium*), bearded area (*Tinea barbae*)

the zoophilic dermatophytes *Trichophyton verrucosum*, *T. mentagrophytes* var. *mentagrophytes*, and *T. mentagrophytes* var. *erinacei* (Kwon-Chung and Bennett 1992).

15.2.4.2 Tinea faciei

This represents the superficial dermatophyte infection limited to the glabrous skin of the face. In pediatric and female patients, the infection may appear on surface of face. The causative agents are same as tinea barbae infection in men.

15.2.4.3 Tinea capitis

Members of the two genera *Microsporum* and *Trichophyton* cause the infection of the scalp, Tinea capitis. The infection may range from mild to severe, almost subclinical, with slight erythema and a few patchy areas of scaling with dull gray hair stumps, to a highly inflammatory reaction with folliculitis, kerion formation, and extensive areas of scarring and alopecia, sometimes accompanied by fever, malaise, and regional lymphadenopathy. Both the skin surface and hairs are involved. Infection of the hair may be described as ectothrix (sheath of arthroconidia formed on the outside of the hair shaft) or endothrix (arthroconidia formed within the hair shaft). The current predominant cause of tinea capitis in most of North, Central, and South America is *T. tonsurans* (endothrix), replacing *M. audouinii* (ectothrix) (Rippon 1988).

15.2.4.4 Tinea corporis

Commonly known as ringworm of the body, Tinea corporis involves the shoulders, trunk, or limbs, and occasionally the face (excluding the bearded area). It may be caused by any dermatophyte. The infection may range from mild to severe, commonly appearing as annular, scaly patches with sharply marginated, raised erythematous vesicular borders.

15.2.4.5 Tinea cruris (Jock Itch)

Infection of adult men in the groin, perianal, and perineal areas, and occasionally the upper thighs is known as Tinea cruris. Etiologic agents are *T. rubrum* and *E. floccosum*. Lesions are erythematous to yellowish-brown, and covered with thin, dry scales. They are usually bilateral and often asymmetric, extending down the sides of the inner thigh and exhibiting a raised, sharply marginated border that is frequently studded with small vesicles.

15.2.4.6 Tinea favosa

Tinea favosa, generally caused by *Trichophyton schoenleinii*, is severe and chronic; presence on the scalp and glabrous skin of yellowish, cup-shaped crusts called scutula, which is composed of epithelial debris and dense masses of mycelium, is a characteristic feature of it.

15.2.4.7 Tinea imbricata

Tinea imbricata, a specialized manifestation of tinea corporis, is a chronic infection which is characterized by concentric rings of overlapping scales scattered throughout the body. *T. concentricum* is the only etiologic agent.

15.2.4.8 Tinea manuum

The causative agent is *T. Rubrum*. Palmar and interdigital areas of the hand are the regions affected, the infection most often presenting as unilateral diffuse hyperkeratosis with prominence of the flexural creases.

15.2.4.9 Tinea pedis (Athlete's Foot)

In tinea pedis, the feet (soles and toe webs) are the most frequently infected regions. The most common clinical manifestation is the intertriginous form, which presents with maceration, peeling, and fissuring, mainly in the spaces between the fourth and fifth toes.

Another common presentation is the chronic, squamous, hyperkeratotic type in which fine silvery scales cover pinkish skin of the soles, heels, and sides of the foot (moccasin foot). An acute inflammatory condition, characterized by the formation of vesicles, pustules, and sometimes bullae, is most frequently caused by *T. mentagrophytes*. The more chronic agents of tinea pedis are *T. rubrum*, *T. mentagrophytes* var. *interdigitale*, and *E. floccosum*.

15.2.4.10 Tinea ungium

Invasion of the nail plate by a dermatophyte is referred to as tinea unguium; infection of the nail by non-dermatophytic fungi is called onychomycosis (nail infection). There are two main types of nail involvement: invasive subungual (distal and proximal) and superficial white mycotic infection (leukonychia trichophytica).

It is important to note that non-dermatophytes and yeasts may infect the sites mentioned above. For example, tinea unguium is only a subset of the onychomycoses, which include other types of fungal infections of the nails. Similarly, tinea

corporis refers only to dermatophyte infection of the skin, and not to other superficial fungal infections such as candidiasis. Although tinea versicolor is commonly called a tinea, it is caused by the non-dermatophyte *Malassezia furfur* (also referred to as *Pityrosporum orbiculare* and *Pityrosporum ovale*), and is not a true tinea infection (Roberts 1969).

15.3 Fungal Infections and Immunity

Patients with weak immunity are much susceptible to opportunistic fungal infections. In this regard, patients with neoplasms, AIDS, cancer, and autoimmune diseases, and transplant recipients are particularly at risk, due to their weak/altered immunity. Only a small percentage of the 100,000 fungal species in existence is known to cause human infection. Patients with hematological or solid malignancies and transplant recipients are especially vulnerable. Filamentous fungi, including species of *Aspergillus* and *Fusarium*, the Zygomycetes, and the dark-walled fungi, generally cause invasive disease in neutropenic hosts and solid organ transplant recipients. At highest risk are patients with prolonged and profound neutropenia after treatment with highly cytotoxic chemotherapy for hematological malignancies, and recipients of hematopoietic stem cell transplantation. In the latter group, infections with filamentous fungi are increasingly encountered during the post-engraftment period. The immune response varies depending on the fungal species encountered. The relative importance of specific innate and adaptive defense mechanisms depends upon the organism and anatomical site of infection. Within a species, the fungal morphotype (e.g., yeast, pseudohyphae and hyphae of *Candida albicans*) may be an important determinant of the host response. Whereas yeasts and spores are often effectively phagocytosed, the larger size of hyphae precludes effective ingestion. Pathogenic fungi have developed mechanisms to elude and subvert host defences. Some fungi have evolved as intracellular parasites, and can survive within phagocytes by using them to evade fungal killing and to disseminate throughout the host. Major characteristics of the immune response are the interdependence of various arms of the immune system and the interplay between host defenses and fungal pathogenic mechanisms. Several shared defense mechanisms are operative in response to a range of fungi. Neutrophils, macrophages and monocytes are fundamentally important antifungal effector cells. Phagocytes, already residing in target organs at the time of infection, attempt to kill or damage fungi. Additional effector cells, including neutrophils and monocytes, are recruited to sites of infection by the action of inflammatory signals, such as cytokines, chemokines, and complement components. Fungi are killed or damaged by production and/or release of reactive oxygen intermediates and AMPs (Diamond et al. 1980; Mambula et al. 2000). Whether the cells use intracellular or extracellular antifungal mechanisms depends upon the infecting species, morphotype, and route of exposure (Diamond et al. 1978; Kan and Bennett, 1988). Dendritic cells (DC) initiate innate and adaptive immunity to a range of microorganisms (Huang et al. 2001). These cells capture and

process antigens, express lymphocyte co-stimulatory molecules, migrate to lymphoid organs, and secrete cytokines to initiate immune responses (Banchereau and Steinman 1998). DCs play a vital role in linking innate and adaptive responses to a range of pathogenic fungi, including *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *C. albicans* (Bauman et al. 2000; Braedel et al. 2004). Signals transmitted by DCs can vary depending upon the encountered fungus or morphotype, with resultant differences in the nature of adaptive immune responses elicited. Differentiation of CD4+ T cells along a T-helper (Th) cell type 1 (Th_1) or type 2 (Th_2) pathway, and development of specific Th responses, are essential determinants of the host's susceptibility or resistance to invasive fungal infections. Development of Th_1 responses is influenced by the concerted action of cytokines, such as interferon (IFN)-γ, interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-12, in the relative absence of Th_2 cytokines, such as IL-4 and IL-10 (Romani 2002). The predominance of Th_1 over Th_2 type cytokines correlates with protection against various mycoses (Romani et al. 1994; Roilides et al. 1999). Within this framework, however, are subtleties relating to quantitative and temporal production of cytokines and the ultimate development of particular T-cell responses, as well as a role for modulation of immunity so as to limit autoimmune injury.

15.4 Therapy of Fungal Infection and Immune Response

This is a great problem in drug development against any pathogen which resembles the host; as we know, both human and fungal cells are eukaryotic, as opposed to bacteria, which are prokaryotes. There should be some molecules or anatomic sites different from the host to be targeted by the drug. Thus, in addition to anatomic similarities (such as possessing a nucleus surrounded by a nuclear membrane, 80S ribosomes, and Golgi apparati), fungal and human cells have similar mechanisms for DNA, RNA, and protein synthesis. This greatly limits the number of potential antifungal drug targets, because the vast majority of compounds that inhibit fungi are also toxic to human cells. Therefore, antifungal drugs tend to target the few features of fungal cells that differ from human cells; they are very few in number. Most fungal cell membranes contain ergosterol, rather than cholesterol, which is the sterol found in human cell membranes. Amphotericin B directly binds to ergosterol, whereas the "azoles" and terbinafine target ergosterol synthesis. However, the major distinguishing feature is that fungal cells have a rigid cell wall containing chitin, mannans and glucans (the target of the echinocandin class of antifungal drugs). The fungal cell wall imparts physical protection to the fungus, making it resistant to certain host defenses, such as complement-mediated lysis.

Innate immune defenses, including β-glucan receptors, mannose receptors, and toll-like receptors (TLRs), have evolved to recognize and respond to components of fungal cell walls. For example, at the phagocytic cell surface there are TLRs that identify conserved molecular patterns found on microbial (including fungal) products (Akira et al. 2001; Mambula et al. 2002; Levitz 2004). These receptors are

composed of an extracellular domain that distinguishes microbial products, and a cytoplasmic domain that transmits signals to intracellular adapter proteins. One such adapter, MyD88, initiates a signaling cascade leading to the expression of microbicidal molecules and cytokines. The proportional role of individual receptors, such as TLR2, TLR4, and TLR9, in MyD88 activation varies depending upon the infecting fungus and the site of infection. Specific receptors differentially activate antifungal functions, which may result in dissimilar responses and susceptibility to infection (Bellocchio et al. 2004; Braedel et al. 2004).

15.5 Skin and Innate Immunity

Human skin is the largest organ of the human body, and it is the first to bear all kind of challenges from its surroundings. Dermatophytes may contaminate or colonize the skin surface without always producing evidence for clinical infection. This limitation in the infectious process largely relies on the effect of the innate ability controlling the skin surface biocene (Braff et al. 2005; Izadpanah and Gallo 2005). Sebum is another skin product weakening dermatophyte growth. By contrast, it stimulates the growth of lipophilic yeasts. The natural AMPs are evolutionarily conserved compounds of the innate immune system. More than 500 peptides have been described as part of the innate immunity in both plants and animals. In humans, they encompass various defensins as well as cathelicidins, complement and toll-like receptors in the epidermis (Harder et al. 2004; Baroni et al. 2006; Bowdish et al. 2006; Kanzler et al. 2007, and Zheng et al. 2007). They are able to inactivate a broad spectrum of microorganisms, including bacteria, fungi, and viruses. Mammalian defensins constitute one of the most common AMP families. They encompass the two subgroups of α-and β-defensins. These compounds differ from one another by the spacing and the connections of their six cysteine residues (Harder et al. 2004; Sawaki et al. 2002). Human α-defensins are predominantly found in neutrophils granules, whereas the human β-defensins hBD1, hBD2 and hBD3 are localized at the surface of epithelial cells (Abiko et al. 2002; Hubert et al. 2007). The AMP hBD1 is constitutively expressed in normal human skin. By contrast, hBD2 and hBD3 are induced predominantly following contact with microbial products such as endotoxins or proinflammatory cytokines, and are highly expressed by differentiated keratinocytes at sites of inflammation and infection (Pernet et al. 2005). Overexpression of β-defensins is associated *in vivo* with activation of keratinocytes and release of epidermal cytokines (Roilides et al. 1999; Akira et al. 2001; Mambula et al. 2002), such as the macrophage inflammatory Protein-3α (MIP3α/CCL20), a member of the CC-chemokine family. MIP3α is further produced by keratinocytes activated by proinflammatory cytokines (Roeder et al. 2004; Droschner et al. 2004). MIP3α as well as β-defensins bind to, and activate the chemokine receptor CCR6, which is expressed by immature DCs and memory T cells. Thus, they promote adaptive immune responses by recruiting DCs and T cells to the site of microbial invasion. The findings that defensins act as

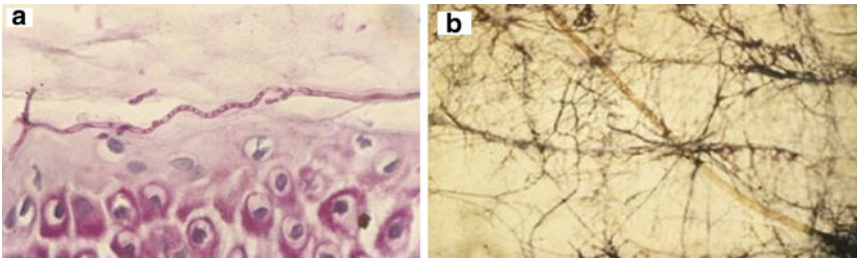

Fig. 15.3 **a** Chain of arthroconidia of a dermatophyte confined to the stratum corneum (periodic acid–Schiff stain). **b** Dermatophytes inside the stratum corneum as seen in a cyanoacrylate skin surface stripping (toluidine blue–basic fuschin stain). Adapted from (Quatresooz et al. 2008)

chemokines, and the *in vivo* co-expression of hBD2 and MIP3α, suggest an important and complementary role in both the innate and adaptive immune response against some microbial invasions, including fungal cells (Jensen et al. 2007; Donnarumma et al. 2004; Quatresooz et al. 2008; Pierard et al. 2006).

15.5.1 Stratum corneum

The stratum corneum is the specific host for dermatomycoses. Superficial dermatomycoses are confined to the SC, with possible extensions to hairs and nails. Very rarely, deeper infections are encountered in immunocompetent patients. The cornified structures exhibit specific characteristics, permitting the adhesion followed by the invasion of a series of dermatophytes, yeasts, and more rarely nondermatophyte molds (Degreef 2008). Various workers have performed antifungal susceptibility tests (AST), using a corneocyte-based or -enriched growth substrate to assess the efficacy of antifungals against pathogens responsible for dermatomycoses. Indeed, AST relevant to the *in vivo* situation should combine the triple association between the specific fungi, the corneocytes, and the antifungals (Knight 1972; Knight 1973; Faergemann 1989; Rurangirwa et al. 1989; Osborne et al. 2004). This work has proved that the SC is a privileged growth medium for keratinophilic fungi. This permissive effect, however, can be limited by the presence of molecular compounds inhibiting fungal growth (Droschner et al. 2004; Jensen et al. 2007; Kawai et al. 2006). This opposite dual situation influences both the spontaneous ability of fungal cells to invade the tissue, and the effect of both antifungal drugs and possibly their active metabolites reaching the SC (Pierard et al. 2007, 2008) (Fig. 15.3).

15.6 Pathogenesis and Innate Immunity

The kinetics of adherence to the skin or nail surface has been investigated in several *Trichophyton* and *Microsporum* species, using different experimental models and microscopy techniques. These studies showed a time-dependent

increase in the number of adhering spores, followed by germination and invasion of the SC by hyphae growing in multiple directions. Some workers have observed that maximum adherence of *Trichophyton* spp. arthroconidia to keratinocytes in suspension occurred within 3–4 h (Zurita and Hay 1987). Use of stripped sheets of SC or separate keratinocytes demonstrated that adherence of *Trichophyton* mentagrophytes arthroconidia is at its maximum after 6 h, and that germination of these spores begins in the first 4 h (Aljabre et al. 1992; Aljabre et al. 1993). In a nail plate model, adherence and germination of *T. mentagrophytes* arthrospores were observed at 6 h and side branches at 16 h (Rashid et al. 1995). The early stages of *T. mentagrophytes* infection were investigated using skin explants of full epidermis thickness (Duek et al. 2004). Adherence was maximum at 12 h, germination had started by 24 h, and penetration of the SC occurred after 3 days. Little is known about the factors that mediate adherence of dermatophytes. The ability of *T. rubrum* to adhere to epithelial cells has been attributed to carbohydrate-specific adhesins, expressed on the surface of microconidia (Esquenazi et al. 2004). From a morphological point of view, fibrillar projections have been observed in *T. mentagrophytes* during the adherence phase. At the skin surface, long and thin fibrils connect fungal arthroconidia to keratinocytes and to each other, while in the inner skin layers, newly formed arthroconidia show thin and short appendices covering their entire surface; the latter begin to vanish as a large contact area is established between conidia and skin tissue (Aljabre et al. 1993; Kaufman et al. 2007). Based on the findings made in the yeast *C. albicans*, where secreted aspartic proteases (Saps) have been shown to play a fundamental role in fungal adherence to epithelia (Ollert et al. 1993; Monod and Borg-von Zepelin 2002; De Bernardis et al. 2007), some workers formulated the hypothesis that dermatophytic-secreted proteases could facilitate or even be necessary for efficient adherence, using the reconstructed feline epidermis (RFE). In that context, *M. canis*-secreted subtilisins, metalloproteases, and dipeptidyl-peptidases are now being investigated for their potential involvement in adherence and early invasion steps, using the RFE model and several specific inhibitors such as protein prosequences. The unique dipeptidyl-peptidase IV (DppIV) has been identified in *Trichophyton* spp. (Monod et al. 2005). Although this phenomenon is independent from enzymatic activity, membrane-associated DppIV from this pathogenic bacterium mediates adherence to fibronectin.

Dermatophytes are provided with an arsenal of proteases aimed at the digestion of the keratin network into assimilable oligopeptides or amino acids. These fungi secrete multiple serine and metallo-endoproteases (subtilisins and fungalysins respectively) (Brouta et al. 2002; Descamps et al. 2002; Jousson et al. 2004a, b), formerly called keratinases. In contrast, little information is available about other hydrolases, such as lipases and a ceramidase, produced by these fungi (Hellgren and Vincent 1981; Giddey et al. 2007). A direct relationship between keratinases and pathogenicity was established by Viani et al. (Viani et al. 2001). They showed that, unlike for other hydrolases, *M. canis* strains with the highest keratinolytic activities *in vitro* were responsible for the more symptomatic infections; meanwhile, since the more severe lesions also resolved faster, these results raise the

question whether keratinases or skin damage they cause are linked with inflammation and immunity.

The importance of dermatophytic, keratinolytic proteases for pathogenicity is thus well-established. Nevertheless, they cannot act before disulfide bridges are reduced within the compact protein network that constitutes keratinized tissues (Kunert 1992). This was recently shown to depend upon a sulfite efflux pump encoded by the Ssu1 gene (Lechenne et al. 2007). Sulfite excretion by this transporter allows sulfitolysis of proteins, rendering them accessible for proteases, and functions in the same time as a possible detoxification pathway. It could therefore be a target for new anti-fungal treatments. Fungal-secreted proteases are produced at high levels when the sole available carbon and nitrogen source is made of complex proteins as opposed to glucose or peptidic digests (Jousson et al. 2004a, b; Mignon et al. 1998). The mechanisms by which the expression of the corresponding genes are induced or repressed are not clearly elucidated. Keratinolytic activity of dermatophytes is probably induced, at least in part, by a restricted supply of assimilable nutrients. The switch in gene activation that would occur at this moment could be controlled by a transcription factor from the GATA family. Indeed, in many fungi, these zinc-finger transcription factors induce the expression of a whole series of genes in response to a change in nitrogen source (Marzluf 1997; Scazzocchio 2000). Among these factors are the products of the areA and nit-2 genes from *Aspergillus nidulans* and *Neurospora crassa*, respectively; they induce the production of numerous enzymes and permeases, including extracellular proteases, conferring the ability to utilize a more complex substrate. Furthermore, in several human and plant pathogenic fungi, areA/ nit-2-like genes have been incriminated in pathogenicity (Hensel et al. 1998; Limjindaporn et al. 2003; Pellier et al. 2003); however, this is not always the case (Froeliger and Carpenter 1996). Recently, the equivalent of these genes in *M. canis*, dnr1, was isolated and functionally investigated by gene disruption. Its inactivation led to impaired growth when keratin was the sole nitrogen source (Yamada et al. 2006). In *T. rubrum*, expression of endoproteases is upregulated by another zinc-finger transcription factor, PACC, which is activated by elevated pH. Disruption of the PACC gene hindered both the secretion of keratinolytic activity and the fungal growth on nail fragments as the sole substrate (Ferreira-Nozawa et al. 2006). Alternatively, Kaufman et al. proposed that constitutively expressed enzymes could release inducers from host skin proteins (Kaufman et al. 2007). AMPs play a very important role as a barrier to the spread of fungal infections, but some superinfections diminish the production of AMPs and defensins, and alter the cytokine profile (Howell et al. 2006). In particular, Th_2 cytokines, such as IL-4 and IL-13, suppress the induction of AMPs and contribute to a disturbed cutaneous antimicrobial response. Thus, in this disorder a decrease in the amount of AMPs released by the skin barrier contributes to disease.

The different ways in which dermatophytes may counter the immune system, or induce damage via immune defenses, are briefly described here. They include lymphocyte inhibition by cell-wall mannans, macrophage function alteration, differential activation of keratinocytes and, putatively, differential secretion of

proteases. Some dermatophytes, such as *T. rubrum* and *T. tonsurans*, are highly adapted to humans, and can evade or silence the immune response, causing chronic dermatophytosis. *Trichophyton rubrum* cell-wall mannans (TRM) seem to be involved in an immunosuppression phenomenon. In a dose-dependent manner, TRM are able to inhibit *in vitro* lymphoproliferative response of mononuclear leukocytes in response to several antigens (dermatophytic or not) and mitogens (Blake et al. 1991a, b); paradoxically, they are a major T-cell antigen (MacCarthy et al. 1994). Although specific suppressor T cells are eventually activated during persistent infections, target cells for TRM action appear to be monocytes rather than lymphocytes (Grando et al. 1992a). TRM may also inhibit SC turnover, directly or via lymphocyte function alteration (Grando et al. 1992b; Dahl 1993). The fine chemical structure of *T. rubrum* and *T. mentagrophytes* mannans has been further characterized by Ikuta et al. (Ikuta et al. 1997), who pointed out potentially significant differences when comparing these to mannans from other fungal species. Moreover, Blake et al. indicated that the amount and inhibitory properties of TRM were different from those of other dermatophyte species (Blake et al. 1991a, b). Despite these numerous investigations, the mechanism underlying immunomodulatory properties of TRM remains poorly understood. This point was recently investigated by Campos et al., who suggested that the observed inhibitory effect could simply result from the saturation of mannose receptors at the surface of macrophages, and subsequent impairment of phagocytosis (Campos et al. 2006).

15.7 Treatment

The fungal cell wall imparts physical protection to the fungus, making it resistant to certain host defences, such as complement-mediated lysis. Innate immune defences, including β-glucan receptors, mannose receptors, and TLRs, have evolved to recognize and respond to components of fungal cell walls. For example, at the phagocytic cell surface are TLRs that identify conserved molecular patterns found on microbial (including fungal) products (Akira et al. 2001; Mambula et al. 2002; Levitz 2004). These receptors are composed of an extracellular domain that distinguishes microbial products and a cytoplasmic domain that transmits signals to intracellular adapter proteins. One such adapter, MyD88, initiates a signaling cascade, leading to the expression of microbicidal molecules and cytokines. The proportional role of individual receptors, such as TLR2, TLR4, and TLR9, in MyD88 activation varies depending upon the infecting fungus and the site of infection. Specific receptors differentially activate antifungal functions, which may result in dissimilar responses and susceptibility to infection (Bellocchio et al. 2004; Braedel et al. 2004).

Dermatomycosis is typically confined to the superficial keratinized tissue and, thus, can often be treated with topical antifungal medications. Because these agents do not penetrate hair or nails, tinea capitis, tinea barbae, and tinea unguium usually

require systemic therapy (Dei Cas and Vernes 1986; Gupta et al. 1998). An increase in antibiotic resistance and the emergence of new pathogens has led to an urgent need for alternative approaches to infection management. Immunomodulatory molecules that do not target the pathogen directly, but rather selectively enhance and/or alter host defense mechanisms, are attractive candidates for therapeutic development. Natural cationic host defense peptides represent lead molecules that boost innate immune responses and selectively modulate pathogen-induced inflammatory responses. Cationic host defense peptides are diverse in their sequence and structures. They are generally amphipathic (having hydrophobic and charged, hydrophilic patches on their surfaces) and small (12–50 amino acids), and have at least two positive charges (as arginine or lysine residues). They can be broadly distributed into four classes based on structure; (1) amphipathic α-helical, (2) β-sheet structures stabilized with two or three disulphide bonds, (3) extended structures, and (4) loop structures with one disulphide bond. Cationic amphipathic peptides are able to interact with and insert into biomembranes, a property that is influenced by their hydrophobicity and net positive charge, conformational flexibility, and secondary structure. Cationic host defense peptides are also known as antimicrobial proteins (AMPs), and were first described in the 1970s and 1980s as gene-encoded molecules responsible for disease resistance in plants and insects. Later, with the identification of defensins in neutrophil granules, it became clear that similar peptides might also be important to the mammalian immune system. Cationic host defense peptides are gene-encoded, and in mammals are expressed in a variety of cell types, including monocyte/macrophages, neutrophils, epithelial cells, keratinocytes, and mast cells. They are generally expressed as pro-peptides that undergo subsequent proteolytic processing to release the biologically active, mature host defence peptide. We now know of more than 20 AMPs in the skin, including cathelicidins, β-defensins, substance P, RANTES, RNase 2,3,7, S100A7, and several others (Braff et al. 2005). Cutaneous production of AMPs is a primary system for protection, and expression of some AMPs further increases in response to microbial invasion.

Cathelicidins are unique AMPs that protect the skin through two distinct pathways: (1) direct antimicrobial activity, and (2) initiation of a host response, resulting in cytokine release, inflammation, angiogenesis, and reepithelialisation (Fig. 15.4). Some may play a specific role against certain microbes in normal skin, whereas others act only when the skin is injured and the physical barrier disrupted. Some other peptides may play a larger role to signal host responses through chemotactic, angiogenic, growth factor, and immunosuppressive activity.

Neutrophils produces the cathelicidin, including the human cathelicidin cationic antimicrobial protein (hCAP)3-18 and the neutrophil defensins. Human CAP-18, the only human cathelicidin identified to date, was first identified in neutrophils (Sørensen et al. 1997), and later shown to be expressed in various squamous epithelia (Frohm et al. 1999), surface epithelial cells of the conducting airways, and serous and mucous cells of the submucosal glands (Bals et al. 1998), by keratinocytes in inflamed skin (Frohm et al. 1997) and by specific lymphocyte and monocyte populations (Agerberth et al. 2000).

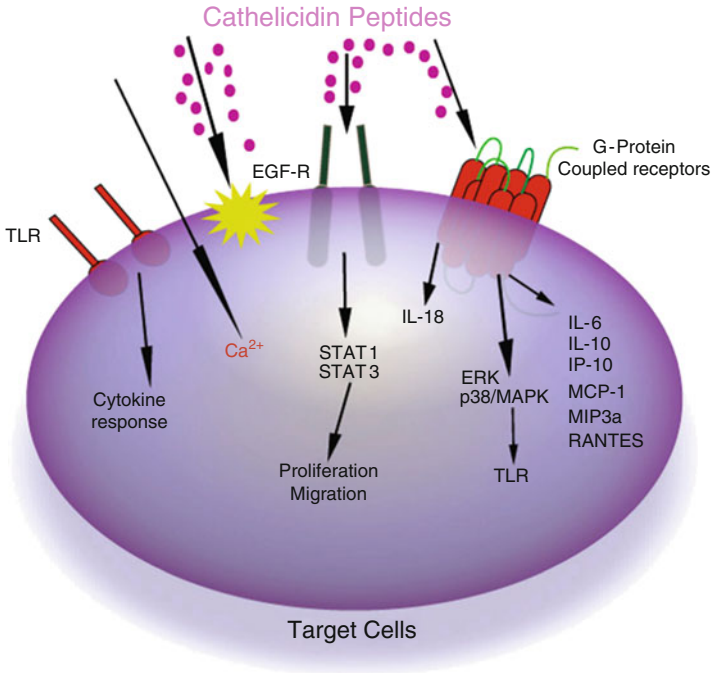

Fig. 15.4 Models for cell activation by cathelicidins. Multiple mechanisms have been proposed for cathelicidins to stimulate a cellular response. Responses are dependent on activation of G protein-coupled receptors and transactivation of the epidermal growth factor receptor or secondary to intracellular Ca21 mobilization or a change in cell membrane function, leading to alterations in receptor responses. Finally, cathelicidins can influence the function of TLRs through both direct and indirect pathways. *EGF-R*, epidermal growth factor receptor; *IP-10*, IFN-g-inducible protein 10; *MCP-1*, monocyte chemoattractant protein 1; *MIP3a*, macrophage inflammatory protein 3a; *ERK*, extracellular signal-regulated kinase; *MAPK*, mitogen-activated protein kinase; *STAT*, signal transducer and activator of transcription

Neutrophils that have been stimulated to degranulate may release these peptides and other granule components into the extracellular environment. They belong to the cathelicidin family of AMPs that are characterized by a conserved N-terminal cathelin domain and a variable C-terminal antimicrobial domain. This C-terminal domain can be cleaved off from the precursor by proteinases, releasing the active peptide. Exocytosed material from neutrophils contains hCAP-18 that has been proteolytically cleaved by proteinase-3, yielding the 4.5 kD active helical peptide LL-37 (Sørensen et al. 2001). LL-37 displays antimicrobial activity against a broad spectrum of microorganisms (Zanetti et al. 1995), and neutralizes LPS bioactivity (Larrick et al. 1995; Nagaoka et al. 2001). The importance of cathelicidins for an effective host defense against infection is illustrated by a recent study showing that mice deficient in the murine cathelicidin-related AMP suffer from more severe bacterial skin infections (Nizet et al. 2001). The LL-37 activates the airway epithelial cells, as demonstrated by ERK1/2 activation and increased IL-8 release

(Van Wetering et al. 1997). Furthermore, this activation involves EGFR activation, metalloproteinase activity, and EGFR ligands. In addition to antimicrobial and chemotactic activity, LL-37 might play an important role in innate immunity by activating airway epithelial cells, suggesting that LL-37 plays a regulatory function in the antimicrobial and inflammatory response of airway epithelial cells. Gallo et al. proposed three different alternative models for the activation of epithelial cells by LL-37 (Hiemstra et al. 1998). These observations, and recent findings that show an association between LL-37 expression and human disease, suggest that LL-37 may form a target for new antimicrobial and anti-inflammatory treatment strategies for skin.

Three major mechanisms have been proposed to explain how LL-37 activates mammalian cells (Fig. 15.5). In the transactivation model, LL-37 stimulates the release of a membrane-bound growth factor. This then binds its high affinity receptor and activates it. In the receptor-binding model, LL-37 serves as a surrogate ligand for a specific receptor. This direct binding initiates receptor activation. In the receptor-activation model, LL-37 associates with and modifies the membrane containing the receptor. This membrane activity indirectly results in a change in receptor function, such that it can signal without a ligand or becomes insensitive to binding by its specific ligand. Evidence to support the transactivation model has

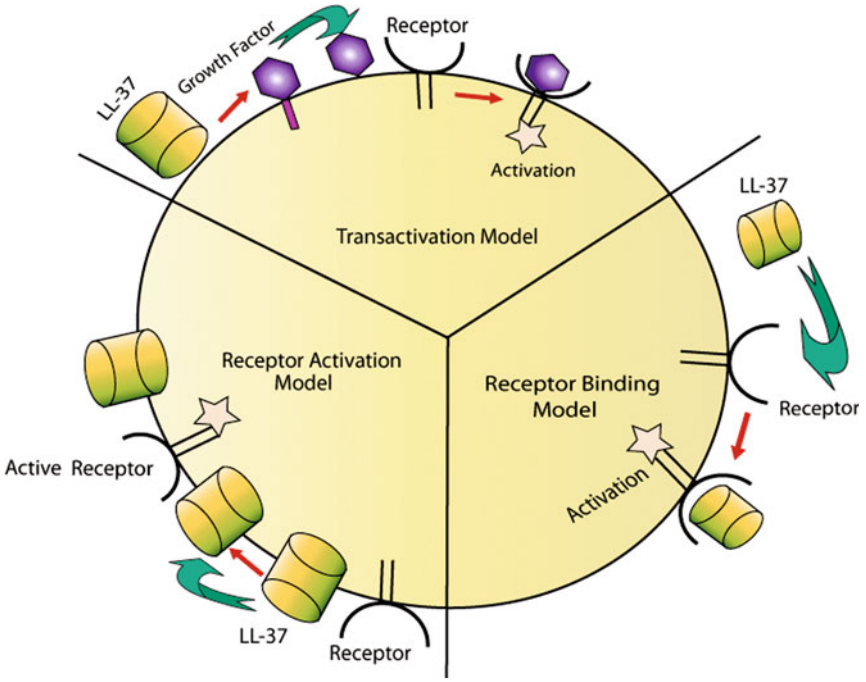

Fig. 15.5 Alternative models for cell activation by antimicrobial peptides. Antimicrobial peptides such as LL-37 are typically small and cationic, and can interact with hydrophobic membranes

been reported in keratinocytes and pulmonary epithelial cells; receptor binding is supported in monocytes and endothelia, and receptor activation in DCs.

15.8 TLR and Dermatomycosis

The AMPs including cathelicidin induce various molecules, which in turn activate the TLRs. Here, we are describing the same process, particularly in concern with innate immunity. Innate immune responses begin with TLR [or other pattern recognition receptors (PRR)] recognition of specific microbial components widely expressed by bacteria, fungi, protozoa, and viruses. Pathogen-encoded TLR ligands are divided into three broad categories: lipids and lipopeptides (TLR2/TLR1; TLR2/TLR6; TLR4), proteins (TLR5), and nucleic acids (TLR3, TLR7, TLR8, TLR9) (Akira et al. 2006; Akira and Takeda 2004; Marshak-Rothstein 2006). They recognize groups of structurally similar and widely distributed molecules, in contrast to the highly selective molecular-level recognition of T- and B-cell receptors. Therapeutic applications to date have used either synthetic versions of natural TLR ligands with optimized pharmacologic properties, or small molecule agonists derived from drug screening efforts.

15.8.1 The TLR and Functions of the Innate Immune System

The major roles played by TLR in regulation of immune and inflammatory responses should be understood very carefully to evaluate the potential of drugs that stimulate or inhibit TLR signaling. Pathogen recognition through TLR and other PRR serves three distinct, although intertwined functions: (1) sensing the presence and type of the pathogen, (2) provoking an immediate antipathogen response, and (3) stimulating the development of long-lasting adaptive response with effector functions appropriate to the type of pathogen (Janeway and Medzhitov 2002; Sansonetti 2006; Akira et al. 2006).

15.8.1.1 Sensory Functions

The detection and classification of invading pathogens by TLR and other PRR are carried out largely by cell types such as DCs and macrophages that continually sample both the external environment at skin and mucosal surfaces, and the internal environment through cells circulating in the blood and lymph (Iwasaki and Medzhitov 2004). Epithelial cells at portals of entry can similarly sense pathogen invasion by PRR (Hornef and Bogdan 2005). Recognition mediates rapid production of cytokines and chemokines that signal the presence of a pathogen, and the specific combination of PRR engaged can convey basic information concerning the type

of pathogen encountered (for example, TLR4 stimulation indicates a gram-negative bacterial infection).

15.8.1.2 Effector Functions

This localized response to infection through TLR initiates rapid recruitment of inflammatory cells to the site of infection, and activates them to induce an arsenal of antimicrobial functions (Hayashi et al. 2003). The migration of leukocytes from the blood into tissue requires the induction of a variety of chemotactic and cell surface adhesion molecules. TLR signals induce these molecules in both circulating hematopoietic cells and vascular endothelial cells, both directly and through TLR-induced cytokines, principally IFNs and TNF-α. Each of the leukocytes develops a characteristic set of effector functions: for neutrophils, reactive oxygen radicals; for macrophages, AMPs and cytokines, reactive nitrogen radicals, and active phagocytosis; for NK cells, perforin-mediated killing. Some functions are specific for microorganisms or viruses, others are less discriminating, and some, such as NK killing, specifically target infected host cells. Consequently, innate responses have the potential for causing significant tissue damage, locally and systemically. Most often, a rapid defense justifies this cost; however, responses that are too intense or prolonged can lead to acute or chronic inflammatory disease states.

15.9 Conclusion

The dermatophytes infect the epidermal layer and are projected to the SC. The epidermis functions at least in part as a protective barrier, and the majority of the protection occurs at the level of the SC. The SC is formed by lipid-depleted corneocytes and a lipid-rich extracellular matrix. This creates a barrier that is able to keep water within the body and prevent the entrance of pathogens and allergens. In addition to the SC, there are so many innate immune factors that blocks the easy entrance and spreading of these dermatophytes. Innate immune defenses, including β-glucan receptors, mannose receptors, and TLRs, have evolved to recognize and respond to components of fungal cell walls. Neutrophils produce the reactive oxygen radicals; macrophages produce the AMPs, various kind of cytokines, and reactive nitrogen radicals, and most importantly they phagocytose the altered cells; NK cells are responsible for perforin-mediated killing of infected cells. The capability of DCs and macrophages to recognize pathogens as foreign particles using TLRs and PRRs initiates the signal for innate immunity. The cathelicidins, a specific AMP, can provoke a prominent innate immunity, and can be used for the therapy purpose against dermatomycosis. From the details given above, it is very clear that innate immunity has a very significant role in checking both fungal and bacterial growth. Although, theoretically, all kinds of infection can be partially or completely blocked by innate immunity; but it is not so in patients,

hence, there should be some cause of spread and growth of dermatophytes in the presence of innate immunity. Some facts like alteration, weakness, and a failure to provoke innate immunity on time are responsible for their free growth and dispersal. Immunocompromised individuals are very susceptible to these infections.

The positive aspect of choosing innate immunity for treatment of any kind of dermatophyte is that it does not need any specific antigen for the activation, i.e., it is not specific for any particular organism; if it is provoked at the perfect time, then it can be helpful for the host. Although much study have been done on innate immunity, nevertheless studies pertaining to dermatomycoses and innate immunity are still to be accomplished. If we are searching for a cure for dermatophytes with the help of innate immunity, a lot of effort is needed to reveal details about the dermatophytes such as their metabolomics, their resistant capability against innate immunity, their interaction with host, and their susceptibility to various kinds of immune factors. When the unrevealed facts emerge, they will help us to control these pathogens either from local or systemic infections.

References

Abiko Y, Jinbu Y, Noguchi T et al. (2002) Upregulation of human beta-defensin 2 peptide expression in oral lichen planus, leukoplakia and candidiasis, an immunohistochemical study. Pathol Res Pract 198:537–542

Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jornvall H, Wigzell H, Gudmundsson GH (2000) The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 96:3086

Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4:499–511

Akira S, Takeda K, Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2:675–680

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124:783–801

Aljabre SH, Richardson MD, Scott EM, Shankland GS (1992) Germination of *Trichophyton mentagrophytes* on human stratum corneum *in vitro*. J Med Vet Mycol 30:145–152

Aljabre SH, Richardson MD, Scott EM, Rashid A, Shankland GS (1993) Adherence of arthroconidia and germlings of anthropophilic and zoophilic varieties of Trichophyton mentagrophytes to human corneocytes as an early event in the pathogenesis of dermatophytosis. Clin Exp Dermatol 18:231–235

Bals R, Wang X, Zasloff M, Wilson JM (1998) The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc Natl Acad Sci U S A 95:9541

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–252

Baroni A, Orlando M, Donnarumma G et al. (2006) Toll-like receptor 2 (TLR2) mediates intracellular signalling in human keratinocytes in response to Malassezia furfur. Arch Dermatol Res 297:280–288

Bauman SK, Nichols KL, Murphy JW (2000) Dendritic cells in the induction of protective and nonprotective anticryptococcal cell-mediated immune responses. J Immunol 165:158–167

Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, Vecchi A, Mantovani A, Levitz SM, Romani L (2004) The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens *in vivo*. J Immunol 172:3059–3069

Blake JS, Cabrera RC, Dahl MV, Herron MJ, Nelson RD (1991a) Comparison of the immunoinhibitory properties of cell wall mannan glycoproteins from Trichophyton rubrum and Microsporum canis. J Invest Dermatol 96:601

Blake JS, Dahl MV, Herron MJ, Nelson RD (1991b) An immunoinhibitory cell wall glycoprotein (mannan) from *Trichophyton rubrum*. J Invest Dermatol 96:657–661

Borelli D (1965) *Microsporum racemosum* nova species. Acta Med Venez 12:148–151

Bowdish DM, Davidson DJ, Hancock RE (2006) Immunomodulatory properties of defensins and cathelicidins. Curr Top Microbiol Immunol 306:27–66

Braedel S, Radsak M, Einsele H, Latge JP, Michan A, Loeffler J, Haddad Z, Grigoleit U, Schild H, Hebart H (2004) *Aspergillus fumigatus* antigens activate innate immune cells via toll-like receptors 2 and 4. Br J Haematol 125:392–399

Braff MH, Bardan A, Nizet V, Gallo RL (2005) Cutaneous defense mechanisms by antimicrobial peptides. J Invest Dermatol 125:9–13

Brouta F, Descamps F, Monod M, Vermout S, Losson B, Mignon B (2002) Secreted metalloprotease gene family of *Microsporum canis*. Infect Immun 70:5676–5683

Campos MR, Russo M, Gomes E, Almeida SR (2006) Stimulation, inhibition and death of macrophages infected with *Trichophyton rubrum*. Microbes Infect 8:372–379

Dahl MV (1993) Suppression of immunity and inflammation by products produced by dermatophytes. J Am Acad Dermatol 28:S19–S23

De Bernardis F, Liu H, O'Mahony R, La Valle R, Bartollino S, Sandini S, Grant S, Brewis N, Tomlinson I, Basset RC, Holton J, Roitt IM, Cassone A (2007) Human domain antibodies against virulence traits of *Candida albicans* inhibit fungus adherence to vaginal epithelium and protect against experimental vaginal candidiasis. J Infect Dis 195(149):57

Degreef H (2008) Clinical forms of dermatophytosis (ringworm infection). Mycopathologia 166:257–265

Dei Cas E, Vernes A (1986) Parasitic adaptation of pathogenic fungi to mammalian hosts. Crit Rev Microbiol 13:173–218

Descamps F, Brouta F, Monod M, Zaugg C, Baar D, Losson B, Mignon B (2002) Isolation of a Microsporum canis gene family encoding three subtilisin-like proteases expressed *in vivo*. J Invest Dermatol 119:830–835

Diamond RD, Krzesicki R, Epstein B, Jao W (1978) Damage to hyphal forms of fungi by human leukocytes *in vitro*. A possible host defense mechanism in aspergillosis and mucormycosis. Am J Pathol 91:313–328

Diamond RD, Clark RA, Haudenschild CC (1980) Damage to Candida albicans hyphae and pseudohyphae by the myeloperoxidase system and oxidative products of neutrophil metabolism *in vitro*. J Clin Invest 66:908–917

Donnarumma G, Paoletti I, Buommino E et al. (2004) Malassezia furfur induces the expression of b-defensins-2 in human keratinocytes in a protein kinase C-dependent manner. Arch Dermatol Res 295:474–481

Droschner RA, Lopez-Garcia B, Massie J et al (2004) Innate immune defense of the nail unit by antimicrobial peptides. J Am Acad Dermatol 50:343–348

Duek L, Kaufman G, Ulman Y, Berdicevsky I (2004) The pathogenesis of dermatophyte infections in human skin sections. J Infect 48:175–180

Esquenazi D, Alviano CS, de Souza W, Rozental S (2004) The influence of surface carbohydrates during *in vitro* infection of mammalian cells by the dermatophyte *Trichophyton rubrum*. Res Microbiol 155:144–153

Faergemann J (1989) A new model for growth and filament production of *Pityrosporum ovale* (orbiculare) on human stratum corneum *in vitro*. J Invest Dermatol 92:117–119

Fearon DT, Locksley RM (1996) The instructive role of innate immunity in the acquired immune response. Science 272:50–53

Ferreira-Nozawa MS, Silveira HC, Ono CJ, Fachin AL, Rossi A, Martinez-Rossi NM (2006) The pH signaling transcription factor PacC mediates the growth of *Trichophyton rubrum* on human nail *in vitro*. Med Mycol 44:641–645

Froeliger EH, Carpenter BE (1996) NUT1, a major nitrogen regulatory gene in *Magnaporthe grisea*, is dispensable for pathogenicity. Mol Gen Genet 251:647–656

Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, Gudmundsson GH (1997) The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. J Biol Chem 272:15258–15263

Frohm NM, Sandstedt B, Sørensen O, Weber G, Borregaard N, Stahle-Backdahl M (1999) The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. Infect Immun 67:2561

Fuentes CA (1956) A new species of *Microsporum*. Mycologia 48:613–614

Fuller LC, Child FC, Midgley G, Higgins EM (2003) Scalp ringworm in south-east London and an analysis of a cohort of patients from a paediatric dermatology department. Br J Dermatol 148:985–988

Gallo RL, Huttner KM (1998) Antimicrobial peptides: an emerging concept in cutaneous biology. J Invest Dermatol 111:739–743

Gallo RL, Ono M, Povsic T et al. (1994) Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. Proc Natl Acad Sci U S A 91:11035–11039

Georg LK, Ajello L, Friedman L, Brinkman SA (1962) A new species of *Microsporum* pathogenic to man and animals. Sabouraudia 1:189–196

Ghannoum M, Isham N, Hajjeh R, Cano M, Al-Hawasi F, Yearick D, Warner J, Long L, Jessup C, Elewski B (2003) *Tinea capitis* in Cleveland: survey of elementary school students. J Am Acad Dermatol 48:189–193

Giddey K, Monod M, Barblan J, Potts A, Waridel P, Zaugg C, Quadroni M (2007) Comprehensive analysis of proteins secreted by *Trichophyton rubrum* and *Trichophyton violaceum* under *in vitro* conditions. J Proteome Res 6:3081–3092

Grando SA, Herron MJ, Dahl MV, Nelson RD (1992a) Binding and uptake of *Trichophyton rubrum* mannan by human epidermal keratinocytes: a time-course study. Acta Derm Venereol 72:273–276

Grando SA, Hostager BS, Herron MJ, Dahl MV, Nelson RD (1992b) Binding of *Trichophyton rubrum* mannan to human monocytes *in vitro*. J Invest Dermatol 98:876–880

Gropp R, Frye M, Wagner TO, Bargon J (1999) Epithelial defensins impair adenoviral infection: implication for adenovirus-mediated gene therapy. Hum Gene Ther 10:957–964

Gupta AK, Summerbell RC (1998) Increased incidence of *Trichophyton tonsurans* tinea capitis in Ontario, Canada between 1985 and 1996. Med Mycol 36:55–60

Gupta AK, Einarson TR, Summerbell RC, Shear NH (1998) An overview of topical antifungal therapy in dermatomycoses. A North American perspective. Drugs 55:645–674

Harder J, Bartels J, Christophers E, Schroder JM (1997) A peptide antibiotic from human skin. Nature 387:861

Harder J, Meyer-Hoffert U, Wehkamp K et al. (2004) Differential gene induction of human b-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid. J Invest Dermatol 123:522–529

Hay RJ, Robles W, Midgley G, Moore MK, European Confederation of Medical Mycology Working Party on Tinea Capitis (2001) Tinea capitis in Europe: new perspective on an old problem. J Eur Acad Dermatol Venereol 15:229–233

Hayashi F, Means TK, Luster AD (2003) Toll-like receptors stimulate human neutrophil function. Blood 102:2660–2669

Hellgren L, Vincent J (1981) Lipolytic activity of some dermatophytes. II. Isolation and characterisation of the lipase of *Epidermophyton floccosum*. J Med Microbiol 14:347–350

Hensel M, Arst HN Jr, Aufauvre-Brown A, Holden DW (1998) The role of the *Aspergillus fumigatus* areA gene in invasive pulmonary aspergillosis. Mol Gen Genet 258:553–557

Hiemstra PS, Van Wetering S, Stolk J (1998) Neutrophil serine proteinases and defensins in chronic obstructive pulmonary disease: effects on pulmonary epithelium. Eur Respir J 12:1200

Hornef MW, Bogdan C (2005) The role of epithelial Toll-like receptor expression in host defense and microbial tolerance. J Endotoxin Res 11:124–128

Howell MD, Gallo RL, Boguniewicz M, Jones JF, Wong C, Streib JE et al (2006) Cytokine milieu of atopic dermatitis skin subverts the innate immune response to vaccinia virus. Immunity 24:341–348

Huang Q, Liu D, Majewski P, Schulte LC, Korn JM, Young RA, Lander ES, Hacohen N (2001) The plasticity of dendritic cell responses to pathogens and their components. Science 294:870–875

Hubert P, Herman L, Maillard C et al. (2007) Defensins induce the recruitment of dendritic cells in cervical human papillomavirus-associated (pre)neoplastic lesions formed *in vitro* and transplanted *in vivo*. FASEB J 21:2765–2775

Ikuta K, Shibata N, Blake JS, Dahl MV, Nelson RD, Hisamichi K, Kobayashi H, Suzuki S, Okawa Y (1997) NMR study of the galactomannans of *Trichophyton mentagrophytes* and *Trichophyton rubrum*. Biochem J 323:297–305

Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. Nat Immunol 5:987–995

Izadpanah A, Gallo RL (2005) Antimicrobial peptides. J Am Acad Dermatol 52:381–390

Jackson SM, Elias PM (1993) Skin as an organ of protection. In: Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF (eds) Dermatology in general medicine, 4th edn. McGraw-Hill, New York, pp 241–253

Janeway CA Jr, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216

Jensen JM, Pfeiffer S, Akaki T et al. (2007) Barrier function, epidermal differentiation, and human beta-defensin 2 expression in tinea corporis. J Invest Dermatol 127:1720–1727

Jousson O, Lechenne B, Bontems O, Capoccia S, Mignon B, Barblan J, Quadroni M, Monod M (2004a) Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. Microbiology 150:301–310

Jousson O, Lechenne B, Bontems O, Mignon B, Reichard U, Barblan J, Quadroni M, Monod M (2004b) Secreted subtilisin gene family in *Trichophyton rubrum*. Gene 339:79–88

Kan VL, Bennett JE (1988) Lectin-like attachment sites on murine pulmonary alveolar macrophages bind *Aspergillus fumigatus* conidia. J Infect Dis 158:407–414

Kanzler H, Barrat FJ, Hessel EM, Coffman RL (2007) Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med 13:552–559

Kaufman G, Horwitz BA, Duek L, Ullman Y, Berdicevsky I (2007) Infection stages of the dermatophyte pathogen *Trichophyton*: microscopic characterization and proteolytic enzymes. Med Mycol 45:149–155

Kawai M, Yamazaki M, Tsuboi R et al. (2006) Human betadefensin-2, an antimicrobial peptide, was elevated in scales collected from tinea pedis patients. Int J Dermatol 45:1389–1390

King RD, Khan HA, Foye JC, Greenberg JH, Jones HE (1975) Transferrin, iron, and dermatophytes. I. Serum dermatophyte inhibitory component definitively identified as unsaturated transferrin. J Clin Med 86:204–212

Knight AG (1972) Culture of dermatophytes upon stratum corneum. J Invest Dermatol 59:427–431

Knight AC (1973) Human models for *in vivo* and *in vitro* assessment of topical antifungal agents. Br J Dermatol 89:509–514

Kunert J (1992) Effect of reducing agents on proteolytic and keratinolytic activity of enzymes of *Microsporum gypseum*. Mycoses 35:343–348

Kwon-Chung KJ, Bennett JE (1992) Medical mycology. Lea and Febiger, PA

Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC (1995) Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. Infect Immun 63:1291

Lechenne B, Reichard U, Zaugg C, Fratti M, Kunert J, Boulat O, Monod M (2007) Sulphite efflux pumps in *Aspergillus fumigatus* and dermatophytes. Microbiology 153:905–913

Lehrer RI, Lichtenstein AK, Ganz T (1993) Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu Rev Immunol 11:105–128

Levitz SM (2004) Interactions of Toll-like receptors with fungi. Microbes Infect 6:1351–1355

Limjindaporn T, Khalaf RA, Fonzi WA (2003) Nitrogen metabolism and virulence of *Candida albicans* require the GATA-type transcriptional activator encoded by GAT1. Mol Microbiol 50:993–1004

MacCarthy KG, Blake JS, Johnson KL, Dahl MV, Kalish RS (1994) Human dermatophyte-responsive T-cell lines recognize cross-reactive antigens associated with mannose-rich glycoproteins. Exp Dermatol 3:66–71

Mambula SS, Simons ER, Hastey R, Selsted ME, Levitz SM (2000) Human neutrophil-mediated nonoxidative antifungal activity against *Cryptococcus neoformans*. Infect Immun 68:6257–6264

Mambula SS, Sau K, Henneke P, Golenbock DT, Levitz SM (2002) Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. J Biol Chem 277:39320–39326

Marshak-Rothstein A (2006) Toll-like receptors in systemic autoimmune disease. Nat Rev Immunol 6:823–835

Marzluf GA (1997) Genetic regulation of nitrogen metabolism in the fungi. Microbiol Mol Biol Rev 61:17–32

Mignon B, Swinnen M, Bouchara JP, Hofinger M, Nikkels A, Pierard G, Gerday C, Losson B (1998) Purification and characterization of a 31.5 kDa keratinolytic subtilisin-like serine protease from *Microsporum canis* and evidence of its secretion in naturally infected cats. Med Mycol 36:395–404

Monod M, Borg-von Zepelin M (2002) Secreted aspartic proteases as virulence factors of Candida species. Biol Chem 383:1087–1093

Monod M, Lechenne B, Jousson O, Grand D, Zaugg C, Stocklin R, Grouzmann E (2005) Aminopeptidases and dipeptidyl-peptidases secreted by the dermatophyte *Trichophyton rubrum*. Microbiology 15:145–155

Nagaoka I, Hirota S, Niyonsaba F, Hirata M, Adachi Y, Tamura H, Heumann D (2001) Cathelicidin family of antibacterial peptides CAP18 and CAP11 inhibit the expression of TNF-α by blocking the binding of LPS to $CD14^+$ cells. J Immunol 167:3329

Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, Gallo RL (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414:454

Ollert MW, Sohnchen R, Korting HC, Ollert U, Brautigam S, Brautigam W (1993) Mechanisms of adherence of *Candida albicans* to cultured human epidermal keratinocytes. Infect Immun 61:4560–4568

Osborne CS, Leitner I, Favre B, Ryder NS (2004) Antifungal drug response in an *in vitro* model of dermatophyte nail infection. Med Mycol 42:159–163

Pellier AL, Lauge R, Veneault-Fourrey C, Langin T (2003) CLNR1, the AREA/NIT2-like global nitrogen regulator of the plant fungal pathogen *Colletotrichum lindemuthianum* is required for the infection cycle. Mol Microbiol 48:639–655

Pernet I, Reymermier C, Guezennec A et al. (2005) An optimized method for intensive screening of molecules that stimulate b-defensins 2 or 3 (hBD2 or hBD3) expression in cultured normal human keratinocytes. Int J Cosmet Sci 27:161–170

Pierard GE, Quatresooz P, Arrese JE (2006) Spotlight on nail histomycology. Dermatol Clin 24:371–374

Pierard GE, Arrese JE, Quatresooz P, Pierard-Franchimont C (2007) Emerging antifungal agents for onychomycosis. Exp Opin Emerg Drugs 12:345–353

Pierard GE, Pierard-Franchimont C, Vroome V et al. (2008) Established and emerging oral antifungals in dermatology. In: Walters HA, Roberts MS (eds) Dermatological and cosmeceutical development. Marcel Dekker, New York, pp 283–296

Quatresooz P, Pierard-Franchimont C, Arrese JE, Pierard GE (2008) Clinicopathologic presentations of dermatomycoses in cancer patients. J Eur Acad Dermatol Venereol 22:407–417
Rashid A, Scott E, Richardson MD (1995) Early events in the invasion of the human nail plate by *Trichophyton mentagrophytes*. Br J Dermatol 133:932–940
Rippon JW (1988) Medical mycology: the pathogenic fungi and the pathogenic actinomycetes, 3rd edn. W.B. Saunders, PA
Roberts SO (1969) Pityriasis versicolor: a clinical and mycological investigation. Br J Dermatol 81:315–326
Roeder A, Kirschning CJ, Rupec RA et al. (2004) Toll-like receptors as key mediators in innate antifungal immunity. Med Mycol 42:485–498
Roilides E, Tsaparidou S, Kadiltsoglou I, Sein T, Walsh TJ (1999) Interleukin-12 enhances antifungal activity of human mononuclear phagocytes against *Aspergillus fumigatus*: implications for a gamma interferon-independent pathway. Infect Immun 67:3047–3050
Romani L (2002) Immunology of invasive candidiasis. In: Calderone RA (ed) Candida and Candidiasis. ASM Press, Washington, DC, pp 223–241
Romani L, Mencacci A, Tonnetti L, Spaccapelo R, Cenci E, Puccetti P, Wolf SF, Bistoni F (1994) IL-12 is both required and prognostic *in vivo* for T helper type 1 differentiation in murine candidiasis. J Immunol 153:5167–5175
Rurangirwa A, Pierard-Franchimont C, Pierard GE (1989) Culture of fungi on cyanoacrylate skin surface strippings — a quantitative bioassay for evaluating antifungal drugs. Clin Exp Dermatol 59:425–428
Sansonetti PJ (2006) The innate signaling of dangers and the dangers of innate signaling. Nat Immunol 7:1237–1242
Sawaki K, Mizukawa N, Yamaai T, Fukunaga J, Sugahara T (2002) Immunohistochemical study on expression of alpha-defensin and beta-defensin-2 in human buccal epithelia with candidiasis. Oral Dis 8:37–41
Scazzocchio C (2000) The fungal GATA factors. Curr Opin Microbiol 3:126–131
Sørensen OE, Arnljots K, Cowland JB, Bainton DF, Borregaard N (1997) The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. Blood 90:2796
Sørensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, Borregaard N (2001) Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood 97:3951
Stern RS (1999) The epidemiology of cutaneous disease. In: Freedberg IM, Fitzpatrick TB (eds) Fitzpatrick's dermatology in general medicine, 5th edn. McGraw-Hill, New York, pp 7–12
Stolzenberg ED, Anderson GM, Ackermann MR, Whitlock RH, Zasloff M (1997) Epithelial antibiotic induced in states of disease. Proc Natl Acad Sci U S A 94:8686–8690
Van Wetering S, Mannesse-Lazeroms SP, Van Sterkenburg MA, Daha MR, Dijkman JH, Hiemstra PS (1997) Effect of defensins on interleukin-8 synthesis in airway epithelial cells. Am J Physiol 272:L888
Viani FC, Dos Santos JI, Paula CR, Larson CE, Gambale W (2001) Production of extracellular enzymes by *Microsporum canis* and their role in its virulence. Med Mycol 39:463–468
Weitzman I, Summerbell RC (1995) The dermatophytes. Clin Microbiol Rev 8:240–259
Yamada T, Makimura K, Abe S (2006) Isolation, characterization, and disruption of dnr1, the areA/nit-2-like nitrogen regulatory gene of the zoophilic dermatophyte, *Microsporum canis*. Med Mycol 44:243–252
Zanetti M, Gennaro R, Romeo D (1995) Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett 374:1
Zheng Y, Niyonsaba F, Ushio H et al. (2007) Cathelicidin LL-37 induces the generation of reactive oxygen species and release of human a-defensins from neutrophils. Br J Dermatol 157:1124–1131
Zurita J, Hay RJ (1987) Adherence of dermatophyte microconidia and arthroconidia to human keratinocytes *in vitro*. J Invest Dermatol 89:529–534

Chapter 16
Cytokine Therapy: Possible Tools in Management of Fungal Infection

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Abstract Despite the availability of several new antifungal agents, invasive fungal infections (IFIs) continue to escalate. In the past few years, currently available antifungal chemotherapies have not been optimal, especially for diseases in immunocompromised patients. However, coinciding with the aggressive use of antibiotics, immunosuppressive treatments, and acquired immunodeficiency syndrome, the frequency of severe fungal infections and the diversity of the causal agents have continued to increase until the present day. Hence, there has been increased interest in the use of immunomodulation, primarily with cytokines, as an adjunct to antifungal agents. In this chapter, we endeavor to find the potential role for cytokines and their therapy in treating IFIs, and also mechanisms for synergy between antifungal therapy and immune responses. It is hoped that this will definitely stimulate further discussions and researches on this important aspect.

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16.1 Introduction

Candida and *Aspergillus* species have been consistently renowned as the most important fungal pathogens. A large autopsy series on patients with cancer found that 58% of fungal infections were caused by *Candida* species and 30% by *Aspergillus* species. A previous study of bone marrow transplantation (BMT) patients showed that the incidence of invasive aspergillosis (IA) among them increased from 5.7% to 11.2%, while in another study of patients with hematologic malignancies it increased from 7% to 21%. The mortality associated with IA in BMT patients, even after engraftment, was close to 90% despite maximal antifungal therapy. Such findings are not encouraging and reflect the severe loss of host defenses in these patients, as well as the relatively poor performance of currently available antifungal drugs in such infectious situations. Thus, novel therapeutic strategies are urgently needed (Rodriguez-Adrian et al. 1998; Trick et al. 2002).

Despite the availability of several old and new antifungal agents, invasive fungal infections (IFIs) continue to escalate (Chen and Sorrell, 2007). These agents include: (1) polyenes: amphotericin B deoxycholate, amphotericin B lipid formulations (liposomal amphotericin B, amphotericin B colloidal dispersion, amphotericin B lipid complex), aerosolized amphotericin B, and liposomal nystatin, (2) azoles such as imidazoles (clotrimazole, miconazole and ketoconazole), (3) triazole agents: first generation triazole agents (fluconazole, itraconazole) and second generation triazoles (voriconazole, posaconazole, ravuconazole), (4) echinocandins such as caspofungin, micafungin and anidulafungin, (5) allylamines and thiocarbamates (terbinafine), (6) nucleoside analogs such as flucytosine, (7) newer antifungal agents such as pneumocandins, pradimicins and benanomicins, nikkomycins, sordarins, and cationic peptides, and (8) antibiotics such as echinocandin-related lipopeptides, benzo-(α)-nepthacenequinones, cyclic depsipeptides, polyoxins and nikkomycins). In the past few years, currently available antifungal chemotherapies have not been optimal, especially for diseases in immunocompromised patients. However, coinciding with the aggressive use of antibiotics, immunosuppressive treatments, and acquired immunodeficiency syndrome (AIDS), the frequency of severe fungal infections (such as candidiasis, cryptococcosis, aspergillosis, and zygomycosis), and the diversity of the causal agents have continued to increase until the present day. Hence, there has been increased interest in the use of immunomodulation, primarily with cytokines, as an adjunct to antifungal agents.

16.2 Immune System in Fungal Infections

The presence of an activated innate immunity, such as the ability of phagocytic cells to inhibit fungal growth, is required for the induction of CD41 Th1 cells (Mencacci et al. 1998a). However, an activated innate immune system is not always sufficient to clear the infection. Thus, a proper integration between the innate and

the adaptive immune systems is required for efficient control of *Candida albicans* infections. Resistance to *C. albicans* infection is determined by phagocytic mechanisms, the activity of which is augmented or reinforced by Th1 cytokines and impaired by Th2 cytokines. It has been observed that cytokines produced by Th1 cells can activate phagocytes to a fungicidal state, whereas those produced by Th2 cells exacerbate the disease because of their deactivating properties for fungicidal effector cells (Romani et al. 1997a). Th1 and Th2 cells are instrumental in mobilizing and activating the proper anticandidal effector mechanism at the site of the infection. Studies in humans have strengthened this concept, by showing that acquired immunity to *C. albicans* correlates with the expression of local or peripheral Th1 reactivity (La Sala et al. 1996). Susceptibility to infection can be seen in thermally injured patients (Kobayashi et al. 1998), and also in patients with human immunodeficiency virus (HIV) infection (Leigh et al. 1998), or in patients with chronic mucocutaneous (Kobrynski et al. 1996). This susceptibility correlates with a biased Th2 response to the fungus. The complexity of the spectrum of *Candida* diseases in humans with the deterministic and apparently reductionist approach can be provided by the Th1/Th2 paradigm in experimental animals. The highly polarized cytokine responses which have been induced by injection of large yeast inocula into inbred mice with widely different degrees of susceptibility can reflect the extremes of testing conditions. This explains the yeast commensalistic relationship with humans and its ability to modulate the host's response, and it may also clarify several aspects of fungal pathogenicity and immunopathology mechanisms. Human studies have revealed several effector mechanisms that result in *Candida* killing. No convincing link has yet been established between a particular clinical condition and any specific effector mechanism (Ashman and Papadimitriou 1995), despite the long-recognized associations between systemic candidiasis and neutrophil deficiency, and between chronic mucosal infections and abnormalities in the cell-mediated response (Odds 1988). Therefore, despite recent evidence indicating some differences in cell and cytokine requirements for expression of resistance at mucosal or systemic levels (Wagner et al. 1996), this regulation emphasizes the fact that the anticandidal responses that have been characterized in systemic and mucosal infections are not unique to either condition (Romani 2000).

16.3 Role of Cytokine in Regulation of Immune Cells in Fungal Infections

16.3.1 Lymphocytes

Cytokines play a major role, acting not only as modulators of antifungal effector functions but also as key regulators in the development of the different Th subsets from precursor Th cells. The development of protective anticandidal Th1 responses requires the concerted actions of several cytokines, such as interferon (IFN)-γ

(Cenci et al. 1998), transforming growth factor (TGF)-β (Spaccapelo et al. 1995), interleukin (IL)-6 (Romani et al. 1996), tumor necrosis factor (TNF)-α (Mencacci et al. 1998a), and IL-12 (Romani et al. 1994), in the absence of inhibitory Th2 cytokines, such as IL-4 and IL-10, which inhibit development of Th1 responses (Tonnetti et al. 1995). Studies have suggested that immunomodulatory functions of IL-12 can play a role in promoting endogenous protective responses during infections. IL-12 can facilitate the development of T-helper type 1 (Th1) lymphocytes required for late protection of fungi. Negative relation of IL-12 during infections can play a key role to establish the chronic infection and protect against harmful excessive cellular immune response (Romani 2000). Studies on mice have suggested that mice with TNF/lymphotoxin (LT)-α and IL-6 deficiencies are highly susceptible to *C. albicans* infections. Resistance to primary and secondary infections cannot be impaired in the absence of IL-1b or IL-10, as occurs in IL-1-converting enzyme (ICE)- or IL-10-deficient mice respectively (Del Sero et al. 1999; Mencacci et al. 1998b). Resistance or susceptibility to infections can be correlate with the levels of *Candida* growth in target organs, and also with the type of Th cytokine production by specific CD41 T-lymphocytes. Lower production of IL-4 and IL-10 and higher production of IFN-γ and IL-2 can be observed in mice that resist primary and secondary infections such as ICE- and IL-10-deficient mice. In contrast, high-level production of IL-4 and IL-10 and low-level production of Th1 cytokines can be observed in TNF/lymphotoxin (LT)-α- and IL-6-deficient mice succumbing to primary infection, and in IL-12p40-, IFN-γR-, and IL-4-deficient mice succumbing to secondary infection (Mencacci et al. 1998c). These data demonstrate that susceptibility to primary and secondary *C. albicans* infections in cytokine-deficient mice results in failure to develop anticandidal, protective Th1 responses and in the occurrence of unprotective IL-4- and IL-10-producing Th2 cells. The production of some proinflammatory cytokines (TNF-α and IL-6) rather than others (IL-1b) can to be essential for the successful control of infection and the resulting protective Th1-dependent immunity. IL-12 production is required for the development of Th1-cell responses, which is maintained in the presence of physiological levels of IL-4 and IL-10 (Mencacci et al. 1998a,c). Thus, a regulated balance of directive cytokines, such as IL-4, IL-10, and IL-12, rather than the relative absence of opposing cytokines, appears to be required for optimal development and maintenance of Th1 reactivity in mice with candidiasis (Romani 2000).

16.3.2 Neutrophils

In candidiasis, the initial handling of fungal pathogen by cells of the innate immune system plays a major role in determining CD41 Th development. Indeed, qualitative or quantitative defects of antifungal effector and immunoregulatory functions of phagocytic cells result in the development of anticandidal Th2, rather than Th1, cell responses (Romani et al. 1997a). Regulation of the early fungal burden (Mencacci et al. 1996), cytokine production (Romani et al. 1997b), and expression

of costimulatory molecules (Mencacci et al. 1998a) are possible pathways through which the innate immune system may control CD41 Th development. Professional mononuclear phagocytes (Vasquez-Torres and Balish 1997) and phagocytes such as epithelial cells (Fidel and Sobel 1998) have been found to have an important role in primary and acquired Th1 reactivity to *C. albicans*. However, an important immunoregulatory role has recently been attributed to neutrophils. Neutrophils have the ability to produce directive cytokines such as IL-10 and IL-12. Most importantly, it has been found that IL-12 released in response to a low-virulence *Candida* strain that initiates Th1 development *in vivo*, but IL-10 released in response to a virulent strain (Romani et al. 1997b). Human neutrophils can also produce bioactive IL-12 in response to a mannoprotein fraction of *C. albicans*, which is capable of inducing Th1 cytokine expression in peripheral blood mononuclear cells (Cassone et al. 1997). Production of IL-12 by neutrophils occurred independently of TNF-α (Mencacci et al. 1998b) and IFN-γ (Cenci et al. 1998). It was impaired upon iron overload (Mencacci et al. 1997) but increased upon *in vitro* priming with IL-4 (Mencacci et al. 1998c) through upregulation of IL-4 receptor expression. Thus, the IL-12-promoting activity of IL-4 may account for its requirement in sustaining memory Th1-cell responses to the fungus (Mencacci et al. 1998c). Cytokine production by neutrophils has also been demonstrated *in vivo* in infected mice; Th1-mediated resistance is increased upon IL-12 administration in neutropenic mice or IL-10 neutralization in non-neutropenic mice. Because of the large number of neutrophils present in the blood or inflammatory tissues in infection (Romani et al. 1997c). Neutrophil production of cytokines can influence the development and maintenance of the Th cell in response to *C. albicans*. It has recently been shown that neutrophils can quickly release *Candida* antigens upon phagocytosis (Ashley et al. 1997). Thus, it is likely that the immunoregulatory role of neutrophils in candidiasis may go beyond its cytokine production, to include signaling through antigen presentation and costimulation. Human studies have shown the multiple and complex role of neutrophils in candidiasis. First, risk factors for IFIs are not the same in all neutropenic patients (Walsh et al. 1994). Secondly, chronic systemic candidiasis initiated by neutropenia may persist against normal neutrophil counts and adequate antifungal therapy (Bodey and Anaissie 1989). Thirdly, some patients, particularly transplant recipients who have adequate or even normal neutrophil counts, may be at high risk for invasive mycoses (Bow et al. 1995; La Rocco and Burgert 1997; Romani 2000).

16.3.3 Dendritic Cells

The recognition that the dendritic cells (DC) are uniquely able to initiate responses in Naïve T cells and that they also participate in Th-cell response (Benchereau and Steinman 1998) and prompted investigators to investigate whether DC interact with *C. albicans*, in its different forms, and to find out the possible mechanisms and importance of this interaction. The fungus behaves as a commensal and true

pathogen of skin and mucosal surfaces, which are known to be highly enriched with DC. For adaptive immune responses against fungi, it has been found necessary that DC be phagocytic at some stage in their life cycle (Romani 1997). The advantage of an immature myeloid DC cell line established from fetal mouse skin is to stimulate T cells *in vitro* and *in vivo* upon cytokine treatment (Girolomoni et al. 1995; Lutz et al. 1996). Devoid of contaminating cells, DC ingest yeasts and hyphae of the fungus through different phagocytic mechanisms. Engulfment of yeasts can occur via coiling or overlapping phagocytosis, eventually leading to phagolysosome formation, where different stages of progressive yeast degradation can be seen. In contrast, internalization of hyphae has been found to occur through a more conventional zipper-type phagocytosis. After phagocytosis of yeasts or hyphae, the downstream cellular events are clearly different. Ingestion of yeasts, but not hyphae, can activate DC for IL-12 and nitric oxide production. *In vivo*, generation of antifungal protective immunity can be observed upon injection of DC ex vivo-pulsed with *C. albicans* yeasts but not hyphae (d'Ostiani et al. 2000). These results indicate that dendritic cells fulfill the requirement of a cell uniquely capable of sensing the two forms of *C. albicans* in terms of the type of immune response elicited. This accumulated evidence points to the unique role of dendritic cells in infections, because they are regarded as a protector of innate recognition and an initiator of Th-cell differentiation and functional commitment (Reis e Sousa et al. 1999). In candidiasis, this behavior requires that DC be exquisitely sensitive to the different forms of the fungus (Kopp and Medzhitov 1999), considering that DC are phagocytos to *C. albicans* and can activate T-cell responses to the fungus (Romani 2000). Thus, these findings provide important and novel insights into the general mechanisms of immunoregulation in fungal infections. Moreover, as the morphogenesis of *C. albicans* is activated *in vivo* by a wide range of signals (Brown and Gow 1999), DC may also act as key regulators of Th reactivity in saprophytism (Romani 2000).

16.4 Role of Cytokines in Treating IFIs

16.4.1 Immunomodulatory Role of Cytokine

Antifungal agents, selective towards ergosterol, can modulate the function (polyenes) or biosynthesis (azoles) of mammalian sterols, and also the host immune response. Azoles have been found to inhibit chemotaxis and superoxide production by polymorphonuclear leukocytes (PMNLs) (Ray and Anand 2000). It has been observed that azoles can sensitise fungal cells to oxidative metabolites produced by phagocytes by inhibiting P-450_{DM} (Georgopapadakou and Walsh 1996). Augmentation of the host defense response, improvement of the underlying disease, and resolution of the principal immune impairment are paramount for successful treatment of invasive mycoses in immunocompromised patients. Systemic mycoses are associated with high morbidity and mortality rates, despite advances in antifungal

chemotherapy (Ray and Anand 2000). Immunomodulatory strategies can be applied in two different modes:

1. *Prevention*: In this mode, a specific patient population at risk for an IFI receives an immunomodulator, such as a vaccine.
2. *Therapy of established disease*: In this therapy, an immunomodulator is used to treat invasive fungal disease. The immunomodulator is used as adjunctive therapy in combination with an antifungal agent.

Immunomodulatory strategies that target innate and antigen-specific immunity are described as follows (Walsh and Giri 1997):

1. To increase the number of neutrophils in recombinant hematopoietic human cytokines (RHHCs) such as CSFs (G-CSF and GM-CSF), granulocyte transfusions, myeloid progenitors (common myeloid progenitors, granulocyte–monocyte progenitors), and thymosin-α1 are useful.
2. To activate of neutrophils RHHCs such as CSFs (G-CSF and GM-CSF), IFN-γ, chemokines, TLR activation can be essential.
3. For activation macrophages and dendritic cells, RHHCs such as colony-stimulating factors (macrophage colony-stimulating factor and GM-CSF), IFN-γ, and TRL activation are essential
4. To heighten cellular immunity, RHHCs such as IFN-γ, TLR activation, pentraxin 3, thymosin-α1, and vaccines are required.
5. To heighten humoral immunity, RHHCs such as vaccines, antibody administration (e.g., monoclonal antibody 18B7 for *Cryptococcus neoformans*) are useful for augmenting the immune response to fungal infection.

Though several immunomodulatory strategies exist which target innate and antigen-specific immunity, evidence of efficacy in well-designed clinical trials is sparse (Walsh and Giri 1997).

16.4.2 Therapeutic Role of Cytokines

The innate host defense against fungal diseases is based on the action of phagocytic cells such as PMNLs and macrophages (Rodriguez-Adrian et al. 1998). The colony-stimulating factors (CSFs) can regulate both the number and the function of these cells. On the other hand, acquired defense involves cellular and humoral immunity that requires interactions between antigen-presenting cells, T-lymphocytes, B-lymphocytes, and NK cells which can be driven and regulated by cytokines such as IL-2 and IFN-γ. The potential role of immune activation via cytokines in the host defense against opportunistic fungi is the subject of several studies and has raised some intriguing questions about novel antifungal strategies for *Candida* and *Aspergillus* infections (Rodriguez-Adrian et al. 1998). Different potential roles of cytokines have been described. First, exposure to fungi and their antigens may include release of IL-2, INF-γ, TNF-α, and also some CSFs such as granulocyte

colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) (Rodriguez-Adrian et al. 1998). These cytokines can in turn activate or enhance the antifungal function of phagocytes against *Candida* and *Aspergillus* species (Rodriguez-Adrian et al. 1998). Exogenous administration of some cytokines is now possible, thus permitting therapeutic bypass needed for direct host response. Cytokines can play an indirect role, via activation of a leukocyte, rather than by any direct action on the fungus; they can be most effective if adequate numbers of circulating leukocytes are present. Cytokines can be used to ensure adequate numbers of leukocytes by speeding recovery of the patient's bone marrow function as well as by permitting novel approaches to transfusion therapy (Rodriguez-Adrian et al. 1998).

16.4.2.1 IFN-γ

INF-γ, a polypeptide secreted by T-lymphocytes, NK cells, alveolar macrophages, and fibroblasts, is the main cytokine produced during a Th1-type response. IFN-γ is approved for administration to humans and also for enhancing the oxidative metabolism and antifungal activity of human macrophages and PMNLs *in vitro* against a broad range of relevant fungal pathogens: *Candida* species, *Aspergillus fumigatus*, *C. neoformans*, *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis* (Rodriguez-Adrian et al. 1998). IFN-γ activates antigen-presenting cells, stimulates proliferation of T- and B-lymphocytes, and enhances cytotoxicity by T-lymphocytes and NK cells. It can be beneficial as an *in vivo* adjunct when given either prophylactically or after establishment of experimental infections with *A. fumigatus* and *C. neoformans* (Rodriguez-Adrian et al. 1998). IFN-γ can promote TNF-α production and can also enhance PMNLs and mononuclear cell-induced damage by increasing the oxidative burst of PMNLs in response to stimuli such as nonopsonized hyphae of *A. fumigatus*. Both IFN-γ and G-CSF can enhance the oxidative bursts and fungicidal activity *in vitro* of human PMNLs against *A. fumigatus* hyphae, with the combination of the two cytokines showing an additive effect. IFN-γ can also restore the corticosteroid-suppressed fungicidal activity of human PMNL and elutriated monocytes. IFN-γ-treated human monocytes can enhance oxygen radical production and damage to *A. fumigatus* hyphae (Steinbach and Stevens 2003). Exogenous administration of IFN-γ and TNF-α has resulted in protective effects in a murine model of IA by decreasing mortality and the number of organs affected by *Aspergillus*. Conversely, IFN-γ and TNF-α neutralization resulted in increased disease and increased expression of IL-10 (Steinbach and Stevens 2003). Although IFN-γ is better than G-CSF or GM-CSF at enhancing PMNL hyphal damage, and both IFN-γ and GM-CSF result in enhanced hyphal damage by PMNLs *in vitro*, combination treatment does not increase damage. One recent case report details use of liposomal amphotericin B and both GM-CSF and IFN-γ in successful

treatment of sinocerebral aspergillosis, with the addition of the IFN-γ temporally related to clinical resolution (Steinbach and Stevens 2003).

16.4.2.2 The CSFs

Three CSFs relevant to phagocytes have been approved for administration to humans: G-CSF, GM-CSF, and M-CSF (Rodriguez-Adrian et al. 1998). CSFs are responsible not only for the replication of bone marrow stem cells but also for the differentiation of granulocytes and monocytes into their mature forms. Cellular sources of these powerful cytokines include monocytes, T-lymphocytes, fibroblasts, and endothelial cells (Rodriguez-Adrian et al. 1998). CSFs enhance chemotaxis, phagocytosis, and killing activity of precursor and mature effector cells (Rodriguez-Adrian et al. 1998). All three CSFs have been observed in multiple studies to enhance the activity of phagocytic cells against *Candida* species, *A. fumigatus*, *C. neoformans*, and *Histoplasma capsulatum* (Rodriguez-Adrian et al. 1998). Despite the several similarities among these cytokines, the CSFs also have some distinctive features, as described below:

Granulocyte Colony-Stimulating Factor

Human recombinant G-CSF has been accepted for clinical use since 1991 (Welte et al. 1996). The potential of exogenously administered G-CSF therapy seems to maintain the innate signal for longer production of PMNLs, or to initiate that signal earlier if endogenous production is decreased or is insufficient during a specific time, such as during neutropenia after BMT (Hartung 1998). One fear is the unwanted side-effect of increased inflammatory products, such as the problematic release of reactive oxygen species and lysosomal contents, with G-CSF use (Hartung 1999). However, *in vivo* and human studies have shown that G-CSF can reduce the production of inflammatory mediators such as IL-1, TNF-α, and IFN-γ (Hartung 1998). In addition to increasing the number of mature circulating PMNLs, G-CSF can enhance phagocytic activity and oxidative burst metabolism. Human G-CSF affects the function of granulocytes only, but not macrophages, and also has a protective effect in murine models of IA. Prophylaxis with human G-CSF and amphotericin B or itraconazole has shown some additive effect in neutropenic animal models of IA but not in those immunosuppressed with cortisone, which has a greater effect against macrophages. In a neutropenic (cyclophosphamide-induced) murine model, human G-CSF alone was found ineffective, but with amphotericin B it showed synergy in survival greater than with itraconazole and G-CSF (Polak-Wyss 1991). Pretreatment of neutrophils with G-CSF and/or IFN-γ can attenuate the inhibitory effect of corticosteroids on PMNL-induced hyphal damage (Roilides et al. 1993). G-CSF administered to human volunteers can increase the fungicidal activity through enhanced respiratory bursts of their PMNLs against *Aspergillus* conidia of their PMNLs by

fourfold (Liles et al. 1997). However, no clear evidence has been reported that G-CSF benefits patients with aspergillosis (Steinbach and Stevens 2003).

Granulocyte–Macrophage Colony-Stimulating Factor

This factor almost doubles the life span of neutrophils, exerts a stimulatory effect upon them, and enhances their attachment to endothelial cells and epithelial cell membranes (Rodriguez-Adrian et al. 1998). It also extends the lifespan and enhances the antibody-dependent cytotoxicity of eosinophils (Lopez et al. 1986; Rodriguez-Adrian et al. 1998). GM-CSF can promote differentiation, proliferation, and activation of cells in the macrophage/ monocyte system, prevents the defective *in vitro* antifungal activity of corticosteroid-treated monocytes (Roilides et al. 1996; Steinbach and Stevens 2003), and also enhances the phagocytic activity of PMNLs (Rodriguez-Adrian et al. 1998; Steinbach and Stevens 2003). GM-CSF has been found to increase spleen cellularity in mice, indicating potent stimulation of hematopoietic cells, and increased IFN-α production by concanavalin-A compared with control cells (Khatami et al. 2001). In a study of a murine model, the antifungal activity of bronchoalveolar macrophages treated with dexamethasone was observed to be less than that of macrophages from dexamethasone plus GM-CSF-treated mice. It has also been demonstrated that GM-CSF administered before dexamethasone blocked the deleterious effects but, if given after dexamethasone, GM-CSF could not reverse the outcome on macrophages (Brummer et al. 2001). In a further study, both murine and human GM-CSF were shown to counteract dexamethasone suppression of murine macrophage function (Brummer et al. 2002). GM-CSF can act synergistically with TNF-α (Bodey 1994). GM-CSF has been revealed to offer some protection against IA in a clinical trial of patients with acute myelogenous leukemia, decreasing the fungal infection-related mortality from 19% to 20% (Rowe et al. 1995). One study of GM-CSF in combination with amphotericin B (1 mg/kg/day) for treatment of proven fungal infection included two patients with refractory aspergillosis. One patient with pulmonary IA who underwent BMT because of breast cancer showed a partial response, whereas the other patient, with acute myelogenous leukemia and sinopulmonary IA, had treatment failure (Bodey et al. 1993). In another case, a child with IA received GM-CSF to overcome neutropenia and, after bone marrow recovery, developed pulmonary cavitation and fatal hemoptysis (Groll et al. 1996). Bone marrow recovery may lead to liquefaction of pulmonary foci and to potential erosive bleeding resulting from an increased inflammatory response, especially in the first week following cavitation (Albelda et al. 1985; Steinbach and Stevens 2003). Thus, GM-CSF would have a theoretical advantage in infections where monocyte–macrophage function is critical, although formal proof is lacking (Rodriguez-Adrian et al. 1998).

Macrophage Colony-Stimulating Factor

M-CSF can modulate mononuclear phagocyte functions such as H_2O_2 production and phagocytosis, and enhances production of IL-1, IFN-γ, and TNF-α (Roilides et al. 1995, 1998a). M-CSF has been found to enhance the nonoxidative mechanism of macrophages to inhibit germination, but while it can draft more macrophages to ingest conidia, it cannot be found to effect ingestion of more conidia (Roilides et al. 1995). A neutropenic rabbit model demonstrated that prophylactic administration occurred 3 days before inoculation, and then neutropenia augmented the pulmonary host defense against IA. Rabbits receiving M-CSF have been found to have increased survival rates and greater numbers of activated pulmonary alveolar macrophages than as observed in control animals (Gonzalez et al. 2001). A phase I trial of M-CSF has suggested some benefit in patients with *Aspergillus* infections, but an insufficient number of patients have been treated to show a statistical benefit (Nemunaitis et al. 1991; Steinbach and Stevens 2003).

16.4.2.3 TNF-α

TNF-α is a pro-inflammatory cytokine secreted by various macrophage populations and is demonstrated as a critical initiator in innate immunity against respiratory pathogens including *A. fumigatus* (Mehrad et al. 1999; Steinbach and Stevens 2003). *In vitro* TNF-α has shown an increase in early host defense against *Aspergillus* invasion, with a slight increase in oxygen radical production by macrophages, upregulation and activation of alveolar macrophage phagocytosis, and also enhanced production of other cytokines such as GM-CSF. By producing oxygen radicals, it can increase a late defense with increased PMNL hyphal damage (Roilides et al. 1998a, b). *In vitro* GM-CSF and TNF-α administration has been observed to hinder dexamethasone-induced immunodeficiency (Steinbach and Stevens 2003). The mechanism by which TNF-α inhibition can augment susceptibility to IFIs includes inhibition of IFN-γ production, decreased expression of pattern-recognition receptors, and leukocyte apoptosis (van der Meer et al. 2005). After revelation to a fungal antigen, naïve T cells have been found to differentiate into distinct helper T-cell subsets (Th1 and Th2), which can generate signature cytokines to determine the nature of the effector response. Consequently, the Th1-cell response can be defined by production of the cytokine IFN-γ, as well as IL-2 and lymphotoxin, and stimulates cell-mediated effector responses and IgG2a production, while Th2 cells have been known to produce IL-4, IL-5, IL-9, and IL-13 and to promote mastocytosis, eosinophilia, and production of IgE and IgG1 (Tsiodras et al. 2008). IFN-γ is crucial for proper activation of phagocytosis and killing of intracellular pathogens, and its production is stimulated by TNF-α (Djeu et al. 1986; Bekker et al. 2001). Hence, TNF-α blockade leads to decreased IFN-γ levels and subsequently to a defective cellular immune response. Both TNF-α and IFN-γ potentiate the expression of toll-like receptor 4 (TLR-4) on the cell

membrane (Bosisio et al. 2002). TLR-4 expression is essential for recognition of such fungal pathogens as *C. albicans* and *A. fumigatus* by host cells such as dendritic cells and macrophages (Tsiodras et al. 2008). Patients receiving anti-TNF-α therapy could be at augmented risk for IFIs because of an inability to recognize fungal antigens through toll-like receptor signaling. TNF-α blockade has been found to reduce TLR-4 expression on dendritic cells from patients with rheumatoid arthritis and from healthy control participants (Netea et al. 2003). TNF-α is essential for granuloma formation and maintenance, and to defend the host against intracellular pathogens such as several fungi (Allendoerfer and Deepe 2000). TNF-α blockers are associated with increased apoptosis of peripheral blood monocytes which can be observed shortly after introduction of such agents as infliximab (Lügering et al. 2001). Regulation of monocyte chemokine and matrix metalloproteinase secretion is partially mediated by TNF-α and IL-1 in granulomatous mycobacterial diseases (Wright and Friedland 2004). Failure to classify mycobacteria in granulomas results in disseminated disease and poor outcome and could be operative in IFIs associated with TNF-α blockade (Tsiodras et al. 2008).

16.4.2.4 RANTES

RANTES (regulated on activation, normal T cell expressed and secreted), has been found to have a crucial role in trafficking and activating leukocytes toward sites of infection and inflammation (Schall 1991; Sozzani et al. 1997). RANTES adheres to inflamed vascular endothelium, causing accumulation of leukocytes (von Hundelshausen et al. 2001). Nonhematological patients dying with severe sepsis have lower circulating RANTES concentrations in comparison to survivors, suggesting a protective role for it (Cavaillon et al. 2003). No information has been observed in patients with hematological malignancy. Platelets are detected as a major source of RANTES. These patient groups are characterized by profound thrombocytopenia and contain higher risk for IFI morbidity and mortality. Research has been conducted into alternatives to RANTES in those patients with IFIs (Ellis et al. 2005).

16.5 Which Patients Are Likely to Respond to Cytokine Therapy?

Despite the abundance of preclinical *in vitro* and *in vivo* data on the role of cytokines in the host response against IFIs, current evidence from their use in the treatment of fungal infections in clinical practice is limited, involving mainly the hematopoietic growth factors (HGFs) and IFN-γ. Both G-CSF and GM-CSF have been approved by the US Food and Drug Administration (FDA) in 1991. Current indications of their use include the reduction of duration of neutropenia

and neutropenic fever in AML patients receiving induction therapy and, in HSCT recipients, the mobilization of hematopoietic progenitors into peripheral blood for stem cell harvest, as well as the reduction of neutropenic complications in patients with severe chronic neutropenia (Komrokji and Lyman 2004). In contrast to G-CSF and GM-CSF, which are widely used worldwide, clinical experience with M-CSF remains limited (Hubel et al. 2002). G-CSF and GM-CSF are available as recombinant human products. Depending on the vector used for the recombinant DNA production (*Escherichia coli*, yeast, Chinese hamster ovary cells), G-CSF is available in two preparations, filgrastim and lenograstim, while GM-CSF is available as sargramostim, molgramostim, and regramostim. They may be administered subcutaneously or intravenously, with the former route providing prolonged delivery. They are metabolized by binding to their receptors, with subsequent internalization, by liver enzymes and by renal clearance (Komrokji and Lyman 2004). Recently a pegylated, long-acting form of G-CSF (pegfilgrastim) has become available. Pegylation of filgrastim results in a large-size molecule that prevents renal clearance. The elimination of the drug is thus predominantly through neutrophil-mediated clearance, with a self-regulating mechanism allowing stimulation of neutrophil production during neutropenia and rapid clearance as neutrophil counts recover (Komrokji and Lyman 2004). In oncology, HGFs have been used as primary and secondary prophylaxis and for treatment of neutropenia. Primary prophylaxis refers to their use before the onset of neutropenia, during the first cycle of chemotherapy. Secondary prophylaxis implies the use of HGFs after the occurrence of severe or febrile neutropenia in a previous chemotherapy cycle where no HGFs were used. Treatment of neutropenia implies the use of HGFs after establishment of neutropenia in order to shorten its duration, prevent infectious complications, and reduce associated morbidity and mortality (Komrokji and Lyman 2004). Both G-CSF and GM-CSF have been shown to be effective in reducing the incidence and duration of neutropenia associated with malignancy or hematological disorders (Hubel et al. 2002, Komrokji and Lyman 2004). Despite the documented efficacy of HGFs in enhancing neutrophil recovery, their routine use as prophylaxis or treatment for neutropenia is not advocated in the guidelines of the American Society of Clinical Oncology (ASCO), which separately address the use of these cytokines in certain groups of hematological patients [acute lymphoblastic leukemia (AML), myelodysplastic syndrome and HSCT patients] (Ozer et al. 2000). The ASCO guidelines, updated in 2000, however, suggest that the use of HGFs in patients with febrile neutropenia should be considered in the presence of certain risk factors, including profound neutropenia, uncontrolled primary disease, pneumonia, hypotension, multi-organ dysfunction (sepsis syndrome), and IFIs (Ozer et al. 2000). One study conducted by the Eastern Cooperative Oncology Group has suggested that the mortality in eight acute lymphoblastic leukemia (AML) patients aged >55 years with documented IFIs, mainly aspergillosis and candidiasis, who received GM-CSF, was significantly lower than the mortality of 12 patients of the placebo group (Rowe 1998). In a prospective comparative study of empiric amphotericin B versus amphotericin B with G-CSF in 59 neutropenic adults with hematological malignancies and antimicrobial-refractory fever or clinical evidence of IFIs, patients

who received adjunctive treatment with G-CSF had a significantly higher response rate (Hazel et al. 1999). In a small noncomparative study, administration of GM-CSF was found to associate with a complete or partial response in six of eight neutropenic patients with documented IFIs (Bodey et al. 1994). The survival of bone marrow transplant patients with invasive candidiasis and a Karnofsky score >20% who received recombinant human M-CSF as adjunctive therapy was significantly higher than that of historical controls (Nemunaitis et al. 1993). In a noncomparative pilot study of AIDS patients with fluconazole-refractory oropharyngeal candidiasis, adjunctive treatment with GM-CSF (sargramostim) appeared to exert a beneficial effect (Vazquez et al. 2000). There have been a number of case reports on the use of G-CSF or GM-CSF as adjunctive treatment for IFIs (Abu Jawdeh et al. 2000; Ma et al. 2001). Compared with studies of HGF use as adjunctive therapy, systematic evaluation of their efficacy as prophylaxis against IFIs has been even more limited. In a retrospective study of GM-CSF use among 141 autologous bone marrow transplant recipients, a trend towards fewer fungal infections and significantly fewer days of amphotericin B treatment was observed in the GM-CSF-treated group compared with control patients (Nemunaitis et al. 1998). A role for GM-CSF in the prevention of IFIs was suggested by another retrospective study of patients receiving high-dose chemotherapy with or without autologous HSCT (Peters et al. 1996). In a randomized trial, 119 patients with hematological malignancies and febrile neutropenia received either antibiotics alone or antibiotics and G-CSF. Although only four IFIs occurred, they were all encountered in the group receiving antibiotics alone (Avilés et al. 1996). Prophylactic administration of G-CSF (filgrastim) in AML patients has been observed to associate with a reduction in the requirement for systemic antifungal therapy and with a nonsignificant reduction of mortality related to fungal infections (Heil et al. 1997). In another study of G-CSF (lenograstim) administration in elderly patients with AML, IFIs occurred in four patients from the lenograstim group but in no patients from the placebo group (Dombret et al. 1995). Apart from their direct administration in patients suffering from or at risk for IFIs, an additional role for HGFs has recently been developed in the field of granulocyte transfusions. Granulocyte transfusion therapy is another approach which has long been considered for the prevention or treatment of infectious diseases in severely neutropenic patients. The efficacy of this strategy in early studies, however, was compromised by limitations in collecting adequate doses of leucocytes from healthy steroid-mobilized donors. The use of G-CSF has markedly enhanced the yield of leucocytes from healthy donors, thus renewing interest in granulocyte transfusions. GM-CSF treatment has been associated with a greater frequency of side-effects, compared with G-CSF, presumably as a consequence of stimulation by GM-CSF of the Th1 cytokine responses (Khatami et al. 2001). Higher toxicity can be seen with GM-CSF prepared in *E. coli* as compared with yeast. Several studies have addressed theoretical concerns about the potential stimulation of leukaemic cells by HGFs. Currently there is no evidence of stimulation of leukemia growth or enhanced drug resistance associated with the clinical use of HGFs (Ozer et al. 2000). Careful monitoring for the development of myelodysplastic syndrome or AML is advised for patients with severe chronic neutropenia receiving

long-term G-CSF therapy, although it is still uncertain whether G-CSF contributes to the malignant transformation in these patients (Dale et al. 2003). Recombinant IFN-γ administered subcutaneously has been used clinically as prophylaxis or adjunctive treatment for both bacterial and fungal infections, although evidence for its efficacy against IFIs is again limited. In 1990, the FDA approved IFN-γ for the prevention of infectious complications in patients with chronic granulomatous disease (CGD). Indeed, in a randomized, double-blind, placebo-controlled trial, prophylactic administration of IFN-γ in CGD patients was associated with a significant reduction in the incidence of serious infections. A trend towards reduced incidence of *Aspergillus pneumonia* has been observed in the patients who received IFN-γ (Antachopoulos and Roilides 2005). In another study involving patients with advanced HIV disease, prophylactic administration of IFN-γ was associated with a trend towards decreased incidence of Candida and other opportunistic infections (Riddell et al. 2001). A recent phase 2, double-blind, placebo-controlled trial evaluated the efficacy of IFN-γ as adjunctive therapy for AIDS-related acute cryptococcal meningitis (Pappas et al. 2004). Although the number of patients in this pilot study was too small to demonstrate a statistically significant difference in the primary efficacy endpoint [clearance of *C. neoformans* from cerebrospinal fluid (CSF) at 2 weeks], the results showed a trend towards more rapid sterilization of CSF and improved combined clinical and mycological response in IFN-γ recipients (Pappas et al. 2004). Several case studies have also been published on the use of IFN-γ as adjunctive treatment in immunocompromised patients with hepatosplenic or refractory oropharyngeal candidiasis, cryptococcal meningitis, aspergillosis, scedosporiosis, and zygomycosis (Antachopoulos and Roilides 2005). The use of IFN-γ is generally well tolerated, with adverse events occurring more frequently with higher doses (Pappas et al. 2004). In a series of 32 HSCT recipients, IFN-γ was tolerated without serious adverse reactions (Safdar et al. 2005). The safety of use of IFN-γ over a prolonged period of prophylaxis was recently demonstrated in a large series of CGD patients (Marciano et al. 2004). Apart from the data on HGFs and IFN-γ, there is a paucity of clinical data on the safety and efficacy of immunomodulatory therapy for most of the other cytokines involved in the host innate and adaptive responses against IFIs. Two phase I trials have evaluated single-dose and multiple-dose regimens of recombinant human IL-12 administration in HIV patients (Jacobson et al. 2002). Administration of IL-12 was generally safe and the maximum tolerated dose appeared to be related to the patient's $CD4^+$ T cell count. Adverse effects were usually mild, transient and reversible, although a dose-dependent increase in their frequency and severity could be demonstrated. In the single-dose study, IL-12 administration induced significant increases in serum IFN-γ concentrations and in the absolute numbers of peripheral NK cells and $CD8^+$ T cells (Jacobson et al. 2000). Interestingly, however, in the multiple-dose study no increase in serum IFN-γ levels or in the absolute numbers of NK, $CD8^+$ or $CD4^+$ cells was observed (Jacobson et al. 2002). While the administration of cytokines may play a role in the treatment or prevention of IFI, measurement of their levels could theoretically have prognostic value in patients at risk or suffering from fungal infections (Roilides et al. 2001). In a small

series of non-neutropenic patients with IA, low or decreasing IL-10 serum concentrations were associated with a favorable outcome, while increasing IL-10 was found in patients with disease progression (Roilides et al. 2001). However, a systematic evaluation of serial cytokine measurements as a prognostic/therapeutic assessment tool in patients with IFIs has not yet been reported (Antachopoulos and Roilides 2005).

16.6 Mechanisms for Synergy Between Antifungal Therapy and Immune Responses

Antifungal drug therapy and the host immune response are the principal determinants of the outcome of fungal infections (Stevens 2008). Newly available antifungal agents are the echinocandins. They act by inhibiting fungal glucan synthase interfering with production of β-1-3-glucan synthase, an essential enzyme located on the growing filaments (i.e., hyphae) of *A. fumigatus* (Stevens 2008; Hohl et al. 2008; Lamaris et al. 2008). β-1-3-Glucan reveals potent immunostimulatory properties arbitrated by the innate immune receptor Dectin-1. Dectin-1 (the mammalian receptor) binds to β-1-3-glucan from *Pneumocystis carinii*, *C. albicans*, *A. fumigatus*, and *Coccidioides posadasii*, and activates fungicidal responses that include phagocytosis, release of inflammatory mediators, and generation of reactive oxygen intermediates (Hohl et al. 2008; Lamaris et al. 2008).

Glucans have been recognized as immune stimulators, although, as a caution, it should be noted that they can be immunosuppressive (Stevens 2008). Recent studies with macrophages and with neutrophils have demonstrated that caspofungin-treated *Aspergillus* hyphae stimulate effector cells *in vitro* to produce such inflammatory mediators as tumor necrosis factor (TNF) and CXCL2. *In vivo*, this result could have immunostimulatory and anti-infective effects at the site of infection (Hohl et al. 2008; Lamaris et al. 2008). Echinocandin-treated conidia (the infectious form of the fungus) and germlings (the initial step in the formation of the invasive hyphae) induce reduced secretion of TNF and CXCL2 in murine macrophages, simultaneously with diminished β-glucan surface exposure, in contrast to the hyphal effects (Hohl et al. 2008; Stevens 2008). β-Glucan is responsible for the enhanced cytokine secretion stimulated by hyphae. It was found that secretion reduces as the hyphae grow in the absence of the drug, but that it increases over time if the hyphae are treated with the drug. Cytokine effect is not related to a direct effect of the drug on macrophages. The drug effect is reduced when the amount of drug present is greater than the growth-inhibitory concentration, which may relate to altered wall remodeling (Stevens 2008). The increase in inflammatory response induced by drug-treated hyphae occurs despite the inhibition of drug-induced fungal growth (Stevens 2008).

Recent studies have provided evidence that the phagocytes are arbitrated either (1) through the mammalian cell receptor for β-glucan, Dectin-1, by neutrophil

expression studies demonstrating upregulation of Dectin-1 mRNA after exposure to drug-treated *Aspergillus* species (not with *Scedosporium prolificans*), or (2) directly by blocking the effect on cytokines with treatment of macrophages with anti-Dectin-1. Activation of Dectin-1 would presumably lead to enhanced cytokine production via such triggering transcription factors as nuclear factor kB and activator protein-1 (Stevens 2008).

It is unclear what the cytokines are doing to result in fungal damage, but it is possible that:

1. The drugs alter the fungi in ways that make the fungi more susceptible to toxic oxygen or nitrogen products produced by the phagocytes.
2. The damage to altered fungi could occur even without an upregulation mediated by the cytokines (Stevens 2008).

If increased cytokine production is viewed as an anti-infective asset, then the reduction in cytokines that occurs when conidia and germlings are treated with caspofungin might suggest a deleterious drug effect in the early stages of infection (Hohl et al. 2008; Stevens 2008).

Recent studies reported that *Aspergillus* species and caspofungin may act cooperatively to upregulate expression of a toll-like receptor, and that caspofungin and other cytokines, such as GM-CSF, may act cooperatively on effector cell function (Stevens 2008). Antifungals can act on phagocyte oxidative burst either directly or mediated through cytokines or by effector cell priming in the following ways: (1) enhancement of drug penetration into phagocytes by cytokine stimulation, (2) reversal by cytokines of depression of phagocyte function by antifungals, (3) reversal by antifungals of immune depression caused by fungal products, (4) cooperation of antifungals with proinflammatory cytokines to polarize the host response toward a Th1 path, (5) alteration of fungal cell membranes by antifungals whose actions affect the membrane (resulting in increased susceptibility to toxic oxygen or nitrogen products), or (6) cooperation of antifungals with innate mammalian constituents, such as chitinase or collectins (Stevens et al. 2000; Rex and Stevens 2005). For further studies kindly refer to Stevens (2008), Hohl et al. (2008) and Lamaris et al. (2008).

16.7 Conclusions and Future Prospects

Unmet needs for improved diagnosis, treatment, and prevention of fungal infections remain formidable. New antifungal drugs and combinations of existing agents should be explored further. Several challenges exist if promising immune-based therapies developed in the laboratories are to be brought to clinical trial. Experimental immunology has provided significant regulatory role for cytokines in both innate and adaptive immune responses to fungal pathogens. The identification of cytokines with the ability to enhance antifungal activities of immune effector cells is significant, since they may have a therapeutic role in the treatment of IFI in the

severely immunocompromised host. The effect of T-lymphocyte (Th1 and Th2) responses on the outcome of fungal infections has been described. Most cytokines have been demonstrated to participate in either Th1 or Th2 responses. Cytokines, such as G-CSF, GM-CSF, M-CSF, IFN-γ and TNF-α, which show promise as adjuvant therapy for patients with proven fungal infections and which were previously known to affect innate immunity, have been demonstrated to be involved in the differential development of adaptive responses. The safety and efficacy of the clinical use of cytokines in the treatment or prevention of IFIs are limited mainly to HGFs and IFN-γ. Even these cytokines are lacking in appropriately designed clinical trials to explore their efficacy.

Future research into the role of cytokines in fungal infections should include an even more comprehensive understanding of the mechanisms of action, pleiotropic effects, and interactions of the cytokines involved, as there is still much to learn. Effective ways for *in vivo* augmentation of antifungal effector cell activity and protective Th1 responses, taking into account the timing of immunomodulatory intervention during the course of infection, should be further investigated. The upregulation of Th1 adaptive immunity, either through administration of Th1 cytokines or via neutralization of Th2 cytokines, should aspire at controlling fungal infections without the undesirable physiological and pathological effects of an overwhelming immune response. The enhancement of clinical efficacy of cytokine use in the prevention or treatment of IFIs is going to be challenging. Cytokines such as the HGFs and IFN-γ have undergone phase 1 or 2 studies. The ideal approach would be through adequately powered prospective, randomized, double-blind and controlled trials where the choice and dosing of cytokine, the timing of intervention, the study population, and endpoints have been selected based on the existing preclinical and clinical evidence. However, clinical studies involving fungal infections face important difficulties, such as the problematic diagnosis and documentation of IFIs, the low accrual of patients, and the evaluation of response to treatment. Clinical trials of cytokine used as an adjunct to conventional antifungal agents for the treatment of IFIs should be designed to show superiority, rather than equivalence, of the combined therapeutic regimen, since it would not make sense to add a compound with possible adverse effects and substantial cost in order to achieve an equivalent outcome. It has been documented that the cytokine administered may modulate the patient's immune response in an unpredictable or even deleterious way and the study design should allow for detection and management of such unwanted effects. Future clinical investigation, by understanding the basic molecular, metabolic, and immunological properties of fungal organisms, could cover studies of pre-emptive administration of cytokines and antifungal therapy in patients with presumed early diagnosed IFIs, based on polymerase chain reaction or antigen-detection assays, and evaluation studies of serial cytokine measurements as a therapeutic tool in patients with IFIs. Therefore, there is now a need for the application of high-throughput approaches for evaluating cytokine responses to IFIs.

References

Abu Jawdeh L, Haidar R, Bitar F, Mroueh S, Akel S, Nuwayri-Salti N, Dbaibo GS (2000) Aspergillus vertebral osteomyelitis in a child with a primary monocyte killing defect: response to GM-CSF therapy. J Infect 41:97–100

Albelda SM, Talbot GH, Gerson ST, Miller WT, Cassileth PA (1985) Pulmonary cavitation and massive hemoptysis in invasive pulmonary aspergillosis. Influence of bone marrow recovery in patients with acute leukemia. Am Rev Respir Dis 131:115–120

Allendoerfer R, Deepe GS Jr (2000) Regulation of infection with *Histoplasma capsulatum* by TNFR1 and -2. J Immunol 165:2657–2664

Antachopoulos C, Roilides E (2005) Cytokine and fungal infections. Br J Haematol 129:583–596

Ashley C, Morhart M, Rennie R, Ziola B (1997) Release of *Candida albicans* yeast antigens upon interaction with human neutrophils *in vitro*. J Med Microbiol 46:747–755

Ashman RB, Papadimitriou JM (1995) Production and function of cytokines in natural and acquired immunity to *Candida albicans* infection. Microbiol Rev 59:646–672

Avilés A, Guzmán R, García EL, Talavera A, Díaz-Maqueo JC (1996) Results of a randomized trial of granulocyte colony-stimulating factor in patients with infection and severe granulocytopenia. Anticancer Drugs 7:392–397

Bekker LG, Freeman S, Murray PJ, Ryffel B, Kaplan G (2001) TNF-α controls intracellular mycobacterial growth by both inducible nitric oxide synthase-dependent and inducible nitric oxide synthase-independent pathways. J Immunol 166:6728–6734

Benchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–252

Bodey GP (1994) The potential role of granulocyte–macrophage colony stimulating factor in therapy of fungal infections: a commentary. Eur J Clin Microbiol Infect Dis 13:363–366

Bodey GP, Anaissie EJ (1989) Chronic systemic candidiasis. Eur J Clin Microbiol Infect Dis 8:855–857

Bodey GP, Anaissie E, Gutterman J, Vadhan-Raj S (1993) Role of granulocyte macrophage colony-stimulating factor as adjuvant therapy for fungal infections in patients with cancer. Clin Infect Dis 17:705–707

Bodey GP, Anaissie E, Gutterman J, Vadhan-Raj S (1994) Role of granulocyte–macrophage colony-stimulating factor as adjuvant treatment in neutropenic patients with bacterial and fungal infection. Eur J Clin Microbiol Infect Dis 13:S18–S22

Bosisio D, Polentarutti N, Sironi M, Bernasconi S, Miyake K, Webb GR, Martin MU, Mantovani A, Muzio M (2002) Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-γ: a molecular basis for priming and synergism with bacterial lipopolysaccharide. Blood 99:3427–3431

Bow EJ, Loewen R, Cheang MS, Schacter B (1995) Invasive fungal disease in adults undergoing remission-induction therapy for acute myeloid leukemia: the pathogenetic role of the antileukemic regimen. Clin Infect Dis 21:361–369

Brown AJP, Gow NAR (1999) Regulatory networks controlling *Candida albicans* morphogenesis. Trends Microbiol 7:333–338

Brummer E, Maqbool A, Stevens DA (2001) *In vivo* GM-CSF prevents dexamethasone suppression of killing of *Aspergillus fumigatus* conidia by bronchoalveolar macrophages. J Leukoc Biol 70:868–872

Brummer E, Maqbool A, Stevens DA (2002) Protection of peritoneal macrophages by granulocyte/macrophage colony-stimulating factor (GMCSF) against dexamethasone suppression of killing of *Aspergillus,* and the effect of human GM-CSF. Microbes Infect 4:133–138

Cassone A, Chiani P, Quinti I, Torosantucci A (1997) A possible participation of polymorphonuclear cells stimulated by microbial immunomodulators in cytokine dysregulated patterns of AIDS patients. J Leukoc Biol 62:60–66

Cavaillon JM, Adib-Conquy M, Fitting C, Adrie C, Payen D (2003) Cytokine cascade in sepsis. Scand J Infect Dis 35:535–544

Cenci E, Mencacci A, del Sero G, d'Ostiani CF, Mosci P, Bacci A, Montagnoli C, Kopf M, Romani L (1998) IFN-g is required for IL-12 responsiveness in mice with *Candida albicans* infection. J Immunol 161:3543–3550

Chen SCA, Sorrell TC (2007) Antifungal agents. Med J Aust 187:404–409

d'Ostiani CF, del Sero G, Bacci A, Montagnoli C, Spreca A, Mencacci A, Ricciardi-Castagnoli P, Romani L (2000) Dendritic cells discriminate between yeasts and hyphae of the fungus *Candida albicans*: implications for initiation of Th immunity *in vitro* and *in vivo*. J Exp Med 15:1661–1674

Dale DC, Cottle TE, Fier CJ, Bolyard AA, Bonilla MA, Boxer LA, Cham B, Freedman MH, Kannourakis G, Kinsey SE, Davis R, Scarlata D, Schwinzer B, Zeidler C, Welte K (2003) Severe chronic neutropenia: treatment and follow-up of patients in the Severe Chronic Neutropenia International Registry. Am J Hematol 72:82–93

del Sero G, Mencacci A, Cenci E, D'Ostiani CF, Montagnoli C, Bacci A, Mosci P, Kopf M, Romani L (1999) Antifungal T helper type 1 responses are upregulated in IL-10-deficient mice. Microbes Infect 1:1169–1180

Djeu JY, Blanchard DK, Halkias D, Friedman H (1986) Growth inhibition of *Candida albicans* by human polymorphonuclear neutrophils: activation by interferon-γ and tumor necrosis factor. J Immunol 137:2980–2984

Dombret H, Chastang C, Fenaux P, Reiffers J, Bordessoule D, Bouabdallah R, Mandelli F, Ferrant A, Auzanneau G, Tilly H, Yver A, Degos L, The AML Cooperative Study Group (1995) A controlled study of recombinant human granulocyte colony-stimulating factor in elderly patients after treatment for acute myelogenous leukemia. AML Cooperative Study Group. N Engl J Med 332:1678–1683

Ellis M, al-Ramadi B, Hedström U, Alizadeh H, Shammas V, Kristensen J (2005) Invasive fungal infections are associated with severe depletion of circulating RANTES. J Med Microbiol 54:1017–1022

Fidel PL, Sobel JD (1998) Protective immunity in experimental *Candida* vaginitis. Res Immunol 149:361–373

Georgopapadakou NH, Walsh TJ (1996) Antifungal agents: chemotherapeutic targets and immunologic strategies. Antimicrob Agents Chemother 40:270–291

Girolomoni G, Lutz MB, Pastore S, Assmann CU, Cavani A, Ricciardi-Castagnoli P (1995) Establishment of a cell line with features of early dendritic cell precursors from fetal mouse skin. Eur J Immunol 25:2163–2169

Gonzalez CE, Lyman CA, Lee S, del Guercio C, Roilides E, Bacher J, Gehrt A, Feuerstein E, Tsokos M, Walsh TJ (2001) Recombinant human macrophage colony-stimulating factor augments pulmonary host defences against *Aspergillus fumigatus*. Cytokine 15:87–95

Groll AH, Shah PM, Mentzel C, Schneider M, Just-Nuebling G, Huebner K (1996) Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. J Infect 33:23–32

Hartung T (1998) Anti-inflammatory effects of granulocyte colony-stimulating factor. Curr Opin Hematol 5:221–225

Hartung T (1999) Granulocyte colony-stimulating factor: its potential role in infectious disease. AIDS 13:S3–S9

Hazel DL, Newland AC, Kelsey SM (1999) Malignancy: granulocyte colony stimulating factor increases the efficacy of conventional amphotericin in the treatment of presumed deep-seated fungal infection in neutropenic patients following intensive chemotherapy or bone marrow transplantation for haematological malignancies. Hematology 4:305–311

Heil G, Hoelzer D, Sanz MA, Lechner K, Liu Yin JA, Papa G, Noens L, Szer J, Ganser A, O'Brien C, Matcham J, Barge A (1997) A randomized, double-blind, placebo-controlled, phase III study of filgrastim in remission induction and consolidation therapy for adults with de novo acute myeloid leukemia. The International Acute Myeloid Leukemia Study Group. Blood 90:4710–4718

Hohl TM, Feldmesser M, Perlin DS, Pamer EG (2008) Caspofungin modulates inflammatory responses to *Aspergillus fumigatus* through stage-specific effects on fungal β-glucan exposure. J Infect Dis 198:176–185

Hubel K, Dale DC, Liles WC (2002) Therapeutic use of cytokines to modulate phagocyte function for the treatment of infectious diseases: current status of granulocyte colony-stimulating factor, granulocyte–macrophage colony-stimulating factor, macrophage colony-stimulating factor, and interferon-gamma. J Infect Dis 185:1490–1501

Jacobson MA, Hardy D, Connick E, Watson J, DeBruin M (2000) Phase 1 trial of a single dose of recombinant human interleukin-12 in human immunodeficiency virus-infected patients with 100–500 CD4 cells/microL. J Infect Dis 182:1070–1076

Jacobson MA, Spritzler J, Landay A, Chan E, Katzenstein D, Schock B, Fox L, Roe J, Kundu S, Pollard R, AACTG 325 Protocol Team (2002) A phase I, placebo-controlled trial of multi-dose recombinant human interleukin-12 in patients with HIV infection. AIDS 16:1147–1154

Khatami S, Brummer E, Stevens DA (2001) Effects of granulocyte–macrophage colony stimulating factor (GM-CSF) *in vivo* on cytokine production and proliferation by spleen cells. Clin Exp Immunol 125:198–201

Kobayashi M, Kobayashi H, Herndon DN, Pollard RB, Suzuki F (1998) Burn-associated *Candida albicans* infection caused by CD30+ type 2 T cells. J Leukoc Biol 63:723–731

Kobrynski LJ, Tanimune L, Kilpatrick L, Campbell DE, Douglas SD (1996) Production of T-helper cell subsets and cytokines by lymphocytes from patients with chronic mucocutaneous candidiasis. Clin Diagn Lab Immunol 3:740–745

Komrokji RS, Lyman GH (2004) The colony-stimulating factors: use to prevent and treat neutropenia and its complications. Expert Opin Biol Ther 4:1897–1910

Kopp EB, Medzhitov R (1999) The Toll-receptor family and control of innate immunity. Curr Opin Immunol 11:13–18

La Rocco M, Burgert SJ (1997) Fungal infections in the transplant recipient and laboratory methods for diagnosis. Rev Iberoam Micol 14:143–146

La Sala A, Urbani F, Torosantucci A, Cassone A, Ausiello CM (1996) Mannoproteins from *Candida albicans* elicit a Th1-type-1 cytokine profile in human *Candida* specific long-term T cell cultures. J Biol Regul Homeost Agents 10:8–12

Lamaris GA, Lewis RE, Chamilos G, May GS, Safdar A, Walsh TJ, Raad II, Kontoyiannis DP (2008) Caspofungin-mediated β-glucan unmasking and enhancement of human polymorphonuclear neutrophil activity against *Aspergillus* and non-*Aspergillus* hyphae. J Infect Dis 198:186–192

Leigh JE, Steele C, Wormerly FL Jr, Luo W, Clark RA, Gallaher W, Fidel PL Jr (1998) Th1/Th2 cytokine expression in saliva of HIV-positive and HIV-negative individuals: a pilot study in HIV-positive individuals with oropharyngeal candidiasis. J Acquir Immune Defic Syndr Hum Retrovirol 19:373–380

Liles WC, Huang JE, van Burik JA, Bowden RA, Dale DC (1997) Granulocyte colony-stimulating factor administered *in vivo* augments neutrophil-mediated activity against opportunistic fungal pathogens. J Infect Dis 175:1012–1015

Lopez AF, Williamson DJ, Gamble JR, Begley CG, Harlan JM, Klebanoff SJ, Waltersdorph A, Wong G, Clark SC, Vadas MA (1986) Recombinant human granulocyte macrophage colony-stimulating factor stimulates *in vitro* mature human neutrophil and eosinophil function, surface receptor expression, and survival. J Clin Invest 78:1220–1228

Lügering A, Schmidt M, Lügering N, Pauels HG, Domschke W, Kucharzik T (2001) Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. Gastroenterology 121:1145–1157

Lutz MB, Assmann CU, Girolomoni G, Ricciardi-Castagnoli P (1996) Different cytokines regulate antigen uptake and presentation of a precursor dendritic cell line. Eur J Immunol 26:586–594

Ma B, Seymour JF, Januszewicz H, Slavin MA (2001) Cure of pulmonary *Rhizomucor pusillus* infection in a patient with hairy cell leukemia: role of liposomal amphotericin B and GM-CSF. Leuk Lymphoma 42:1393–1399

Marciano BE, Wesley R, de Carlo ES, Anderson VL, Barnhart LA, Darnell D, Malech HL, Gallin JI, Holland SM (2004) Long-term interferon-gamma therapy for patients with chronic granulomatous disease. Clin Infect Dis 39:692–699

Mehrad B, Strieter RM, Standiford TJ (1999) Role of TNF-alpha in pulmonary host defense in murine invasive aspergillosis. J Immunol 162:1633–1640

Mencacci A, Spaccapelo R, del Sero G, Enssle KH, Cassone A, Bistoni F, Romani L (1996) CD4+ T-helper-cell responses in mice with low-level *Candida albicans* infection. Infect Immunol 64:4907–4914

Mencacci A, Cenci E, Boelaert JR, Bucci P, Mosci P, Fè d'Ostiani C, Bistoni F, Romani L (1997) Iron overload alters innate and T helper cell responses to *Candida albicans* in mice. J Infect Dis 175:1467–1476

Mencacci A, Cenci E, del Sero G, Fè d'Ostiani C, Mosci P, Montagnoli C, Bacci A, Bistoni F, Quesniaux VF, Ryffel B, Romani L (1998a) Defective co-stimulation and impaired Th1 development in tumor necrosis factor/lymphotoxin-α double-deficient mice infected with *Candida albicans*. Int Immunol 10:37–48

Mencacci A, Cenci E, del Sero G, Fé d'Ostiani C, Mosci P, Trinchieri G, Adorini L, Romani L (1998b) IL-10 is required for development of protective CD41 T helper type 1 cell responses to *Candida albicans*. J Immunol 161:6228–6237

Mencacci A, del Sero G, Cenci E, d'Ostiani CF, Bacci A, Montagnoli C, Kopf M, Romani L (1998c) Endogenous IL-4 is required for development of protective CD4+ T helper type 1 cell responses to *Candida albicans*. J Exp Med 187:307–317

Nemunaitis J, Meyers JD, Buckner CD, Shannon-Dorcy K, Mori M, Shulman H, Bianco JA, Higano CS, Groves E, Storb R et al (1991) Phase I trial of recombinant human macrophage colony-stimulating factor (rhM-CSF) in patients with invasive fungal infections. Blood 78:907–913

Nemunaitis J, Shannon-Dorcy K, Appelbaum FR, Meyers J, Owens A, Day R, Ando D, O'Neill C, Buckner D, Singer J (1993) Long-term follow-up of patients with invasive fungal disease who received adjunctive therapy with recombinant human macrophage colony-stimulating factor. Blood 82:1422–1427

Nemunaitis J, Buckner CD, Dorsey KS, Willis D, Meyer W, Appelbaum F (1998) Retrospective analysis of infectious disease in patients who received recombinant human granulocyte–macrophage colony-stimulating factor versus patients not receiving a cytokine who underwent autologous bone marrow transplantation for treatment of lymphoid cancer. Am J Clin Oncol 21:341–346

Netea MG, Radstake T, Joosten LA, van der Meer JW, Barrera P, Kullberg BJ (2003) Salmonella septicemia in rheumatoid arthritis patients receiving anti-tumor necrosis factor therapy: association with decreased interferon-γ production and toll-like receptor 4 expression. Arthritis Rheum 48:1853–1857

Odds FC (1988) *Candida* and candidosis: a review and bibliography, 2nd edn. Baillière-Tindall, London, UK, pp 68–92

Ozer H, Armitage JO, Bennett CL, Crawford J, Demetri GD, Pizzo PA, Schiffer CA, Smith TJ, Somlo G, Wade JC, Wade JL III, Winn RJ, Wozniak AJ, Somerfield MR, The American Society of Clinical Oncology Growth Factors Expert Panel (2000) 2000 Update of recommendations for the use of hematopoietic colonystimulating factors: evidence-based, clinical practice guidelines. J Clin Oncol 18:3558–3585

Pappas PG, Rex JH, Sobel JD, Filler SG, Dismukes WE, Walsh TJ, Edwards JE (2004) Guidelines for treatment of candidiasis. Clin Infect Dis 38:161–189

Peters BG, Adkins DR, Harrison BR, Velasquez WS, Dunphy FR, Petruska PJ, Bowers CE, Niemeyer R, McIntyre W, Vrahnos D, Auberry SE, Spitzer G (1996) Antifungal effects of yeast-derived rhu-GM-CSF in patients receiving high-dose chemotherapy given with or without autologous stem cell transplantation: a retrospective analysis. Bone Marrow Transplant 18:93–102

Polak-Wyss A (1991) Protective effect of human granulocyte colony-stimulating factor (hG-CSF) on *Cryptococcus* and *Aspergillus* infections in normal and immunosuppressed mice. Mycoses 34:205–215

Ray A, Anand S (2000) Recent trends in antifungal therapy: focus on systemic mycoses. Indian J Chest Dis Allied Sci 42:357–366

Reis e Sousa G, Sher A, Kaye P (1999) The role of dendritic cells in the induction and regulation of immunity to microbial infection. Curr Opin Immunol 11:392–399

Rex JH, Stevens DA (2005) Systemic antifungal agents. In: Mandell GE et al (eds) Principles and Practice of Infectious Disease, 6th edn. Churchill and Livingstone, Philadelphia, PA, pp 502–514

Riddell LA, Pinching AJ, Hill S, Ng TT, Arbe E, Lapham GP, Ash S, Hillman R, Tchamouroff S, Denning DW, Parkin JM (2001) A phase III study of recombinant human interferon gamma to prevent opportunistic infections in advanced HIV disease. AIDS Res Hum Retroviruses 17:789–797

Rodriguez-Adrian LJ, Grazziutti ML, Rex JH, Anaissie EJ (1998) The potential role of cytokine therapy for fungal infection in patients with cancer: Is recovery from neutropenia all that is needed? Clin Infect Dis 26:1270–1278

Roilides E, Sein T, Holmes A, Chanock S, Blake C, Pizzo PA, Walsh TJ (1995) Effects of macrophage colony stimulating factor on antifungal activity of mononuclear phagocytes against *Aspergillus fumigatus*. J Infect Dis 172:1028–1034

Roilides E, Blake C, Holmes A, Pizzo PA, Walsh TJ (1996) Granulocyte macrophage colony-stimulating factor and interferon-gamma prevent dexamethasone-induced immunosuppression of antifungal monocyte activity against *Aspergillus fumigatus* hyphae. J Med Vet Mycol 34:63–69

Roilides E, Dimitriadou-Georgiadou A, Sein T, Kadiltsoglou I, Walsh TJ (1998a) Tumor necrosis factor alpha enhances antifungal activities of polymorphonuclear and mononuclear phagocytes against *Aspergillus fumigatus*. Infect Immunol 66:5999–6003

Roilides E, Dignani MC, Anaissie EJ, Rex JH (1998b) The role of immunoreconstitution in the management of refractory opportunistic fungal infections. Med Mycol 36:12–25

Roilides E, Sein T, Roden M, Schaufele RL, Walsh TJ (2001) Elevated serum concentrations of interleukin-10 in nonneutropenic patients with invasive aspergillosis. J Infect Dis 183:518–520

Roilides E, Uhlig K, Venzon D, Pizzo PA, Walsh TJ (1993) Prevention of corticosteroid-induced suppression of human polymorphonuclear leukocyte–induced damage of *Aspergillus fumigatus* hyphae by granulocyte colony-stimulating factor and gamma interferon. Infect Immunol 61: 4870–4877

Romani L (1997) The T cell response to fungi. Curr Opin Immunol 9:484–490

Romani L (2000) Innate and adaptive immunity in *Candida albicans* infections and saprophytism. J Leukoc Biol 68:175–178

Romani L, Mencacci A, Tonnetti L, Spaccapelo R, Cenci E, Wolf S, Puccetti P, Bistoni F (1994) Interleukin-12 but not interferon-g production correlates with induction of T helper type-1 phenotype in murine candidiasis. Eur J Immunol 24:909–915

Romani L, Mencacci A, Cenci E, Spaccapelo R, del Sero G, Nicoletti I, Trinchieri G, Bistoni F, Puccetti P (1996) Impaired neutrophil response and $CD4^+$ T helper cell 1 development in interleukin-6-deficient mice infected with *Candida albicans*. J Exp Med 183:1–11

Romani L, Bistoni F, Puccetti P (1997a) Initiation of T helper cell immunity to *Candida albicans* by IL-12: the role of neutrophils. Chem Immunol 68:110–135

Romani L, Mencacci A, Cenci E, Spaccapelo R, Del Sero G, Nicoletti I, Trinchieri G, Bistoni F, Puccetti P (1997b) Neutrophils production of IL-12 and IL-10 in candidiasis and efficacy of IL-12 therapy in neutropenic mice. J Immunol 158:5349–5356

Romani L, Mencacci A, Cenci E, del Sero G, Bistoni F, Puccetti P (1997c) An immunoregulatory role for neutrophils in CD4+ T helper subset selection in mice with candidiasis. J Immunol 158:2356–2362

Rowe JM (1998) Treatment of acute myeloid leukemia with cytokines: effect on duration of neutropenia and response to infections. Clin Infect Dis 26:1290–1294

Rowe JM, Andersen JW, Mazza JJ, Bennett JM, Paietta E, Hayes FA, Oette D, Cassileth PA, Stadtmauer EA, Wiernik PH (1995) A randomized, placebo-controlled phase III study of granulocyte–macrophage colony-stimulating factor in adult patients (155 to 70 years of age) with acute myelogenous leukemia: a study of the Eastern Cooperative Oncology Group (E1490). Blood 86:457–462

Safdar A, Rodriguez G, Ohmagari N, Kontoyiannis DP, Rolston KV, Raad II, Champlin RE (2005) The safety of interferongamma-1b therapy for invasive fungal infections after hematopoietic stem cell transplantation. Cancer 103:731–739

Schall TJ (1991) Biology of the RANTES/SIS cytokine family. Cytokine 3:165–183

Sozzani S, Luini W, Borsatti A, Sozzani S, Luini W, Borsatti A, Polentarutti N, Zhou D, Piemonti L, D'Amico G, Power CA, Wells TN, Gobbi M, Allavena P, Mantovani A (1997) Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. J Immunol 159:1993–2000

Spaccapelo R, Romani L, Tonnetti L, Cenci E, Mencacci A, del Sero G, Tognellini R, Reed SG, Puccetti P, Bistoni F (1995) TGF-b is important in determining the *in vivo* susceptibility or resistance in mice infected with *Candida albicans*. J Immunol 155:1349–1360

Steinbach WJ, Stevens DA (2003) Review of newer antifungal and immunomodulatory strategies for invasive aspergillosis. Clin Infect Dis 37:S157–S187

Stevens DA (2008) A possible mechanism for synergy between antifungal therapy and immune defenses. J Infect Dis 198:159–162

Stevens DA, Brammer HA, Meyer DW, Steiner BH (2000) Recombinant human chitinase. Curr Opin Anti-Infect Investig Drugs 2:399–404

Tonnetti L, Spaccapelo R, Cenci E, Mencacci A, Puccetti P, Coffman RL, Bistoni F, Romani L (1995) Interleukin-4 and -10 exacerbate candidiasis in mice. Eur J Immunol 25:1559–1565

Trick WE, Fridkin SK, Edwards JR, Hajjeh RA, Gaynes RP, National Nosocomial Infections Surveillance System Hospitals (2002) Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989–1999. Clin Infect Dis 35:627–630

Tsiodras S, Samonis G, Boumpas DT (2008) Fungal infections complicating tumor necrosis factor α blockade therapy. Mayo Clin Proc 83:181–194

van der Meer JW, Popa C, Netea MG (2005) Side effects of anticytokine strategies. Neth J Med 63:78–80

Vasquez-Torres A, Balish E (1997) Macrophages in resistance to candidiasis. Microbiol Mol Rev 61:170–192

Vazquez JA, Hidalgo JA, de Bono S (2000) Use of sargramostim (rh-GM-CSF) as adjunctive treatment of fluconazole-refractory oropharyngeal candidiasis in patients with AIDS: a pilot study. HIV Clin Trials 1:23–29

von Hundelshausen P, Weber KS, Huo Y, Proudfoot AE, Nelson PJ, Ley K, Weber C (2001) RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. Circulation 103:1772–1777

Wagner RD, Vasquez-Torres A, Jones-Carson J, Warner T, Balish E (1996) B-cell knockout mice are resistant to mucosal and systemic candidiasis of endogenous origin but susceptible to experimental candidiasis. J Infect Dis 174:589–597

Walsh TJ, Giri N (1997) Pradimicins: a novel class of broad spectrum antifungal compounds. Eur J Clin Microbiol Infect Dis 16:93–97

Walsh TJ, Hiemenz J, Pizzo PA (1994) Evolving risk factors for invasive fungal infections: all neutropenic patients are not the same. Clin Infect Dis 18:793–798

Welte K, Gabrilove J, Bronchud MH, Platzer E, Morstyn G (1996) Filgrastim (r-metHuG-CSF): the first 10 years. Blood 88:1907–1929

Wright KM, Friedland JS (2004) Regulation of monocyte chemokine and MMP-9 secretion by proinflammatory cytokines in tuberculous osteomyelitis. J Leukoc Biol 75:1086–1092

Chapter 17
Immunomodulators: Potential in Treatment of Systemic Fungal Infections

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Abstract Innate immunity mediates strong resistance to fungal pathogens and contributes to host defense against opportunistic fungal infections such as candidiasis, aspergillosis, and other rare infections. Immune factors such as cytokines and effector immune cells work synergistically with antifungal agents to restrict fungal growth. However, in immunocompromised hosts, the defectiveness of immune functions that should cooperate with antifungal drugs to clear the pathogens seems to be a critical factor that impedes the effectiveness of these drugs. The renovation or augmentation of immune responses is now considered as one of the foundations of effective antifungal therapy. Immunomodulation represents a novel approach to antimicrobial therapy that depends on boosting host immunity, rather than direct antimicrobial activity. Immunopotential therapy therefore offers a rational approach to the treatment of fungal infections, because it is intended to enhance immune functions in general. Major advances in the field of experimental immunology have provided insight into the important regulatory role of cytokines in both innate and adaptive immunity to fungal pathogens. Exploration has also begun with immunotherapy, with use of cytokines and immunomodulators alone or in combination with antifungal therapy. The administration of cytokines to patients, together with antifungal agents, offers promising immuno-therapeutic modalities

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for further research. The diverse array of natural, synthetic, and recombinant immunomodulators discussed in this chapter succinctly demonstrates the potential of these agents to stimulate host defense mechanisms for prophylaxis and treatment of various fungal infections.

17.1 Introduction

Fungal organisms are ubiquitous in nature. Although there are an estimated 250,000 fungal species, fewer than 150 have been described as human pathogens. Several reasons have been proposed for the increase in invasive fungal infections, including the use of antineoplastic and immunosuppressive agents, broad-spectrum antibiotics, and prosthetic devices and grafts, and more aggressive surgery. Patients with burns, neutropenia, human immunodeficiency virus (HIV) infection, and pancreatitis are also predisposed to fungal infection (Eggimann et al. 2003). Systemic fungal infections get into the blood stream and cause life-threatening infections, and opportunistic infections are mainly found in people with a weakened immune system that could be caused by any systemic or superficial infections. *Candida*, *Cryptococcus*, *Aspergillus* and pneumocystic fungi are some potent organisms involved in systemic fungal infections.

The AIDS epidemic is one of the most important factors which have contributed to the rising incidence of fungal diseases. Prior to the widespread usage of highly active antiretroviral therapy (HAART) in developed countries, up to 80% of HIV-infected persons developed mucosal candidiasis, while others developed cryptococcosis, histoplasmosis, or coccidioidomycosis during the course of their disease (Hajjeh and Warnock 2003). Analysis of U.S. National Center for Health Statistics (NCHS) death records showed that fungal infections were the seventh most common cause of infectious disease- related mortality in 1992, and that mycotic disease-related fatalities had increased more than threefold since 1980 (Pinner et al. 1996). Additional analysis revealed that candidiasis and aspergillosis were the two specific diseases that accounted for most of these deaths (McNeil et al. 2001).

Augmentation of the host defense response, improvement of the underlying disease, and resolution of the principal immune impairment are paramount for successful treatment of invasive mycoses in immuno-compromised patients. Systemic mycoses are associated with high morbidity and mortality rates despite advances in antifungal chemotherapy. Recent studies have shown that upregulating the host immune response by immunological adjuncts could be helpful. Immunostimulants enhance the overall immunity of the host, and present a nonspecific immune response against the microbial pathogens. They also work to heighten humoral and cellular immune responses, by either enhancing cytokine secretion, or by directly stimulating B- or T-lymphocytes. Immunotherapy offers many therapeutic advantages through the availability of a wide range of recombinant cytokines that exert their effects indirectly through leukocyte activation rather than directly on the fungus. Immunotherapy is designed to increase the

number of phagocytic cells and shorten the duration of neutropenia, modulate the kinetics or actions of those cells at the site of infection, and/or activate the fungicidal activity of phagocytes to kill fungal cells more efficiently (Latge 1999; Roilides and Pizzo 1992). Administration of recombinant hematopoietic human cytokines, such as granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interferon-γ, interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α), have been shown to decrease the duration of neutropenia, increase the microbicidal action of neutrophils, monocytes, and macrophages, and reduce the duration of cytotoxic chemotherapy in systemic mycoses (Roilides and Pizzo 1992; Kullberg 1997). In this chapter we will deal with immunomodulators (synthetic and natural).

17.2 Immunomodulators

The immune system can be manipulated specifically by vaccination or nonspecifically by immunomodulation (Masihi 1994a, b, 1996, 1997; Masihi and Lange 1988, 1990). Immunomodulators are biological or synthetic substances capable of altering the immune response by augmenting or reducing any of the components of the immune system including both innate and adaptive arms of the immune response. Immunomodulators are usually products of the immune system (Committee on New Directions in the Study of Antimicrobial Therapeutics 2006). Basically, immunomodulators are agents that alter the immune response by suppression (immunosuppressive) or enhancement (immunostimulant) (Saunders Comprehensive Veterinary Dictionary 2007). Synonymous terms for immunomodulators include biological response modifiers, immune-augmentors, or immunorestoratives. Microbial products, drugs of natural and synthetic origin, and proteins derived from the immune system represent some of the immunomodulators that are currently in use.

Immunomodulators correct weak immune systems and temper immune systems that are overactive, but they do not boost the immune system the way immune stimulants such as *Echinacea* do. Immunomodulators are recommended for people with auto-immune diseases and they are widely used in chronic illness to restore immune system health in people who have been on lengthy courses of antibiotics or antiviral therapies. Certain antibiotics can, in addition to their antibacterial properties, also modulate the immune response (Labro 1998; Stevens 1996). Some immunomodulators are naturally present in the body, and certain of these are available in pharmacologic preparations.

Approaches to immunomodulation can be divided into those that are specific to pathogens (pathogen-specific) and those that are not (nonspecific). Specific immunomodulators are administered together with antigen, for instance in vaccines, where they are known as immunological adjuvants, and boost the immune response to the vaccine candidates (Bomford 1988). In principle it is possible to imagine a

specific immunosuppressant which, when given together with antigen, would induce a state of specific nonresponsiveness or tolerance to the antigen.

Pathogen-specific immunomodulators include antibody reagents and vaccines. With the exception of the rabies and varicella zoster vaccines, currently licensed vaccines are administered to prevent acute infectious diseases rather than for therapy and are not discussed further here (Pirofski and Casadevall 2006). Nonspecific immunostimulants are given on their own in order to elicit a generalized state of resistance to pathogens which in many cases is believed to depend on the activation of macrophages (Bomford 1988). Nonspecific immunosuppressants reduce the capacity of the immune system to respond to antigens either by the blunderbuss approach of killing dividing cells with cytotoxic drugs, or by interfering with the function of cells of the immune system in a more selective way. Nonspecific immunomodulators include cytokines, antimicrobial peptides, certain antimicrobial drugs, and microbes such as probiotics. At present, clinical experience with nonspecific immunomodulators as antimicrobial tools has been predominantly limited to cytokines.

Immunostimulatory agents do not directly affect immune memory cells, as activation and differentiation of memory cells require precise cell–cell and MHC–antigen interactions. However, they are specific in that immunostimulants enhance particular immune responses to combat specific pathogens. Immunostimulating activities may be divided into those that (1) enhance phagocytic activities, and (2) effect cell-mediated and humoral immunity (Tan and Vanitha 2004).The modulation of the immune response has a number of important implications. For example, the adjuvant action of the cytokine, lymphokine, hormone, or growth factor can increase the concentration of protective antibodies produced against the antigenic portion of the conjugate in the vaccinated organism. Likewise, antibody production against antigens coadministered with the conjugate can be increased. As a result, effective (i.e., protective) vaccination can be achieved with a smaller quantity of conjugated antigen and/or co-administered antigen than would be normally required. This reduction in the required amount of conjugated antigen and co-administered antigen may lead to more widespread use of vaccines which are difficult or costly to prepare or which are weakly immunogenic. This is especially true in the developing nations that face such epidemics as malaria and cholera, with very limited health care budgets. It may also provide for safer vaccination when the antigen is toxic at the concentration normally required for effective immunization. By reducing the amount of antigen, the risk of toxicity may be reduced.

17.3 Combinational Trends of Antifungals with Immunomodulators

Since fungal infections occur mainly in immunosuppressed patients, it is reasoned that adding an immunomodulator or stimulator to an antifungal agent may improve the chance of a successful outcome. Consequently, researchers have sought to

determine the effects of adding immune factors (e.g., granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor) or effector cells [e.g., primarily amacrophages, polymorphonuclear neutrophils (PMNs), and monocytes] to antifungal drug regimens in an attempt to manipulate both innate and adaptive host defenses. Effective antifungal agents may act in collaboration with host effector cells at various intracellular and extracellular locations, or during different postinfection intervals corresponding to different phases and mechanisms of host defense response (Chiller et al. 2001, 2002; Stevens 1998). Overall, various antifungals combined with immunomodulators against candidiasis have been shown to be generally more effective than monotherapy (Chiller et al. 2002; Coste et al. 2002; Kuhara et al. 2000; Mencacci et al. 2000a; Vora et al. 1998a, b). Adjunctive immunotherapy using antibody-based therapies has been investigated for *Cryptococcus neoformans* and *A. fumigatus* infections and generally shows enhanced activity with combination therapies (Clemons and Stevens 2001; Feldmesser et al. 1996; Mukherjee et al. 1994, 1995; Nassar et al. 1995; Roilides et al. 2002; Vora et al. 1998a, b). Different studies in this field have been summarized in previous reviews (Stevens 1998; Stevens et al. 1998, 2000).

Some case reports have been described supporting the notion that immunomodulators can influence the efficacy of antifungal agents (Chiller et al. 2001; Ellis et al. 2002). These studies suggested that immunomodulators may be acting via neutrophils (Th_1 response) or monocytes (inducing tumor necrosis factor and macrophage inflammatory protein 1). In separate studies, voriconazole, posaconazole, and itraconazole enhanced the antifungal functions of human PMNs against hyphae of *S. prolificans* and *S. apiospermium* (Gil-Lamaignere et al. 2002b). Similarly, AmB lipid complex plus PMN displayed a significant additive effect against both *Scedosporium* species (22% for *S. prolificans* and 81% for *S. apiospermum*) (Gil-Lamaignere et al. 2002a). Efficacies of L-AmB plus granulocyte colony-stimulating factor have also been demonstrated *in vivo* by using an immunosuppressed murine model of disseminated *Scedosporium* infection (Ortoneda et al. 2002). Recently, Steinbach et al. (Steinbach et al. 2004) used disk diffusion, microdilution checkerboard, and gross and microscopic morphological analyses to demonstrate that a combination of the immunosuppressants cyclosporine or tacrolimus (FK506) with CAS exhibits a positive interaction against *A. fumigatus*.

In candidemia, fluconazole remains the drug of choice in neutropenic and nonneutropenic patients in whom *C. krusei* is unlikely and who have received no prior treatment with fluconazole. However, AmB is the agent of choice when infection is due to a fluconazole-resistant organism or *C. krusei*, or in patients who develop candidemia while on fluconazole therapy (Sheehan et al. 1999). Combination therapy of various drugs is also recommended, for example fluconazole is the drug of choice for the treatment of cryptococcal meningitis and is also the agent of choice for prophylaxis against cryptococcal meningitis in AIDS patients following initial therapy with AmB with or without flucytosine. High-dose fluconazole in combination with amphotericin B and flucytosine is the current treatment approach for the disseminated trichosporonosis in an immunocompromised host (Groll and Walsh 1999).

Antifungal drugs such as fluconazole and amphotericin B have shown broad immunomodulatory properties (Yamaguchi et al. 1993). Cytokines, effector cells, and antifungals seem to work synergistically to restrict fungal growth in immunocompetent persons (Stevens 1998). In immunocompromised hosts, the lack of effector functions that cooperate with antifungal drugs to clear the pathogens seems to be a crucial factor in impeding the effectiveness of the drug (Stevens 1998; Roilides et al. 1998a). Antifungal chemotherapy in conjunction with immunostimulatory molecules such as IL-12, IFN-γ and GM-CSF has been found to show enhanced efficacy against many fungal pathogens (Casadevall and Pirofski 2001). In *in vivo* murine models of *S. prolificans* infection, combined administration of liposomal amphotericin B and G-CSF has been reported to be effective (Ortoneda et al. 2002).

Taken together, these studies show that combining an antifungal agent with concomitant improvement of host immune response through the use of an immunostimulator is a promising area that needs to be investigated through experimental animal systems and clinical trials. A clear demonstration of the clinical relevance of this approach is the decrease in the incidence of esophageal candidiasis in the HIV/AIDS setting, resulting from host immune reconstitution brought about by the use of HAART (Ghannoum 2001). Although combining an antifungal with another therapeutic class has shown promise, more studies are needed to determine whether these combinations have widespread clinical relevance.

17.4 Tuftsin: A Highly Effective Immunomodulator

Tuftsin ($C_{21}H_{40}N_8O_6$) is a tetrapeptide (Thr^{289}-Lys^{290}-Pro^{291}-Arg^{292}) produced by enzymatic cleavage of the Fc-domain of the heavy chain of immunoglobulin G. It is produced primarily in the spleen. Tuftsin was first identified in 1970 by scientists Najjar and Nishioka. It was named after Tufts University where the peptide was discovered. Tuftsin binds to specific receptors on the surface of macrophages and polymorphonuclear leukocytes, stimulating their migration, phagocytic, bactericidal, and tumoricidal activity. It also influences antibody formation. Tuftsin deficiency, either hereditary or following splenectomy, results in increased susceptibility to certain infections, for example those caused by capsulated organisms such as *H. influenzae*, pneumococci, meningococci, and salmonella (Constantopoulos et al. 1972, 1973; Najjar 1981). Tuftsin has been chemically synthesized and it is considered for use in immunotherapy (Fig. 17.1).

Tuftsin, due to its hydrophilic character, cannot be grafted on the surface of liposomes without being attached to a sufficiently long hydrophobic anchor. Structure–function studies of this tetrapeptide indicate that its binding and subsequent activation of the mononuclear phagocyte system (MPS) is dependent upon rather strict conservation of its molecular structure. Thus, modifications of the peptide at its N-terminus or within the chain lead to a significant reduction or even loss of its biological activity (Fridkin and Gottlieb 1981). However, the activity is largely

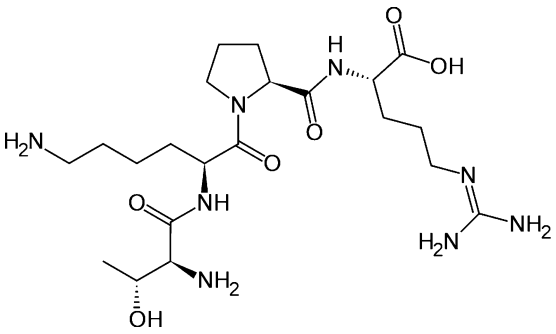

Fig. 17.1 Molecular skeleton of tuftsin [Pubchem (ID 24780)]

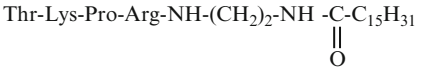

Fig. 17.2 Structure of palmitoyl tuftsin (I)

retained if modifications are restricted only to the C-terminus (Gottlieb et al. 1982). All the modifications are therefore limited to the carboxyl group of the Arg residue. Direct attachment of a fatty acyl group to the Arg residue, without any spacer arm, leads to modified tuftsin, which does not allow formation of liposomes, presumably due to perturbation of the phospholipid polar head group packing by the bulky Arg residue (Singhal et al. 1984). This problem is, however, circumvented by introducing an ethylenediamine spacer arm between the Arg residue and the hydrophobic anchor (Fig. 17.2).

Liposomes containing palmitoyl tuftsin (I) specifically recognize macrophages and PMN leukocytes (Singhal et al. 1984). Treatment of macrophages with these liposomes considerably increases their respiratory burst activity (Singh et al. 1992). Pretreatment of animals with tuftsin-bearing liposomes enables them to resist malaria (Gupta et al. 1986); leishmania (Guru et al. 1989); antifungal (Owais et al. 1993), and antifilarial (Owais et al. 2003) drugs in liposomes containing palmitoyl tuftsin is shown to increase the therapeutic efficacy of drugs against these infections. Gupta and Haq (2005) described procedures for preparation of I as well as the liposomes that contain I in their bilayers, entrapment of various drugs in these liposomes, and their delivery to experimental animals with infected *L. donovani*, *M. tuberculosis*, or *Aspergillus*.

Immunomodulator-based therapy seems likely to be more beneficial for treatment of fungal infectious diseases. The co-administration of tuftsin increased the efficiency of liposomised-polyene antibiotics (nystatin and amphotericin B) against experimental murine candidiasis in immunocompromised Balb/c mice. Pretreatment with liposomised tuftsin prior to *C. albicans* infection clearly enhanced protection against candidiasis, suggesting a prophylactic role of tuftsin in normal and temporarily neutropenic mice (Khan et al. 2004). One of the pioneer researches by Khan et al. (2005a) showed the immunopotentiating efficacy of tuftsin against experimental murine aspergillosis in both normal and immunodebilitant BALB/c

mice. They found that co-administration of the immunomodulator tuftsin and liposomised-amphotericin B was highly effective in the treatment of systemic infection of *A. fumigatus* in both cases, resulting in successful elimination of fungal pathogen (Khan et al. 2005b). In another study, Khan and Owais (2005) evaluated the combination of liposomal amphotericin B (lip-Amp B) and immunomodulator tuftsin to cure *C. neoformans* infection in BALB/c mice. Pretreatment of mice with liposomal tuftsin before challenging them with the *C. neoformans* infection resulted in 100% survival of the treated animals followed by treatment with lip-Amp B. In another set of experiments, they conducted the same study in leukopenic mice and found that incorporation of tuftsin in liposomes resulted in increased anticryptococcal activity of liposomal amphotericin B compared with amphotericin B deoxycholate and conventional liposomal amphotericin B formulations (Khan et al. 2005b).

Interestingly, tuftsin also increased the stability of liposomal amphotericin B. Our group has also demonstrated that co-administration of immunomodulator tuftsin along with liposomal formulations of amphotericin B successfully minimizes toxicity, as well as other side-effects of the drug (Masood and Owais 2006). The pharmacokinetics of amphotericin B in *Candida albicans*-infected mice treated with conventional and tuftsin-loaded amphotericin B liposomes was evaluated and was found to exhibit superior efficacy, safety, and favorable pharmacodynamics, therefore suggesting their potential therapeutic value in the management of fungal infections (Khan and Owais 2006).

Tuftsin-bearing nystatin was found to be effective in eliminating a strain of *C. albicans* less susceptible to amphotericin B (*C. albicans* JMCR) in Balb/c mice, but it may not be recommended due to toxicity constraints (Khan et al. 2003). Treatment with tuftsin-loaded nystatin liposomes was most effective in eliminating fungal burden from lung tissues of infected mice compared to those treated with free nystatin or nystatin liposomes without tuftsin. (Khan et al. 2006).

17.5 Cytokines as Nonspecific Immunomodulators

Invasive fungal infections (IFI) constitute a major threat for immunocompromised hosts. Particularly susceptible to IFI are patients with hematological malignancies and either disease- or treatment-related immunosuppression, including acute leukemia, especially acute myeloid leukemia (AML), chronic leukemia, lymphomas, and multiple myeloma, and recipients of allogeneic hematopoietic stem cell transplants (HSCT). The increased susceptibility of these patients to IFI has been attributed to several factors, including the underlying hematological malignancy, prolonged neutropenia, and impairment of host defense mechanisms because of intensive cytotoxic therapy or corticosteroid use, ablative radiotherapy, severe gastro-intestinal mucosal damage, delayed engraftment or graft-versus-host disease (GVHD) (Viscoli et al. 1999; Pagano et al. 2001; Martino and Subira 2002).

Preclinical studies have convincingly demonstrated that immunomodulation with cytokines can enhance the antifungal activity of neutrophils and monocytes/macrophages as well as upregulate protective T-helper type 1 adaptive immune responses. There is some evidence that Th_1 immune responses may be necessary for the optimal control of fungal infections (Brieland et al. 2001; Centeno-Lima et al. 2002). In this regard, immune interventions to polarize the immune response toward a Th_1 type may be beneficial. Evidence of Th_1/Th_2 dysimmunoregulation in hepatosplenic candidiasis (Roilides et al. 1998b) and invasive aspergillosis (Roilides et al. 2001), characterized by increased circulating levels of IL-10, has been demonstrated in humans. The utility of adjunctive therapy using immune modulating agents, such as hematopoietic growth factors (HGFs) or granulocyte transfusions, continues to be a matter of debate. No definitive randomized studies have been performed. Up to now, studies have only justified the safety of immunomodulating therapy, with anecdotes suggesting efficacy.

17.5.1 Hematopoietic Growth Factors (HGF)

HGF are able to augment the number of circulating phagocytes and their precursors. Of the HGFs, Granulocyte colony-stimulating factor (G-CSF) stimulates the proliferation and differentiation of myeloid progenitor cells to PMN leucocytes. Apart from increasing the number of mature neutrophils, G-CSF also enhances their phagocytic activity *in vitro* against a variety of pathogenic fungi, including *Candida*, *Aspergillus*, and *Fusarium* spp. (Roilides et al. 1993; Natarajan et al. 1997; Gaviria et al. 1999). Furthermore, ex vivo incubation with G-CSF was shown to enhance the impaired respiratory burst of neutrophils derived from transplant recipients against *Candida* and *Cryptococcus* yeasts as well as *Aspergillus* and *Rhizopus* conidia (Pursell et al. 2003). Recent studies, however, have demonstrated an additional important role of G-CSF in the regulation of adaptive T helper-cell responses. In particular, G-CSF promotes the "nonprotective" Th_2 responses through functional G-CSF receptors in T cells and monocytes (Boneberg et al. 2000; Franzke et al. 2003). In ex vivo lipopolysaccharide-stimulated whole blood, G-CSF treatment attenuated the release of IL-12, IL-1β, IFN-γ and TNF-α (Boneberg et al. 2000).

Macrophage colony-stimulating factor (M-CSF) accelerates the proliferation and differentiation of monocyte myeloid progenitors, and enhances chemotaxis, phagocytosis, and secondary cytokine production in mature monocytes and macrophages (Nemunaitis 1998). Incubation of macrophages with M-CSF enhances the killing of *Candida* spp. and *Cryptococcus* spp. Treatment of chronic disseminated candidiasis in rats with M-CSF has been shown to reduce the outgrowth of *C. albicans*. It enhances monocyte/macrophage antifungal activity against *C. albicans*, *A. fumigatus*, *H. capsulatum*, and *T. asahii* (Khemani et al. 1995; Roilides et al. 1995b, Sasaki et al. 2000; Gonzalez et al. 2001). These *in vitro* data were in agreement with animal studies of invasive candidiasis, aspergillosis, and trichosporonosis, where

M-CSF treatment was associated with improved survival and reduced fungal burden (Cenci et al. 1991; Sasaki et al. 2000; Gonzalez et al. 2001).

Granulocyte–macrophage colony-stimulating factor (GM-CSF) accelerates haemopoiesis in the early steps of differentiation of myeloid cells, resulting in increased production of neutrophils, monocytes, and eosinophils. It also stimulates a variety of functional activities in these cells, including phagocytosis of fungal organisms by neutrophils or monocytes/macrophages (Armitage 1998). GM-CSF enhances TLR2 expression (important for response to yeast zymosan) and TLR2-mediated IL-8 responses in neutrophils (Kurt-Jones et al. 2002). It also enhances the expression of Dectin-1, which is the major receptor for the β-glucans of fungal cell wall, in murine macrophages (Willment et al. 2003).

17.5.2 Anti-inflammatory Cytokines

IFN-γ produced by T and Natural Killer (NK) cells is a key cytokine both in the innate and adaptive immune response to IFI. It stimulates migration, adherence, and antifungal activity of neutrophils and/or macrophages against *C. albicans*, *A. fumigatus*, *F. solani*, *T. beigelii*, and *P. marneffei* (Lyman et al. 1994; Gaviria et al. 1999; Kudeken et al. 1999; Mencacci et al. 2000b) The observed augmentation of antifungal activity by IFN-γ *in vitro* was in agreement with results of animal studies of experimental candidiasis and aspergillosis (Mencacci et al. 2000b). IL-12 is required for the development of protective Th1 responses against fungal infections. This important regulatory role is partly mediated by IL-12 induction of IFN-γ and IL-18 production (Romani et al. 1997; Kawakami et al. 2000a) and has been demonstrated in animal models of invasive candidiasis, aspergillosis, cryptococcosis, and paracoccidiosis (Romani et al. 1994; Cenci et al. 1998; Decken et al. 1998; Brieland et al. 2001; Arruda et al. 2002). Interferon gamma may be superior at enhancing the antifungal activity of phagocytes (Roilides et al. 1995a; Gaviria et al. 1999). The efficacy of adjunctive interferon-gamma 1b (IFN-γ 1b) with amphotericin B was studied in a Phase II, double-blind placebo-controlled trial for AIDS-associated cryptococcal meningitis (Pappas et al. 2004). The rationale for interferon therapy for cryptococcosis has a strong basis in preclinical studies in mice (Lutz et al. 2000) and in a human study showing an association between cerebrospinal fluid levels of IFN-γ and treatment in HIV-infected patients with cryptococcal meningitis (Siddiqui et al. 2005).

Two other cytokines, IL-15 and IL-18, play a role in the protective adaptive or innate immune response against IFI. IL-18 is involved in the development of Th_1 response through its stimulatory effect on the production of IFN-γ (Stuyt et al. 2002). It was shown to protect against *C. albicans* or *C. neoformans* infection in animal models and to restore defective Th_1 immunity to *C. albicans* in caspase 1-deficient mice (Kawakami et al. 2000b; Mencacci et al. 2000c; Stuyt et al. 2004). IL-15 is involved in the innate immunity against fungal infections by enhancing the antifungal activity of polymorphonuclear or monocyte cells against *C. albicans* and

A. fumigatus (Musso et al. 1998; Vazquez et al. 1998; Winn et al. 2003). An additional role of IL-15 in NK cell activation has recently been demonstrated (Tran et al. 2003).

17.5.3 Pro-inflammatory Cytokines

IL-4 is one of the cytokines associated with the development of Th2 response against fungal pathogens. It also suppresses phagocytic activity of monocytes/macrophages against C. albicans (Cenci et al. 1993; Roilides et al. 1997). IL-4 was shown to impair host resistance to *A. fumigatus*, *H. capsulatum*, *C. neoformans* and Paracoccidioides braziliensis in animal models (Cenci et al. 1999; Kawakami et al. 1999a; Gildea et al. 2003; Pina et al. 2004). The suppressive effect of IL-10 on the innate and protective Th_1 antifungal responses was demonstrated in mouse models of invasive candidiasis, aspergillosis, and histoplasmosis (Tonnetti et al. 1995; Del Sero et al. 1999; Vazquez-Torres et al. 1999; Clemons et al. 2000; Deepe and Gibbons 2003). Furthermore, it was recently shown that IL-10 produced from dendritic cells is required for activation of $CD4^+$ $CD25^+$ T_{reg} cells (Montagnoli et al. 2002). Taken together, the data presented for IL-10, IL-4 and IL-12 suggest that, for optimal development and maintenance of protective responses against fungal pathogens, a finely regulated balance of these directive cytokines, rather than the relative absence of opposing cytokines, appears to be required (Mencacci et al. 2000b).

TNF-α is a pro-inflammatory cytokine necessary for the development of effective innate and adaptive immunity to fungal infections. It stimulates antifungal effector functions of neutrophils and/or macrophages against *C. albicans*, *A. fumigatus*, and *C. neoformans* (Roilides et al. 1998c; Kawakami et al. 1999b; Mencacci et al. 2000b; Netea et al. 2004). It also induces a number of other cytokines, including IFN-γ IL-1, IL-6 and IL-12 (Netea et al. 2004). The role of TNF-α in the development of protective Th_1 responses was demonstrated in animal models of candidiasis, aspergillosis, and cryptococcosis (Mencacci et al. 2000b; Bauman et al. 2003).

17.5.4 Cytokine Therapy in Neutropenic Hosts

During the past two decades, invasive fungal infections have emerged as a major threat to immunocompromised hosts. Patients with neoplastic diseases are at significant risk for such infections as a result of their underlying illness and its therapy. *Aspergillus*, *Candida*, *Cryptococcus*, and emerging pathogens, such as the zygomycetes, dark walled fungi, *Trichosporon*, and *Fusarium*, are largely opportunists, causing infection when host defenses are breached. The immune response varies with respect to the fungal species and morphotype encountered. The risk for particular infections differs, depending upon which aspect of immunity is impaired.

Shortening the duration of neutropenia by use of recombinant human cytokines permits more intensive cytotoxic chemotherapy, thereby decreasing the duration and frequency of invasive fungal infections.

G-CSF and GM-CSF are used frequently in patients who are neutropenic and have invasive fungal infections. Adjunctive immunotherapy may be especially important for treatment of mould infections characterized by a large circulating fungal burden and relative resistance to antifungal drugs, as with disseminated fusariosis. In addition, other reports emphasize that outcomes of therapy for zygomycosis are improved with rapid resolution of neutropenia (Kontoyiannis et al. 2000). The potential utility of neutrophil transfusions as adjunctive therapy has been rejuvenated with the development of G-CSF-primed community donor transfusions. Studies evaluating the safety and efficacy of such transfusions, and the use of interferon-gamma for adjunctive therapy of aspergillosis in neutropenic patients are either ongoing or in development.

Reconstitution of the effector cells both numerically and functionally by treatment with leucocyte transfusions (WBCTx) from donors treated with G-CSF has also been attempted. Some patients with persistent neutropenia and infections refractory to conventional antifungal antibiotics appear to respond to adjuvant WBCTx (Roilides et al. 1998a). But, WBCTx may cause severe adverse reactions in the recipient. Therefore, careful selection of the donor, collection technique, and recipient are important. Recent studies with GM-CSF suggest that this recombinant cytokine may be active as an adjunctive therapy in the management of invasive fungal infections in cancer patients. The American Society for Clinical Oncology recently provided guidelines for patients receiving G-CSF and GM-CSF. These cytokines should be used when the expected incidence of febrile neutropenia is >40% in order to avoid infectious complications and to maintain dose intensity in subsequent treatment cycles. These cytokines were also recommended, in combination with autologous progenitor cells transplantation, after high dose chemotherapy.

Recovery from neutropenia is considered critical in cases of *S. prolificans* infection because this infection has a poor outcome (mortality rate approaching 100%) in persistently immunosuppressed patients despite aggressive systemic antifungal therapy (Barbaric and Shaw 2001; Revankar et al. 2002). Early detection, surgical removal of infected tissue (if possible), and immunorestoration appear to be the major means of halting progression of this devastating infection (Rippon 1988; Perfect and Schell 1996). *In vitro*, interferon-γ and GM-CSF can enhance neutrophil superoxide production, increasing the damage of *S. prolificans* hyphae by neutrophils and enhancing the fungicidal activity of macrophages-monocytes, thereby showing a positive immunomodulatory effect against this hyalohyphomycete (Groll and Walsh 2001; Gil-Lamaignere et al. 2001). Both G-CSF and GM-CSF accelerate myelopoiesis and decrease the duration of neutropenia, but they are different cytokines with different targets and immunomodulatory effects. Both *in vitro* and *ex vivo*, G-CSF, GM-CSF, and M-CSF have been shown to increase the fungicidal action of phagocytes against *Candida* and *Aspergillus* in a variety of experimental systems (Roilides et al. 1995a, b).

17.5.5 Recombinant Cytokines

Various biopotent molecules have been studied for their potential to modulate and restore impaired immune functions required to resist fungal infections. Recombinant cytokines and cationic peptides are two classes of low-molecular-weight compounds that have shown promise in this area of research. These include recombinant human cytokines including granulocyte colony-stimulating factor (rHuG-CSF), recombinant human macrophage colony-stimulating factor (rHuM-CSF), interferons, etc., some of which have shown encouraging results (Shukla et al. 1992). The addition of cytokines and other immunomodulatory approaches to antifungal therapy of cryptococcosis are actively being explored (Casadevall and Pirofski 2001; Lutz et al. 2000; Clemons et al. 2001). Studies are currently ongoing in animal models and phase I/II human trials with recombinant human gene product interferon (IFN)-γ, and monoclonal antibodies directed against cryptococcal capsular polysaccharide (Lutz et al. 2000; Clemons et al. 2001; Pappas et al. 2001; Larsen et al. 2002).

17.6 Plant Components as Immunomodulatory Agents

The use of plant products as immunostimulants has a traditional history. However, the isolation of the active principals involved did not gain momentum till the nineteenth century. Plants synthesize chemicals as part of their defense against pathogens. Many such compounds occur in nature as anti-feedant and anti-infectant chemicals, and are found effective against microbes.

> "Four vegetables are indispensable for the well being of man: Wheat, the Grape, the Olive and the Aloe. The first nourishes him, the second raises his spirit, the third brings him harmony and the fourth cures him".
>
> Christopher Columbus

Among the natural (plant) products studied in Central Drug Research Institute (CDRI) for immune modulating activity, iridoid glucosides from *Nyctanthes arbortristis* showed a promising immunomodulatory effect against systemic candidiasis in mouse (Khan et al. 1995). The ethanol (50%) extracts of seeds, roots, and flowers of *N. arbortristis* (arbortristosides A and C) showed immune stimulant activity based on enhanced haemagglutinating antibody (HA) titre, plague forming cells (PFC) counts, delayed type hypersensitivity (DTH), and macrophage migration inhibition (MMI). The immune stimulant effect of seed was, however, more significant in ethanol extract compared to *n*-butanol fraction of all the plant parts. The protective effect of these extracts/fractions was found to be possibly due to immune stimulatory activity of arbor-tristoside A and C elicited by significant (<0.001) increase in humoral and DTH response to sheep red blood cells (SRBCs) and MMI in Balb/c mouse (Khan et al. 1995).

Aloe vera, also known as the medicinal aloe, is a species of succulent plant that probably originated in Northern Africa. *Aloe vera* has been used as an

immunostimulant having antibacterial and antifungal activities. *Aloe vera* extracts have been shown to inhibit the growth of fungi that cause tinea (Shamim et al. 2004). Topical application of *Aloe vera* may be effective for genital herpes and psoriasis (Vogler and Ernst 1999). However, it is not effective for the prevention of radiation-induced injuries, nor does it offer protection from sunburn or suntan (Feily and Namazi 2004). In a double-blind clinical trial the group using an *Aloe vera* containing dentifrice and the group using a fluoridated dentifrice both demonstrated a statistically significant reduction of gingivitis and plaque (de Oliveira et al. 2008).

Acemannan, the major fraction of aloe polysaccharides, has been extensively studied for immunomodulatory effects. Reports showed that these β (1,4)-linked acetylated mannans are able to increase phagocytic activities (Egger et al. 1996; Jae et al. 2001). CARN 750, an acemannan, stimulated leukocytes and lymphocytes in a dose-dependant manner, as well as triggered the release of IL-1, IL-6 and TNF-α. Administrations of CARN 750 also showed a positive influence on lymphocyte proliferation in the spleen and bone marrow (Egger et al. 1996), both of which are essential lymphoid organs that produce and differentiate lymphocytes. In fact, earlier reports mentioned the ability of acemannans to stimulate Th_2 cells. It has been postulated that the actions of acemannan may be attributed to the residual presence of aloerides (Pugh et al. 2001). In accord with this postulate, polysaccharides from crude extracts have been shown to enhance transcription of cytokines. High concentrations of aloeride also seemed to enhance macrophage activities (Pugh et al. 2001), and may be a contributing factor for the increased phagocyte stimulation by acemannan.

Traditionally, *Angelica sinensis,* because of its high phytoestrogenic content, is reputed to have a stabilizing effect on the female hormonal system, making it useful in treating menstrual problems. In China, it is often referred to as "female ginseng". Constituents of *A. sinensis* include ligustilide, butylidene phthalide, and β-sitosterol (Bensky and Gamble 1993). Essential oil extracts from *Angelica* were shown to inhibit selected pathogens (Elgayyar et al. 2001), and polysaccharides were shown to induce activation of both specific and nonspecific immune components (Ahn et al. 1998). In a later study, a polysaccharide, angelan, isolated from roots of *Angelica gigas*, was shown to trigger the release of cytokines IL-2, -4, -6, and INF-γ from macrophages. Cytokine release was found to occur in a sequential manner, with IL-6 presenting an almost immediate increase, followed by IL-4, with IL-2 having the slowest rate of increase (Sang et al. 1998). The increase in IL-2 may be attributed to the preceding increase in IL-6. In accord with the type of cytokines released, it can be postulated that with the initial rapid rise in mediators that activate Th_2 cells, the primary effect of angelan is the enhancement of T cell-dependent antibody production.

Ginger is a domestic remedy also known for its anti-infectant effects. Essential oil constituents from rhizomes of *Z. officinale* were found to decrease growth rate of a variety of bacteria and fungi, including *Staphylococcus* and *Candida* (Martins et al. 2001). The most effective antimicrobial constituent was found to be citral. Curcumene, a sesquiterpene, from ginger oil was found to inhibit *Rhizoctonia solani* (Agarwal et al. 2001). Another structurally characterized compound, 1,7-bis(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one also showed inhibitory effects on

Pyricularia oryzae (Ramos et al. 1996). Ethanol soluble extracts from the rhizomes of *Z. officinale* were tested for their action on cytokines and found to promote the secretion of IL-1 and IL-6 in a time- and dose-dependant manner (Hori et al. 2003).

17.7 Monoclonal Antibody-Based Immunomodulator

Currently, there is only one antibody reagent licensed for use against an infectious disease in the United States — Palivizumab, although it is not used against fungal infections. Licensed in 1998, Palivizumab is a neutralizing, humanized monoclonal antibody (mAb) to protein F on respiratory syncytial virus (RSV). Because the antiviral activity of Palivizumab was associated with a reduction in inflammatory mediator release in a murine model of RSV (Mejias et al. 2004), its mechanism of action probably involves immunomodulation.

Recently, Mycograb, a human recombinant antibody fragment, was shown to significantly improve the response to amphotericin B in patients with invasive candidiasis (Pachl et al. 2006). Patients who received Mycograb and Amphotericin B showed a higher rate of complete overall response on day 10 of therapy, a significantly better mycological response and less *Candida*-attributable mortality than patients who received amphotericin B and a placebo. Mycograb is a recombinant antibody fragment lacking an Fc region, and is produced from a human anti-Hsp90 (heat-shock protein 90) cDNA library with an epitope that inhibits fungal Hsp90, NILKVIRKNIVKK (Matthews and Burnie 2001). Nonetheless, the *in vitro* activity of Mycograb (with amphotericin B and other antifungal agents) against resistant *Candida* and other fungal species (Matthews et al. 2003; Nooney et al. 2005) suggests it could hold promise as a broadly active antifungal agent.

The first mAb used to treat a fungal disease in humans was the mouse mAb 18B7, which binds to the cryptococcal capsular polysaccharide glucuronoxylomannan (Larsen et al. 2005; Casadevall et al. 1998). Extensive preclinical testing revealed that 18B7 augmented host defense mechanisms against *C. neoformans*, *in vitro* and *in vivo*, which has been reviewed by Casadevall et al. (2005). Although there is concern that mAbs could have limited usefulness for microbes that demonstrate high antigenic variation and mutability, combinations of mAbs have shown promise in overcoming this limitation (Ter et al. 2006).

17.8 Immune Peptides as Immunomodulators

Among the various categories of immunomodulating agents reported so far, certain peptides seem to hold better promise (Shukla et al. 1992). At least nine immune-defense peptide products are commercially available with annual sales of over $4 billion (Latham 1999). Six novel peptides viz., hexapeptide (89/215), glycopeptides (89/729, 90/341), pentapeptide (SP-5) and lipopeptides (86/450, 84/201),

synthesized have been evaluated in CDRI for potent immunostimulant activity (Khan and Jain 2000). Hexapeptide 89/215, lipopeptide 86/450 and glycopeptide 90/341 provided marked protection to mice against systemic candidiasis. The peptides 86/450 and 84/201 also stimulated antibody and DTH in guinea pigs in the presence of Freund's complete adjuvant (Shukla et al. 1992). The lipopeptide (86/450) gave a sevenfold increase in HA titre, 135% increase in plaque-forming cells (PFC) and 218% increase in sheep red blood cells (SRBCs) in a mouse model. The 86/450 also induced nonspecific immunostimulation in the treated animals, as evidenced by the macrophage migration inhibition (MMI) and phagocytosis of (^{14}C) labeled *E. coli* of the peritoneal macrophage, and enhanced uptake of ^{3}H thymidine by the splenocytes of treated versus untreated normal mice (Djeu et al. 1986). Thus, 86/450 may have a direct stimulating effect on the lymphocytes.

17.9 Conclusion

As the world's immunodeficient population grows as a result of the HIV pandemic and increased use of highly immune suppressive regimens to treat a variety of illnesses, the challenges of mycotic infections are expected to continue. The increase in the population of compromised hosts, coupled with exciting biotechnology advances, has spurred on research into the immune response to fungi. The relative importance and interconnected responses of innate and adaptive immune in protection are actively being investigated. In order to develop a prospective chemotherapeutic agent against opportunistic infections, it is important to know that host factors such as degree of immunological debility as well as recovery of immune functions to normality may contribute significantly to a successful elimination of the pathogens. Concomitantly, methods of immune manipulation and reconstitution have become promising areas of research activity. Future therapies for invasive fungal may include agents that augment the antifungal activity of effector cells and alter Th balance. While there is some clinical experience with the use of recombinant cytokines as an adjunct to antifungal drug therapy (Roilides and Walsh 2004), clinical trials in highly compromised hosts are needed, as many questions remain regarding safety, efficacy, and optimal use. Another potential approach is manipulation of cellular signaling cascades.

During the last decade, immunomodulators have evolved to become a viable adjunct to established therapeutic modalities in infectious diseases. Immunomodulators of natural, synthetic, and recombinant origin can stimulate host defense mechanisms for prophylaxis and treatment of diverse viral, bacterial, parasitic, and fungal diseases. Many immunomodulators act by inducing endogenous production of cytokines. The therapeutic value of cytokines in infectious diseases is increasingly being recognized. Overall, the use of cytokines as therapeutic tools in the setting of infections has given rise to an optimistic view of the use of such reagents. Approaches based on neutralization of immunosuppressive cytokines in infectious diseases are

also an area of considerable promise. Limitations of therapy with exogenous cytokines, however, have to be recognized. These are associated with the inherent toxicity of such material, their unclear pharmacological behavior, and their pleiotropic effects. Efficacy of exogenous cytokines capable of potentiating normal host defense mechanisms may be curtailed in immunocompromised patients lacking pertinent effector cells or having disease-related factors which prevent lymphocyte activation. In view of the short half-life of cytokines and high doses necessary to achieve therapeutic benefits, stimulation by chemically well-characterized immunomodulators of endogenous cytokines may be more advantageous. Selective stimulation by suitable immunomodulators of discrete lymphocyte subpopulations and cytokines important in protective effector mechanisms against a given infection will play an increasingly important role. Some immunomodulator preparations are already licensed for use in patients. Other compounds are being extensively investigated in preclinical and clinical studies. Nonantibiotic agents such as immunomodulators possessing antimicrobial activity offer a novel approach as an adjunct modality for the treatment of infectious and malignant conditions in the coming decades.

The future use of adjunctive immunomodulators for infectious diseases requires a better understanding of microbial pathogenesis and the relative need for immune activation versus immune modulation in the context of the immune response of the affected individual. In light of the fact that certain infectious diseases reflect an insufficient response, whereas others reflect an overly exuberant response, different types of interventions are likely to be required, depending on the immune status of the patient.

References

Agarwal M, Walia S, Dhingra S, Khambay BPS (2001) Insect growth inhibition, antifeedant and antifungal activity of compounds isolated/derived from *Zingiber officinale* Roscoe (ginger) rhizomes. Pest Manag Sci 57:289–300

Ahn K, Woong SS, Hwan MK, Sang BH, Kim IH (1998) Biotech letters 20(1):5–7

Armitage JO (1998) Emerging applications of recombinant human granulocyte-macrophage colony-stimulating factor. Blood 92:4491–4508

Arruda C, Franco MF, Kashino SS, Nascimento FR, Fazioli Rdos A, Vaz CA, Russo M, Calich VL (2002) Interleukin-12 protects mice against disseminated infection caused by *Paracoccidioides brasiliensis* but enhances pulmonary inflammation. Clin Immunol 103:185–195

Barbaric D, Shaw PJ (2001) *Scedosporium* infection in immunocompromised patients: successful use of liposomal amphotericin B and itraconazole. Med Pediatr Oncol 37:122–125

Bauman SK, Huffnagle GB, Murphy JW (2003) Effects of tumor necrosis factor alpha on dendritic cell accumulation in lymph nodes draining the immunization site and the impact on the anticryptococcal cell-mediated immune response. Infect Immun 71:68–74

Bensky D, Gamble A (1993) Chinese herbal medicine; materica medica (revised edition). Eastland Press, Seattle, USA

Bomford R (1988) Immunomodulators from plants and fungi. Phytother Res 2(4):159–164

Boneberg EM, Hareng L, Gantner F, Wendel A, Hartung T (2000) Human monocytes express functional receptors for granulocyte colony-stimulating factor that mediate suppression of monokines and interferon-gamma. Blood 95:270–276

Brieland JK, Jackson C, Menzel F, Loebenberg D, Cacciapuoti A, Halpern J et al (2001) Networking in lungs of immunocompetent mice in response to inhaled *Aspergillus fumigatus*. Infect Immun 69:1554–1560

Casadevall A, Pirofski LA (2001) Adjunctive immune therapy for fungal infections. Clin Infect Dis 33:1048–1056

Casadevall A, Cleare W, Feldmesser M, Glatman-Freedman A, Kozel TR, Lendvai N, Mukherjee J, Pirofski L, Rivera J, Rosas AL et al (1998) Characterization of a murine monoclonal antibody to *C. neoformans* polysaccharide which is a candidate for human therapeutic studies. Antimicrob Agents Chemother 42:1437–1446

Cenci E, Bartocci A, Puccetti P, Mocci S, Stanley ER, Bistoni F (1991) Macrophage colony-stimulating factor in murine candidiasis: serum and tissue levels during infection and protective effect of exogenous administration. Infect Immun 59:868–872

Cenci E, Romani L, Mencacci A, Spaccapelo R, Schiaffella E, Puccetti P, Bistoni F (1993) Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of *Candida albicans*. Eur J Immunol 23:1034–1038

Cenci E, Mencacci A, Fe d'Ostiani C, Del Sero G, Mosci P, Montagnoli C, Bacci A, Romani L (1998) Cytokine- and T helper-dependent lung mucosal immunity in mice with invasive pulmonary aspergillosis. J Infect Dis 178:1750–1760

Cenci E, Mencacci A, Del Sero G, Bacci A, Montagnoli C, d'Ostiani CF et al (1999) Interleukin-4 causes susceptibility to invasive pulmonary aspergillosis through suppression of protective type I responses. J Infect Dis 180:1957–1968

Centeno-Lima S, Silveira H, Casimiro C, Aguiar P, do Rosario VE (2002) Kinetics of cytokine expression in mice with invasive aspergillosis: lethal infection and protection. FEMS Immunol Med Microbiol 32:167–173

Chiller T, Farrokhshad K, Brummer E, Stevens DA (2001) The interaction of human monocytes, monocyte-derived macrophages, and polymorphonuclear neutrophils with caspofungin (MK-0991), an echinocandin, for antifungal activity against *Aspergillus fumigatus*. Diagn Microbiol Infect Dis 39:99–103

Chiller T, Farrokhshad K, Brummer E, Stevens DA (2002) Effect of granulocyte colony-stimulating factor and granulocyte–macrophage colony stimulating factor on polymorphonuclear neutrophils, monocytes or monocyte-derived macrophages combined with voriconazole against *Cryptococcus neoformans*. Med Mycol 40:21–26

Clemons KV, Stevens DA (2001) Overview of host defense mechanisms in systemic mycoses and the basis for immunotherapy. Semin Respir Infect 16:60–66

Clemons KV, Lutz JE, Stevens DA (2001) Efficacy of recombinant gamma interferon for treatment of systemic cryptococcosis in SCID mice. Antimicrob Agents Chemother 45:686–689

Clemons KV, Grunig G, Sobel RA, Mirels LF, Rennick DM, Stevens DA (2000) Role of IL-10 in invasive aspergillosis: increased resistance of IL-10 gene knockout mice to lethal systemic aspergillosis. Clin Exp Immunol 122:186–191

Committee on New Directions in the Study of Antimicrobial Therapeutics (2006) Immunomodulation: treating infectious diseases in a microbial world: report of two workshops on novel antimicrobial therapies. National Academies Press, Washington, DC

Constantopoulos A, Najjar VA, Smith JW (1972) Tuftsin deficiency: a new syndrome with defective phagocytosis. J Pediat 80:564–572

Constantopoulos A, Najjar V, Wish JB, Necheles TH, Stolbach LL (1973) Defective phagocytosis due to tuftsin deficiency in splenectomized subjects. Am J Dis Child 125:663–665

Coste A, Linas MD, Cassaing S, Bernad J, Chalmeton S, Seguela JP, Pipy B (2002) A sub-inhibitory concentration of amphotericin B enhances candidastatic activity of interferon-gamma- and interleukin-13- treated murine peritoneal macrophages. J Antimicrob Chemother 49:731–740

Decken K, Kohler G, Palmer-Lehmann K, Wunderlin A, Mattner F, Magram J, Gately MK, Alber G (1998) Interleukin-12 is essential for a protective Th1 response in mice infected with *Cryptococcus neoformans*. Infect Immun 66:4994–5000

Deepe GS Jr, Gibbons R (2000) Recombinant murine granulocyte–macrophage colony-stimulating factor modulates the course of pulmonary histoplasmosis in immunocompetent and immunodeficient mice. Antimicrob Agents Chemother 44:3328–3336

Deepe GS Jr, Gibbons RS (2003) Protective and memory immunity to *Histoplasma capsulatum* in the absence of IL-10. J Immunol 171(10):5353–5362

de Oliveira SM, Torres TC, Pereira SL, Mota OM, Carlos MX (2008) Effect of a dentifrice containing *Aloe vera* on plaque and gingivitis control: A double-blind clinical study in humans. J Appl Oral Sci 16(4):293–296

Del Sero G, Mencacci A, Cenci E, d'Ostiani CF, Montagnoli C, Bacci A et al (1999) Antifungal type 1 responses are upregulated in IL-10-deficient mice. Microbes Infect 1:1169–1180

Djeu JY, Blanchard DK, Halkias D et al (1986) Growth inhibition of *C. albicans* by human polymorphonuclear neutrophils; activation by interferon and tumour necrosis factor. J Immunol 137:2480–2484

Egger SF, Brown GS, Kelsey LS, Yates KM, Rosenberg LJ, Talmadge JE (1996) Int J Immunopharm 18(2):113–126

Eggimann P, Garbino J, Pittet D (2003) Management of *Candida* species infections in critically ill patients. Lancet Infect Dis 3(12):772–785

Elgayyar M, Draughon FA, Golden DA, Mount JRJ (2001) Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. J Food Prot 64(7):1019–1024

Ellis M, Watson R, McNabb A, Lukic ML, Nork M (2002) Massive intracerebral aspergillosis responding to combination high dose liposomal amphotericin B and cytokine therapy without surgery. J Med Microbiol 51:70–75

Feily A, Namazi MR (2009) *Aloe vera* in dermatology: a brief review. G Ital Dermatol Venereol 144:84–91

Feldmesser M, Mukherjee J, Casadevall A (1996) Combination of 5-flucytosine and capsule-binding monoclonal antibody in the treatment of murine *Cryptococcus neoformans* infections and *in vitro*. J Antimicrob Chemother 37:617–622

Franzke A, Piao W, Lauber J, Gatzlaff P, Konecke C, Hansen W et al (2003) G-CSF as immune regulator in T cells expressing the G-CSF receptor: implications for transplantation and autoimmune diseases. Blood 102:734–739

Fridkin M, Gottlieb P (1981) Tuftsin, Thr-Lys-Pro-Arg. Anatomy of an immunologically active peptide. Mol Cell Biochem 41:73–97

Gaviria JM, van Burik JA, Dale DC, Root RK, Liles WC (1999) Comparison of interferon-gamma, granulocyte colony-stimulating factor, and granulocyte–macrophage colony-stimulating factor for priming leukocyte-mediated hyphal damage of opportunistic fungal pathogens. J Infect Dis 179:1038–1041

Ghannoum MA (2001) *Candida:* a causative agent of an emerging infection. J Investig Dermatol 6:188–196

Gildea LA, Gibbons R, Finkelman FD, Deepe GS Jr (2003) Overexpression of interleukin-4 in lungs of mice impairs elimination of Histoplasma capsulatum. Infect Immun 71:3787–3793

Gil-Lamaignere C, Maloukou A, Winn RM, Panteliadis C, Roilides E (2001) The Eurofung Network. Effects of interferon-gamma and granulocyte–macrophage colony-stimulating factor on human neutrophil-induced hyphal damage of *Scedosporium* spp. Abstracts of the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago. Abstract J-469

Gil-Lamaignere C, Roilides E, Maloukou A, Georgopoulou I, Petrikkos G, Walsh TJ (2002a) Amphotericin B lipid complex exerts additive antifungal activity in combination with polymorphonuclear leucocytes against *Scedosporium prolificans* and *Scedosporium apiospermum*. J Antimicrob Chemother 50:1027–1030

Gil-Lamaignere C, Roilides E, Mosquera J, Maloukou A, Walsh TJ (2002b) Antifungal triazoles and polymorphonuclear leukocytes synergize to cause increased hyphal damage to *Scedosporium prolificans* and *Scedosporium apiospermum*. Antimicrob Agents Chemother 46:2234–2237

Gonzalez GM, Tijerina R, Najvar LK, Bocanegra R, Luther M, Rinaldi MG, Graybill JR (2001) Correlation between antifungal susceptibilities of *Coccidioides immitis in vitro* and antifungal treatment with caspofungin in a mouse model. Antimicrob Agents Chemother 45: 1854–1859

Gottlieb P, Beretz A, Fridkin M (1982) Tuftsin analogs for probing its specific receptor site on phagocytic cells. Eur J Biochem 125:631–638

Groll AH, Walsh TJ (2001) Uncommon opportunistic fungi: new nosocomial threats. Clin Microbiol Infect 7(Suppl 2):8–24

Groll AH, Walsh TJ (1999) Azoles: Triazoles. In: Yu VL et al (eds) Antimicrobial Therapy and Vaccines, 1st edn. Williams and Wilkins, Philadelphia, pp 1158–1165

Gupta CM, Haq W (2005) Tuftsin-bearing liposomes as antibiotic carriers in treatment of macrophage infections. Methods Enzymol 391:291–304

Gupta CM, Puri A, Jain RK, Bali A, Anand N (1986) Protection of mice against *Plasmodium berghei* infection by a tuftsin derivative. FEBS Lett 205:351–354

Guru PY, Agrawal AK, Singha UK, Singhal A, Gupta CM (1989) Drug targeting in *Leishmania donovani* infections using tuftsin-bearing liposomes as drug vehicles. FEBS Lett 245: 204–208

Hajjeh RA, Warnock DW (2003) Epidemiology of systemic fungal diseases: overview. In: Dismukes WE, Pappas PG, Sobel JD (eds) Clinical Mycology. Oxford University Press, New York, pp 23–30

Hori Y, Miura T, Hirai Y, Fukumura M, Nemoto Y, Toriizuka K, Ida Y (2003) Pharmacognostic studies on ginger and related drugs – part 1: five sulfonated compounds from Zingeberis rhizome (Shokyo). Phytochemistry 62:613–617

Jae KL, Myung KL, Yeo PY, Young SK, Jong SK, Yeong SK, Kyung JK, Seong SH, Chong-kil L (2001) Acemannan purified from Aloe vera induces phenotypic and functional maturation of immature dendritic cells. Int Immunopharm 1(7):1275–1284

Kawakami K, Qureshi MH, Koguchi Y, Zhang T, Okamura H, Kurimoto M, Saito A (1999a) Role of TNF-alpha in the induction of fungicidal activity of mouse peritoneal exudate cells against *Cryptococcus neoformans* by IL-12 and IL-18. Cell Immunol 193:9–16

Kawakami K, Qureshi MH, Zhang T, Koguchi Y, Xie Q, Kurimoto M, Saito A (1999b) Interleukin-4 weakens host resistance to pulmonary and disseminated cryptococcal infection caused by combined treatment with interferon-gamma-inducing cytokines. Cell Immunol 197:55–61

Kawakami K, Koguchi Y, Qureshi MH, Kinjo Y, Yara S, Miyazato A et al (2000a) Reduced host resistance and Th1 response to *Cryptococcus neoformans* in interleukin-18 deficient mice. FEMS Microbiol Lett 186:121–126

Kawakami K, Qureshi MH, Zhang T, Koguchi Y, Yara S, Takeda K et al (2000b) Involvement of endogenously synthesized interleukin (IL)-18 in the protective effects of IL-12 against pulmonary infection with *Cryptococcus neoformans* in mice. FEMS Immunol Med Microbiol 27:191–200

Khan ZK, Jain P (2000) Antifungal agents and Immunomodulators in systemic mycoses. Indian J Chest Dis Allied Sci 42:345–3551

Khan MA, Owais M (2005) Immunomodulator tuftsin increases the susceptibility of *Cryptococcus neoformans* to liposomal amphotericin B in immunocompetent BALB/c mice. J Drug Target 13(7):423–429

Khan MA, Owais M (2006) Toxicity, stability and pharmacokinetics of amphotericin B in immunomodulator tuftsin-bearing liposomes in a murine model. J Antimicrob Chemother 58(1):125–132

Khan MA, Syed FM, Nasti HT, Saima Dagger K, Haq W, Shehbaz A, Owais M (2003) Use of tuftsin bearing nystatin liposomes against an isolate of *Candida albicans* showing less *in vivo* susceptibility to amphotericin B. J Drug Target 11(2):93–99

Khan MA, Nasti TH, Saima K, Mallick AI, Firoz A, Wajahul H, Ahmad N, Mohammad O (2004) Co-administration of immunomodulator tuftsin and liposomised nystatin can combat less susceptible *Candida albicans* infection in temporarily neutropenic mice. FEMS Immunol Med Microbiol 41(3):249–258

Khan MA, Ahmad N, Moin S, Mannan A, Wajahul H, Pasha ST, Khan A, Owais M (2005a) Tuftsin-mediated immunoprophylaxis against an isolate of *Aspergillus fumigatus* shows less *in vivo* susceptibility to amphotericin B. FEMS Immunol Med Microbiol 44(3): 269–276

Khan MA, Nasti TH, Owais M (2005b) Incorporation of amphotericin B in tuftsin-bearing liposomes showed enhanced efficacy against systemic cryptococcosis in leucopenic mice. J Antimicrob Chemother 56(4):726–731

Khan MA, Faisal SM, Mohammad O (2006) Safety, efficacy and pharmacokinetics of tuftsin-loaded nystatin liposomes in murine model. J Drug Target 14(4):233–241

Khan ZK, Manglani A, Shukla PK et al. (1995) Immunomodulatory effect of plant extracts and iridoid glucosides from *Nyctanthes arbortristis* against systemic candidiasis in mice. Int J Pharmacog 33:297–304

Khemani S, Brummer E, Stevens DA (1995) *In vivo* and *in vitro* effects of macrophage colony-stimulating factor (M-CSF) on bronchoalveolar macrophages for anti-histoplasmal activity. Int J Immunopharmacol 17:49–53

Kontoyiannis D, Wessel V, Bodey G, Rolston K (2000) Zygomycosis in the 1990s in a tertiary care center. Clin Infect Dis 30:851–856

Kudeken N, Kawakami K, Saito A (1999) Cytokine-induced fungicidal activity of human polymorphonuclear leukocytes against *Penicillium marneffei*. FEMS Immunol Med Microbiol 26:115–124

Kuhara T, Uchida K, Yamaguchi H (2000) Therapeutic efficacy of human macrophage colony-stimulating factor, used alone and in combination with antifungal agents, in mice with systemic *Candida albicans* infection. Antimicrob Agents Chemother 44:19–23

Kullberg BJ (1997) Trends in immunotherapy of fungal infections. Eur J Clin Microbiol Infect Dis 16:51–55

Kurt-Jones EA, Mandell L, Whitney C, Padgett A, Gosselin K, Newburger PE, Finberg RW (2002) Role of toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. Blood 100: 1860–1868

Labro MT (1998) Antibacterial agents — phagocytes: new concepts for old in immunomodulation. Int J Antimicrob Agents 10:11–21

Larsen RA, Pappas P, Perfect J, Aberg JA, Casadevall A, Dismukes WE (2002) The National Institute of Allergy and Infectious Diseases (NIAID) and the Mycoses Study Group. Passive immunization for therapy: the MSG 43 study. Fifth International Conference on *Cryptococcus* and Cryptococcosis, Adelaide, Australia. Abstract S3.3

Larsen RA, Pappas PG, Perfect J, Aberg JA, Casadevall A, Cloud GA, James R, FIller S, Dismukes WE (2005) Phase I evaluation of the safety and pharmacokinetics of murine-derived anticryptococcal antibody 18B7 in subjects with treated cryptococcal meningitis. Antimicrob Agents Chemother 49:952–958

Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12:310–350

Latham PW (1999) Therapeutic peptides revisited. Nat Biotechnol 17:755–757

Lutz JE, Clemons KV, Stevens DA (2000) Enhancement of antifungal chemotherapy by interferon-gamma in experimental systemic cryptococcosis. J Antimicrob Chemother 46:437–442

Lyman CA, Garrett KF, Pizzo PA, Walsh TJ (1994) Response of human polymorphonuclear leukocytes and monocytes to Trichosporon beigelii: host defense against an emerging opportunistic pathogen. J Infect Dis 170:1557–1565

Martino R, Subira M (2002) Invasive fungal infections in hematology: new trends. Ann Hematol 81:233–243

Martins AP, Salguelro L, Goncalves MJ, Proenca da Cunha A, Vila R, Cafigueral S, Mazzoni V, Tomi F, Casanova J (2001) Essential oil composition and antimicrobial activity of three Zingiberaceae from S. Tomee principe. J Planta Med 67:580–584

Masihi KN (1994a) Cytokines and immunomodulators: promising therapeutic agents. Parasitol Today 10:1–2

Masihi KN (ed) (1994b) Immunotherapy of Infections. Marcel Dekker, New York

Masihi KN (1996) Immunotherapy of microbial diseases. In: Hadden JW, Szentivanyi A (eds) Immunopharmacol reviews. Plenum, New York, pp 157–199
Masihi KN (ed) (1997) Special issue: First European Conference on Immunopharmacology. 26–29 May 1997, Berlin. Int J Immunopharmacol 19:463–617
Masihi KN, Lange W (1988) Immunomodulators and nonspecific host defence mechanisms against microbial infections. Pergamon, Oxford
Masihi KN, Lange W (eds) (1990) Immunotherapeutic prospects of infectious diseases. Springer, Berlin
Masood AK, Owais M (2006) Toxicity, stability and pharmacokinetics of amphotericin B inimmunomodulator tuftsin-bearing liposomes in a murine model. J Antimicrob Chemother 58:125–132
Matthews R, Burnie J (2001) Antifungal antibodies: a new approach to the treatment of systemic candidiasis. Curr Opin Investig Drugs 2:472–476
Matthews RC, Rigg G, Hodgetts S, Carter T, Chapman C, Gregory C, Illidge C, Burnie J (2003) Preclinical assessment of the efficacy of Mycograb, a human recombinant antibody against fungal HSP90. Antimicrob Agents Chemother 47:2208–2216
McNeil MM, Nash SL, Hajjeh RA, Phelan MA, Conn LA, Plikaytis BD, Warnock DW (2001) Trends in mortality due to invasive mycotic diseases in the United States, 1980–1997. Clin Infect Dis 33:641–647
Mejias A, Chavez-Bueno S, Rios AM, Saavedra-Lozano J, Fonseca AM, Hatfield J, Kapur P, Gomez AM, Jafri HS, Ramilo O (2004) Anti-respiratory syncytial virus (RSV) neutralizing antibody decreases lung inflammation, airway obstruction, and airway hyper-responsiveness in a murine RSV model. Antimicrob Agents Chemother 48:1811–1822
Mencacci A, Bacci A, Cenci E, Montagnoli C, Fiorucci S, Casagrande A et al (2000a) Interleukin 18 restores defective Th1 immunity to *Candida albicans* in caspase 1-deficient mice. Infect Immun 68:5126–5131
Mencacci A, Cenci E, Bacci A, Montagnoli C, Bistoni F, Romani L (2000b) Cytokines in candidiasis and aspergillosis. Curr Pharm Biotech 1:235–251
Mencacci A, Cenci BA, Bistoni F, Romani L (2000c) Host immune reactivity determines the efficacy of combination immunotherapy and antifungal chemotherapy in candidiasis. J Infect Dis 181:686–694
Montagnoli C, Bacci A, Bozza S, Gaziano R, Mosci P, Sharpe AH, Romani L (2002) B7/CD28-dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to *Candida albicans*. J Immunol 169:6298–6308
Mukherjee J, Scharff MD, Casadevall A (1994) *Cryptococcus neoformans* infection can elicit protective antibodies in mice. Can J Microbiol 40:888–892
Mukherjee J, Feldmesser M, Scharff MD, Casadevall A (1995) Monoclonal antibodies to *Cryptococcus neoformans* glucuronoxylomannan enhance fluconazole efficacy. Antimicrob Agents Chemother 39:1398–1405
Musso T, Calosso L, Zucca M, Millesimo M, Puliti M, Bulfone-Paus S, Merlino C et al (1998) Interleukin-15 activates proinflammatory and antimicrobial functions in polymorphonuclear cells. Infect Immun 66:2640–2647
Najjar VA (1981) Biochemical aspects of tuftsin deficiency syndrome. Med Biol 59:134–138
Nassar F, Brummer E, Stevens DA (1995) Different components in human serum inhibit multiplication of *Cryptococcus neoformans* and enhance fluconazole activity. Antimicrob Agents Chemother 39:2490–2493
Natarajan U, Brummer E, Stevens DA (1997) Effect of granulocyte colony-stimulating factor on the candidacidal activity of polymorphonuclear neutrophils and their collaboration with fluconazole. Antimicrob Agents Chemother 41:1575–1578
Nemunaitis J (1998) Use of macrophage colony-stimulating factor in the treatment of fungal infections. Clin Infect Dis 26:1279–1281
Netea MG, Kullberg BJ, Van der Meer JW (2004) Proinflammatory cytokines in the treatment of bacterial and fungal infections. BioDrugs 18:9–22
Nooney L, Matthews RC, Burnie JP (2005) Evaluation of Mycograb(R), amphotericin B, caspofungin, and fluconazole in combination against *Cryptococcus neoformans* by checkerboard and time-kill methodologies. Diagn Microbiol Infect Dis 51:19–29

Ortoneda M, Capilla J, Pujol I, Pastor FJ, Mayayo E, Fernandez-Ballart J, Guarro J (2002) Liposomal amphotericin B and granulocyte colony-stimulating factor therapy in a murine model of invasive infection by *Scedosporium prolificans*. J Antimicrob Chemother 49:525–529

Owais M, Ahmed I, Krishnakumar B, Jain RK, Bachhawat BK, Gupta CM (1993) Tuftsin-bearing liposomes as drug vehicles in the treatment of experimental aspergillosis. FEBS Lett 326:56–58

Owais M, Misra-Bhattacharya S, Haq W, Gupta CM (2003) Immunomodulator tuftsin augments antifilarial activity of diethylcarbamazine against experimental brugian filariasis. J Drug Target 11:247–251

Pachl J, Svoboda P, Jacobs F, Vandewoude K, van der HB, Spronk P, Masterson G, Malbrain M, Aoun M, Garbino J et al (2006) A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. Clin Infect Dis 42:1404–1413

Pagano L, Girmenia C, Mele L, Ricci P, Tosti ME, Nosari A, Buelli M et al (2001) Infections caused by filamentous fungi in patients with hematologic malignancies. A report of 391 cases by GIMEMA Infection Program. Haematologica 86:862–870

Pappas PG, Perfect JR, Cloud GA, Larsen RA, Pankey GA, Lancaster DJ et al (2001) Cryptococcosis in human immunodeficiency virus-negative patients in the era of effective azole therapy. Clin Infect Dis 33:690–699

Pappas PG, Bustamante B, Ticona E, Hamill RJ, Johnson PC, Reboli A, Aberg J, Hasbun R, Hsu HH (2004) Recombinant interferon gamma 1b as adjunctive therapy for AIDS-related acute cryptococcal meningitis. J Infect Dis 189:2185–2191

Perfect JR, Schell WA (1996) The new fungal opportunists are coming. Clin Infect Dis 22 (Suppl 2):S112–S118

Pina A, Valente-Ferreira RC, Molinari-Madlum EE, Vaz CA, Keller AC, Calich VL (2004) Absence of interleukin-4 determines less severe pulmonary paracoccidioidomycosis associated with impaired Th2 response. Infect Immun 72:2369–2378

Pinner RW, Teutsch SM, Simonsen L, Klug LA, Graber JM, Clarke MJ, Berkelman RL (1996) Trends in infectious diseases mortality in the Unites States. JAMA 275:189–193

Pirofski L, Casadevall A (2006) Immunomodulators as an antimicrobial tool. Curr Opin Microbiol 9:489–495

Pugh N, Ross SA, ElSohly MA, Pasco DS (2001) Characterisation of aloeride, a new high-molecular- weight polysaccharide from *Aloe vera* with potent immunostimulatory activity. J Agric Food Chem 49:1030–1034

Pursell K, Verral S, Daraiesh F, Shrestha N, Skariah A, Hasan E, Pitrak D (2003) Impaired phagocyte respiratory burst responses to opportunistic fungal pathogens in transplant recipients: *in vitro* effect of r-metHuG-CSF (Filgrastim). Transplant Infect Dis 5:29–37

Ramos RA, De la Torre RA, Alonso N, Villaescusa A, Betancourt J, Vizoso AJ (1996) Screening of medicinal plants for induction of somatic segregation activity in *Aspergillus nidulans*. J Ethnopharmacol 52:123–127

Revankar SG, Patterson JE, Sutton DA, Pullen R, Rinaldi MG (2002) Disseminated phaeohyphomycosis: review of an emerging mycosis. Clin Infect Dis 34:467–476

Rippon JW (1988) Pseudallescheriasis. In: Rippon JW (ed) Medical mycology, 3rd edn. WB Saunders Company, Philadelphia, pp 651–680

Roilides E, Pizzo PA (1992) Modulation of host defence by cytokines: Evolving adjuncts in prevention and treatment of serious infections in immunocompromised hosts. Clin Infect Dis 15:508–524

Roilides E, Walsh T (2004) Recombinant cytokines in augmentation and immunomodulation of host defenses against *Candida* spp. Med Mycol 42:1–13

Roilides E, Uhlig K, Venzon D, Pizzo PA, Walsh TJ (1993) Enhancement of oxidative response and damage caused by human neutrophils to *Aspergillus fumigatus* hyphae by granulocyte colony-stimulating factor and gamma interferon. Infect Immun 61:1185–1193

Roilides E, Holmes A, Blake C, Pizzo PA, Walsh TJ (1995a) Effects of granulocyte colony-stimulating factor and interferon-gamma on antifungal activity of human polymorphonuclear

neutrophils against pseudohyphae of different medically important *Candida* species. J Leukoc Biol 57:651–656

Roilides E, Sein T, Holmes A, Chanock S, Blake C, Pizzo PA, Walsh TJ (1995b) Effects of macrophage colony-stimulating factor on antifungal activity of mononuclear phagocytes against *Aspergillus fumigatus*. J Infect Dis 172:1028–1034

Roilides E, Kadiltsoglou I, Dimitriadou A, Hatzistilianou M, Manitsa A, Karpouzas J, Pizzo PA, Walsh TJ (1997) Interleukin-4 suppresses antifungal activity of human mononuclear phagocytes against *Candida albicans* in association with decreased uptake of blastoconidia. FEMS Immunol Med Microbiol 19:169–180

Roilides E, Dignani MC, Anaissie EJ et al (1998a) The role of immunoreconstitution in the management of refractory opportunistic fungal infections. Med Mycol 36(Suppl 1):12–25

Roilides E, Sein T, Schaufele R, Chanock SJ, Walsh TJ (1998b) Increased serum concentrations of interleukin-10 in patients with hepatosplenic candidiasis. J Infect Dis 178:589–592

Roilides E, Dimitriadou-Georgiadou A, Sein T, Kadiltsoglou I, Walsh TJ (1998c) Tumor necrosis factor alpha enhances antifungal activities of polymorphonuclear and mononuclear phagocytes against *Aspergillus fumigatus*. Infect Immun 66:5999–6003

Roilides E, Maloukou A, Gil-Lamaignere C, Winn RM, Panteliadis C, Walsh TJ (2001) Differential effects of interleukin 15 on hyphal damage of filamentous fungi induced by human neutrophils. Abstracts of the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago. Abstract J-468

Roilides E, Lyman CA, Filioti J, Akpogheneta O, Sein T, Lamaignere CG, Petraitiene R, Walsh TJ (2002) Amphotericin B formulations exert additive antifungal activity in combination with pulmonary alveolar macrophages and polymorphonuclear leukocytes against *Aspergillus fumigatus*. Antimicrob Agents Chemother 46:1974–1976

Romani L, Mencacci A, Tonnetti L, Spaccapelo R, Cenci E, Puccetti P, Wolf SF, Bistoni F (1994) IL-12 is both required and prognostic *in vivo* for T helper type 1 differentiation in murine candidiasis. J Immunol 153:5167–5175

Romani L, Puccetti P, Bistoni F (1997) Interleukin-12 in infectious diseases. Clin Microbiol Rev 10:611–636

Sang BH, Young HK, Chang WL, Sun MP, Hae YL, Kyung SA, Ik HK, Hwan MK (1998) Characteristic immunostimulation by angelan isolated from *Angelica gigas*. Nakai Immunopharm 40(1):39–48

Sasaki E, Tashiro T, Kuroki M, Seki M, Miyazaki Y, Maesaki S et al (2000) Effects of macrophage colony-stimulating factor (M-CSF) on anti-fungal activity of mononuclear phagocytes against *Trichosporon asahii*. Clin Exp Immunol 119:293–298

Saunders Comprehensive Veterinary Dictionary (2007) 3rd edn. Elsevier

Shamim SS, Waseemuddin Ahmed, Iqbal Azhar (2004) Antifungal activity of *Allium*, *Aloe*, and *Solanum* species. Pharmaceutical Biology 42(7):491–498

Sheehan DJ, Hitchcock CA, Sibley CM (1999) Current and emerging azole antifungal agents. Clin Microbial Rev 12:40–79

Shukla PK, Khan ZK, Mathur KB (1992) Immunomodulatory effect of novel peptides against systemic candidiasis in mice. 32nd Annual Conf Assoc Microbiol India, Madurai, India

Siddiqui AA, Brouwer AE, Wuthiekanun V, Jaffar S, Shattock R, Irving D, Sheldon J, Chierakul W, Peacock S, Day N et al (2005) IFN gamma at the site of infection determines rate of clearance of infection in cryptococcal meningitis. J Immunol 174:1746–1750

Singh SP, Chhabra R, Srivastava VML (1992) Respiratory burst in peritoneal exudate cells in response to a modified tuftsin. Experientia 48:994–996

Singhal A, Bali A, Jain RK, Gupta CM (1984) Specific interactions of liposomes with PMN leukocytes upon incorporating tuftsin in their bilayers. FEBS Lett 178:109–113

Steinbach WJ, Schell WA, Blankenship JR, Onyewu C, Heitman J, Perfect JR (2004) *In vitro* interactions between antifungals and immunosuppressants against *Aspergillus fumigatus*. Antimicrob Agents Chemother 48:1664–1669

Stevens DL (1996) Immune modulatory effects of antibiotics. Curr Opin Infect Dis 9:165–169

Stevens DA (1998) Combination immunotherapy and antifungal chemotherapy. Clin Infect Dis 26:1266–1269

Stevens DA, Walsh TJ, Bistoni F, Cenci E, Clemons KV, Del Sero G, Fe d'Ostiani C, Kullberg BJ, Mencacci A, Roilides E, Romani L (1998) Cytokines and mycoses. Med Mycol 36 (Suppl 1):174–182

Stevens DA, Kullberg BJ, Brummer E, Casadevall A, Netea MG, Sugar AM (2000) Combined treatment: antifungal drugs with antibodies, cytokines or drugs. Med Mycol 38(suppl 1):305–315

Stuyt RJ, Netea MG, Verschueren I, Fantuzzi G, Dinarello CA, Van Der Meer JW, Kullberg BJ (2002) Role of interleukin-18 in host defense against disseminated *Candida albicans* infection. Infect Immun 70:3284–3286

Stuyt RJ, Netea MG, van Krieken JH, van der Meer JW, Kullberg BJ (2004) Recombinant interleukin-18 protects against disseminated *Candida albicans* infection in mice. J Infect Dis 189:1524–1527

Tan BKH, Vanitha J (2004) Immunomodulatory and antimicrobial effects of some traditional Chinese medicinal herbs: a review. Curr Med Chem 11:1423–1430

Ter MJ, van den Brink EN, Poon LL, Marissen WE, Leung CS, Cox F, Cheung CY, Bakker AQ, Bogaards JA, van DE et al (2006) Human monoclonal antibody combination against SARS coronavirus: synergy and coverage of escape mutants. PLoS Med 3:e237

Tonnetti L, Spaccapelo R, Cenci E, Mencacci A, Puccetti P, Coffman RL, Bistoni F, Romani L (1995) Interleukin-4 and -10 exacerbate candidiasis in mice. Eur J Immunol 25:1559–1565

Tran P, Ahmad R, Xu J, Ahmad A, Menezes J (2003) Host's innate immune response to fungal and bacterial agents *in vitro*: up-regulation of interleukin-15 gene expression resulting in enhanced natural killer cell activity. Immunology 10:9263–9270

Vazquez N, Walsh TJ, Friedman D, Chanock SJ, Lyman CA (1998) Interleukin-15 augments superoxide production and microbicidal activity of human monocytes against *Candida albicans*. Infect Immun 66:145–150

Vazquez-Torres A, Jones-Carson J, Wagner RD, Warner T, Balish E (1999) Early resistance of interleukin-10 knockout mice to acute systemic candidiasis. Infect Immun 67:670–674

Viscoli C, Girmenia C, Marinus A, Collette L, Martino P, Vandercam B, Doyen C, Lebeau B, Spence D, Krcmery V, De Pauw B, Meunier F (1999) Candidemia in cancer patients: a prospective, multicenter surveillance study by the Invasive Fungal Infection Group (IFIG) of the European Organization for Research and Treatment of Cancer (EORTC). Clin Infect Dis 28:1071–1079

Vogler BK, Ernst E (1999) *Aloe vera*: a systematic review of its clinical effectiveness. Br J Gen Prac 49:823–828

Vora S, Chauhan S, Brummer E, Stevens DA (1998a) Activity of voriconazole combined with neutrophils or monocytes against *Aspergillus fumigatus*: effects of granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor. Antimicrob Agents Chemother 42:2299–2303

Vora S, Purimetla N, Brummer E, Stevens DA (1998b) Activity of voriconazole, a new triazole, combined with neutrophils or monocytes against *Candida albicans:* effect of granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor. Antimicrob Agents Chemother 42:907–910

Willment JA, Lin HH, Reid DM, Taylor PR, Williams DL, Wong SY, Gordon S, Brown GD (2003) Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. J Immunol 171:4569–4573

Winn RM, Gil-Lamaignere C, Roilides E, Simitsopoulou M, Lyman CA, Maloukou A, Walsh TJ (2003) Selective effects of interleukin (IL)-15 on antifungal activity and IL-8 release by polymorphonuclear leukocytes in response to hyphae of *Aspergillus* species. J Infect Dis 188:585–590

Yamaguchi H, Abe S, Tokuda Y (1993) Immunomodulating activity of antifungal drugs. Ann N Y Acad Sci 685:447–457

Chapter 18
Fungal Vaccines: Recent Trends

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Abstract The concept that fungal vaccines could be useful is no longer merely an illusion. There is a need for either preventive or therapeutic vaccines to curb the rising incidence of fungal infections. With the advent of AIDS and the use of increasingly potent immunosuppressive therapies to combat autoimmune diseases, malignancies, and transplant rejection, the incidence of opportunistic fungal infection has increased. To combat such conditions, an appropriate fungal vaccine needs to be developed apart from antifungal therapy. Prior to the development of any fungal vaccine, one should know the mechanism of immune response elicited by a fungal infection, i.e., both humoral and cell-mediated. In addition, there are dormant states of infection. Because a high proportion of the fungal diseases are thought to involve reactivation of dormant foci, therapeutic immunization becomes a compelling approach to eliminate dormant organisms or to heal active infection, especially in life-threatening disease. In animal models of fungal infection, protective responses have been elicited with vaccines composed of whole organisms, soluble cell-free fractions, purified proteins, glucans, and nucleic acids. Methods to boost the immune response to vaccination include the use of adjuvants and antigen-loaded

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dendritic cells (DCs). The recognition that antibodies are effective against fungal pathogens has spawned interest in developing vaccines that elicit antibody-mediated protection. Recently, a novel polysaccharide–protein conjugate vaccine that uses the algal antigen laminarin was shown to elicit antibodies to β-glucan in fungal cell walls, and to mediate protection against both experimental candidiasis and aspergillosis. Conventional methodologies of vaccination are giving way to newer approaches, of which DNA vaccination represents one of the most exciting, not only for fungal diseases but also for all diseases mediated by pathogenic microbes. The primary method at present is to use plasmid-encoded DNA that does not replicate and is delivered by intramuscular injection producing Th1 response, thus clearing the pathogenic fungi. DNA vaccination holds promise in the field of mycology. One of the major controversies and challenges surrounding fungal vaccination is the case of the immunocompromised host. The challenge will be to enhance the effect of a vaccine when the immune system is dysfunctional. One approach would be to link the vaccine with attempts to restore the integrity of the immune system, i.e., the delivery of a vaccine in combination with cytokine or cytokines that are known to enhance the immune system. Another approach would be to link the administration of the vaccine with infusion of immunocompetent T cells or B cells to promote the immunogenicity of the vaccine.Therefore, newer methods are necessary to identify who is at highest risk, in order to determine who would benefit the most from vaccination or a combination of vaccination and immunorestorative therapy.

18.1 Fungal Vaccine

The concept that fungal vaccines could be useful is no longer merely an illusion. Previously it did not attract much attention because of the relatively low incidence of infection, and the limited geographic distribution of several fungi compared to many viral and bacterial diseases. But now the trend has changed, as fungal diseases are no longer merely arcane infections; they have gained international recognition as important causes of morbidity and mortality. There is a need for either prevention or therapeutic vaccines to curb the rising incidence of life-threatening fungal infections (Boneberger and Korting 2008; Cassone 2008; Deepe 1997; Segal 1987).

The rising incidence may be attributed to the following factors.

18.1.1 Prolonged Antibacterial Therapy

If antibacterial therapy is prolonged, it may predispose to fungal infections even in immunocompetent hosts. For example, superficial *Candida* infections involving the mucous membranes, especially the vaginal epithelium, are very common. *Aspergillus* infections, particularly the invasive forms, are also more common in patients subjected to prolonged antibiotic therapy, i.e., those suffering from chronic sinusitis, chronic bronchitis, bronchiectasis, and asthma.

18.1.2 Increased Number of AIDS Cases

The increased number of AIDS cases with depressed immunity and longer survival rate through appropriate chemotherapy (antiretroviral therapy, ART) has increased the chances of fungal infection, particularly by *Pneumocystis carinii* (or *Pneumocystis jirovecii*), *Candida albicans*, and *Cryptococcus neoformans*.

18.1.3 Increased Use of Corticosteroids

The use of corticosteroids promotes fungal infection via multiple mechanisms: leukotaxis is depressed, transient T-cell lymphopenia and monocytopenia occur, and alterations in blastogenesis and monocyte functions, and also a decreased response to lymphokines, have been reported. As might be expected from such a broad immunosuppressive effect, infections with *Candida*, *Cryptococcus*, *Histoplasma* and *Aspergillus* species have been reported in steroid-administered patients. The incidence of thrush has been very high in persons who use inhaled steroids for asthma.

18.1.4 More Aggressive Treatment Modalities of Cancer Patients, Particularly Those with Leukemia and Lymphoma

Cytotoxic chemotherapies result in granulocytopenia and defects in cell-mediated immunity, leading to an increased risk of fungal infection in these immunosuppressed patients.

18.1.5 Increased Number of Bone Marrow Transplant and Solid Organ Transplant Procedures

The immunosuppressive therapy used in transplantation primarily depresses cell-mediated immunity. Even with newer immunosuppressive agents such as Cyclosporin A, superficial and systemic candidal infections occur. Cryptococcal infection (common in renal transplants) correlates well with the degree of immunosuppression, particularly when antilymphocyte serum is used to suppress rejection, and aspergillosis in lung and heart–lung transplants is not uncommon.

18.1.6 Catheter-Borne Infections Among Patients

Integumentary breaches, as seen in the placement of intravenous or intra-arterial catheters, predispose the patients primarily to infections with *Candida* species, the source usually being the host's own flora.

18.1.7 Intravenous Drug Abuse

In intravenous drug abuse, the intact skin barrier is directly bypassed, so disseminated infections with *Candida*, *Aspergillus* and *Zygomycetes* have occurred.

18.1.8 Increased Use of Surgical Procedures

Surgical procedures can promote fungal infections in several ways, but the mechanisms are similar in that a normally sterile site is exposed to exogenous or endogenous fungi and the infection follows. Intra-abdominal candidal infections and superficial wound infections due to dressings contaminated with fungal spores have been seen. Complicated surgical patients may have multiple risk factors for fungal infections, including a prolonged course of antimicrobial therapy, poor nutritional status and metabolic derangement, all of which contribute to fungal infections.

18.1.9 The Success of Intensive Care Units in Prolonging the Survival of Highly Immunocompromised Patients

Immunosuppressed conditions of these patients allow overwhelming infection by opportunistic fungi such as *C. albicans* and *Aspergillus fumigatus*. Other fungi such as *Coccidioides immitis*, *Blastomyces dermatitidis*, and *Histoplasma capsulatum* cause more severe disease in immunocompromised hosts.

In addition to the increase in the incidence of fungal infections in recent years, the second reason that stresses the need for fungal vaccines is that, despite recent additions to our antifungal drug armamentarium, success rates for many mycoses remain unacceptably low, due to their inability to sterilize infected organs, and antifungal drug therapy is also often limited by toxicity, resistance, and high cost. So to circumvent these difficulties, alternative approaches in the form of vaccines and passive immunotherapy must be developed (Dan and Levitz 2006; Hamad 2008).

The third reason is that there are escalating advances in biotechnology which offer the hope of new and more effective vaccines.

Many challenges confront the development of fungal vaccines for humans, including differences in host susceptibility, varied pathogenic mechanisms employed by the different species of fungi, and mechanisms of host resistance. Hence, no single antigen can be expected to serve as a pan-fungal vaccine. Instead, it is likely that progress for fungal vaccines will have to be made at the level of each individual organism. In recent years, tremendous strides have been made in understanding the immunopathogenesis of medically important fungal infections and identifying putative vaccine candidates. Such discoveries will facilitate the introduction of fungal vaccines into the therapeutic armamentarium of clinicians (Deepe 2004).

18.2 Historical Perspective

Since the original pioneering work of Jenner and Pasteur, many vaccines have been licensed for viral and bacterial diseases of humans, but none have been licensed for medically important fungi.

The largest clinical trial of a vaccine for a mycosis, specifically coccidioidomycosis, was performed by the Valley Fever Vaccine Study Group (Pappagianis and The Valley Fever Study Group 1993). In this study, nearly 3,000 subjects who were skin-test negative for this fungal infection were randomized blindly to receive either whole spherules killed with formaldehyde or saline. One of the problems with the potential effectiveness of the spherule preparation was that less than 30% of the vaccinated subjects manifested evidence of a response to the spherule preparation. Thus, in almost 70% of subjects, the vaccine was not immunogenic.

Despite the failure of the vaccine for coccidioidomycosis, there are useful fungal vaccines in veterinary medicine. Equines and other mammals can be infected with *Pythia insidiosum*, which causes a debilitating cutaneous, subcutaneous, or systemic disease (de Cock et al. 1987). Two vaccines, Miller's (Miller 1981) and Mendoza's (Mendoza et al. 1992), have been demonstrated to be of use to both prevent and treat horses infected with *P. insidiosum*. The former is composed of sonicated hyphal antigens, whereas the latter is prepared from culture filtrate antigens. Mendoza et al. (2003) suggested that switching from T helper2 (Th2) to a T helper1 (Th1) response was behind the curative properties of the immunotherapeutic agents used.

A live vaccine (LTF130) against *Trichophyton verrucosum* ringworm in cattle was developed and successfully used to reduce infections in cattle herds in the former Soviet Union and some countries in Eastern Europe (Segal 1989). The use of vaccine in humans, however, has not been effective.

18.3 Goals of Immunization (Deepe 1997)

A. The first and foremost goal is that vaccine against medically important fungi should be capable of promoting the capacity of the immune system to sterilize tissues, i.e., prevent the establishment of latent state of the infecting fungus in the host; although the natural host defenses can limit the invasiveness of the infecting fungus, they do not necessarily sterilize tissues. Thus, a fraction of the infecting inoculums may survive for years, and these niduses of infection may serve as reservoirs for reactivation if the host immune system becomes impaired.
B. A useful vaccine would be one that protects against reactivation in individuals who have been infected by fungi that establish a dormant state.
C. The fungal vaccine should be such that it promotes clearance of dormant organisms. This vaccine would considerably reduce the risk of reactivation of disease in immunosuppressed as well as immunocompetent individuals.

18.4 Requirements for a Successful Immunization Program

In parallel with any large-scale institution of a fungal vaccine program, sound and thorough epidemiological data concerning fungal diseases must be accumulated. Identifying who is at risk for infection, and the age at which infection is acquired, are two key elements in the development of a vaccine program. Unfortunately, there is little epidemiological information about the fungal diseases. With a few exceptions (*C. immitis* infection), fungal diseases do not require reporting to state health departments or the Centers for Disease Control and Prevention. Thus, the extent of active cases of mycoses has been determined based on hospital discharges. However, with the increasing emphasis in medical practice on outpatient setting, it is likely that hospital information will tremendously underreport many fungal infections.

Another difficulty confronting acquisition of epidemiological data is the lack of suitable reagents for detecting prior exposure to fungal infection. Although histoplasmin, coccidioidin and spherulin have proven useful in identifying exposed individuals, functional reagents for the other mycoses, especially deep mycoses, are practically nonexistent. For example, *C. neoformans* has a ubiquitous distribution, yet the true prevalence of exposed individuals is not known. Cryptococcin was used as a skin test reagent, but its effectiveness in recognized infected individuals is unclear (Muchmore et al. 1968; Schimpff and Bennett 1975).

A comprehensive reexamination of the epidemiology of the fungi is critical in establishing the foundation for a vaccination program. Introduction of a fungal vaccine program should be united with a systematic survey of the incidence and prevalence of infections.

18.5 Mechanisms of Protection

The design of any vaccine, including those of fungi, must begin with a thorough understanding of the mechanisms that confer protective immunity in the host against a specific pathogen. When that information is available, the search for constituents from the fungus that elicits the particular type of immune response that is necessary for clearance can be undertaken.

18.5.1 Innate Immunity

The first-line host defense is the presence of physical barriers in the form of intact skin and mucous membranes (Blanco and Garcia 2008). The cellular components of innate immunity play an important role in host defenses because of their phagocytic and microbicidal properties.

Polymorphonuclear leukocytes (PMNs) are an important arm of the effector limb of immunity. These cells secrete several cytokines, including Interleukin-1 (IL-1), IL-12, tumor necrosis factor α (TNF-α), granulocyte–macrophage colony-stimulating factor (GM-CSF), and macrophage-CSF (M-CSF), which are key elements in the afferent limb of the immune response (Biron 1994; Fearon and Locksley 1996; Lloyd and Oppenheim 1992). Although these cells are short-lived (usually <48 h), they may be triggered to release these cytokines by components within a fungal vaccine or an adjuvant. For example, killed *C. neoformans* can induce human neutrophils to produce TNF-α and IL-1 (Retini et al. 1996). Similarly, killed *C. albicans* stimulates PMNs to release IL-1, IL-6, and TNF-α (Cassone et al. 1993; Djeu et al. 1990).

Natural killer (NK) cells release TNF-α after exposure to *C. albicans* (Djeu et al. 1988). Moreover, killed *C. neoformans* can trigger the release of IL-12 and TNF-α by monocytes or macrophages (Harrison and Levitz 1996; Levitz et al. 1994a, b). IFN-γ renders murine macrophages highly active in killing *P. marneffei* yeast cells by promoting the release of nitric oxide (Kudeken et al. 1998).

It is reasonable to conclude that the release of cytokines by cells of the innate immune system is central to the evolution of a protective immune response. Thus, the design of vaccines or adjuvant should include elements that can stimulate innate immunity to generate the desired protective response.

18.5.2 Acquired Immunity

T and B cells are the two principal cellular constituents of acquired immunity. Although stimulation of innate immunity by vaccine components may be achieved, it is the humoral and cellular immune systems that must be activated to provide long-lived immunity against fungi. It has been demonstrated that fungal vaccines do elicit both humoral and cell-mediated immunity in the immunized host. For some vaccines (e.g., *H. capsulatum*), a correlation between the induced immunity and protection has been observed, and it was possible to transfer the immunity adoptively (Segal 1987).

Since many species of fungi trigger both arms of the immune response, the challenge in vaccine design is to determine the relative importance of humoral and cellular immunity in host defenses and to identify fungal antigens that will elicit protective antibodies, protective T cells, or both.

18.5.2.1 Cellular Immunity (T-Cell Mediated Immunity)

Immunocompetent T cells are crucial in host defenses against many pathogenic fungi, including *B. dermatitidis, C. immitis, C. neoformans, C. albicans, H. capsulatum, Paracoccidioides brasiliensis, Penicillium marneffei*, and *P. carinii*. Numerous studies have demonstrated that animals that lack T cells are more susceptible to

infection with these fungi than are those with immunocompetent T cells (Balish et al. 1996; Beaman et al. 1979; Beck et al. 1996; Gomez et al. 1988; Hill and Harmsen 1991; Huffnagle et al. 1991; Menacci et al. 1994, 1996; Modlin et al. 1985; Mody et al. 1993; Tewari et al. 1978). Conversely, passive transfer of sensitized T cells can promote elimination of the fungus (Brummer et al. 1982; Cox et al. 1988; Theus et al. 1995).

Both $CD4^+$ and $CD8^+$ T-cell subpopulations have been shown to modify the infection with *H. capsulatum, C. Neoformans, C. albicans*, *P. marneffei*, and *P. carinii* in experimental animals (Balish et al. 1996; Beck et al. 1996; Beno et al. 1995; Deepe 1994; Gomez et al. 1988; Hill and Harmsen 1991; Huffnagle et al. 1991; Theus et al. 1995).

In humans, $CD4^+$ T cells are essential for host defenses. Most patients who manifest one of these infections often have profound depletion of $CD4^+$ T cells to $<200/\mu l$ (as seen in AIDS patients). The principal mechanism by which $CD4^+$ T cells influence host resistance is by production of cytokines. $CD4^+$ T cells can be separated into two functional categories: T helper1 (Th1) and T helper2 (Th2). The Th1 cells produce Interferon gamma (IFN-γ) and IL-2, and are the primary mediators of host defenses associated with the activation of phagocytes which is required for the clearance of fungal infection (Blanco and Garcia 2008; Seder and Paul 1994). Th2 cells, on the other hand, produce IL-4, IL-5, IL-10, and IL-13; all these cytokines are involved in antibody production, and result in susceptibility to infection (Blanco and Garcia 2008; Seder and Paul 1994).

Another effector mechanism is the cytotoxic activity. This function is contained chiefly within $CD8^+$ T-cell subpopulation, although $CD4^+$ T cells also can express cytolytic activity (Morrison et al. 1986). $CD8^+$ T cells can damage *C. albicans* and kill *C. neoformans* directly (Beno et al. 1995; Levitz et al. 1994a, b, 1995; Murphy et al. 1993).

The suggested mechanism of action is that the cytolytic T cell, through its T-cell receptor, engages a cell infected with fungi and responds to peptides bound to class I or class II MHC (major histocompatibility complex) on the cell surface, thus causing lysis. Concomitantly, recognition of the peptide–MHC complex by the T-cell receptor prompts the T cell to release macrophage-activating cytokines (e.g. IFN-γ) that can stimulate surrounding phagocytes to express antifungal activity.

Another primary contribution of T cells is the production of antibodies. These cells collaborate with B cells to initiate the synthesis and release of antibodies. T cells that are Th1 promote the synthesis of IgG2a and IgG3, whereas Th2 cells enhance the production of IgM, IgG1, IgA, and IgE (Seder and Paul 1994). Thus, the type of Th cell generated in response to infection or vaccination becomes a key issue, since the ability of an antibody to promote fungal elimination may be dependent on the isotype produced. In experimental cryptococcosis, the hierarchy of efficacy among monoclonal antibodies in fungal clearance is IgG1 > IgG3 > IgM > IgA (Mukherjee et al. 1994). Thus, both Th1 and Th2-generated antibodies are efficacious in this infectious model, although the Th2-associated antibody IgG1 appears to be most effective.

18.5.2.2 Humoral Immunity

Innate and cell-mediated immunity have been acknowledged as the primary mediators of host resistance to the fungi, but there is a growing body of evidence that antibodies are key elements of the protective immune response to some fungi.

Several mechanisms can explain the influential role of antibody (Casadevall 1995). First, antibodies may act as opsonins that enhance the phagocytosis of fungi. Ingestion of antibody-opsonized fungi may direct the organism into cellular compartments (e.g., phagolysosomes) that are inimical to survival of the fungus. Also, these molecules may promote agglutination of fungal elements, thus constraining the ability of clumped cells to disseminate. Antibodies may bind to circulating fungal substances, such as mannan from *C. albicans* or polysaccharide from the capsule of *C. neoformans*, which modulate the expression of cellular immunity. Antibodies to fungal surfaces may block binding to host cells, which may limit the extent of infection, and they may exert cidal activity or alter morphogenesis (Casadevall 1995). Hence, antibodies can exert multiple functions that augment the protective immune response.

There were early studies in which passively transferred polyclonal antisera from immunized animals failed to confer protection, because it is now known that polyclonal antisera may be comprised of protective, irrelevant, and harmful antibodies (Casadevall and Scharff 1994; Casadevall et al. 1994). Thus, the effect of a particular antiserum is dependent upon summation of the biological activities of the three types of antibodies.

Studies with antibodies to *C. albicans* and *C. neoformans* represent the best example of the powerful influence of antibody immunity to fungi. For both organisms, the administration of polyclonal antisera to animals has produced mixed results. Thus, immune sera have been protective in some but not all studies (Casadevall 1995).

The advent of monoclonal antibody technology has facilitated the concept of mapping determinants to identify those that evoke a protective antibody response. Defining the smallest epitope or epitopes that can elicit protective antibodies has the advantage of eliminating unnecessary or unwanted antibodies produced in response to complex antigenic substances. Both protective and nonprotective monoclonal antibodies to antigens from *C. albicans* and *C. neoformans* have been identified (Casadevall 1995; Mukherjee et al. 1994a; Mukherjee et al. 1994b; Mukherjee et al. 1994c).

The recognition that antibodies are effective against fungal pathogens has spawned interest in developing vaccines that elicit antibody-mediated protection. Recently, a novel glycoconjugate (polysaccharide–protein conjugate) vaccine that uses the algal antigen laminarin (a well-characterized but poorly immunogenic β-glucan preparation from the brown algae *Laminaria digitata*) conjugated with diphtheria toxoid CRM 197. This Lam-CRM conjugate was shown to elicit antibodies to β-glucan in fungal cell walls, and thus mediate protection against both experimental candidiasis and aspergillosis. Remarkably, vaccine-induced antibodies manifested direct antifungal effects, suggesting that vaccine efficacy might not require cellular or other components of the immune system (Casadevall and Pirofski 2006; Cassone and Torosantucci 2006; Torosantucci et al. 2005).

In none of the vaccine formulations under study has β-glucan been considered as a vaccine component, though this polysaccharide is critical for fungal viability and is present in all human pathogenic fungi. The study mentioned above showed that a vaccine composed of β-glucan would best fit the medical need, possibly allowing simultaneous immunization against more than one fungal infection.

Other glycoconjugate vaccines against *C. neoformans* and *Candida* have been generated (Deepe 2004; Mochon and Cutler 2005). For both fungi, the conjugated polysaccharide is the external capsular or cell-wall material (glucuronoxylmannan for *C. neoformans* or mannan for *Candida*).

β-glucan constituents are present on fungal cell surfaces accessible to antibodies, which therefore could optimize the cells and facilitate complement deposition. The importance of this process (e.g., rapid opsonization of *Blastomyces dermititidis* cells, which notoriously expose β-glucan on their surface) for antifungal protection has been highlighted in several studies (Zhang et al. 2001).

In a recent study, anti-β-glucan antibodies have been shown to increase the candidacidal activity of macrophages *in vitro* (Ishibashi et al. 2005). In a more subtle fashion, these antibodies may alter the host inflammatory responses, of which β-glucans may be a strongly inductive component (Brown and Gordon 2005), and shift the cytokine profile toward the protective Th1 pattern, as demonstrated in experimental cryptococcosis (Yuan et al. 1997).

The anti β-glucan antibodies could also be exploited for immunotherapy, particularly given the recent biotechnological developments making more feasible the *in vitro* production of human antibodies with preselected specificity (Traggiai et al. 2004). There are certain mucosal pathologies (e.g., *Candida vaginitis*) that affect millions of apparently non-immunocompromised women for whom a vaccine inducing protective antibodies would be of utmost usefulness.

18.6 Fungal Immunogens

If the goal is to create a subunit vaccine composed of inert molecules, one of the most formidable challenges to the production of a useful vaccine is the identification of one or more immunogens from a fungus (Table 18.1).

We will be dealing now with individual fungi, discussing their immunogens and any proposed vaccine against each specific fungus:

18.6.1 Blastomyces dermititidis

18.6.1.1 Immunogens

B. dermatitidis is endemic primarily to the south eastern and mid western United States. One protein-containing antigen, WI-1, now known as BAD1 (Blastomyces

Table 18.1 Fungal agents and their immunogenic components

Fungal agents	Immunogens
Yeast, yeast-like and opportunistic fungi	
Candida albicans	Mannan, enolase, mannoprotein
Cryptococcus neoformans	Capsule, cell wall, and cell membrane.
Pneumocystis carinii	MSG, p55
Dimorphic fungi	
Histoplasma capsulatum	Cell wall and cell membrane, ribosomal–protein complex
Blastomyces dermititidis	WI-1 (now known as BAD1)
Coccidioides immitis	C-ASWS, complement-fixing antigen/ Chitinase, antigen 2, 4-HPPD, 33 kDa antigen, SOW
Paracoccidioides brasiliensis	gp43
Penicillium marneffei	Mp1p

adhesion), is found on the surface of *B. dermatitidis* yeast cells (Klein and Jones 1994). It is highly immunogenic, and stimulates a humoral and T-cell mediated response. It also serves as a ligand between phagocyte and fungus, and is recognized by the CR3 and CD14 receptors (Newman et al. 1995). Immunization produced delayed-type hypersensitivity response as well as a humoral response. The magnitude of response was directly correlated with the amount of antigen injected.

18.6.1.2 Vaccine

More recently, Wuthrich et al. (2000) described a genetically engineered attenuated strain of *B. dermatitidis* that harbors a targeted deletion of BAD1. The vaccine confers sterilizing immunity against infection with isogenic and nonisogenic strains in a murine model. T cells rather than B cells are largely responsible for vaccine immunity, particularly $CD4^+$ T cells. Cytokines including IFN-γ, TNF-α, and granulocyte–macrophage colony-stimulating factor (GM-CSF) are instrumental in immunity mediated by these cells in normal hosts (Wuthrich et al. 2002). There is sufficient redundancy in immune-deficient hosts so that an absence of one or more of these products can be offset by a compensatory response in one of the others. This theme has been extended to studies in $CD4^+$ T-cell-deficient hosts (Wuthrich et al. 2003), where $CD8^+$ T cells subsume the role of T-cell help, producing cytokines IFN-γ and TNF-α needed for host defense and sterilizing immunity against *B. dermititidis*. These findings raise the prospect that engineered vaccines can protect against infection with *B. dermititidis* or related fungi, possibly even in immune-deficient hosts (Casadevall and Pirofski 2003).

18.6.2 Candida albicans

18.6.2.1 Immunogens

C. albicans has become a major cause of morbidity among patients infected with human immunodeficiency virus (HIV), and morbidity and mortality among those in intensive care units or those who have undergone aggressive chemotherapy for malignant diseases or transplantation, especially liver transplantation (Fraser et al. 1992). Both humoral and cell-mediated immunity are involved in host defenses to this pathogen.

To date, no single protein has been shown to mediate protection; one candidate protein is enolase. This protein is recognized by lymphocytes from *C. albicans*-exposed animals, and it induces a humoral response (Sundstrom et al. 1994). However its protective efficacy has not been determined.

Surface mannans of *C. albicans* induce a protective immune response in a model of disseminated candidiasis, and this ability has been associated with the presence of circulating agglutinins to the mannan fraction. Antiserum from vaccinated mice was able to passively transfer protection as well as monoclonal antibody to the mannan fraction (Han and Cutler 1995).

A mannoprotein fraction (MP-F2) from the cell wall of *C. albicans* yeasts is immunogenic in mice. This fraction preferentially stimulates $CD4^+$ T cells to produce IFN-γ but not IL-4 (Menacci et al. 1994, 1996). Moreover, immunization of mice with MP-F2 conferred a modest reduction in candidal colony-forming units (CFU) and prolongation of mean survival time compared to controls (Menacci et al. 1994).

In a recent study it has been shown that, *C. albicans* expresses CR3-related protein antigenically, structurally and functionally related to human adhesion glycoprotein, also known as Mac-1, iC3b receptor or complement receptor type 3. Active immunization with CR3-RP glycoconjugate resulted in immunoenhancement with respect to expression of IL-2 receptor subunit CD25 on B-lymphocytes, and inductive increase of the $CD4^+/CD8^+$ ratio (Paulovicova et al. 2008).

18.6.2.2 Vaccines

It should be noted that despite the efforts made, no clinically available anticandida vaccine of proven efficacy has yet been obtained. The challenge for vaccine development is the commensal nature of this organism; humans are colonized during the early postnatal period. Some recent developments seem promising.

In a study conducted by Han and Cutler (1995), it was shown that the cell surface mannans of *C. albicans* (which function as adhesins), when encapsulated into liposomes and used to vaccinate mice over 5–6 week period, circulating agglutinins specific for the mannan fraction, increased resistance to disseminated candidiasis. Antiserum also protected severe combined immunodeficiency disease (SCID) mice against disseminated disease. Two monoclonal antibodies (MAbs) specific for

candidal surface determinants were obtained: MAbB6.1 and MAbB6. MAbB6.1 is specific for a mannan epitope in the adhesin fraction, and MAbB6 is specific for a different epitope in the fraction. Both MAbs are immunoglobulin M, and both strongly agglutinate candidal cells, but only MAbB6.1 protected both normal and SCID mice against disseminated candidiasis.

In another study by Han and colleagues using a conjugate vaccine consisting of *C. albicans* mannan extract–protein (bovine serum albumin) conjugates without liposomes showed that this conjugate vaccine can induce protective antibody responses against experimental disseminated candidiasis and candidal vaginal infections (Han et al. 1999).

Cutler (2005) in his study showed that antibodies specific for short-chain β-linked oligomannosides (*Candida* mannan component) are protective against candidiasis caused by *C. albicans* and its serotypes and other important species such as *C. tropicalis* and *C. glabrata*. Although the mechanism of protection against vaginal infection requires further investigation, experimentally the ability of antibody to rapidly deposit high amounts of complement factor C3 onto the yeast cell wall is requisite for enhancing resistance against disseminated candidiasis.

Spellberg and colleagues in their study showed that vaccination of mice with rAls3p-N induces a broader antibody response than rAls1p-N (recombinant N terminus of Als1p i.e. agglutinin-like sequence-1), and a similar cell-mediated immune response. The rAls3p-N vaccine was equally as effective as rAls1p-N against disseminated candidiasis, but was more effective than rAls1p-N against oropharyngeal or vaginal candidiasis. Thus it may be concluded that rAls3p-N vaccine is a promising new candidate for further exploration to prevent systemic and mucosal candidal infections (Spellberg et al. 2006).

A recent study done by Xin et al. (2008) showed that the first fully synthetic glycopeptide vaccines combining β-mannan and peptide epitopes induce protection against disseminated candidiasis in mice. Six T cell peptides found in *C. albicans* cell-wall proteins were selected by algorithm peptide epitope searches; each was synthesized and conjugated to the fungal cell wall β-mannan trisaccharide by novel saccharide–peptide linker chemistry to create glycopeptides conjugates. This approach based on fully synthetic chemically defined immunogens should be generally useful in vaccine development.

A specific *Candida* antigen, the heat shock protein 90 (HSP 90), has been identified as an immunodominant antigen that elicits protective antibodies (Matthews et al. 1991, 1995; Raska et al. 2008).

The *Candida* ribosomal vaccine induces protection against systemic candidiasis, and elicits humoral and cell-mediated immune responses, the latter appearing to be correlated with protection (Eckstein et al. 1997; Segal 1987, 1991). This vaccine has the potential for human use.

Use of molecular engineering technology enabled production of a human antibody (known as "Mycograb"), which is being evaluated in human clinical trials (Matthews et al. 2000; Matthews and Burnie 2001; Rigg et al. 2001) as an adjunct to antifungal therapy and possible immunoprophylaxis in compromised patients (Matthews and Burnie 2001).

18.6.3 Coccidioides immitis

18.6.3.1 Immunogens

Several potential immunogens have been identified from *C. immitis*. One of these is the enzyme 4-hydroxyphenylpyruvate dioxygenase (4-HPPD), which converts the 4-hydroxyphenylpyurvate to homogenizate (Wyckoff et al. 1995).

Extract from the spherule outer wall (SOW) contains two proteins of 58 kDa and 66 kDa that stimulate T cells. These proteins, though not yet identified, are potential vaccine candidates.

An alkali-soluble, water-soluble antigen (C-ASWS) from *C. immitis* mycelia has successfully been used to vaccinate mice, protecting the mice against intranasal challenge with 50 or 500 arthroconidia (Lecara et al. 1983).

A 33 kDa antigen has been isolated from the wall of mature spherules. This antigen is recognized by sera from humans who have recovered from infection and subjects who were vaccinated with killed spherules (Galgiani et al. 1996). It also stimulates human T cells.

Additional immunogens from *C. immitis* have been cloned and sequenced. These include antigen 2 (Zhu et al. 1996) and the complement fixation/chitinase gene (Pishko et al. 1995; Yang et al. 1996; Zimmermann et al. 1996).

18.6.3.2 Vaccines

Some candidate subcellular vaccines that have exhibited protection against respiratory challenge in mice include derivatives of mechanically disrupted spherules (Zimmermann et al. 1998), a soluble proline-rich antigen (PRA) developed by Galgiani and colleagues (Shubitz et al. 2002), which proved to be the same as antigen 2 (Ag2) developed by Cox and colleagues (Zhu et al. 1996), and antigens including coccidioides-specific antigen (CSA) of Cole et al. (2004). Recombinant peptide forms of Ag2/PRA and CSA, when mixed or chemically combined, have the appropriate properties and immunogenicity that may permit them to be tested in non-human primates and humans (Cox and Magee 2004).

18.6.4 Cryptococcus neoformans

18.6.4.1 Immunogens

There has been much progress in the development of a vaccine for *C. neoformans* infection. The discovery that monoclonal antibodies to the capsular polysaccharide could mediate protection has led to rapid advances in the field. The capsular polysaccharide, which is innately poorly immunogenic, has been conjugated to

tetanus toxoid. Injection of mice with this immunogen stimulates the production of antibodies that possess the same specificity as antibodies generated during active infection. Both sources of antibodies bind to the same antigenic determinant on glucuronoxylomannan (Casadevall et al. 1992).

Proteins in the cell wall and cell membrane of this fungus are stimulatory for lymphocytes from adult humans and fetal cord blood. Thus, a mitogen or superantigen may be present in this fraction of *C. neoformans* (Mody et al. 1996).

18.6.4.2 Vaccines

Immunization is an attractive form of preventing cryptococcosis. A cryptococcal glucuronoxylomannan tetanus toxoid conjugate vaccine was developed, and it appeared to be highly immunogenic in murine models (Devi et al. 1991). Thus, progress toward vaccine development is being made. Trials in humans have not yet been carried out with any product, but it seems that, for patients at highest risk for cryptococcosis, any vaccine will have to be administered at a time when their immune systems are competent enough to make a protective response. Without a standardized skin test, it is more difficult to determine who is at risk and the magnitude of potential need.

18.6.5 Histoplasma capsulatum

18.6.5.1 Immunogens

Two early reports identified protective immunogens from *H. capsulatum*. One was ethylenediamine extract from cell wall and cell membrane (CW/M) of this fungus, and the other was the ribosomal–protein complex (Garcia and Howard 1971; Tewari et al. 1978).

18.6.5.2 Vaccines

Feit and Tewari (1974) demonstrated that immunization of mice with *H. capsulatum* yeast ribosomes or ribosomal proteins elicited a high degree of immunity, protecting up to 90% of animal from lethal challenge. The immunity was comparable to that obtained by a sublethal infection with live yeast cells (Tewari 1975). Recently, Deepe and his associates isolated a glycoprotein (HIS-62) from the cell walls and cell membranes of *H. capsulatum* var *capsulatum* yeast cells, cloned the gene encoding this antigen, and prepared a recombinant protein that was shown to be a member of the heat shock protein HSP 60 family. Immunization of mice with the native as well as with the recombinant protein conferred significant protection against a lethal challenge with *H. capsulatum* yeast cells (Gomez et al. 1991,

1995). In view of the concern of using heat shock proteins as vaccine in humans, four recombinant polypeptides were prepared from HSP 60, but these recombinants did not confer significant protection against *Histoplasma capsulatum* (Deepe et al. 1996).

18.7 *Paracoccidioides brasiliensis*

18.7.1 Immunogens

The major diagnostic antigen, a glycoprotein with a molecular mass of 43 kDa (**gp43**), has shown promise as a potential protective antigen. Gp43 elicits an immunodominant humoral response as well as a cellular immune response (Travassos et al. 1995).

18.7.2 Vaccines

Chemotherapy is the basis of treatment of paracoccidioidomycosis. Depending on the *Paracoccidioides brasiliensis* virulence, the status of host immunity, the degree of tissue involvement and fungal dissemination, treatment can be extended for long periods with an alarming frequency of relapses. Association of chemotherapy with a vaccine to boost the cellular immune response seemed a relevant project not only to reduce the duration of treatment but also to prevent relapses and improve the prognosis of anergic cases.

Gp43 (candidate immunogen), and more specifically its peptide derivative P10 carrying the $CD4^+$ T-cell epitope, were used as peptide vaccines in mice (Travassos et al. 2008). P10 showed additive protective effects in drug-treated mice, stimulating a Th1 type immune response with high IFN-γ and IL-12.

18.8 *Pneumocystis carinii* (or *P. jirovecii*)

A major surface antigen of *P. carinii*, known as major surface glycoprotein (MSG) or glycoprotein A, is an immunodominant antigen that is recognized by sera from animals or humans exposed to this fungus, and subserves an adhesin function (O'Riordan et al. 1995). Injection of MSG into rats prior to administration of corticosteroids leads to reduction in the burden of *P. carinii* compared to that in controls (Theus et al. 1996).

Similarly, immunization with recombinant p55, a protein derived from rat pneumocystis without a defined function, also protects corticosteroid-treated rats (Smulian and Theus 1996). The mechanism whereby these antigens promote the

protective immune response is likely to be humoral in nature, as T-cell function in steroid-treated animals is greatly depressed. Thus, these two antigens provide a promising beginning in the search for a vaccine for this opportunistic pathogen.

18.9 *Penicillium marneffei*

18.9.1 Immunogen

Cell-wall mannoprotein Mp1p (Cao et al. 1998), is highly antigenic and a potent immunogen, and promotes protective response by stimulating the secretions of IL-12 by human macrophages.

18.9.2 Vaccine

Recently, recombinant Mp1p protein vaccine was used for generation of protective immune responses against *P. marneffei* infection using a mouse model, and compared with *MP1* DNA vaccine (*MP1* gene encodes Mp1p) delivered by live-attenuated *Salmonella typhimurium* (Wong et al. 2002). The intramuscular *MP1* DNA vaccine offered the best protection after challenge with *P. marneffei* yeast cells intravenously.

18.10 Approaches to Vaccination

18.10.1 Conventional Methods

The most common approach to experimental fungal vaccination to date has been to identify inert fungal substances such as proteins or carbohydrates for vaccination. These types of components are safest, since they do not involve the use of replicating microbes whose virulence may be altered. However, native protein or carbohydrate purification has limitations because of yield and purity concerns; therefore, many investigators have employed recombinant technology to generate large quantities of a protein-containing immunogen. Thus, HSP 60 from *H. capsulatum*, MSG and p55 from *P. carinii,* BAD1 from *B. dermatitidis*, and 4-hydroxyphenylpyruvate dioxygenase from *C. immitis* represent several examples of recombinant fungal proteins that are expressed by prokaryotes and are undergoing examination of their immunobiological function.

Immunogenic carbohydrates such as glucuronoxylomannan from the capsule of *C. neoformans* or mannans from *C. albicans* still require conventional biochemical

purification for isolation. Carbohydrates are weakly immunogenic, and they are useful only for eliciting a humoral response. Furthermore, they often require a carrier to make them potent. Cryptococcal glucuronoxylomannan has been chemically conjugated to tetanus toxoid. This linkage converts the polysaccharide from a T-cell-independent to a T-cell-dependent antigen, thus enhancing substantially the immunogenicity of the polysaccharide. Mannans from *C. albicans* have been incorporated into liposomes to create an immunogenic substance.

18.10.2 Adjuvants

Soluble proteins when delivered intradermally or intramuscularly require an adjuvant. The selection of an adjuvant becomes a critical issue in the endeavor to generate an appropriate immune response for each fungus. Aluminum salts (alum) were one of the first adjuvants to be used, and they remain the only one for human use. Their efficacy is limited largely to promoting antibody responses to immunogens (Gupta and Siber 1995).

Most investigators have used conventional adjuvants with fungal immunogens such as Freund's, monophosphoryl lipid A plus streptococcal cell-wall skeleton or other oil-based adjuvants. None have been found to be superior.

Many adjuvants often elicit a predominant $CD4^+$ cell response. In fungi, this subpopulation of cells appears to be the central T-cell family that confers protection either through effector function or by collaborating with B cells to stimulate humoral immunity.

18.10.2.1 Cytokines as Adjuvants

Cytokines are an exciting new addition to the armamentarium of adjuvants. As an example, the monokine IL-12 can act as an adjuvant. The mechanism of action appears to be that IL-12 enhances the Th1 response and as a consequence the production of IFN-γ, which is a key in the generation of a protective immune response.

18.10.3 DNA Vaccine

Conventional methodologies of vaccination are giving way to newer approaches, of which DNA vaccination represents one of the most exciting, not only for fungal diseases but also for all diseases mediated by pathogenic microbes. The primary method at present is to use plasmid-encoded DNA that does not replicate and inject it into the muscle, with or without an irritant such as cardiotoxin (Doe et al. 1996; Tascon et al. 1996; Ulmer et al. 1993). Bombardment into the skin of DNA-bound particles is another delivery system that is available for immunization. One of the recent developments noted with DNA delivery by a gene gun is that a Th2 response

is dominant, whereas DNA delivered by intramuscular injection produces Th1 response (Feltquate et al. 1997). Thus for pathogenic fungi that require a Th1 response for clearance, vaccination with a gene gun may not be suitable.

DNA vaccination holds promise in the field of mycology. At present, however, no laboratory has reported the utility of DNA vaccination in a medically important fungal infection.

18.10.4 Dendritic Cell Vaccination

A third method that has been reported is to transfect dendritic cells, which are potent antigen-presenting cells, with naked DNA, and assess the impact on protective immunity (Condon et al. 1996). Dendritic cells transfected with fungal RNA also restore antifungal resistance in hematopoietic transplantation (Bozza et al. 2004). A vaccine with antigen-exposed dendritic cells as adjuvants appears to be particularly promising for use in patients suffering from invasive aspergillosis (Feldmesser 2005).

18.10.5 Altered Fungus (Crippled Fungus) as Vaccine

The use as a vaccine of a fungal strain with diminished if not absent virulence certainly has potential, but several risks are involved. First, the traits that make the fungus less virulent would have to be stable. In addition, for deep mycoses, the vaccine fungus would have to be eliminated by the host, since it otherwise could form a nidus for reactivation. Genes that encode the replication cycle would have to be deleted or damaged by molecular manipulation. As an example, treatment of the mycelia phase of *H. capsulatum* with the sufhydryl blocking agent, p-chloromercuryphenylsulfonic acid, inhibits the ability of fungal elements to convert to the yeast phase. These crippled fungal elements, when injected into mice, protect the animals from a challenge with a lethal inoculum of yeast cells (Medoff et al. 1986). Thus, it may be possible to incapacitate important components of the fungal cell cycle that allow propagation yet maintain immunogenicity.

18.11 Therapeutic Vaccination

The use of an immunogen to treat an infectious disease rather than just to prevent it has emerged as an addition to the potential use of vaccine candidates.

The most likely mechanism to account for the activity of the immunogen is that it boosts antigen-specific immunity during active infection.

Because a high proportion of fungal diseases are thought to involve reactivation of dormant foci, therapeutic immunization becomes a compelling approach to

eliminate dormant organisms or to heal active infection, especially in life-threatening diseases.

18.12 Vaccination of the Immunocompromised Host

One of the major controversies and challenges surrounding fungal vaccination is the case of the immunocompromised host. As many fungal infections develop in patients whose immune system has been altered by pharmaceutical agents or by HIV infection, it is these patients who need the vaccine. It is clear that vaccines that rely on a competent immune system to function may not be efficacious in these patients. Certainly, vaccines that elicit protective antibodies may be more useful in these conditions. However, even antibody-centered vaccines would eventually lose efficacy in patients whose immune systems are compromised over months to years.

The challenge will be to enhance the effect of a vaccine when the immune system is dysfunctional.

One approach will be to link the vaccine with attempts to restore the integrity of the immune system; for example, delivery of a vaccine in combination with cytokine or cytokines that are known to enhance the immune system.

Another approach would be to link the administration of the vaccine with infusion of immunocompetent T cells or B cells to promote the immunogenicity of the vaccine.

Another dilemma that must be confronted is that clinicians do not yet have the means to identify those immunocompromised individuals who will develop fungal infections. Although risk factors are known, not every individual at risk manifests a serious fungal infection.

As recently discussed by Deepe (2004) and Mochon and Cutler (2005), the development of antifungal vaccines requires overcoming several major obstacles, one of which could be the need to identify and vaccinate persons at risk before they become so immunocompromised as to be unresponsive to immunization. Several categories of patients at risk of invasive candidiasis could benefit from a safe and effective immunoprophylactic vaccine (Spellberg and Edwards 2003; Stevens 2004).

Therefore, newer methods are necessary to identify who is at highest risk in order to determine who would benefit the most from vaccination and immunorestorative therapy.

References

Balish E, Vazquez-Torres FA, Jones-Carson J, Wagner RD, Warner T (1996) Importance of β_2-microglobulin in murine resistance to mucosal and systemic candidiasis. Infect Immun 64:5092–5097

Beaman LV, Pappagianis D, Benjamini E (1979) Mechanisms of resistance to infection with *Coccidioides immitis* in mice. Infect Immun 23:681–685

Beck JM, Newbury RL, Palmer BE, Warnock ML, Byrd PK, Kaltreider HB (1996) Role of $CD8^+$ lymphocytes in host defense against *Pneumocystis carinii* in mice. J Lab Clin Med 128:477–487

Beno DW, Stover AG, Matthews HL (1995) Growth inhibition of *Candida albicans* hyphae by $CD8^+$ lymphocytes. J Immunol 154:5273–5281

Biron CA (1994) Cytokines in the generation of immune responses to, and resolution of, virus infection. Curr Opin Immunol 6:530–538

Blanco JL, Garcia ME (2008) Immune response to fungal infections. Vet Immunol Immunopathol 125:47–70

Boneberger S, Korting HC (2008) Therapeutic or prophylactic antifungal vaccination: problems and solutions. Hautarzt 59:828–832

Bozza S, Montagnoli C, Gaziano R, Rossi G, Nkwanyuo G, Bellocchio S, Romani L (2004) Dendritic cell-based vaccination against opportunistic fungi. Vaccine 22:857–864

Brown GD, Gordon S (2005) Immune recognition of fungal beta-glucans. Cell Microbiol 7:471–479

Brummer E, Morozumi PA, Stevens DA (1982) Protection against pulmonary blastomycosis: adoptive transfer with T lymphocytes, but not serum from resistant mice. Cell Immunol 73:349–359

Cao L, Chan CM, Lee C, Wong SSY, Yuen KY (1998) MP1 encodes an abundant and highly antigenic cell wall mannoprotein in the pathogenic fungus *Penicillium marneffei*. Infect Immun 66:966–973

Casadevall A (1995) Antibody immunity and invasive fungal infections. Infect Immun 63:4211–4218

Casadevall A, Pirofski LA (2003) Exploiting the redundancy in the immune system: vaccines can mediate protection by eliciting 'unnatural' immunity. J Exp Med 197:1401–1404

Casadevall A, Pirofski LA (2006) Polysaccharide-containing conjugate vaccines for fungal diseases. Trends Mol Med 12:6–9

Casadevall A, Scharff MD (1994) Serum therapy revisited: animal models of infection and development of passive antibody therapy. Antimicrob Agents Chemother 38:1695–1702

Casadevall A, DeShaw M, Fan M, Dromer F, Kozel TR, Pirofski LA (1994) Molecular and idiotypic analysis of antibodies to *Cryptococcus neoformans* glucuronoxylomannan. Infect Immun 62:3864–3872

Casadevall A, Mukherjee J, Devi SJN, Schneerson R, Robbins JB, Scharff MD (1992) Antibodies elicited by a *Cryptococcus neoformans*-tetanus toxoid conjugate vaccine have the same specificity as those elicited in infection. J infect Dis 165:1086–1093

Cassone A (2008) Fungal vaccines: real progress from real challenges. Lancet Infect Dis 8: 114–124

Cassone A, Torosantucci A (2006) Opportunistic fungi and fungal infections: the challenge of a single, general antifungal vaccine. Expert Rev Vaccines 5:859–867

Cassone A, Palma C, Djeu JY, Aiuti F, Quinti I (1993) Anticandidal activity and interleukin-1 beta and interleukin-6 production by polymorphonuclear leukocytes are preserved in subjects with AIDS. J Clin Microbiol 31:1354–1357

Cole GT, Xue JM, Okeke CN, Tarcha EJ, Basrur V, Schaller RA, Herr RA, Yu JJ, Hung CY (2004) A vaccine against coccidioidomycosis is justified and attainable. Med Mycol 42:189–216

Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD Jr (1996) DNA-based immunization by *in vivo* transfection of dendritic cells. Nat Med 2:1122–1128

Cox RA, Magee DM (2004) Coccidioidomycosis: host immune response and vaccine development. Clin Microbiol Rev 17:804–839

Cox RA, Kennell W, Boncyk L, Murphy JW (1988) Induction and expression of cell-mediated immune responses in inbred mice infected with *Coccidioides immitis*. Infect Immun 56:13–17

Cutler JE (2005) Defining criteria for anti-mannan antibodies to protect against candidiasis. Curr Mol Med 5:383–392

Dan JM, Levitz SM (2006) Prospects for development of vaccines against fungal diseases. Drug Resist Updat 9:105–110

de Cock AW, Mendoza L, Padhye AA, Ajello L, Kaufman L (1987) *Pythium insidiosum* sp. nov., the etiologic agent of pythiosis. J Clin Microbiol 25:344–349

Deepe GS Jr (1994) Role of $CD8^+$ T cells in host resistance to systemic infection with *Histoplasma capsulatum* in mice. J Immunol 152:3491–3500

Deepe GS Jr (1997) Prospects for the development of fungal vaccines. Clin Microbiol Rev 10:585–596

Deepe GS Jr (2004) Preventive and therapeutic vaccines for fungal infections: from concept to implementation. Expert Rev Vaccines 3:1–9

Deepe GS Jr, Gibbons R, Brunner GD, Gomez FJ (1996) A protective domain of heat-shock protein 60 from *Histoplasma capsulatum*. J Infect Dis 174:828–834

Devi SJ, Scheerson R, Egan W, Ulrich TJ, Bryla D, Robbins JB, Bennett JE (1991) *Cryptococcus neoformans* serotype A glucuronoxylomannan protein conjugate vaccine: synthesis, characterization, and immunogenicity. Infect Immun 59:3700–3707

Djeu JY, Blanchard DK, Richards AL, Friedman H (1988) Tumor necrosis factor induction by *Candida albicans* from human natural killer cells and monocytes. J Immunol 141:4047–4052

Djeu JY, Serbousek D, Blanchard DK (1990) Release of tumor necrosis factor by human polymorphonuclear leukocytes. Blood 76:1405–1409

Doe B, Selby M, Barnett S, Baenziger J, Walker CM (1996) Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. Proc Natl Acad Sci USA 93:8578–8583

Eckstein M, Barenholz Y, Bar LK, Segal E (1997) Liposomes containing *Candida albicans* ribosomes as a prophylactic vaccine against disseminated Candidiasis in mice. Vaccine 15:220–224

Fearon DT, Locksley RM (1996) The instructive role of innate immunity in the acquired immune response. Science 272:50–54

Feit C, Tewari RP (1974) Immunogenicity of ribosomal preparations from yeast cells of *Histoplasma capsulatum*. Infect Immun 10:1091

Feldmesser M (2005) Prospects of vaccine for invasive aspergillosis. Med Mycol 43:571–587

Feltquate DM, Heaney S, Webster RG, Robinson HL (1997) Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunizations. J Immunol 158:2278–2284

Fraser VJ, Jones M, Dunkel J, Storfer S, Medoff G, Dunagan WC (1992) Candidemia in a tertiary care hospital: epidemiology, risk factors, and predictors of mortality. Clin Infect Dis 15:414–421

Galgiani JN, Peng T, Lewis ML, Cloud GA, Pappagianis D (1996) Cerebrospinal fluid antibodies detected by ELISA against a 33-kDa antigen from spherules of *Coccidioides immitis* in patients with coccidioidal meningitis. J Infect Dis 173:499–502

Garcia JP, Howard DH (1971) Characterization of antigens from the yeast phase of *Histoplasma capsulatum*. Infect Immun 4:116–125

Gomez AM, Bullock WE, Taylor CL, Deepe GS Jr (1988) The role of $L3T4^+$ T cells in host defense against *Histoplasma capsulatum*. Infect Immun 56:1685–1691

Gomez FJ, Allendoerfer R, Deepe GS Jr (1995) Vaccination with recombinant heat shock protein 60 from *Histoplasma capsulatum* protects mice against pulmonary histoplasmosis. Infect Immun 63:2587–2595

Gomez FJ, Gomez AM, GS Deepe Jr (1991) Protective efficacy of a 62-kilodalton antigen, HIS-62, from the cell wall and cell membrane of *Histoplasma capsulatum* yeast cells. Infect Immun 59:4459–4464

Gupta RK, Siber GR (1995) Adjuvants for human vaccines — current status, problems and future prospects. Vaccine 13:1263–1276

Hamad M (2008) Antifungal immunotherapy and immunomodulation: a double-hitter approach to deal with invasive fungal infections. Scand J Immunol 67:533–543

Han Y, Cutler JE (1995) Antibody response that protects against disseminated candidiasis. Infect Immun 63:2714–2719

Han Y, Ulrich MA, Cutler JE (1999) *Candida albicans* mannan extract–protein conjugates induce a protective immune response against experimental candidiasis. J Infect Dis 179:1477–1484

Harrison TS, Levitz SM (1996) Role of IL-12 in peripheral blood mononuclear cell responses to fungi in persons with and without HIV infection. J Immunol 156:4492–4497

Hill JO, Harmsen AG (1991) Intrapulmonary growth and dissemination of an avirulent strain of *Cryptococcus neoformans* in mice depleted of $CD4^+$ or $CD8^+$ T cells. J Exp Med 173: 755–758

Huffnagle GB, Yates JL, Lipscomb MF (1991) Immunity to a pulmonary *Cryptococcal neoformans* infection requires both $CD4^+$ and $CD8^+$ T cells. J Exp Med 173:793–800

Ishibashi KI, Yoshida M, Nakabayashi I, Shinohara H, Miura NN, Adachi Y, Ohno N (2005) Role of anti-β-glucan antibody in host defense against fungi. FEMS Immunol Med Microbiol 44:99–109

Klein BS, Jones JM (1994) Purification and characterization of the major antigen, WI-1, from *Blastomyces dermititidis* yeasts and immunological comparison with A antigen. Infect Immun 62:3890–3900

Kudeken N, Kawakami K, Saito A (1998) Different susceptibilities of yeasts and conidia of *Penicillium marneffei* to nitric oxide-mediated fungicidal activity of murine macrophages. Clin Exp Immunol 112:287–293

Lecara G, Cox RA, Simpson RB (1983) *Coccidioides immitis* vaccine: potential of an alkali-soluble, water-soluble cell wall antigen. Infect Immun 39:473–475

Levitz SM, Dupont MP, Smail EH (1994a) Direct activity of human T lymphocytes and natural killer cells against *Cryptococcus neoformans*. Infect Immun 62:194–202

Levitz SM, Matthews HL, Murphy JW (1995) Direct antimicrobial activity of T cells. Immunol Today 16:387–391

Levitz SM, Tabuni A, Kornfeld H, Reardon CC, Golenbock DT (1994b) Production of tumor necrosis factor alpha in human leukocytes stimulated by *Cryptococcus neoformans*. Infect Immun 62:1975–1981

Lloyd AR, Oppenheim JJ (1992) Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. Immunol Today 13:169–172

Matthews RC, Burnie JP, Rigg G, Hodgetts SJ, Nelson JC (2000) Human recombinant antibody to hsp 90 in the treatment of disseminated candidiasis. ASM Abstract No. 063

Matthews R, Burnie J (2001) Anifungal antibodies: a new approach to treatment of systemic candidiasis. Curr Opin Investig Drugs 2:472–476

Matthews R, Hodgetts S, Burnie J (1995) Preliminary assessment of a human recombinant antibody fragment to hsp 90 in murine invasive candidiasis. J Infect Dis 171:1668–1671

Matthews RC, Burnie JP, Howat D, Rowland T, Walton F (1991) Autoantibody to heat-shock protein 90 can mediate protection against systemic candidosis. Immunology 74:20–24

Medoff G, Sacco M, Maresca B, Schlessinger D, Painter A, Kobayashi GS, Carratu L (1986) Irreversible block of the mycelia-to-yeast phase transition of *Histoplasma capsulatum*. Science 231:476–479

Menacci A, Spaccapelo R, del Sero G, Enssle KH, Cassone A, Bistoni F, Romani L (1996) $CD4^+$ T-helper-cell responses in mice with low-level *Candida albicans* infection. Infect Immun 64:4907–4914

Menacci A, Torosantucci A, Spaccapelo R, Romani L, Bistoni F, Cassone A (1994) A mannoprotein constituent of *Candida albicans* that elicits different levels of delayed-type hypersensitivity, cytokine production, and anticandidal protection in mice. Infect Immun 62:5353–5360

Mendoza L, Mandy W, Glass R (2003) An improved *Pythium insidiosum*-vaccine formulation with enhanced immunotherapeutic properties in horses and dogs with pythiosis. Vaccine 21:2797–2804

Mendoza L, Villalobos J, Calleja CE, Solis A (1992) Evaluation of two vaccines for the treatment of pythiosis insidiosi in horses. Mycopathologia 119:89–95

Miller RI (1981) Treatment of equine phycomycosis by immunotherapy and surgery. Aust Vet J 30:377–382

Mochon AB, Cutler JE (2005) Is a vaccine needed against *Candida albicans*? Med Mycol 43:97–115

Modlin RL, Segal GP, Hofman FM, Walley MS, Johnson RH, Taylor CR, Rea TH (1985) In situ localization of T lymphocytes in disseminated coccidioidomycosis. J Infect Dis 151:314–319

Mody CH, Chen GH, Jackson C, Curtis JL, Toews GB (1993) Depletion of murine $CD8^+$ T cells *in vivo* decreases pulmonary clearance of a moderately virulent strain of *Cryptococcus neoformans*. J Lab Clin Invest 121:765–773

Mody CH, Sims KL, Wood CJ, Syme RM, Spurrell JC, Sexton MM (1996) Proteins in the cell wall and membrane of *Cryptococcus neoformans* stimulate lymphocytes from both adults and fetal cord blood to proliferate. Infect Immun 64:4811–4819

Morrison LA, Lukacher AE, Braciale VL, Fan DP, Braciale TJ (1986) Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. J Exp Med 163:903–921

Muchmore HG, Felton FG, Salvin SB, Rhoades ER (1968) Delayed hypersensitivity to cryptococcin in man. Sabouraudia 6:285–288

Mukherjee J, Scharff MD, Casadevall A (1994a) *Cryptococcus neoformans* infection can elicit protective antibodies in mice. Can J Microbiol 40:888–892

Mukherjee J, Zuckier LS, Scharff MD, Casadevall A (1994b) Therapeutic efficacy of monoclonal antibodies to *Cryptococcus neoformans* glucuronoxylomannan alone and in combination with amphotericin B. Antimicrob Agents Chemother 38:580–587

Mukherjee S, Lee S, Mukherjee J, Scharff MD, Casadevall A (1994c) Monoclonal antibodies to *Cryptococcus neoformans* capsular polysaccharide modify the course of intravenous infection in mice. Infect Immun 62:1079–1088

Murphy JW, Hidore MR, Wong SC (1993) Direct interactions of human lymphocytes with yeast-like organism, *Cryptococcus neoformans*. J Clin Invest 91:1553–1566

Newman SL, Chaturvedi S, Klein BS (1995) The WI-1 antigen of *Blastomyces dermatitidis* yeasts mediates binding to human macrophage CD11b/CD18 (CR3) and CD14. J Immunol 154:753–761

O'Riordan DM, Standing JE, Limper AH (1995) *Pneumocystis carinii* glycoprotein A binds macrophage mannose receptors. Infect Immun 63:779–784

Pappagianis D, The Valley Fever Study Group (1993) Evaluation of the protective efficacy of the killed *Coccidioides immitis* spherule vaccine in humans. Am Rev Respir Dis 148:656–660

Paulovicova E, Bystricky S, Machova E, Bujdakova H (2008) Immune responsiveness of a novel peptidoglycan conjugate prepared from surface Candida immunogens: mannan and CR3-related protein. FEMS Immunol Med Microbiol 53:421–428

Pishko EJ, Kirkland TN, Cole GT (1995) Isolation and characterization of two chitinase-encoding genes (cts1, cts2) from the fungus *Coccidioides immitis*. Gene 167:173–177

Raska M, Belakova J, Horynova M, Krupka M, Novotny J, Sebestova M, Weigl E (2008) Systemic and mucosal immunization with *Candida albicans* hsp90 elicits hsp90-specific humoral response in vaginal mucosa which is further enhanced during experimental vaginal candidiasis. Med Mycol 46:411–420

Retini C, Vecchiarelli A, Monari C, Tascini C, Bistoni F, Kozel TR (1996) Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. Infect Immun 64:2897–2903

Rigg G, Matthews RC, Gregory C, Illedge C, Hodgetts S, Burnie JP (2001) Antibodies in the treatment of disseminated candidosis. Mycoses 44(Suppl 1):63–64

Schimpff SC, Bennett JE (1975) Abnormalities in cell-mediated immunity in patients with *Cryptococcus neoformans* infection. J Allergy Clin Immunol 55:430–431

Seder RA, Paul WE (1994) Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. Annu Rev Immunol 12:635–674

Segal E (1987) Vaccines against fungal infections. CRC Crit Rev Microbiol 14:229–270

Segal E (1989) Vaccines for the management of dermatophyte and superficial yeast infection. Clin Microbiol Rev 8:317–335

Segal E (1991) Immunizations against fungal disease in man and animals. In: Arora DK, Ajello L, Mukherji KG (eds) Handbook of applied mycology, vol 2. Marcel Dekker, New York, pp 341–368

Shubitz L, Peng T, Perrill R, Simons J, Orsborn K, Galgiani JN (2002) Protection of mice against *Coccidioides immitis* intranasal infection by vaccination with recombinant antigen 2/PRA. Infect Immun 70:3287–3289

Smulian AG, Theus SA (1996) Active immunization with recombinant 55 kDa antigen (rp55) and rp55 fragments provides partial protection against subsequent *Pneumocystis carinii* (Pc) infection, Abstr. 130. In: Abstracts of the joint meeting of The American Society of Parasitologists and The Society of Protozoologists 1996. The American Society of Parasitologists and The Society of Protozoologists, Lawrence, KS, USA

Spellberg BJ, Ibrahim AS, Avanesian V, Fu Y, Myers C, Phan QT, Filler SG, Yeaman MR, Edwards JE Jr (2006) Efficacy of the anti-Candida rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. J Infect Dis 194:256–260

Spellberg DA, Edwards JE Jr (2003) The pathophysiology and treatment of *Candida* sepsis. Curr Infect Dis Rep 4:387–399

Stevens DA (2004) Vaccinate against aspergillosis! A call to arms of the immune system. Clin Infect Dis 38:1131–1136

Sundstrom P, Jensen J, Balish E (1994) Humoral and cellular immune responses to enolase after alimentary tract colonization of intravenous immunization with *Candida albicans*. J Infect Dis 170:390–395

Tascon RE, Colston MJ, Ragno S, Stavropoulos E, Gregory D, Lowrie DB (1996) Vaccination against tuberculosis by DNA injection. Nat Med 2:888–892

Tewari RP, Sharma DK, Mathur A (1978) Significance of thymus-derived lymphocytes in immunity elicited by immunization with ribosomes of live yeast cells. J Infect Dis 138: 605–613

Tewari RP (1975) The immune system and infectious diseases. Karger, Basel, Switzerland, p 441

Theus SA, Andrews RP, Steele P, Walzer PD (1995) Adoptive transfer of lymphocyte sensitized to the major surface glycoprotein of *Pneumocystis carinii* confers protection in the rat. J Clin Invest 95:2587–2593

Theus SA, Steele P, Andrews RP, Walzer PD (1996) A protective response is elicited following immunization with major surface glycoprotein of *Pneumocystis carinii*. Abstr. 129. In: Abstracts of the joint meeting of The American Society of Parasitologists and The Society of Protozoologists 1996. The American Society of Parasitologists and The Society of Protozoologists, Lawrence, KS, USA

Torosantucci A, Bromuro C, Chiani P, de Bernardis F, Berti F, Galli C, Norelli F, Bellucci C, Polonelli L, Costantino P, Rappuoli R, Cassone A (2005) A novel glycol–conjugate vaccine against fungal pathogens. J Exp Med 202:597–606

Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, Murphy BR, Rappuoli R, Lanzavecchia A (2004) An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. Nat Med 10:871–875

Travassos LR, Puccia R, Cisalpino P, Taborda C, Rodrigues EG, Rodrigues M, Silveira JF, Almeida IC (1995) Biochemistry and molecular biology of the main diagnostic antigen of *Paracoccidioides brasiliensis*. Arch Med Res 26:297–304

Travassos LR, Rodrigues EG, Iwai LK, Taborda CP (2008) Attempts to a peptide vaccine against paracoccidioidomycosis, adjuvant to chemotherapy. Mycopathologia 165:341–352

Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Stanislaw H, Deck RR, DeWitt CM, Friedman A, Hawe LA, Leander KR, Martinez D, Perry HC, Shiver JW, Montgomery DL, Liu MA (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259:1745–1749

Wong LP, Woo PC, Wu AY, Yuen KY (2002) DNA immunization using a secreted cell wall antigen Mp1p is protective against *Penicillium marneffei* infection. Vaccine 20:2878–2886

Wuthrich M, Filutowicz HI, Klein BS (2000) Mutation of the WI-1 gene yields an attenuated *Blastomyces dermatitidis* strain that induces host resistance. J Clin Invest 106:1381–1389

Wuthrich M, Filutowicz HI, Warner T, Klein BS (2002) Requisite elements in vaccine immunity to *Blastomyces dermatitidis*: plasticity uncovers vaccine potential in immune-deficient hosts. J Immunol 169:6969–6976

Wuthrich M, Filutowicz HI, Warner T, Deepe GS Jr, Klein BS (2003) Vaccine immunity to pathogenic fungi overcomes the requirement for CD4 help in exogenous antigen presentation to $CD8^+$ T cells: implications for vaccine development in immune-deficient hosts. J Exp Med 197:1405–1416

Wyckoff EE, Pishko EJ, Kirkland TN, Cole GT (1995) Cloning and expression of a gene encoding a T-cell reactive protein from *Coccidioides immitis*: homology to 4-hydroxyphenylpyruvate dioxygenase and the mammalian F antigen. Gene 161:107–111

Xin H, Dziadek S, Bundle DR, Cutler JE (2008) Synthetic glycopeptides vaccines combining beta-mannan and peptide epitopes induce protection against candidiasis. Proc Natl Acad Sci U S A 105:13526–13531

Yang C, Zhu Y, Magee DM, Cox RA (1996) Molecular cloning and characterization of the *Coccidioides immitis* complement fixation/chitinase antigen. Infect Immun 64:1992–1997

Yuan RR, Casadevall A, Oh J, Scharff MD (1997) T cells cooperate with passive antibody to modify *Cryptococcus neoformans* infection in mice. Proc Natl Acad Sci USA 94:2483–2488

Zhang MX, Brandhorst TT, Kozel TR, Klein BS (2001) Role of glucan and surface protein BAD1 in complement activation by *Blastomyces dermatitidis* yeast. Infect Immun 69:7559–7564

Zhu Y, Yang C, Magee DM, Cox RA (1996) Molecular cloning and characterization of *Coccidioides immitis* antigen 2-cDNA. Infect Immun 64:2695–2699

Zimmermann CR, Johnson SM, Martens GW, White AG, Pappagianis D (1996) Cloning and expression of the complement fixation antigen-chitinase of *Coccidioides immitis*. Infect Immun 64:4967–4975

Zimmermann CR, Johnson SM, Martens GW, White AG, Zimmer BL, Pappagianis D (1998) Protection against lethal murine coccidioidomycosis by a soluble vaccine from spherules. Infect Immun 66:2342–2345

Chapter 19
Antifungal Activity of Medicinal Plant Extracts and Phytocompounds: A Review

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Abstract The epidemiological data suggest that the incidence and prevalence of serious mycoses continues to be a public health problem. The increased use of antifungal agents has resulted in the development of resistance to these drugs. The spread of multidrug-resistant strains of fungus and the reduced number of drugs available make it necessary to discover new classes of antifungals from natural products including medicinal plants. Historically, herbs and spices have enjoyed a rich tradition of use for their medicinal properties and provide unlimited opportunities for new drug leads because of the huge chemical diversity. Assays of bioactive compounds have been reported with good antifungal properties *in vitro* or *in vivo*. It is almost impossible to discuss the various characteristics of these plants such as mode of action and extraction of active compounds in a single review. Therefore, we have focussed here mainly on the antifungal plant extracts, their use against pathogeinc and drug resistant fungi. The various classes of compounds such as phenolics, terpenoids, saponins, and alkaloids, etc., are discussed in detail. The new emerging classes of antifungal proteins and peptides are also reviewed briefly. In this chapter, we also describe the technical aspects related to the methodology for screening and identification of antifungal compounds. The technical aspects regarding the use of reliable methodology of extraction, screening, bioautography, and identification of pure compounds from crude extracts and fractions are also discussed here.

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19.1 Introduction

Infectious diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide (World Health Organization 1998). Despite the significant progress in human medicine, infectious diseases caused by microorganisms such as fungi are still a major threat to public health. The impact is even more in developing countries due to the unavailability of medicine and the emergence of widespread drug resistance (Okeke et al. 2005).

Infections induced by pathogenic fungi are increasingly recognized as an emerging threat to public health (Wu 1994; Walsh et al. 1996). The increase in occurrence of fungal infections during recent years is due to a growth in the immunocompromised population, such as organ transplant recipients and cancer and HIV patients (Portillo et al. 2001). Certain commensal fungi, such as *Candida* species, cause infections when their human hosts become immunocompromised (Cannon et al. 1995). These problems are also associated with resistance to antibiotics and toxicity during prolonged treatment with several antifungal drugs (Giordani et al. 2001).

There are many synthetic and natural product-based drugs available for treating fungal infections, but they are not consistently effective (Lazar and Wilner 1990; Gearhart 1994; Goa and Barradell 1995). Furthermore, the development of resistance in fungi against most of the drugs has now been reported for several years (Cuenca-Estrella et al. 2000). The use of amphotericin B, known as the "gold standard", is limited because of its infusion-related problems and nephrotoxicity (Grasela et al. 1990; Fanos and Cataldi 2000). In addition, the low efficacy, and side-effects and resistance associated with the existing drugs, highlight the advent of safe, novel, and effective antifungal drugs. Plants produce a great deal of secondary metabolites, many of them with antifungal activity. Similarly, traditional medicine has made use of many different plant extracts for treatment of fungal infection and many of these have been tested for *in vitro* antifungal activity. Based on the knowledge that plants develop their own defense against fungal pathogens (Gurgel et al. 2005), they appear as an interesting source for antifungal compounds.

Medicinal plants have also been reported in traditional systems of medicine for the treatment of both human and animal mycoses, and are considered to be a valuable source for the discovery of new antifungal drugs (Mathias-Mundy and McCorkle 1995). Many books have reported and recorded the use of medicinal plants in the traditional system of medicine. Gerard's *Herball*, first published in 1597, has so far yielded 16 currently prescribed drugs (Cox 1998). In a study, Fabricant and Farnsworth (2001) reported that 94 species of plants are utilized for the production of 122 single-agent natural products that are being used as single-agent drugs around the world. Thus, even with this very incomplete database of global ethnomedical information, there is abundant opportunity for the discovery of new medicinal agents.

According to an estimate by the World Health Organization (WHO), about 80% of the world's population in developing countries depend essentially on plants for

their primary health care. However, only a few plants have been scientifically studied for the assessment of their quality, safety, and efficacy. In addition to these, recent evidence from the pharmaceutical companies shows that, for some complex diseases, natural products still represent an extremely valuable source for the production of new chemical entities, since they represent privileged structures selected by evolutionary mechanisms over a period of millions of years (Newman et al. 2003; Clardy and Walsh 2004; Koehn and Carter 2005).

19.2 Screening of the Crude Extracts and Antifungal Activity

Many studies have been carried out to screen medicinal plants for their antifungal activity. Various groups all over the world initiated antifungal programs for plants used in traditional medicine or as antiinfectious agents. The most extensive screening was sponsored by the National Cancer Institute (NCI, USA). However, the main focus of this study was not antifungal screening. In this program, around 30,000 species were screened from 1960 to 1981 (Douros and Suffness 1980, 1881; Cragg et al. 1997, 1994). The Central Drug Research Institute (CDRI), Lucknow, India conducted the second largest study and evaluated more than 2,000 plant extracts for several biological activities including antifungal, antibacterial, antidiabetic, antifertility, antihypercholesteremic, anti-inflammatory, antitumor, cardiovascular, central nervous-system depressant, cytotoxicity, diuretic, and others (Bhakuni et al. 1969, 1971; Dhar et al. 1968, 1973, 1974; Dhawan et al. 1977, 1980). A large number of known and novel bioactive compounds were isolated from the plants (Rastogi and Dhawan 1982). After these programs, various workers conducted research in different parts of the world, in both academic and pharmaceutical institutions. Some of the screening studies are summarized here.

Seventy-eight plants were selected from Indian traditional medicines on the basis of their use in the treatment of infectious diseases. Only 10% of ethanol extracts showed the activity at 1.6 mg/ml; however, 44% were active when tested at concentration of 6.25 mg/ml (Valsaraj et al. 1997).

Mehmood et al. (1999) demonstrated the activity of aqueous and alcoholic extracts of 37 Indian medicinal plants against the pathogenic yeast, *Candida albicans*, and dermatophytes, *Epidermophyton floccosum*, *Microsporum gypseum*, and *Trichophyton rubrum*. In this study, all the plants showed activity; however, 21 plants showed stronger activity against *C. albicans* and at least one dermatophyte. When compared, only 16 aqueous extracts showed the activity. Further, they demonstrated that MIC values of the five most active extracts ranged between 4 and 9 mg/ml.

In another extensive study, Ahmad and Beg (2001) screened ethanolic extracts of 45 Indian medicinal plants used in the traditional system of Indian medicine for their antimicrobial activity against certain drug resistant bacteria and a yeast, *C. albicans*, of clinical origin. Of these, anticandidal activity was detected in 24 plant extracts. Qualitative phytochemical tests, thin-layer chromatography, and

TLC bioautography of certain active extracts demonstrated the presence of common phytocompounds including phenols, tannins, and flavonoids as major active constituents. In addition to this, no correlation was observed between susceptibility of test strains with plant extracts and antibiotic resistance behavior of the microbial strains (*C. albicans*).

Similarly, Aqil and Ahmad (2003) demonstrated the activity of ethanolic extracts of 22 traditionally used Indian medicinal plants for their antifungal activity against five filamentous fungi (*Aspergillus niger, Alternaria alternata*, *Fusarium chlamydosporum*, *Rhizoctonia bataticola*, and *Trichoderma viride*) and a yeast, *C. albicans*, of clinical origin. Seventeen and nine plant extracts showed antifungal and anticandidal activity respectively.

Eleven traditionally used Argentinian medicinal plants were assayed for antifungal activity against yeasts, hialohyphomycetes, as well as dermatophytes, using the broth microdilution method. Of the tested methanolic extracts, *Eupatorium buniifolium*, *Terminalia triflora*, *T. mentagrophytes*, and *T. rubrum* were the most susceptible species, with MICs ranging from 100 to 250 μg/ml. *Lithrea molleoides* showed the broadest spectrum of action inhibiting all the tested dermatophytes at MIC of 250 μg/ml (Muschietti et al. 2005).

In another study, Turchetti et al. (2005) screened leaf extracts of *Camellia sinensis*, *Cupressus sempervirens*, and *Pistacia lentiscus* and the seed extract of *Glycine soja* against yeast and yeast like species implicated in human mycoses. Of these extracts, only *C. sinensis* exhibited broad activity towards *C. glabrata, Clavispora lusitatiae, Cryptococcus laurentii, Filobasidiella neoformans, Issatchenkia orientalis, Saccharomyces cerevisiae*, and *Prototheca wickerhamii* strains. MICs ranging from 300 to 4,800 μg/ml of extract (corresponding to 130–2,010 μg/ml total polyphenols) were observed.

In the same year Lamidi et al. screened 77 crude extracts from leaves and stem barks of 15 Gabonese plants used in traditional medicine for their cytotoxic, antileishmanial, and antifungal activities. The methanolic extract of *Polyalthia suaveolens* displayed a strong antifungal activity on all the tested strains ($IC_{50} < 1$ mg/ml). This extract was divided into six fractions; however, the antifungal activity observed for the crude extract could not be recovered in the corresponding fractions (Lamidi et al. 2005).

A total of 65 crude methanol extracts belonging to 56 plant species of 38 families from Tanzania were screened, using the broth microdilution method, for antifungal susceptibility testing of yeasts. Among the tested plant species, 45% (25 species) showed antifungal activity against one or more test fungi. The most susceptible yeasts were *C. neoformans*, followed by *C. krusei*, *C. tropicalis*, and *C. parapsilosis*. The least susceptible were *C. albicans* and *C. glabrata*. Strong antifungal activity was exhibited by extracts of *Clausena anisata*, *Sclerocariya birrea*, *Turraea holstii*, *Sterculia africana*, *Acacia robusta* sub sp. Usambarensis, *Cyphosterma hildebrandti, Elaeodendron buchannanii*, *A. nilotica*, *Jatropha multifida*, and *Pteridium aquilinum* (Hamza et al. 2006).

Braga et al. (2007) screened 20 plants used in Brazilian traditional medicine for their antifungal activity against *C. albicans* and *C. neoformans*. Among the 20

tested methanolic extracts, *Schinus terebintifolius*, *O. gratissimum*, *Cajanus cajan*, and *Piper aduncum* extracts were the most active against *C. albicans* (MIC of 1.25 mg/ml) whereas *Bixa orellana*, *O. gratissimum*, and *Syzygium cumini* exhibited the best activity against *C. neoformans* (MIC of 0.078 mg/ml).

Masoko et al. (2007) screened hexane, dichloromethane, and methanol leaf extracts of the 24 South African *Combretum* species against five fungal animal pathogens *C. albicans*, *C. neoformans*, *A. fumigatus*, *M. canis*, and *Sporothrix schenckii*. They reported MIC values in the range of 20–60 μg/ml; substantially better values have been reported in the literature for crude extracts. Methanol extracted the highest quantity from leaves, but the acetone extracts had the highest antifungal activity in practically all cases. The methanolic extracts of *C. moggii* and *C. petrophilum* were, however, most active against all the pathogens. All extracts of *C. nelsonii* were also very effective against all the pathogens. They also report time-dependent antifungal activity, and the MIC values determined after 48 h were usually 2 times higher than values determined after 24 h.

Screening of the antifungal activity of plants from the north-east of Mexico against some of the main etiological agents inducing pulmonary mycoses, *C. albicans*, *A. fumigatus*, *Histoplasma capsulatum*, and *Coccidioides immitis*, was conducted *in vitro*. Ten hydroalcoholic extracts from the 15 plants evaluated showed antifungal activity against at least one of these fungi. Furthermore, a differential extraction was conducted with solvents of different polarities, and 16 extracts showed activity ranging from 16 to 125 μg/ml against the different fungi (Alanís-Garza et al. 2007).

Extracts from 50 plant parts obtained from 39 different plants belonging to 22 families used to treat infectious diseases in Bunda district, Tanzania, were tested against *A. niger* and *C. albicans*. Only *Balanites aegyptiaca* stem bark exhibited a high antifungal activity against *C. albicans* (MIC 125 μg/ml and MFC 250 μg/ml) (Maregesi et al. 2008).

In another study conducted in Mexico, 14 plants were tested for their antifungal activity. Of these, *Fragaria virginiana*, *Epilobium angustifolium*, and *Potentilla simplex* demonstrated strong antifungal potential overall. *Fragaria virginiana* had some degree of activity against all of the fungal pathogens. *Alnus viridis, Betula alleghaniensis*, and *Solidago gigantea* also demonstrated a significant degree of activity against many of the yeast isolates (Webster et al. 2008).

In a study from Krisch et al. 2009, fruit juices and pomace (skin, seeds) extracts from blackcurrant (*Ribes nigrum*), gooseberry (*R. uva-crispa*), and their hybrid plant (jostaberry, *Ribes* × *nidigrolaria*) were evaluated against the 12 most frequently isolated human pathogenic *Candida* species. Growth of most of the *Candida* species was inhibited, with the exception of *C. albicans, C. krusei, C. lusitaniae and C. pulcherrima. R. nigrum.*

Uma et al. 2009 have also shown the anticandidal activity of *Asparagus racemosus*. In their study, the *in vitro* anticandidal activity of *A. racemosus* roots and tubers extract was investigated against *C. albicans, C. tropicalis, C. krusei, C. guillermondii, C. parapsilosis*, and *C. stellatoida,* which are isolated from vaginal thrush patients. The extract of *A. racemosus* showed a high degree of

activity against all the *C*. strains. The inhibitory effect of the extract against all the Candida tested was found comparable with that of standard antibiotics used.

Furthermore, a study from Tayel and El-Tras (2009) revealed the anticandidal activity of pomegranate. In this study, anticandidal activity of pomegranate peel extracts (PPE) and application of PPE aerosol as sanitizer agent against *C*. *albicans* contamination were investigated. PPE exhibited potent anticandidal activity against *C*. *albicans* strains compared with standard fungicides in both susceptibility techniques used. Methanol, ethanol and water extracts were the most effective for inhibiting *C*. *albicans* growth. PPE aerosol was an efficient method for complete sanitizing of semi-closed places against *C*. *albicans* growth.

19.3 Classes of Compounds and Their Antifungal Activity

Several papers and reviews have been published on the occurrence of antifungal compounds in relation to their role in plant resistance (Ingham 1973). However, literature and systematic reviews on the natural products as an alternative to antifungal drugs are still scanty. The distribution of antifungal compounds can be defined either on the basis of their taxonomic distribution or on the basis of their chemical classes. Table 19.1 shows the antifungal natural products belonging to all major classes of secondary metabolites such as phenolics, alkaloids, terpenoids, saponins, flavonoids, proteins, and peptides, etc. The importance of these groups of compounds against pathogenic/nonpathogenic fungi is described below.

19.3.1 Terpenoids

Terpenoids (or isoprenoids), a subclass of the prenyllipids (terpenes, prenylquinones, and sterols), represent the oldest group of small molecular products synthesized by plants and are probably the most widespread group of natural products. It has been reported in the literature that aglycones of terpenoids are more stable and active as compared to the glycosides. For instance four nonglycosidic iridiods were discovered in *Aliertial macrophylla* and two of the 1α and 1β hydroxydihdrocornin aglycones showed fungitoxicity against a range of *Clostridium* and *Aspergillus* species (Young et al. 1992). Some representative structures of this group are shown in Fig. 19.1 (bold numbers 1 to 8 in brackets below refer to these).

Six antifungal sesquiterpenes [5- and 7-hydroxycalamenene, drimenol (**1**), drimenal, viridiflorol, gymnomitrol, and chloroisopiagiochin D] were isolated by Scher et al. (2004) from a dichloromethame and methanol extract of the liverwort *Bazzania trilobata* (L.). These compounds showed antifungal activity against various fungi including *Botrytis cinerea*, *Cladosporium cucumerinum*, *Phythophthora infestans*, *Pyricularia oryzae*, and *Septoria tritici*. Similarly polygodial, a sesquiterpene isolated from *Polygonum punctatum* (Polygonaceae), was

Table 19.1 Antifungal activity of plant extracts and their active principle

S. No.	Plant name (Family)	Part used	Chemical class	Compound (s)	Microorganism tested	References
1.	*Ajania fruticulosa* (Asteraceae)	Fruits	Xantholides	Seven different xantholides	*Candida albicans, C. glabrata, A. fumigetus*	Lavault et al. (2005)
2.	*Alibertia macrophylla* (Rubiaceae)	Leaves	Nonglycosidic iridoid	l z- and Ijl-Hydroxydihydrocornin aglycones	*Cladosporium sphaeros- permum; C. cladosporioides; A. niger; Colletotrichum qloeosporioides*	Young et al. (1992), Luciano et al. (2004)
3.	*Aniba panurensis* (Lauraceae)	Whole plant	Alkaloid	6,8-didec-(1Z)-enyl-5,7-dimethyl-2,3-dihydro-1Hindolizinium	*C. albicans*	Klausmeyer et al. (2004)
4.	*Aquilegia vulgaris* (Ranunculaceae)	Leaves and stems	Flavonoid	4′-methoxy-5,7-dihydroxyflavone 6-C-glucoside	*A. niger*	Bylka et al. (2004)
5.	*Avena sativa* (Poaceae)	Root	Triterpenoid saponin	Avenacins	*Geumannomyces graminis var. tritici*	De Bertoldi et al. (2009)
6.	*Bauhinia manea* (Leguminosae)	Wood	Chalcone	Isoliquiritigenin; isoliquiritigenin 2′-methyl ether; echinatin	*Borryris cinerea; Saprolegnia asterophora and three other fungi*	Achenbach et al. (1988)
			Flavan Diphenylpropene	(2S)-7,4′ -Dihydroxyfiavan; (2S)-3,4′ -dih ydroxy-7-methoxyfiavan; (2S)-7, 4′-Dihydroxy-3′ -methoxyflavan; Obtustyrene		
7.	*Blumea balsamifera* (Asteraceae)	Leaves	Flavonoid	Luteolin	*A. niger*, *Trichophyton mentagrophytes* and *C. albicans*	Ragasa et al. (2005)
8.	*Camellia japonica* (Theaceae)	Leaf	Saponin	Camellidins I and II	*Pesraloria longisera*	Nagata et al. (1985), Kim et al. (2001)
9.	*Camptotheca acuminata* (Cornaceae)	Leaves	Flavonoid	Trifolin and hyperoside	*Alternaria alternata*, *Epicoccum nigrum*, *Pestalotia guepinii*, *Drechslera* spp. and *Fusarium avenaceum.*	Li et al. (2005)
10.	*Cassia tora* (Leguminoceae)	Seeds	Anthraquinone	Emodin	*Botrytis cinerea, Erysiphe graminis, Phytophora infestans, Puccinia recondita, Pyricularia grisea and Rhizoctonia solani*	Kim et al. (2004)
11.	*Celastrus hypoleucus* (Celastraceae)	Root	Triterpenoid	Pristimerin, celastrol	*Glomerelia cinguiata: R. solani*	Luo et al. (2005)
12.	*Centaurea sulphurea* (Asteraceae)		Sesquiterpene lactone	Costunolide, dehydrocostunolide	*C. sulphurea*	Barrero et al. (2000)
13.	*Chamaecyparis pisifera* (Cupressaceae)	Leaves and twigs	Diterpene	Pisiferic acid	*P. oryzae*	Kobayashi et al. (1987), Xiao et al. (2001)
14.		Fruits				

(*continued*)

Table 19.1 (continued)

S. No.	Plant name (Family)	Part used	Chemical class	Compound (s)	Microorganism tested	References
	Chisocheton paniculatus (Meliaceae)		Meliacin-type nortriterpenoid	1,2-Dihydroxy-6Cl-aceroxyazadirone and three similar meliacins	*Curvularia verruciformis; Dreschlera oryzae; Alternaria solani*	Bordoloi et al. (1993), Phongmaykin et al. (2008), Yang et al. (2009)
15.	*Combretum apiculatum* (Combretaceae)	Heartwood	Phenanthrene Dihydrostilbene	4,7-Dihydroxy-2,3,6-trimethoxyphenanthrene; 2,7-dihydroxy-3,4,6-trimethoxydihydro-phenanthrene; 4,4′-dihydroxy-3,5-dimethoxydihydrostilbene	*Penicillium expansum*	Malan and Swinny (1993), McGaw et al. (2001), Bisoli et al. (2008)
16.	*Commiphora rostrata* (Burseraceae)	Bark of stem	Alkanone	2-decanone; 2-undecanone; 2-dodecanone	*Aspergillus; Penicillium sp.*	Mcdowell et al. (1988), Hanus et al. (2005)
17.	*Croton lacciferus* (Euphorbiaceae)	Root	Benzoquinone	2,6- Dimethoxy benzoq uinone	*C. cladosporioides*	RatnayakeBandara and Wimalasiri (1988), Mohamed et al. (2009)
18.	*Datura metel* (Solanaceae)	Whole plant	Alkaloid	2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate	*C. albicans, C. tropicalis, A. fumigatus, A. flavus* and *A. niger*	Dabur et al. (2005)
19.	*Detarium microcarpum* (Leguminaceae)	Pulp	Diterpene	Clerodane diterpene	*C. cucumerinum*	Cavin et al. (2006)
20.	*Dioscorea balatas* (Dioscoreaceae)	Tuber	Oxygenated bibenzyl and Phenanthrene	3-Hydroxy-5-methoxybibenzyl 3,2′-dihydroxy-5-methoxybibenzyl (batatasin IV) (4{}); 6-hydroxy-2,4,7-trimethoxyphenanthrene (batatasin I) (41); 6,7-dihydroxy-2,4-dimethoxy- phenanthrene; 2,7-dihydroxy-4,6-dimethoxy-phenanthrene	Showed activity against 24 fungi	Takasugi et al. (1987)
21.	*Dolichos kilimandscharicus* (Lehuminodae)	Roots	Triterpenoid saponin	3-0-glucosides of hederagenin, bayogenin and medicagenic aeid	*C. cucumerinum*	Marston et al. (1988)
22.	*Dolichos lablab* (Fabaceae)	Fruits	Protien	Dolichin	*R. solani*	Lee et al. (2003)
23.	*Ecballium elaterium* (Cucurbitaceae)	Fruit	Cucurbitacin	Cucurbitacin I	*Boitylis cinerea*	Har-Nur and Meyer (1990)

24.	*Euodia lunu-ankenda* (Lauraceae)	Root bark	Phenylethanone	1-[2′,4′ -Dihydroxy-6′(3″-methyl-Z″ -butcnyloxy}-5′(3″-methyl- 2″-butenyl)Jphenylethanone and related compound	*C. cladosporioides*	Kumar et al. (1990), Kadavul and Dixit (2009)
25.	*Eupatorium riparium* (Asteraceae)	Roots	Chromene	Methylripariochromene A	*C. gloeosporioides*	RatnayakeBandara et al. (1992))
26.	*Glycosmis cyanocarpa* (Rutaceae)	Leaves	Sulfur-containing amide	Sinharine; methylsinharine	*C. cladosporioides*	Greger et al. (1992), Pacher et al. (2001)
27.	*Glycosmis mauritiana* (Rutaceae)	Leaves	Sulfur-containing amide	IIlukumbin; methylillukumbins A and B	*C. cladosporioides*	Greger et al. (1993), Pacher et al. (2001)
28.	*Haplophyllum sieversii* (Rutaceae)	Aerial part	Alkaloid	flindersine, anhydroevoxine and haplamine	*C. fragariae*, *C. gloeosporioides* and *C. acutatum*	Cantrell et al. (2005)
29.	*Helichrysum decumbens* (Asteraceae)	Leaf surface	Prenylated phenol	Phloroglucinol derivatives (25–27)	*C. herb arum*	Aiyegoro and Okoh (2009)
30.	*Helichrysum nitens* (Asteraceae)	Leaf surface	Methoxylated flavone and flavonol	Chrysin dimethyl ether; galangin trimethyl ether; baicalin trimethyl ether; five more highly rnethoxylated flavonoids	*C. cucumerinum*	Tornas-Barberan et al. (1988), Aiyegoro and Okoh (2009)
31.	*Hordeum vulgare* (Poaceae)	Leaves	Indole alkaloid	Gramine	*Erysiphe qraminis*	Wippich and Wink (1985)
		Inner epidermal cells	Phenylpropanoid	Unidentified	*Erysiphe graminis f.sp hordei*	Zarnowski et al. (2002)
32.	*Humulus lupulus* (Cannabaceae)	Resin	Flavanone	6-Isopentenylnaringenin	*T. rubrum*	Mizobuchi and Sate (1984), Natarajan et al. (2008)
			Chalcone	xanthohumol	*T. mentaqrophytes*	
33.	*Lupinus albus* (Legominosea)	Leaf surface	Isoflavone	Luteone; wighteone	*Helminthosporium carbonum*	Ingham et al. (1983), Bednarek et al. (2003), Erdemoglu et al. (2007)
		Roots	Isoflavone	Luteone; wighteone; licoisoflavones A and B; parvisoflavone B	*C. herbarum*	Tahara et al. (1984), Bednarek et al. (2003), Erdemoglu et al. (2007)
34.	*Lycopersicon esculentum* (Solanaceae)	Green fruits	Steroidal alkaloid	Tomatine	*Fusarium solani*	Defago and Kern (1988), Soylu et al. (2006), Vinaya et al. (2009)
35.	*Mammea longifolia* (Clusiaceae)	–	Coumarin	Surangin B	*R. solani and B. cinerea*	Deng and Nicholson (2005)

(*continued*)

Table 19.1 (continued)

S. No.	Plant name (Family)	Part used	Chemical class	Compound (s)	Microorganism tested	References
36.	*Mangifera indica* (Anacardiaceae)	Peel and pulp	Alkylated phenols	5-(12-*cis*-Heptadecenyl)-resorcinol; 5-pentadecenylresorcinol	*A. alternata*	Cojocaru et al. (1986), Kabuki et al. (2000), Bbosa et al. (2007)
37.	*Melia azedarach* (Meliaceae)	Seeds carnels	Hydroxy coumarins	Scopoletin	*Fusarium verticillioides*	Carpinella et al (2005)
38.	*Mollugo pentaphylla* (Molluginaceae)	Aerial parts	Triterpenoid	Mollugenol A	*C. cucumerinum*	Hamburger et al. (1989), Kim et al. (2008)
39.	*Musa* (Musaceae)	Unripe fruit peel	Amine	Dopamine (oxidation products)	*C. musae*	Muirhead and Deverall (1984), Mokbel and Hashinaga (2005)
40.	*Mutisia friesiana* (Asteraceae)	Aerial part	Coumarins	5-methylcoumarins, mutisicoumarones C and D.	*C. cucumerinum*	Viturro et al (2004)
41.	*Nicotiana tabacum* (Solanaceae)	Leaf surface	Diterpenoid	1.- and tJ-4.S,I3-Duvatriene-L3-diols	*Peronospora rabacina*	Reuveni et al. (1987), Akinpelu and Obuotor (2000)
42.	*Oryza officinalis* (Poaceae)	Leaves	Modified fatty acid	Jasmonic acid, Hydroxybenzoic acid	*P. oryzae*	Cho et al. (1998)
43.	*Persea americana* (Lauraceae)	Peel of unripe fruit	Long-chain alcohol	cis, cis l-Acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene	*C. gloeosporioides*	Prusky et al. (1983), Adikaram et al. (1992), Gomez-Flores et al. (2008)
		Peel of unripe fruit	Long-chain alcohol	1,2,4- Trihydroxyheptadec-l6-yne; 1,2,4-trihydroxyheptadec-16-ene; l-acetoxy-2,4-dih ydroxy-heptadec-Ie-yne	*C. cladosporioides*	
44.	*Picea sirchellsis* (Pinaceae)	Bark	Stilbene	Astringin; rhaponticin	*Paeolus schweinitzii; Sparassis crispa*	Woodward and Pearce (1988)
45.	*Pinus radiara* (pinaceae)	Needle surface	Long-chain fatty acid	Stearic acid: (!)-Hydroxydodecanoic acid; co-hydroxytetradecanoic ' acid; c;-hydroxyhexadecanoic acid	*Dothistroma pini*	Franich et al. (1983)
			Oxidized diterpene acid	7-Ketodehydroabietic acid ; 7-hydroxydehydroabietic acid; 15-hydroxypodocarpic acid		
46.	*Piper aduncwn* (Piperaceae)	Leaves	Chromene	Methyl 8-hydroxy-2,2-dimethyl-2H-chromene-6- carboxylate; 2,2-din:;etbyl-8-{3-metbyl-2-butenyl}-2H-chromene-6-carboxylic acid	*P. oxalicum*	Orjala et al. (1993), Guerrini et al. (2009)

47.	*P. regnellii* (*Piperaceae*)	Leaves	Neolignan	eupamatenoid-3, eupamatenoid-5	*Tricophyton rubrum, Tricophyton mentagrophytes ; Microsporum canis, M. gypsium*	Koroishi et al. (2008)
48.	*Polygala nicaeensis* (Polygalaceae)	Roots	Xanthone	1,7-Dihydroxy-4-methoxyxanthone; 1,7-dihydroxy-3,5.6-trimetboxyxanthone	*C. cucumerinum*	Marston et al. (1993)
49.	*Polygonum punctatum* (Polygonaceae)	–	Sesquiterpene	polygodial	*Zygosaccharomyces bailli*	Fujita and Kubo (2005)
50.	*Populus deltoides* (Saliaceae)	Leaf glands	Flavanone	Pinocembrin	*Melampsora medusae*	Shain and Miller (1982), Hoof et al. (2008)
51.	*Prunus yedoensis* (Rosaceae)	Leaves	Phenol Coumarin	Benzylalcobol	*C. herbarum*	Ito and Kumazawa (1992)
52.	*Psidium acutangulum* (Myrtaceae)	Twigs and leaves	Dihydrochalcone	3′ -F ormyl-2′.4′.6′ -trihydroxy-dihydrochalcone	*R. solani; Helminthosporium ceres*	Miles et al. (1991), Nowakowska (2007)
53.	*Rapanea melanophloeos* (Myrsinaceae)	Leaves	Trirerpenoid saponin	Sakurasosaponin	*C. cucumerinum*	Ohtani et al. (1993)
54.	*Rosmarinus officinalis* (Lamiacae)	Leaves and callus cultures	Enol ester of hydroxy-cinnamic acid	2-(3,4-Dihydroxyphenyl) ethenyl esters of caffeic acid (24)	*C. herb arum*	Banthorpe et al. (1989), Pintore et al. (2001), Oluwatuyi et al. (2004)
55.	*Rubia tinctorum* (Rubiaceae)	Root	Anthraquinone aglycone	Alizarin and emodin	*A. niger, Alternaria alternaria, P. verrucosum, Mucor mucebo, Doratomyces stemonitis and P. verrucosum*	Manojlovic et al. (2005)
56.	*S. woronowii* (Lamiaceae)	Aerial parts	Neo-clerodane diterpenoid	Jodrellin B	*Fusarium oxysporum f.sp. yeo persici*	Cole et al. (1991), Springob and Kutchan (2009)
57.	*Scutellaria violacea* (Lamiaceae)	Aerial parts	Neo-clerodane diterpenoid	Oerodin	*Fusarium oxysporum f.sp. yeo persici*	Cole et al. (1991), Springob and Kutchan (2009)
58.	*Sesamum angolense* (Pedaliaceae)	Root bark	Naphthoquinone	Naphthoxirenes	*C. cucumerinum*	Potterat et al. (1987)
59.	*Sorghum cultivars* (Poaceae)	Leaves and grains	Leuco-anthocyanidin	Flavan-4-ols	*Fusarium moniliforme; Curvularia lunara*	Jambunathan et al. (1986), Boddu et al. (2004), Kil et al. (2009)
60.	*Stemonoporus canaliculatus* (Dipterocarpaceae)	Bark	Stilbene trimer	Canaliculatol	*C. cIadosporioides*	Bokel et al. (1988), Springob and Kutchan (2009)
61.	*Theobroma cacao* (Sterculiaceae)	Flush shoot tissue	Proanthocyanidin	Polymeric procyanidin	*Crinipellis perniciosa*	Brownlee et al. (1990), Duke (2004)

(*continued*)

Table 19.1 (continued)

S. No.	Plant name (Family)	Part used	Chemical class	Compound (s)	Microorganism tested	References
62.	*Tithonia diversifolia* (Asteraceae)	–	Isocoumarins	Tithoniamarin	*Microbotryum violaceum, Chlorella fusca*	Yemele-Bouberte et al. (2006))
63.	*Triticum aestivum* (poaceae)	Leaves	A1kadienal	E-triticene; P-triticene, puroindoline	*C. cucumerinum*	Spendley et al. (1982), Liyana-Pathirana and Shahidi (2005), Dhatwalia et al. (2009)
64.	*Vernonanthura tweedieana (Asteraceae)*	Root	Sesquiterpene	6-cinnamoyloxy-1-hydroxyeudesm-4-en-3-one	*T. mentagrophytes*	Portillo et al. (2005)
65.	*Wedelia biflora* (Asteracea)	Leaf surface	Flavonol	7,3′-Di-O-methylquercetin	*R. solani*	Miles et al. (1993), Yoganandam et al. (2009)
66.	*Zingiber officinale* (Zingiberaceae)	Rhizomes	Diarylheptenone	Gingerenones A, B and C: isogingerenone B	*P. oryzae*	Endo et al. (1990), Samy (2005), Park et al. (2008)

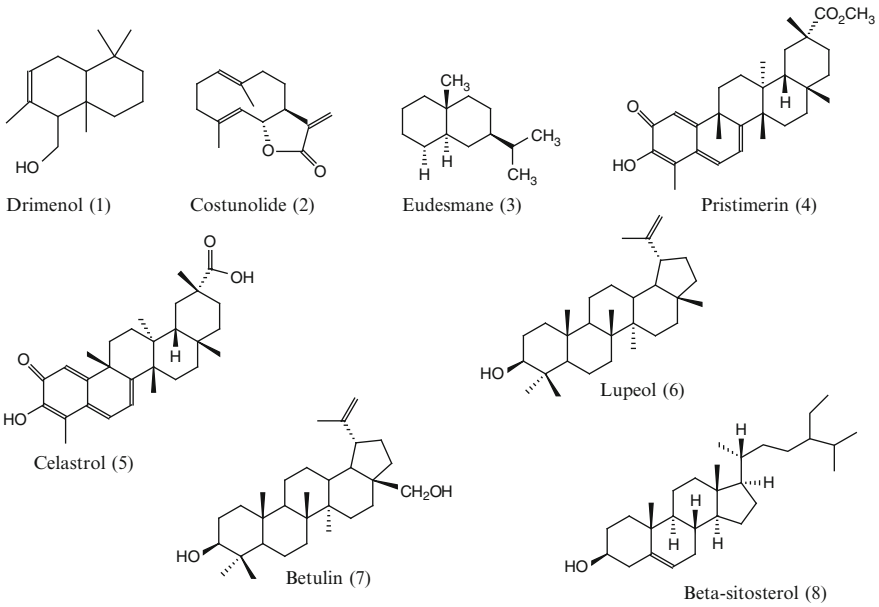


Fig. 19.1 Structures of antifungal terpenoids

found to exhibit a fungicidal activity against food spoilage yeast, *Zygosaccharomyces bailii* (Fujita and Kubo 2005).

The activity of the sesquiterpene lactones isolated from the *Centaurea* plants against the fungus *Cunninghamella echinulata* was evaluated. Costunolide (**2**) and dehydrocostunolide showed noticeable IC_{50} values, while more polar lactones were inactive. These results suggest that a relatively low polarity is one of the molecular requirements for the antifungal activity of sesquiterpene lactones. From other *Centaurea* species, *Centaurea thessala* and *C. attica*, two new eudesmanolides, 4-*epi*-sonchucarpolide and their 8-(3-hydroxy-4-acetoxy-2-methylene-butanoyloxy) derivative, and one new eudesmane (**3**) derivative named atticin were isolated (Skaltsa et al. 2000). Fractionation of *Detarium microcarpum* (Leguminoseae) extract led to the isolation of four new clerodane diterpenes which showed antifungal activity against *C. cucumerinum* (Cavin et al. 2006).

Many diterpenoids show antifungal activity; a few of them are phytoalexins, while others are preformed. The preformed diterpenoids have been reported to be involved in the resistance of several conifers against phytopathogenic fungi (Franich et al. 1983). Two new diterpenes, fusicoserpenol A and dolabeserpenoic acid A, were isolated from leaves of *Hypoestes serpens* (Acanthaceae). Both of the compounds showed good antifungal activity using TLC bioautography at 10 μg concentration against *C. cucumerinum* (Rasoamiaranjanahary et al. 2003).

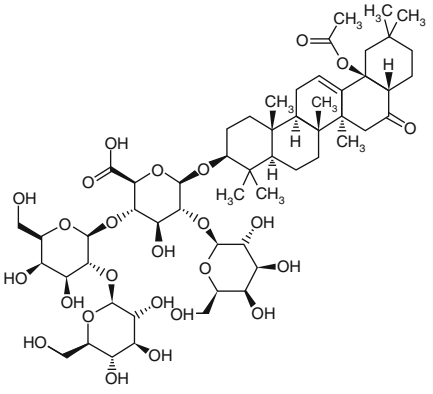
Diterpenoids 16α-hydroxy-cleroda-3,13-(14)-*Z*-diene-15,16-olide and 16-oxo-cleroda-3,13-(14)-*E*-diene-15-oic acid, isolated from the hexane extract of the seeds of *P. longifolia* (Annonaceae), also demonstrated significant antifungal

activity (Marthanda et al. 2005). Adou et al. (2005) isolated five new diterpenoids through bioactivity-guided fractionation, namely humirianthone, 1-hydroxy-humirianthone, 15*R*-humirianthol, patagonol, and patagonal. All five diterpenes showed activity against phytopathogenic fungi.

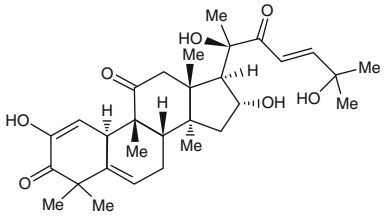
Two triterpenoid, pristimerin (**4**) and celastrol (**5**), isolated from the roots of *Celastrus hypoleucus* (Celastraceae), exhibited an inhibitory effect against diverse pathogenic fungi such as *R. solani* and *Glomerelia cinguiata* (Luo et al. 2005). Similarly Mollugenol A, a triterpenoid isolated from the aerial part of *Mollugo pentaphylla*, showed antifungal activity against *C. cucumerium* (Hamburger et al. 1989). In another study, four triterpenes, namely lupeol (**6**), betulin (**7**), β-sitosterol (**8**), 20(29)-lupene-3β-isoferulate, and two naphthoquinones, shinanolone and octahydroeuclein, isolated from the ethanolic extract of *E. natalensis* root bark, were investigated for their antifungal activity against *A. flavus*, *A. niger*, *C. cladosporioides*, and *Phytophthora* sp. Only β-sitosterol, 20(29)-lupene-3β-isoferulate, and shinanolone showed significant inhibition of *A. niger* at 10 μg/ml. Of these tested compounds, only octahydroeuclein was found to be significantly effective against *Phytophthora* sp. at 10 μg/ml. β-Sitosterol and octahydroeuclein inhibited the growth of *C. cladosporioides* significantly at the same concentration (Lall et al. 2006).

19.3.2 Saponins

An important source of constitutive antifungals is the saponins. Figure 19.2 shows two of these (bold numbers in brackets 9 and 10 below). Saponins are natural detergents that are effective antimicrobial, cholesterol-lowering anticancer compounds. These compounds chemically related to the triterpenoid group such as



Camillidin (9)



Cucurbitacin I (10)

Fig. 19.2 Structures of antifungal saponins

triterpene saponins, together with steroidal saponins, were also isolated as antifungal constituents.

A novel triterpene saponin, CAY-I, from the *Capsicum frutescens* L. (Solanaceae) plant commercially known as cayenne pepper, was investigated to determine its *in vitro* antifungal activity. CAY-1 showed activity against 16 different fungal strains, including *Candida* spp. and *A. fumigatus*, and showed highest activity against *C. neoformans*. Importantly the mechanism for this activity appears by disrupting the membrane integrity of fungal cells. The triterpenoid saponins camellidin I (**9**) and II from the leaves of *C. japonica* display antifungal activity against *Pestalocia longisate* (Nagata et al. 1985). In another study, glucoside of triterpenoid saponins 3-O-glucosides of hederegenins was isolated from Dolicus, and showed good activity against *Clostridium cucumerinum* (Tahara et al. 1984).

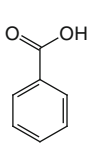
Gonzalez et al. (2004) isolated one novel saponin along with three others from the leaves of *Solanum hispidum*. All isolated compounds showed antifungal activity against both *T. mentagrophytes* and *T. rubrum*. However 6α-*O*-[β-*D*-xylopyranosyl-(1→3)-β-*D*quinovopyranosyl]-(25,*S*)-5α-spirostan-3β-ol, was found most active with IC_{50} values of 25 μg/ml. Similarly five saponins were isolated using bioactivity guided fractionation from *S. chrysotrichum*. All the isolated compounds showed antimycotic activity against *T. mentagrophytes*, *T. rubrum*, *A. niger*, and *C. albicans*.

A very important triterpenoidal saponin is cucurbitacin I (**10**). Although the whole family of cucurbitacins (cucurbitacin A, B, Q, I, and E, etc.) has very high activity, they have at the same time also shown potential toxicity. Cucurbitacin has been isolated from *Ecbellium elaterium* and found to be a very good antifungal against *B. cinera* (Har-Nun and Meyer 1990). Similarly Sauton et al. (2004) have isolated and demonstrated antifungal activity of steroid saponins from *Dioscorea cayenensis* against the human pathogenic yeasts *C. albicans*, *C. glabrata* and *C. tropicalis*. The saponins have been isolated from *A. racemosus*, *Astragalus verrucosus* Moris, *A. auriculiformis* and from *Hedera taurica* (Araliaceae), and have shown very good antifungal activity against *C. albicans*, *C. krusei*, and *C. tropicalis* (Pistelli et al. 2002; Mel'nichenko et al. 2003; Mandal et al. 2005; Rukayadi et al. 2008; Uma et al. 2009) (Table 19.1).

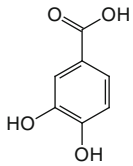
19.3.3 Phenolic Compounds

In recent years, a large number of studies have been published on antimicrobial activity of the phenolics compounds of natural origin. In many cases, these substances serve as plant defense mechanisms against microorganisms and insects, etc. Some plants give their odors like terpenoids, however others (quinones and tannins) are responsible for plant pigment. A large number of aromatic plants show antibacterial and often antifungal activity. These compounds include simple and alkylated phenols, phenolic acid, phenyl propanoids, coumarins, quinines, anthraquinones, and xanthones, etc. (Fig. 19.3: bold numbers in brackets 11 to 14 below refer).

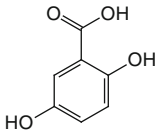
Fig. 19.3 Structures of antifungal phenolics



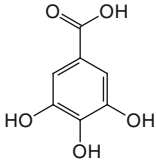
Benzoic acid (11)



Protocatechuic acid (12)



Gentisic acid (13)



Gallic acid (14)

Benzoic acid (**11**), protocatechuic acid (**12**) and gentisic acid (**13**), along with some other phenolic acids, have been reported as constitutive antifungal compounds (Tahara et al. 1984). However, gallic acid (**14**) is inhibitory to both pathogenic and saprophytic fungi. Phenylpropanoid or hydroxycinnamic acid include p-coumaric, ferulic, ferulic synaptic, and chlorogenic, etc. All these phenolics have been reported as strong antifungals against a number of plant and human pathogenic fungi (Kuc et al. 1956). Some new phenolic acid derivatives (crassinervic acid, aduncumene, hostmaniane, and gaudichaudanic acid) were isolated from four *Piper* species, *P. crassinervium*, *P. aduncum*, *P. hostmannianum*, and *P. gaudichaudianum* respectively, as major secondary metabolites. These compounds were reported to be fungitoxic against *C. cladosporioides* and *C. sphaerospermum* (Lago et al. 2004).

De-Leo et al. (2004)) isolated three new phenolic compounds together with a few known compounds from the leaves of *Baseonema acuminatum* (Asclepiadaceae). The compounds showed antifungal activity against two clinically isolated *C. albicans* strains in the range of 25–100 μg/ml. In the same year Lee et al. isolated four phenolic amides, dihydro-N-caffeoyltyramine, *trans*-N-feruloyloctopamine, *trans*-N-caffeoyltyramine, and *cis*-N-caffeoyltyramine, from an ethyl acetate extract of the root bark of *Lycium chinense* Miller. All had an antifungal effect against *C. albicans* with the potency of 5–10 μg/ml and showed no toxicity (hemolytic activity) against human erythrocyte cells (Lee et al. 2004). In another study two compounds were isolated from the essential oils of *Pulicaria odora*, a Moroccan medicinal plant, and identified as 2-isopropyl-4-methylphenol and isobutyric acid 2-isopropyl-4-methylphenylester. The study showed that the crude essential oil and the 2-isopropyl-4-methylphenol exhibited a very significant antibacterial and antifungal activity, while the isobutyric acid 2-isopropyl-4-methylphenylester was inactive for all tested strains (Ezoubeiri et al. 2005).

A bioassay-directed purification from a sunflower by column chromatography followed by HPLC allowed the isolation of a new compound, 3-acetyl-4-acetoxyacetophenone, and known compounds, demethoxyencecalin and 3-acetyl-4-hydroxyacetophenone. The new compound, 3-acetyl-4-acetoxyacetophenone, showed antifungal activity similar to the coumarin ayapin, previously described as a potent *Sclerotinia* inhibitor. A screening of seven sunflower genotypes in a field experiment showed a correlation between these compounds and resistance to *Sclerotinia* (Prats et al. 2007). Naldoni et al (2009) isolated benzophenones 7-epiclusianone and guttiferone-A from the pericarp and seeds of fruits of *Garcinia brasiliensis*. The pericarp hexane extract (PHE), seed ethanol extract (SEE), and both the compounds showed varying levels of activity against *C. albicans* (Table 19.1).

19.3.3.1 Flavones, Flavonoids, and Flavonols

Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol, and flavonoids are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (Cowan 1999). In the flavonoid group, reports on antifungal compounds from medicinal plants mainly concern those isolated from species of the fabaceae and moraceae families. However, reports on the antifungal activity of flavonoids of several medicinal plants belonging to other botanical families have also been found in the literature (Abad et al. 2007). Figure 19.4 shows a number of these (bold numbers in brackets 15 and 16 below), together with some of the coumarins from the next section (bold numbers in brackets 17 to 24).

Two new flavones from *Artemisia giraldi*, identified as 6,7,4′-trihydroxy-3′,5′-dimethoxyflavone and 5,5′-dihydroxy-8,2′,4′-trimethoxyflavone, together with 5,7,4′-trihydroxy-3′,5′-dimethoxy-flavone, have been reported to exhibit activity against *A. flavus* (Zheng et al. 1996). Galangin (**15**), a flavonol commonly found in propolis samples, has been shown to have inhibitory activity against *A. tamarii, A. flavus, C. sphaerospermum, Penicillium digitatum*, and *P. italicum* (Afolayan and Meyer 1997). A new prenylated flavanone recently isolated from the shrub *Eysenhardtia texana* has been identified as 5,7,4′-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2S)-flavanone and shown to possess activity against the opportunistic pathogen *C. albicans* (Wachter et al. 1999).

Alcerito et al. (2002) analyzed the epicuticular wax of the leaves of *Arrabidaea brachypoda* for its flavonoid content and isolated four compounds: 3′,4′-dihydroxy-5,6,7-trimethoxyflavone, cirsiliol, cirsimaritin, and hispidulin. They are known flavonoids, and showed antifungal activity against *C. sphaerospermum*. Eighteen prenylated flavonoids, purified from five different medicinal plants, were evaluated for their antimicrobial activity by determination of MIC using the broth

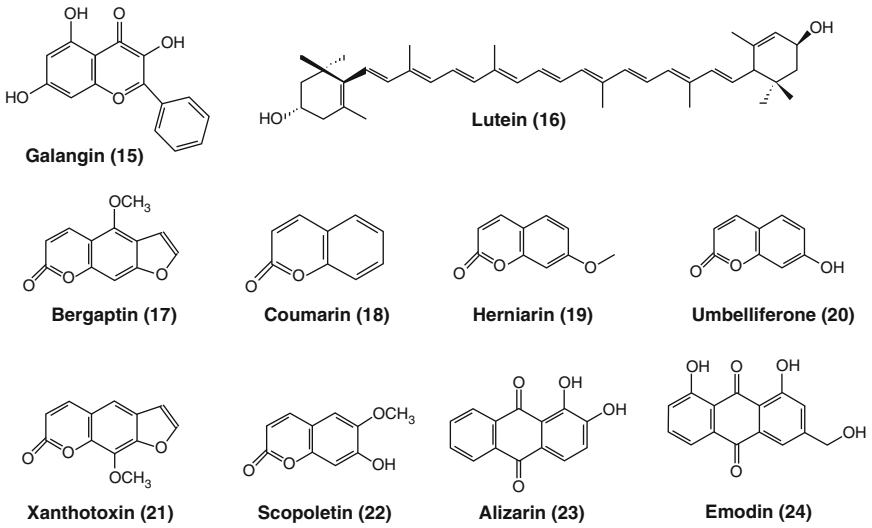


Fig. 19.4 Structures of antifungal flavonoids and coumarins

microdilution methods against four bacterial and two fungal strains (*C. albicans, Saccaromyces cerevisiae*). Of these isolated flavonoids, papyriflavonol A, kuraridin, sophoraflavanone D, and sophoraisoflavanone A exhibited a good antifungal activity with strong antibacterial activity. However, broussochalcone A was effective to *C. albicans* only (Sohn et al. 2004).

The leaves of *Blumea balsamifera* afforded icthyothereol acetate, cryptomeridiol, lutein (**16**), and beta-carotene. Antimicrobial tests indicated that the first compound has moderate activity against the fungi *A. niger, T. mentagrophytes,* and *C. albicans*, while two have low activity against *A. niger, T. mentagrophytes,* and *C. albicans* (Ragasa et al. 2005). The new kaempferol 3-O-β-d-glucopyranosyl (1→2)-O-β-d-glucopyranosyl (1→2)-O-[α-l-rhamnopyranosyl-(1→6)]-β-d-glucopyranoside (1) has been isolated from carnation (*Dianthus caryophyllus*) along with two known C- and O-flavonoid glycosides. The isolated compounds exhibited antifungal activity against different *F. oxysporum* f. sp. *dianthi* pathotypes (Galeotti et al. 2008). In addition to studies into simple phenolic derivatives and flavonoids, those into other antifungal phenolic compounds from natural sources also included coumarins and anthraquinones.

19.3.3.2 Coumarins

Various coumarins isolated from the traditionally used medicinal plants in Brazil showed that the isolated coumarins are active either alone or in combination against a number of fungi, *C. neoformans*, *M. gypseum*, *T. rubrum*, and *T. mentagrophytes* (Stein et al. 2006).

Ojala et al. (2000) tested six commercial coumarins, bergaptin (**17**), coumarin (**18**), herniarin (**19**), umbelliferone (**20**), xanthotoxin (**21**), and scopoletin (**22**), and found that they are active against *F. culmorum* (Fig. 19.4). Tithoniamarin is a new isocoumarin dimer isolated from *Tithonia diversifolia* (Hemsl) Gray (Asteraceae). Preliminary studies showed that tithoniamarin has antifungal and herbicidal activities against *Microbotryum violaceum* and *Chlorella fusca* (Yemele-Bouberte et al. 2006). Alizarin (**23**) and emodin (**24**), anthraquinone aglycone of *Rubia tinctorum*, has shown antifungal activity (Manojlovic et al. 2005).

19.3.4 Alkaloids

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. There are many reports on alkaloids showing activity against human pathogens, for example, the isoquinoline alkaloid jatrorrhizine, a range of peptide alkaloids, the quinolizidine alkaloid dietamnine and the pyrrolizidine alkaloids, etc., (Harborne and Baxter 1993). Berberine (**25**), a well-known alkaloid, has been reported for its strong antifungal activity against a number of organisms including human pathogenic fungi *C. albicans, T. mentagrophytes*, *M. canis*, *T. rubrum*, *E. floccosum*, and *M. gypseum* (Greathouse and Walkins 1938; Freile et al. 2006). Similarly, tomatine (**26**) from tomato and α-solanine from potato are examples of steroidal glycoalkaloids, and have very strong antifungal activity (Grayer and Harborne 1994). A few alkaloids are shown in Table 19.1 and Fig. 19.5 (bold numbers in brackets 25 to 28 refer).

A new alkaloid, puntarenine, which was isolated along with berberine from the organic extract of the whole plant *Xanthorhiza simplicissima*, was found to exhibit good activity against the dermatophytic fungus *T. mentagrophytes* and the yeast *S. cerevisiae* (Okunade et al. 1994). Dabur and co-workers isolated a novel alkaloid, 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate, from the plant *Datura metel* L. (Solanaceae). The compound was found to be active against all the species tested, namely *C. albicans*, *C. tropicalis*, *A. fumigatus*, *A. flavus,* and *A. niger* (Dabur et al. 2005). Klausmeyer et al. (2004) isolated a novel alkaloid, 6,8-didec-(1*Z*)-enyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium, from organic extract of *Aniba panurensis* using bioactivity-guided fraction. In vitro bioassay demonstrated the activity of this alkaloid against a drug-resistant strain of *C. albicans*.

Slobodníková et al. (2004) tested the crude extract of *Mahonia aquifolium* along with its two main alkaloids, berberine and jatrorrhizine, for their *in vitro* antifungal activity against 20 strains of *Candida* sp. isolated from chronic vulvovaginal candidoses. Both the compounds show varying levels of minimum inhibitory concentrations, illustrating strong antifungal activity. Bioassay-guided fractionation of the extract showing growth-inhibition against *Colletotrichum fragariae*, *C. gloeosporioides*, and *C. acutatum* has led to the isolation of the bioactive alkaloids, flindersine (**27**), anhydroevoxine, and haplamine (**28**). Of these,

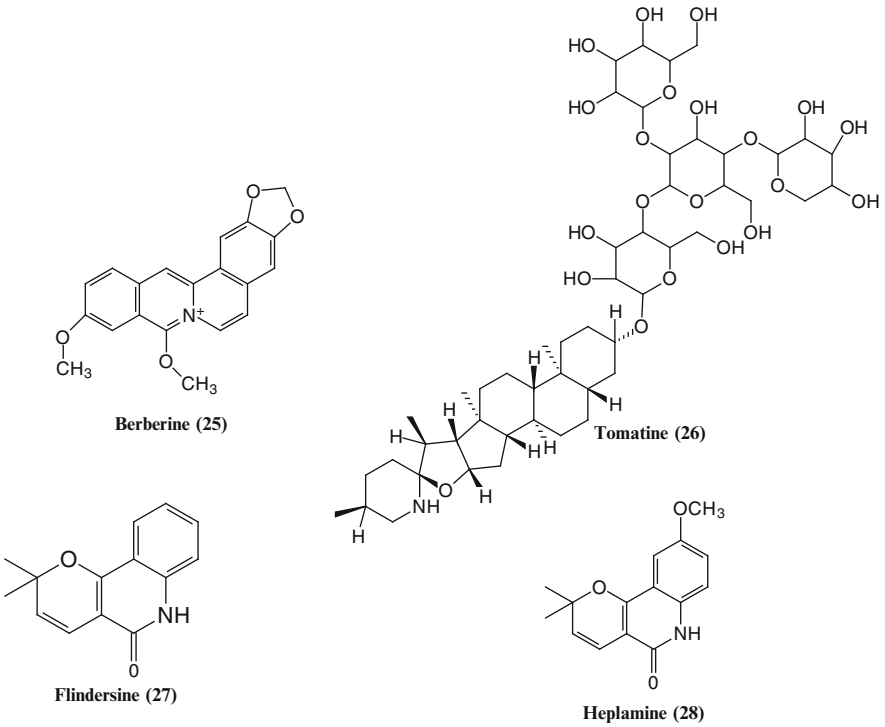


Fig. 19.5 Structures of antifungal alkaloids

flindersine and haplamine demonstrated the highest level of antifungal activity (Cantrell et al. 2005).

Bahçeevli et al. (2005) isolated A beta-carboline-, a tryptamine-, and two phenylethylamine-derived alkaloids along with three known aromatic compounds from the aerial parts and roots of *Cyathobasis fruticulosa*. The one new alkaloid, N-methyl-N-formyl-4-hydroxy-beta-phenylethylamine, showed marginal antifungal activity.

Rao et al. (2009) isolated a novel fibrecisine alkaloid [1,2-methylenedioxy-8-hydroxy-6a(*R*)-aporphine] with 21 known alkaloids including berberines, tetrahydroberberines, and aporphine derivatives. The bioassay result indicated that the berberines showed more potent activity than aporphine derivatives against the test *Candida* strains, while tetrahydroberberines showed very weak activity against *C. neoformans*. Similarly Meng et al. (2009) showed antifungal activity of the benzo[c]phenanthridine alkaloids from *Chelidonium majus* Linn against resistant clinical yeast isolates. Of the six compounds determined, 8-hydroxydihydro-sanguinarine and 8-hydroxydihydrochelerythrine demonstrated potent activity with the MIC ranges of 2–80 and 4–100 μg/ml, respectively. Dihydrosanguinarine, dihydrochelerythrine, sanguinarine, and chelerythrine had some degree of antifungal activity.

19.3.5 Proteins and Peptides

There are hundreds of antifungal peptides and proteins known, with more being discovered almost daily. They are produced by a multitude of organisms including leguminous flowering plants, nonleguminous flowering plants, gymnosperms, fungi, bacteria, insects, and mammals. In the last decade alone, many reports have been published on antifungal proteins and peptides.

Wong and Ng (2005a) isolated an antifungal peptide, vulgarinin, from the seeds of haricot beans (*Phaseolus vulgaris*) by using a simple protocol consisting of affinity chromatography on Affi-gel blue gel and gel filtration on Superdex 75. Vulgarinin manifested an antifungal activity toward fungal species such as *F. oxysporum, Mycosphaerella arachidicola, Physalospora piricola,* and *B. cinerea* (Wong and Ng 2005b). In the same year these authors reported lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*P. lunatus* L.), that has very good antifungal activity against *F. oxysporum, M. arachidicola*, and *B. cinerea*. Actinchinin, an antifungal protein, was isolated from the gold kiwi fruit. This protein exerts antifungal activity against *F. oxysporum* (Xia and Ng 2004).

Defensin has been isolated from the legume *Trigonella foenum-graecum*. The protein is characterized by the presence of eight cysteine residues, conserved in the various plant defensins and forming four disulphide bridges which stabilize the mature peptide. The protein exhibited antifungal activity against the broad host range fungus, *R. solani* and the peanut leaf spot fungus, *Phaeoisariopsis personata* (Olli and Kirti 2006). In addition to these, the work of Tzi Bun Ng (Ng 2004) has demonstrated the isolation of a number of antifungal peptides that have tremendous antifungal properties against a series of fungi.

19.4 Experimental Approaches

19.4.1 Selection of Plants

The choice of method for selecting plants for phytochemical and biological activity screening is often difficult. The large number of plants mentioned in traditional systems of medicine from all over the world requires a rationale for the discovery of interesting biologically active chemicals. Different approaches to chemical discovery can be distinguished; however, Fabricant and Farnsworth (2001) defined four standard approaches for selecting plants — (1) random selection followed by chemical screening, (2) random selection followed by antimicrobial assays, (3) follow-up of antimicrobial activity reports, and (4) follow-up of ethnomedical or traditional uses of plants against infectious diseases.

The first approach that defines searches for classes of secondary metabolites containing various antimicrobial substances (e.g., alkaloids, glycosides, isothiocyanates, etc.) is still very popular because the tests are easy to perform; however,

false-positive tests often render results difficult to interpret. The second approach for plant selection is quite labor-intensive, as it requires collection, identification, and analysis of all available plant parts, irrespective of prior knowledge and experience. However, third approach based on the follow up of current scientific literature available. The fourth approach includes plants used in traditional systems of medicine such as Ayurveda, Unani, Siddha, Kampo, and traditional Chinese medicine, Herbalism, folklore, and Shamanism and involves the use of databases, etc. A fifth, nonsystematic approach can be serendipity, where plant selection is based on ethnomedical use, but where the recovered bioactivity is new or unexpected, for example the anticancer compounds vinblastine and taxol (Clardy and Walsh 2004). Irrespective of the adopted plant collection strategy, when a plant has been identified, the first task should be the identification of bioactive compounds to the level of pure compound or to the level of major group of compounds.

19.4.2 Scheme for the Extraction and Identification of Active Principle

After selection of the plants, a major problem is the processing of the plant material that will be used in the panel of screening. Best strategies should be used to guarantee that the active principle is not destroyed, lost or altered. The first important measure should be the extraction period and efficiency. The extraction period can be shortened by grinding the plants finer and by shaking at a very rapid rate (Eloff and McGaw 2006). Extraction efficiency can be increased by sonicating the samples in a bath-type sonicator. Three to five extractions (10 min each, using a sonic bath) of very finely ground plant material with organic solvent of high polarity can extract up to 80% of the compounds on a dry-weight basis.

Choice of the solvent for extraction is also very important. Initial screening of the plants for biological activity starts with the use of aqueous or organic extracts followed by various fractionations. In one study Kotze and Eloff (2002) used 11–12 different extractants to extract and analyze the biological activity of *Combretum mycrophyllum*. Methanol, methylene dichloride, and tetrahydrofurane extract most components; however, diisopropyl ether, ethanol, ethyl ether, acetone, and ethyl acetate showed most biological activity with low quantity of other nonactive components. For hydrophilic compounds, polar solvents such as methanol, ethanol, or ethyl-acetate should be used; however, for extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol 1:1 should be used. In some instances, extraction in hexane is used to remove chlorophyll.

Various extraction and isolation strategies for pure compounds are reported in the literature, but successive fractionation using solvents of different polarity is commonly used. Figure 19.6 shows the simplified extraction and identification flow chart to be used in order to achieve/optimize bioactive phytochemical identification. After extracts/fractionation, a concentration step is usually required, which

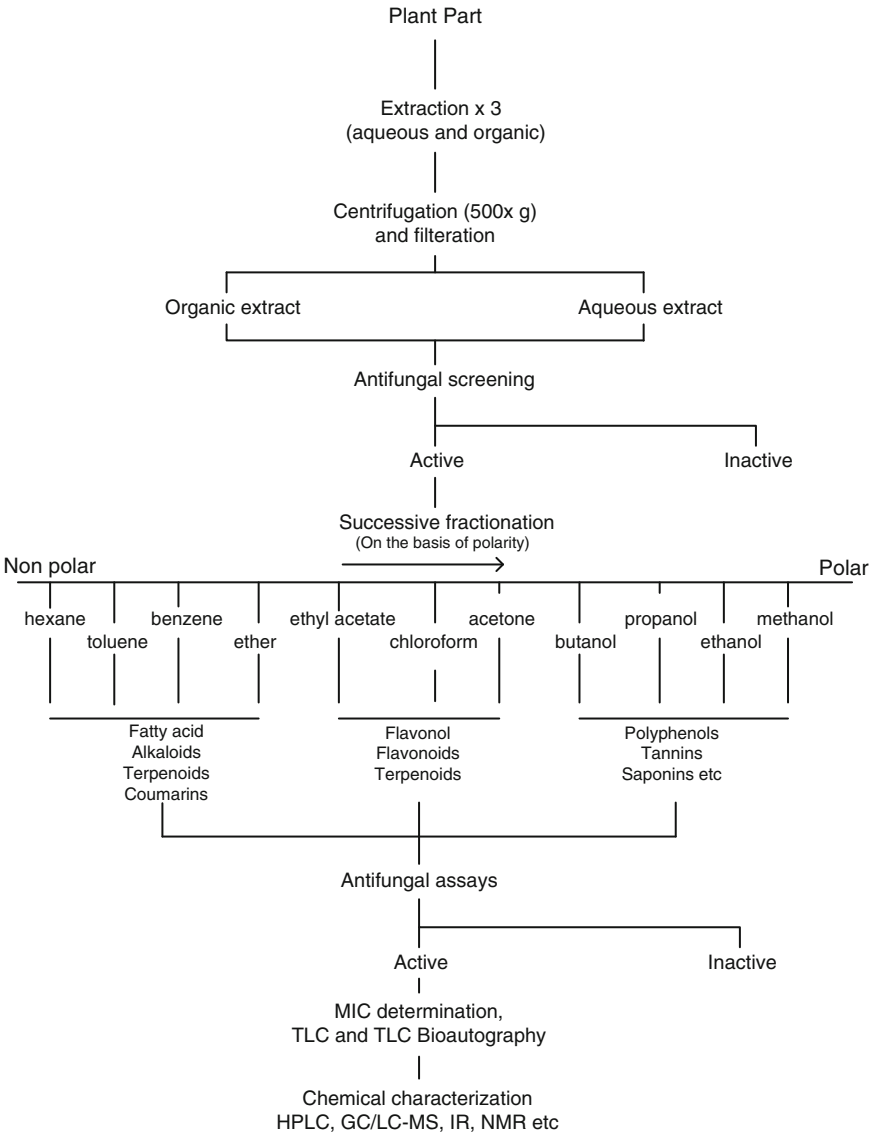


Fig. 19.6 Proposed scheme of extraction and identification of antifungal compounds from medicinal plants

should be based on evaporation of the solvent under vacuum at low temperature to avoid any loss of thermolabile constituent. In most cases, it is better if screening can be done prior to separating polar from less-polar constituents by the sequential use of solvents from high to low polarity. It saves labor and resources in the case of low or no activity.

19.4.3 Solvent for Testing

The most frequently used solvents to make up test compound solutions for quantitative purposes include dimethyl sulfoxide (DMSO), methanol, and ethanol. However, methanol and ethanol have the disadvantage of rapid evaporation. Solution in 100% DMSO is a better option as it can avoid the contamination and has good compatibility with most of the compounds. But it is also important to note that DMSO is potentially toxic for cells and many microorganisms, so the in-test concentration of DMSO should not exceed 1%.

On the other hand, sometimes dried extracts are not freely soluble even if the same solvent is used. In these conditions, it is better not to dry the extract. The aliquots of the extract can be dried after activity evaluation, and active concentration can be achieved by back calculation (Eloff and McGaw 2006). It is always preferable to store dry extracts/compounds at −20°C. However, extracts of a few plants in methanol and acetone are reported to retain activity, so may be supposed to be stable when stored at room temperature in dissolved form (Eloff et al. 2001).

19.4.4 Antifungal Assays

Several methods for detecting activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method. There are several methods available for antifungal activity testing, which can be classified into three main groups, i.e., diffusion, dilution, and bioautographic methods.

19.4.4.1 Agar Well Diffusion Method

The agar well diffusion method is the most widely used technique for assaying plant extracts for their antimicrobial activity. In this technique, a well or reservoir containing the test compound at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir (zone inhibition diameter) is measured at the end of the incubation period. Different types of reservoirs can be used, such as filter paper disks or holes punched in the medium. Of the various methods to conduct the assays described in the literature, however, the agar well diffusion method as described by Perez et al. (1990) can be easily used. We have adopted this method and used it since 1998 without any difficulty (Ahmad et al. 1998; Mehmood et al. 1999; Ahmad and Beg 2001; Aqil and Ahmad 2003; Aqil et al. 2005; Ahmad and Aqil 2007; Aqil and Ahmad 2007).

Small sample requirements and the possibility of testing six–eight extracts per plate against a single microorganism are specific advantages. In this assay 0.1 ml of diluted inoculum (10^5 CFU/ml) of test organism is spread on Saboroud Dextrose

agar plates. Wells of 8 mm diameter are punched into the agar medium and sealed with molten agar to prevent leaching of the compound. The wells are filled with 100 μl of plant extract of known concentration and solvent blank (≤1% DMSO) separately. The plates are incubated at 28 ± 2°C, for 72 h. The zone of inhibition of test organism growth around each well can be measured in mm. In order to enhance the detection limit, the inoculated plates can be kept at a lower temperature for several hours before incubation. In this way, compound diffusion can be enhanced over microbial growth, and better inhibition diameter is expected.

The agar well diffusion method also has some demerits. This method is not appropriate for the testing of nonpolar samples or samples that do not easily diffuse into agar, and it is not a preferred method for the testing of essential oils because of their volatile nature. The extra potency reported with this method is much less than for the dilution method, because the compound which is in contact with the test organism is much less than the loaded dose.

19.4.4.2 Broth Dilution Method

In this method, test compounds are mixed with a suitable medium and inoculated with the test organisms. Although it can be carried out in liquid as well as in solid media, the better way to perform it is in liquid media. The inhibition of fungal growth can be measured in a number of ways, for example by measuring optical density, by plating the known volume on the agar plates, or by the streak-plate method (Eloff and Mcgaw 2006; Aqil and Ahmad 2007). However, the most frequently used method measures turbidity and employs redox-indicators. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 405 nm. However, test samples that are not fully soluble may interfere with turbidity readings. In these cases, individual plants or plant samples may give better results.

The major benefit of this assay is that it allows determining whether a phytocompound or extract has a fungicidal or fungistatic action at tested concentration. This can be determined by plating the samples from the test mixture and assessing growth or no growth. Kostiala and Kostiala (1984) compared the disk diffusion method with the broth dilution method using different antibiotics against *C. albicans*. They concluded that disk diffusion assay is equally good or better if short incubation (24 h) is used; however, the broth dilution method was found to be more sensitive when incubation was extended to 48 h.

Eloff (1998) developed microdilution and macrodilution techniques using 96-well microplates and sugar tubes respectively. This technique uses the redox indicator p-iodonitro tetrazolium violet (MTT) and resazurin to quantify fungal growth (Jahn et al. 1995; Pelloux-Prayer et al. 1998). Resazurin has the advantage of not precipitating upon reduction, allowing direct reading. Visual readings can also be used if a spectrophotometer is not available, as these compounds produce an intense red color.

19.4.4.3 TLC-Bioautography Technique

Bioautography can explain how many biologically active compounds are present in an extract. The best use of this technique is that it permits localizing of antimicrobial activities of an extract on the chromatogram; it supports a quick search for new antimicrobial agents through bioassay-guided isolation. This can be achieved using one of two approaches; (1) contact bioautography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, or (2) agar overlay bioautography, where a seeded agar medium is applied directly onto the TLC plate.

For direct bioautographic assay, we used the agar overlay assay approach as described by Slusarenko et al. (1989): 5–10 µl of each plant extract are spotted on E-MERCK chromatographic silica gel G f_{254}, 0.25 mm-thick plates of 3 × 8 cm. The chromatograms are developed using different solvent systems. One milliliter of spore suspension (10^5 spores/ml) is used for each 10 ml of media. Developed chromatograms are placed in autoclaved petri plates. Spores of test fungi in molten agar medium are poured over the chromatograms. The plates are incubated at 28°C for 72 h. A zone of inhibition of bacterial growth could be seen around the active chromatogram spot. The spot was also confirmed by flooding the plates with 0.02 mg/ml solution of *p*-iodonitro-tetzolium.

Although the technique is very sensitive, its applicability is limited to fungi that easily grow on TLC plates. The bioautography technique is more difficult with fungi, because they grow more slowly and contamination can be a problem. Another problem is the need for complete removal of residual low volatile solvents.

19.4.4.4 Media, Inoculum and Organisms

Sabouraud (SAB) agar or broth is a general growth media for fungi. Yeast inoculates can be prepared from overnight cultures or from existing biofreeze stocks. It is recommended that collection is made from cultures during the logarithmic growth phase, and that four or five colonies of a pure culture on agar shold always be taken, to avoid selecting an atypical variant (Anon 2003). In dilution methods, an inoculum of about 10^4 spores/ml is adequate for most yeasts and fungi (Hadacek and Greger 2000). However in our laboratory we have used 10^5 spores/ml for both the agar well diffusion (0.1 ml) and the broth dilution methods, and found good response in all the studies.

The choice of test organisms always depends on the specific purpose of the investigation. However in a primary screening, the use of drug-sensitive reference strains is preferable, which should represent common pathogenic species of different classes. A small set of reference fungi is used for primary screening and includes *T. mentagrophytes* and *E. floccosum* as representatives of the dermatophytes. As opportunistic filamentous fungi, *A. niger* and *F. solani* are listed (Cos et al. 2006).

19.5 Conclusion

There is a growing body of evidence indicating the benefits of medicinal plants for their use against pathogenic microorganisms. Plant-based remedies used in human and animal medicine are an essential part of the primary health care system in many countries. Extensive screening programs of plants used mainly in traditional medicine have resulted in the discovery of thousands of phytochemicals with inhibitory effects on different types of microorganisms *in vitro*. Studies from all over the world have indicated that several plant extracts and their phytocompounds have been identified in an effort to supplement the relatively sparse portfolio of antifungal drugs. There is a need to exploit these bioactive compounds in disease caused by pathogenic fungi.

Although hundreds of antifungal compounds have been identified, it is important to keep in mind that while *in vitro* activity does not necessarily verify the efficacy of a plant extract, it does provide a preliminary indication of the usefulness and potential toxicity of the plant. Problems such as narrow spectrum of activity, susceptibility to efflux pumps, protein binding, serum inactivation, and poor pharmaceutical properties are associated with most of these compounds. Therefore, new anti-infective agents must act on drug-resistant pathogens and may work on multiple targets.

For antifungal activities, follow-up of bioassay-guided fractionation can be performed with an assay of choice. Whenever possible, final reporting of active extracts or pure compounds should be done by applying the broth-dilution method. Stringent endpoint criteria with IC_{50}-values generally below 100 μg/ml for extracts and below 25 μM for pure compounds should be set. Those bioactive compounds which show promising *in vitro* activity should be subjected to *in vivo* studies to determine their efficacy, stability, and bioavailability.

References

Abad MJ, Ansuategui M, Bermejo M (2007) Active antifungal substances from natural sources. ARKIVOC 7:116–145

Achenbach H, Stocker M, Constenla MA (1988) Flavonoid and other constituents of *Bauhinia manca*. Phytochemistry 27:1835–1841

Adikaram NKB, Ewing DF, Karunaratne AM, Wijcratne EMK (1992) Antifungal compounds from immature avocado fruit peel. Phytochemistry 31:93–96

Adou E, Williams RB, Schilling JK, Malone S, Meyer J, Wisse JH, Frederik D, Koere D, Werkhoven MC, Snipes CE, Werk TL, Kingston DG (2005) Cytotoxic diterpenoids from two lianas from the Suriname rainforest. Bioorg Med Chem 13:6009–6014

Afolayan AJ, Meyer JJ (1997) The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. J Ethnopharmacol 57:177–181

Ahmad I, Aqil F (2007) In vitro efficacy of bioactive extracts of 15 medicinal plants against ESβL-producing multidrug-resistant enteric bacteria. Microbiol Res 162:264–275

Ahmad I, Beg AZ (2001) Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. J Ethnopharmacol 74:113–123

Ahmad I, Mehmood Z, Mohammad F (1998) Screening of some Indian medicinal plants for their antimicrobial properties. J Ethnopharmacol 62:183–193

Aiyegoro OA, Okoh AI (2009) Phytochemical screening and polyphenolic antioxidant activity of aqueous crude leaf extract of *Helichrysum pedunculatum*. Int J Mol Sci 10(11):4990–5001

Akinpelu DA, Obuotor EM (2000) Antibacterial activity of *Nicotiana tabacum* leaves. Fitoterapia 71:199–200

Alanís-Garza GM, González-González R, Salazar-Aranda N, Waksman de Torres VM, Rivas-Galindo (2007) Screening of antifungal activity of plants from the northeast of Mexico. J Ethnopharmacol 114:468–471

Alcerito T, Barbo FE, Negri G, Santos DYAC, Meda CI, Young MCM, Chávez D, Blatt CTT (2002) Foliar epicuticular wax of *Arrabidaea brachypoda*: flavonoids and antifungal activity. Biochem Syst Ecol 30:677–683

European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (2003) Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin Microbiol Infection 9:1–97

Aqil F, Ahmad I (2003) Broad-spectrum antibacterial and antifungal properties of certain traditionally used Indian medicinal plants. World J Microbiol Biotechnol 19:653–657

Aqil F, Ahmad I (2007) Antibacterial properties of traditionally used Indian medicinal plants. Methods Find Exp Clin Pharmacol 29:79–92

Aqil F, Khan MSA, Owais M, Ahmad I (2005) Effect of certain bioactive plant extracts on clinical isolates of β-lactamase producing methicillin resistant *Staphylococcus aureus*. J Basic Microbiol 45:106–114

Bahçeevli AK, Kurucu S, Kolak U, Topçu G, Adou E (2005) Alkaloids and aromatics of *Cyathobasis fruticulosa* (Bunge) Aellen. J Nat Prod 68:956–958

Banthorpe DV, Bilyard HJ, Brown OD (1989) Enol esters of caffeic acid in several genera of the labiatae. Phytochemistry 28:2109–2113

Barrero AF, Oltra JE, Alvarez M, Raslan DS, Saude DA, Akssira M (2000) New sources and antifungal activity of sesquiterpene lactones. Fitoterapia 71:60–64

Bbosa GS, Kyegombe DB, Ogwal-Okeng J, Bukenya-Ziraba R, Odyek O, Waako P (2007) Antibacterial activity of *Mangifera indica* (L.). Afr J Ecol 45:13–16

Bednarek P, Kerhoas L, Einhorn J, Franski R, Wojtaszek P, Rybus-Zaj M, Stobiecki M (2003) Profiling of flavonoid conjugates in *Lupinus albus* and *Lupinus angustifolius* responding to biotic and abiotic stimuli. J Chem Ecol 29:1127–1142

Bhakuni DS, Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN (1969) Screening of Indian plants for biological activity. II. Indian J Exp Biol 7:250–262

Bhakuni DS, Dhar ML, Dhar MM, Dhawan BN, Gupta B, Srimal RC (1971) Screening of Indian plants for biological activity. III. Indian J Exp Biol 9:91–102

Bisoli E, Garcez WS, Hamerski L, Tieppo C, Garcez FR (2008) Bioactive pentacyclic triterpenes from the stems of *Combretum laxum*. Molecules 13:2717–2728

Boddu J, Svabek C, Sekhon R, Gevens A, Nicholson RL, Jones AD, Pedersen JF, Gustine DL, Chopra S (2004) Expression of a putative *flavonoid 3′-hydroxylase* in sorghum mesocotyls synthesizing 3-deoxyanthocyanidin phytoalexins. Physiol Mol Plant Pathol 65:101–113

Bokel N, Diyasenu MNC, Gunatilaka AAL, Kraus W, Sotheeswaran S (1988) Canaliculatol, an antifungal resveratrol trimer from *Stemonoporous canaliculatus*. Phytochemistry 27: 377–380

Bordoloi M, Saikia B, Mathur RK, Goswumi BN (1993) A meliacin from *Chisocheton paniculatus*. Phytochemistry 34:583–584

Braga FG, Bouzada MLM, Fabri RL, Matos MO, Moreira FO, Scio E, Coimbra ES (2007) Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. J Ethnopharmacol 111:396–402

Brownlee HE, McEuen AR, Hedger J, Scott IM (1990) Anti-fungal effects of cocoa tannin on the witches' broom pathogen *Crinipellis pernicious*. Physiol Mol Plant Pathol 36:39–48

Bylka W, Szaufer M, Matlawska J, Goslinska O (2004) Antimicrobial activity of isocytisoside and extracts of *Aquilegia vulgaris*. Lett Appl Microbiol 39:93–97

Cannon RD, Holmes AR, Mason AB, Monk BC (1995) Oral *candida*: clearance, colonization or candidiasis? J Dent Res 74:1152–1161

Cantrell CL, Schrader KK, Mamonov LK, Sitpaeva GT, Kustova TS, Dunbar C (2005) Isolation and identification of antifungal and antialgal alkaloids from *Haplophyllum sieversii*. J Agric Food Chem 53:7741–7748

Carpinella MC, Ferravoli CG, Palacios SM (2005) Antifungal synergistic effect of scopoletin, a hydroxycoumarin isolated from melia azedarach L. Fruits. J Agric Food Chem 53: 2922–2227

Cavin AL, Hay AE, Marston A, Stoeckli H, Scopelliti R, Diallo D, Hostettmann K (2006) Bioactive diterpenes from the fruits of *Detarium microcarpum*. J Nat Prod 69:768–773

Cho JY, Moon JH, Seon KY, Park KH (1998) Antimicrobial activity of 4-Hydroxybenzoic acid and *trans* 4-hydroxycinnamic acid isolated and identified from rice hull. Biosci Biotechnol Biochem 62:2273–2276

Clardy J, Walsh C (2004) Lessons from natural molecules. Nature 432:829–837

Cojocaru M, Droby S, Glotter E, Goldman A, Gottlieb HE, Jacoby B, Prusky D (1986) 5-(12-Heptadecenyl)-resorcinol, the major component of the antifungal activity in the peel of mango fruit. Phytochemistry 25:1093–1095

Cole MD, Bridge PD, Dellar JE, Fellows LE, Cornish MC, Anderson JC (1991) Antifungal activity of neo-clerodane diterpenoids from Scutellaria. Phytochemistry 30:1125–1127

Cos P, Vlietinck AJ, Berghe DV, Maes L (2006) Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. J Ethnopharmacol 106:290–306

Cowan MM (1999) Plant products as antimicrobial agents. Clin Microbiol Rev 12:564–582

Cox PA (1998) The promise of Gerard's Herball: new drugs from old books. Endeavour 22:51–53

Cragg GM, Boyd MR, Cardellina JH, Newman DJ, Snader KM, McCloud TG (1994) Ethnobotany and drug discovery: the experience of the US National Cancer Institute. Ciba Found Symp 185:178–190

Cragg GM, Newman DJ, Snader KM (1997) Natural products in drug discovery and development. J Nat Prod 60:52–60

Cuenca-Estrella M, Mellado E, Diaz-Guerra TM, Monzon A, Rodriguez-Tudela JL (2000) Susceptibility of fluconazole-resistant clinical isolates of *Candida* spp. to echinocandin LY303366, itraconazole and amphotericin B. J Antimicrob Chemother 46:475–477

Dabur R, Chhillar AK, Yadav V, Kamal PK, Gupta J, Sharma GL (2005) In vitro antifungal activity of 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate, a dihydro–pyrrole derivative. J Med Microbiol 54:549–552

de Bertoldi C, de Leo M, Braca A, Ercoli L (2009) Bioassay-guided isolation of allelochemicals from *Avena sativa* L.: allelopathic potential of flavone *C*-glycosides. Chemoecology 19:169–176

de Leo M, Braca A, De Tomasi N, Norscia I, Morelli I, Battinelli L, Mazzanti G (2004) Phenolic compounds from *Baseonema acuminatum* leaves: isolation and antimicrobial activity. Planta Med 70:841–846

Defago G, Kern H (1988) Induction of *Fusarium solani* mutants insensitive to tomatine, their pathogenicity and aggressiveness to tomato fruits and pea plants. Physiol Plant Pathol 22:29–37

Deng Y, Nicholson RA (2005) Antifungal properties of surangin B, a coumarin from *Mammea longifolia*. Planta Med 71:364–365

Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN, Ray C (1968) Screening of Indian plants for biological activity. Indian J Exp Biol 6:232–247

Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN, Srimal RC, Tandon JS (1973) Screening of Indian plants for biological activity. IV. Indian J Exp Biol 11:43–54

Dhar ML, Dhawan BN, Prasad CR, Rastogi RP, Singh KK, Tandon JS (1974) Screening of Indian plants for biological activity. V. Indian J Exp Biol 12:512–523

Dhatwalia VK, Sati OP, Tripathi MK, Kumar A (2009) Isolation, characterization and antimicrobial activity at diverse dilution of wheat puroindoline protein. World J Agric Sci 5:297–300

Dhawan BN, Patnaik GK, Rastogi RP, Singh KK, Tandon JS (1977) Screening of Indian plants for biological activity. VI. Indian J Exp Biol 15:208–219

Dhawan BN, Dubey MP, Mehrotra BN, Rastogi RP, Tandon JS (1980) Screening of Indian plants for biological activity. IX. Indian J Exp Biol 18:594–606

Douros J, Suffness M (1881) New natural products under development at the National Cancer Institute. Recent Results Cancer Res 76:153–175

Douros J, Suffness M (1980) The National Cancer Institute's Natural Products Antineoplastic Development Program. Recent Results Cancer Res 70:21–44

Duke (2004) Biological activity summary for cocoa (*Theobroma cacao* L.). J Med Food 3:115–119

Eloff JN (1998) Sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extracts for bacteria. Planta Med 64:711–713

Eloff JN, Mcgaw LJ (2006) Plant extracts used to manage bacterial, fungal and parasitic infections in South Africa. In: Ahmad I, Aqil F, Owais M (eds) Modern phytomedicine: turning medicinal plants into drugs. Wiley, Germany, pp 97–119

Eloff JN, Jager AK, van Staden JS (2001) The stability and relationship between anti-inflammatory activity and antibacterial activity of South African *combretum* species. S Afr J Sci 97:291–293

Endo K, Kanno E, Oshima Y (1990) Structures of antifungal diarylheptenones, gingerenones A, B, C and isogingerenone B, isolated from the rhizomes of *Zingiber officinale*. Phytochemistry 29:797

Erdemoglu N, Ozkan S, Tosun F (2007) Alkaloid profile and antimicrobial activity of *Lupinus angustifolius* L. alkaloid extract. Phytochem Rev 6:197–201

Ezoubeiri A, Gadhi CA, Fdil N, Benharref A, Jana M, Vanhaele M (2005) Isolation and antimicrobial activity of two phenolic compounds from *Pulicaria odora* L. J Ethnopharmacol 99:287–292

Fabricant DS, Farnsworth NR (2001) The value of plants used in traditional medicine for drug discovery. Environ Health Perspect 109:69–75

Fanos V, Cataldi L (2000) Amphotericin B-induced nephrotoxicity: a review. J Chemother 12:463–470

Franich RA, Gadgil PD, Shain L (1983) Fungistatic effects of *Pinus radiata* needle epicuticular fatty and resin acids on *Dothistroma pini*. Physiol Plant Pathol 23:183–195

Freile M, Giannini M, Sortino M, Zamora M, Juarez A, Zacchino S, Enriz D (2006) Antifungal activity of aqueous extracts and of Berberine Isolated from *Berberis heterophylla*. Acta Farm Bonaerense 25:83–88

Fujita K, Kubo I (2005) Naturally occurring antifungal agents against *Zygosaccharomyces bailii* and their synergism. J Agric Food Chem 53:5187–5191

Galeotti F, Barile E, Curir P, Dolci M, Lanzotti V (2008) Flavonoids from carnation (*Dianthus caryophyllus*) and their antifungal activity. Phytochem Lett 1:44–48

Gearhart MO (1994) Worsening of lung function with fluconazole and review of azole antifungal hepatotoxicity. Ann Pharmacother 28:1177–1181

Giordani R, Trebaux J, Masi M, Regli P (2001) Enhanced antifungal activity of ketoconazole by *Euphorbia characias* latex against *Candida albicans*. J Ethnopharmacol 78:1–5

Goa KI, Barradell LB (1995) Fluconazole: an update of its pharmacodynamic and pharmacokinetic properties and therapeutic use in major superficial and systemic mycoses in immunocompromised patients. Drugs 50:658–690

Gomez-Flores R, Arzate-Quintana C, Quintanilla-Licea R, Tamez-Guerra P, Tamez-Guerra R, Monreal-Cuevas E, Rodríguez-Padilla C (2008) Antimicrobial activity of *Persea americana* Mill (Lauraceae) (Avocado) and *Gymnosperma glutinosum* (Spreng.) Less (Asteraceae) leaf extracts and active fractions against *Mycobacterium tuberculosis*. Am Eur J Sci Res 3:188–194

Gonzalez M, Zamilpa A, Marquina S, Navarro V, Alvarez L (2004) Antimycotic spirostanol saponins from *Solanum hispidum* leaves and their structure–activity relationships. J Nat Prod 67:938–941

Grasela TH, Goodwin SD, Walawander MK (1990) Prospective surveillance of intravenous amphotericin B use patterns. Pharmacother 10:341–348

Grayer RJ, Harborne JB (1994) A survey of antifungal compounds from higher plants 1982–1993. Phytochemistry 37:19–42

Greathouse GA, Walkins GH (1938) Berberine as a factor in the resistance of Mahonia trifoliata and M. swaseya to Phymatotrichum root-rot. Am J Bot 25:743

Greger H, Hofer O, Kiihlig H, Wurz G (1992) Sulfur containing cinnamides with antifungal activity from *Glycosmis cyanocarpa*. Tetrahedron 48:1209–1218

Greger H, Zechner G, Hofer O, Hadacek F, Wurz G (1993) Sulphur-containing amides from Glycosmis species with different antifungal activity. Phytochemistry 34:175–179

Guerrini A, Sacchetti G, Rossi D, Paganetto G, Muzzoli M, Andreotti E, Tognolini M, Maldonado ME, Bruni R (2009) Bioactivities of *Piper aduncum* L. and *Piper obliquum* Ruiz & Pavon (Piperaceae) essential oils from Eastern Ecuador. Environ Toxicol Pharmacol 27:39–48

Gurgel LA, Sidrim JJC, Martins DT, Filho CV, Rao VS (2005) In vitro antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. J Ethnopharmacol 97:409–412

Hadacek F, Greger H (2000) Testing of antifungal natural products: methodologies, comparability of results and assay choice. Phytochem Anal 11:137–147

Hamburger M, Dudan G, Nair AGN, Jayaprakasam R, Hostettmann K (1989) An antifungal triterpenoid from *Mollugo pentaphylla*. Phytochemistry 28:1767–1768

Hamza OJM, Beukel CJPB, Matee MIN, Moshi MJ, Mikx FHM, Selemani O, Mbwambo ZH, Van der Ven AJAM, Verweij PE (2006) Antifungal activity of some Tanzanian plants used traditionally for the treatment of fungal infections. J Ethnopharmacol 108:124–132

Hanus LO, Rezanka T, Dembitsky VM, Moussaieff A (2005) Myrrh–commiphora chemistry. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 149:3–27

Harborne JB, Baxter H (eds) (1993) Phytochemical dictionary. Taylor and Francis, London

Har-Nun N, Meyer AM (1990) Cucurbitacins protect cucumber tissue against infection by *Botrytis cinerea*. Phytochemistry 29:787–791

Hoof LV, Berghe DAV, Vlietinck AJ (2008) Screening of poplar trees for antibacterial, antifungal and antiviral activity. Biol Plant 22:265–273

Ingham JL (1973) Disease resistance in higher plants. Phytopath Z 78:314

Ingham JL, Tahara S, Harborne JB (1983) Fungitoxic isoflavones from *Lupinus albus* and other *Lupinus* species. Z Naturforsch 38c:194–200

Ito T, Kumazawa K (1992) Antifungal substances from mechanically damaged cherry leaves (*Prumus yedoensis* matsumura). Biosci Biotechnol Biochem 56:1655

Jahn B, Martin E, Stueben A, Bhakdi S (1995) Susceptibility testing of *Candida albicans* and *Aspergillus* species by a simple microtitre menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. J Clin Microbiol 33:661–667

Jambunathan R, Butler LG, Bandsyopadkyay R, Mughogho K (1986) Polyphenol concentrations in grain, leaf and callus tissues of mold-susceptible and mold-resistant sorghum cultivars. J Agric Food Chem 34:425–429

Kabuki T, Nakajima H, Arai M, Ueda S, Kuwabara Y, Dosako S (2000) Characterization of novel antimicrobial compounds from mango (*Mangifera indica* L.) kernel seeds. Food Chem 71:61–66

Kadavul K, Dixit AK (2009) Ethnomedicinal studies of the woody species of Kalrayan and Shervarayan Hills, Eastern Ghats, Tamil Nadu. Indian J Trad Knowl 8:592–597

Kil HY, Seong ES, Ghimire BK, Chung M, Kwon SS, Goh EJ, Heo K, Kim MJ, Lim JD, Lee D, Yu CY (2009) Antioxidant and antimicrobial activities of crude sorghum extract. Food Chem 115:1234–1239

Kim KY, Davidson PM, Chung HJ (2001) Antibacterial activity in extracts of *Camellia japonica* L. petals and its application to a model food system. J Food Prot 64:1255–1260

Kim YM, Lee CH, Kim HG, Lee HS (2004) Anthraquinones Isolated from *Cassiatora* (Leguminosae) seed show an antifungal property against phytopathogenic fungi. J Agric Food Chem 52:6096–6100

Kim SS, Kim JY, Lee NH, Hyun CG (2008) Antibacterial and anti-inflammatory effects of Jeju medicinal plants against acne-inducing bacteria. J Gen Appl Microbiol 54:101–106

Klausmeyer P, Chmurny GN, McCloud TG, Tucker KD, Shoemaker RH (2004) A novel antimicrobial indolizinium alkaloid from *Aniba panurensis*. J Nat Prod 67(10):1732–1735

Kobayashi K, Nishino C, Tomita H, Fukushima M (1987) Antifungal activity of pisiferic acid derivatives against the rice blast fungus. Phytochemistry 26:3175–3179

Koehn FE, Carter GT (2005) The evolving role of natural products in drug discovery. Nat Rev Drug Discov 4:206–220

Koroishi AM, Foss SR, Cortez DAG, Nakamura TU, Nakamura CV, Filho BPD (2008) In vitro antifungal activity of extracts and neolignans from *Piper regnellii* against dermatophytes. J Ethnopharmacol 117:270–277

Kostiala AAI, Kostiala I (1984) Broth dilution and disc diffusion methods in the susceptibility testing of pathogenic *Candida albicans* against four antimycotics. Mycopathologia 87:121–127

Kotze M, Eloff JN (2002) Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). S Afr J Bot 68:62–67

Krisch J, Ordogh L, Galgoczy L, Papp T, Vagvolgyi C (2009) Anticandidal effect of berry juices and extracts from *Ribes* species. Cent Eur J Biol 4:86–89

Kuc J, Henze RE, Ullstrup A1, Quacken-Bush FW (1956) Chlorogenic and caffeic acids as fungistatic agents produced by potatoes in response to inoculation with *Helminthosporium carbonium*. J Am Chem Soc 78:3123–3125

Kumar V, Karunaratne V, Sanath MR, Meegalle K, Macleod JK (1990) Two fungicidal phenylethanones from Euodia lunu-ankenda root bark. Phytochemistry 29:243–245

Lago JH, Ramos CS, Casanova DC, Morandim AA, Bergamo DC, Cavalheiro AJ, Bolzani VS, Furlan M, Guimaraes EF, Young MC, Kato MJ (2004) Benzoic acid derivatives from *Piper* species and their fungitoxic activity against *Cladosporium cladosporioides* and *C. sphaerospermum*. J Nat Prod 67:783

Lall N, Weiganand O, Hussein AA, Meyer JJM (2006) Antifungal activity of naphthoquinones and triterpenes isolated from the root bark of *Euclea natalensis*. S Afr J Bot 72:579–583

Lamidi M, Digiorgio C, Delmas F, Favel A, Egele C, Rondi ML, Ollivier E, Uze L, Balansard G (2005) In vitro cytotoxic, antileishmanial and antifungal activities of ethnopharmacologically selected Gabonese plants. J Ethnopharmacol 102:185

Lavault M, Landreau A, Larcher G, Bouchara JP, Pagniez F, Le Pape P, Richomme P (2005) Antileishmanial and antifungal activities of xanthanolides isolated from *Xanthium macrocarpum*. Fitoterapia 76:363–366

Lazar JD, Wilner KD (1990) Drug interactions with fluconazole. Rev Infect Dis 12:S327–S333

Lee HB, Lee HK, Ahn YJ (2003) Antibacterial activity of oriental medicinal plant extracts toward *Helicobacter pylori*. Agric Chem Biotechnol 46:97–99

Lee DG, Park Y, Kim MR, Jung HJ, Seu YB, Hahm KS, Woo ER (2004) Anti-fungal effects of phenolic amides isolated from the root bark of *Lycium chinense*. Biotechnol Lett 26:1125–1130

Li S, Zhang Z, Cain A, Wang B, Long M, Taylor J (2005) Antifungal activity of camptothecin, trifolin, and hyperoside isolated from *Camptotheca acuminata*. J Agric Food Chem 53:32–37

Liyana-Pathirana CM, Shahidi F (2005) Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions. J Agric Food Chem 53:2433–2440

Luciano JHS, Lima MAS, de Souza EB, Silveira ER (2004) Chemical constituents of *Alibertia myrciifolia* Spruce ex K. Schum. Biochem Syst Ecol 32:1227–1229

Luo DQ, Wang H, Tian X, Shao NJ, Liu JK (2005) Antifungal properties of pristimerin and celastrol isolated from *Celastrus hypoleucus*. Pest Manag Sci 61:85–90

Malan E, Swinny E (1993) Substituted bibenzyls, phenanthrenes and 9, 10-dihydrophenan-threnes from the heartwood of *Combretum apiculatum*. Phytochemistry 34:1139–1142

Mandal P, Sinha SP, Mandal NC (2005) Antimicrobial activity of saponins from *Acacia auriculiformis*. Fitoterapia 76:462–465

Manojlovic NT, Solujic S, Sukdolak S, Milosev M (2005) Antifungal activity of *Rubia tinctorum, Rhamnus frangula* and *Caloplaca cerina*. Fitoter 76:244–246

Maregesi SM, Pieters L, Ngassapa OD, Apers S, Vingerhoets R, Cos P, Vanden Berghe DA, Vlietinck AJ (2008) Screening of some Tanzanian medicinal plants from Bunda district for antibacterial, antifungal and antiviral activities. J Ethnopharmacol 119:58–66

Marston A, Gafner F, Dossagi SF, Hostettmann K (1988) Fungicidal and molluscicidal saponins from *Dolichos kilimandscharicus*. Phytochemistry 27:1325–1326

Marston A, Hamburger M, Sordat-Disercns I, Msonthi JD, Hostettmann K (1993) Xanthones from *Polygala nyikensis*. Phytochemistry 33:809–812

Marthanda M, Subramanyan M, Hima M, Annapurna J (2005) Antimicrobial activity of clerodane diterpenoids from *Polyalthia longifolia* seeds. Fitoterapia 76:336

Masoko P, Picard J, Eloff JN (2007) The antifungal activity of twenty-four Southern African *Combretum* species (Combretaceae). S Afr J Bot 73:173–183

Mathias-Mundy E, McCorkle C (1995) Ethnoveterinary medicine and development: a review of the literature. In: Warren DM, Surrerwer L, Broshenka D (eds) The cultural dimension of indigenous knowledge systems. Intermediate Technology Publications, London, pp 488–498

McDowell PG, Lwande W, Deans SG, Waterman PG (1988) Volatile resin exudate from stem bark of *Commiphora rostrata*: potential role in plant defence. Phytochemistry 27:2519–2521

McGaw LJ, Rabe T, Sparg SG, Jager AK, Eloff JN, van Staden J (2001) An investigation on the biological activity of *Combretum* species. J Ethnopharmacol 75:45–50

Mehmood Z, Ahmad I, Mohammad F, Ahmad S (1999) Indian medicinal plants: a potential source for anticandidal drugs. Pharmaceut Biol 37:237–242

Mel'nichenko EG, Kirsanova MA, Grishkovets VI, Tysh LV, Krivorutchenko IL (2003) Antimicrobial activity of saponins from *Hedera taurica* Carr. Mikrobiol Z 65:8–12

Meng F, Zuo G, Hao X, Wang G, Xiao H, Zhang J, Xu G (2009) Antifungal activity of the benzo [c]phenanthridine alkaloids from *Chelidonium majus* Linn against resistant clinical yeast isolates. J Ethnopharmacol 125:494–496

Miles OH, Rosa de Medeiros JM, Chittawong Y, Hedin PA, Swithenbunk C, Lidert Z (1991) 3′-formyl-2′, 4′,6′-trihydroxydihydrochalcone from *Psidium acutangulum*. Phytochemistry 30:1131–1132

Miles DH, Chittawong V, Hedin PA, Kokpol U (1993) Potential agrochemicals from leaves of *Wedelia biflora*. Phytochemistry 32:1427–1429

Mizobuchi S, Sate Y (1984) A new flavanone with antifungal activity isolated from hops. Agric Biol Chem 48:2771–2775

Mohamed IE, Nur EBEE, Choudhary MI, Khan SN (2009) Bioactive natural products from two Sudanese medicinal plants *Diospyros mespiliformis* and *Croton zambesicus*. Rec Nat Prod 3:198–203

Mokbel MS, Hashinaga F (2005) Antibacterial and antioxidant activities of banana (*Musa*, AAA cv. Cavendish) fruits peel. Am J Biochem Biotechnol 1:125–131

Muirhead IF, Deverall BJ (1984) Evaluation of 3, 4-dihydroxybenzaldehyde, dopamine and its oxidation products as inhibitors of *Colletotrichum musae* (Berk. and Curt.) arx in green banana fruits. Aust J Bot 32:375–382

Muschietti L, Derita M, Sülsen V, Muñoz JD, Ferraro G, Zacchino S, Martino V (2005) In vitro antifungal assay of traditional Argentine medicinal plants. J Ethnopharmacol 102:233–238

Nagata T, Tsushida T, Hamaya E, Enoki N, Manabe S, Nishino C (1985) Camellidins: antifungal saponins isolated from *Camellia japonica*. Agric Biol Chem 49:1181–1186

Naldoni FJ, Claudino AL, Cruz JW Jr, Chavasco JK, Faria e Silva PM, Veloso MP, Dos Santos MH (2009) Antimicrobial activity of benzophenones and extracts from the fruits of *Garcinia brasiliensis*. J Med Food 12:403–407

Natarajan P, Katta S, Andrei I, Ambati VBR, Leonida M, Haas GJ (2008) Positive antibacterial co-action between hop (*Humulus lupulus*) constituents and selected antibiotics. Phytomedicine 15:194–201

Newman DJ, Cragg GM, Snader KM (2003) Natural products as sources of new drugs over the period 1981–2002. J Nat Prod 66:1022–1037

Ng TB (2004) Antifungal proteins and peptides of leguminous and non-leguminous origins. Peptides 25:1215–1222

Nowakowska Z (2007) A review of anti-infective and anti-inflammatory chalcones. Eur J Med Chem 42:125–137

Ohtani K, Mavi S, Hostettmann K (1993) Molluscicidal and antifungal triterpenoid saponins from *Rapanea melanophloeos* leaves. Phytochemistry 33:83–86

Ojala T, Remes S, Haansuu P, Vuorela H, Hiltunen R, Haahtela K, Vuorela P (2000) Antimicrobial activity of some coumarin containing herbal plants growing in Finland. J Ethnopharmacol 73:299–305

Okeke IN, Laxmaninarayan R, Bhutta ZA, Duse AG, Jenkins P, O'Brien TF, Pablos-Mendez A, Klugman KP (2005) Antimicrobial resistance in developing countries. Part 1: recent trends and current status. Lancet Infect Dis 5:481–493

Okunade AL, Hufford CD, Richardson MD, Peterson JR, Clark AM (1994) Antimicrobial properties of alkaloids from *Xanthorhiza simplicissima*. Pharm Sci 83:404–406

Olli S, Kirti PB (2006) Cloning, characterization and antifungal activity of defensin Tfgd1 from *Trigonella foenum-graecum* L. J Biochem Mol Biol 39:278–283

Oluwatuyi M, Kaatz GW, Gibbons S (2004) Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochemistry 65:3249–3254

Orjala J, Erdelmeier CAJ, Wright AD, Rali T, Sticher O (1993) Two chromenes and a prenylated benzoic acid derivative from *Piper aduncum*. Phytochemistry 34:813–818

Pacher T, Bacher M, Hofer O, Greger H (2001) Stress induced carbazole phytoalexins in *Glycosmis* species. Phytochemistry 58:129–135

Park M, Bae J, Lee DS (2008) Antibacterial activity of [10]-gingerol and [12]-gingerol isolated from ginger rhizome against periodontal bacteria. Phytother Res 22:1446–1449

Pelloux-Prayer AL, Priem B, Joseleau JP (1998) Kinetic evaluation of conidial germination of *Botrytis cinerea* by a spectrofluorometric method. Mycol Res 102:320–322

Perez C, Pauli M, Bazerque P (1990) An antibiotic assay by the well agar method. Acta Biol Med Exp 15:113–115

Phongmaykin J, Kumamoto T, Ishikawa T, Suttisri R, Saifah E (2008) A new sesquiterpene and other terpenoid constituents of *Chisocheton penduliflor*u. Arch Pharm Res 31:21–27

Pintore G, Usai M, Bradesi P, Juliano C, Boatto G, Tomi F, Chessa M, Cerri R, Casanova J (2001) Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. oils from Sardinia and Corsica. Flavor Fragrance J 17:15–19

Pistelli L, Bertoli A, Lepori E, Morelli I, Panizzi L (2002) Antimicrobial and antifungal activity of crude extracts and isolated saponins from *Astragalus verrucosus*. Fitoterapia 73:336–339

Portillo A, Vila R, Freixa B, Adzet T, Cañigueral S (2001) Antifungal activity of Paraguayan plants used in traditional medicine. J Ethnopharmacol 76:93–98

Portillo A, Vila R, Freixa B, Ferro E, Parello T, Casanova J, Cañigeral S (2005) Phenylethanoid glycosides from Cistanches salsa inhibit apoptosis induced by 1-methyl-4-phenylpyridinium ion in neurons. J Ethnopharmacol 97:49

Potterat O, Stoeckli-Evans H, Msonthi JD, Hostettmann K (1987) Two new antifungal naphthoxirene derivatives and their glucosides from Sesamum angolense WELW. Helv Chim Acta 70:1551–1557

Prats E, Galindo JC, Bazzalo ME, León A, Macías FA, Rubiales D, Jorrín JV (2007) Antifungal activity of a new phenolic compound from capitulum of a head rot-resistant sunflower genotype. J Chem Ecol 33:1245–1253

Prusky D, Keen NT, Eaks J (1983) Further evidence for the involvement of a pre-formed antifungal compound in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. Physiol Mol Plant Pathol 22:189–198

Ragasa CY, Co AL, Rideout JA (2005) Antifungal metabolites from *Blumea balsamifera*. Nat Prod Res 19:231–237

Rao GX, Zhang S, Wang HM, Li ZM, Gao S, Xu GL (2009) Antifungal alkaloids from the fresh rattan stem of *Fibraurea recisa* Pierre. J Ethnopharmacol 123:1–5

Rasoamiaranjanahary L, Marston A, Guilet D, Schenk K, Randimbivololona F, Hostettmann K (2003) Antifungal diterpenes from *Hypoestes serpens* (Acanthaceae). Phytochemistry 62:333–337

Rastogi RP, Dhawan BN (1982) Research on medicinal plants at the Central Drug Research Institute, Lucknow (India). Indian J Med Res 76:27–45

RatnayakeBandara BM, Wimalasiri WR (1988) Diterpene alcohols from *Croton lacciferus*. Phytochemistry 27:225–226

RatnayakeBandara BM, Hewage CM, Karun-aratne V, Wannigama P, Adikaram NKB (1992) An antifungal chromene from *Eupatorium riparium*. Phytochemistry 31:1983–1985

Reuveni M, Tuzun S, Cole JS, Siegel R, Nesmith WC, Kuc J (1987) Removal of duvatrienediols from the surface of tobacco leaves increases their susceptibility to blue mold. Physiol Mol Plant Pathol 30:441

Rukayadi Y, Shim JS, Hwang JK (2008) Screening of Thai medicinal plants for anticandidal activity. Mycoses 51:308–312

Samy RP (2005) Antimicrobial activity of some medicinal plants from India. Fitoterapia 76:697–699

Sauton M, Mitaine AC, Miyamoto T, Dongmo A, Lacaille MA (2004) Antifungal steroid saponins from *Dioscorea cayenensis*. Planta Med 70:90–92

Scher JM, Speakman JB, Zapp J, Becker H (2004) Bioactivity guided isolation of antifungal compounds from the liverwort *Bazzania trilobata* (L.) S.F. Gray. Phytochemistry 65:2583–2588

Shain L, Miller JB (1982) Pinocembrin: an antifungal compound secreted by leaf glands of eastern cottonwood. Phytopathology 72:877–880

Skaltsa H, Lazari D, Panagouleas C, Georgiadov E, Garcia B, Sokovic M (2000) Sesquiterpene lactones from *Centaurea thessala* and *Centaurea attica:* antifungal activity. Phytochemistry 55:903–908

Slobodníková L, Kost'álová D, Labudová D, Kotulová D, Kettmann V (2004) Antimicrobial activity of Mahonia aquifolium crude extract and its major isolated alkaloids. Phytother Res 18:674–676

Slusarenko AJ, Longland AC, Whitehead IM (1989) Convenient, sensitive and rapid assay for antibacterial activity of phytoalexins. Bot Halvetica 99:203–207

Sohn HY, Son KH, Kwon CS, Kwon GS, Kang SS (2004) Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L. *Morus mongolica* Schneider, *Broussnetia papyrifera* (L.) Vent, *Sophora flavescens* Ait and *Echinosophora koreensis* Nakai. Phytomedicine 11:666–672

Soylu EM, Soylu S, Kurt S (2006) Antimicrobial activities of the essential oils of various plants against tomato late blight disease agent Phytophthora infestans. Mycopathologia 161:119–128

Spendley PJ, Bird PM, Ride JP, Leworthy DP (1982) Two novel antifungal alka-2,4-dienals from Triticum aestivum. Phytochemistry 21:2403–2404

Springob K, Kutchan TM (2009) Introduction to the different classes of natural products. In: Osbourn AE, Lanzotti V (eds) Plant-derived natural products synthesis, function, and application. Springer, New York, pp 3–50

Stein AC, Alvarez S, Avancini C, Zacchino S, von Poser G (2006) Antifungal activity of some coumarins obtained from species of Pterocaulon (Asteraceae). J Ethnopharmacol 107:95–98

Tahara S, Ingham JL, Nakahara S, Mizutani 1, Harborne JB (1984) Fungitoxic dihydrofurano-isoflavones and related compounds in white lupin, *Lupinus albus*. Phytochemistry 23:1889–1900

Takasugi M, Kawashima S, Mende K, Katsui N, Masarnune T, Shirata A (1987) Antifungal compounds from *Dioscorea batatas* inoculated with *Pseudomonas cichorii*. Phytochemistry 26:371–375

Tayel AA, El-Tras WF (2009) Anticandidal activity of pomegranate peel extract aerosol as an applicable sanitizing method. Mycoses. doi:10.1111/j.1439-0507.2008.01681.x

Tornas-Barberan FA, Msonthi JD, Hostettmann K (1988) Antifungal epicuticular methylated flavonoids from *Helichrysum nitens*. Phytochemistry 27:753–755

Turchetti B, Pinelli P, Buzzini P, Romani A, Heimler D, Franconi F, Martini A (2005) In vitro antimycotic activity of some plant extracts towards yeast and yeast-like strains. Phytother Res 19:44–49

Uma B, Prabhakar K, Rajendran S (2009) Anticandidal activity of *Asparagus racemosus*. Indian J Pharm Sci 71:342–343

Valsaraj R, Pushpangadan P, Smitt UW, Adsersen A, Nyman U (1997) Antimicrobial screening of selected medicinal plants from India. J Ethnopharmacol 58:75–83

Vinaya K, Kavitha R, Ananda Kumar CS, Benaka Prasad SB, Chandrappa S, Deepak SA, Nanjunda Swamy S, Umesha S, Rangappa KS (2009) Synthesis and antimicrobial activity of 1-benzhydryl-sulfonyl-4-(3-(piperidin-4-yl) propyl)piperidine derivatives against pathogens of Lycopersicon esculentum: a structure-activity evaluation study. Arch Pharm Res 32:33–41

Viturro CF, Dela-Fuente JR, Maier MS (2004) 5-Methylcoumaranones from *Mutisia friesiana* and their bioactivity. J Nat Prod 67:778

Wachter GA, Hoffmann JJ, Furbacher T, Blake ME, Timmermann BN (1999) Antibacterial and antifungal flavanones from Eysenhardtia texana. Phytochemistry 52:1469–1471

Walsh TJ, Gonzalez C, Lyman CA, Chanock SJ, Pizzo PA (1996) Invasive fungal infections in children: recent advances in diagnosis and treatment. Adv Pediatr Infect Dis 11:187–290

Webster D, Taschereau P, Belland RJ, Sand S, Rennie RP (2008) Antifungal activity of medicinal plant extracts; preliminary screening studies. J Ethnopharmacol 115:140–146

Wippich C, Wink M (1985) Biological properties of alkaloids. Influence of quinolizidine alkaloids and gramine on the germination and development of powderly mildew, *Erysiphe graminis* f. sp. hordei. Experientia 41:1477–1479

Wong JH, Ng TB (2005a) Vulgarinin, a broad-spectrum antifungal peptide from haricot beans (*Phaseolus vulgaris*). Int J Biochem Cell Biol 37:1626–1632

Wong JH, Ng TB (2005b) Lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*Phaseolus lunatus* L.). Peptides 11:2086–2092

Woodward S, Pearce RN (1988) The role of stilbenes in resistance of Sitka spruce (*Picea sitchensis* (Bong) Carr) to entry of fungal pathogens. Physiol Mol Plant Pathol 33:127

World Health Organization (1998) The World Health report. Life in the 21st century: a vision for all, 2 Measuring health. World Health Organization, Geneva, Switzerland, pp 39–60

Wu TC (1994) On the development of antifungal agents: perspective of the US Food and Drug Administration. Clin Infect Dis 19:S54–S58

Xia L, Ng TB (2004) Actinchinin, a novel antifungal protein from the gold kiwi fruit. Peptides 25:1093–1098

Xiao D, Kuroyanagi M, Itani T, Matsuura H, Udayama M, Murakama M, Umehara K, Kawhara N (2001) Studies on constituents from *Chamaecyparis pisifera* and antibacterial activity of diterpenes. Chem Pharm Bull 49:1479–1481

Yang MH, Wang JS, Luo JG, Wang XB, Kong LY (2009) Tetranortriterpenoids from *Chisocheton paniculatus*. J Nat Prod 72:2014–2018

Yemele-Bouberte M, Krohn K, Hussain H, Dongo E, Schulz B, Hu Q (2006) Tithoniamarin and tithoniamide: a structurally unique isocoumarin dimer and a new ceramide from *Tithonia diversifolia*. Nat Prod Res 20:842–849

Yoganandam GP, Gowri R, Biswas D (2009) Evaluation of *Wedelia Biflora* (Linn) D.C for anthelmintic and antimicrobial activity. J Pharm Res 2:375–377

Young MCM, Braga MR, Dietrich SMC, Gottlieb HE, Trevisan LMV, Bolzani VS (1992) Fungitoxic non-glycosidic iridoids from *Alibertia macrophylla*. Phytochemistry 31:3433

Zarnowski R, Suzuki Y, Yamaguchi I, Pietr SJ (2002) Alkylresorcinols in barley (*Hordeum vulgare* L. *distichon*) grains. Z Naturforsch 57:57–62

Zheng WF, Tan RX, Yang L, Liu ZL (1996) Two flavones from Artemisia giraldii and their antimicrobial activity. Planta Med 62:160–161

Chapter 20
Novel Drug Delivery Systems for Antifungal Compounds

Qamar Zia, Mohammad Farzuddin, Mairaj Ahmad Ansari, Maroof Alam, Azmat Ali, Iqbal Ahmad, and Mohammad Owais

Abstract Development of new approaches for treatment of invasive fungal infections encompasses new delivery systems for approved and investigational compounds. Novel delivery systems consisting of cyclodextrins (CDs), cochleates, nanoparticles, and long-circulating ("stealth") liposomes modulate the pharmacokinetics of existing drugs, and may also be useful to enhance the delivery of antifungal agents to sites of infection. Among several promising new drug-delivery systems, liposomes represent an advanced technology for site-directed delivery of active molecules. Research on liposome technology has progressed from conventional vesicles ("first-generation liposomes") to "second-generation liposomes", in which long-circulating liposomes are obtained by modifying the surface of liposomes using several molecules, such as glycolipids, sialic acid, or synthetic polymer poly-(ethylene glycol) (PEG), resulting in prolonged reticulo-endothelial system uptake and serum half-life, thus increasing the therapeutic efficacy of drugs. At present, several formulations for amphotericin B are in clinical use for fungal infections in Europe and the United States. Nanoformulations have also been applied as drug delivery systems (DDSs), with great success. Finally, progress in the design of DDSs has led to the development of carriers targeted to specific tissues and cells. Efforts are now going on to improve their stability in the biological environment, to mediate the biodistribution of active compounds, and to improve drug loading, targeting, transport, release, and interaction with biological barriers. This chapter discusses the state of the art in the field of DDSs, used for control of systemic fungal infections.

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20.1 Introduction

At present, clinical medicine possesses a plethora of different pharmaceutical products, with the list increasing rapidly every year as the understanding of molecular mechanisms of diseases develops. The scientific community is never satisfied only with a favorable drug action against the disease under treatment; for them, the task of avoiding undesirable drug actions on normal tissues and minimizing side-effects of the therapy is equally important. Clinically, the therapeutic efficacy of an antifungal drug relies not only on its intrinsic antifungal activity, but also on the bioavailability, that is, penetration and distribution of the drug at the infection site (Pierard et al. 2007), as well as the host's immune response. Many azole drugs have low aqueous solubility because of their hydrophobic structures. For example, miconazole, ketoconazole, itraconazole, and posaconazole are all very slightly soluble (<1 μg/ml) or insoluble at neutral pH, whereas the aqueous solubilities of fluconazole and voriconazole are thousands of times higher. Generally, low aqueous solubility is associated with low oral bioavailability (Chan and Stewart 1996). Thus, the means to deliver antifungals are critical for effective prophylaxis and treatment of fungal infections. Recently, the emergence of biotechnology research has generated great interest in developing novel drug delivery systems (DDSs) (Fig. 20.1), to improve both the pharmacological and therapeutic properties of parenterally administered drugs (Moses et al. 2003).

The clinical utility of most conventional chemotherapeutics is hampered either by the inability to deliver therapeutic drug concentrations to the target tissues, or by severe and harmful toxic effects on normal organs and tissues. In the development of novel therapeutics, the ability to devise a suitable pharmaceutical formulation for delivery is of utmost importance. The history of antifungal chemotherapy is replete with examples where serious problems in finding the appropriate vehicle for a drug have led to considerable limitations in its clinical usefulness, or even to the discontinuation of clinical development (Groll et al. 1998). Different approaches have been attempted to overcome these problems, by providing "selective" delivery to the affected area; the ultimate solution would be to target the drug only to those organs, tissues or cells affected by the disease. The ideal way out of such problems lies in the targeting of drugs using suitable carriers such as serum proteins, immunoglobulins, synthetic polymers, liposomes, niosomes, microspheres, erythrocytes, reverse micelles, pharmacosomes, monoclonal antibodies (MAb), etc. (Gregoriadis 1977). Nanoparticles, submicron-sized particles (3–200 nm), devices, or systems, applied as DDSs can be made using a variety of materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes), viruses (viral nanoparticles), and even organometallic compounds (nanotubes). Among these carriers, liposomes show great potential for effective delivery of drugs to the site of action, and for controlling the release of these drugs at a predetermined rate. On the other hand, however, antifungal drugs have been at the forefront of the successful development of novel carrier systems, including

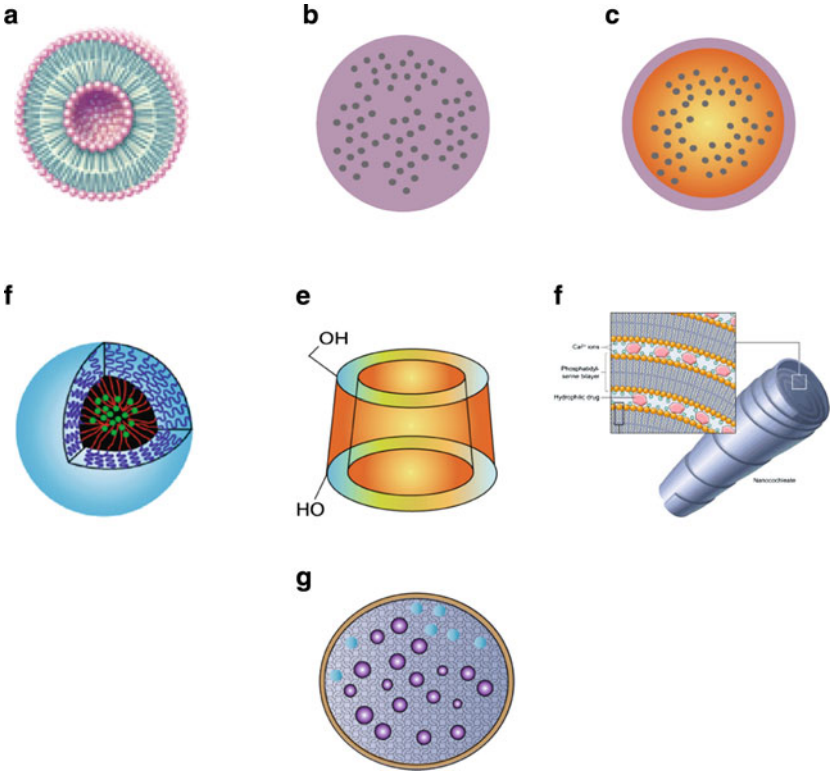

Fig. 20.1 Structure of various drug delivery systems: **a** liposomes/niosomes, **b** nanospheres, **c** nanocapsules, **d** micelles, **e** cyclodextrins, **f** cochleates, **g** microspheres

the lipid carriers and CDs, and further innovative approaches are currently the subject of intensive investigation.

For the pharmaceutical industry, novel drug-delivery technologies have become a strategic tool for expanding drug markets. Drug-delivery-based technology can address the very basic issues associated with current pharmaceuticals, such as extending product life, or can add to their performance and acceptability, either by increasing efficacy or by improving safety and patient compliance (Roco and Bainbridge 2002). In addition, with the completion of the human genome project, and seeking the help of computational biology approaches, the knowledge gained is being transformed into the development of newer drugs that require appropriate DDSs for their effective use. This technology permits the delivery of drugs that are highly water-insoluble or unstable in the biological environment. It is expected that novel DDSs can make a significant contribution to the global pharmaceutical market. This is illustrated by the fact that approximately 13% of the current global pharmaceutical market is accounted for by sales of products incorporating a DDS (Roco and Bainbridge 2002). As exemplified by the lipid formulations of AmB and cyclodextrin (CD) itraconazole, antifungal compounds have assumed a pioneering

role for the clinical development of novel carrier systems. As science continues to move forward and we learn more about the complexities of the human body, the "repositioning" of old drugs will continue, and in coming years there will be a great rise in designing suitable DDSs. In fact, the quest to find the ideal delivery strategy will continue until a system that can deliver drugs with maximum efficacy and selectivity, with no side-effects, is found. Since there is an armamentarium of delivery systems available, we have restricted our discussion to those nanocarriers that are used to deliver various antifungal agents. We also discuss various formulations based on these delivery systems available in the market or under research.

20.2 Liposomes

Liposomes (Fig. 20.1a), self-assembling closed colloidal vesicular structures of varying size, consisting of one or more concentric lipid bilayers and an aqueous inner compartment (Maurer et al. 2001), are lyotropic liquid crystals composed of relatively biocompatible and biodegradable materials, These spherical structures can have diameters ranging from 80 nm to 100 μm (Sharma and Sharma 1997). A liposome may be composed of naturally derived phospholipids with mixed lipid chains (such as egg phosphatidylethanolamine), or of pure surfactant components such as dioleoylphosphatidylethanolamine (DOPE) and cholesterol bilayer. The distinct advantage of liposomes is their ability to encapsulate various materials, combined with their structural versatility. Liposomes can be classified according to their lamellarity (uni-, oligo-, and multilamellar vesicles) (Fig. 20.2), size (small, intermediate, or large), and preparation method (such as reverse-phase evaporation vesicles, VETs). Unilamellar vesicles comprise one lipid bilayer (diameters 50–250 nm), contain a large aqueous core, and are preferentially used to encapsulate water-soluble drugs. Multilamellar vesicles comprise several concentric lipid bilayers in an onion-skin arrangement with diameters of 1–5 μm. The high lipid content allows these multilamellar vesicles to passively entrap lipid-soluble drugs.

In 1965, Bangham et al. first used a liposomal structure as a model to study the effect of narcotics on lipid bilayer membranes (Bangham et al. 1965). Nine years later, Allison and Gregoriadis described the use of a similar liposomal system as an immunological adjuvant (Allison and Gregoriadis 1974). Since these early experiments, liposomes have become an established carrier and delivery system in the field of pharmaco- and immuno-therapy. Drugs with widely varying lipophilicities can be encapsulated in liposomes, either in the phospholipid bilayer, in the entrapped aqueous core, or at the bilayer interface. Liposomes composed of natural lipids are biodegradable, biologically inert, weakly immunogenic (van Rooijen and van Nieuwmegen 1980), produce no antigenic or pyrogenic reactions, and possess limited intrinsic toxicity (Campbell 1983). Therefore, drugs encapsulated in liposomes are expected to be transported without rapid degradation, and with minimum side-effects to the recipients. Moreover, efforts have been made to assess the specificity of drug carriers to the target organs, cells or compartments within the

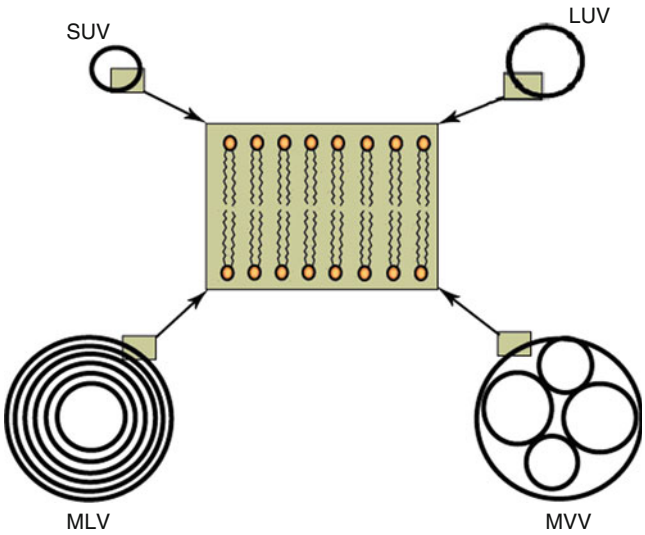

Fig. 20.2 Schematic illustration of liposomes of different size and number of lamellae. Small unilamellar vesicles (*SUV*), large unilamellar vesicles (*LUV*), multilamellar vesicles (*MLV*), multivesicular vesicles (*MVV*)

cells (Gregoriadis 1977). Liposomes are better suited for assessing their targetable properties, because of the ease of modifying their surface when compared to other drug carriers such as nanoparticles (Grislain et al. 1983) and microemulsions (Mizushima et al. 1982). Liposomes have emerged as versatile drug carriers, which can be used to control retention of entrapped drugs in the presence of biological fluids, maintain controlled vesicle residence in the systemic circulation or other compartments in the body, and provide enhanced vesicle uptake by target cells (Gregoriadis and Florence 1993).

Liposomes have been investigated for delivery of chemotherapeutic agents for cancer (Harrington et al. 2002), vaccines for immunological protection (Zho and Neutra 2002), radiopharmaceuticals for diagnostic imaging (Matteucci and Thrall 2000), and nucleic acid-based medicines for gene therapy (Mady et al. 2004). These delivery systems are becoming increasingly utilized by the pharmaceutical industry to deliver certain vaccines, enzymes, or drugs for treatment or prevention of a variety of diseases. Several sophisticated formulations have been developed, and their superior properties demonstrated with regard to stability, pharmacokinetics, biodistribution, and toxicity. The compounds entrapped in the liposomes are protected from the action of external media, particularly enzymes (Chaize et al. 2004) and inhibitors. Moreover, liposomes possess a unique opportunity to deliver the drugs into cells by fusion or endocytosis mechanism, and practically any drug can be entrapped into liposomes irrespective of its solubility. Encouraging results of liposomal drugs in the treatment or prevention of a wide spectrum of diseases in experimental animals and in humans indicate that more liposome-based products

for clinical and veterinary applications may be forthcoming in recent years. New liposomal delivery systems formulated with new types of phospholipids such as cardiolipin (NeoPharm) and sphingomyelin (Inex) have now been evaluated for the delivery of chemotherapeutic agents (Zhang et al. 2004; Waterhouse et al. 2005).

20.2.1 Liposomes as Drug-Delivery Vehicles

Ideally, a liposomal formulation of a therapeutic compound includes three main features. First, the formulated substance in circulation needs to be stable and inert, to protect the delivered material from degradation and to extend its availability at the desired concentration. Second, specific interaction with and accumulation in the target tissue need to occur, thereby reducing the effective dosage and possible side-effects linked to high systemic concentrations. Third, a mechanism for controlled drug release must be present that is exclusively active in the target tissue, and mediates delivery of the payload to the appropriate cell type and subcellular compartment.

Liposome applications in drug delivery depend, and are based on their physicochemical and colloidal characteristics such as composition, size, loading efficiency, and the stability of the carrier, as well as their biological interactions with the cells. For drug delivery, liposomes can be formulated as a suspension, as an aerosol or in a (semi) solid form such as a gel, cream or dry powder; *in vivo*, they can be administered using different routes of application, such as parenteral, oral, or topical. After systemic (usually intravenous) administration, which seems to be the most promising route for this carrier system, liposomes are typically recognized as foreign particles and consequently endocytosed by cells of the mononuclear phagocytic system (MPS), mostly fixed Kupffer cells in the liver and spleen (Lasic and Martin 1995; Gregoriadis 1995). This fate may be useful for delivering drugs to these cells but, in general, excludes other applications, including site-specific drug delivery by using ligands expressed on the liposome surface in order to bind to receptors (over)expressed on the diseased cells (Lasic 1993). For this reason, substances that prolonged liposome blood circulation times which could evade rapid uptake by the MPS were discovered (Allen and Chonn 1987; Gabizon and Papahadjopoulos 1988), culminating in the development of PEG-coated, sterically stabilized liposomes (Papahadjopoulos et al. 1991; Senior et al. 1991; Lasic and Martin 1995). Many other approaches have been attempted to achieve targetable properties, including noncovalent association of cell specific antibodies with liposomes (Gregoriadis and Neerunjun 1975), coating of liposomes with heat aggregated immunoglobulins M (IgM) (Weissmann et al. 1975), and covalent attachment of poly and MAb to the liposomes, which selectively deliver the drug to the desired sites of action (Koning et al. 2002; Moribe and Maruyama 2002). Liposomal targeting through the integration of specific ligands and a mechanism of controlled drug release result in the accumulation of the drug at the site of action. Specific conditions found in the target tissues (e.g., a low pH in inflammatory tissues and tumors) can be used as a trigger for controlled release by the integration of

pH-sensitive components into the liposomal bilayer. A similar mechanism, pH-dependent release via membrane fusion, can be used to escape endolysosomal degradation after endocytosis, and to deliver active molecules or antigens to the cytoplasm of target cells (Drummond et al. 2000).

20.2.2 Specialized Liposomes (Long-Circulating Liposomes)

Liposomes face certain limitations. First, they possess a short circulation half-life after intravenous administration (Bakker-Woudenberg et al. 1994). Second, they are prone to adhere to each other and fuse to form larger vesicles in suspension, which may result in inclusion leakage (Taylor et al. 2005; Zhang and Granick 2006). Therefore, stability is a general problem with lipid vesicles. Moreover, SUVs possess the disadvantage of low aqueous entrapment volume, and the use of charged liposomes can be toxic. Several different strategies have been envisaged to overcome these shortcomings with the surface modification of liposomes (Oku and Namba 1994). The first strategy studied was the preparation of liposomes mimicking the erythrocyte membrane; in this case, the liposome surface was modified with gangliosides and sialic acid derivatives, such as monosialoganglioside (GM1) (Allen et al. 1989). Figure 20.3 shows liposomes with different modifications in order to increase their stability. Different types of biocompatible

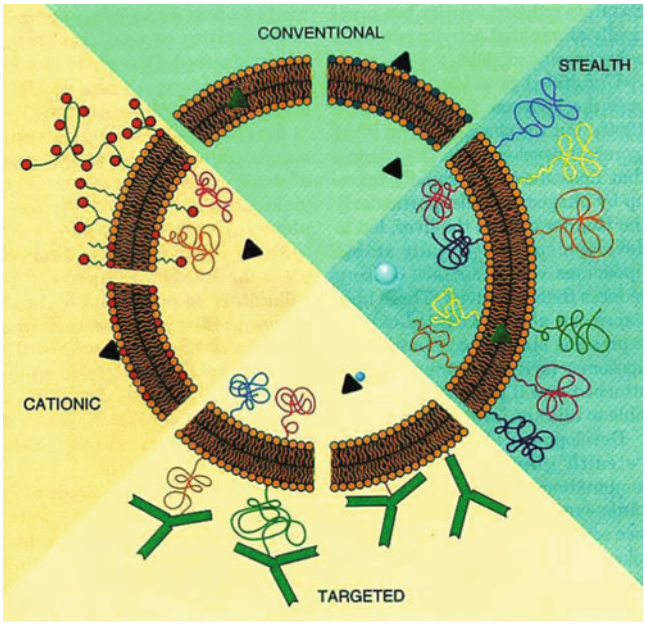

Fig. 20.3 Schemetic representation of liposomes modified with different molecules (either for targeting or for stabilization)

polymers such as chitosan can be employed to improve the efficiency of conventional liposomal systems (Perugini et al. 2000). Several authors have reported the use of chitosan as a liposome coating to increase the stability of drug release (Henriksen et al. 1997) and for targeting purposes (Takeuchi et al. 2003; Guo et al. 2003).

The subsequent step was to increase the hydrophilicity of the liposomal surface by using hydrophilic polymers. The mechanism whereby steric stabilization of liposomes increases their longevity in circulation has been extensively discussed (Drummond et al. 1999). The molecules found to be active in prolonging liposome circulation time include amphiphatic polyethylene glycol derivatives of phosphatidylethanolamine such as PE-PEG, (Maruyama et al. 1994) monosialoganglioside (GM_1) (Allen and Chonn 1987), and others (Torchilin et al. 1994). This coating allows the liposomes to evade RES uptake and remain in the systemic circulation for a long period of time (Maruyama et al. 1992). The basic concept is that a hydrophilic polymer or a glycolipid, such as PEG or GM1, possessing a flexible chain that occupies the space immediately adjacent to the liposome surface ("periliposomal layer"), tends to exclude other macromolecules from this space. Consequently, access and binding of blood plasma opsonins to the liposome surface are hindered, and thus interactions of MPS macrophages with such liposomes are inhibited. By reducing MPS uptake, long-circulating liposomes can passively accumulate inside other tissues or organs.

20.2.2.1 GM1 and Glucoronide Liposomes

Several glycolipids have been tested in studies of MPS uptake of liposomes after i.v. injection: the glycolipid GM1 (a brain-tissue-derived monosialoganglioside) significantly decreased MPS uptake when incorporated on the liposome surface, and the formulation remained in blood circulation for several hours. GM1 grafted liposomes with a diameter in the 90–200 nm range have longer blood retention (Liu et al. 1992). Large liposomes with a diameter of >300 nm preferentially accumulate in the spleen, whereas those with a diameter of <40 nm probably penetrate the interstitial spaces of the liver. The degree of macrophage uptake depends on the concentration of GM1 in PC liposomes: a concentration of 10 mol% decreased MPS uptake by 90%. This MPS-avoiding effect was reversed by the removal of the sialic acid moiety, demonstrating the important role played by this molecule in MPS-trapping avoidance (Yamauchi et al. 1994). Mora et al. (2002) observed higher brain-tracer uptake for GM1 liposomes than for control liposomes in the cortex, basal ganglia, and mesencephalon of both hemispheres; conversely, no significant changes were observed in liver uptake or blood concentration of the tracer. He et al. (1998) synthesized galactose derivatives Gal-β-$(CH_2\text{-}CH_2\text{-}O)_3$-$C_{14}H_{29}$ with oxygen-ethyl, which had specific affinity to the liver, as strengthened targeted material. Oku and Namba (Oku and Namba 2005) recently summarized their research on glucuronate-modified long circulating liposomes. Other recent interesting applications of GM-coated liposomes involve their use for oral

administration and delivery to the brain. In particular, Taira et al. (2004) suggest that among liposomal formulations used as oral drug carriers, those containing GM1 and GM type III have better possibilities of surviving through the gastrointestinal tract. Liposomes with different glycosyls combining on their surface can get different distributions *in vivo*. For example, liposomes could be taken up by liver parenchymal cells when carrying galactose residues, and could be taken up by Kupffer cells when carrying mannose residues. They are intensively distributed in the pulmonary (Zhang 2006) when carrying derivatives of amino mannose. The addition of the strengthened targeted material did not affect the physical and chemical properties of liposomes.

20.2.2.2 PEG-Coated Liposomes (Stealth Liposomes)

In order to improve the blood circulation time of liposomes, PEG has been extensively used as a polymeric steric stabilizer. Surface modification of liposomes with PEG can be achieved in several ways: by physically adsorbing the polymer onto the surface of the vesicles, by incorporating the PEG-lipid conjugate during liposome preparation, or by covalently attaching reactive groups onto the surface of preformed liposomes. For incorporation into the liposomal bilayer, numerous lipid derivatives of PEG have been made using lipids that, for example, contain a primary amino group (particularly PE), an epoxy group, or a diacylglycerol moiety (Allen and Hansen 1991; Allen et al. 1991a,b; Kirpotin et al. 1996; Woodle 1998); the most widely used method being to anchor the polymer in the liposomal membrane via a cross-linked distearoylphosphatidyl ethanolamine (DSPE) (Allen et al. 1991a, b, 2002). Alternatively, activated PEG can be anchored to reactive phospholipid groups of preformed liposomes (Senior et al. 1991). Another strategy has utilized the transfer of PEG-phospholipid conjugates from the micellar phase into the lipid bilayer of preformed vesicles (Uster et al. 1996). The PEG lipids can also be incorporated in to the outer surface only, utilizing the so-called post-insertion technique (Awasthi et al. 2004). This coating of liposomes by PEG attracts water to the surface of the liposome and inhibits opposing absorption and uptake by the MPS, resulting in prolonged circulation and, potentially, increased accumulation at sites of infection (Allen 1998). PEG coating on the surface of the liposomes substantially reduces the binding of blood proteins (van Etten et al. 1998a).

Moghimi and Szebeni (Moghimi and Szebeni 2003) critically examined the supposed mechanisms that contribute to prolonged circulation times of sterically protected liposomes. The behavior of PEGylated liposomes depends on the characteristics and properties of the specific PEG linked to the surface. Figure 20.4 represents the regimens proposed by deGennes, when polymers are attached to the liposome surface, depending on the graft density of the polymer (de Gennes 1980). Although the accepted opinion is that PEG increases circulation longevity of drug carriers by reducing or preventing protein binding and/or by inhibiting cell binding/uptake, there is sufficient conflicting data to warrant a reassessment of the mechanism(s) by which surface-grafted PEGs improve liposome properties (Allen et al. 2002).

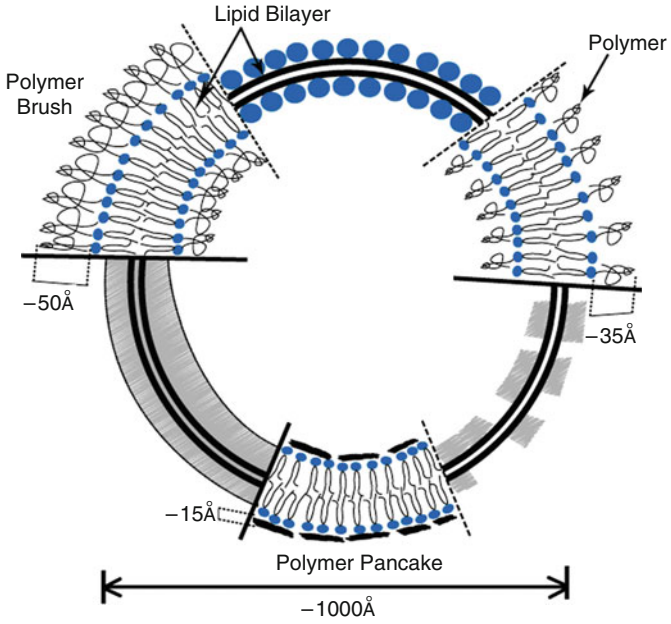

Fig. 20.4 Schematic diagrams of poly-(ethylene glycol) (PEG) configurations regime (mushroom, brush and pancake) for polymer grafted onto surface of liposomes

Laverman et al. (2001) and Ishida et al. (2002) reported that intravenous injection in rats of PEG-grafted liposomes may significantly alter the pharmacokinetic behavior of a second dose when this second dose is administered after an interval of several days. This phenomenon, called "accelerated blood clearance" (ABC), appears to be inversely related to the PEG content of liposomes. Current research on PEG liposomes has concentrated on attaching PEG to the liposome surface in a removable fashion, in order to facilitate subsequent liposome capture by the cells.

20.2.2.3 Targeted Liposomes/Immmunoliposomes

To increase liposomal drug accumulation in the desired tissues, producing higher and more selective therapeutic activity, the use of targeted liposomes has been suggested. This involves the coupling of targeting moieties capable of recognizing target cells, binding to them, and inducing the internalization of liposomes or encapsulated drugs. Targeting moieties include MAb or fragments, peptides, growth factors, glycoproteins, carbohydrates, or receptor ligands (Sapra and Allen 2003; Medina et al. 2004). One of the most compelling advantages is the dramatic increase in the amount of drug which can be delivered to the target. Furthermore, the number of ligand molecules exposed on the liposome surface can be increased, improving ligand avidity and degree of uptake.

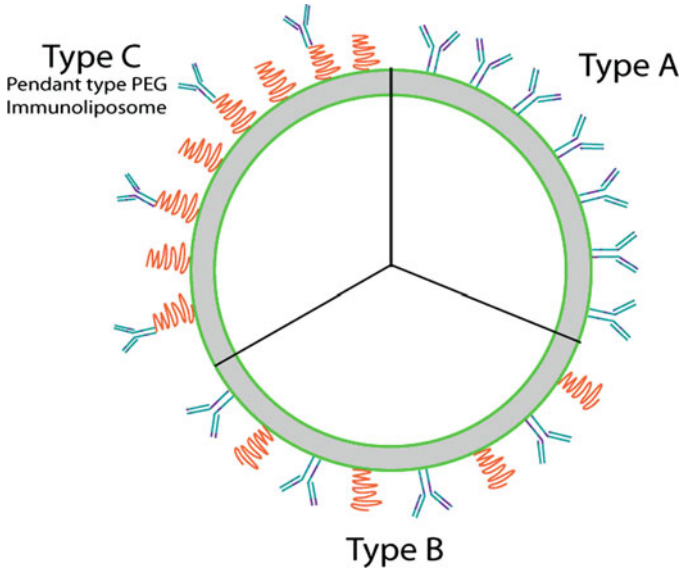

Fig. 20.5 Schematic illustration of immobilization of antibody on liposomes. *Type A*: PEG-free immunoliposomes with antibody covalently linked to the short anchor; *Type B*: PEG-immunoliposomes with antibody covalently linked to anchor; *Type C*: PEG-immunoliposomes with antibody attached to the distal terminal of anchor molecule (pendant-type PEG-immunoliposomes)

Immunoliposomes can also be built using the stealth technique, in such a fashion as to be long-circulating and nonimmunogenic, as shown in Fig. 20.5 (Maruyama et al. 1995). Type A is a PEG-free immunoliposome with the antibody covalently linked to a short anchor. Type B is a PEG-immunoliposome with the antibody covalently linked to the short anchor. Antibody molecules and PEG molecules coexist on the liposome surface. A new type of long-circulating immunoliposome, i.e., polyethyleneglycol (PEG) immunoliposome-attached antibodies at the distal end of PEG chain, the so-called Type C or pendant-type immunoliposomes, show much higher targetability than the ordinary immunoliposomes. The presence of free PEG does not interfere with the binding of the terminally linked antibody to the antigen. Ideally, the coupling method should be both simple and rapid, producing a stable, nontoxic bond. During conjugation, the antibody should retain antigen recognition, and the liposomes should not lose their structural integrity. The pendant-type immunoliposomes can escape from the gaps between adjacent endothelial cells and openings at the vessel termini during tumor angiogenesis, by passive convective transport much higher than ligand-directed targeting. A future possibility is to conjugate other ligands to the free PEG terminals of Fab′-Type C, in order to direct the liposomes to surface receptors known to be internalized at a high rate by cells. The ultimate goal is the incorporation of a fusogenic molecule that would induce fusion of Type C immunoliposomes following their binding to the target cells, or their internalization by endocytosis.

20.2.2.4 pH-Sensitive Liposomes

Liposomes exhibiting pH sensitivity, and composed of phosphatidyl ethanolamine and protonatable amphiphiles, have been developed and used for the intracellular delivery of charged water-soluble compounds (Chu et al. 1990). PE-based pH-sensitive liposomes have been shown to efficiently deliver diverse molecules to the cytoplasm (Drummond et al. 2000). Liposomes that destabilize at mildly acidic pH (pH-sensitive liposomes) can deliver highly charged encapsulated materials into cells more efficiently than non-pH-sensitive liposomes (Torchilin et al. 1993). After liposomes enter cells via endocytosis, the acidic pH inside the endosomes causes pH-sensitive liposomes to release their aqueous contents into the cytoplasm, most likely by destabilizing the endosome membrane (Collins 1995). Like other non-sterically stabilized liposomes, however, pH-sensitive liposomes have very short circulation times *in vivo* (Liu et al. 1989).

Several investigators have tried to construct serum-stable pH-sensitive liposomes (Kono et al. 1994; Viani et al. 1993). Vladimir et al. (1997) found that addition of PEG-PE to the membrane of pH-sensitive liposomes composed of cholesteryl hemisuccinate (CHEMS) and DOPE confers steric stability to these vesicles. Bilayer destabilization in liposomes containing DOPE can result in both liposome fusion with adjacent membranes and content release (Hope et al. 1998). The pH sensitivity of the liposomes decreased as the mole percentage of PEG-PE was increased (Vladimir et al. 1997). pH-sensitive vesicles can also be produced by coating liposomes with pH-responsive polymers (Chen et al. 1999). One group has successfully synthesized pH-sensitive formulations bearing hydrophobically modified copolymers of N-isopropylacrylamide (NIPAM) and methacrylic acid (MAA) (Zignani et al. 2000). Roux et al. demonstrated that terminally alkylated pH-sensitive NIPAM copolymers could provide, at neutral pH, a steric barrier sufficient enough to significantly increase circulation time of the liposomes (Roux et al. 2002, 2003). In another study, Rox et al. prepared pH-sensitive liposomes using a terminally alkylated copolymer of NIPAM and MAA and PEG-phospholipid derivative (Roux et al. 2004). Unfortunately, although the addition of PEG-PE efficiently prolonged liposome circulation time, it also led to a significant decrease in liposome pH-sensitivity.

20.2.3 Liposomal Formulations

Four types of lipid-based formulations of amphotericin B incorporated into liposomes, sheets or disks are under intensive clinical investigation. These lipid-based formulations differ in size, structure, shape, lipid composition and molar amphotericin B content. Despite all these differences, they are all less toxic than Fungizone® to mammalian cells, animal and human. Two of them (Amphocil and Abelcet) are nonliposomal formulations (not discussed here), while one is based on lipid nanosphere (discussed later). Only one liposomal formulation of Amphotericin

B (AmBisome™), which is most widely used, is discussed below. Animal studies have shown that liposomal formulations lead to higher concentrations of AmB in the lungs than Fungizone® (Ruijgrok et al. 2005) or AmB directly solubilized in fluorocarbons (Vyas et al. 2005). Clinical trials have shown that the lipid formulations are easier to aerosolize and better tolerated than Fungizone® (Perfect et al. 2004; Drew 2006). Macrophages may function as reservoirs of amphotericin B for intracellular and extracellular antimicrobial action (Brajtburg and Bolard 1996). Similarly, drug concentrations of liposomes loaded with AmB and coated with mannan or pullanan were higher than those delivered by unmodified liposomes, when aimed to target alveolar macrophages (Vyas et al. 2005). Recently, a novel lipid-based oral formulation, comprising of AmB, DSPE-PEG, monoglycerides, and diglycerides (licensed to iCo Therapeutics Inc., Vancouver, BC, Canada) was developed, protecting the acid labile AmB from destruction in the stomach and thus presenting more AmB to the intestinal mucosa for absorption (Sachs-Barrable et al. 2008). This results in sustained plasma concentrations that approximate conventional AmB intravenous therapy (Fungizone®) without the significant drug and infusion-related side-effects (Wasan et al. 2009).

The most well-established of the lipid-complexed formulations of amphotericin B is AmBisome™ (NeXstar Pharmaceutical Inc.), which is now widely available. This is the only true liposomal formulation. It is being used increasingly in Europe for the treatment of serious, life-threatening fungal infection. It is composed of small, unilamellar vesicles which yield a very stable lipid bilayer, in the gel state at physiological temperature. AmB is incorporated into this bilayer at 10 moles% (Table 20.1). The size of the liposomes (about 80 nm) means that they have a long circulating half-life and a good penetration into tissues. AmB is anchored tightly in the AmBisome™ bilayer, due to favorable interactions of the macrolide with the surrounding lipids. DSPG probably interacts directly with AmB; cholesterol may also play a role. The stable bilayer composition reduces exchange with lipoproteins and contributes to the very low toxicity of this formulation (Adler-Moore and Proffitt 2002). In the early 1990s, three studies (Mills et al. 1994; Meunier et al. 1991; Ringden et al. 1991) demonstrated the safety and efficacy of the AmBisome™. According to some clinical reports (Hay 1994), AmBisome™ showed a low adverse reaction (0–5%) and good tolerance with high-dose or long-term administration in clinical use.

The use of AmBisome™ is clinically supported by extensive published data (Ng and Denning 1995). AmBisome™ appears to be effective (with a response rate up to 80%) and well-tolerated in the treatment of invasive fungal infections in immune-compromised patients. Furthermore, the response rate has been 40–50% for infections with *Aspergillus* and *Candida* spp. (Ng and Denning 1995). AmBisome™ appears to have an underlying immunomodulating effect by enhancing effector cell function against fungal cells (Abu-Salah 1996). Over six times more amphotericin B in AmBisome than in Fungizone® could be delivered in mice without any acute toxicity. A phase 3, multicenter, randomized, double-blind study of the safety and efficacy of an liposomal AmB loading dose regimen versus a standard liposomal AmB regimen was performed by Cornely (2005), to determine the appropriate

Table 20.1 Various properties of commercially available lipid formulation of amphotericin B (AmBisome™)

Properties	AmBisome™
Lipid components	Hydrogenated soy PC:Chol:DSPG:AMB 2:1:0.8:0.4
AMB/lipid (mol%)	10
Charge	Negative
Structure	Small unilamellar liposomes
Particle size (nm)	45–80 (in diameter)
Available as	Lyophilized powder
Toxicity (as compared to amphotericin B)	70–80 times less toxic
Safety profile	Adverse effects in <5% of the patients
Dosage	5–7.5 mg/kg/day
Acute LD_{50} murine	>175 mg/kg
Bioavailability (as compared to fungizone)	Greater
V_d (l/kg)	0.11
C_{max} (mg/l)	83
AUC (mg h/l)	555
Plasma half-life (h) (5 mg/kg)	8.6
Treatment (7 days) (5 mg/kg/day)	
Plasma concentration (mg/l)	62.90
CSF concentration (mg/l)	0.024
Brain concentration (μg/g)	1.99
Nephrotoxicity as measured by serum creatinine (mg/l)	
AmBisome™ (3 mg/kg)	13
AmBisome™ (5 mg/kg)	12

V_d, volume of distribution
C_{max}, maximum plasma concentration
AUC, area under concentration-time curve

daily dose for the initial treatment of invasive aspergillosis and other filamentous fungal infections in immunocompromised patients. The standard regimen of 3 mg/kg/day for 14 days had a favorable overall response rate of 50% and a 12-week survival rate of 72%, comparable to those previously reported for voriconazole in a similarly designed trial (Herbrecht et al. 2002). However, the high-dose regimen did not demonstrate any improvement in overall response or survival. Finally, the cost-effectiveness of AmBisome™ has been compared with Fungizone® for empirical treatment of febrile neutropenic patients (Hiemenz et al. 1998). AmBisome™ is probably the only agent which can reasonably be considered for dose escalation or prophylactic therapy, because toxicity is reduced in all measured parameters.

Leenders et al. (1997) compared a 21-day course of AmBisome™ 4 mg/kg/day with Fungizone® 0.7 mg/kg/day on a small number of HIV-positive patients with cryptococcal meningitis. The overall results were roughly the same, in that the difference in 21-day rates of Cerebrospinal Fluid (CSF) culture conversion did not reach statistical significance. In another study by Leenders et al. (1998), AmBisome™ 5 mg/kg/day was compared with Fungizone® 1 mg/kg/day in a severely neutropenic patient population who had proven or probably invasive fungal infections. Complete responses with AmBisome™ were better than Fungizone®.

A multicentre study by Walsh et al. (1999) compared AmBisome™ 3 mg/kg/day with Fungizone® 0.6 mg/kg/day. The results showed that there were significantly fewer proven breakthrough fungal infections in the AmBisome™ treatment group.

van Etten et al. (1995b) reported that the therapeutic efficacy of liposomal AmB coated with PEG (PEG-L-AmB) was better than that of AmBisome™ against invasive candidiasis in neutropenic mice. In comparison to L-AmB, it conferred a significantly prolonged blood residence time, and displayed dose-independent pharmacokinetics over a large range of dosages (van Etten et al. 1998b). In a leukopenic mouse model of disseminated candidiasis, PEG L-AmB had enhanced antifungal efficacy as compared to the nonPEGylated product and L-AmB (AmBisome™) (van Etten et al. 1995b, 1998b). In another study, immunoliposomes of PEG-L-AmB provided enhanced survival and tissue clearance in a murine model of pulmonary aspergillosis (Otsubo et al. 1998). These carriers served to improve the therapeutic index of AmB, and were found to be more effective than AmB integrated with long-circulating liposomes (100% vs 83.3% survival rate) (Otsubo et al. 1998). Likewise, treatment of murine candidiasis and cryptococcosis with AmB integrated with immunoliposomes proved enhanced activity compared to that with conventional L-AmB (Belay et al. 1991; Dromer et al. 1990). In mice with pulmonary inflammation (aspergillosis), 34A-PEG-LAmB immunoliposomes (Maruyama et al. 1995) showed highest concentrations of AmB in lungs. 34A-PEG-LAmB exhibited good therapeutic efficacy in low doses, in which AmBisome™ did not show a satisfactory effect against pulmonary aspergillosis in immunosuppressed mice (Kohno et al. 1997).

In vivo studies have shown encouraging results for the association of liposomal AmB with both voriconazole and echinocandins, in models as different as rat models of invasive aspergillosis (Kirkpatrick et al. 2006), a murine model of cerebral aspergillosis (Clemons et al. 2005), or a murine model of *Candida glabrata* systemic infection (Olson et al. 2005). In humans, a few case reports and a small series of results of associations have been reported, but none are randomized studies (Aliff et al. 2003; Kontoyiannis et al. 2003; Marr et al. 2004).

NyotranÒ (Aronex Pharmaceuticals), an intravenous multilamellar liposomal nystatin formulation (having size 0.1–3 μm), contains dimyristoyl phosphotidyl choline and dimyristoyl phosphatidyl glycerol in a 7:3 ratio (Arikan and Rex 2001). Liposomal nystatin appeared to be as active as free nystatin, with minimal inhibitory concentrations (MICs) and MLCs that were similar to, or lower than, those of the latter against *Aspergillus* spp., *Candida* spp., and *Cryptococcus neoformans*. Neither formulation of nystatin was as active as amphotericin B deoxycholate (Fungizone®) or Amphotericin B lipid complex (Abelcet), but both were more effective than liposomal amphotericin B (AmBisome™) (Elizabeth et al. 1998). Its *in vivo* efficacy may be superior compared to the parent compound, nystatin, due to the enhanced entrapment of the drug in the reticuloendothelial system and thus increased delivery to the site of infection (Arikan and Rex 2001). Another study showed that pH-sensitive nystatin liposomes possess enhanced antifungal activity in terms of increased survival rate, as well as reduced fungal burden in brain and liver (Nasti et al. 2006).

The co-administration of tuftsin increased the efficiency of liposomised-polyene antibiotics (nystatin and amphotericin B) against experimental murine candidiasis in immunocompromised Balb/c mice (Khan et al. 2004a). Our group has demonstrated immunopotentiating efficacy of tuftsin against experimental murine aspergillosis in both normal and immunodebilitant BALB/c mice, and found that co-administration of the immunomodulator tuftsin and liposomised-amphotericin B was highly effective in the treatment of systemic infection of *Aspergillus fumigatus* in both cases, resulting in successful elimination of fungal pathogen (Khan et al. 2005a). Tuftsin-loaded nystatin liposomes exerted less toxicity to human erythrocytes than free nystatin, and showed a higher level of the drug in the systemic circulation (Khan et al. 2006). Another study demonstrated higher efficacy of tuftsin-loaded Amp B liposomes against experimental murine cryptococcosis, in terms of enhanced survival rate and reduced fungal burden in organs (lungs and brain) of the treated mice (Khan and Owais 2005). A study by Ahmad et al. (2005) evaluated effectiveness of various forms of clove oil against vaginal candidiasis, and found that topical administration of liposomized clove oil was highly effective in combating the disease.

Chloroquine entrapped in phosphotidyl serine (PS) liposomes shows increased activity against *C. neoformans* infection in both *in vitro* and *in vivo* studies. Chloroquine in PS liposomes was found to be more effective than the same dose of free chloroquine or chloroquine entrapped in neutral liposomes (Khan et al. 2004b, 2005b). In another study, we demonstrated that treatment with Diallyl Sulphide (DAS)-bearing liposome significantly decreased residual fungal load in vital organs of experimental animals against infection caused by *Candida albicans* (Maroof et al. 2009). It also resulted in the highest survival rate in animals. The liposomal DAS was also found to be free of toxic manifestations, as revealed by erythrocyte lysis test and liver/kidney function tests (Maroof et al. 2009).

Liposomal clotrimazole (e.g., clotrimazole: egg phospholipid: cholesterol = 2:7:3, molar ratio) for vaginal administration were developed to reduce dosing frequency and drug toxicity, which are issues associated with currently available formulations (Ning et al. 2005). A heptaene polyene macrolide SJA-95 isolated from a new strain, *Streptomyces sp*. S-24, has shown some promising antifungal activity *in vitro* against yeasts, filamentous fungi, and clinical isolates, including plant pathogens (Naik et al. 2007). Also reported is/are its possible mechanism(s) of antifungal activity (Desai and Naik 2008a). Studies have indicated that Lip SJA-95 treatment led to prolonged survival time, effective microbiological clearance, and reduced toxicity in the mice model of Candidiasis (Desai and Naik 2008b).

20.3 Nanoparticles (Polymer–Drug Conjugates)

Nanoparticles (including nanospheres and nanocapsules of size 10–200 nm) (Fig. 20.1b, c) are biodegradable polymeric particles in the solid state, and are either amorphous or crystalline. They serve as drug carriers, with the active

principle either dissolved, entrapped, encapsulated, adsorbed, or chemically attached, thus protecting it against chemical and enzymatic degradation (Yuan and Yi 2003). Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. Nanoparticles as drug carriers can be formed from both biodegradable polymers and nonbiodegradable polymers. Nanoparticles can be administered by various routes, are easily produced and stored, and, in contrast to liposomes, inexpensive (Kreuter 1996). Nanoparticles enhance the delivery of certain drugs across membranes, and may enhance the oral bioavailability of poorly absorbable drugs and tissue uptake after parenteral administration through adherence to the capillary wall. Because of their small size, they have the potential to leave the vascular system at sites of inflammation (Davis 1997). Nanoparticles are subject to phagocytosis and endocytosis: due to their hydrophobic surface, they are rapidly coated ("opsonized") by plasma proteins and taken up by the MPS of liver, spleen, and bone marrow. Coating with hydrophilic copolymers or polyethylene glycol (PEG), however, results in increased hydrophilicity, prolonged circulation in the bloodstream and thereby, potentially enhanced uptake in non-MPS organs, and accumulation at sites of inflammation (Davis 1997). The size of nanoparticles for crossing different biological barriers is dependent on the tissue, target site, and circulation (Brannon-Peppas and Blanchette 2004).

Self-assembled nanocarriers are generally characterized of a hydrophobic core and a hydrophilic shell, i.e., the hydrophobic core acts as a drug incorporation part and the outer shell acts as a safeguard from attack by the reticuloendothelial system. Therefore, they are considered as superior drug carriers, and have been developed by several researchers (Kataoka et al. 1993; La et al. 1996; Gref et al. 1994; Jeong et al. 1998; Yokoyama et al. 1991). Polymeric nanoparticles have been synthesized using various methods (Pinto et al. 2006), according to application needs and the type of drugs to be encapsulated. Such nanoparticles provide a controlled/sustained release property, subcellular size, and biocompatibility with tissue and cells (Panyam and Labhasetwar 2003). Encapsulating drugs within NPs can improve their solubility and pharmacokinetics, and, in some cases, enable further clinical development of new chemical entities that have stalled because of poor pharmacokinetic properties (Alexis et al. 2008). Apart from this, these nanomedicines are stable in blood, nontoxic, nonthrombogenic, nonimmunogenic, noninflammatory, do not activate neutrophils, are biodegradable, and are applicable to various molecules such as drugs, proteins, peptides, or nucleic acids (Des Rieux et al. 2006). Drug-loading into nanoparticles (Fig. 20.6) is achieved by two methods: (1) by incorporating the drug at the time of nanoparticle production, or (2) by adsorbing the drug after the formation of nanoparticles by incubating them in the drug solution (Soppimath et al. 2001). Drugs can be loaded onto nanoparticles by adding them to a solution that contains previously prepared nanoparticles, or by adding them to the reaction mixture during the polymerization process (Soppimath et al. 2001).

The major carrier materials of nanoparticles are synthetic biodegradable high molecular polymer and natural polymer. The former usually include poly-α-cyanoacrylate

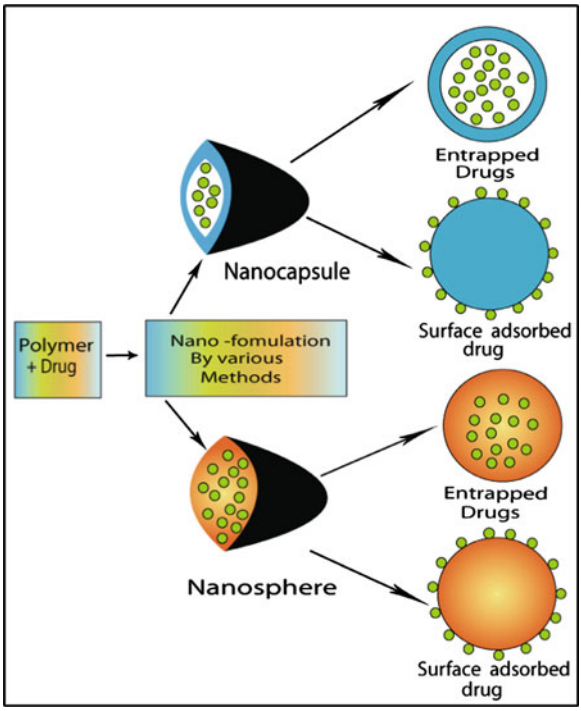

Fig. 20.6 Drug loading into nanoparticles: The drug molecules are either entrapped inside or adsorbed on the surface. (Originally adapted from (Tiyaboonchai 2003) but modified)

alkyl esters, polyvinyl alcohol, polylactic acid (PLA), and polyglycolic acid, etc. The latter is usually divided into two classes: proteins (albumin, collagen, gelatin, and vegetable protein) and polysaccharides (cellulose, starch and its derivatives, alginate, chitin, and chitosan, etc.) (Xiao and Li 2002). Synthetic biodegradable material can hydrolyze under mild physiological conditions. These types of material possess better biocompatibility and lower immunogenicity than the natural biopolymer. Furthermore, their properties are easy to control by chemical or physical modification. The most common synthetic materials are polyesters (Hao and Liang 2002), such as PLA, PGA, polylactic acid/glycolic acid (PLGA), lactic acid–polyethylene glycol (polypropylene glycol) copolymer, etc. Other materials include N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA), polystyrene-maleic anhydride copolymer, and poly-L-glutamic acid (PGA). Among synthetic polymers, PGA was the first biodegradable polymer to be used for conjugate synthesis (Li 2002). Several representative chemotherapeutics that are used widely in the clinic have been tested as conjugates with PGA *in vitro* and *in vivo*, and have shown encouraging abilities to circumvent the shortcomings of their free-drug counterparts (Li 2002). Owing to their small size and excellent biocompatibility, nanosized polymer nanoparticles can circulate in the bloodstream for long periods of time, allowing them to reach the target site.

20.3.1 Nanoparticle Formulations

A lipid nanosphere formulation of AmB (NS-718), composed of AmB, soybean oil, lecithin, and maltose, was developed that has an average particle size of 25–50 nm (Kohno et al. 1995). NS-718 showed efficacy against pulmonary aspergillosis in rats and pulmonary cryptococcosis in mice. The renal and nephrotic toxicity of NS-718 was estimated to be lower than that of AmB from the results of the toxicity study in the rat infusion model (Maesaki et al. 1999a). In vitro, the compound had similar activity against *C. albicans*, *C. neoformans*, and *A. fumigatus*, and was more effective against murine pulmonary cryptococcosis and aspergillosis than equimolar dosages of D-AmB and L-AmB (Hossain et al. 1998; Maesaki et al. 1999b; Otsubo et al. 1999). NS-718 exhibited reduced uptake by the MPS, prolonged residence in the bloodstream and distribution to other extravascular sites, as evidenced by higher peak concentrations and area under the curve (AUC)-values in lung tissue (Otsubo et al. 1999). In a tissue distribution study, the concentration of NS-718 in the liver was lower than that of Fungizone®. The concentration of AmB in pleural exudate after NS-718 was intravenously injected was higher than that obtained with Fungizone®. These results showed that NS-718 easily permeates leaky blood vessels at sites of inflammation (Fukui et al. 1996). Thus, NS-718 has a broad spectrum of antifungal activities, AmBisome™ showed weaker antifungal activity than that of NS-718, because the release of AmB from AmBisome™ was slow and slight. In another study, the antifungal activity of lipid nanosphere LNS-AmB against *C. albicans* was found to be similar to that of Fungizone® and dimethyl sulfoxide-solubilized AmB. In a mouse model of systemic candidiasis, LNS-AmB (1.0 mg/kg) was much more effective than AmBisome™ (8.0 mg/kg) or Fungizone® (1.0 mg/kg) (Hiroshi et al. 2003). Recently, nanoparticulate carriers of amphotericin B were prepared in different gelatins (GNPs) (type A or B) by the two-step desolvation method and assessed for controlled delivery as well as reduced toxicity (Nahar et al. 2008). AmB nanoparticles prepared by a nanoprecipitation method demonstrated some improvement in toxicity factors when compared to Fungizone®. Also, the AUC for AmB nanoparticles indicates an eight-fold increase over orally administered Fungizone® (Italia et al. 2009).

In order to improve the oral bioavailability of two clinically important antifungal drugs — clotrimazole and econazole — researchers have encapsulated them in nanoparticles of a synthetic polymer (polylactide-co-glycolide, PLG) or a natural polymer (alginate stabilized with chitosan). Overall, the alginate formulation appeared to be better than the PLG formulation, with better encapsulation efficiency, biodistribution, and pharmacokinetic data (Pandey et al. 2005). One group suggests that PLGA nanoparticles loaded with voriconazole (PNLV) could improve voriconazole antifungal efficacy, as shown by VRC tests against fungi (Peng et al. 2008). Nanoparticle compositions of itraconazole for deep lung delivery have been designed to achieve sufficient drug concentrations at the infected sites. Particle engineering technologies, including evaporative precipitation of aqueous solution (EPAS) (Elder et al. 2006), spray freezing into liquid (SFL) (Vaughn et al. 2005)

and ultra-rapid freezing (URF) (Overhoff et al. 2007), have been used to prepare nanostructured particles containing itraconazole. Recently, a novel nanostructured itraconazole solid solution (itraconazole: mannitol: lecithin = 1:0.5:0.2 weight ratio, URF–itraconazole) formulation employing FDA-approved excipients for pulmonary delivery was developed by URF technology (Yang et al. 2008a).

20.4 Micelles (Amphiphilic Block Copolymers)

Polymeric micelles (Fig. 20.1d), formed of amphiphilic block copolymers (ABCs) (5–50 nm), self-assemble to form a nanoscopic supramolecular core/shell structure in aqueous media (Kwon and Okano 1996), and are of great interest for drug delivery applications (Torchilin 2001). ABC micelles have a hydrophobic core surrounded by a hydrophilic outer shell. The hydrophobic core region serves as a reservoir for hydrophobic drugs, and can act as a nanodepot for these agents. This drug-loaded inner core is protected by a corona of biocompatible, hydrophilic polymeric chains exposed to the aqueous environment (Kataoka et al. 2001), that stabilizes the hydrophobic core and renders the polymers water-soluble, making the particle an appropriate candidate for i.v. administration (Adams et al. 2003). The self-assembly of amphiphilic block copolymers in water is based on nonpolar and hydrophobic interactions between the lipophilic core-forming polymer chains. The process is concomitantly driven by a gain in entropy of the solvent molecules as the hydrophobic components withdraw from the aqueous media (Dufresne et al. 2003). The hydrophilic shell contributes greatly to the pharmaceutical behavior of polymeric formulations by maintaining the micelles in a dispersed state, as well as by decreasing undesirable drug interactions with cells and proteins through steric-stabilization effects. The size of polymeric micelles ranges from ~10 to ~100 nm, and usually the size distribution is narrow (Kataoka et al. 2001). The polymer concentration at which the association first takes place, sometimes known as the critical association concentration (CAC), is lower by several orders of magnitude than typical surfactant CMC values. Thus, polymeric micelles are more stable towards dilution in biological fluids. They can increase drug bioavailability and retention, since the drug is well-protected from possible inactivation under the effect of their biological surroundings (Kwon 2002). Substitution of block copolymer micelles with specific ligands is a very promising strategy for a broader range of sites of activity with a much higher selectivity (Torchilin 2001). Usually, amphiphilic block copolymers of the A–B or B–A–B type, where A represents a hydrophilic block and B represents a hydrophobic block, are used to construct polymeric micelles. After conjugation with drug, they can form a core–shell micellar structure with the hydrophobic drug-bound B segments in the hydrophobic core and the hydrophilic A segments as a hydrated outer shell, as shown in Fig. 20.7.

There are two typical methods of entrapping a poorly water-soluble drug in the hydrophobic core of a micelle: (a) a block copolymer and a poorly water-soluble

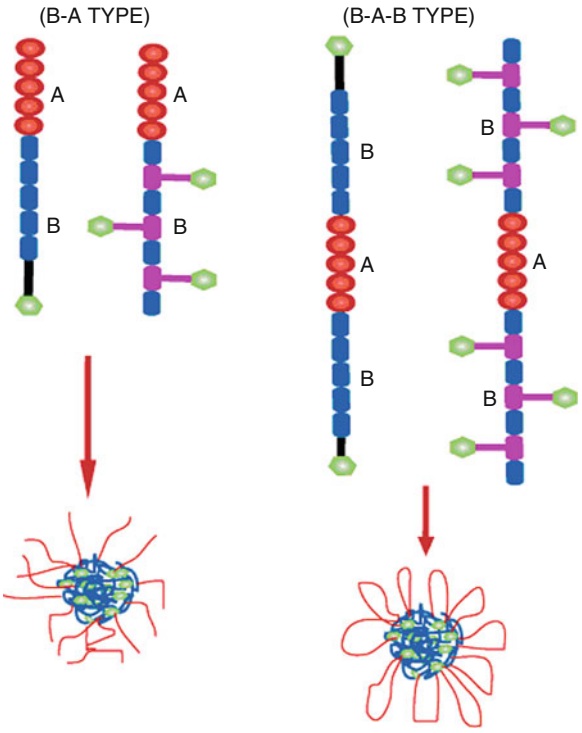

Fig. 20.7 Selected types of polymer–drug conjugate, where *A* represents a hydrophilic block and *B* represents a hydrophobic block

drug are dissolved in a water-miscible organic solvent, such as ethanol or N,N-dimethyl formamide (DMF), and the solution is dialyzed in water (Dialysis Method), or (b) a drug solution of a water-immiscible organic solvent, such as dichloromethane or chloroform, is added to an aqueous polymeric solution and the organic solvent is evaporated from the solution mixture (O/W Emulsion-Solvent Evaporation Method). In addition, the drug can be loaded into a polymeric micelle by physical encapsulation (physically entrapped in the core of block copolymer micelles and transported at concentrations that can exceed their intrinsic water-solubility: see Batrakova et al. 1996) or chemical covalent attachment (Nakanishi et al. 2001). Physical methods of drug encapsulation in polymeric micelles include the dialysis method, the oil/water emulsion method, the solvent evaporation method, co-solvent evaporation, and the freeze-drying method (Aliabadi and Lavasanifar 2006). Moreover, the hydrophilic blocks can form hydrogen bonds with the aqueous surroundings and form a tight shell around the micellar core. As a result, the contents of the hydrophobic core are effectively protected against hydrolysis and enzymatic degradation (Bae et al. 2003). In addition, the corona may prevent recognition by the reticulo-endothelial system, and therefore preliminary elimination of the micelles from the bloodstream (Packhaeuser et al. 2004).

20.4.1 Formulations Based on Micelles

To increase its therapeutic index, AmB has been solubilized in micelles based on poly(ethylene oxide)-block-poly(β-benzyl-L-aspartate) (PEO-block-PBLA at a drug: carrier ratio of 1:19 and a mean particle size of 2,694 nm, using a dialysis method of drug loading. The AmB diblock copolymer formulation showed a lower degree of aggregation of the AmB molecules than D-AmB, was less hemolytic and displayed increased antifungal activity *in vitro* (Yu et al. 1998a, b). The antifungal activity of the AmB-loaded PEO-block-PBLA micelles was found to be four to eight times higher than Fungizone® in terms of MICs. Finally, AmB-loaded PEO-PBLA micelles can be freeze-dried and easily reconstituted in water (Yu et al. 1998a,b). A new type of conjugate of AMB with block copolymer poly(ethylene glycol)-b-poly(L-lysine) (PEG-b-PLL), which is considered to be fully biocompatible and nontoxic, was prepared by Sedlák et al. (2007a, b). Choi et al. (2008) showed that AmpB-encapsulated polymeric micelles of poly (d,l-lactide-co-glycolide) (PLGA) grafted-dextran (DexLG) copolymer can be considered to be potential antifungal agent carriers. Lavasanifar et al. (2002) assessed the effect of fatty acid substitution of a micelle forming poly(ethylene oxide)-block-poly(*N*-hexyl stearate-Laspartamide) (PEO-*b*-PHSA) on the encapsulation, hemolytic properties and antifungal activity of amphotericin B (AmB), and found that PEO-*b*-PHSA micelles with a high level of stearic acid side-chain substitution can effectively solubilize AmB and reduce its hemolytic activity, yet retain its potent antifungal effects. In another study, a block copolymer poly(2-ethyl-2-oxazoline)-block-poly(aspartic acid) (PEOz-b-PAsp) increased not only the solubility of AmB but also simultaneously the drug potency. The prolonged release of AmB from micelles effectively inhibited the growth of *C. albicans* even after 3 days of administration. Moreover, the *in vitro* cytotoxicity of AmB-loaded micelles was less than that of Fungizone® (Wang et al. 2009).

Itraconazole (ITZ-PM) monomethoxy poly(ethylene glycol)-*b*-poly(lactic acid) and poly(lactic acid) formulation showed remarkably increased solubility in aqueous media. Toxicity studies and pharmacokinetic profiles of ITZ-PM for itraconazole and its major metabolite, hydroxy-itraconazole, were comparable to those of the CD formulations (Sporanox® injection and oral solution) in rats and dogs. These results suggest that ITZ-PM can be an advantageous formulation for both intravenous and oral routes (Yi et al. 2007).

20.5 Cyclodextrins

CDs are unique molecules with "pseudo-amphiphilic" structure, and several members of this family are used industrially in pharmaceutical and allied applications (Fig. 20.1e). CDs are cyclic oligosaccharides manufactured from starch (Bilensoy and Hincal 2009). The degradation of starch by the enzyme glucosyl-transferase

generates, by chain splitting and intramolecular rearrangement, primary products that are cyclic oligomers of α-1,4-D-glucopyranoside, or CDs. Major natural CDs are crystalline, homogeneous, nonhygroscopic substances that have a torus-like macro-ring shape built up from glucopyranose units (Loftsson and Duchene 2007; Uekama 2004). CDs derive their system of nomenclature from the number of glucose residues in their structure, such that the glucose hexamer is referred to as α-CD, the heptamer as β-CD and the octomer as γ-CD. There are literally thousands of variations of CDs that have variable ring size and random or site-specific chemical functionalization. A comprehensive overview of all aspects of CDs is available (Atwood et al. 1996).

CDs are "enabling" vehicles, and can be used for oral and intravenous (i.v.) delivery. When utilized as a vehicle for oral administration, CDs enhance the bioavailability of insoluble drugs by molecular dispersion, protection from degradation, and delivery to the surfaces of the intestinal wall. Given as parenteral vehicles, they serve as solubilizers for complex hydrophobic drugs, but they do not alter their pharmacokinetics (Thompson 1997). Their three-dimensional form is that of a truncated hollow cylinder with a hydrophilic outside and a hydrophobic inside. The internal hydrophobic domain can accommodate poorly water-soluble molecules, while the hydrophilic outside facilitates solubility in the aqueous environment (Thompson 1997; Stella and Rajewski 1997). Figure 20.8 depicts CD–drug complexation at various ratios. Because carrier and drug are associated by nonspecific hydrophobic forces only, they can easily dissociate at sites of greater affinity, i.e., at the lipid-rich surface of the intestinal wall after oral administration (Stevens 1999) and after contact with plasma proteins when administered i.v. (Stella and Rajewski 1997). Thus far, most research has focused on the β-CDs. The natural β-CD has a low solubility in water and also considerable nephrotoxicity after i.v. administration; following chronic dosing, CD cholesterol crystals are deposited in

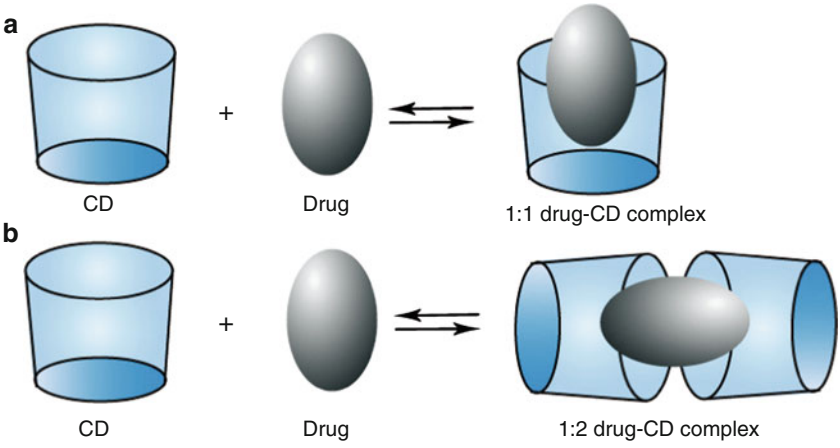

Fig. 20.8 Scheme depicting cyclodextrin–drug complexation. **a** 1:1 drug–CD complex. **b** 1:2 drug–CD complex

proximal renal tubular cells, leading to cell damage and ultimately, cell necrosis (Thompson 1997). The major advantage of amphiphilic CDs is their self-alignment or packaging properties at interfaces, which is sufficient to form nanoparticles spontaneously without the presence of a surfactant, along with their demonstrated ability of forming inclusion complexes with various drugs in their cavity and within the long aliphatic chains (Memisoglu-Bilensoy et al. 2005; Bilensoy et al. 2007). For manufacturing amphiphilic CDs, three different techniques have been reported in the literature, viz. the nanoprecipitation technique (Fessi et al. 1997; Wouessidjewe et al. 1996; Lemos-Senna et al. 1998a), the emulsion/solvent evaporation technique (Lemos-Senna et al. 1998b), and the detergent removal technique (Lemos-Senna 1998). Among these techniques, the nanoprecipitation technique is generally preferred, because it is a simple technique resulting in unimodal distribution with good reproducibility.

20.5.1 Cyclodextrin (CD) Formulations

Several attempts to improve the therapeutic efficacy of miconazole by formation of inclusion complexes (prepared by freeze-drying and kneading) with CD and their derivatives have been reported (Hostetler et al. 1992; Pedersen 1994). Among these, the miconazole/hydroxypropyl β-CD (HP-β-CD) complex demonstrated a 2.3-fold increase in oral bioavailability in rats, compared to an aqueous miconazole nitrate particulate suspension (Tenjarla et al. 1998). Likewise, complexes of ketoconazole and clotrimazole with various CDs have been investigated (Taneri et al. 2002, 2003a, b; Yong et al. 2007). Only moderately enhanced oral bioavailability of ketoconazole/HP-β-CD in mice was reported (Hostetler et al. 1992). Among clotrimazole/CD complexes, the complexation with gamma-CD had greater antifungal activity against *C. albicans* than the corresponding physical mixture, or clotrimazole alone (Yang et al. 2008b). Complexation of sulconazole with CD offers the possibility to improve the aqueous solubility of the antifungal drug without modification of its original structure. The inclusion complex showed an acute toxicity smaller than the pure drug, due to higher solubility and bioavailability of the complexed drug. Also, the *in vitro* antifungal activity of the complexed drug was shown to be higher (MIC50 is half and MIC90 is a quarter) than that of the pure biological active compound (Miron et al. 2009).

Three CD formulations of antifungal agents are currently in clinical use. They include the HP-β-cyclodextrin (HP-β-CD) itraconazole oral suspension, the HP-β-CD itraconazole intravenous solution, and the investigational intravenous sulphobutyl ether β-CD (SBE-β-CD) formulation of voriconazole. Itraconazole is commercially available in numerous dosage forms under the brand name Sporanox® (Ortho-McNeil, NJ, USA). The capsule-based oral dosage form consists of beads having a sugar core, coated with a mixture of itraconazole and hydroxypropylmethylcellulose (HPMC), sealed with an outer coating layer. The bioavailability from the oral capsule is low, with considerable intra- and inter-individual variability

(Poirier and Cheymol 1998), because this formulation requires an acidic environment and the presence of food for adequate absorption (Barone et al. 1993). HP-β-CD was chosen as the functional excipient to enable both of these formulations (Peeters et al. 2002). The oral and intravenous formulations that were developed proved to be safe and effective in numerous clinical trials, and this resulted in their market introduction in the United States and Europe (the oral solution in 1997 and the intravenous product in 1999) (De Beule and Van Gestel 2001; Slain et al. 2001; Willems et al. 2001).

An alternative oral solution, the itraconazole/HP-β-CD complex containing 10 mg/ml of itraconazole and 400 mg/ml of HP-β-CD at a target pH of 2, has also been developed. In comparison to the capsule form, the oral HP-β-CD itraconazole suspension confers enhanced oral bioavailability (55%), with a mean increase in the AUC 0–24 of approximately 30% (Barone et al. 1998) and with more consistent plasma concentrations (Willems et al. 2001). The carrier has minimal-to-absent systemic effects, due to the lack of systemic absorption. It may, however, exert osmotic activity in the intestinal lumen, which may lead to gastrointestinal intolerance (Stevens 1999). Hydroxybutenyl cyclodextrin (HBen-β-CD), a chemically modified CD with higher aqueous solubility than HP-β-CD, has been used to formulate itraconazole complexes, including liquid and lyophilized solid forms (Buchanan et al. 2007). After intravenous administration of HP-β-CD itraconazole or SBE-β-CD voriconazole, the CD vehicle is cleared very rapidly from the plasma; within 24 h, more than 95% of given dose are excreted in unchanged form into the urine (Thompson 1997; Irie and Uekama 1997). The sulphobutyl ether derivatives of β-CD (SBE-β-CD) represent the newest CD derivative to be approved (Thompson 1997; Zia et al. 2001; Rajewski et al. 1995; Kim et al. 1998). An SBE-β-CD intravenous formulation of the antifungal agent voriconazole (Vfend; Pfizer) resulted in a dosage form containing 3.2 g of the CD and 200 mg of the active principle. The future of CDs and CD-containing polymers in the pharmaceutical industry seems to be bright. There are numerous traditional and nontraditional applications that are on the horizon for commercialization.

20.6 Cochleate Lipid Cylinders

Cochleate delivery vehicles represent a new technology platform for oral and systemic delivery of clinically important drugs. Cochleates (Fig. 20.1f) are a novel lipid-based system that has potential as a delivery system for molecules with important therapeutic biological activities, including drugs (Zarif et al. 1999a, 2000; Graybill et al. 1999; Santangelo et al. 2000) and genes (Zarif and Mannino 2000) for therapeutic purposes, and antigens for vaccine applications (Mannino and Gould-Fogerite 1997). Cochleates are stable, negatively charged phospholipid–cation precipitates, composed of naturally occurring materials, for example, phosphatidylserine and calcium. They are defined multilayered structures of solid continuous lipid bilayer sheets, rolled up in a spiral, with little or no internal aqueous space. This anhydrous, unique structure provides protection from degradation in the

gastrointestinal tract for associated molecules, and offers a new way of stabilizing biopharmaceuticals such as proteins, peptides, nucleic acids and other sensitive moieties. Papahadjopoulos et al. first described cochleates in 1975 as an intermediate in the preparation of large unilamellar vesicles (Papahadjopoulos et al. 1975).

Cochleates are formed by the precipitation of safe products, a negatively charged lipid (phosphatidylserine) and a cation (calcium). Addition of Ca^{2+} to negatively charged, unilamellar phosphatidyl serine vesicles induces fusion and formation of planar sheets, as calcium interaction with negatively charged lipid displaces H_2O and condenses lipid. Formation of stable drug–cochleate nanocrystal takes place as calcium lipid sheets "roll-up" to form spiral multilamellar, cochleate cylinders with no aqueous space (Papahadjopoulos et al. 1975), in order to minimize contact with water, excluding H_2O and O_2. Incubation of these lipid cylinders with the chelating agent EDTA restored the negative charge of the phospholipids, and resulted in the formation of very large unilamellar vesicles that became a useful tool for studying the properties of biological membranes. Incorporated into the multilayered system of phospholipid membranes, the active principle is shielded from the harsh environment of the gastrointestinal tract, and directed to and efficiently taken up by the membranes of the intestinal epithelium. Ca^{2+}-mediated membrane perturbation and fusion phenomena have been proposed as mechanisms for uptake (Zarif et al. 1999a). It is unclear, however, whether and how the cochleates reach the systemic circulation, or whether the drug is delivered for absorption with the carrier staying behind. Cochleate formulation technology is particularly applicable to macromolecules and small-molecule drugs that are hydrophobic, amphiphilic, negatively or positively charged and possess poor oral bioavailability. Various procedures have been developed allowing the control of cochleate particle size, including the trapping and hydrogel methods, which use either a direct addition or a slow diffusion of calcium into the negatively charged liposome/drug suspension.

20.6.1 Antifungal Formulations of Cochleates

Amphotericin B cochleates represent an alternative approach to the treatment of disseminated fungal infections via oral administration of drug, as all currently available formulations of AmB are only administered through intravenous infusion. AmB-cochleate formulations (C-AmB) have an advantage over existing formulations, because cochleates are resistant to degradation in the gastrointestinal tract. Proof-of-principle studies for cochleate-mediated oral delivery have been carried out in a murine model with amphotericin B (Edwards and Filler 1992). A cochleate lipid formulation of amphotericin B (AmB), consisting of Ca^{2+}, dioleoyl phosphatidyl serine and AmB in a 10:1 (lipid:drug) molar ratio and having a mean particle diameter of less than 500 nm, has been developed and is undergoing preclinical characterization. The rearrangement between AmB and phosphatidylserine molecules seems to have no impact on the structure of cochleate. Sheets of

AmB-DOPS appear to roll up into cylinders when a lipid/AmB molar ratio of 10/1 is used.

Compared to deoxycholate amphotericin B (D-AmB), C-AmB had at least 50-fold reduced hemolytic potential, and was tolerated with 100% survival in healthy mice at repeat daily dosages given by the oral (p.o.) or intraperitoneal (i.p.) route. In preclinical studies, amphotericin B cochleates exhibited comparative antifungal activity, but with reduced toxicity compared to injectable forms of amphotericin B (Zarif and Perlin 2002).

C-AmB has been shown to be highly protective in a mouse candidiasis model following parenteral administration (Graybill et al. 1999). Cochleates containing AMB (C-AmB) inhibit the growth of *C. albicans*, and the *in vivo* therapeutic efficacy of C-AmB administered orally has been evaluated in a mouse model of systemic candidiasis (Santangelo et al. 2000). C-AMB was highly effective at blocking cell growth of *C. albicans in vitro*, at a level comparable to that of D-AmB (Zarif et al. 2000). In vivo, after i.p. administration to non-immunosuppressed mice systemically infected with *C. albicans*, C-AmB was as effective as D-AmB and liposomal amphotericin B (L-AmB), as measured by survival and the residual fungal burden in kidney tissue (Graybill et al. 1999). In a lethal, non-immunosuppressed mouse model of disseminated candidiasis, C-AmB produced 100% survival at low dosages, and displayed dose-dependent clearance of the organism from kidney and lung (Zarif et al. 1999a). Initial biodistribution studies of C-AmB administered orally in a mouse model showed that cochleates delivered therapeutic levels of AMB to target organs (Zarif et al. 1999b).

Cochleate technology has been used to develop an oral formulation of Amphotericin B (Bioral™ amphotericin B, BioDelivery Sciences International (BDSI), NC, USA) for antifungal treatment. This oral cochleate formulation has shown promising data in mouse models of aspergillosis and candidiasis, as shown by the outcome of the Phase I trial released in February 2009 on the company website (Thornton and Wasan 2009). The Bioral™ technology is being evaluated as a new means of overcoming the poor oral absorption of drugs such as the antifungal drug Amphotericin B. Several preclinical studies have demonstrated the potential value of agents delivered using the Bioral® technology. It seems that C-AmB promises to be an ideal safe system for delivering amphotericin B orally (Delmas et al. 2002).

20.7 Niosomes

Niosomes (nonionic surfactant vesicles) (Fig. 20.1a) are one of the DDSs, which resemble liposomes in their architecture and can be used as an effective alternative to liposomal drug carriers (Uchegbu and Vyas 1998). The self-assembly of niosomes (Uchegbu and Vyas 1998) in aqueous media, resulting in closed bilayer structures, represents an interesting opportunity to achieve vesicular colloidal drug carriers, and can entrap both hydrophilic and lipophilic drugs, either in an aqueous layer or in vesicular membrane (Carafa et al. 1998). These nonionic surfactant

vesicles appear to be similar to liposomes in terms of their physical properties, structures and methods of preparation. Niosomes alleviate the disadvantages associated with liposomes (Florence and Baillie 1989; Beugin et al. 1998), such as chemical instability, variable purity of phospholipids and high cost (Vora et al. 1998), and can show entrapment of more substances.

Niosomes may be made up of a variety of amphiphiles bearing sugar, polyoxyethylene, polyglycerol, crown ether, and amino acid hydrophilic head groups, and these amphiphiles typically possess one to two hydrophobic alkyl, perfluoroalkyl or steroidal groups (Arunothayanun et al. 2000; Manconi et al. 2002). Nonionic surfactants used to prepare vesicles are polyglycerol alkyl ethers, glucosyl dialkyl ethers, crown ethers, ester-linked surfactants, polyoxyethylene alkyl ether (Handjani-vila et al. 1979; Echegoyen et al. 1988; Hunter et al. 1988), Brij (Bhaskaran and Panigrahi 2002), and a series of Spans and Tweens (Bhaskaran and Panigrahi, 2002; Namdeo and Jain 1996). The latest generation of surfactants is represented by amphiphiles having two polar crown ether headgroups linked to both ends of a long alkyl chain (Muzzalupo et al. 1996). These amphiphiles, generally referred to as bolaform surfactants, can be anionic, cationic, zwitterionic or nonionic as a function of the kind of ion, which can be complexed by the two-crown ether headgroups (Muzzalupo et al. 1996), and have recently been used as components for the preparation of niosomes (Muzzalupo et al. 2005). Niosomes can be prepared by the same procedure as that for liposomes, e.g., conventional chloroform film method (Bangham et al. 1965), reverse-phase evaporation (Szoka and Papahadjopoulos 1978) and ethanol injection (Batzri and Korn 1973). Most methods require large amounts of organic solvents that are toxic to human and environments and have multisteps. In some preparation methods, even without using organic solvents, such as the heating method (Mozafari et al. 2007) and the polyol dilution method (Kikuchi et al. 1994), there are problems in using a high temperature, which is not suitable for heat-labile substances. Niosomes face no restrictions on the route of delivery, and can be delivered by oral (Attia et al. 2007; Varshosaz and Najafabadi 2003), parenteral (Jain and Vyas 1995), nasal (Gayathri and Udupa 2000), and topical routes (Shahiwala and Misra 2002).

Niosomes consist of two components, which are the main component and membrane additives. The main component is composed mainly of nonionic surfactants. Membrane additives are substances that are added in the formulation in order to stabilize the niosomes. The most common additive found in niosomal systems is cholesterol, which is known to abolish the gel-to-liquid phase transition of liposomal and niosomal systems, resulting in less leakiness of the vesicles. However, it may have effects on membrane permeability, encapsulation efficiency, bilayer rigidity, ease of rehydration of freeze-dried niosomes, and toxicity. In general, it has been found that a molar ratio of 1:1 between cholesterol and nonionic surfactants is an optimal ratio for the formulation of physically stable niosomal vesicles. There have been several trials which attempted to find other substances in substitution to cholesterol. One of the methods used to stabilize niosomes is to add a charged molecule to the bilayer. Dicetylphosphate (DCP) and phosphatidic acid are known as negatively charged molecules, while stearylamine (STR) and

cetylpyridinium chloride are positively charged molecules, which are both commonly used for preventing aggregation of niosomes. Tween 80 and Span 80 are pharmaceutically acceptable, innocuous, nonionic surfactants (Gianasi et al. 1997). PEG, Tween 80 and Span 80 can be used to prepare highly stable niosomes (Liu and Guo 2005), which are provided with the hydrotrope-solubilization action to both hydrophilic and hydrophobic drugs.

Niosomes have been proposed for a number of potential therapeutic applications, i.e., as immunological adjuvants (Jain et al. 2005), anticancer and anti-infective drug targeting agents (Balasubramaniam et al. 2002; Gude et al. 2002), carriers of anti-inflammatory drugs (Shahiwala and Misra 2002), and as diagnostic imaging agents (Uchegbu and Vyas 1998). In addition, niosomes are versatile carrier systems that can be administered through various routes. Particular efforts have been aimed at using niosomes as effective transdermal DDSs (Barry 2001; Choi and Maibach 2005).

Antifungal drug, ketoconazole niosomes prepared by the thin-film hydration technique using surfactant (Tween 40 or 80), cholesterol and drug in five different ratios were formulated in FAPG base and tested for *in vitro* antifungal activity (cup-plate method) (Satturwar et al. 2001). Incorporation of DAS in niosomes enhances its antifungal efficacy, and significantly reduced fungal load and mortality in treated animals compared with the free form of DAS. Also, niosomal DAS was found to be free of toxic manifestations, as revealed by histopathological studies as well as liver/kidney function tests (Alam et al. 2009). Niosomal formulation of clotrimazole (e.g., clotrimazole:span 40:cholesterol = 1:8:2, molar ratio) for vaginal administration were developed in order to reduce dosing frequency and drug toxicity (Ning et al. 2005)

20.8 Microspheres

Particulate agents with usual size of 1–300 μm are a tiny spherical entity, in which drugs are wrapped by polymer materials or dispersed in polymer materials, the solid skeleton structure being called microspheres (Fig. 20.1g). Administration of medication via such systems is advantageous, because microspheres can be ingested or injected; they can be tailored for desired release profiles and used for site-specific delivery of drugs, and in some cases can even provide organ-targeted release (Sanli et al. 2009). According to biodegradability, it can be divided into biodegradable microspheres and nonbiodegradable microspheres. Biodegradable microspheres include albumin microspheres, modified starch microspheres, gelatin microspheres, polypropylene dextran microspheres and PLA microspheres, etc. The most widely used techniques for microsphere preparation are: (1) spray drying, (2) double emulsion, and (3) phase separation–coacervation (Sinha and Tehan 2003). The release from the microspheres is dependent both on diffusion through the polymer matrix and on polymer degradation (Sinha and Tehan 2003). Biodegradation of

polymeric biomaterials involves cleavage of hydrolytically or enzymatically sensitive bonds in the polymer, leading to polymer erosion (Katti et al. 2002).

According to the current literature reports on nonbiodegradable microspheres, PLA is the only polymer approved for use by people as a controlled-release agent (Wang et al. 2003). In addition, reports on immune microsphere and magnetic microsphere are also common in recent years. Immune microsphere possesses immunocompetence because antibody and antigen were coated or adsorbed on the polymer microspheres. Immunomagnetic microspheres can be prepared by combining two kinds of microspheres, which could significantly improve its targeting. Magnetic nanoparticles possess a unique magnetic feature, namely their reaction to a magnetic force. Magnetic nanoparticles have generally been coated with protective shells as magnetic polymer microparticles (Chen et al. 2009).

Recently, amphotericin B microsphere formulations, with and without the addition of polyethylene glycol 2000 in cross-linked bovine serum albumin, have been prepared, characterized and assessed for their antifungal activity *in vitro* (Angra et al. 2009). A new formulation of amphotericin B, consisting of drug-containing albumin microspheres (prepared by a spray-drying process) was evaluated by Marine et al. (2009) for its efficacy against murine-disseminated infections by *C. glabrata* or *Candida tropicalis*. It showed a similar efficacy to that of amphotericin B deoxycholate and liposomal amphotericin B in the treatment of both disseminated murine infections.

20.9 Conclusion

The success of a newly developed pharmaceutical formulation is related to the fact that it is able to deliver the active substance to the target organ at therapeutically relevant levels, with negligible discomfort and side-effects, increasing patient compliance to the therapeutics. The current focus of research has generated a broad spectrum of carriers, with the majority showing rather poor biological performance in terms of both circulation time and target specificity, and there are numerous formulations that are on the horizon for commercialization. However, many questions still remain to be answered, including optimal dosage levels, duration of treatment, and which agents to combine in what setting. Again, the concepts of drug incorporation and release from such particles have been poorly investigated. Also, the ultimate fate of the constituent materials of the vehicles remains undefined. Toxicity is also a major safety concern for using nanoDDSs in the clinical setting. In designing long-circulating particles; attention must also be paid to their application to a relevant pathology. Here, it is still unclear which approach to targeting is likely to yield the best results. The advances in azole antifungal drug formulations are promising. However, many of these data are limited to *in vitro* or animal studies. Further work is needed to translate these findings into the clinical arena, in order to benefit patients at risk for or suffering from fungal infections. That is why the future of drug therapeutics may not lie in the

development of new chemical entities but in the modification of the existing drug molecules, using suitable carriers to eliminate toxicity and improve activity, the principle of new lives for old drugs (Juliano and Lopez-Berestein 1985).

To date, synthetic polymers have been the materials of choice in the construction of long-circulating colloids. A deficiency in this field which has to be rectified is the precise characterization of these macromolecules in terms of molecular weight and purity. The problem with molecular weight determination is due to technical difficulties associated with the techniques available at present. For instance, aqueous gel permeation chromatography, used for the separation and molecular weight determination of water-soluble polymers, is a relatively new technique, and its use is hindered by the fact that there are very few molecular weight standards that can be used to calibrate the system. Also, little work has been done in determining the nature of polymeric degradation products, their biological activity and ultimate fate following parenteral administration. Finally, a major factor that has been ignored is genetics. Future considerations must be given to the immunogenetic and pharmacogenetic differences and related polymorphisms.

With a better understanding of the biological interactions associated with nanomaterials, and the improvements in surface chemistry modifications, it is not a matter of if, but when, such hurdles will be cleared. We can envision a future where tiny nanoDDSs travel through our bloodstream searching for sites of infection or disease processes, and once any irregular cellular activities are found, the triggered release of the nanoDDS' cargo repairs damaged tissues or kills foreign invaders. Every step along the way is meticulously executed in an intelligent automatic manner. This is the future of nanotechnology.

References

Abu-Salah KM (1996) Amphotericin B: an update. Br J Biomed Sci 53:122–133

Adams ML, Lavasanifar A, Kwon GS (2003) Amphiphilic block copolymers for drug delivery. J Pharm Sci 92:1343–1355

Adler-Moore JP, Proffitt R (2002) AmBisome: liposomal formulation, structure, mechanism of action and pre-clinical experience. J Antimicrob Ther 49(Suppl 1):21–30

Ahmad N, Alam MK, Shehbaz A, Khan A, Mannan A, Hakim SR, Bisht V, Owais M (2005) Antimicrobial activity of clove oil and its potential in the treatment of vaginal candidiasis. J Drug Target 13(10):555–561

Alam M, Dwivedi V, Khan AA, Mohammad O (2009) Efficacy of niosomal formulation of diallyl sulfide against experimental candidiasis in Swiss albino mice. Nanomedicine 4(7):713–724

Alexis F, Basto P, Levy-Nissenbaum E, Radovic-Moreno AF, Zhang LF, Pridgen E Wang AZ, Marein SL, Westerhof K, Molnar LK, Farokhzad OC (2008) Her-2-targeted nanoparticle–affibody bioconjugates for cancer therapy. Chem Med Chem 3:1839–1843

Aliabadi HM, Lavasanifar A (2006) Polymeric micelles for drug delivery. Expert Opin Drug Deliv 3(1):139–162

Aliff TB, Maslak PG, Jurcic JG, Heaney ML, Cathcart KN, Sepkowitz KA, Weiss MA (2003) Refractory Aspergillus pneumonia in patients with acute leukemia: successful therapy with combination caspofungin and liposomal amphotericin. Cancer 97:1025–1032

Allen C, Dos SN, Gallagher R, Chiu GNC, Shu Y, Li WM, Johnstone SA, Janoff AS, Mayer LD, Webb MS, Bally MB (2002) Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol). Biosci Rep 22:225–250

Allen TM (1998) Liposomal drug formulations. Rationale for development and what we can expect for the future. Drugs 56:747–756

Allen TM, Chonn A (1987) Large unilamellar liposomes with low uptake by the reticuloendothelial system. FEBS Lett 223:42–46

Allen TM, Hansen C (1991) Pharmacokinetics of stealth verses conventional liposomes: effect of dose. Biochim Biophys Acta 1068:133–141

Allen PD, TM GA, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C et al (1991b) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci U S A 88:11460–11464

Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A (1991a) Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. Biochim Biophys Acta 1066:29–36

Allen TM, Hansen C, Rutledge J (1989) Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. Biochim Biophys Acta 981:27–35

Allison AG, Gregoriadis G (1974) Liposomes as immunological adjuvants. Nature 252:252

Angra PK, Oettinger C, Pai SB, D'Souza MJ (2009) Amphotericin B microspheres: a therapeutic approach to minimize toxicity while maintaining antifungal efficacy. J Microencapsul 26(7): 580–587

Arikan S, Rex JH (2001) Nystatin LF. Curr Opin Investig Drugs 2:488–495

Arunothayanun P, Bernard MS, Craig DQ, Uchegbu IF, Florence AT (2000) The effect of processing variables on the physical characteristics of non-ionic surfactant vesicles (niosomes) formed from a hexadecyl diglycerol ether. Int J Pharm 201:7–14

Attia IA, El-Gizawy SA, Fouda MA, Donia AM (2007) Influence of a niosomal formulation on the oral bioavailability of acyclovir in rabbits. AAPS Pharm Sci Tech 8(4):E1–E7

Atwood JL, Davies JED, MacNicol DD, Vogtle F (eds) (1996) Comprehensive supramolecular chemistry, vol 3, Cyclodextrins. Pergamon, Oxford

Awasthi VD, Garcia D, Klipper R, Goins BA, Phillips WT (2004) Neutral and anionic liposome-encapsulated hemoglobin: effect of postinserted poly(ethylene glycol)–distearoyl phosphatidyl ethanolamine on distribution and circulation kinetics. J Pharmacol Exp Ther 309: 241–248

Bae Y, Fukushima S, Harada A, Kataoka K (2003) Design of environment-sensitive supramolecular assemblies for intracellular drug delivery: polymeric micelles that are responsive to intracellular pH change. Angew Chem Int Ed 42:4640–4643

Bakker-Woudenberg IA, Storm G, Woodle MC (1994) Liposomes in the treatment of infections. J Drug Target 2:363–371

Balasubramaniam A, Kumar VA, Pillai KS (2002) Formulation and *in vivo* evaluation of niosome-encapsulated daunorubicin hydrochloride. Drug Dev Ind Pharm 28:1181–1193

Bangham AD, Standish MM, Miller N (1965) Cation permeability of phospholipid model membranes: effect of narcotics. Nature 208:1295–1297

Barone JA, Koh JG, Bierman RH et al (1993) Food interaction and steady-state pharmacokinetics of itraconazole capsules in healthy male-volunteers. Antimicrob Agents Chemother 37(4): 778–784

Barone JA, Moskovitz BL, Guarnieri J, Hassell AE, Colaizzi JL, Bierman RH, Jessen L (1998) Enhanced bioavailability of itraconazole in hydroxypropyl-beta-cyclodextrin solution versus capsules in healthy volunteers. Antimicrob Agents Chemother 42:1862–1865

Barry BW (2001) Novel mechanisms and devices to enable successful transdermal drug delivery. Eur J Pharm Sci 14:101

Batrakova EV, Dorodnych TY, Klinskii EY, Kliushnenkova EN, Shemchukova OB, Goncharova ON et al (1996) Anthracycline antibiotics non-covalently incorporated into the block copolymer micelles: *in vivo* evaluation of anti-cancer activity. Br J Cancer 74:1545–1552

Batzri S, Korn ED (1973) Single bilayer liposomes prepared without sonication. Biochim Biophys Acta 289:1015–1019

Belay T, Hospenthal DR, Rogers AL, Patterson MJ (1991) Evaluation of antibody-bearing liposomal amphotericin B in the treatment of systemic candidiasis in a neutropenic murine model. J Med Vet Mycol 29:419–421

Beugin S, Edwards K, Karlsson G, Ollivon M, Lesieur S (1998) New sterically stabilized vesicles based on nonionic surfactant, cholesterol and poly(ethylene glycol)–cholesterol conjugates. Biophys J 74:3198–3210

Bhaskaran S, Panigrahi L (2002) Formulation and evaluation of niosomes using different non-ionic surfactants. Indian J Pharm Sci 64(1):63–65

Bilensoy E, Dogan AL, Sen M, Hincal AA (2007) Complexation behaviour of antiestrogen drug tamoxifen citrate with natural and modified cyclodextrins. J Inclusion Phenom Macrocyclic Chem 57:651–654

Bilensoy E, Hincal AA (2009) Recent advances and future directions in amphiphilic cyclodextrin nanoparticles. Expert Opin Drug Deliv 6(11):1161–1173

Brajtburg J, Bolard J (1996) Carrier effects on biological activity of amphotericin B. Clin Microbiol Rev 9:512–531

Brannon-Peppas L, Blanchette JO (2004) Nanoparticle and targeted systems for cancer therapy. Adv Drug Deliv Rev 56(11):1649–1659

Buchanan CM, Buchanan NL, Edgar KJ et al (2007) Pharmacokinetics of itraconazole after intravenous and oral dosing of itraconazole–cyclodextrin formulations. J Pharm Sci 96(11): 3100–3116

Campbell PI (1983) Toxicity of some charged lipids used in liposome preparation. Cytobios 37:21–26

Carafa M, Santucci E, Alhaique F, Coviello T, Murtas E, Riccieri FM, Lucania G, Torrisi MR (1998) Preparation and properties of new unilamellar non-ionic surfactant vesicles. Int J Pharm 160:51–59

Chaize B, Colletier JP, Winterhalter M, Fournier D (2004) Encapsulation of enzymes in liposomes: high encapsulation efficiency and control of substrate permeability. Artif Cells Blood Substit Immobil Biotechnol 32:67–75

Chan OH, Stewart BH (1996) Physicochemical and drug-delivery considerations for oral drug bioavailability. Drug Discov Today 1(11):461–473

Chen AZ, Kang YQ, Pu XM, Yin GF, Li Y, Hu JY (2009) Development of Fe_3O_4-poly(llactide) magnetic microparticles in supercritical CO_2. J Colloid Interface Sci 330:317–322

Chen T, Choi LS, Einstein S, Klippenstein MA, Scherrer P, Cullis PR (1999) Proton-induced permeability and fusion of large unilamellar vesicles by covalently conjugated poly (2-ethylacrylic acid). J Liposome Res 9:387–405

Choi KC, Bang JY, Kim PI, Kim C, Song CE (2008) Amphotericin B-incorporated polymeric micelles composed of poly(d,l-lactide-co-glycolide)/dextran graft copolymer. Int J Pharm 355(1–2):224–230

Choi MJ, Maibach HI (2005) Liposomes and niosomes as topical drug delivery systems. Skin Pharmacol Physiol 18:209–219

Chu CJ, Dijkstra J, Lai MZ, Hong K, Szoka FC (1990) Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture. Pharmacol Res 7:824–834

Clemons KV, Espiritu M, Parmar R, Stevens DA (2005) Comparative efficacies of conventional amphotericin B, liposomal amphotericin B (AmBisome), caspofungin, micafungin, and voriconazole alone and in combination against experimental murine central nervous system aspergillosis. Antimicrob Agents Chemother 49:4867–4875

Collins D (1995) pH-Sensitive liposomes as tools for cytoplasmic delivery. In: Philippot JR, Schuber F (eds) Liposomes as tools in basic research and industry. CRC, Boca Raton, FL, pp 201–214

Cornely O (2005) Ambiload. Am Soc Hematol 55:727–734

Davis SS (1997) Biomedical applications of nanotechnology — implications for drug targeting and gene therapy. Trends Biotechnol 15:217–224

de Beule K, Van Gestel J (2001) Pharmacology of itraconazole. Drugs 61(Suppl 1):27–33
de Gennes PG (1980) Conformations of polymers attached to an interface. Macromolecules 13:1069–1075
Delmas G, Park S, Chen ZW, Tan F, Kashiwazaki R, Zarif L, Perlin DS (2002) Efficacy of orally delivered cochleates containing amphotericin B in a murine model of aspergillosis. Antimicrob Agents Chemother 46(8):2704–2707
Des Rieux A, Fievez V, Garinot M, Schneider YJ, Preat V (2006) Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J Control Release 116(1): 1–27
Desai SK, Naik SR (2008a) Probable mechanism(s) of antifungal activity of SJA-95, a heptaene polyene antibiotic. Indian J Pharm Sci 70(2):165–169
Desai SK, Naik SR (2008b) Preparation, relative toxicity, chemotherapeutic activity, and pharmacokinetics of liposomal SJA-95: a new polyene macrolide antibiotic. J Liposome Res 18:279–292
Drew R (2006) Potential role of aerosolized amphotericin B formulations in the prevention and adjunctive treatment of invasive fungal infections. Int J Antimicrob Agents 27S:S36–S44
Dromer F, Barbet J, Bolard J, Charreire J, Yeni P (1990) Improvement of amphotericin B activity during experimental cryptococcosis by incorporation into specific immunoliposomes. Antimicrob Agents Chemother 34:2055–2060
Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D (1999) Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev 51:691–743
Drummond DC, Zignani M, Leroux J-C (2000) Current status of pH-sensitive liposomes in drug delivery. Prog Lipid Res 39:409–460
Dufresne MH, Fournier E, Jones M-C, Ranger M, Leroux JC (2003) Block copolymer micelles–engineering versatile carriers for drugs and biomacromolecules. In: Gurny R (ed) Challenge in drug delivery for the new millennium Gattefosse Bulletin Technique, vol 96. Gattefosse, Saint-Priest, France, pp 87–102
Echegoyen LE, Hernandaz JC, Kaifer AE, Gokel GW, Echegoyen L (1988) J Chem Soc Chem Commun 12:836
Edwards JE Jr, Filler SG (1992) Current strategies for treating invasive candidiasis: emphasis on infections in nonneutropenic patients. Clin Infect Dis 14(suppl 1):S106–S113
Elder EJ, Hitt JE, Rogers TL et al (2006) Particle engineering of poorly water soluble drugs by controlled precipitation. ACS Symp Ser 924:292–304
Elizabeth MJ, Joshua OO, Szekely A, Wallace TL, David WW (1998) Comparison of *in vitro* antifungal activities of free and liposome-encapsulated nystatin with those of four Amphotericin b formulations. Antimicrob Agents Chemother 42(6):1412–1416
Fessi HC, Devissaguet JP, Puisieux F, Thies C (1997) Process for the preparation of dispersible colloidal systems of a substance in the form of nanoparticles. US Patent 5118528
Florence AT, Baillie AJ (1989) Non-ionic surfactant vesicles, alternatives to liposomes in drug delivery? In: Prescott LF, Nimmo WS (eds) Novel drug delivery and its therapeutic application. Wiley, New York, pp 281–296
Fukui H, Koike T, Saheki A, Sonoke S, Yoshikawa H, Sasaki H, Tomii Y, Seki J (1996) A novel antifungal drug delivery system: lipid nano-sphere incorporating amphotericin B (LNS-AmB). Proceedings of the 23rd International Symposium on Controlled Release of Bioactive Materials, Kyoto, Japan, p 655. Abstr. 5026
Gabizon A, Papahadjopoulos D (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 85:6949–6953
Gayatri Devi S, Venkatesh P, Udupa N (2000) Niosomal sumatriptan succinate for nasal administration. Int J Pharm Sci 62:479–481
Gianasi E, Cociancich F, Uchegbu IF, Florence AT, Duncan FR (1997) *Pharmaceutical and biological characterization of a doxorubicin-polymer conjugate* (*PK1*) *entrapped in* sorbitan monostearate Span 60 niosomes. Int J Pharm 148:139–148
Graybill JR, Najvar L, Bocanegra R, Scolpino A, Mannino RJ, Zarif L (1999) A new lipid vehicle for amphotericin B. In: Abstract 39th ICAAC, San Francisco, 26–29 September, p 583

Gref R, Minamitake Y, Peracchia MT, Trubetskoy V, Torchilin V, Langer R (1994) Biodegradable long-circulating polymeric nanospheres. Science 263:1600–1603
Gregoriadis G (1977) Targeting of drugs. Nature 265:407–411
Gregoriadis G (1995) Engineering liposomes for drug delivery: progress and problems. Trends Biotechnol 13:527–537
Gregoriadis G, Florence AT (1993) Liposomes in drug delivery: clinical, diagnostic and ophthalmic potential. Drugs 45:15–28
Gregoriadis G, Neerunjun ED (1975) Homing of liposomes to target cells. Biochem Biophys Res Commun 65:537–544
Grislain L, Couvreur P, Lenaerts V, Roland M, Depreg-Decampeneere D, Speiser P (1983) Pharmakokinetics and distribution of a biodegradable drug-carrier. Int J Pharmacol 15:335–338
Groll AH, Piscitelli SC, Walsh TJ (1998) Clinical pharmacology of systemic antifungal agents. Adv Pharmacol 44:343–500
Gude RP, Jadhav MG, Rao SG, Jagtap AG (2002) Effects of niosomal cisplatin and combination of the same with theophylline and with activated macrophages in murine B16F10 melanoma model. Cancer Biother Radiopharm 17:183–192
Guo J, Ping Q, Jiang G, Huang L, Tong Y (2003) Chitosan-coated liposomes: characterization and interaction with leuprolide. Int J Pharm 260:167–173
Handjani-vila RM, Ribier A, Rondot B, Vanlerberghe G (1979) Dispersions of lamellar phases of non-ionic lipids in cosmetic products. Int J Cosmet Sci 1:303–314
Hao H, Liang GZ (2002) Polymer nanoparticle applicated in drug delivery systems. Ion Exch Adsorption 18:380–384
Harrington KJ, Syrigos KN, Vile RG (2002) Liposomally-targeted cytotoxic drugs in the treatment of cancer. J Pharm Pharmacol 54:1573–1600
Hay RJ (1994) Liposomal amphotericin B, AmBisome. J Infect 28:35–43
He W, Yang JX, Yang J, Yin WH (1998) The research on liver targeting delivery system of cantharidin polyphase liposome modified by strengthened targeting material. J China Pharm Univ 20:413–417
Henriksen I, Vagen SR, Sande SA, Smitad G, Karlsen J (1997) Interactions between liposomes and chitosan II: effect of selected parameters on aggregation and leakage. Int J Pharm 146:193–204
Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, Kern WV, Marr KA, Ribaud P et al (2002) Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 347:408–415
Hiemenz JW, Cagnoni P, Tong K (1998). A cost-effectiveness study comparing ambisome versus amphotericin B deoxycholate in the empirical treatment of persistently febrile neutropenic patients. Focus on Fungal Infections 8, Orlando (Abstract)
Hiroshi F, Tomohiro K, Takashi N, Akira S, Satoru S, Yoshifumi T, Junzo S (2003) Comparison of LNS-AmB, a novel low-dose formulation of amphotericin B with lipid nano-sphere (LNS®), with commercial lipid-based formulations. Int J Pharm 267:101–112
Hope MJ, Mui B, Ansell S, Ahkong QF (1998) Cationic lipids, phosphatidyl-ethanolamine and the intracellular delivery of polymeric, nucleic acid based drugs. Mol Membr Biol 15:1–14
Hossain MA, Maesaki S, Kakeya H, Noda T, Yanagihara K, Sasaki E, Hirakata Y, Tomono K, Tashiro T, Kohno S (1998) Efficacy of NS-718, a novel lipid nanosphere-encapsulated amphotericin B against *Cryptococcus neoformans*. Antimicrob Agents Chemother 42: 1722–1725
Hostetler JS, Hanson LH, Stevens DA (1992) Effect of cyclodextrin on the pharmacology of antifungal oral azoles. Antimicrob Agents Chemother 36(2):477–480
Hunter CA, Dolan TF, Coombs GH, Baillie AJ (1988) Vesicular systems (niosomes and liposomes) for delivery of sodium stibogluconate in experimental murine visceral leishmaniasis. J Pharm Pharmacol 40(3):161–165
Irie T, Uekama K (1997) Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J Pharm Sci 86:147–162

Ishida T, Harashima H, Kiwada H (2002) Liposome clearance. Biosci Rep 22:197–224
Italia JL, Yahya MM, Singh D, Ravi Kumar MN (2009) Biodegradable nanoparticles improve oral bioavailability of Amphotericin B and show reduced nephrotoxicity compared to intravenous Fungizone®. Pharm Res 26:1324–1331. doi: 10.1007/s11095-009-9841-2
Jain CP, Vyas SP (1995) Preparation and characterization of niosomes containing rifampicin for lung targeting. J Microencapsul 12(4):401–407
Jain S, Singh P, Mishra V, Vyas SP (2005) Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against Hepatitis B. Immunol Lett 101:41–49
Jeong YI, Cheon JB, Kim SH, Nah JW, Lee YM, Sung YK, Akaike T, Cho CS (1998) Clonazepam release from core-shell type nanoparticles *in vitro*. J Control Release 51:169–178
Juliano RL, Lopez-Berestein G (1985) New lives for old drugs — liposomal drug delivery systems reduce the toxicity but not the potency of certain chemotherapeutic agents. Pharm Int 45:15–28
Kataoka K, Harada A, Nagasaki Y (2001) Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv Drug Deliv Rev 47:113–131
Kataoka K, Kwon GS, Yokohama M, Okano T, Sakurai Y (1993) Block copolymer micelles as vehicles for drug delivery. J Control Release 24:119–132
Katti DS, Lakshmi S, Langer R, Laurencin CT (2002) Toxicity, biodegradation and elimination of polyanhydrides. Adv Drug Deliv Rev 54:933–961
Khan MA, Ahmad N, Moin S, Mannan A, Wajahul H, Pasha ST, Khan A, Owais M (2005a) Tuftsin-mediated immunoprophylaxis against an isolate of *Aspergillus fumigatus* shows less *in vivo* susceptibility to amphotericin B. FEMS Immunol Med Microbiol 44(3):269–276
Khan MA, Faisal SM, Mohammad O (2006) Safety, efficacy and pharmacokinetics of tuftsin-loaded nystatin liposomes in murine model. J Drug Target 14(4):233–241
Khan MA, Jabeen R, Mohammad O (2004a) Prophylactic role of liposomized chloroquine against murine cryptococcosis less susceptible to fluconazole. Pharm Res 21(12):2207–2212
Khan MA, Jabeen R, Nasti TH, Mohammad O (2005b) Enhanced anticryptococcal activity of chloroquine in phosphatidylserine-containing liposomes in a murine model. J Antimicrob Chemother 55(2):223–228
Khan MA, Nasti TH, Saima K, Mallick AI, Firoz A, Wajahul H, Ahmad N, Mohammad O (2004b) Co-administration of immunomodulator tuftsin and liposomised nystatin can combat less susceptible *Candida albicans* infection in temporarily neutropenic mice. FEMS Immunol Med Microbiol 41(3):249–258
Khan MA, Owais M (2005) Immunomodulator tuftsin increases the susceptibility of *Cryptococcus neoformans* to liposomal amphotericin B in immunocompetent BALB/c mice. J Drug Target 13(7):423–429
Kikuchi H, Yamauchi H, Hirota S (1994) A polyol dilution method for mass production of liposome. J Liposome Res 4:71–91
Kim Y, Oksanen DA, Massefski W, Blake JF, Duffy EM, Chrunyk B (1998) Inclusion complexation of ziprasidone mesylate with β-cyclodextrin sulfobutyl ether. J Pharm Sci 87:1560–1567
Kirkpatrick WR, Coco BJ, Patterson TF (2006) Sequential or combination antifungal therapy with voriconazole and liposomal amphotericin B in a guinea pig model of invasive aspergillosis. Antimicrob Agents Chemother 50:1567–1569
Kirpotin D, Hong KL, Mullah N, Papahadopoulos D, Zalipsky S (1996) Liposomes with detachable polymer coating. Destabilization and fusion of dioleoylphosphatidyl ethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol). FEBS Lett 388:115–118
Kohno S, Otsubo T, Hara K, Tomii Y, Seki J (1995) A new antifungal drug delivery system, lipid nano-sphere encapsulated amphotericin B (LNSAmB), its evaluation in the rat model of invasive pulmonary aspergillosis. In: Program and Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, DC, p 131. Abstr. F109
Kohno S, Otsubo T, Tanakab E, Maruyama K, Hara K (1997) Amphotericin B encapsulated in polyethylene glycol-immunoliposomes for infectious diseases. Adv Drug Deliv Rev 24:325–329

Koning GA, Kamps JA, Scherphof GL (2002) Efficient intracellular delivery of 5-fluoro-deoxyuridine into colon cancer cells by targeted immunoliposomes. Cancer Detect Prev 26:299–307

Kono K, Zenitani KI, Takagishi T (1994) Novel pH-sensitive liposomes: liposomes bearing a poly (ethylene glycol) derivative with carboxyl groups. Biochim Biophys Acta 1193:1–9

Kontoyiannis DP, Hachem R, Lewis RE, Rivero GA, Torres HA, Thornby J, Champlin R, Kantarjian H, Bodey GP, Raad II (2003) Efficacy and toxicity of caspofungin in combination with liposomal amphotericin B as primary or salvage treatment of invasive aspergillosis in patients with hematologic malignancies. Cancer 98:292–299

Kreuter J (1996) Nanoparticles and microparticles for drug and vaccine delivery. J Anat 189:503–505

Kwon GS (2002) Block copolymer micelles as drug delivery systems. Adv Drug Deliv Rev 54:167–252

Kwon GS, Okano T (1996) Polymeric micelles as new drug carriers. Adv Drug Deliv Rev 21:107–116

La SB, Okano T, Kataoka K (1996) Preparation and characterization of the micelle-forming polymeric drug indomethacin-incorporated poly(ethylene oxide)-poly(beta-benzyl L-aspartate) block copolymer micelles. J Pharm Sci 85:85–90

Lasic DD (1993) Liposomes: from physics to applications. Elsevier, Amsterdam

Lasic DD, Martin FJ (eds) (1995) Stealth liposomes. CRC, Boca Raton, FL

Lavasanifar A, Samuel J, Sattari S, Glen SK (2002) Block copolymer micelles for the encapsulation and delivery of Amphotericin B. Pharm Res 19(4):418–422

Laverman P, Carstens MG, Boerman OC, Dams ET, Oyen WJ, van Rooijen N, Corstens FH, Storm G (2001) Factors affecting the accelerated blood clearance of polyethylene glycol liposomes upon repeated injection. J Pharmacol Exp Ther 298:607–612

Leenders AC, Daenen S, Jansen RL, Hop WC, Lowenberg B, Wijermans PW, Cornelissen J, Herbrecht R, van der Lelie H, Hoogsteden HC, Verbrugh HA, de Marie S (1998) Liposomal amphotericin B compared with amphotericin B deoxycholate in the treatment of documented and suspected neutropenia-associated invasive fungal infections. Br J Haematol 103:205–212

Leenders ACAP, Reiss P, Portegies P, Clezy K, Hop WCJ, Borleffs HJ, JCC AT, Kauffmann RH, Jones P, Kroon FP, Verbrugh HA, de Marie S (1997) Liposomal amphotericin B (AmBisome) compared with amphotericin B both followed by oral fluconazole in the treatment of AIDS-associated cryptococcal meningitis. AIDS 11:1463–1471

Lemos-Senna E (1998) Contribution a l'étude pharmacotechnique et physico-chimique de nanosphères de cyclodextrins amphiphiles comme transporteurs de principes actifs. PhD thesis, Université Paris-Sud

Lemos-Senna E, Wouessidjewe D, Lesieur S, Duchene D (1998a) Preparation of amphiphilic cyclodextrin nanospheres using the emulsion solvent evaporation method, influence of the surfactant on preparation and hydrophobic drug loading. Int J Pharm 170:119–128

Lemos-Senna E, Wouessidjewe D, Lesieur S, Puisieux F, Couarrazze G, Duchene D (1998b) Evaluation of the hydrophobic drug loading characteristics in nanoprecipitated amphiphilic cyclodextrins nanospheres. Pharm Dev Technol 3:1–10

Li C (2002) Poly(L-glutamic acid)-anticancer drug conjugates. Adv Drug Deliv Rev 54:695–713

Liu D, Mori A, Huang L (1992) Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. Biochim Biophys Acta 1104:95–101

Liu D, Zhou F, Huang L (1989) Characterization of plasma-stabilized liposomes composed of dioleoylphosphatidylethanolamine and oleic acid. Biochem Biophys Res Commun 162:326–333

Liu TQ, Guo R (2005) Preparation of a highly stable niosome and its hydrotrope-solubilization action to drugs. Langmuir 21:11034–11039

Loftsson T, Duchene D (2007) Cyclodextrins and their pharmaceutical applications. Int J Pharm 329(1–2):1–11

Mady MM, Ghannam MM, Khalil WA, Repp R, Markus M, Rascher W, Muller R, Fahr A (2004) Efficient gene delivery with serum into human cancer cells using targeted anionic liposomes. J Drug Target 12:11–18

Maesaki S, Hossain MA, Sasaki E, Hashiguchi K, Higashiyama Y, Yoshitsugu Y, Tomono K, Tashiro T, Kohno S (1999a) The future of antifungal agents. Non azole antifungal agents. Nippon Ishinkin Gakkai Zasshi 40(3):157–161

Maesaki S, Miyazaki Y, Higashiyama Y, Tomono K, Kohno S (1999b) The efficacy and attenuation of nephrotoxicity of NS-718, a novel lipid nanosphere encapsulated amphotericin B against *Cryptococcus neoformans* infection. In: Abstracts of the 39th International Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 26–29 September 1999; Washington, DC, American Society for Microbiology, Abstract 2013

Manconi M, Sinico C, Valenti D, Loy G, Fadda AM (2002) Niosomes as carriers for tretinoin I. Preparation and properties. Int J Pharm 234:237–248

Mannino RJ, Gould-Fogerite S (1997) Antigen cochleate formulations for oral and systemic vaccination. In: Levine MM (ed) New generation vaccines, 2nd edn. Marcel Dekker, New York, pp 229–239

Marine M, Espada R, Torradob J, Pastora FJ, Guarroa J (2009) Efficacy of a new formulation of amphotericin B in murine disseminated infections by *Candida glabrata* or *Candida tropicalis*. Int J Antimicrob Agents 34:566–569

Maroof A, Farazuddin M, Owais M (2009) Potential of liposomal diallyl sulphide in treatment of experimental murine candidiasis. Biosci Rep Jul 31 [Epub ahead of print]

Marr KA, Boeckh M, Carter RA et al (2004) Combination antifungal therapy for invasive aspergillosis. Clin Infect Dis 39:797–802

Maruyama K, Okuizumi S, Ishida O, Yamauchi H, Kikuchi H, Iwatsuru M (1994) Phosphatidyl polyglycerols prolong liposome circulation *in vivo*. Int J Pharm 111:103–107

Maruyama K, Takizawa T, Yuda T, Kennel SJ, Huang L, Iwatsuru M (1995) Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycols) conjugated at their distal terminal to monoclonal antibodies. Biochim Biophys Acta 1234:74–80

Maruyama K, Yuda T, Okamoto A, Kojima S, Suginaka A and Iwatsuru M (1992) Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). Biochim Biophys Acta 1128:44–49

Matteucci ML, Thrall DE (2000) The role of liposomes in drug delivery and diagnostic imaging: a review. Vet Radiol Ultrasound 41:100–107

Maurer N, Fenske DB, Cullis PR (2001) Developments in liposomal drug delivery systems. Expert Opin Biol Ther 1(6):923–947

Medina OP, Zhu Y, Kairemo K (2004) Targeted liposomal drug delivery in cancer. Curr Pharm Des 10:2981–2989

Memisoglu-Bilensoy E, Bochot A, Trichard L et al (2005) Amphiphilic cyclodextrins and microencapsulation. In: Benita S (ed) Microencapsulation second and revised edition. Taylor & Francis, New York, pp 269–295

Meunier F, Prentice HG, Ringden O (1991) Liposomal amphotericin B (Ambisome): safety data from a phase II: III clinical trial. J Antimicrob Chemother 28B:83–91

Mills W, Chopra R, Linch DC, Goldstone AH (1994) Liposomal amphotericin B in the treatment of fungal infections in neutropenic patients: a single centre experience of 133 episodes in 116 patients. Br J Haematol 86:754–760

Miron L, Mares M, Nastasa V, Spulber M, Fifere A, Pinteala M, Harabagiu V, Simionescu BC (2009) Water soluble sulconazole-*b*-cyclodextrin complex: physico-chemical characterization and preliminary pharmacological studies. J Incl Phenom Macrocycl Chem 63:159–162

Mizushima Y, Hamano T, Yokoyama K (1982) Use of a lipid emulsion as a novel carrier for corticosteroids. J Pharm Pharmacol 34:49–53

Moghimi SM, Szebeni J (2003) Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog Lipid Res 42:463–478

Mora M, Sagrista ML, Trombetta D, Bonina FP, de Pasquale A, Saija A (2002) Design and characterization of liposomes containing long-chain N-acylPEs for brain delivery: penetration of liposomes incorporating GM1 into the rat brain. Pharm Res 19:1430–1438

Moribe K, Maruyama K (2002) Pharmaceutical design of the liposomal antimicrobial agents for infectious disease. Curr Pharm Des 8:441–454

Moses MA, Brem H, Langer R (2003) Advancing the field of drug delivery: taking aim at cancer. Cancer Cell 4:337–341

Mozafari MR, Reed CJ, Rostron C (2007) Cytotoxicity evaluation of anionic nanoliposomes and nanolipoplexes prepared by the heating method without employing volatile solvents and detergents. Die Pharmazie 62:205–209

Muzzalupo R, Ranieri GA, La Mesa C (1996) Translational diffusion and other physicochemical properties of a bolaform surfactant in solution. Langmuir 12:3157–3161

Muzzalupo R, Trombino S, Iemma F, Puoci F, La Mesa C, Picci N (2005) Preparation and characterization of bolaform surfactant vesicles. Colloid Surf B Biointerfaces 46:78–83

Nahar M, Mishra D, Dubey V, Jain NK (2008) Development, characterization, and toxicity evaluation of amphotericin B–loaded gelatin nanoparticles. Nanomedicine: NBM 4:252–261. doi:10.1016/j.nano.2008.03.007

Naik SR, Desai SK, Nanda RK, Narayanan MS (2007) Fermentation, isolation, purification, and biological activity of SJA-95, a heptaene polyene macrolide antibiotic produced by *Streptomyces* sp. strain S-24. Arzneim Forsch (Drug Res) 57(3):171–179

Nakanishi T, Fukushima S, Okamoto K, Suzuki M, Matsumura Y, Yokoyama M, Okano T, Sakurai Y, Kataoka K (2001) Development of the polymer micelle carrier system for doxorubicin. J Control Release 74:295–302

Namdeo A, Jain NK (1996) Niosomes as drug carriers. Indian J Pharm Sci 58(2):41–46

Nasti TH, Khan MA, Owais M (2006) Enhanced efficacy of pH-sensitive nystatin liposomes against *Cryptococcus neoformans* in murine model. J Antimicrob Chemother 57:349–352

Ng TTC, Denning DW (1995) Liposomal amphotericin B (AmBi-some) therapy in invasive fungal infections. Evaluation of United Kingdom compassionate use data. Arch Intern Med 155:1093–1098

Ning MY, Guo YZ, Pan HZ et al (2005) Preparation, *in vitro* and *in vivo* evaluation of liposomal/niosomal gel delivery systems for clotrimazole. Drug Dev Ind Pharm 31(4–5): 375–383

Oku N, Namba Y (1994) Long-circulating liposomes. Crit Rev Ther Drug Carrier Syst 11(4): 231–270

Oku N, Namba Y (2005) Glucuronate-modified, long-circulating liposomes for the delivery of anticancer agents. Methods Enzymol 391:145–162

Olson JA, Adler-Moore JP, Smith PJ, Proffitt RT (2005) Treatment of *Candida glabrata* infection in immunosuppressed mice by using a combination of liposomal amphotericin B with caspofungin or micafungin. Antimicrob Agents Chemother 49:4895–4902

Otsubo T, Maesaki S, Hossain MA, Yamamoto Y, Tomono K, Tshiro T, Seki J, Tomi Y, Sonoke S, Kohno S (1999) In vitro and *in vitro* activities of NS-718, a new lipid nanosphere incorporating amphotericin B, against *Aspergillus fumigatus*. Antimicrob Agents Chemother 43:471–475

Otsubo T, Maruyama K, Maesaki S, Miyazaki Y, Tanaka E, Takizawa T, Moribe K, Tomono K, Tashiro T, Kohno S (1998) Long-circulating immunoliposomal amphotericin B against invasive pulmonary aspergillosis in mice. Antimicrob Agents Chemother 42:40–44

Overhoff KA, Engstrom JD, Chen B, Scherzer BD, Milner TE, Johnston KP, Williams RO (2007) Novel ultra-rapid freezing particle engineering process for enhancement of dissolution rates of poorly water-soluble drugs. Eur J Pharm Biopharm 65(1):57–67

Packhaeuser CB, Schnieders J, Oster CG, Kissel T (2004) In situ forming parenteral drug delivery systems: an overview. Eur J Pharma Biopharm 58:445–455

Pandey R, Ahmad Z, Sharma S, Khuller GK (2005) Nano-encapsulation of azole antifungals: potential applications to improve oral drug delivery. Int J Pharm 14:268–276

Panyam J, Labhasetwar V (2003) Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev 55(3):329–347

Papahadjopoulos D, Vail WJ, Jacobson K, Poste G (1975) Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. Biochim Biophys Acta 394:483–491

Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C et al. (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 88:11460–11464

Pedersen M (1994) Isolation and antimycotic effect of a genuine miconazole beta-cyclodextrin complex. Eur J Pharm Biopharm 40(1):19–23

Peeters J, Neeskens P, Tollenaere JP, van Remoortere P, Brewster ME (2002) Characterization of the interaction of 2-hydroxypropyl-β-cyclodextrin with itraconazole at pH 2, 4 and 7. J Pharm Sci 91:1414–1422

Peng H-S, Liu X-J, LV Gui-xiang, Sun B, Kong Q-F, Zhai D-X, Wang Q, Zhao W, Wang G-Y, Wang D-D, Li H-L, Jin L-H, Kostulas N (2008) Voriconazole into PLGA nanoparticles: improving agglomeration and antifungal efficacy. Int J Pharm 352:29–35

Perfect JR, Dodds Ashley E, Drew R (2004) Design of aerosolized Amphotericin B formulations for prophylaxis trials among lung transplant recipients. Clin Infect Dis 39(Suppl 4): S207–S210

Perugini P, Genta I, Pavanetto F, Conti B, Scalia S, Baruffini A (2000) Study on glycolic acid delivery by liposomes and microspheres. Int J Pharm 196:51–61

Pierard GE, Arrese JE, Quatresooz P et al (2007) Emerging therapeutic agents for onychomycosis. Expert Opin Emerg Drugs 12(3):345–353

Pinto RC, Neufeld RJ, Ribeiro AJ, Veiga F (2006) Nanoencapsulation I. Methods for preparation of drug loaded polymeric nanoparticles. Nanomedicine 2(1):8–21

Poirier JM, Cheymol G (1998) Optimisation of itraconazole therapy using target drug concentrations. Clin Pharmacokinet 35(6):461–473

Rajewski RA, Traiger G, Bresnahan A, Jaberaboansari P, Stella VJ, Thompson DO (1995) Preliminary safety evaluation of parenterally administered sulfoalkyl ether β-cyclodextrin derivatives. J Pharm Sci 84:927–932

Ringden O, Meunier F, Tollemar J, Ricci P, Tura S, Kuse E, Viviani MA, Gorin NC, Klastersky J, Fenaux P, Prentice HG, Ksionski G (1991) Efficacy of Amphotericin F encapsulated in liposomes (Ambisome) in the treatment of invasive fungal infections in immunocompromised patients. J Antimicrob Chemother 28B:73–82

Roco MC, Bainbridge WS (2002) Converging technologies for improving human performance. National Science Foundation and Department of Commerce Report. Kluwer, Boston, MA

Roux E, Lafleur M, Lataste E, Moreau P, Leroux J-C (2003) On the characterization of pH-sensitive liposome/polymer complexes. Biomacromolecules 14:240–248

Roux E, Passirani C, Scheffold S, Benoit J-P, Leroux J-C (2004) Serum-stable and long-circulating, PEGylated, pH-sensitive liposomes. J Control Release 94:447–451

Roux E, Stomp R, Giasson S, Pezolet M, Moreau P, Leroux J-C (2002) Steric stabilization of liposomes by pH-responsive Nisopropylacrylamide copolymer. J Pharm Sci 91:1795–1802

Ruijgrok RJ, Fens MH, Bakker-Woudenberg IA et al (2005) Nebulization of four commercially available amphotericin B formulations in persistently granulocytopenic rats with invasive pulmonary aspergillosis: evidence of long-term biological activity. J Pharm Pharmacol 57:1289–1295

Sachs-Barrable K, Lee SD, Wasan EK et al (2008) Enhancing drug absorption using lipids: a case study presenting the development and pharmacological evaluation of a novel lipid-based oral amphotericin B formulation for the treatment of systemic fungal infections. Adv Drug Deliv Rev 60(6):692–701

Sanli O, Karaca I, Isiklan N (2009) Preparation, characterization, and salicylic acid release behavior of chitosan/poly(vinyl alcohol) blend microspheres. J Appl Polym Sci 111: 2731–2740

Santangelo R, Paderu P, Delmas G, Chen ZW, Mannino RJ, Zarif L, Perlin D (2000) Oral efficacy of cochleate-amphotericin B (CAMB) in a mouse model with systemic candidiasis. Antimicrob Agents Chemother 44(9):2356–2360

Sapra P, Allen TM (2003) Ligand-targeted liposomal anticancer drugs. Prog Lipid Res 42:439–462

Satturwar PM, Khandare JN, Nande VS (2001) Niosomal delivery of ketoconazole. Indian drugs 12:620–624

Sedlák M, Pravda M, Kubicová L, Mikulcíková P, Ventura K (2007a) Synthesis and characterisation of a new pH-sensitive amphotericin B-poly(ethylene glycol)-b-poly(L-lysine) conjugate. Bioorg Med Chem Lett 17(9):2554–2557

Sedlák M, Pravda M, Staud F, Kubicová L, Týcová K, Ventura K (2007b) Synthesis of pH-sensitive amphotericin B-poly(ethylene glycol) conjugates and study of their controlled release *in vitro*. Bioorg Med Chem 15(12):4069–4076

Senior J, Delgado C, Fisher D, Tilcock C, Gregoriadis G (1991) Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. Biochim Biophys Acta 1062:77–82

Shahiwala A, Misra A (2002) Studies in topical application of niosomally entrapped Nimesulide. J Pharm Pharmaceut Sci 5:220–225

Sharma A, Sharma U (1997) Liposomes in drug delivery: progress and limitations. Int J Pharm 154:123–140

Sinha VR, Tehan A (2003) Biodegradable microspheres for protein delivery. J Control Release 90:261–280

Slain D, Rogers PD, Cleary JD, Chapman SW (2001) Intravenous itraconazole. Ann Pharmacother 35:720–729

Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE (2001) Biodegradable polymeric nanoparticles as drug delivery devices. J Control Release 70(1–2):1–20

Stella VJ, Rajewski RA (1997) Cyclodextrins: their future in drug formulation and delivery. Pharm Res 14:556–567

Stevens DA (1999) Itraconazole in cyclodextrin solution. Pharmacotherapy 19:603–611

Szoka FC Jr, Papahadjopoulos D (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Natl Acad Sci USA 75:4194–4198

Taira MC, Chiaramoni NS, Pecuch KM, Alonso-Romanowski S (2004) Stability of liposomal formulations in physiological conditions for oral drug delivery. Drug Deliv 11:123–128

Takeuchi H, Matsui Y, Yamamoto H, Kawashima Y (2003) Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. J Control Release 86:235–242

Taneri F, Guneri T, Aigner Z et al (2002) Improvement in the physicochemical properties of ketoconazole through complexation with cyclodextrin derivatives. J Incl Phenom Macro 44 (1–4):257–260

Taneri F, Guneri T, Aigner Z et al (2003a) Improvement of the physicochemical properties of clotrimazole by cyclodextrin complexation. J Incl Phenom Macro 46(1–2):1–13

Taneri F, Guneri T, Aigner Z et al (2003b) Influence of cyclodextrin complexation on the physicochemical and biopharmaceutical properties of ketoconazole. J Incl Phenom Macro 47 (1–2):15–23

Taylor TM, Davidson PM, Bruce DB, Weiss J (2005) Liposomal nanocapsules in food science and agriculture. Crit Rev Food Sci Nutr 45:1–19

Tenjarla S, Puranajoti P, Kasina R et al (1998) Preparation, characterization, and evaluation of miconazole-cyclodextrin complexes for improved oral and topical delivery. J Pharm Sci 87(4):425–429

Thompson DO (1997) Cyclodextrins-enabling excipients: their present and future use in pharmaceuticals. Crit Rev Ther Drug Carrier Syst 14:1–104

Thornton SJ, Wasan KM (2009) Amphotericin B for oral administration to treat systemic fungal infections and visceral leishmaniasis. Expert Opin Drug Deliv 6(3):271–284
Tiyaboonchai W (2003) Chitosan nanoparticles: a promising system for drug delivery. Naresuan Univ J 11:51
Torchilin VP (2001) Structure and design of polymeric surfactant- based drug delivery systems. J Control Release 73:137–172
Torchilin VP, Shtilman MI, Trubeyskoy VS, Whiteman K, Milstein AM (1994) Amphiphilic vinyl polymers effectively prolong liposome circulation time *in vivo*. Biochim Biophys Acta 1195:181–184
Torchilin VP, Zhou F, Huang L (1993) pH-sensitive liposomes. J Liposome Res 3:201–255
Uchegbu IF, Vyas SP (1998) Non-ionic surfactant based vesicles (niosomes) in drug delivery. Int J Pharm 172:33–70
Uekama K (2004) Design and evaluation of cyclodextrin-based drug formulation. Chem Pharm Bull 52(8):900–915
Uster PS, Allen TM, Daniel DB, Mendez CJ, Newman MS, Zhu GZ (1996) Insertion of poly (ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged *in vivo* circulation time. FEBS Lett 386:243–246
van Etten EW, Snijders SV, van Vianen W, Bakker-Woudenberg IA (1998a) Superior efficacy of liposomal amphotericin B with prolonged circulation in blood in the treatment of severe candidiasis in leukopenic mice. Antimicrob Agents Chemother 42:2431–2433
van Etten EWM, van Vianen W, Janneke H, Bakker-Woudenberg IAJM (1998b) Activity of liposomal Amphotericin B with prolonged circulation in blood versus those of AmBisome and Fungizone against intracellular *Candida albicans* in murine peritoneal macrophages. Antimicrob Agents Chemother 42:2437–2439
van Etten EWM, van Vianen W, Tijhuis RHG, Storm G, Bakker-Woudenberg IAJM (1995b) Sterically stabilized amphotericin B-liposomes: toxicity and biodistribution in mice. J Control Release 37:123–129
van Rooijen N, van Nieuwmegen R (1980) Liposomes in immunology: multilamellar phosphatidylcholine liposome as a simple biodegradable and harmless adjuvant without any immunogenic activity of its own. Immunol Commun 9:243–256
Varshosaz J, Najafabadi RA (2003) Development and physical characterization of sorbitan monoester niosome for insulin oral delivery. Drug Deliv 10:251–262
Vaughn JM, Gao X, Yacaman MJ, Johnston KP, Williams RO III (2005) Comparison of powder produced by evaporative precipitation into aqueous solution (EPAS) and spray freezing into liquid (SFL) technologies using novel Z-contrast STEM and complimentary techniques. Eur J Pharm Biopharm 60(1):81–89
Viani P, Cervato G, Gatti P, Cestaro B (1993) Plasma dependent pH sensitivity of liposomes containing sulfatide. Biochim Biophys Acta 1147:73–80
Vladimir A, Slepushkin SS, Paul D, Mary SN, Luke SG, Maria CPL, Nejat D (1997) Sterically stabilized pH-sensitive liposomes: intracellular delivery of aqueous contents and prolonged circulation *in vivo*. J Biol Chem 272:2382–2388
Vora B, Khopade AJ, Jain NK (1998) Proniosome based transdermal delivery of levonorgestrel for effective contraception. J Control Release 54:149–165
Vyas SP, Quraishi S, Gupta S et al (2005) Aerosolized liposome-based delivery of amphotericin B to alveolar macrophages. Int J Pharm 296:12–25
Walsh TJ, Finberg RW, Arndt C, Hiemenz J, Schwartz C, Bodensteiner D et al (1999) Liposomal amphotericin B for empirical therapy in patients with persistent fever and neutropenia. New Eng J Med 340:764–771
Wang CH, Wang WT, Hsiue GH (2009) Development of polyion complex micelles for encapsulating and delivering amphotericin. Biomaterials 19:3352–3358
Wang XC, He J, Yang CM, Yuan Y (2003) New progress of the research on passive targeting western and Chinese medicine agents. China Hosp Pharm J 23:238–240

Wasan EK, Bartlett K, Gershkovich P, et al (2009) Development and characterization of oral lipid-based Amphotericin B formulations with enhanced drug solubility, stability and antifungal activity in rats infected with *Aspergillus fumigatus* or *Candida* albicans. Int J Pharm (2009) 372:76–84 doi:10.1016/j.ijpharm.2009.01.003

Waterhouse DN, Madden TD, Cullis PR et al (2005) Preparation, characterization and biological analysis of liposomal formulation of vincristine. Meth Enzym 391:40–57

Weissmann G, Bloomgerden D, Kaplan R, Cohen C, Hoffstein S, Collins T, Gotlieb A, Nagle D (1975) A general method for introduction of enzymes, by means of immunoglobulin coated liposomes into lysosomes of deficient cells. Proc Natl Acad Sci U S A 72:88–94

Willems L, Van der Geest R, De Beule K (2001) Itraconazole oral solution and intravenous formulations: A review of pharmacokinetics and pharmacodynamics. J Clin Pharm Ther 26:159–169

Woodle MC (1998) Controlling liposome blood clearance by surface-grafted polymers. Adv Drug Deliv Rev 32:139–152

Wouessidjewe D, Skiba M, Leroy-Lechat F, Lemos-Senna E, Puisieux F, Duchêne D (1996) A new concept in drug delivery based on 'skirt-shaped cyclodextrins aggregates' present state and future prospects. STP Pharma Sci 6:21–26

Xiao L, Li B (2002) Drug-loaded nanoparticle and TCM modernization. Chinese Traditional Herbal Drugs 33:385–388

Yamauchi H, Yano T, Kato T, Tanaka I, Nakabayashi S, Higashi K, Miyoshi S, Yamada H (1994) Effects of sialic acid derivative on long circulating time and tumor concentration of liposomes. Int J Pharmacol 113:141–148

Yang W, Tam J, Miller DA, Zhou J, McConville JT, Johnston KP et al (2008a) High bioavailability from nebulized itraconazole nanoparticle dispersions with biocompatible stabilizers. Int J Pharm 361(1–2):177–188

Yang W, Wiederhold NP, Williams RO (2008b) Drug delivery strategies for improved azole antifungal action. Expert Opin Drug Deliv 5(11):1199–1216

Yi Y, Yoon HJ, Bong OK, Myungseob S, Sun-Ok K, Sung-Joo H, Min HS (2007) A mixed polymeric micellar formulation of itraconazole: Characteristics, toxicity and pharmacokinetics. J Control Release 117:59–67

Yokoyama M, Okano T, Sakurai Y, Ekimoto H, Shibazaki C, Kataoka K (1991) Toxicity and antitumor activity against solid tumors of micelleforming polymeric anticancer drug and its extremely long circulation in blood. Cancer Res 51:3229–3236

Yong CS, Li DX, Prabagar B et al (2007) The effect of beta-cyclodextrin complexation on the bioavailability and hepatotoxicity of clotrimazole. Pharmazie 62(10):756–759

Yu BG, Okano T, Kataoka K, Kwon G (1998a) Polymeric micelles for drug delivery: solubilization and haemolytic activity of amphotericin B. J Control Release 30:131–136

Yu BG, Okano T, Kataoka K, Sardari S, Kwon GS (1998b) In vitro dissociation of antifungal efficacy and toxicity for amphotericin B-loaded poly(ethylene oxide)-block-poly(β-benzyl-aspartate) micelles. J Control Release 56:285–291

Yuan DF, Yi YM (2003) Advance of the research on liver-targeted nanoparticles. Her Med 22:113–114

Zarif L, Graybill JR, Perlin D, Najvar L, Bocanegra R, Mannino RJ (2000) Antifungal activity of amphotericin B cochleates against *Candida albicans* infection in a mouse model. Antimicrob Agents Chemother 44:1463–1469

Zarif L, Jin T, Scolpino A, Mannino RJ (1999a) Are cochleates the new lipid-based carrier for oral drug delivery? In: Abstracts of the 39th *International Conference on Antimicrobial Agents and Chemotherapy,* San Francisco, CA, 26–29 September 1999; Washington, DC, American Society for Microbiology, Abstract 1939 p 344

Zarif L, Mannino RJ (2000) Cochleates, lipid-based vehicles for gene delivery – Concept, achievements and future development. In: Habib N (ed) Cancer gene therapy: past achievements and future challenges. Plenum, London, pp 83–94

Zarif L, Perlin D (2002) Amphotericin B nonocholeates: from formulation to oral efficacy. Drug Deliv Technol 2(4):89

Zarif L, Segarra L, Jin T, Scolpino A, Hyra D, Perlin DS, Graybill JR, Mannino RJ (1999b) Oral and systemic delivery of AmB mediated by cochleates. AAPS PharmSci 1:S–453

Zhang JA, Xuan T, Parmar M et al (2004) Development and charaterization of a novel liposome-based formulation of SN-38. Int J Pharm 270(1–2):93–107

Zhang LF, Granick S (2006) How to stabilize phospholipid liposomes (using nanoparticles). Nano Lett 6:694–698

Zhang Y (2006) Advances in research on target-oriented drug delivery system of Chinese material medical. Chinese Traditional Herbal Drugs 37:641–647

Zho F, Neutra MR (2002) Antigen delivery to mucosa-associated lymphoid tissues using liposomes as a carrier. Biosci Rep 22:355–369

Zia V, Rajewski RA, Stella VJ (2001) Effect of cyclodextrin charge on complexation of neutral and charged substrates: comparison of (SBE)7M-β-CD to HP-β-CD. Pharm Res 18:667–673

Zignani M, Drummond DC, Meyer O, Hong K, Leroux J-C (2000) In vitro characterization of a novel polymeric-based pH-sensitive liposome system. Biochim Biophys Acta 1463:383–394

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