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Antifungal Resistance Mechanisms in Dermatophytes

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Abstract Although fungi do not cause outbreaks or pandemics, the incidence of severe systemic fungal infections has increased significantly, mainly because of the explosive growth in the number of patients with compromised immune system. Thus, drug resistance in pathogenic fungi, including dermatophytes, is gaining importance. The molecular aspects involved in the resistance of dermatophytes to marketed antifungals and other cytotoxic drugs, such as modifications of target enzymes, over-expression of genes encoding ATP-binding cassette (ABC) transporters and stress-response-related proteins are reviewed. Emphasis is placed on the mechanisms used by dermatophytes to overcome the inhibitory action of terbinafine and survival in the host environment. The relevance of identifying new molecular targets, of expanding the understanding about the molecular mechanisms of resistance and of using this information to design new drugs or to modify those that have become ineffective is also discussed.

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Introduction

Dermatophytes are pathogenic fungi specialized in the infection of skin, hair and nails that utilize keratinous substrates as the carbon, nitrogen and sulphur sources. They belong to three anamorphic (asexual or imperfect) genera, *Epidermophyton, Microsporum* and *Trichophyton*, and have long been classified as anthropophilic, zoophilic and geophilic species on the basis of their primary habitat associations. Thus, anthropophilic dermatophytes are associated with humans and rarely infect other animals, zoophilic dermatophytes usually infect animals or are associated with animals, but occasionally infect humans, and geophilic dermatophytes are primarily associated with keratinous materials such as hair, feathers, hooves and horns, but as a part of their decomposition process. When dermatophytes infect humans, they colonize the keratinized outermost layer of the skin, and usually do not invade the living tissue. These infections include tinea capitis (infection of scalp), tinea pedis (infection of feet), tinea cruris (infection of the groin area), and tinea unguium or onychomycosis (infection of nails). However, the disease generally known as tinea or

ringworm may be a consequence of the inflammatory reaction of the host to the enzymes secreted by the fungus during its invasive process [1].

In the beginning of the last century, the major causes of human death were infectious diseases, but their incidence started to decrease with the improvement of basic sanitation conditions and with the discovery and widespread use of vaccines and antimicrobial agents, among others. Although fungi do not cause outbreaks or pandemics, the incidence of severe systemic fungal infections has increased significantly, mainly because of the explosive growth in the number of patients with compromised immune system. Opportunistic fungal infections are common among patients who have acquired immunodeficiency syndrome (AIDS) or who have had medical procedures that suppress the immune system, such as organ transplantation and chemotherapy. The indiscriminate use of antibiotics also contributes to the worsening of this picture, leading to the installation of fungal infections. Hence, fungal infections may become an important cause of human death or at least a significant cause of reduced quality of human living standards. On this basis, it is necessary to have antifungals available for the efficient control of fungal infections.

The focus of this review is mainly on the molecular aspects involved in the resistance of dermatophytes to marketed antifungals and survival in the host environment. For doing so, it is also crucial to understand the biology, growth physiology, mechanisms of adaptation to different types of stress and the physiology of the interaction of these microorganisms with the host environment. If specific examples do not exist for dermatophytes, model fungi, which have been extensively studied in terms of biochemistry, physiology, genetics and antifungal resistance in well-documented investigations, will be mentioned.

Infection and Survival in the Host Environment

The successful initiation of infection is a process closely related to the capability of the infecting dermatophyte to overcome the host resistance mechanisms. Cutaneous barriers against dermatophytes adherence, germination of arthroconidia and hyphae penetration into the *stratum corneum* include the intact keratinized layers of the skin and mucosal surfaces, the presence of fungistatic fatty acids on the skin—undecanoic acid, for instance—and other effectors such as skin pH. The adherence capability has been attributed to the presence of glycoproteins containing mannans in the cell wall of these fungi, which is presumed to correlate with virulence [2]. In addition, for dermatophytes to be successful in their installation in the host, the arthroconidia must germinate very rapidly and the hyphae must penetrate the body surface; otherwise they will be eliminated by the continuous desquamation of the epithelium. Once installed, the dermatophytes must scavenge nutrients for growth, a process based on the induction of structural proteins, permeases and enzymes of the cell wall, in addition to the secretion of a variety of proteins and hydrolytic enzymes such as nucleases, lipases, nonspecific proteases and keratinases, among others, which occur in response to a short supply of essential nutrients in the host [3, 4]. The de-repression of this metabolic machinery also responds to environmental pH signalling, which allows the dermatophyte to use macromolecules within a broad ambient pH range. Dermatophytes de-repress unspecific proteolytic enzymes and keratinases with optimum activity at acidic pH during the initial stages of infection probably because human skin has an acidic pH [5, 6]. It is noteworthy that proteinases with an optimal activity at acidic pH are also important virulence factors of dermatophytes [7]. These proteases act on keratinous and nonkeratinous substrates in the *stratum corneum*, releasing peptides that are hydrolysed to amino acids by putative peptidases. Indeed, growth of the dermatophyte *Trichophyton rubrum* in vitro is dependent on the initial culture pH, with an apparent optimum at pH 4.0 [8]. Furthermore, the pH of the medium changes during cultivation as a function of the nutrient utilized reaching values that range from pH 8.3–8.9 [8, 9], an environment in which most of the known keratinases have optimal enzymatic activity. It is well documented that extracellular pH is a key environmental signal that governs growth, differentiation, physiology and viability of all living organisms, and also that cells change their homeostatic response as a function of ambient pH sensing. The adaptive responses to ambient pH seem to be universal since they have been identified in microorganisms, invertebrates and mammals [10, 11]. Despite its

widespread characteristic, the molecular knowledge of the adaptive response to ambient pH is limited to the cloning and characterization of some genes in few living species. The *pacC* gene from *T. rubrum* [12], which encodes a putative protein that is homologous to the PacC/Rim101p family of pH signalling transcription regulators [13, 14], was isolated and sequenced. The disruption of *pacC* gene decreased both the secretion of keratinolytic proteases and the ability of the mutant *pacC-1* to grow on human nail fragments as the sole source of nutrition. Henceforth, the keratinolytic proteases secreted by *T. rubrum*, apparently required to its complete installation and to remain in the host, are somehow regulated by PacC protein [12].

Although dermatophytes have this powerful metabolic machinery for survival, they still need to overcome many other pitfalls imposed by the host. Thus, dermatophytes have developed mechanisms that allow them to avoid the host response such as, for example, the immunosuppressive action of fungal mannans that causes reduction of inflammation and phagocytosis. Phagocytic cells can also be avoided by the expression of the iC3b receptor, thereby competing for C3b utilized by polymorphonuclear cells [2, 15]. These responses limit the infection to superficial tissue layers, where changes such as skin scales, vesicles, pustules, annular dermatitis and a severe inflammatory response can be observed. The chemical composition of the cell wall of dermatophytes also plays an important role in pathogenicity because of the existing correlation between alteration in the chemical composition of the cell wall, cell dimorphism and virulence [16].

Antifungal Drugs and Targets

The topic antifungal medications used by the end of the nineteenth century in the treatment of superficial mycoses consisted of some inorganic salts such as potassium permanganate, lead arsenate, mercuric chloride and potassium iodide in various cream or ointment bases. Acriflavin, gentian violet and the acids benzoic, acetylsalicylic, undecanoic, undecylenic, among others, were introduced in medical practice early in the last century as the first organic antifungal medications of topic use. It is noteworthy that the search for new antifungals was influenced by the discovery of penicillin and its clinical use during the 1940s, a time in which the idea of synthetic chemotherapy was being introduced. Systemic antifungal agents to treat mycoses were rare until the advent of modern chemotherapy. Although the number of antifungal drugs was small, fungal infections were easily treated before the 1980s because they were often limited to superficial mycoses, athlete's foot, thrush caused by *Candida albicans*, cryptococcosis, ringworms (keratomycoses) and a few cases of deep-seated mycoses [17].

A reasonable number of antifungal agents exist currently on the pharmaceutical market, together with some derivatives of these drugs that have become less toxic, with enhanced potencies and improved pharmacokinetics. However, their cellular targets are limited because of the similarity existing between fungi and hosts, i.e., both are eukaryotic organisms. With some exceptions (e.g., griseofulvin, flucytosine, caspofungin and ciclopiroxolamine), the antifungal drugs in common usage are directed against the ergosterol biosynthetic pathway (Table 1). Ergosterol, a cholesterol analogue, is the major sterol of the fungal plasma membrane and contributes to a variety of cellular functions such as fluidity and integrity. The proper functioning of many membrane-bound enzymes, including chitin synthase, which is crucial for cell growth and division, is also dependent on ergosterol for the maintenance of the membrane's native conformation [18, 19]. However, the significant incidence of fungal infections on the growing population of immunocompromised patients and the emerging resistance to existing drugs emphasize the importance of molecular studies concerning antifungal resistance, which in turn may be valuable in the search for new targets and in the improvement of the existing antifungals.

Antifungal Susceptibility Testing

Tests designed to ascertain the minimal amount of drug needed to inhibit the growth of fungal strains in culture (minimum inhibitory concentration or MIC) are generally used to determine the relative effectiveness of different antifungal agents and to detect the development of drug-resistant organisms [20]. However, the MIC value for any drug depends on the quality of the specimen, quantity of the inoculum,

Table 1 Common antifungal drugs in the market and their cellular targets

Chemical classes	Drugs	Targets
Allylamines and thiocarbamates	Terbinafine	Ergosterol synthesis
	Naftifine	
	Tolnaftate	
	Liranaftate	
Azoles	Tioconazole	Ergosterol synthesis
	Clotrimazole	
	Econazole	
	Miconazole	
	Ketoconazole	
	Fluconazole	
	Itraconazole	
	Terconazole	
	Voriconazole	
	Posaconazole	
Polyenes	Amphotericin B	Ergosterol (membrane function)
	Nystatin	
Antibiotic	Griseofulvin	Microtubule assembly (mitosis)
Pyrimidine	Flucytosine	DNA and RNA synthesis
Echinocandins	Caspofungin	Glucan synthesis
	Micafungin	
	Anidulafungin	
Oxaboroles/ hydroxamic acid	Ciclopiroxolamine	Iron chelator

composition and pH of the medium, temperature and time of incubation, drug solvent and growth curve [21]. In addition, the conidiation of some dermatophytes is very poor on standard fungal media. The Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-Forming Filamentous Fungi (M38-A) [22], standardized by the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards, or NCCLS), does not explicitly address the antifungal susceptibility of dermatophytes [23]. Nevertheless, successful adaptations of the M38-A protocol for susceptibility testing of dermatophytes, which gave excellent reproducibility of MIC data, were adopted [21, 23–25]. However, it has also been reported that MICs of antifungals obtained with hyphal-fragments inocula from other filamentous fungi were substantially higher than those obtained with conidial inocula [26].

The correlation between the in vitro dermatophyte MICs and the clinical outcomes remains unclear [23]. Systematic susceptibility testing of clinical isolates from patients with onychomychosis, who failed on therapy with terbinafine, did not reveal any correlation between the MIC of terbinafine and the clinical failure [27]. In fact, dermatophytes often produce arthroconidia in vivo. These cellular structures, defined as a disarticulation of septate hyphae to produce a chain of conidia, are considered to be the primary cause of skin and nail infections in humans and animals [28]. It was demonstrated that arthroconidia from *T. rubrum* experimentally induced in vitro (10% of CO_2 at 37°C, pH 7.5), and therefore presumably in vivo, are more resistant to antifungals than hyphae [29, 30], probably because of the thickness of their cell wall. Interestingly, hyperkeratosis, which often occurs in onychomycosis, may lead to a decrease in local O_2 concentration and an increase in CO_2 concentration [31], an effect similar to that used for induction of arthroconidia in vitro.

Another aspect to be considered in the susceptibility to antimycotics in vivo is the formation of biofilms. Biofilms are differentiated masses of microbes that adhere to surfaces and are surrounded by a matrix of extracellular polymers, increasing resistance to standard antimicrobials. It is well known that *C. albicans* biofilms are highly resistant to most of the antifungals [32]. The concept of biofilm for dermatophyte was introduced by Burkhart et al. [33] to explain dermatophytomas, a form of onychomycosis refractory to standard antifungal therapies. Dermatophytomas are characterized by a dense white mass of fungus tenaciously adherent to the surrounding nail plate, which may require surgical removal [33].

Antifungal Resistance Mechanisms

Historically, the clinical resistance of microbes has been defined as the persistence or progression of an infection despite appropriate antimicrobial therapy. In vivo, resistance is also correlated with antifungal misuse because patients often fail to finish the full course of treatment. Thus, the inadequate use or dosage of drugs contributes to the failure in eliminating the disease agent completely, encouraging growth of the

most resistant strains, which may lead to hard-to-treat fungal infections. The in vitro resistance of an isolate can be classified as either intrinsic or acquired. Intrinsic resistance allows all normal members of a species to tolerate a particular drug. In this case, a specific characteristic responsible for resistance is inherent to the species and has arisen through the process of evolution. Acquired resistance is a term used when a resistant strain emerges from a population that was previously drug-sensitive [34]. Even considering a low frequency of gene mutation, the selective pressure exerted by the constant use of antifungal agents selects eventual resistant strains that will become predominant in the population. Unless the mutation that rendered the fungus resistant also reduces its adaptability, the mutant strain will persist even in the absence of selective pressure of the drug.

Various biochemical mechanisms contribute to the phenotype of drug resistance in fungi. The most frequent ones involve a decrease in drug uptake, structural alterations in the target site and an increase in drug efflux or in intracellular target levels. From a molecular viewpoint, these biochemical changes can result from gene amplification, gene transfer, gene deletion, point mutations, loss of cis- and trans-acting regulatory elements and transcriptional activation. All these effects could be on genes directly involved in the combat against the cytotoxic compounds and/or could be on genes involved in their regulation or processing [34]. However, other molecular mechanisms may be involved in the resistance to inhibitory agents, in addition to specific variations possibly occurring in the mechanisms mentioned above. Only few reports have addressed the drug resistance mechanism in dermatophytes, and most of them have been described in *T. rubrum* (Table 2). It is noteworthy that resistance to a particular drug can be achieved by more than one mechanism and probably, under certain circumstances, they are activated simultaneously.

Modifications Involving Target Enzymes

Although enzymes are one of the major target classes for drugs, only few of them, including squalene epoxidase (SE), are focused as drug targets in fungi. Also, a small number of drugs acting as inhibitors of these enzymes are marketed antifungals [35]. SE is a key enzyme of the ergosterol biosynthetic pathway that catalyses the stereo-specific epoxidation of squalene to 2,3-oxidosqualene in fungi [36]. This enzyme is a microsomal mono-oxygenase, which requires the presence of molecular oxygen and a reducing cofactor, NADH or NADPH, for its enzymatic activity, and is stimulated by FAD [37, 38]. Fungal SEs are structurally similar to their mammalian counterparts, but the mammalian SEs have a NH_2-terminal extension absent in fungal proteins, and the SEs from *Saccharomyces cerevisiae*, *C. albicans* and *Neurospora crassa* have a stretch of 32–34 residues that is missing in *T. rubrum*, *Schizosaccharomyces pombe* and *Homo sapiens* proteins. All of them contain the three consensus considered as the hallmark of flavoprotein hydroxylases [39–41]. Terbinafine, a member of the allylamine group, has higher affinity for fungal SEs probably due to variations in the enzyme conformation [42, 43]. Fungal SEs are inhibited by terbinafine in a noncompetitive manner with regard to squalene and the mammalian SEs are inhibited competitively [44]. Thus, terbinafine specifically inhibits fungal SE by blocking the synthesis of 2,3-oxidosqualene leading to depletion of ergosterol and accumulation of squalene causing growth inhibition, whose effect is of medical importance [45, 46]. In fact, dermatophytes are the major clinical target of terbinafine, an antifungal drug used in the treatment of skin, nail and hair infections.

Fungi utilize several mechanisms to overcome the inhibition by terbinafine, such as mutations in the SE gene leading to substitutions in amino acids which are probably involved in the binding of terbinafine to SE (Fig. 1). Single amino acid exchanges in this region of the protein Erg1 (or ErgA) conferred high resistance to terbinafine in fungi and yeasts [39, 47–50]. However, the function of SE in the ergosterol biosynthesis is probably preserved in these mutants because all of the *A. nidulans* terbinafine-resistant strains grew and conidiated as wild type in the absence of terbinafine [51]. Terbinafine-resistant mutant strains carrying amino acid substitutions in the terbinafine binding domain can be generated by UV mutagenesis [51] and by site-directed mutagenesis [50], or isolated from patients [27, 49].

Cloning and sequencing of the SE gene from *T. rubrum* revealed an open reading frame coding for 489 residues, with an equivalent similarity (57%) to both yeast and mammalian SEs [39]. An amino acid substitution (L393F) in the SE of a terbinafine-resistant *T. rubrum* clinical isolate was identified

Table 2 Putative mechanisms of cytotoxic drug resistance in *Trichophyton rubrum*

Drugs	Mechanism of action	Putative resistance mechanisms [references]
Terbinafine	Inhibition of squalene epoxidase	Modification of target enzyme by mutation [39, 49]
		Increased drug efflux [62, 68]
		Stress adaptation[a] [68]
		Over-expression of salicylate mono-oxygenase (drug degradation) ? [83]
Fluconazole	Inhibition of cytochrome P450 14α-lanosterol demethylase	Increased drug efflux [58, 62, 68]
		Stress adaptation[a] [68]
Imazalil	Inhibition of cytochrome P450 14α-lanosterol demethylase	Increased drug efflux [58, 62]
Itraconazole	Inhibition of cytochrome P450 14α-lanosterol demethylase	Increased drug efflux [58, 62]
Ketoconazole	Inhibition of cytochrome P450 14α- lanosterol demethylase	Increased drug efflux [58, 62]
		Over-expression of lanosterol-14α-demethylase [54]
Tioconazole	Inhibition of cytochrome P450 14α- lanosterol demethylase	Increased drug efflux [62]
		Stress adaptation[a] [69]
Amphotericin B	Binding to ergosterol and destabilization of cell membrane functions	Increased drug efflux [54]
		Stress adaptation[a] [54]
Griseofulvin	Inhibition of mitosis	Increased drug efflux [58, 62, 68]
		Stress adaptation[a] [68, 69]
Acriflavin	Topoisomerases inhibition/intercalating RNA and DNA	Increased drug efflux [62, 68]
		Stress adaptation[a] [68]
Undecanoic acid	Unspecific cellular interactions	Stress adaptation[a] [68]
Benomyl	Inhibition of mitosis. Binding to tubulin	Increased drug efflux [62]
Ethidium bromide	Intercalating RNA and DNA	Increased drug efflux [58, 62]
4NQO	DNA modification, Mutagenic action	Increased drug efflux [62]
PHS11A	Fungal fatty acid synthase inhibitor	Increased drug efflux [67]
		Stress adaptation[a] [67]

[a] Stress adaptation: nonspecific responses to drug challenge

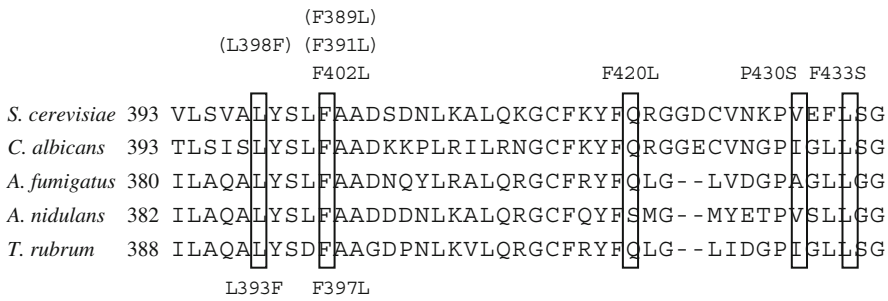

Fig. 1 Alignment of amino acid sequence from a conserved domain of *T. rubrum* squalene epoxidase (SE) with selected fungal SE family members, where amino acid substitutions affected fungal susceptibility to terbinafine. Four amino acid substitutions in Erg1 protein that conferred resistance to terbinafine in *S. cerevisiae* are shown above the sequences [48]. The F402L amino acid substitution corresponds to the F389L and F391L substitutions in SEs respectively from *A. fumigatus* and *A. nidulans*, which conferred resistance to terbinafine [50]. The L398F amino acid substitution in the *C. albicans* SE conferred a terbinafine-resistance phenotype to *S. cerevisiae* after gene transformation [39]. The position of amino acid substitutions L393F and F397L observed in two *T. rubrum* clinical isolates resistant to terbinafine is indicated [39, 49]

[39], being the mutant about 1,000-fold less susceptible to terbinafine than normal strains, as well as other SE inhibitors [27]. Also, in the same sequence region, a substitution at amino acid 397 (F397L) in another *T. rubrum* clinical isolate conferred higher resistance to terbinafine (and other SE inhibitors) [49]. Interestingly, these two amino acids are conserved in SE from other organisms, including humans (Fig. 1). The identification of three other amino acid substitutions in the same domain of *S. cerevisiae* SE that conferred resistance to terbinafine, F420L, P430S and F433S, suggests that the corresponding substitutions in *T. rubrum*, F415, P423 and L426, respectively, can also confer resistance to terbinafine in this dermatophyte. Also, the combination of more than one mutation in the same strain will probably cause the double mutant to become highly resistant to terbinafine. Indeed, interaction of genes in a double mutant of *A. nidulans* showing high resistance to terbinafine was constructed [51], with the substitution F391L in SE [50] (corresponding to F397L in *T. rubrum*), being one of these mutations. The second mutation was located by classical genetics in a different chromosome [51]. Furthermore, the epistatic relationship existing between these two genes [51] reveals that the mechanism of resistance to this drug may be more complex than expected.

The over-expression of target enzymes is another molecular mechanism developed by several fungi to overcome the inhibitory action of antifungals either by gene amplification or up-regulation of the corresponding gene. Transformation-based approaches have been used to introduce extra copies of genes encoding target enzymes in other fungi to study gene dose-dependent resistance to antifungals [52]. This approach revealed that *Aspergillus fumigatus* carrying extra copies of the gene encoding SE exhibited a high level of resistance to terbinafine [53]. Therefore, this is a potential mechanism for resistance that can also be revealed in dermatophytes and that can be, in the near future, the reason for lack of clinical response to terbinafine. This hypothesis is supported by reports confirming the over-expression of several genes of the ergosterol biosynthetic pathway in response to ketoconazole in *T. rubrum*. Azoles inhibit the lanosterol-14α-demethylase, encoded by the *ERG11* gene that showed a 3.6-fold induction in response to ketoconazole [54].

Drug Efflux Events

Multidrug efflux transporters are membrane proteins found in all living organisms. These proteins bind to a variety of structurally and chemically dissimilar compounds and actively extrude them from the cells [55]. Substrates for multidrug transporters in fungi include antifungal and cytotoxic drugs, which make these transporters responsible for failure in various antimicrobial treatments. In spite of this, the relevance of the drug resistance phenomenon for dermatophytes in vivo remains to be determined. A clinical isolate of *T. rubrum* submitted to a mutagen treatment showed simultaneous resistance to griseofulvin and tioconazole in vitro, suggesting that a multidrug resistance MDR-like phenomenon might be involved in this event [56, 57]. Several years later the *T. rubrum TruMDR1* gene was identified, which encodes an ATP-binding cassette (ABC) transporter. This gene is over-expressed in the presence of various nonstructurally related drugs (Table 3), suggesting its participation in drug efflux events in this dermatophyte [58]. Sequence analysis of the *TruMDR1* gene revealed an open reading frame coding for 1511 amino acids with homology to several other ABC transporters from *A. nidulans*, and to known members of the PDR (pleiotropic drug resistance) subclass of ABC transporters such as Cdr1p and Cdr2p of *C. albicans* and Snq2p of *S. cerevisiae* [59–61].

In addition, the *T. rubrum TruMDR2* gene, which encodes another ABC transporter, revealed an increased level of transcription when the fungus was exposed to griseofulvin, tioconazole and various other antifungal drugs (Table 3). The encoded protein TruMdr2p showed 1331 amino acids with homology to members of the multidrug-resistance (MDR) class of ABC transporters [62]. Although ethidium bromide and 4-nitroquinoline *N*-oxide (4NQO) are not antifungal drugs, they were assayed because they are substrates for some multidrug-resistance pumps in other microorganisms [63, 64]. Disruption of the *TruMDR2* gene rendered the mutant strain more sensitive to terbinafine, 4NQO and ethidium bromide than the control strain, suggesting that this transporter has a function in modulating drug susceptibility in *T. rubrum*. In contrast, no differences were observed in the level of sensitivity between the Δ*TruMDR2* mutant and the control strain when both strains were submitted to other drugs that induced the expression

Table 3 Multidrug resistance genes of *Trichophyton rubrum* and their transcriptional profile in response to cytotoxic drugs

GenBank accession number	Gene symbol	Putative identification	Drug [references]	Induction/ Repression
AF525740	*TruMDR1*	Pleiotropic drug resistance protein, ABC superfamily	EB, KTC, CHX, FLC, GRS, IMZ, ITRA [58]	I
DW692605			PHS11A [67]	
DW680156			KTC [54]	
AF291822	*TruMDR2*	ABC-type multidrug transporter	ACR, BEN, EB, KTC, CAP, GRS, FLC, IMZ, ITRA, MTX, 4NQO, TRB, TIO [62]	I
DW694247			ACR, FLC, GRS, TRB, UDA [68]	
			PHS11A [67]	
DW005358		ABC-type multidrug transporter	FLC, GRS, TRB [68]	I
DW696331		ABC-type multidrug transporter	PHS11A [67]	I
EB801596		MFS drug transporter	PHS11A [67]	I
DW690470		Multidrug efflux RND transporter	AMB [54]	I
EB801566		ABC-type multidrug transport system	PHS11A [67]	R
DW698195		ABC-type multidrug transport system	AMB [54]	R
DW701717		Na+-driven multidrug efflux pump	AMB [54]	R

Drugs: acriflavin, ACR; amphotericin B, AMB; benomyl, BEN; chloramphenicol, CAP; cycloheximide, CHX; ethidium bromide, EB; fluconazole, FLC; griseofulvin, GRS; imazalil, IMZ; itraconazole, ITRA; ketoconazole, KTC; methotrexate, MTX; 4-nitroquinoline *N*-oxide, 4NQO; tioconazole, TIO; terbinafine, TRB; undecanoic acid, UDA and PHS11A. Putative identification: ATP-binding cassette, ABC; Major facilitator superfamily, MFS; and resistance nodulation cell division, RND

of *TruMDR2* gene. However, we cannot rule out the possibility that *TruMDR2* gene has a function in modulating the susceptibility to drugs that increase the levels of *TruMDR2* transcripts. This is because the presence of additional forms of ABC transporter proteins in *T. rubrum* may compensate for the deletion of the *TruMDR2* gene by acting as an efflux pump, causing resistance to these toxic compounds. This could be the case for itraconazole, an antimycotic agent used to control dermatophytosis, which strongly induced transcription of *TruMDR2* and to which the Δ*TruMDR2* mutant strain presented no change in itraconazole sensitivity. In fact, *TruMDR1* gene is also induced by many of these drugs, including itraconazole [58], although TruMdr1p showed only 29% of identity to TruMdr2p. Increased sensitivity to some antifungals could be observed in *C. albicans* only if a double-deletion strain was constructed for genes *CDR1* and *CDR2* (ABC transporters) because Cdr1p was able to compensate for the lack of Cdr2p [59]. Nevertheless, although *CDR1* and *CDR2* were up-regulated in *C. albicans* cells cultivated in the presence of subinhibitory conditions of ciclopiroxolamine, no change in susceptibility to ciclopiroxolamine could be observed for mutants disrupted for both *CDR1* and *CDR2* compared to the parent wild-type strain, suggesting that these genes do not affect susceptibility to this drug [65, 66]. In addition, some expressed sequence tags (ESTs) revealed other putative multidrug efflux transporters in *T. rubrum* (Table 3) [54, 67, 68]. Identification of genes potentially involved in cellular detoxification in dermatophytes is the first step to ascertain the molecular events related to antifungal resistance.

Cellular Responses to Stress

Microorganisms respond to sublethal doses of chemical and physical agents by synthesizing a variety of specific proteins and low molecular weight compounds that are thought to act as protectors or signalling effectors to promote the development of defensive reactions or tolerance [69]. Antibiotic

concentrations below the minimal inhibitory concentrations (sub-MIC) modulate the expression of various virulence factors in bacteria [70], by increasing for instance the production of soluble capsular polysaccharide [71].

Fungi also use numerous signal-transduction pathways to sense and ensure appropriate physiological mechanisms to adapt to environmental stress, which is characterized by eliciting changes in the expression of cell stress genes. Antifungal drugs induce cell stress responses that are required to overcome the toxic effects of these drugs allowing fungal survival. For example, the electrophoretic pattern of the intracellular esterases of *T. rubrum* is altered when the fungus is cultured in the presence of subinhibitory concentrations of tioconazole or griseofulvin. Several strains (original isolate or antimycotic resistant mutants) presented five clearly visible bands when cultivated on medium containing sub-MICs of tioconazole or griseofulvin, and only two bands when cultivated in the absence of antimycotics. Although griseofulvin and tioconazole are drugs with distinct mechanisms of action, they trigger the appearance of the same esterase bands [69], suggesting a common mechanism of adaptation. The additional forms of esterase produced by the cells in the presence of these antimycotics may be a nonspecific response to cellular stress, or may participate in cellular detoxification processes in *T. rubrum.* Overproduction of esterase in response to environmental stress has been described as a mechanism of cellular detoxification in other organisms [72].

The exposure of *T. rubrum* to sub-MIC of amphotericin B increased the transcription of genes that encode stress-response-related proteins, including Hsp70, Hsp104 and ubiquitin, oxidative-stress proteins such as glutathione synthase, and ethanol-stress proteins such as glyceraldehyde-3-phosphate dehydrogenase [54]. Interestingly, amphotericin B repressed the transcription of other stress-response-related genes [54], suggesting the existence of a complex mechanism for the survival of the fungus under stress conditions. Different genes encoding stress-response proteins were also induced or repressed when *T. rubrum* was submitted to PHS11A, a fungal fatty acid synthase (FAS) inhibitor [67]. Catalytic activities and functional domain organization among human and fungal FAS differ significantly. One structural distinction between human and fungal FAS is that human FAS occurs in a monodimeric form, arranged in a "head-to-tail" manner to form two palmitate-synthesizing sites [73], whereas fungal FAS operates as heteromultimeric protein aggregates containing both alpha (FAS1) and beta (FAS2) subunits [74–77]. Thus, these basic structural differences between the mammalian and fungal FAS render them as potential antifungal drug targets [17].

Several genes were over-expressed after *T. rubrum* exposure to acriflavin, fluconazole, griseofulvin, terbinafine or undecanoic acid, which represent different classes of antifungal agents. These genes were induced by more than one drug suggesting that most of them could be involved in nonspecific responses to cellular stress [68]. The up-regulation of two of these genes encoding a carboxylic ester hydrolase and a Pol protein supports this hypothesis. The carboxylic ester hydrolase was differentially expressed in all treatments, except under undecanoic acid [68]. Although the physiological role of some esterases is not clear, it is known that these enzymes are secreted [78] and present a specific electrophoretic pattern when *T. rubrum* is exposed to griseofulvin or tioconazole as mentioned above [69]. Also, the identification of a gene similar to *pol* gene of *Cg*ret retrotransposon element from *Glomerella cingulata* (anamorph: *Colletotrichum gloeosporioides*), up-regulated under fluconazole, griseofulvin, acriflavin and terbinafine, suggests that a transposable element (TE) has a function in *T. rubrum* drug stress response [68]. In fact, transposition in response to environmental stress has been proposed as an adaptive response of the genome from various organisms [79, 80]. Thus, although the retrotransposons were identified in other pathogenic fungi, for the first time a retrotransposon activity was observed in dermatophytes evidencing that drug stress might be associated with TE activation [68].

The identification of transcripts differentially expressed during exposure of fungi to cytotoxic drugs helps to understand the mechanisms of action and, mostly, the mechanism of stress adaptation to these drugs. In many of these experiments, a large number of these putative genes have no significant matches in databases, probably because they are exclusive to *T. rubrum* or at least to dermatophytes. In addition, many of these transcripts are also over-expressed when *T. rubrum* grows in keratin as the

carbon source, which mimics the host–pathogen interaction [9]. Considering the relevance of these genes in the survival of *T. rubrum* under stress conditions and in the interaction with the host, the proteins encoded by these genes can be potential drug targets in dermatophytes.

Alternative Mechanism of Terbinafine Resistance

Modification and degradation of drugs are important antibiotic resistance mechanisms in a variety of bacteria and eukaryotic systems [81, 82]. The classical example is the presence in the bacterial R plasmid of genes encoding enzymes that modify the antibiotics leading to their inactivation. There are only few studies focusing on antifungal alteration or degradation by fungi as resistance mechanisms. One of them describes an alternative mechanism of terbinafine resistance through over-expression of the *A. nidulans* salicylate 1-monooxygenase, a well-characterized naphthalene-degrading enzyme. It has been hypothesized that resistance can follow degradation of the naphthalene nucleus contained in the molecular structure of terbinafine [83]. In addition, plasmids containing the *A. nidulans* salicylate 1-monooxygenase gene were able to transform a *T. rubrum* strain from being sensitive to being resistant to terbinafine, probably by a multicopy effect (F. Segato, personal communication). Nevertheless, the clinical relevance of this putative mechanism of terbinafine resistance in dermatophytes is to be determined.

Putative Molecular Targets for New Drug Development

Antifungal agents have been the focus of pharmaceutical industries, since life-threatening and irritating superficial mycoses are increasing worldwide. Although terbinafine, itraconazole and griseofulvin have been used in the treatment of dermatophytosis, there is a necessity for more effective management of these drugs and discovery of new broad-spectrum antifungals. One of the major challenges in developing antifungal drugs lies in the similarities shared between fungi and their hosts. To develop new drugs, it is necessary to have in mind that an efficient antifungal should act in a wide range of fungi having no or low toxicity to the host. Furthermore, especially for the treatment of onychomycosis, it is also interesting and required that the drug accumulates in the nail plate and persists there for a long time to be effective in therapy [84, 85]. On the other hand, an adequate cellular target is assigned to be essential and conserved in a variety of fungi, but not displaying a counterpart in the host [86].

Current strategies to identify new antimicrobial drugs include the screening for natural or synthetic products that inhibit fungal growth and the designing of new molecules capable of interfering with a target in the fungus, affecting its viability. The first strategy is assessed by in vitro susceptibility tests, while the latter one requires bioinformatics and molecular biology approaches, to search and validate new molecules regarding their effectiveness in interacting with a cellular target. This strategy is also used in the searching for new molecular and biochemical targets [87].

Recent advances in fungal genomics and proteomics, and the outcome of bioinformatic tools, turned these areas into the forefront in the discovery of cellular targets and novel antifungal agents. Genomic sequence information and comparative genomics are used to assist the search for new molecular targets through the identification of essential genes and the reconstruction of cellular pathways that are then compared between different pathogens and screened in the host genome, where it should be absent. However, molecular genetics regarding both the biology and pathogenic processes of dermatophytes are scarce. The inclusion of five dermatophytes in the sequencing project by the Fungal Genome Initiative (The Broad Institute of Harvard and MIT/National Institute of Health) and the recent release of a transcription profile of *T. rubrum* developmental phases [88, 89] will render possible the *in silico* search for molecular targets, in a near future.

Comparative genomics revealed eight nonhumans *A. nidulans* ESTs that are essential and homologous to yeast genes: phosphoglyceromutase, fructose biphosphate aldolase, UTPG-1-phosphate uridylyltransferase, trehalose phosphate synthase, histidine kinase, FAS and two homologues of unknown function [17]. Furthermore, a novel FAS inhibitor affected transcription of some genes involved in lipid metabolism, cAMP and MAPK pathways, and multidrug resistance [67]. However, various other tests, such as toxicity to animals and humans, are essential before it can be used in therapy.

Moreover, genes and proteins required during infection of host tissues by dermatophytes, or virulence factors, are important targets to be revealed and validated to assist the development of new drugs. Virulence is defined as the relative infectiousness of a microorganism causing disease combined with the capability to overcome the host natural immune defences [90]. The most important virulence factors for dermatophytes described so far are the enzymes released during *stratum corneum* and nail invasion, including keratinases, proteinases, lipases, mucinolytic enzymes, elastases and DNases [4, 6]. However, the potential exploitation of molecules involved in pathogenesis as drug target has some issues that must be concerned. Generally, many virulence factors act simultaneously and the interference in just one of them may not have the expected effect. Another point is that these molecules might be specific to a species or genera and subsequently have a low potential for the development of broad-spectrum antifungal agents. A potential target filling these requisites is the transcription factor PacC, a wide-domain regulatory protein involved in pathogenicity events [12] and present in several species of three genera of dermatophytes [91].

Recently, sulphite transporters were proposed as interesting targets for antifungal drugs in dermatology, because of their role in the proteolytic digestion of hard keratin. Sulphite is secreted by filamentous fungi as a reducing agent during keratin degradation, allowing the cystine in keratin to be cleaved into cysteine and S-sulphocysteine, and thereby to become accessible to hydrolysis by a variety of secreted endo- and exoproteases. Therefore, efflux-pump-mediated sulphite detoxification and sulphitolysis may be considered as complementary functions in the digestion of compact keratinous tissue [92]. The authors identified a gene encoding a sulphite transporter in *T. rubrum* (*TruSSU1*), which belongs to the tellurite-resistance/dicarboxylate transporter (TDT) family. Given the absence of TDT transporters in humans, sulphite transporters could be a target to treat dermatophyte infection with minimal toxicity.

Functional genomics analysis will be very powerful to determine the role of regulatory genes and virulence factors in dermatophytes, and to analyse the effects caused by the perturbation of metabolic pathways targeted by antifungals. Promising methodologies to achieve this goal comprise gene expression analyses by microarrays, or subtractive hybridization, gene disruption, insertion-mutagenesis and gene inactivation by small interference RNA (siRNA) or antisense RNA [93]. Another interesting strategy is the computer-based models to predict protein structures and for modelling the interactions between protein and small-molecule inhibitors, also termed molecular docking approach [94]. However, even in well-known sequenced organisms, this kind of *in silico* analysis is still limited [86].

Conclusions and Perspectives

Although many antifungal drugs have been developed during the last two decades, they are confined to a relatively few chemical classes. In addition, the occurrence of resistance in clinical isolates leads to failure in the treatment of mycosis. Thus, the effective control of dermatophytes will necessarily involve the development of a new generation of potent broad-spectrum antifungals with selective action against new targets in the fungal cells, without irreversible side effects in the host. However, to search for new molecular targets, it is essential to have a full understanding about the biology of pathogenic fungi and about the mechanisms of fungal-host interactions. In addition, knowledge of the antifungal resistance mechanisms is also essential, since the development of resistant strains to any new drug is inevitable.

The metabolic responses that govern homeostatic pH and extracellular pH sensing represent an interesting area to be studied in dermatophytes, since these mechanisms are possibly involved in the installation, development and survival of dermatophytes in humans. The complete sequencing of dermatophyte genomes and the analysis of the promoters of the genes involved in this adaptive response will allow the discovery of new genes, providing new functional information through the study of their expression, and perhaps identifying molecular targets for the design of new antifungals.

The growing number of genomic sequence data generated from nonpathogens aids the genome-based drug discovery, improving functional assignment by adding breadth to protein family trees, as well as serving to highlight the unique features of dermatophytes, e.g., essential genes for the installation of infection. The large number of already existing possibilities for potential drug targets encoded in

fungal genomes (cell wall, virulence factors, proteins involved in fungal-host interactions, proteins that modulate DNA topology, gene transcription, mRNA translational and post-transcriptional modifications) should be explored and validated for the successful production of new fungal inhibitors. Extensive genome sequencing in both host and fungi coupled with the development of bioinformatics has enabled a more direct approach towards target discovery. Systematic comparative genomics can reveal intrinsic differences between fungal and human genomes, generating an expanded nonhuman gene list for drug-screening experiments, useful to identify targets selective to fungi and harmless to man. Furthermore, the detection of potential targets by bioinformatics and the role of genetics in target validation will be of fundamental importance to design specific drugs against fungal infections. The recent efforts in dermatophyte genome sequencing, transcriptome and proteome analysis provide effective ways to find new possibilities to cure and prevent diseases caused by fungi.

In a nutshell, a combination of technologies will provide the optimum platform for antifungal discovery to treat dermatophytosis and other mycosis. High-throughput screening of chemical libraries identifies candidate inhibitor classes, whose structure can be adapted according to results obtained *in silico*. Also, in vitro interaction assays, synthetic modifications and effectiveness tests should be performed, so that a successful inhibitor can emerge. The availability of public databases and computer-based methodologies to predict the properties of drugs and targets encourages confidence that many of the difficulties in taking compounds from target inhibitors through clinically effective drugs should be overcome. In the future, genomic and computer-based approaches to antifungal discovery will eventually be carried out. Functional genomics will provide information about gene function and regulation, allowing the comprehension of dermatophyte biology and pathogenicity. Nevertheless, since resistance in clinical isolates may occur and it can be a process that involves more than one mechanism, the understanding about the mechanisms that confer antifungal resistance is essential for designing modifications in the currently used antifungal.

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