Shiburaj Sugathan · N.S. Pradeep
Sabu Abdulhameed *Editors*

Bioresources and Bioprocess in Biotechnology

Volume 2 : Exploring Potential Biomolecules

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Dedicated to the memory of
Dr. Christopher Augur (Chris) whose life
and scientific contributions in the area of
bioresources peaked great on a narrow base.

Dr. Christopher Augur
(1960–2009)

Foreword

I am delighted to introduce Vol. II of "Bioresources and Bioprocess in Biotechnology: *Exploring potential biomolecules",* edited by Dr. Shiburaj Sugathan, Dr. N S Pradeep and Dr. Sabu Abdulhameed. In this regard, I would like to highlight its specific features in the most possible brief form so that the peers may quickly lay their hands on the collection of erudite essays with guiding commentaries and reviews. To be exact, I am happy to say that the editors have done a commendable job in an area of several bio-applications. Technically speaking, there may be other similar edited volumes very coarsely comparable to the present one produced in the past. However, the present compilation makes a better composition, considering its scope and the extent of recent and emerging areas in bioresources and bioprocess technology. The salient features of the outstanding collection of reviews are the much-needed single volume for students, researchers and industrialists in the field of biotechnology, particularly hitherto apparently neglected areas of knowledge with transformational potential. The present volume will be of use to researchers in the fields of antimicrobials particularly toward mycobacterium, plant-based alternative medicines, enzymes, anticancer and anti-inflammatory molecules, medicinal significance of polyphenol-containing fermented products, etc.

The editors must be congratulated for bringing out such an extensive volume beautifully written for universal appeal. The following areas are dealt with utmost care and scholarship. They are chemical alterations of compounds (e.g., a drug) occurring within the body, as by enzymatic activity; plant biosynthetic pathway assemblies for engineering microbial systems to produce targeted chemical compounds; biodiversity of plants ensuring resources for new food crops and medicines; ever nascent ethnopharmacology; etc. No doubt, this volume will be of great use to one and all in the fields of biological resources and biotechnology and

materials research for solving the maladies presently limiting sustainable and comfortable life to humans in a conserved environment with equal rights to all life forms.

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Preface

In an era of growing awareness about the threats of biodiversity loss, the society is witnessing an unprecedented interest in novel bioresources, which are increasingly prized for their potential use in many applications. The loss of bioresources is occurring at an alarming rate, a consequence of increasing population pressure, agricultural land degradation, urbanization and above all neglect. Deforestation and forest degradation are large-scale problems in developing countries.

To meet the demands of the society in industrial, healthcare, food and other sectors, the utilization of bioresources is an absolute requirement. At the same time, we need to make sure that the overexploitation should never result in biodiversity loss. To manage this situation, we need to have more knowledge on rarely used or unused resources which are available in bulk and are easy to propagate rapidly. Modern biotechnology is armed with techniques for sustainable utilization of bioresources to meet the increasing demand.

The concept of sustainable development indicates that economic and environmental protection are inseparably linked and that the quality of present and future life fails in meeting basic human needs without destroying the environment on which the life depends. There is a growing recognition worldwide that conservation and sustainable management of bioresources are the need of the hour. The use of biotechnological tools and bioprospecting will open new vistas in many fields viz. agriculture, medicine, horticulture, environment, etc. Since we cannot do without exploiting the available bioresources to our advantage, there has to be a balance between uses of resources and their conservation.

There is an increasing realization that bioresources especially medicinal plants and microbes can provide cheaper means of disease management by analyzing further their functional potential. This interest has led to a better understanding of the role of plant and microbial bioactives in health promotion and disease prevention. Generation of high-throughput data and the study of molecular mechanisms of diseases have all contributed to this effort.

Kerala, India

Shiburaj Sugathan
N.S. Pradeep
Sabu Abdulhameed

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Part I

Enzymes

Enzymes for Bioenergy

1

Rajeev K. Sukumaran, Amith Abraham, and Anil K. Mathew

Abstract

Lignocellulosic ethanol is emerging as the prominent candidate for renewable liquid transportation fuels, and the conversion of biomass to ethanol requires enzymatic hydrolysis. Enzymes that hydrolyze biomass have been the subject of several studies, since the cost estimations of second-generation ethanol show significant contributions by this single consumable. The chapter introduces biomass-hydrolyzing enzymes in the context of biorefineries and provides an overview on the current knowledge and understanding of these enzymes with respect to their types, mode of action, regulation of gene expression, and synergies. The changing concepts about the role of individual enzymes and the new discoveries on lignocellulose breakdown are presented to highlight the developments in biomass hydrolysis paradigm. It also covers the current strategies employed for commercial production of different lignocellulose-hydrolyzing enzymes and their blending to derive efficient cocktails. Finally, the importance of cost reduction in production and usage of biomass hydrolysis enzymes for a cost-effective bioethanol technology is discussed along with the current approaches in addressing this.

Keywords

Cellulase • Hemicellulase • Xylanase • Biomass hydrolysis • Biorefineries • Bioethanol • Bioenergy

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1.1 Introduction

World energy consumption is on the rise, and a significant growth in energy demand of about 48% is projected for the year 2040 from the base value in 2012 (EIA 2016). Economic growth along with accompanying changes can significantly influence energy consumption, and improvement in living standards brings with it a rapidly growing demand for energy. While renewable energy is the world's fastest growing source of energy, fossil fuels continue to provide most of the world's energy, and liquid transportation fuels represent a major share of this (EIA 2016). Even with efforts worldwide on alternative energy resources, it is projected that the demand for fossil fuels will be on the rise at least for the next few decades with serious impacts on the global environment and climate. It is in this context that the renewable liquid transportation fuels including biodiesel and bioethanol gain prime importance as the existing alternatives to petroleum-based transportation fuels. Lignocellulose is inarguably the world's most abundant renewable source of energy and this justifies the enormous efforts put into developing plant biomass-based fuels – primarily bioethanol. While several of the second-generation (2G) ethanol programs claim to have gone commercial, it still is not a reality at consumer level. The major limitation in commercialization of 2G ethanol is the cost of its production. Lignocellulose contains mainly the sugar polymers – cellulose and hemicellulose, and a significant fraction as lignin. Both the sugar polymers can be broken down to their component sugars, which then can be fermented by microbial action to produce bioethanol. The hydrolysis/saccharification of biomass can be achieved by chemical agents (e.g., acids) or through enzymatic hydrolysis. The latter is often much more efficient and requires only ambient conditions, whereas the former needs higher temperature and is plagued by issues like generation of sugar breakdown products, and the need to deal with acidic waste streams (Visser et al. 2015). The seemingly simple enzymatic process is made difficult by the recalcitrance of lignocellulose and the cost of biomass-hydrolyzing enzymes. Recalcitrance of biomass to enzymatic hydrolysis stems from the highly organized structure of lignocellulose, which prevents access of the enzymes to cellulose. Biomass pretreatments are aimed at making the cellulose more accessible to the enzymes and can bring significant improvements in digestibility. The chapter is primarily focused on biomass-hydrolyzing enzymes in the context of bioenergy, and specifically bioethanol. Microorganisms producing cellulase, their regulation at molecular levels, production strategies, enzyme cocktails for biomass hydrolysis, and the emerging strategies for improving production and efficiencies of biomass-hydrolyzing enzymes are discussed.

1.2 Biomass-Hydrolyzing Enzymes and Their Role in Biofuels Production

Plant biomass consists of three major structural biopolymers, namely, cellulose, hemicellulose, and lignin, each having a unique and complex structure. Cellulose is the major component and is a homopolymer of β-1,4-linked glucose units which can have a degree of polymerization (DP) up to 10,000. Often the cellulose chains are

organized into microfibrils and there is cross-linking between adjacent chains through hydrogen bonds leading to crystalline and amorphous domains (Pu et al. 2013). Cellulose can make up to 15–30% of primary cell walls and up to 40% of secondary cell walls (Sticklen 2008). Hemicellulose on the other hand is a diverse group of short chain, branched, substituted polymer of sugars with a DP of ~70–200 (Zhao et al. 2012). The sugar monomers in hemicelluloses can be xylose, mannose, galactose, rhamnose, and arabinose, with xylose being the most abundant one. They can also contain the sugar acids like glucuronic or galacturonic acid and the hydroxyl groups of sugars can be partially substituted with acetyl groups (Gírio et al. 2010). Lignin is a class of complex cross-linked phenyl propane units, primarily comprising the monomeric units—p-coumaryl, coniferyl, and sinapyl alcohols. Typical plant cell wall structure consists of cellulose microfibrils embedded in a matrix of lignin interspersed with hemicellulose fibers forming a very rigid and organized structure, which is rather difficult to break (Fig. 1.1).

Nature's arsenal for breaking plant cell wall structures includes enzymes that can hydrolyze all of these compounds, and these catalysts play a very important role in the recycling of organic carbon on the globe. Different microorganisms are capable of producing enzymes that can degrade cellulose, hemicellulose, or lignin, or a combination of all. Recently, cellulases have taken the center stage in enzyme research, primarily because of their important role in second-generation ethanol (bioethanol) from lignocellulosic biomass. Sugar polymers in the lignocellulosic biomass are linked through β-1,4 glycosidic linkages that can be hydrolytically cleaved to release monomeric sugars. Enzymatic hydrolysis primarily employs cellulases derived from filamentous fungi – especially strains of *Trichoderma, Penicillium*, and *Aspergillus*, mostly in a crude concentrated form. While cellulases have been around for several decades, the enzymes tailored for efficient biomass hydrolysis are a recent development and involve deliberate blending of multiple enzymes from different sources so as to achieve maximum hydrolytic efficiency. Natural cellulases are slow acting and are affected by several parameters from the reaction environment. While recent research has been successful in improving the efficiencies of biomass-hydrolyzing enzymes and their reaction rates, the same cannot be said for their cost of production. It has been realized that the cost of biomass-hydrolyzing enzymes is a major hurdle for developing an economically viable

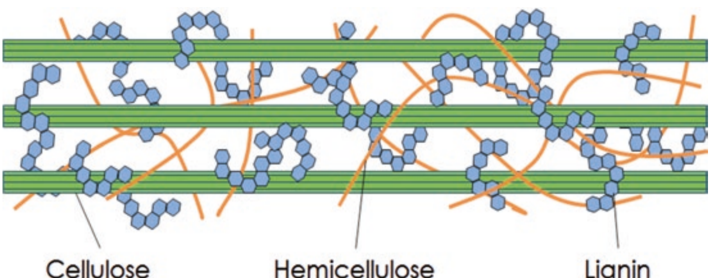

Fig. 1.1 Schematic representation of the lignocellulose structure

cellulosic ethanol industry (Banerjee et al. 2010), and there have been numerous efforts toward bringing down the cost of cellulases at the level of both production strategies and organism engineering. Enzyme majors of the world—Novozymes and Dupont (Genencor)—have been able to bring down the cost of enzyme per unit volume of ethanol produced to levels 10–12 folds lower, but a marketable commercial Lignocellulosic (LC) ethanol remains elusive. This is despite the fact that there are claims from companies on successful running of lignocellulosic ethanol plants. Several research studies have repeatedly highlighted the contribution of enzyme cost to the cost of lignocellulosic biofuels, and have shown that the cost of producing enzymes was much higher than that commonly assumed in the literature (Marcuschamer et al. 2012). A recent report puts the cost of cellulase per liter of ethanol at US$ 0.72 (48% of the production cost) based on the actual purchase price of cellulase in the industrial enzyme market (Liu et al. 2016). The above discussions highlight the importance of cellulase cost reduction in bioethanol production and active research efforts are now directed toward this cause worldwide. The strategies range from using cost-effective carbon sources and onsite production of enzymes (Johnson 2016) to developing genetically modified source organisms (Seiboth et al. 2012a; Fuji et al. 2013). However, reducing the production cost of cellulase alone is not the solution, and the ways to reduce the cost of cellulase for bioethanol production involve a range of possible solutions including development of efficient pretreatment regimes that allow better access of enzymes to the biomass, preventing lignin redeposition on biomass after pretreatment, use of surface active agents to aid hydrolysis, etc. A better understanding of these strategies would require knowledge on the enzymes and their mechanism of action.

1.2.1 Enzymes for Biomass Hydrolysis – Types and Mode of Action

While there are a multitude of proteins aiding in biomass hydrolysis, the major enzymes involved in biomass hydrolysis can be grouped as cellulases, hemicellulases, and lignin-degrading enzymes. Also there are a large number of accessory enzymes and proteins that are involved in helping the deconstruction, which cannot be grouped into any general categories. The following discussions will introduce the biomass-hydrolyzing enzymes and the major emphasis will be on cellulases since these are the major enzymes which are directly involved in breaking down the carbohydrate polymers to fermentable sugars and therefore important in biofuel production. Hemicellulases and lignin-degrading enzymes shall also be introduced.

1.2.1.1 Cellulases

Cellulases are enzymes, which hydrolyze the β-1,4-D-glucan linkages in cellulose and produce as primary products glucose, cellobiose, and cello-oligosaccharides. Cellulases are produced by a number of microorganisms and comprise several different enzyme classifications. Three major types of cellulase enzymes are involved

in the hydrolysis of native cellulose, namely, endoglucanases (EG), exoglucanases/cellobiohydrolases (CBH), and β-glucosidase (BGL) (Schulein 1998). In cellulase-producing organisms, there are multiple enzymes under each of these classifications, which act synergistically to break down cellulose. The classical model for cellulase hydrolysis emerged from the work done on *Trichoderma reesei* in the late 1990s by several groups as reviewed in Payne et al. (2015). In this model, EGs (Cel7B in *Trichoderma reesei*) attack the amorphous regions on the surface of cellulose microfibril revealing new reducing and nonreducing ends in the cellulose chain, which then serve as sites for attack by exoglucanases. The exoglucanases can also attack the available free ends of the cellulose chains. In *T. reesei*, the exoglucanase that attacks the reducing end of the cellulose chain is cellobiohydrolase I (CBHI/Cel7A) and that which attacks the non-reducing end is cellobiohydrolase II (CBHII/Cel6A). The cellobiose released by exoglucanases is cleaved to glucose units by the final enzyme in the cascade – beta glucosidase. The role of each cellulase and its synergism is described below.

1.2.1.2 Endoglucanase

Endoglucanases or endo 1,4-β-D-glucan glucanohydrolases (EC 3.2.1.4) are enzymes which randomly act on the cellulose polymer producing nicks in the amorphous regions of cellulose (endo-initiating) to expose the reducing and nonreducing ends by cleaving the endo β-1,4 linkages between adjacent glucose units. They are generally measured by detecting the reducing groups released from the soluble carboxymethylcellulose substrate (Sheehan and Himmel 1999). Endoglucanases are classically considered as the initiators of cellulose hydrolysis by the cellulase complex, since their action is essential for exposing the reducing and nonreducing ends in the cellulose polymer, essential for the action of exoglucanases/cellobiohydrolases. Recent view on endoglucanases also proposes its role to help cellobiohydrolases to overcome blockage at amorphous regions of cellulose (Payne et al. 2015). Endoglucanases are represented in several glycosyl hydrolase (GH) families, and in the model organism *T. reesei* there are six endoglucanases represented in families GH5, GH7, GH12, GH45, and GH74 (Kubicek 2012).

1.2.1.3 Exoglucanases

Exoglucanases or exocellulases are of two types, namely, cellulose 1,4-β-D-cellobiosidase (reducing end) EC 3.2.1.176 (cellobiohydrolase I/CBHI) and cellulose 1,4-β-D-cellobiosidase (nonreducing end) EC 3.2.1.91 (cellobiohydrolase II/CBHII). These enzymes attack the available reducing or nonreducing free ends or the ends generated by the action of EGs to liberate cellobiose units. While CBHI attacks the reducing ends of the chain, CBHII attacks the nonreducing ends (Cantarel et al. 2009). The current view on exoglucanases does not consider them as having exclusive exoglucanase action, but as exoglucanases with endo-initiating action (Kurasin and Valjamae 2011). In the model organism *T. reesei*, cellobiohydrolases are represented in glycosyl hydrolases families GH6 and GH7.

1.2.1.4 Beta Glucosidases

Beta glucosidases or cellobiases (EC 3.2.1.21) are enzymes that catalyze the hydrolysis of terminal, nonreducing beta-D-glucosyl residues with release of beta-D-glucose (Leah et al. 1995). Beta glucosidases (BGLs) catalyze the final reaction in cellulose hydrolysis, namely, the hydrolysis of cellobiose to two molecules of glucose, and are responsible for the regulation of the cellulolytic cascade through their own feedback inhibition by their reaction product glucose. Most of the microbial BGLs employed in biomass hydrolysis belong to GH family 3, while they can be found in families 1, 3, 9, 30, and 116 (Teugjas and Väljamäe 2013). BGL action is considered as a critical step in cellulose hydrolysis since the substrate of BGL – cellobiose – is a strong inhibitor of CBHs and its hydrolysis is essential to overcome product inhibition of the exoglucanases. Since glucose accumulation can lead to BGL inhibition which in turn leads to CBH inhibition through accumulation of cellobiose, the regulation of cellulase production in response to the hydrolysis of cellulose is of critical importance in most of the organisms producing these enzymes. In several cases the BGLs are also inhibited by their substrate, believed to be caused by the transglycosylation reaction capable of being performed by these enzymes (Bohlin et al. 2013).

1.2.1.5 Other Cellulolytic Enzymes and Accessory Proteins

It has long been recognized that the hydrolysis of the dense crystalline lattices of cellulose has to be mechanically disrupted for access of the hydrolytic enzymes and the role of a “swelling factor” which was nonhydrolytic and was proposed as early as 1950 (Reese et al. 1950). “Swollenin”, a protein with sequence similarity to plant expansions, was described in *T. reesei* by Saloheimo et al. (Saloheimo et al. 2002). It was believed that swollenin and similar nonhydrolytic swollenin-like proteins act like a zipper opening up the cross-linking of cellulose microfibrils just like plant expansins (Arantes and Saddler 2010). It was also proposed originally that these proteins lack hydrolytic activity since only negligible quantities of sugar release were observed with their independent action, while they enhanced hydrolysis of cellulosic substrates (Gourlay et al. 2012). The mechanism of promoting cellulose breakdown was speculated to be through a nonhydrolytic weakening of hydrogen bonding (Jäger et al. 2011, Gourlay et al. 2012). However, the most recent works have indicated that the protein does have hydrolytic activity and shows a unique mode of action with similarities to the action of both endoglucanases and exoglucanases (Andberg et al. 2015). Apart from swollenin, the “disrupting” or “amorphogenesis inducing” class of biomass-degrading proteins includes expansins, bacterial expansin-like proteins, fungal expansin-like proteins, loosenin, etc. (Arantes and Saddler 2010; Gourlay et al. 2013).

Revolutionary changes in the conventional cellulose deconstruction paradigm have emerged with the discovery of a class of enzymes that share conserved structural features binding a metal ion and following a hitherto undescribed oxidative mechanism (Vaaje-Kolstad et al. 2010). These types of enzymes which are now considered ubiquitous have been termed as Lytic Polysaccharide Mono Oxygenases (LPMOs). The most important feature of these enzymes is their ability to attack the

highly crystalline regions of cellulose where EGs are unable to bind productively. Thus they are able to synergize with glycosyl hydrolases, likely as endo-acting enzymes that act directly on the surface of crystalline cellulose. It is now known that LPMOs require a reducing agent and molecular oxygen and a copper ion in the active site (Payne et al. 2015). The electron donor can also be a co-secreted enzyme like cellobiose dehydrogenase (CDH), the only known example of a secreted flavo-cytochrome (Dimarogona et al. 2012).

1.2.1.6 Mechanism of Cellulose Hydrolysis by Cellulases

With more and more studies on cellulase action being undertaken, it is now becoming clearer that our understanding of cellulose hydrolysis is probably not complete, and there are paradigms not yet characterized. However, decades of research in this field have given insights into a generally appreciated mechanism of action, and recent discoveries like that of the LPMOs have improved that understanding. The following mode of hydrolysis is a summary of what is currently accepted as the mechanism of cellulose breakdown by cellulases. Cellulose structure is complex with crystalline array of cellulose microfibrils with glucan chains interlinked through hydrogen bonds. There are regions of disorder in the arrangement of glucan chains along the cellulose microfibrils, which are called the amorphous regions. The biomass-degrading enzymes work at the solid liquid interfaces, which implies that a high concentration of catalytic units is required at the surface for efficient hydrolysis of the polymer. This is achieved by the unique adaptation of several of the endo- and exoglucanases in having a three-domain structure with a carbohydrate binding module (CBM) which attaches to the cellulose surface, a catalytic module which does the actual hydrolysis, and a linker which serves mobility and also aids the enhanced binding of the enzyme to the cellulose surface. A detailed description of the structural features of cellulose-hydrolyzing enzymes is beyond the scope of this chapter and the readers are directed to Payne et al. (2015) for a comprehensive discussion on this topic. The typical three-domain structure aids in the processivity of the exoglucanases and endoglucanases that possess this structure. Processive enzymes are those which catalyze consecutive reactions without release of their substrate, and in the cases of cellulases with this modular structure, they help to keep the catalytic domain near the substrate (Teeri et al. 1998).

On recognition of a free chain end, the cellulase threads the chain into the tunnel (exoglucanase) in the catalytic domain of the enzyme to form a catalytically active complex (CAC). Hydrolysis occurs following a retaining or an inverting mechanism (Davies and Henrissat 1995) depending on the type of enzyme and the product is expelled. The processive cycle is continued with multiple events of hydrolysis before finally dissociating from the chain and reinitiating the processive cycle at a new site (Payne et al. 2015). The processive mechanism for exocellulase-mediated cellulose hydrolysis is represented in Fig. 1.2.

In the case of endoglucanases, the processive cycle is different in that the chain threading and product expulsion are omitted. The binding site of endoglucanase has a cleft instead of a tunnel, which allows chain acquisition without threading. It is now known that cellulose hydrolysis by the exoglucanases proceeds by movement

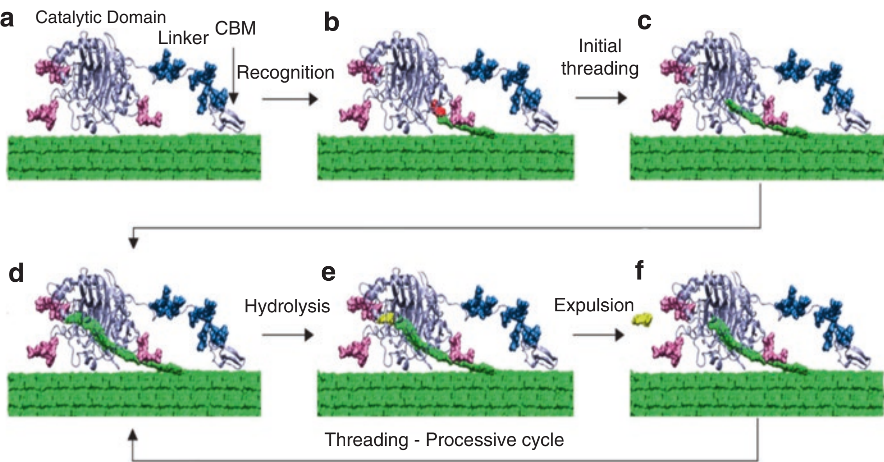

Fig. 1.2 Mechanism of processive cellulose hydrolysis by *T. reesei* cellobiohydrolase (Cel7A). (**a**) Enzyme binding to cellulose (**b**) recognition of the reducing end of a glucan chain (**c**) initial threading of the glucan chain into the catalytic tunnel (**d**) formation of CAC by threading (**e**) processive hydrolytic cycle showing product formation (cellobiose shown in *yellow*) (**f**) product expulsion (Reproduced from Beckham et al. (2011), with permission from Elsevier)

of the enzyme through the cellulose surface while the glucan chain is threaded to the active site tunnel, and this movement requires that the cellulose chain is threaded and is being hydrolyzed (Igarashi et al. 2009). It is considered that the rate-limiting step in processive CBH action is the dissociation from cellulose chains. Processively acting CBH molecules can get stalled at amorphous regions of cellulose and this leads to a diminished hydrolysis rate (Praestgaard et al. 2011). The renewed concept on the role of EGs is that they are acting not only to generate reducing and nonreducing ends and thus helping CBH to attach, but also to help CBH dissociate from the cellulose chain when they encounter amorphous regions during their processive action (Jalak et al. 2012).

In light of the above findings, the roles of different cellulases may have to be redefined. EGs and LPMOs do the endo-initiation in the amorphous and crystalline regions of cellulose respectively by breaking down the glycosidic bonds. Endo-initiation is also aided by the exoglucanases. The liberated reducing and nonreducing ends are attacked respectively by cellobiohydrolase I and II, which act in a processive fashion to liberate cellobiose units, and the cellobiose units are eventually cleaved to glucose by the beta glucosidases (Fig. 1.3).

1.2.1.7 Hemicellulases

Hemicellulose is a hetero-polysaccharide made up of various carbohydrate monomers having different linkages and substitutions, and its structure and composition changes with the plant source and geographical origin (Juturu and Wu 2012). The different types of hemicelluloses recognized include xyloglucans, xylans, ferulate

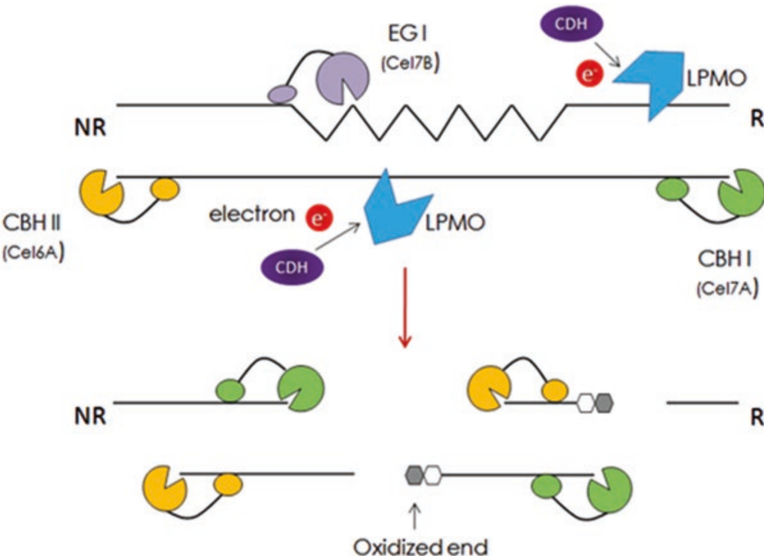

Fig. 1.3 Mechanism of cellulose hydrolysis. Present concept on the hydrolysis of cellulose by filamentous fungi incorporating hydrolytic and oxidative breakdown. Action of LPMO requires an electron donor which in this case is the cellobiose dehydrogenase (CDH) enzyme. LPMO action liberates a new chain end which is oxidized. *R* reducing end, *NR* Nonreducing end

esters, mannans, glucomannans, and β-1,3 and β-1,4 glucans (Scheller and Ulvskov 2010). While xylans are the major components of hemicellulose in hardwood and herbaceous plants, mannans form the major component in the hemicellulose of softwoods. All of the xylans of the higher plants are based on a β-1,4-linked xylopyranose backbone which is substituted with acetyl groups and other sugar residues (Fig. 1.4) (Dodd and Cann 2009). The heterogeneous nature of hemicellulose necessitates the requirement of multiple enzymes that act synergistically and/or sequentially. Different microorganisms employ different strategies for degradation of hemicellulose. Several of the biomass-degrading filamentous fungi secrete an entire cocktail of hemicellulases together, and these act synergistically on the hemicellulose to break down the polymer into its monomers. On the other hand, aerobic bacteria accomplish this in two stages, where the first step is the secretion of enzymes that break the hemicellulose backbone and release oligomers; the second one is its further cleavage to monomeric sugars by cell-wall-bound or intracellular enzymes. In yet another strategy, anaerobic bacteria uses cellulosome-like structures to hydrolyze hemicellulose (Shallom and Shoham 2003).

The major hemicellulose-degrading enzymes are the enzymes which break down the xylan backbone (endo- and exoxylanases and β-xylosidases) and the side chains (arabinofuranosidases, glucuronidases, acetyl xylan esterases, ferulic acid esterases, and alpha galactosidases). A total degradation of xylan requires the synergistic action of mainly endoxylanases, which cleaves the β-1,4 xylose linkages of xylan backbone; exoxylanases, which hydrolyzes β-1,4 linkages of xylan from the

Fig. 1.4 Xylan structure (Reproduced from Dodd and Cann 2009, with permission from John Wiley & Sons)

reducing ends releasing xylooligosaccharides; and β-xylosidases, which cleaves the xylobiose and xylooligosaccharides to release xylose. In addition, the enzymes α-arabinofuranosidases and glucuronidases remove arabinose and 4-*O*-methyl glucuronic acid substituents from the xylose backbone, and the esterases – acetylxylan esterase, ferulic acid esterase, and ρ-coumaric acid esterase – hydrolyze the ester-bonded substituents – acetic acid, ferulic acid, and ρ-coumaric acid – from the xylan (Sukumaran 2009). An overview of the functions of various hemicellulases as provided by Juturu and Wu (2013) is given in Table 1.1.

Endoxylanase (EX, EC 3.2.1.8) hydrolyzes the xylan backbone and has catalytic cores belonging to GH families 8,10,11,30, and 43 with the most common ones being GH 10 and 11. These differ in their substrate specificities and the GH10 is more active on substituted xylan. Similar to cellulases, they may also contain CBMs (Sweeney and Xu 2012). Endoxylanases randomly cleave the xylan backbone from inside releasing long chain xylooligomers on which the β-xylosidases act. β-Xylosidase or xylan-1,4-β-xylosidase (BX, EC 3.2.1.37) acts on the xylo oligosaccharides and xylobiose released by BX to form xylose. These enzymes have catalytic cores belonging to the GH3, 30, 39, 43, 52, and 54 families. These two enzymes are often collectively called xylanases. A third class of enzyme which acts on the xylan backbone is also recognized and is called the exoxylanase that hydrolyzes short chain xylo oligomers acting from the reducing end. Unlike the BX, these are inactive on xylobiose and are also inert on pure polymeric xylan (Juturu and Wu 2014). Hemicellulases are frequently blended to cellulases in commercial biomass-hydrolyzing enzymes due to their ability to synergize with cellulases. However, the need for hemicellulase addition depends on the type of pretreatment employed as well, since some pretreatments (e.g., acid) remove the hemicellulose portion

Table 1.1 Hemicellulose-degrading enzymes and their native functions

Enzyme type	Native function	Action sites
(I) Glycosyl hydrolases		
Endoxylanase	cleaves β-1,4 bond of xylan backbone releasing xylooligomers	β-1,4 xylan backbone
β-Xylosidase	cleaves exo β-1,4 bond of xylooligomers releasing xylose	β-1,4 xylooligomers
Endo-1,4-mannanase	cleaves β-1,4 bond of mannan releasing mannan oligomers	β-1,4 mannan
β-Mannosidase	cleaves exo β-1,4 bond of mannan oligomers releasing mannose	β-1,4 mannan oligomers
α-L-Arabinofuranosidase	cleaves arabinan at O-2 and O-3 positions on xylan backbone	α-L-arabinofuranosyl oligomers
α-L-Arabinanase	cleaves xylooligomers generating arabinose	α-1,5-arabinan
α-D-Glucuronidase	cleaves α-1, 2 bond between glucuronic acid side chain substitutions releasing glucuronic acid	4-*O*-methyl-α-glucuronic acid
(II) Carbohydrate esterases		
Acetyl xylan esterase	cleaves acetyl side chain substitutions releasing acetic acid	2- or 3-*O*-acetyl xylan
Feruloyl xylan esterase	cleaves ferulic acid side chain substitutions releasing ferulic acid	Ferulic acid substitutions

Table reproduced from Juturu and Wu (2013), with permission from John Wiley & Sons

completely, thereby reducing the requirement of hemicellulases. Nevertheless, hemicellulases are a major component of the biomass-hydrolyzing enzymes, since the pretreatment methods are seldom capable of complete removal of hemicellulose, and there are multitudes of pretreatment regimes that result in intact or near intact hemicellulose component. Also the understanding of hemicellulases cannot be regarded as complete and we still have hemicellulose-active enzymes whose role and mode of action is ambiguous (Tenkanen et al. 2013).

1.2.1.8 Lignin-Degrading Enzymes

Most of the cellulolytic organisms produce oxidoreductases as part of the lignocellulolytic machinery and the main function of these enzymes is considered to be degradation of lignin (Sweeney and Xu 2012). Lignin degradation is important for access of cellulases and hemicellulases to the carbohydrate polymers and also in diminishing the inactivation of these enzymes through nonproductive binding on lignin. In biofuel production, the major applications of lignin-degrading enzymes are considered to be delignification and detoxification. Delignification applies to the pretreatment of biomass to remove lignin, whereas the detoxification is relevant in the context of post-hydrolysis processing of biomass to remove potential inhibitors of fermentation (Placido and Capareda 2015). While there are different

microorganisms that produce lignin-degrading enzymes, white rot fungi are considered to be the most efficient producers. Most of the current understanding on lignin-degrading enzymes has emerged from studies on the enzymes of white rot fungi. In these organisms the ligninolytic enzyme system consists of three major classes, namely, laccases, manganese peroxidases, and lignin peroxidases.

Laccases (EC 1.10.3.2) or benzene diol oxygen oxidoreductases are oxidoreductases which enjoy wide distribution among microorganisms. These are enzymes having copper in their active site and are generally classified as multicopper oxidases or blue multicopper oxidases (Rodríguez Couto and Toca Herrera 2006). They employ oxygen as an oxidizing agent and cofactor. Laccases have low substrate specificity and therefore can degrade several compounds having phenolic structure (Placido and Capareda 2015). Other major enzymes that act on lignin – lignin peroxidase (LiP) and manganese peroxidase (MnP) – are heme peroxidases having protoporphyrin IX as the prosthetic group. Lignin peroxidases (EC1.11.1.14) are capable of oxidizing sites of very high redox potential including moderately activated aromatic rings of nonphenolic model lignin compounds. Manganese peroxidase (EC 1.11.1.13) on the other hand cannot oxidize nonphenolic lignin model compounds and depend on the generation of Mn3+ as a diffusible charge transfer mediator. Yet another peroxidase is the versatile peroxidase described in the fungus *Phanerochaete chrysosporium* and capable of both LiP and MnP activities (Fischer and Fong 2014).

Direct use of lignin-degrading enzymes in biomass hydrolysis is not practiced and often the applications of these enzymes are in the delignification of biomass as a pretreatment step. Here again, the pretreatment is more often accomplished by whole microorganisms elaborating ligninases rather than use of their enzymes in isolation. Nevertheless, there are several successful reports on the use of enzymes in isolation for delignification (Gutiérrez et al. 2012; Wang et al. 2013). Mostly laccases are employed for such applications, though MnP, LiP, or combinations of these enzymes may be employed. Another major application of ligninases is the detoxification of the biomass hydrolysates. Several of the conventional pretreatment processes generate toxic compounds classified as furan derivatives, sugar degradation products, weak acids, and phenolic compounds from lignin. These compounds can affect the growth and ethanol production by yeasts or other microbes used for bioethanol production and are sometimes removed prior to fermentation so that the ethanol yields are improved. Ligninolytic enzymes are an efficient means of degrading these inhibitors and offer the advantages of reduced or no sugar loss, ambient conditions of operation. Mostly phenolic compounds are removed, while lesser known ligninolytic enzymes like aryl-alcohol oxidases (AAOs) are being investigated for removal of furan derivatives (Carro et al. 2015). Detailed reviews on the ligninolytic enzymes and their applications for biofuels may be found in Placido and Capareda (2015) and Fisher and Fong (2014).

1.3 Microbial Production of Cellulases and the Systems for Cellulose Hydrolysis

While cellulases, hemicellulases, ligninolytic enzymes, and a myriad of different accessory enzymes and proteins are involved in the hydrolysis of lignocellulosic biomass in nature, not all of them are used in the preparations for commercial hydrolysis of biomass for biorefinery applications. In commercial preparations of biomass-hydrolyzing enzymes, enzymes are only used in their crude form, the only processing steps employed being concentration, stabilization, and formulation. Major attention is often only given to cellulases, though it is also implied that other enzymes are present in the preparations, since there are no elaborate purification steps involved. Often cellulase preparations contain hemicellulases, LPMOs, ligninolytic enzymes, etc. depending on the source organisms and techniques employed for production. It may be noted that the most commonly employed microorganism for cellulase production is *Trichoderma reesei*, which is often genetically modified for enhanced cellulase expression and is derepressed for carbon catabolite repression. *T. reesei* is limited in its ability for synthesis of beta glucosidase, and, often in biomass hydrolyzing blends BGL and xylanase from heterogeneous sources are added to make the enzyme more effective. Since cellulases are the major determinants of the efficacy of the biomass-hydrolyzing enzyme cocktails, the current discussion is limited to cellulases. Detailed discussion on hemicellulases and other enzymes may be found in Shalom and Shoham (2003), Juturu and Wu (2013), and Placido and Capareda (2015).

Ability to degrade cellulose is not a common trait among microorganisms and only a few specialized microorganisms – mostly bacteria and filamentous fungi – are capable of cellulose depolymerization (Quiroz-Castañeda and Folch-Mallol 2013). The machinery for cellulose degradation is radically different in the anaerobic bacteria and the rest of the organisms, and these involve cell wall-bound complex structures known as cellulosomes. Aerobic bacteria and filamentous fungi normally secrete a complex array of free enzymes that act synergistically to convert cellulose like the *T. reesei* cellulases. In yet another mechanism recently hypothesized, certain bacteria (e.g., *Fibrobacter succinogenes*) found in the rumen of herbivores use a mechanism involving both cell wall-bound and free enzymes for cellulose hydrolysis (Burnet et al. 2015). The following discussion will describe the free and bound systems of cellulose hydrolysis in the context of microbial degradation of cellulose, and the third mechanism shall be introduced separately.

1.3.1 Cellulose Hydrolysis Through Cell Wall-Bound Enzyme Complexes

Cell wall-bound cellulase-degrading enzyme complexes called cellulosomes are employed by several anaerobic bacteria for breaking down cellulose in nature. These include several Clostridia including the typical strain *Clostridium thermocellum*. The other common anaerobic bacteria include *C. cellulovorans, C.*

cellulolyticum, C. acetobutylicum, Acetivibrio celluloyticus, Bacteriodes cellulosolvans, Ruminococcus albus, R. flavifaciens, etc. (Fontes and Gilbert 2010). There are also anaerobic fungi like *Neocalimastix, Pyromices*, and *Orpinomyces* which employ the cellulosomes for degradation of celluloses (Haitjema et al. 2014). These systems employed by the anaerobic microorganisms are called "complexed systems" as the cellulosomes are multiprotein complexes anchored to the microbial cell wall. Cellulosomes are the largest extracellular enzyme complexes found in nature, and there are polycellulosomes as large as 100 MDa (Doi and Kosugi 2004). Cellulosome contain high-molecular weight noncatalytic proteins called scaffoldin onto which the enzymes are attached. The modular cellulases and hemicellulases produced by anaerobic microbes contain a dockerin appended to the catalytic module (the enzyme) and a noncatalytic carbohydrate binding module (CBM) (Fontes and Gilbert 2010). Dockerins are proteins of ~70 aminoacids usually present in single copy at the C terminal end of cellulolytic enzymes. They serve the purpose of anchoring the enzyme to the large scaffoldin protein which bears modules called cohesins that directly bind the dockerins. Cohesins are modules that are ~150 residues in length and are present as internal repeats in the scaffoldin. Typically about 1–11 cohesin modules are found in a scaffoldin, and it is recognized that the interaction of cohesions with dockerins may not be highly specific allowing different dockerins (bearing different enzymes) to be assembled on the cellulosome complex. Also the scaffoldin molecules contain a cellulose-specific family 3 CBM and a C terminal divergent dockerin which serve respectively the functions of targeting the cellulosome to the cellulose and to the bacterial cell wall (Fontes and Gilbert 2010). A typical cellulosome assembly is represented in Fig. 1.5.

The co-localization of different enzymes and CBMs on the cellulosome allows them to act in close proximity on the cellulose surface, which in turn is proposed to enhance the hydrolytic ability (Resch et al. 2013).

In addition to the anaerobic cellulolytic bacteria inhabiting the rumen or gut microbiomes and aquatic environments, there are anaerobic fungi that are capable of efficient cellulose degradation. It is now known that an early branch of fungi belonging to the order *Neocallimastigomycota* inhabit the digestive tracts of mammalian and reptilian herbivores that consume highly fibrous diets (Haitjema et al. 2014). They are suggested to be responsible for 40–70% of plant biomass digestion in the ruminant and nonruminant herbivores (Akin et al. 1990). These fungi possess both the complexed and the free enzymes and are believed to act by developing a highly branched rhizoidal network of rhizomycelia that penetrates the substrate and exposes it for attack by the secreted cellulases (Haitjema et al. 2014). While the studies on anaerobic fungi have confirmed that the enzymes of these fungi can form large complexes and they encode fungal dockerin domains, more is yet to be known about the cohesins or scaffoldins in them. While most of the studies have identified fungal dockerin domains in the cellulolytic enzymes elaborated by the anaerobic fungi, the type and structure of scaffoldins have largely remained elusive. Recently, scaffoldins have been described in *Neocallimastix* (Wang et al. 2014). It is also known that the dockerins displayed by one enzyme can bind another cellulase from the same organism (Nagy et al. 2007), implying that the mechanism of cellulose

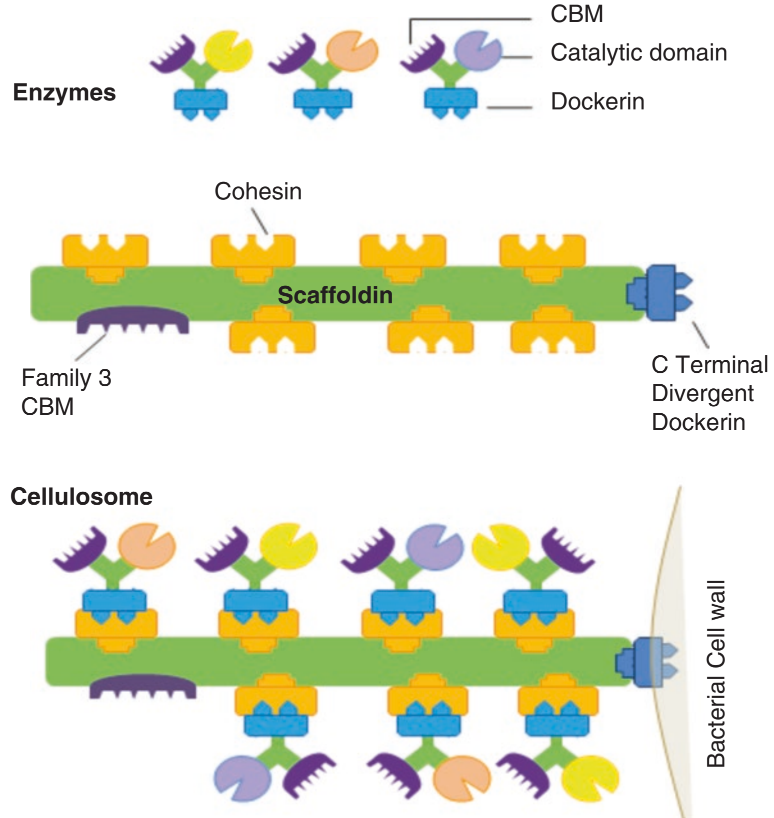

Fig. 1.5 Cellulosome assembly

hydrolysis employed by such fungi includes secreted complexes of enzymes in addition to the cell wall-bound cellulosomal complexes. The implications of cellulose hydrolysis by anaerobic fungi are presumably high because of the unique features displayed by these organisms by virtue of the unique ecosystem in which they survive. Anaerobic fungi are mostly residents of the rumen and alimentary tract of herbivores, and their growth and survival is dependent on their ability to metabolize plant biomass in a highly efficient and fast manner (Gruninger et al. 2014). This would also imply that their enzyme systems are capable of rapid turnovers within the shortest span of time and would make excellent candidates as components in biomass-hydrolyzing enzyme cocktails for biorefineries. In fact, this approach has been tried recently using a defined enzyme cocktail from *Orpinomyces sp.* strain C1A, which indicated that the enzyme cocktail from anaerobic fungus performed better than the commercial biomass-hydrolyzing enzymes at least in certain cases (Morrison et al. 2016). Apparently, the cellulosome paradigm for cellulose hydrolysis may provide newer and efficient means for biomass conversion either alone or in synergy with the free enzyme systems and warrants more focused studies.

1.3.2 Free/Noncomplexed Systems for Cellulose Hydrolysis

Noncomplexed or free cellulase/hemicellulase systems are the most common among known cellulose digestion paradigms. Soluble cellulases are employed by a wide array of cellulolytic bacteria and fungi, and this strategy is characteristic of aerobic microorganisms. Aerobic cellulose-degrading microbes are represented in several bacterial phyla as well as in several classes of fungi, and these are characterized by elaborate mechanisms of induction and repression control of the cellulase gene expression. The free enzyme paradigm represents the case where cellulolytic enzymes diffuse as single catalytic units often containing binding modules covalently attached through linker domains as known in the enzyme suite from the classical model organism for cellulase production – *Trichoderma reesei* (*Hypocrea jecorina*) (Payne et al. 2015). While several fungi can utilize cellulose as an energy source, only few strains are capable of secreting a complex of cellulases that have practical application in the hydrolysis of plant biomass, in the context of a lignocellulose biorefinery. Besides *T. reesei*, the other fungi known to produce high amounts of secreted cellulases include strains of *Aspergillius, Penicillium, Humicola,* and *Phanerocheate* (Sukumaran 2009). However, to date, most of the commercial cellulase preparations for biomass hydrolysis are produced using *Trichoderma reesei* and *Aspergillus niger.* The noncomplexed systems are characterized by the classical cellulose hydrolysis paradigm having secreted endo- and exoglucanases acting on the cellulose, and beta glucosidase cleaving the cellobiose generated by exoglucanase action. Most of the information on the noncomplexed systems has come through studies on the filamentous fungus *Trichoderma reesei*, the genetically modified strains of which are used currently for commercial production of cellulases. In *Trichoderma reesei*, there are two cellobiohydrolases, at least five endoglucanases, and seven beta glucosidases which are under tight regulation through induction and repression mechanisms. These enzymes secreted by the fungus act synergistically for hydrolysis of cellulose. In most of the fungi, the mechanisms of cellulose degradation and the mode of action of cellulases are similar to that in *Trichoderma*, while the regulation of cellulase gene expression might have organism-specific features. A more detailed discussion on the noncomplexed cellulase action is already presented in Sect. 1.2.

Aerobic bacteria are another important class of microorganisms that uses a free enzyme strategy for cellulose hydrolysis. These include the aerobic bacteria belonging to the order *Actinomycetales* (e.g., *Streptomyces lividans*), aerobic gram-positive bacteria (e.g., *Bacillus*, *Cellulomonas, Thermobifida*), aerobic gliding bacteria (e.g., *Cytophaga, Sporocytophaga*), gram negative (e.g., *Cellvibrio*), etc. (Quiroz-Castañeda and Folch-Mallol 2013). While most of the secreted cellulases contain carbohydrate-binding domains, there are several secreted cellulase which do not possess CBMs. Secreted cellulases, though a characteristic of aerobic cellulose-degrading organisms, are not unique to them and have been described even in the

anaerobic cellulosome-bearing microorganism *Clostridium thermocellum* (Berger et al. 2007).

1.3.3 Other Systems for Cellulose Hydrolysis

Certain microorganisms use strategies that do not fit into the complexed or non-complexed cellulose hydrolysis paradigms. Typical examples are the certain members of the genus *Caldicellulosiruptor* and the genus *Fibrobacter*, each of which possess a different strategy for cellulose hydrolysis other than the classical complexed or free enzyme paradigms. In *Caldicellulosiruptor bescii*, the cellulolytic machinery is midway between cellulosomes and free cellulases in that it consists of secreted cellulases that are multimodular, containing multiple binding and catalytic domains. The catalytic domains can be with different activities (Brunecky et al. 2013). The dominant cellulase in this organism – CelA – is a complex thermostable enzyme containing N terminal glycosyl hydrolase family 9 (GH9) endoglucanase domain, 3 GH3 CBMs, and a terminal C terminal GH48 catalytic domain, the latter an exoglucanase. The family 9 and 48 catalytic domains are highly synergistic as described in *Clostridium thermocellum* (Vazana et al. 2010). The multifunctional combination of enzyme activities is functionally distinct from cellulosomes and the *Caldicellulosiruptor* enzymes exist as free enzymes not associated with the cell (Young et al. 2014). CelA is now known to act both by conventional cellulase processivity and excavation of cavities into the surface of the biomass substrate (Brunecky et al. 2013).

Even more intriguing is the entirely different cellulolytic mechanism employed by the rumen bacterium *Fibrobacter succinogenes*. The organism contains genes for endoglucanases, but does not have genes for exoglucanases or processive endoglucanases, both of which are needed for release of cellobiose from cellulose chains (Ransom Jones et al. 2012). Interestingly, the anaerobic cellulose degrader does not possess a cellulosome; nor does it have CBMs on the enzymes it produces (Burnet et al. 2015). It is now known that certain outer membrane proteins in *F. succinogenes* called the fibro-slime proteins are involved (Jun et al. 2007), and these proteins along with type IV pilin structures mediate the attachment of the organism to the substrate (cellulose). Cellulose chains are broken by a protein complex present on the outer cell wall, which includes catalytic function, and the individual cellulose chains released through this are transported into the perisplasmic space via an ABC transporter. Once here, they are depolymerized by endoglucanases, eliminating the need for processive enzymes or exoglucanases (Wilson 2009). While a lot more is yet to be learned about the mechanism of cellulose degradation in *Fibrobacter*, there seems to be a consensus that this is one of the most efficient mechanisms for cellulose degradation among microbes and holds great promise for use in biomass conversion strategies. The different mechanisms employed by microorganisms are represented in Fig. 1.6.

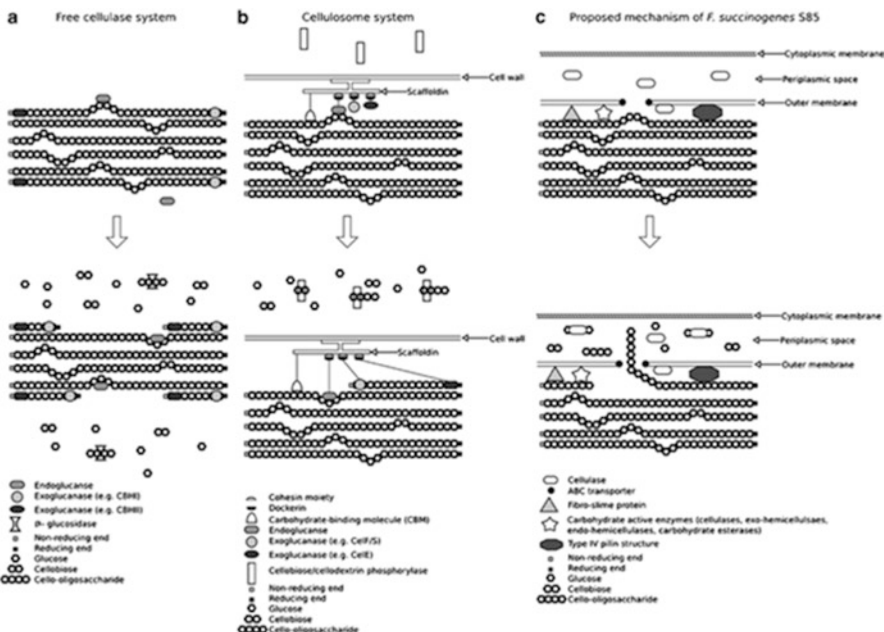

Fig. 1.6 Cellulose hydrolysis mechanisms adopted by microorganisms (Reproduced from Ransom-Jones et al. 2012, with permission from Springer)

1.4 Regulation of Cellulase Production

While cellulases are produced by a large number of bacteria and actinomycetes, fungi have remained the commercial sources of cellulases, since they are capable of secreting copious amounts of highly efficient enzymes. In several cases, it is also possible to mix and match different enzymes from different fungi and still obtain the synergies, and this has been especially true with the cellulases from *Trichoderma reesei* and the beta glucosidases from *Aspergillus niger. T. reesei* has remained the gold standard for industrial cellulase production, with the fungus capable of secreting more than 100 g/L of cellulase and the extracellular titers of protein reaching 30 g/L on the cellulose-inducing substrate – lactose (Durand et al. 1988; Bischof et al. 2016). The organism continues to amaze the scientific fraternity with its newly discovered capabilities, though it contains a remarkably lower number of carbohydrate-active enzymes (CAZymes) compared to several other cellulolytic fungi (Martinez et al. 2008). Because of these and several other advantages offered by this fungus most of the studies on cellulase gene regulation have been made in this organism. The presence of lignocellulose or the polymers derived from it can cause the induction of cellulase and hemicellulase genes in the fungus, and this is probably an adaptation to economize on energy and resources – synthesizing the enzymes only when needed. In the presence of an easily utilizable carbon source like glucose, the genes encoding cellulases and hemicellulases can be switched off – a phenomenon

known as carbon catabolite repression (Aro et al. 2005). Understanding the regulation of cellulase gene expression in fungi is important for designing strategies as well as for genetic manipulations to enhance cellulase production.

Production of most of the cellulases/hemicellulases is coordinately regulated and induced in the presence of natural cellulosic substrates. How the insoluble (hemi) cellulose polymers can induce gene expression was a question that intrigued researchers, and it was speculated from quite early the role of a diffusible small molecule inducer, originally identified as sophorose (2-*O*-β glucopyranosyl glucose), a disaccharide believed to be generated by transglycosylation activity of β-glucosidases (Sternberg and Mandels 1979, 1980). Other pure sugars/oligosaccharides like xylose, xylobiose, gentiobiose, sorbose, galactose, lactose, β-cellobiono 1, 5 lactone, etc. have been reported as inducers of (hemi)cellulases (Aro et al. 2005; Amore et al. 2013). The proposed mechanism of cellulase induction assumes that there is a basal-level expression of cellulases, especially Cel7A and Cel6A (cellobiohydrolase I and II), and the extracellular cellulase activity results in the generation of the soluble inducer. Evidence of this came from the work of Carle-Urioste et al. (1997), who demonstrated that the mRNAs of *cbh1* and *egl1* are transcribed under uninduced conditions and, on induction, the transcript numbers can increase to more than 1000-fold compared to the basal expression. It is proposed that the rate-limiting step in cellulase induction is the initial hydrolysis of cellulose. About 14 h is required for induction and this is thought to be because of the dependency on protein synthesis (Amore et al. 2013). Cel6A (CBH2) is the main surface-bound cellulase in *T. reesei* and is implicated in the synthesis of soluble inducer (Seiboth et al. 1992). Sophorose, the best-known inducer for cellulases, is synthesized from cellobiose through transglycosylation activity of β glucosidase (Vaheri et al. 1979). While the involvement of the major extracellular BGL – *Cel3A* – is proven (Fowler and Brown 1992), it is not the sole BGL that is implicated in the synthesis of sophorose (Amore et al. 2013).

Though the complete pathway of cellulase induction by sophorose is not known, sufficient information exists on the transcription factors that are involved in the regulation of cellulases and hemicellulases. Five transcription factors have been identified to be involved in the regulation of cellulases and these include the positive regulators XYR1, ACE 2, and HAP2/3/5 complex and the negative regulators ACE 1 and CRE1 (Kubicek et al. 2009). There are also recent reports on other factors that regulate the expression of cell wall-degrading enzymes in *T. reesei* which include the transcription factor BglR (Nitta et al. 2012) and the putative methyl transferase LAE1 (Seiboth et al. 2012b). The former is suggested to regulate the BGL genes whereas the latter is considered important for production of cellulases and hemicellulases, while the precise mechanism is still unclear (Häkkinen et al. 2014). Xyr1 acts as the central regulator of plant cell wall degradation in *T. reesei*, and controls the expression of CAZYme genes such as *xyn1*, *xyn2*, *bxl1*, *cbh1*, *cbh2*, *egl1*, and *bgl1*(Stricker et al. 2006). While XYR1 mediates the induction signal from various inducing carbon sources for these genes, its transcription is not induced by any of these inducers (Mach-Aigner et al. 2008). Deletion of *xyr1* results in lack of

induction of cellulases by cellulose and sophorose, indicating that the induction by these compounds requires Xyr1 (Akel et al. 2009). Regulation of *xyr1* expression seems to be mediated only by repression through the carbon catabolite repressor-CRE1 and not by induction mechanisms (Mach-Aigner et al. 2008). ACE 1 is believed to antagonize XYR 1 function by competing for one of its binding sites in the promoter of genes regulated by xyr1. Thus it is a negative regulator of cellulases and its deletion results in an enhanced expression of all the major cellulase and hemicellulase genes in sophorose- and cellulose-induced cultures (Aro et al. 2006). ACE 2 on the other hand is a positive regulator and Ace2 deletion has been shown to decrease the cellulase activity in cellulose-induced cultures, while there are no differences of gene expression in sophorose-induced cultures (Aro et al. 2001). ACE 2 can bind the promoter motif [GGC (T/A)4] in the *cbh1* promoter, which is also recognized by XYR1. It is believed that ACE2 binding to the promoter element requires phosphorylation and dimerization (Stricker et al. 2008). HAP 2/3/5 complex binds the CCAAT box, which is a common *cis* acting element found in the promoter and enhancer region of several eukaryotic genes (Zeilinger et al. 1998). The HAP 2/3/5 complex binding is believed to generate an open chromatin structure for complete transcriptional activation. Sophorose induction can cause the loss of nucleosome positioning in the promoter mediated by binding of the HAP 2/3/5 complex, which in turn makes the TATA box accessible (Zeilinger et al. 2003). Read together, the above details present a model for cellulase regulation in which the master regulator XYR1 is fine-tuned by regulators like ACE1 and ACE2. While XYR1 is nonsubstrate specific, ACE1 and ACE 2 are more specific, helping to fine-tune cellulase gene regulation. The role of the HAP 2/3/5 complex is proposed to be facilitating the binding of other factors to the cellulase promoter by removing nucleosome positioning on the DNA. More recently, the role of another cellulase regulator designated as ACE III has been proposed, which is essential for the expression of several cellulase genes (Häkkinen et al. 2014). Apparently, the knowledge about cellulase regulation has come a long way and is progressing, and this can have serious impacts on our understanding of plant cell wall degradation as well as on how fungal strains are developed for commercial production of biomass-degrading enzymes. The above discussion has been focused on the regulation of cellulases in *T. reesei*, and while it cannot be generalized, similar mechanisms of regulation exist in other fungi. More detailed discussions on the regulation of cellulase gene expression in *T. reesei* and other fungi may be found in Amore et al. (2013).

1.5 Cellulase Production Strategies

Production of cost-effective cellulase preparations for biomass hydrolysis is the major challenge in successful development of biofuels, despite the fact that there have been significant improvements in production technologies that have brought down the cost of enzymes per unit amount of ethanol produced from biomass. Also the cost evaluations of cellulases have been complicated due to the fact that there is scant information available in public about the technologies and raw materials used

for production of cellulase, and because the costs of enzymes are often reported in terms of price per unit quantity of ethanol, rather than the unit cost of enzyme (Marcuschamer et al. 2012; Liu et al. 2016). While techno-economic studies have indicated that rather than just lowering enzyme production costs, feasibility for biomass to ethanol conversion needs a comprehensive and broader addressing of multiple steps in biorefinery operation including better pretreatment, reduction of lignin and phenolics, use of more stable enzymes and reduction of residence times, lower enzyme loadings, and onsite enzyme production (Marcuschamer et al. 2012), the contribution of enzyme production cost is still significant. The following discussions will therefore address the common strategies for production and the recent developments aimed at reducing the cost of enzymes through multifaceted approaches.

1.5.1 Microbes Used for Industrial Production of Cellulases

Filamentous fungi, especially the ascomycetes fungi *Trichoderma reesei* and *Aspergillus niger* or their derivative strains, are used by enzyme companies like Novozymes, Genencor (DuPont), and Iogen for commercial production of cellulases, with the exception of Dyadic, which uses engineered strains of *Chrysoporium lucknowense* (Zhang and Zhang 2013). Most of the *Trichoderma reesei* strains currently in use for production of cellulases have been derived from the original isolate *Trichoderma* sp. strain QM6a isolated by the US Army Research Laboratories at Natick over 70 years ago. The strain was improved through random mutagenesis experiments at the Natick laboratory and Rutgers University, which resulted in the strain RUT-C30 with over 20-fold improvement in cellulase activity. This strain is still the prototype cellulase hyperproducer available in the public domain (Bischof et al. 2016). The titers of extracellular cellulase on cellulase-inducing carbon source – lactose – can reach 30 g/L as demonstrated by Durand et al. (1988). A direct comparison of the levels of cellulase production by different organisms is complicated by the different strategies of production employed, by the different substrates and inducers employed, and in some cases by the different units used for expressing their activity. Several such comparisons have been made, which have mostly concentrated on the published information, and information on the cellulase yields from studies in the public domain may be obtained from these reports (Sukumaran et al. 2005; Mathew et al. 2008; Chandel et al. 2012; Hansen et al. 2015; Cunha et al. 2016). The highest cellulase activities (as filter paper units, FPUs) have been recorded at about 10–15 FPUs/g substrate under solid-state fermentation (SSF) and about 13 FPUs/ml for submerged fermentation (Hansen et al. 2015). Lignocellulose hydrolysis requires the concerted action of several enzymes including the endoglucanases, cellobiohydrolases, β glucosidases, xylanases, β-xylosidases, and several other accessory enzymes; and no single organism can optimally produce all these enzymes. Hence it is a common practice to produce different enzymes using different organisms and the enzymes are blended to form cocktails with better efficiency for biomass hydrolysis. Typically for *T. reesei*

cellulase preparations, BGL from *Aspergillus niger* is blended, which makes up for *T. reesei*'s lower BGL activity and product inhibition. Apparently, the production of biomass-hydrolyzing enzymes is a complex process which has to take care of fungal growth parameters, ideal conditions for the optimal enzyme production, and balancing the costs involved. The following discussions will concentrate on the technologies used for production of cellulases.

1.5.2 Fermentation Strategies

Commercial production of cellulases typically employs the submerged fermentation (SmF) strategy while solid-state fermentation (SSF) processes are also used by companies (Sukumaran et al. 2005). Each method has its own advantages and disadvantages and the question of a best method is rather irrelevant, since the method adopted would depend on the organism employed, substrate used, growth conditions, existing infrastructure if any, need for purification, etc. While SSF is claimed to be the cheaper method for microbial enzyme production by many (e.g., Tengerdy 1996; Castilho et al. 2000, Viniegra-González et al. 2003; Krishna 2005), there are also reports that state the contrary (Nakkeeran et al. 2012). Also it should be noted that the enzymes elaborated by the same organism can be different in SmF and SSF (Hansen et al. 2015), and the cost of production also needs to be read along with the efficiency of the enzyme in catalyzing the desired reaction. In this context the production costs are tightly connected to the productivity of the enzyme-producing strain and the final activity yield. The hydrolytic efficiency of the multienzyme complex depends on both the properties of individual enzymes and synergies between them and their ratio in the cocktail (Gusakov et al. 2014).

1.5.3 Solid-State Fermentation (SSF)

Solid-state fermentation is defined as fermentation with near absence of free water but substrate moistened to support growth of microorganisms (Pandey 2003). The substrate can either be a natural organic material like wheat bran or an inert support material impregnated or coated with the liquid growth medium (Ooijkaas et al. 2000). SSF processes are closer to the natural conditions of growth of most of the filamentous fungi and are hence expected to be ideal for production of enzymes that are required for plant cell wall degradation. There have been a large number of studies on solid-state fermentation for cellulase production reported in the literature. Even earlier studies like those by Chahal (1985) have demonstrated the higher yields of enzyme in SSF compared to SmF, and there have been reports which indicated significant cost reductions for SSF compared to SmF (Tengerdy 1996). Solid-state fermentation is typically performed by inoculating a natural substrate (e.g., wheat bran) moistened/impregnated with the suitable medium that carries essential nutrients – especially nitrogen source since in the majority of cases the substrate itself acts as the carbon source. Additional carbon source may be added for

enhancing the growth rate or the final fungal biomass achieved. Since cellulases are inducible enzymes, an inducer like pure cellulose or a small molecule inducer like lactose/cellobiose may also be added for enhanced production. One of the advantages of SSF for cellulase production is the possibility of using a lignocellulosic substrate for fermentation, which will serve as a carbon source as well as the inducer (Nigam and Singh 1996). The usage of the same raw material as the feedstock to be used in a biorefinery for cellulases production has the added advantage of a more appropriate enzyme cocktail being elaborated by the organism, and this approach has been tested successfully by many (Sukumaran et al. 2009; Roslan et al. 2011; Maeda et al. 2013; Pirota et al. 2014). It should be noted that the choice of carbon source has a significant impact on cellulase production under SSF since differential induction of cellulases in response to the type of carbon sources used is a common phenomenon in cellulase production by fungi.

The major advantages proposed for solid-state fermentation include (1) higher enzyme production associated with higher biomass, (2) lower product inhibition, (3) lower protease activity, (4) ability to use water-insoluble substrates as C and N sources, which allows use of cheap and abundant lignocellulosic biomass, and (5) concentrated enzyme (Viniegra-González et al. 2003; Hölker and Lenz 2005). It has also been shown that SSF offers a situation of lowered catabolite repression in comparison to SmF (Díaz-Godínez et al. 2001). The immobility of typical SSF cultures can be an advantage and disadvantage at the same time. Since this replicates a natural growth environment for filamentous fungi, without any shear (as would be encountered in agitated SmF cultures), there is better growth and less autolysis due to hyphal rupture. At the same time, this also results in mass and heat transfer limitations. As the fermentation proceeds on an organic substrate like wheat bran, the substrate is decomposed leading to collapse of pores and aggregation of particles, which lead to less oxygen supply to cells and lower heat dissipation, both of which affect the growth and enzyme production by fungi (Chen 2013). The classical setup used for solid-state fermentation for large-scale enzyme production is tray reactors, though other designs like drum reactors and packed bed reactors are also employed. A typical tray reactor setup includes several trays of optimal size kept in racks inside a climate-controlled chamber (Fig. 1.7). The trays may be constructed of stainless steel, aluminum, wood, or even plastic and may or may not have lids. The trays and lids in some setups are perforated to allow aeration and heat dissipation. The rooms are maintained at controlled temperature and humidity and often with forced circulation of HEPA filtered air (though this may not be essential). This configuration is easy to set up and easily scalable with low labor intensity.

The mixing and heat transfer issues associated with stationary tray fermenters can be addressed through use of rotating drum-type reactors which are either mixed continuously or intermittently and with or without forced aeration. These types of reactors were patented by the French company Lyven and the Indian company Biocon and have been in use for commercial enzyme production. There are also several other types of reactor designs in use, while tray reactors are the dominant types used in commercial SSF.

Fig. 1.7 Solid-State Fermentation Facility (Koji) Room (Courtesy – CSIR-NIIST, Trivandrum, India) Koji Room (SSF chamber) showing steel racks for keeping trays and climate control system. Trays showing fully grown *Penicillium janthinellum* culture ready for harvest (for cellulase production)

One of the major advantages of SSF is the possibility of using the fermented substrate as such in wet or dry form as the enzyme (Zhuang et al. 2007; Singhania et al. 2015). The elimination of the downstream processing step can result in significant cost reductions, but has other disadvantages like carryover of the spores/mycelia to the biomass hydrolysis step (leading to contamination and lower productivity) and the increase in solids loading in the hydrolysis reactor leading to inefficient mixing. Apparently, the choice of direct use of fermented material as enzyme or after extraction and removal of the fungal spores/mycelia depends on the conditions of hydrolysis. The latter, if performed at higher temperatures, can kill the mycelia and may prevent spore germination. Major disadvantages of SSF for cellulase production include the common disadvantages of SSF, which are the challenges in product purification, inability for complete automation and online monitoring of cell growth and enzyme production, providing heat and mass transfer, difficulty in mixing, necessity to keep the moisture content optimum, increased possibility for contamination (since SSF systems are not fully closed systems), etc. Moreover, heterogeneity and batch variations in solid substrates can have a serious impact on reproducibility. Also there are issues with scalability, with tray reactors occupying a large footprint compared to similar capacity SmF systems. Nevertheless, for production of enzymes like that used for biomass hydrolysis where purity is not a major concern, SSF systems might hold promise, since the enzymes produced on the same substrates to be used as feedstock for biofuels can yield enzymes which are more appropriate for the job, and at higher concentrations. The methodology also has the process advantages of lower water and energy consumption, reduced waste stream, less capital infrastructure, and the ability to use semiskilled labor (Zhuang et al. 2007). Recent reviews on the application of SSF technology for biomass-hydrolyzing enzymes may be found in Yoon et al. (2014), Farinas (2015), and Behera and Ray (2016).

1.5.4 Submerged Fermentation (SmF)

Submerged fermentation is by far the most commonly used technique for production of industrial enzymes, which also includes cellulases. SmF is preferred by the industry since it allows easy online monitoring of the process parameters and allows their control (Hansen et al. 2015). Heat and mass transfer are better in SmF, since the cells are dispersed in a very conductive aqueous environment and also due to the fact that the system allows for regulation of temperature with external heating/cooling besides having efficient mechanisms for mixing. Biomass-hydrolyzing enzymes from the common industrially employed cellulase producers (e.g., *Trichoderma reesei*) are inducible and the best activities are reported when grown in media containing cellulose. Untreated lignocellulosic substrates are generally found to support lesser enzyme yields compared to pure cellulose forms like Solka Floc or Avicell (Mathew et al. 2008). A range of different cellulosic materials has been tried as substrates for cellulase production and this includes pure celluloses (Hendy et al. 1982), paper pulp (Zhuang et al. 2007), corn cob residue (Liming and Xueliang 2004), sugar cane bagasse (Pereira et al. 2013), and even dairy manure (Wen et al. 2005). Most of these natural substrates are capable of inducing the cellulase systems in fungi often at par with known inducers or sometimes even better (Mathew et al. 2008). The choice of raw materials for cellulase production can influence both the yields and the final compositions of the cellulase preparation, which in turn will also affect the cost of enzyme preparations. Agro residues like corn cobs, rice or wheat straws, bagasse, etc. are cheap raw materials that can induce cellulase production due to their content of cellulose and hemicellulose polymers. However, they might need pretreatment for delignification before being used in cellulase production media. In a biorefinery context, especially for onsite enzyme production, this would not pose a big problem since the same feedstock that goes for biofuel production can serve as carbon source/inducer for cellulase production.

Media used for cellulase production have typically followed a composition after the original *T. reesei* medium used by Mandels and Weber (1969), but with modifications as appropriate for the strain/organism. Typically for *T. reesei* the fermentations are carried out in an acidic pH range with the optimal temperature being in the range of 25–30 °C (Mathew et al. 2008). Besides the carbon source, the choice as well as the concentration of nitrogen sources are known to affect cellulase production and a high C/N ratio is proposed to be conductive for enhanced cellulase production by *T. reesei* (Liming and Xueliang 2004). Stirred tank reactors are commonly employed for SmF production of cellulases, which has the advantages of better mixing and aeration. However, fungal fermentation can present very unique problems associated with the control of their morphology. The morphology of fungi in SmF may vary based on various factors including the type of organism, inoculum density, pH, presence of surface active agents, agitation rates, etc., and careful selection of parameters might be essential to maintain the optimal fungal morphology. In typical submerged fermentations, fungi may grow in pelleted form or they may be dispersed as mycelial mats or aggregates. The latter form is often a serious limitation since the aggregates result in mass transfer limitations and cell damage due to

shear. Also the mycelia may get entangled in the baffles of the reactor and can get accumulated on the fermenter wall wherever there is limited turbulence. The aggregates or mats can become really large, disallowing any penetration of substrates or oxygen into the core, leading to death of cells in the center of the aggregates. The pellets are highly entangled dense masses of hyphae which can assume sizes between a few hundred micrometers and several millimeters (Domingues et al. 2000). Pelleted growth is generally preferred in fungal fermentations since it allows better mass and oxygen transfer due to an even distribution and enhanced surface area. Optimal morphologies in fermentations are dependent on operational conditions and knowledge of these can aid in enhancing the productivities in SmF (Cui et al. 1998). It has been observed that higher inoculum densities can lead to smaller pellet size, translating to higher protein secretion and higher filter paper activity (Domingues et al. 2000). Similarly, in the same study it was observed that the presence of surfactant inhibited pellet formation by *T. reesei* RUT-C30. In another study which related cellulase productivity to fungal morphology, it was found that the buffers and pH conditions that promoted compact pellet formation resulted in enhanced enzyme yield (Ferreira et al. 2009). A pH of around 4.8 and 100 mM succinate buffer supported maximum cellulase yield by *T. reesei* RUT-C30 in this study (Ferreira et al. 2009).

High productivities and yields of cellulases at industrial scale require that the fermentation is conducted under carbon flux limitation under either fed batch or continuous mode (Jourdier et al. 2012). Pure forms of cellulose, lignocellulosic substrates like pretreated plant biomass materials, or soluble carbon sources that can induce cellulases like lactose are the common carbon sources used in commercial-scale production of cellulases. The typical processes can be batch, fed batch, or continuous. Lactose is used as the carbon source/inducer in commercial production of cellulases employing *Trichoderma*, the disaccharide being the most affordable among highly potent soluble inducers. While cultivation of the fungus in cellulose/lignocellulose is cheaper, control of glucose concentration becomes a major limitation. At low concentrations of (ligno)cellulose, glucose production might be too slow to meet the metabolic needs of active growth and enzyme production, while at high concentrations, a higher rate of glucose generation compared to its consumption can result in catabolite repression (England et al. 2010). Apparently to maintain the conditions of carbon flux limitation, fed batch or continuous mode becomes helpful (Jourdier et al. 2013). In fact, most of the commercial production of cellulases employs the fed batch strategy where a soluble inducer like lactose or a cellulosic substrate is carefully dosed into the fermentation medium at appropriate intervals. The use of insoluble inducers/carbon sources like pure cellulose, paper pulp, or any lignocellulosic substrate poses the additional challenge of mixing the medium in the production reactors. This is a very serious limitation when batch process has to be employed, and often the production strategy has to ensure a size reduction treatment before the insoluble substrate is fed to the reactor (Shin et al. 2000; Liming and Xueliang 2004). However, this can increase the production cost due to the need for pulverization of substrate. Here again, a fed batch process may help to maintain sufficient mixing, since the concentration of insoluble substrate

can be kept minimum and fed as per demand (Shibuya et al. 1981). The use of a soluble carbon source in lieu of (ligno) cellulose has obvious advantages as this will allow better mixing and aeration and greater control over the fermentation process. Also the fermentation would no longer be dependent on the hydrolysis of lignocellulose (Allen and Mortensen 1981). While sophorose is the best inducer of cellulase in *Trichoderma reesei*, it is also the most expensive and difficult to manufacture. Recently, Danisco Inc. has patented a process using concentrated glucose, containing appreciable quantities of sophorose as carbon source/inducer for cellulase production using *T. reesei* (England et al. 2015). Here the inducing mixture of sugars was generated through transglycosylation activity employing whole cellulase preparations from *T. reesei*.

It may be concluded that the choice of method for cellulase production depends on the end application and the cost evaluations also need to consider the efficacy of the enzyme preparation on the chosen lignocellulosic substrate. While SSF and SmF have their own advantages and disadvantages, it is not prudent to state one method is better than the other, since the choice is often made depending on the context of application, rather than on the mere advantages of a particular method.

1.6 Strategies for Cost-Efficient Cellulase Preparations

Attaining cost efficiency of biomass-hydrolyzing enzymes is a complex R&D problem considering the fact that the cost of cellulases for biomass conversion depends not just on the cost of their production, but also on various factors that determine their efficiency for the hydrolysis of biomass. The production costs of cellulase are tightly connected to the productivity of the enzyme-producing strain and the final activity yield in the fermentation broth. The hydrolytic efficiency of the multienzyme complex depends on both the properties of the individual enzymes and synergies between them, and their ratio in the cocktail (Gusakov et al. 2014). While the knowledge on the number and type of enzymes and proteins that are involved in biomass hydrolysis is far from complete, the minimum set of enzymes required is probably easy to gauge. Moreover, in actual practice, the enzymes used may never be in their pure form, which means that preparations enriched for a component enzyme would still contain other activities and accessory proteins, albeit in smaller amounts. This would probably take care of the requirements of those accessory activities which may be needed only in lesser proportions, in analogy with nature's own arsenal for plant cell wall degradation that uses the accessory activities in far lesser amounts compared to the main enzymes – cellobiohydrolases and endoglucanases. Commercial enzyme cocktails for biomass hydrolysis contain several different enzymes and accessory proteins of suboptimal activities and relative proportions, and it is important to know the optimal concentrations of each enzyme and their relative proportions to develop efficient cocktails for hydrolysis of biomass. For example, the typical *T. reesei* cellulase preparation, though rich in cellobiohydrolase, contains only low concentrations of beta glucosidase, and hence the biomass-hydrolyzing cocktails produced by enzyme manufactures often blend in large

quantities of heterologous β- glucosidase, generally sourced from *Aspergillus niger* (Knauf and Moniruzzaman 2004; Rana et al. 2014). It is often difficult to gauge the optimal combinations of the different component enzymes that give maximal hydrolysis of a given feedstock and hence the highest sugar concentrations. Mostly, the optimizations of enzyme cocktails have been empirical, with different mixtures being analyzed for their efficacy in hydrolysis of the target feedstock. This often presents the issue of having to try several combinations of component enzymes experimentally, before arriving at the optimal one. Recently, high throughput methods have been used for such optimizations and the US Great Lakes Bioenergy Research Center platform called the GENPLAT (Great Lakes Bioenergy Research Center Enzyme Platform) is an approach aimed at addressing this issue. GENPLAT allows rapid assays and optimization of enzyme mixture tailored for different pretreatments, feedstock, and combinations of them (Banerjee et al. 2010).

Cost reduction of commercial enzymes for biomass hydrolysis often has to employ a combination of different strategies that cover use of cost-effective substrates/carbon source, efficient fermentation strategy that minimizes catabolite repression, use of cheaper but efficient inducer(s),enzymes with improved activities and/or stability, enzyme modification to reduce nonspecific binding, development of cocktails with higher efficiency, additives that enhance the enzyme activity, enzyme reuse, and onsite production (Fig. 1.8).

As has been stated previously, the cost reduction of biomass-hydrolyzing enzymes is not just about reducing the production cost of cellulases, but more economy can be achieved through multiple steps that improve the hydrolysis step and through usage of lesser enzyme per unit biomass to be hydrolyzed (Marcuschamer et al. 2012; Liu et al. 2016). Nevertheless, a major contribution can definitely come from reduction of enzyme production cost. Cheaper fermentation strategy like solid-state fermentation may definitely improve the cost of production, but if the enzyme needs elaborate downstream processing, this may not be cost effective. Most of the biomass-hydrolyzing enzyme preparations are not used in their purified form and the only downstream processing steps would be filtration and concentration, since accessory activities are equally important as the main cellulase and hemicellulase activities and crude preparations often perform better for hydrolysis of pretreated biomass. Direct use of the moldy bran for biomass hydrolysis was already described under the section for solid-state fermentation (Zhuang et al. 2007, Singhania et al. 2015). The major advantage of solid-state fermentation can be the use of cost-effective substrate/inducers in the form of native or pretreated lignocellulosic biomass (Cunha et al. 2012). Cellulases being inducible enzymes, appropriate inducers are added in the fermentation medium for production of enzymes. In most of the industrial production scenarios using *T. reesei*, the common inducer added is lactose, which happens to be the only economically viable soluble inducer. It should be noted that the best inducer for *T. reesei* is sophorose, which is very expensive for any commercial-scale production. However, this issue could be resolved by generating the inducer the way nature does it: by transglycosylation of sugars mediated by beta glucosidases. The patent from Danisco describes exactly that, and large quantities of inducer (sophorose) were demonstrated to be generated

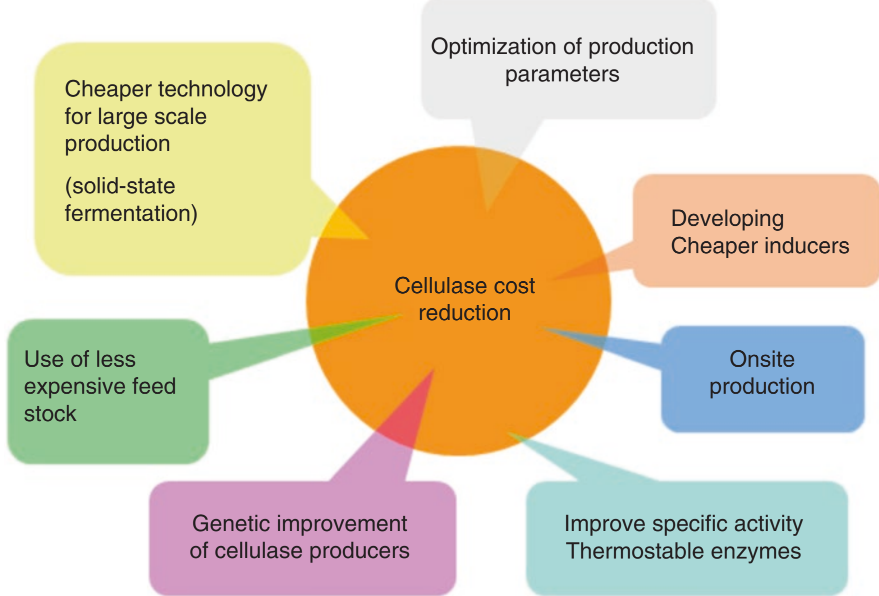

Fig. 1.8 Multipronged approaches for cellulase cost reduction

from sugars, through incubation with whole enzymes from *T. reesei* (England et al. 2015). While there are no studies to demonstrate this in other fungal systems, it may be speculated that similar systems exist in other fungi as well, since in nature, inducers have to be generated by the organisms themselves.

Commercial preparations of cellulase contain several additives which serve different functions, such as stabilization, protection against microbial attack, surface activity, etc., and the type and amount of these additives are often the industry's proprietary information. However, the general principles of protein stabilization and formulation are applicable in the case of cellulases and the major type of additives can very well be described. While an elaborate discussion on the protein stabilization in commercial preparations is beyond the scope of this chapter, the components that really add to the enhancement in cellulase activity either directly or indirectly are definitely worth mentioning. The most important among such additives are the amphiphiles, which are surface-active agents. Surfactants and proteins have been traditionally used in biomass-hydrolyzing enzyme preparations, and as additives in the hydrolysis process for enhancing the hydrolysis. However, the precise mechanisms by which they accomplish this are still obscure. It is believed that the amphiphiles enhance hydrolysis in multiple different ways, which include preventing nonspecific binding of enzymes to lignin, modification of biomass chemical structure, reduction in viscosity, surface tension and consequent reduction in enzyme contact with air liquid interface, etc. (Eckard et al. 2013a). Biomass pretreatment techniques are designed to remove either lignin or hemicellulose in general, and the degradation products and oligomers can seriously inhibit enzymatic hydrolysis.

Surfactants can form emulsions with the hydrophobic lignin and hemicellulose degradation products promoting lignin removal and allowing enzyme access to the feedstock's reaction sites (Tu et al. 2007a; Seo et al. 2011a). The role of surfactants in reducing the nonproductive adsorption of cellulase to nonproductive sites on biomass has long been recognized (Castanon and Wilke 1981) and has been proven in several systems (Zheng et al. 2008; Qing et al. 2010). Surfactants have now been demonstrated to also help the desorption of the nonspecifically bound cellulases, probably through a competitive mechanism (Li et al. 2016). Structural changes to lignocellulose by the surfactant Tween 20 contributing to enhanced hydrolysis were described by Seo et al. (2011b). In another recent study by Eckard et al. (2014), it was found that there is a reformation of the α-helix substructure of cellulase protein, which would be another potent contributor to the observed increase in hydrolysis activity. The same authors have also demonstrated that the surfactants can protect enzymes from thermal deactivation after extended incubation (Eckard et al. 2013b). Apparently, surfactants like Tween 80 and Tween 20 or polymeric surfactants like poly ethylene glycol and even proteins like bovine serum albumin or casein are probably good choices as additives in enzyme preparations as well as in biomass hydrolyses since they can enhance the sugars yields through one or all of the mechanisms described above. A more elaborate discussion on the role of amphiphiles may be found in Eckard et al. (2013a).

Improvement in the enzyme efficacy is yet another method to improve the cost of cellulase preparations, and changing the enzyme's hydrolytic turnovers, thermal stability, or affinities can impact the hydrolytic performance and hence the cost of hydrolysis. The major advantages projected for thermostable enzymes include (1) higher specific activity and stability, which allows the enzyme to perform for longer durations and reduction in the volume/quantity of enzyme to be used per unit amount of biomass; (2) better compatibility with processes and chemicals used for biomass (pre)treatments; (3) lower costs of cooling since hydrolysis can proceed at a higher temperature; (4) reduction in microbial contamination risks due to increased temperature of hydrolysis; (5) decreased fluid viscosity and hence better mass transfer; (6) ability to store enzyme at room temperature; and (7) greater flexibility for biorefinery process configurations (Yeoman et al. 2010). More than three times higher release of sugars has been reported with cellulase cocktails containing engineered thermostable endoglucanase from *T. reesei* at 60 °C (Trudeau et al. 2014). Pretreatment of biomass is usually performed at temperatures near or equal to 200 °C and the biomass needs to be cooled down before hydrolysis can be performed (typically at 50 °C). Having enzymes that perform at higher temperatures can significantly reduce the energy for cooling, thereby reducing the overall cost of ethanol production (Trudeau et al. 2016).

Reducing the nonspecific binding of enzyme to lignin is another important means to improve enzyme efficiency, and recently this has been achieved through modification of the linker peptide that connects the catalytic domain and carbohydrate-binding module in *T. reesei* Cel6A (Scott et al. 2016). This was achieved through modifications of the amino acids in the linker peptide so as to decrease the isoelectric point of the linker peptide and/or increase the ratio of threonine to serine in the

linker peptide, relative to the parental linker peptide. Modification of cellulases so as to have an increased net negative charge compared to the parental form has also been shown to be effective in reducing affinity to lignin and noncellulosic materials, with improved hydrolytic activity (Cascao-Pereira et al. 2013). Iogen Energy Corporation patented a modified cellulase with lower affinity for lignin and increased activity in its presence (Lavigne et al. 2010). The inventors discovered that substitution of a basic or charge-neutral amino acid at position 129, 322, 363, or 410 or of the amino acid at position 186 by a threonine results in a decrease in the extent of deactivation of the modified *T. reesei* Cel6A cellulase by lignin relative to that of a parental Cel6A cellulase from which it is derived. There is at least a 15% reduction in the extent of deactivation by lignin relative to that of a parental form and this decreased lignin inactivation contributes to increased activity for the hydrolysis of lignocellulose (Lavigne et al. 2010). Inhibition by glucose, the end product of biomass breakdown, is a major problem in the enzymatic hydrolysis of lignocellulose, and glucose-tolerant enzymes (enzymes that can act at glucose concentrations that are otherwise inhibitory to normal enzymes) are highly desired. Another patent application from Iogen described cellulase variants with reduced glucose inhibition (Lavigne et al. 2009). In yet another recent report, Cao et al. (2015) described engineering a highly glucose-tolerant beta glucosidase for thermostability and proved its efficacy in enhancing hydrolysis of sugar cane bagasse.

One of the most obvious means of enzyme cost reduction is the reuse of enzymes. After hydrolysis of lignocellulosic substrates, some amount of enzymes remains free in solution, whereas some still remain bound to the unhydrolyzed substrate/residues (Weiss et al. 2013). Ability to recover and reuse these can significantly reduce the cost of biomass conversion, since enzymes are the most costly reagents in biomass hydrolysis. There have been a large number of studies ranging from beta glucosidase immobilization on magnetite (Dekker 1990) to recycling of the entire unhydrolyzed residues (Visser et al. 2015). The major strategies in use for recycling of enzyme used for biomass hydrolysis include (1) immobilization of the enzymes, (2) ultrafiltration to recover free enzymes from hydrolysates, (3) readsorption of free enzymes from hydrolysates using fresh substrates, and (4) reuse of bound enzymes through recycling of residual substrates (Gomes et al. 2015; Visser et al. 2015). Immobilization as a recycling strategy can be applied effectively only to beta glucosidases since the mode of action of endo- and exoglucanases constrains their use in immobilized form. Apparently, most of the studies on immobilization of enzymes for biomass hydrolysis have concentrated on beta glucosidases (Dekker 1990; Tu et al. 2006; Song et al. 2016). Recapturing of free cellulase from hydrolysates through use of ultrafiltration has been described by several authors (Qi et al. 2012; Chen et al. 2013). Free enzymes from hydrolysates can also be captured back by readsorption onto fresh substrates. Here, fresh substrates can be added after completion of hydrolysis and a solid liquid separation step to recover the clear hydrolysates (Tu et al. 2007b; Shang et al. 2014). Alternatively, fresh substrates can be added into an ongoing hydrolysis process so that the enzyme preferentially adsorbs on the fresh substrates due to the higher cellulose content (Du et al. 2014; Huang et al. 2016). In the case of cellulase adsorbed on to the residual solids, recycling of the enzyme is effected by the recycling of part or all of the residues. The residues

after a solid liquid separation step are recovered and resuspended in fresh buffer followed by addition of fresh substrate (Rodrigues et al. 2012). However, direct recycling of residual substrate could be a problem, since with every cycle, the amount of lignin and unhydrolyzable material increases with resultant decrease in efficiency (Gomes et al. 2015). In both cases (recovery from hydrolysates and reuse of residues), the reuse of enzyme requires the addition of fresh beta glucosidase (BGL) since BGL do not have CBMs and hence recovery of this enzyme by adsorption is not practical. In a very recent work, Huang et al. (2016) have demonstrated a workaround by using an engineered organism expressing BGL and using it in simultaneous saccharification and fermentation with enzyme recovery by capture using fresh substrate. Needless to say, the recovery of cellulase from hydrolysates/residues is still a complicated art and needs to evolve to make it economically feasible.

Apart from all the above strategies which are dependent on the technical aspects of enzyme production and hydrolysis, one which is feasible and practiced is the onsite production of enzymes. This can have serious impacts on the costs of hydrolysis operation, since onsite production can do away with all logistic costs and the costs involved for purification and stabilization of enzymes and their storage, since enzyme production can be scheduled as per demand and used directly (Cunha et al. 2016). Different studies have shown the cost advantages of onsite enzyme production. Hong et al. (2013) estimated a 30% reduction in enzyme production cost, whereas Takimura et al. (2013) had estimated up to 70% reduction in cost compared to offsite production. A recent study performing the cost evaluation of cellulases for biomass hydrolysis based on Aspen Plus flow sheet simulation at a scale of 2000 tons of dry corn stover daily revealed that enzyme cost drives the price for bioethanol below the profitable margin, when enzyme is purchased from the market, even with the cheapest enzyme currently available on sale (Liu et al. 2016). The authors suggested that for profitable cellulosic ethanol production, novel and innovative strategies including onsite production should be explored and tested.

1.7 Perspectives and Conclusions

Lignocellulose is considered as one of the best options as a renewable feedstock for energy, especially liquid transportation fuels. Most of the technology options available today propose deconstruction of lignocellulosic biomass using enzymes to generate fermentable sugars which are then converted to ethanol or any other value-added products through microbial fermentation. Major technical limitations of the past are now being overcome and there are now companies claiming the commercial production of second-generation ethanol. While this is true, there is still no bioethanol available as a commercial commodity, and the price of bioethanol is publically disclosed. While a feasible pricing is claimed, several techno-economic analyses performed on the price development of lignocellulosic ethanol point to the high pricing needed to make the operation possible, and a major contributor to the operation cost is the cost of enzymes. Biomass-hydrolyzing enzyme preparations have evolved significantly over the past few decades and highly efficient cocktails

are now available, especially the enzymes from Novozymes and Genencor (Dupont). Still it is considered that the cost of enzymes is much larger than what is normally assumed and reported in the literature, and can be as high as 48% of the production cost of bioethanol. This highlights the importance of further active R&D on the biomass hydrolyzing enzymes, covering various aspects like improvement of enzyme performance and stability, reducing the production cost of enzymes, development of enzymes that do not exist naturally (e.g., enzyme chimeras, artificial cellulosomes), reducing inhibitions by lignin and glucose, reducing non-specific binding to lignin and improvements in the strategies for efficient usage of enzymes that include recovery and reuse of enzymes, and usage of minimal enzyme dosage achieved through predicting optimal cocktails. Several of the current studies show that the strategy cannot be successful by just addressing enzyme production costs, but needs an integrated approach which addresses issues like making the target biomass more susceptible to hydrolysis by adopting appropriate pretreatment strategies and the onsite production of enzyme for reduction of logistic and storage costs. One of the important conclusions that emerge is that the enzymes themselves need to be made more efficient and, for this, the use of modern biotechnological tools can be employed. Thus there could be enzymes with reduced binding to lignin, higher turnovers, and less inhibition by products. All these are possible only through a thorough understanding of the enzyme action and its synergies. It is important to note that there are still possibilities of as yet undiscovered novel activities in nature whose potential may be tapped. Knowledge is being gained on the structure-function relationship of biomass-hydrolyzing enzymes and this information can go a long way in deriving novel activities by engineering the existing enzymes for new features or creating entirely new functions like in the case of enzyme chimeras.

This chapter has tried to address biomass-hydrolyzing enzymes in the context of bioenergy and more specifically bioethanol and has tried to highlight the importance of these enzymes in future biorefineries. An overview is presented about the mode of action of these enzymes, especially the latest knowledge in this domain and also on the aspects of production of the enzymes. It also provides an overview on the regulation of expression of cellulases, the knowledge on which is critical for designing production strategies. From the foregoing discussion on these aspects and about the strategies on cost reduction of enzymatic hydrolysis, it emerges that there are still several gaps in the technologies for enzymatic biomass conversion, especially regarding the technical developments needed for bringing effective cost reduction. There is no single effective solution, and it is apparent that only an integrated approach covering various areas like engineering of enzymes to onsite production would make the enzymatic hydrolysis cost effective.

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Therapeutic Enzymes

2

Swaroop S. Kumar and Sabu Abdulhameed

Abstract

Enzymes as therapeutics hold a few advantages over non-enzymatic drugs with their amazing specificity towards targets as well as multiple substrate conversion. Development of enzyme therapeutics against rare diseases such as lysosomal storage disorders and severe combined immunodeficiency undoubtedly raised the hope of patients and improved their quality of life. Development of enzyme therapeutics against cardiovascular diseases witnessed a tremendous explosion in the past four to five decades and resulted in the development of the first approved genetically engineered drug against cardiovascular diseases (Activase®). Since then many recombinant cardiovascular drugs have been approved for clinical application. Often immunogenicity associated with enzyme drugs and the cost of production are major setbacks for their development. Despite their advantages only a few enzymes were approved by the Food and Drug Administration (FDA).

Keywords

Therapeutic enzymes • Plasminogen activators • Staphylokinase • Nattokinase • Velaglucerase alfa • Alglucosidase alfa • Serrapeptase • Rasburicase

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2.1 Introduction

Therapeutic usage of enzymes was in practice long ago with the application of crude preparations of enzymes as digestive aids for gastrointestinal diseases. The therapeutic potential of enzymes was first described when effective treatment of anthrax by crude secretions from *Bacillus pyocyaneus* was performed, suggesting that enzymes are responsible for the action. Now enzymes find clinical applications as cardiovascular agents, oncolytics, digestive aids, in the replacement therapy of lysosomal storage disorders, wound debridement therapeutics and so on. With the progression of enzymology in the late nineteenth century, therapeutic applications of newly discovered enzymes were explored apart from their use as digestive aids. Investigations on digestive enzyme trypsin resulted in its application in the treatment of diphtheria by the removal of fibrous membrane formed in the throat during the infection. Anti-cancer activity of trypsin was reported by John Beard, who proposed that defence against cancer was represented by pancreatic enzymes which are proteolytic in nature (Beard 1906). Attracted by Beard's hypothesis, physicians started injecting pancreatic enzymes for cancer treatment, but after his death, attention towards Beard's cancer treatment dropped. Trypsin was commercially available for oral administration as well as injection marketed by leading manufactures like Merck and Fairchild in the 1900s. In the 1920s Edward Howell observed that enzyme-rich raw food was reducing digestive load and promoting health. In 1932 he founded the National Enzyme Company for the production of enzyme supplements, substituting the enzymes lost while cooking and thus improving digestion.

The main advantage of an enzyme drug is its specificity. Enzymes specifically bind to target molecules, which make enzymes stand out from any other class of drugs. Also enzymes perform catalysis of multiple molecules. Therapeutic use of an enzyme against genetic diseases was initiated by De Duve for the treatment of lysosomal storage diseases (De Duve 1966). This opened the way for enzyme replacement therapy. In 1983 the Orphan Drug Act was passed in the USA to support the development of therapeutics against rare diseases for which suitable drug and treatment measures were not developed. For diseases such as lysosomal storage disorders due to lack of enzymes, this was a boon. Since then many of the therapeutic enzymes for the treatment of rare diseases have been developed.

The first genetically engineered drug to be approved was a recombinant tissue plasminogen activator alteplase (Activase®) in 1989. Later many cardiovascular enzymes were developed and approved by the USFDA. Adagen, used for the treatment of severe combined immunodeficiency (SCID), was the first therapeutic enzyme application against genetic disease. Enzyme therapies contribute a prominent share in clinical practice these days.

There are some limitations to therapeutic applications of enzymes as well. Primarily the large size makes it difficult to get distributed. This can be evaded by enzyme targeting. Some of the targeting methodologies include covalent linkage with specific molecules such as mannose-6-phosphate or development of an enzyme-monoclonal antibody complex. Secondly, the enzyme upon intravenous infusion was generally treated as a foreign substance by the body. This could elicit

immune response, contributing severe allergic reactions and life-threatening conditions. Covalent modification of enzymes by molecules such as polyethylene glycol is used to circumvent the immunogenicity. Another problem associated with enzymes in therapeutics is their relatively short half-life. Microencapsulation and artificial liposomal entrapment are some of the techniques used to increment the stability and half-life of enzyme drugs. Finally the purity of enzymes used for therapy is also a significant factor. This in turn makes enzyme drugs more expensive. However, advancements in drug development and delivery over the past few decades have revolutionized enzyme therapy. Newer drugs with improved stability and less antigenicity have been developed.

2.2 Enzymes for Treatment of Cardiovascular Diseases

Fibrin is a major protein formed during wound healing and is the end product of the blood clotting cascade. It prevents loss of blood during injuries. Enzymes like plasmin directly degrade fibrin. A balance between fibrin formation and removal is required for normal functioning of the system. However, when the balance shifts towards the improper accumulation of fibrin, thrombolytic diseases such as acute myocardial infarction and stroke occur. Thirty-one per cent of mortality worldwide in 2012 was due to cardiovascular diseases according to the World Health Organization.

Elimination of blood clot or thrombus is the key factor in thrombolytic therapy. Either of the therapeutic approaches, treatment with anti-coagulants (warfarin and heparin) or antiplatelets (dipyridamole and aspirin), or surgical treatment of thrombus or fibrinolytic enzyme therapy can be practised to remove or lyse the clot. Thrombolytic therapy using fibrinolytic enzyme has an advantage over anti-coagulants and antiplatelets, as the enzymes could act upon the existing clot. Thrombolytic enzymes are thus known as clot buster enzymes. Based on the mechanism of action they are of two types, viz. plasminogen activators (e.g. tissue-type plasminogen activator (tPA) and urokinase) and plasmin-like enzymes (e.g. nattokinase and lumbrokinase).

2.2.1 Plasminogen Activators

2.2.1.1 Tissue-Type Plasminogen Activator (tPA)

Tissue plasminogen activator (tPA) is a serine protease enzyme which converts plasminogen to plasmin and thus aids in clot dissolution. Tissue plasminogen activator consists of 527 amino acids with a molecular weight of 70 KDa (Pennica et al. 1983). Normally they are found in endothelial cells and assist in restoring the blood flow after thrombus is formed. Under certain physiological conditions fibrinolysis may not occur because plasminogen activators as well as plasmin are inhibited by circulating plasminogen activator inhibitors and α2 plasmin inhibitors. However,

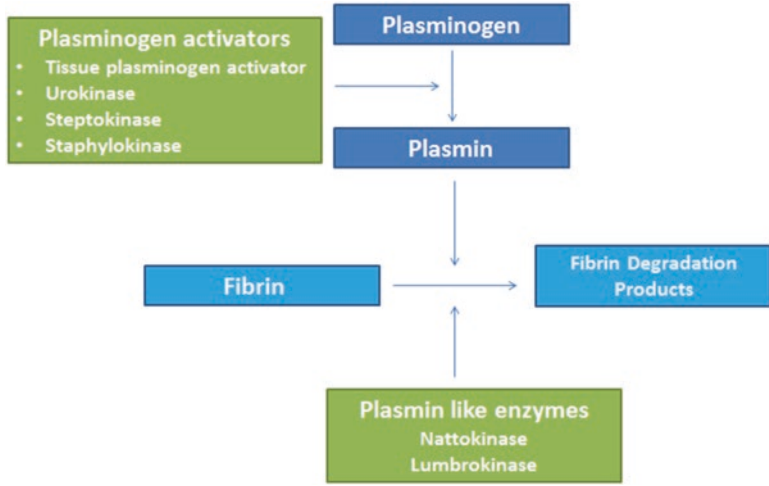


Fig. 2.1 A schematic representation of fibrinolytic enzyme therapy

therapeutic administration of plasminogen activator enzymes can overcome the effect, thus leading to thrombolysis.

Intravenous administration of tPA was approved by the FDA in 1996 and since then it has been in practice for acute stroke therapy. Therapeutic practice of tissue plasminogen activator (tPA) was initially for acute coronary artery occlusion. Recombinant tissue plasminogen activator (rtPA) production was achieved in 1981 by molecular cloning of the human tPA gene (Pennica et al. 1983). Recombinant tissue plasminogen activators (rtPAs) are available for therapeutic usage such as alteplase, reteplase and tenecteplase. Currently available PAs differ in their source, antigenicity, half-life and haemorrhagic potential (Fig. 2.1).

2.2.1.2 Alteplase

Intravenous administration of alteplase is a US Food and Drug Administration (USFDA)-approved medical therapy for the treatment of patients with acute ischaemic stroke, pulmonary embolism and acute myocardial infarction. In 1987 alteplase obtained approval as the first recombinant therapeutic enzyme. Alteplase is restricted to be used within 3 h after ischaemic stroke in patients younger than 80 years under European approval. However, shorter half-life of 4–8 min makes alteplase less suitable for bolus injections (Ali et al. 2014). Alteplase is commercially available as Activase®. Alteplase is also associated with some deleterious effects such as haemorrhagic transformation during ischaemic stroke, although it is efficient in treating the condition.

2.2.1.3 Reteplase

Another recombinant tissue plasminogen activator is reteplase. It is a shortened mutant single-chain polypeptide comprising 355 of 527 amino acid residues of tPA. Reteplase is commercially available as Retavase®. It has lower fibrin specificity

and lacks fibrin-binding domain. Though lower in specificity, it has a higher plasma half-life of around 11–14 min, which makes it better for bolus infusion treatment, and reteplase is much cheaper than tPA (Ali et al. 2014). Intracranial haemorrhage or bleeding problems are associated with reteplase treatment, similar to alteplase.

2.2.1.4 Tenecteplase

Tenecteplase is another recombinant mutant of tissue-type plasminogen activator (tPA) with multiple point mutations. It comprises 527 amino acids. Mutations include Asp103Thr, Glu117Asn and 4 alanine substitutions (Lys296Ala, His297Ala, Arg298Ala and Arg299Ala). These mutations increase half-life, resistance to plasminogen activator inhibitor 1 (80-fold) and fibrin specificity (14-fold) (Smalling 1996). The longer half-life makes it suitable for bolus administration. Also fewer bleeding complications were observed for tenecteplase treatment compared to alteplase. Tenectplase was approved by the USFDA in 2000 for the treatment of myocardial infarction. It is commercially available as TNKase (Fig. 2.2).

2.2.1.5 Urokinase Plasminogen Activator

Urokinase is another plasminogen activator used for treating cardiovascular diseases. An enzyme urokinase (UK) with a molecular weight of 54 kDa was isolated from urine which converts plasminogen to plasmin while situated inside thrombus and thus makes it protected against circulating antiplasmins. Also the absence of urokinase inhibitors in plasma makes it readily available at the site of action. UK has a prolonged half-life of about 15 min (Ali et al. 2014). Urokinase is a serine protease, and a heterodimer with polypeptide chains of 20 kDa and 34 kDa. It is secreted as a precursor molecule pro-urokinase, which is single chain and can be activated to two-chain urokinase by plasmin or kallikrein (Bernik 1973). Pro-urokinase (pro-UK) is effective for around 24 h after administration while urokinase gets inactivated within a few hours. Upon intravenous administration pro-UK attained complete fibrinolysis within 1.5 h while urokinase took 3 h for lysis. Pro-urokinase (pro-UK) is preferred over urokinase due to its improved selectivity towards fibrin and superior half-life over the latter (Zamarron et al. 1984). Recombinant pro-urokinase can be produced either in *Escherichia coli* or in mammalian cells. Prolyse is a recombinant pro-urokinase (rpro-UK) produced in the

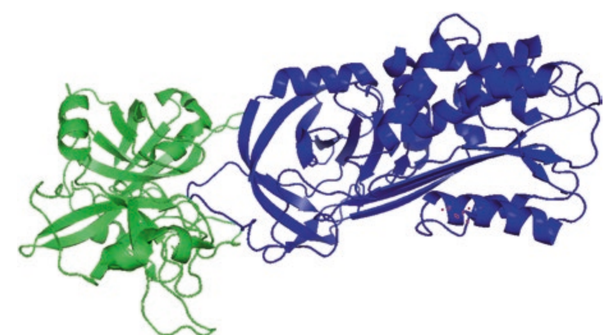


Fig. 2.2 Crystal structure of tissue-type plasminogen activator in complex with plasminogen activators inhibitor-1. Tissue-type plasminogen activator in green colour and plasminogen activators inhibitor-1 blue colour (pdb id: 5BRR)

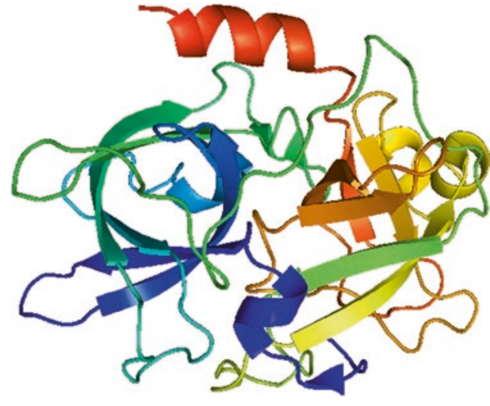


Fig. 2.3 Crystal structure of urokinase type plasminogen activator (pdb id: 1 W11)

murine hybridoma cell line and was found to be effective in stroke and myocardial infarctions (Ali et al. 2014) (Fig. 2.3).

2.2.1.6 Streptokinase (SK)

Streptokinase (SK) is a microbial plasminogen activator produced by different strains of β- haemolytic streptococci. It is a single-chain polypeptide of molecular mass 47 kDa. Streptokinase protein binds with plasminogen and converts it into fibrinolytic plasmin. SK has a better half-life of 18 min compared to tissue plasminogen activator (tPA) (Ali et al. 2014). SK elicits antigenic reactions such as hypotension and anaphylactic reactions. Upon treatment with SK, incidence of intracranial haemorrhage and bleeding complications is common (Goa et al. 1990). Many streptokinase-based thrombolytic drugs are available in the market such as Streptase®, Kabikinase®, Heberkinasa®, etc., and Heberkinasa® is a recombinant streptokinase. Despite substantial limitations with streptokinase therapy, its easy availability and lower cost make it a drug of choice for thrombolytic therapy in many underdeveloped countries.

2.2.1.7 Anistreplase

Anistreplase (anisoylated plasminogen streptokinase activator complex, (APSAC)) is a mixture of human plasminogen with an active site protected by acylation and streptokinase. Upon injection, the acyl group would be removed, leaving the plasminogen-streptokinase complex. This complex has more specificity towards plasminogen bound to clot. Unlike streptokinase which is inactive until circulating plasminogen binds, APSAC is a readily available plasminogen activator and doesn't require prolonged infusion. APSAC has a longer half-life (Smith et al. 1981). Its commercial name is Eminase®. Adverse effects associated with APSAC are similar to those of streptokinase. However, this product has recently been discontinued from application in the USA.

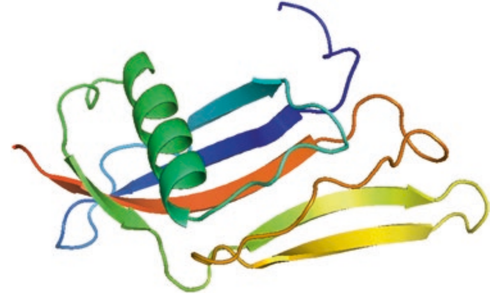


Fig. 2.4 Crystal structure of staphylokinase determined at 1.8 A^0 (pdb id: 2SAK)

2.2.1.8 Staphylokinase (SAK)

Staphylokinase (SAK) is an extracellular protein secreted by the majority of *Staphylococcus aureus* strains. It is a tissue plasminogen activator (tPA) and forms a complex with plasminogen to form plasmin. SAK is a monomer of 136 amino acids without disulphide bridges. Recombinant staphylokinase has shown higher fibrinolytic activity than fibrinogenolytic activity in comparison to streptokinase (SK) (Collen and Van de Werf 1993). Many SAK variants (rSAK) are produced with reduced immunogenicity by site-directed mutagenesis. They show higher fibrin specificity with fewer bleeding complications but haemorrhage may still occur in staphylokinase therapy (Fig. 2.4).

2.2.2 Plasmin-Like Enzymes

Tissue plasminogen activators such as tPA and urokinase are widely accepted for thrombolytic therapy, and their cost and unfavourable after effects such as bleeding complications and haemorrhage demand newer and safer thrombolytics. Plasmin-like fibrinolytic enzymes from various sources have been discovered like lumbrokinase from earthworms and fibrolase from snake venom. They are direct acting and do not require activation of plasmin through plasminogen. They are considered to be fit for oral administration. Most important among the microbial producers are those from the genus *Bacillus*.

2.2.2.1 Nattokinase or Subtilisin NAT

Nattokinase (NK) was isolated from fermented food natto which is an extracellular enzyme produced by *Bacillus subtilis natto* with fibrinolytic potential (Sumi et al. 1987). It is a cysteine-free serine protease with a molecular mass of 28 KDa comprising 275 amino acids. Later similar fibriolytic enzymes were obtained from other fermented foods such as Chungkook-jang soy sauce, doen-jang, douche, etc. Nattokinase or subtilisin NAT is an extracellular protein with fibrinolytic potential secreted by various organisms. Their producers include *Bacillus subtilis, B. amyloliquefaciens, B. licheniformis, B. amylosacchariticus, Flavibacterium, Pseudomonas sp.*, etc. NK shows higher similarity to subtilisin E (99.5%) and subtilisin J (99.3%) (Kurosawa et al. 2015). NK is a direct-acting fibrinolytic enzyme which not only

dissolves fibrin but also activates tPA to form plasmin, thereby increasing inherent fibrinolysis mechanism. Further, it shows higher specificity towards fibrin than fibrinogen. The stability of NK in the gastrointestinal tract makes it a more suitable candidate for oral administration as a potent cardiovascular disease neutraceutical. NK supplementation is also reported to have advantages in the prevention of hypertension. NK was found to be safer in both animal and human healthy volunteers, and oral consumption was not found to cause any side effects (Kim et al. 2008; Lampe and English 2016).

2.2.2.2 Lumbrokinase (LK)

Lumbrokinases are a group of complex protease enzymes isolated from various species of earthworms. Earthworms were used as traditional medicine in Asian countries like China and Korea over thousands of years. They are also referred to as earthworm powder enzymes as they were available in powder formulation. Lumbrokinases are regarded as potential therapeutics against thrombosis. Enzymes with fibrinolytic potential were first isolated and characterized in 1991 from an earthworm species *Lumbricus rubellus*. Later fibrinolytic enzymes were isolated from various species of earthworms such as *Lumbricus bimastus*, *Eisenia fetida*, *E. andrei*, etc other than *L. rubellus*. Six fibrinolytic enzymes were isolated from *L. rubellus* while seven enzymes were isolated from *E. fetida*. Lumbrokinase are more fibrin specific compared to other thrombolytics such as tPA and urokinase. LK also acts as a plasminogen activator by converting plasminogen into plasmin. Apart from clot dissolution it also reduced platelet aggregation and thinning of blood by reducing viscosity. Lumbrokinases have a molecular mass ranging from 20 to 35 KDa and show activity across a wide range of pH ranging from pH 1 to 11(Pan et al. 2010). LK is commercially available as Boluoke® and has been approved by the China Food and Drug Administration. LK is widely used in China against coronary heart disease, deep vein thrombosis, etc. It has also been used as an oral

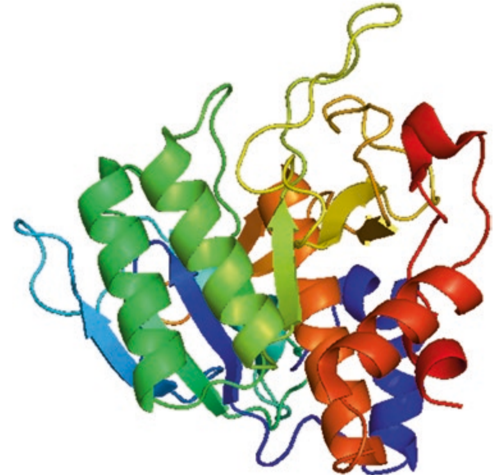


Fig. 2.5 Crystal structure of nattokinase from *Bacillus subtilis natto* (pdb id: 3DWW)

Table 2.1 FDA-approved enzymes for cardiovascular diseases

Enzyme/drugs	Brand name of approved drugs	Classification	Indications	Side effects
Alteplase	Activase®	Tissue-type plasminogen activator (tPA)	Acute myocardial infarction, acute ischemic stroke, pulmonary embolism	Bleeding, vomiting, low blood pressure (hypotension), dizziness
Reteplase	Retavase®	Tissue-type plasminogen activator (tPA)	Acute myocardial infarction	Nausea, vomiting, fever, injection site bleeding
Tenecteplase	TNKase® Metalyse®	Tissue-type plasminogen activator (tPA)	Acute myocardial infarction	Bleeding, nausea or dizziness
Urokinase	Abbokinase®	plasminogen activator	Acute myocardial infarction	Haemorrhage, anaphylaxis
	Kinlytic®			
Streptokinase	Streptase®, Kabikinase®, Heberkinasa®	plasminogen activator	Myocardial infarction, pulmonary embolism, deep vein thrombosis, arterial thrombosis or embolism	Bleeding, anaphylaxis
Anistreplase	Eminase®	plasminogen activator	Acute myocardial infarction	Bleeding, nausea, fever

cardiovascular supplement in many countries such as the USA, Canada and Japan. The main advantage of LK is that it is a safer therapeutic enzyme with no bleeding disorders. So far administration of LK has not been reported to cause any damage to kidney and liver. However, it has not received FDA approval as a thrombolytic drug to date (Fig. 2.5) (Table 2.1).

2.3 Enzymes for Treatment of Cancer

Enzyme therapy is also employed in cancer treatment and increased the survival rate of cancer patients. Cancer therapy involves prevention or destruction of cancer cell proliferation without damaging the normal cells. Enzyme therapy for cancer treatment can be either by the use of antineoplastic enzyme therapy or by the use of enzyme prodrug therapy. Amino acid deprivation methodology is a technique used

for anti-cancer therapy where depletion and thereby the induction of starvation of amino acids is attained in tumour cells which are auxotrophic to particular amino acids. This often reduces tumour proliferation. Enzyme prodrug therapy uses antibody-conjugated enzymes, converting prodrug into cytotoxic drug at tumour cells and thereby killing tumour cells.

2.3.1 Antineoplastic Enzyme Therapy

2.3.1.1 L-asparaginase

L-aspraginase is a widely used antineoplastic enzyme, and it is involved in the hydrolysis of L-asparagine to aspartate and ammonia. L-asparaginase is one of the most potent therapeutic agents against acute lymphoblastic leukaemia (ALL). Reduction of lymphomas was observed when administered with guinea pig serum in mice and it was later found that it was due to L-asparaginase. Tumour cells often depend on circulating L-asparagine for their survival as they lack the ability to synthesize L-asparagine, whereas normal cells with the help of L-asparagine synthetase produce the amino acid. L-asparaginase readily upon administration converts the circulating L-asparagine available. This makes tumour cells starved for the amino acid and eventually prevents cell proliferation. L-asparaginase was known to be isolated from various sources such as bacteria, fungi, plants and animals. Plant sources include *Pisum sativum, Withania somnifera,* etc. L-aspraginase has also been isolated from pancreas, spleen and kidneys of many animals. Bacterial producers include *Erwinia chrysanthemi*, *Escherichia coli*, *Serratia marcescens*, *Proteus vulgaris*, *Vibrio succinogenes*, etc. Pegasparagase enzyme (asparaginase complexed with polyethylene glycol (PEG) has a better half-life, increased stability and is safer for patients with allergy to native enzyme (Kurtzberg et al. 2011). Elspar® and Oncaspar® (Pegasparagase) are two commercially available forms of L-asparaginase. Oncaspar® received USFDA approval in 2006 for treatment of ALL (Fig. 2.6).

2.3.1.2 Arginine Deaminase

Another antineoplastic enzyme therapy is by arginine deprivation of cancer cells. Arginine is a non-essential amino acid synthesized by argininosuccinate synthetase (ASS) and argininosuccinate lyase from citrulline. In normal cases cells do not require arginine supply. But certain cancer cells lack ASS such as melanoma, renal cell carcinomas and hepatocellular carcinomas and they require circulating arginine. Arginine deiminase (ADI) is an enzyme from mycoplasma that converts arginine to citrulline, thereby reducing circulating arginine. This causes arginine deprivation in tumour cells that lack argininosuccinate synthetase, thereby inhibiting tumour progression (Kim et al. 2009). ADI itself is antigenic and enzyme ADI can be modified by attaching polyethylene glycol (PEG) (ADI-PEG 20) to reduce antigenicity and improve half-life. ADI-PEG20 was found to be effective for hepatocellular carcinoma, malignant melanoma and pancreatic cancer cells (Ensor et al. 2002) (Fig. 2.7).

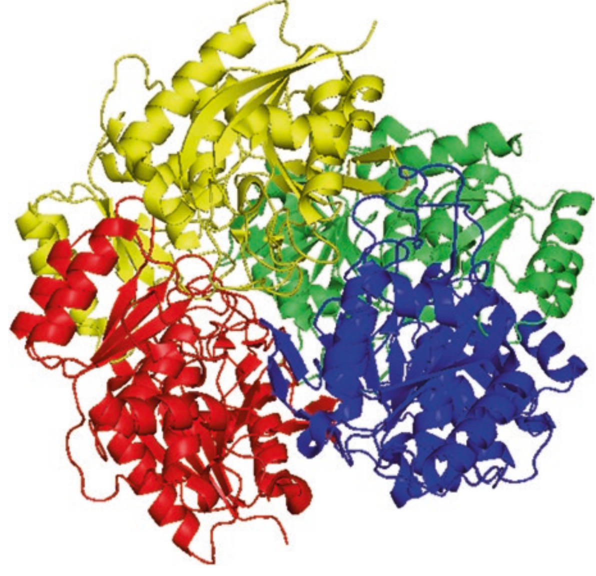


Fig. 2.6 Crystal structure of L-asparaginase homo tetramer from *Erwinia chrysanthemi* (pdb id: 1OJ7)

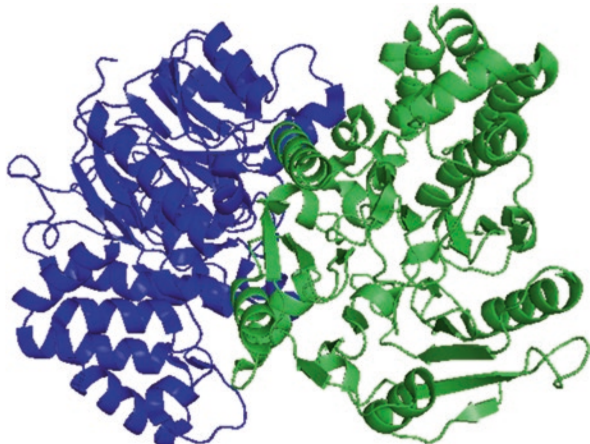


Fig. 2.7 Crystal structure of arginine deiminase homo dimer from *Mycoplasma arginini* (pdb id: 1LXY)

2.3.2 Antibody-Directed Enzyme Prodrug Therapy (ADEPT)

Another method to prove the role of enzymes in cancer treatment is by antibody-directed enzyme prodrug therapy. Due to the lack of tumour specificity of cytotoxic drugs, generating them specifically at tumour sites was considered. ADEPT involves administration of enzyme-conjugated anti-tumour monoclonal antibody which localizes at tumour sites and subsequent inoculation with non-cytotoxic prodrug, which upon reaching tumour sites will get converted to cytotoxic drug and thereby destroy tumour cells. Enzymes for prodrug therapy are mainly of three types: (1) Non-mammalian enzymes (without mammalian homologue); as no mammalian

Table 2.2 Enzymes for antibody-directed enzyme prodrug therapy (ADEPT), prodrugs and active cytotoxic drugs

Enzymes	Prodrug	Cytotoxic drug	Potential Targets
Carboxypeptidase G2	4-[bis-(2-chloroethyl)-amino] benzoyl-L-glutamic acid	4-[bis-(2-chloroethyl)-amino] benzoic acid	Colon cancer
Penicillin V amidase	Doxorubicin-N-p-hydroxyphenoxyacetamide	Doxorubicin	Lung carcinoma
	Melphalan-N-p-hydroxyphenoxyacetamide	Melphalan	Lung carcinoma
β-lactamase	5-fluorouracil-cephalosporin	5-fluorouracil	Colon cancer
Cytosine deaminase	5-fluorocytocine	5-fluorouracil	Colon cancer
β–glucuronidase,	Doxorubicin –glucuronide	Doxorubicin	Lung carcinoma
	Epirubicin-glucuronide	Epirubicin	Lung carcinoma
Carboxypeptidase A	Methotrexate-alpha-phenylalanine	Methotrexate	Lung carcinoma
Alkaline phosphatase	Etoposide phosphate	Etoposide	Colon cancer
	Mitomycin phosphate	Mitomycin	Colon cancer

homologue is present they deliver higher specificity towards the substrate. But as they are from a non-mammalian source, they elicit immunogenicity, which is the major disadvantage of these enzymes. A few of them include carboxypeptidase G2, penicillin G amidase, β-lactamase, cytosine deaminase, etc. (2) Non-mammalian enzymes (with mammalian homologue); though from a non-mammalian source they have their counterpart in humans, having a lower level of expression, so that enzymes administered will have more activity. β–glucuronidase, carboxyantigen and carboxypeptidase A are some among them. The homologous nature of these enzymes makes them generate lower immunogenicity. However, their specificity towards prodrug activation at tumour cells seems reduced. (3) Mammalian enzymes are also used in prodrug therapy where their mammalian nature eliminates risk of immunogenicity. But their major disadvantage is prodrug conversion, which may occur in normal tissues too. Some of the mammalian enzymes are alkaline phosphatase, α-galactosidase, etc (Table 2.2).

2.4 Enzymes as Digestive Aids

Digestive disorders are common, such as malabsorption, lactose intolerance and celiac disease. Enzyme supplementation is used to manage these digestive disorders as they are mostly due to lack of digestive enzymes. From the nineteenth century itself, pancreatic enzyme preparations were used as digestive aids.

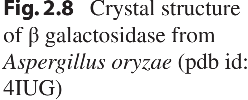
2.4.1 Pancreatic Enzyme Supplements for Malabosrption

Malabsorption is often caused by exocrine pancreatic insufficiency (EPI), referring to the inability or insufficient production of digestive enzymes by pancreas. EPI may occur due to various clinical conditions such as cystic fibrosis, chronic pancreatitis, pancreatic surgeries, etc. Pancreatic enzymes usually comprise amylase, lipase and protease. Protease digests proteins in food to peptides; amylase digests carbohydrates other than cellulose into oligo and disaccharides; while lipase hydrolyses fat into fatty acids and monoglycerides. Pancreatic amylase and protease deficiency are usually compensated by amylase from salivary enzymes, pepsin from gastric juice, peptidases and saccharidases from mucosa of the small intestine, etc. Fat digestion is mostly accomplished by pancreatic lipase, and its insufficiency is a major concern in EPI. Weight loss, steatorrhea, abdominal pain, etc. are some of the symptoms associated with EPI. Apart from this, deficiencies of fat-soluble vitamins also occur in EPI patients. Steatorrhea occurs with pancreatic insufficiency while the volume of pancreatic lipase has declined to less than 10% of normal secretion.

One of the best ways to overcome malabsorption due to EPI is supplemental enzyme therapy or pancreatic enzyme replacement therapy (PERT). Pancreatic enzymes are available from a wide variety of sources including animal, plant and fungal sources. The first pancreatic preparations were freeze-dried hog pancreas itself, which was later replaced by total pancreatic extracts. They usually comprise pancreatic trypsin, amylase and lipase. Lipase is the least stable among all pancreatic enzymes and often gets destroyed by gastric acid, which is the major challenge in PERT. Attempts were made to overcome inactivation of enzymes by co-administration of bicarbonates and antacids. Later acid resistant enteric-coated enzymes were developed but release of lipase from coated preparation was not easy. With the advent of newer techniques, improvement in PERT has been attained. Currently available pancreatic enzyme preparations are from porcine source. Except Viokaze®, a non-enteric-coated pancreatic enzyme of porcine origin, all of them are enteric-coated microspheres which are acid resistant. The enteric-coated microspheres release pancreatic lipase at a pH of 5.5-6 (Baker 2008). Varieties of enzyme preparations are available which differ in particle size and release mechanism (pH related). PERT certainly improved the quality of the patient's life by improving fat absorption and reduction of steatorrhea, weight loss and other symptoms associated with malabsorption. Lipase preparations are available from various microbial sources with improved stability. None of the non-porcine preparations has been licenced for use in humans, though some are in clinical trials. Some of the commercially available pancreatic enzyme preparations are CREON®, Pancreaze®, Zenpep®, Ultresa®, Pertyze® and Viokase®.

2.4.2 β Galactosidase for Lactose Intolerance

Lactose intolerance is the inability to hydrolyse lactose, a disaccharide in mammalian milk. It is a metabolic disorder due to β galactosidase (lactase) enzyme



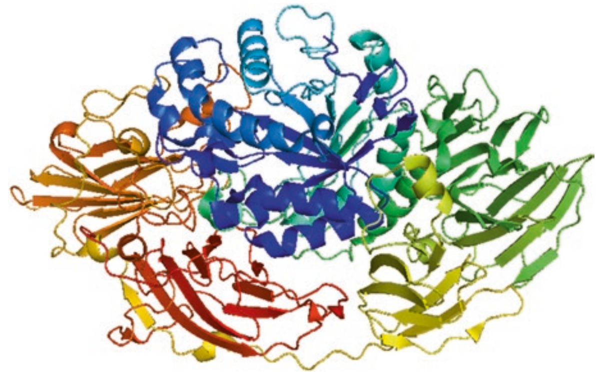


Fig. 2.8 Crystal structure of β galactosidase from *Aspergillus oryzae* (pdb id: 4IUG)

insufficiency. It is one of the most common gastrointestinal disorders. Elimination of milk and dairy products is a common practice to overcome lactose intolerance. But lactose is an excellent source of energy, and avoiding milk products leads to malnutrition, especially calcium and mineral deficiency. Management of lactose intolerance is achieved by enzyme supplementation therapy. β-galactosidase enzymes obtained from various sources are used for this. The sources include yeasts such as *Kluyveromyces fragilis* and *K. lactis*, fungi such as *Aspergillus niger* and *A. oryzae*, and bacteria such as *Bacillus coagulans*. Another approach to overcome lactose intolerance is supplementation of dairy along with probiotics. In probiotics some of the microorganisms producing β-galactosidase are present such *as Lactobacillus, Bifidobacterium, Streptococcus and Lactococcus* (Fig. 2.8).

2.4.3 Glutenases for Celiac Disease

Celiac disease is intolerance to dietary protein gluten in genetically susceptible individuals. It is an autoimmune disease which is an inflammatory response to gluten leading to small intestinal mucosal injury and nutrient malabsorption. Gluten is a dietary protein found in wheat, barley and rye and is rich in glutamine and proline. The only currently available method to overcome celiac disease is following a gluten-free diet. But the abundance of gluten in various diets makes it extremely difficult to avoid. The management of celiac disease other than a gluten-free diet plan is under development. Oral enzyme therapies with certain gluten-specific proteases (glutenase) are promising. Glutamine-specific endopeptidase from barley EP-B2, prolyl endopeptidase from *Sphingomonas capsulata* (SC PEP), prolyl oligopeptidase *Flavobacterium meningosepticum* (FM-POP), prolyl endoprotease from *Aspergillus niger* (AN-PEP), etc. are some of the glutenase with potential to detoxify gluten prior to reaching the small intestine (Gass et al. 2005; Shan et al. 2004). The endopeptidases ALV003 (comprising both EP-B2 and SC PEP) and AN-PEP are two gastrically active glutanases, and ALV003 is currently under phase 2b clinical trials.

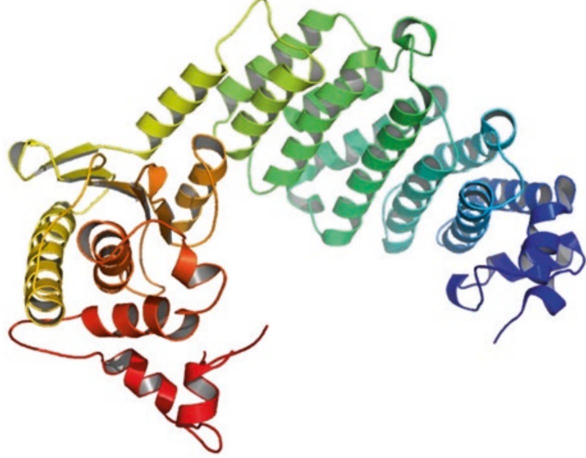
2.5 Enzymes for Wound Debridement

Cleansing and wound debridement are common practices in wound bed preparation from ancient times. Debridement is removal of foreign material, non-viable tissue, excess wound moisture and contaminants from the wound bed until healthy tissue gets exposed. Debridement can be by means of mechanical, chemical (enzymatic), autolytic and surgical methods. Removing necrotic tissue by debridement helps in the healing process and enzymatic wound debridement has many advantages. Enzymatic wound healing is a selective debridement process by which proteolytic enzymes help to remove the necrotic tissue. Proteases from various sources were used for debriding purposes. A few of the debriding agents are considered here such as bacterial collagenase, papain/urea and trypsin.

2.5.1 Collagenase

Collagenase is a proteolytic enzyme that could degrade collagen. It is proteinase specific to collagen which has a triple helical structure whereas none of the other proteinases cleave collagen. Collagen binds to necrotic tissue at the wound surface and dissolutes collagen and thus facilitates wound healing. It is obtained from *Clostridium histolyticum* and is commercially available as Santyl® (McCallon et al. 2014) with an optimum pH between 6 and 8. Dupuytren's contracture and Peyronie's disease are often associated with trauma and deposition of collagen plaques. Collagenase is an FDA-approved drug for treating Dupuytren's contracture and Peyronie's disease (Honig 2014) (Fig. 2.9).

Fig. 2.9 Crystal structure of collagenase G from *Clostridium histolyticum* (pdb id: 2Y50)



2.5.2 Fibrinolysin/DNase

Fibrinolysin/DNase contains fibrinolysin from bovine plasma and DNase from bovine pancreas. Fibrinolysin aids in debridement by dissolution of fibrin. It also inhibits clotting and dilates blood vessels. Fibrinolysin when dissolved in aqueous solution loses it activity within 6–8 h. Deoxyribonuclease (DNase) helps to break down nucleic acid materials and nucleoproteins present in exudates and necrotic tissue (Falabella 2006). It is commercially available as Elase®.

2.5.3 Papain-Based Debridement

Papaya juice has been used for wound debridement since ancient times. Papain, a cysteine protease enzyme isolated from *Carica papaya*, breaks the fibrinous material in necrotic tissue and thus promotes the wound healing process. It is a non-selective enzyme. Papain is used in combination with urea. Papain requires activators and urea helps to expose the enzyme to activators. Another advantage of urea is that it denatures proteins in necrotic tissue and hence makes them easily digestible by papain. Papain/urea-based formulation was found to be effective in wound debridement. Papain produces inflammatory response and thus induces pain. In order to relieve pain chlorophyllin copper complex (CCC) along with papain/urea is used in commercial formulations (Shapira et al. 1973). It has been found to be useful in the treatment of pressure ulcers. Panafil® is a commercially available papain/urea formulation and is active at a wide range of pH from 3 to 12.

2.5.4 Trypsin

Trypsin is an endopeptidase secreted by pancreas and apart from digestion of proteins in food trypsin finds various other applications. Topical application of trypsin is often implemented for wound healing. Trypsin is a mild debriding agent and speeds the healing of dermal wounds (White et al. 2013). Wounding is often associated with clotting, leaving serum on the wound. Trypsin cleaves serum albumin, which in turn initiates fibrocyte differentiation and thereby facilitates wound healing. Xenaderm ® and Granulex ® are two products containing trypsin available for topical application.

2.6 Enzymes to Treat Lysosomal Storage Diseases (LSD)

Lysosomal storage diseases (LSD) are a group of disorders comprising over 50 diseases. Lysosomes are the site of intracellular digestion in eukaryotic cells and can digest almost all kinds of macromolecules such as glycogen, nucleic acids, proteins, glycosphingolipids, mucopolysaccharides, etc. which are engulfed within lysosomes. Lack of enzyme hydrolysis of various macromolecules within

lysosomes is associated with each disorder. This results in accumulation of macromolecules in lysosomes, thereby causing enlargement of cells carrying lysosomes and eventually leading to organ dysfunction. Lysosomal storage diseases are genetic disorders either due to autosomal recessive inheritance or X-linked inheritance, which causes dysfunction or reduced activity of specific lysosomal enzymes. LSDs includes Gaucher disease types 1 and III, Fabry disease, Mucopolysaccharidosis Type I (Hurler, Hurler/Scheie Syndrome), Mucopolysaccharidosis Type II (Hunter Syndrome), Mucopolysaccharidosis Type VI, (Maroteaux-Lamy Syndrome) and Pompe disease.

Christian de Duve was the first to propose the concept of enzyme replacement therapy for lysosomal storage disorders and it was later experimentally supported (De Duve 1966). Though it was proposed during the 1960s its clinical use was not in practice until 1991. Alglucerase (Ceredase®) was the first enzyme for replacement therapy approved by the USFDA in 1991 against a lysosomal storage disease (Gaucher disease).

2.6.1 Gaucher Disease

Gaucher disease is an inherited disease characterized by a defect of lack of glucocerebrosidase enzyme resulting in glucocerebroside accumulation in lysososmes. The lipid-accumulated cells are called Gaucher cells and found in liver, spleen, bone marrow and lung. This makes it a multi-organ disorder. The disease is characterized by hepatosplenomegaly (enlargement of liver and spleen) and skeletal complications. Gaucher disease is classified into three types based on clinical manifestations. In Type I-non-neuronopathic (adult), the central nervous system is not involved. But in the case of Type II-acute neuronopathic (infantile) and Type III-subacute neuronopathic (juvenile) the central nervous system is involved. Most common among them are Type 1 where the central nervous system is not involved. Disease control is either by enzyme replacement therapy (ERT) or substrate reduction therapy. Enzyme replacement therapy was found to be successful in managing Gaucher disesase. As mentioned previously, enzyme replacement therapy was first established for the treatment of Gaucher disease where Alglucerase (Ceredase®), a placental-derived enzyme glucocerebrosidase, was used. ERT reversed symptoms of Gaucher disease. It was later replaced by recombinant glucocerebrosidase (Imiglucerase, Cerezyme®) in 1994. Two other recombinant enzymes, namely velaglucerase alfa (VPRIV®), which received FDA approval in 2010, and taliglucerase alfa (Elelyso®), which received FDA approval in 2012, are also available for ERT. The recombinant enzymes differ from each other in terms of source. Imiglucerase is obtained from Chinese hamster ovary (CHO) cell line, velaglucerase alfa is isolated fromm human cells, and taliglucerase alfa is obtained from carrot cells and shows similar effectiveness (Elstein 2011). Imiglucerace has been in use for a longer period and only 1% of patients develop adverse reactions to imiglucerase ERT (Zimran et al. 2011) (Fig. 2.10).

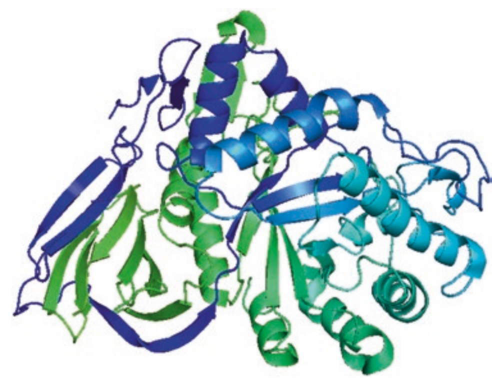


Fig. 2.10 Crystal structure of velaglucerase alfa chain A (pdb id: 2WKL)

2.6.2 Fabry's Disease

The second most frequently occurring lysosomal storage disease is Fabry disease with an occurrence of one in 117,000 births characterized by deficiency of α-galactosidase A (α-gal A). This results in accumulation of globotriaosylceramide (Gb3) in lysosomes. Though it is an X-linked disease and affects mostly hemizygous men, heterozygous women may suffer the same level of severity and decline in lifespan (Mehta et al. 2010). Fabry's disease often affects renal cells, endothelial cells and neuronal cells. Clinical manifestations include renal dysfunction and cardiac problems such as ischaemia, gastrointestinal problems, etc. Enzyme replacement therapy (ERT) for Fabry's disease was introduced in 2001. Fabry's disease is a progressive disorder and enzyme replacement therapy at initial stages was found to be very helpful in disease control. Agalsidase alfa (Replagal®) and agalsidase beta (Fabrazyme®) are two recombinant α-galactosidase enzymes available for ERT. Agalsidase alfa is isolated from cultured human skin fibroblasts whereas agalsidase beta is isolated from Chinese hamster ovary cells. ERT certainly improved quality of life by decreasing organ damage and reducing or even reversing disease progression (Fig. 2.11).

2.6.3 Glycogen Storage Disease Type II (Pompe disease)

Pompe disease is a lysosomal storage disease due to the lack of the enzyme α-glucosidase (GAA). This leads to lysosomal accumulation of glycogen, mostly in cardiac and skeletal muscles. It is also known as glycogen storage disease Type II (GSDII) or acid malatase deficiency. Pompe disease is categorized into two forms, viz. infantile-onset Pompe disease (IOPD) and late-onset Pompe disease (LOPD). In infantile-onset Pompe disease (IOPD) symptoms start within 2 months after birth often with cardiomyopathy, and death occurs before the age of 1 year if it remains untreated. In late-onset Pompe disease (LOPD) symptoms appear in the later stages of life ranging from greater than 1 year to adulthood. LOPD progress slowly and eventually leads to respiratory failure. Pompe disease is currently managed by

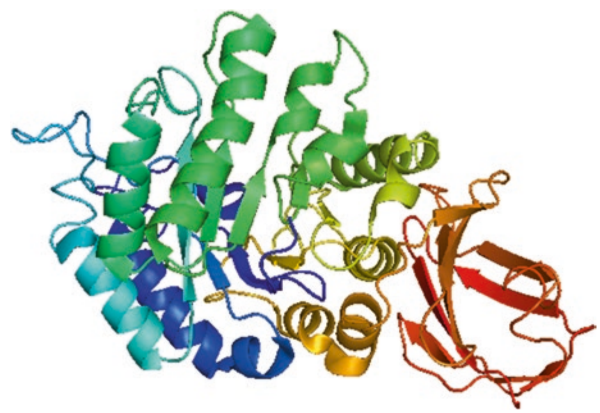


Fig. 2.11 Crystal structure of agalsidase alfa chain A (pdb id: 1R46)

enzyme replacement therapy using recombinant human GAA (rhGAA) produced either in Chinese hamster ovary (CHO) cells or in transgenic rabbit milk (Bijvoet et al. 1999; Van Hove et al. 1996). Myozyme® (alglucosidase alfa) was approved in 2006 for enzyme replacement therapy (ERT) of IOPD and Lumizyme® was approved in 2010 for enzyme replacement therapy (ERT) of LOPD by FDA. Though ERT for Pompe disease has certain limitations such as lifelong administration of enzyme, cost of therapy and loss of administered enzyme prior to reaching target muscles, it has certainly improved the quality of life led by patients with Pompe disease.

2.6.4 Mucopolysacchariodoses (MPSs)

Mucopolysaccharidoses (MPSs) are a group of disorders characterized by deficiency of lysosomal enzymes for degradation of glycosaminoglycans (GAGs) or mucopolysaccharides. There are seven types of MPSs based on enzyme affected, namely Types I, II, III, IV, VI, VII and IX. MPSs are associated with lysosomal progressive accumulation of undigested glycosaminoglycan (GAG) leading to cellular and multiple organ damage. MPSs are multivisceral diseases affecting musculoskeletal, gastrointestinal, neurological, cardiovascular and pulmonary systems leading to death in severe forms. All MPSs except MPS II show autosomal recessive inheritance whereas MPS II shows an X-linked recessive inheritance. Enzyme replacement therapy (ERT) is now available for MPS I, MPS II and MPS VI.

MPS I is a disorder characterized by the deficiency of the enzyme α-L-iduronidase leading to progressive accumulation of glycosaminoglycans (GAGs) in lysosomes, namely heparan sulphate and dermatan sulphate. Based on severity of disease, MPS I is classified into three sub-types: severe (Hurler syndrome), intermediate (Hurler–Scheie syndrome) and mild (Scheie syndrome). The first of its kind to be approved for ERT against MPS was Laronidase (recombinant human α-L-iduronidase) and it has been available in Europe and the USA since 2003. MPS II (Hunter syndrome) is an X-linked recessive disorder which appears mostly in males and very rarely in females. Hunter syndrome is due to absence of the enzyme iduronate-2-sulphatase

(I2S) responsible for breakdown of heparan sulphate and dermatan sulphate triggering subsequent accumulation of them within the lysosomes (Noh and Lee 2014). Idursulphase (recombinant form of human I2S) has been approved for ERT against MPS II and has been available since 2006. MPS VI, also known as Maroteaux-Lamy syndrome, is characterized by a deficiency of arylsulphatase B (N-acetyl galactosamine 4-sulphatase) causing lysosomal accumulation of dermatan sulphate and chondroitin sulphate. Galsulphase (recombinant human arylsulphatase B) has been approved and has been available since 2005 for treatment. ERTs against other MPSs are in development and yet to be approved. ERT is a lifelong treatment leading to improvement in locomotion and respiration and eventually improving the quality of life of patients suffering from MPSs (Table 2.3).

2.7 Treatment of Bleeding Disorders

Haemophilia is an inherited excessive bleeding disorder. It has two types, namely haemophilia A, characterized by insufficiency of clotting factor VIII, and haemophilia B, characterized by insufficiency of clotting factor IX. Haemophilia patients are at high risk during injury and surgery. Mostsurgical procedures are not recommended to them due to post-operative bleeding complications. Intravenous infusion of corresponding clotting factor (FVIII/FIX) is the remedy for this.

2.7.1 Recombinant Factor VIII

As mentioned earlier, haemophilia A is characterized by deficiency of factor VIII. Plasma-derived coagulation factor VIII (FVIII) was initially used for replacement therapy of the disease, which was later replaced by recombinant factor VIII with the advancement of recombinant DNA techniques. A fusion protein was developed by combining a single molecule of recombinant FVIII (rFVIII) and Fc domain of human IgG1 expressed in human embryonic kidney (HEK) 293H cells. Compared to rFVIII, the fusion protein (rFVIIIFc) has increased efficacy, prolonged half-life, etc. (Peters et al. 2013). rFVIIIFc is commercially available as efraloctocog alfa for treatment of haemophilia A. Unfortunately, some patients develop alloantibodies against FVIII, which creates problems in the treatment of patients with antibodies as inhibitors.

2.7.2 Recombinant Factor IX

Haemophilia B treatment involves infusion factor IX (FIX) concentrates. They can be given on demand during an emergency or prophylactically to prevent bleeding (given in minimum dose). Factor IX or Christmas factor is a serine protease, and initially plasma-derived FIX concentrates were given for treatment and later

Table 2.3 Enzyme replacement therapy for lysosomal storage disease: storage material accumulated, deficient enzyme and approved enzymes for treatment

Disorder	Deficient enzyme	Storage material	Approved enzyme for ERT	Genetic inheritance
Gaucher disease	β-glucocerebrosidase	Glucocerebroside	Alglucerase (Ceredase®)	Autosomal recessive
			Imiglucerase, (Cerezyme®)	
			Velaglucerase alfa (VPRIV®)	
			Taliglucerase alfa (Elelyso®)	
Fabry's disease	α-galactosidase A	Globotriaosylceramide	Agalsidase alfa (Replagal®)	X-linked recessive
			Agalsidase beta (Fabrazyme®)	
Pompe disease	α-glucosidase	Glycogen	Alglucosidase alfa (Myozyme®, Lumizyme®)	Autosomal recessive
Mucopolysaccariodoses I (MPS I)	α-L-iduronidase	Heparan sulphate, Dermatan sulphate	Laronidase (Aldurazyme®)	Autosomal recessive
Mucopolysaccariodoses II (MPS II)	Iduronate-2-sulphatase	Heparan sulphate, Dermatan sulphate	Idursulphase (Elaprase®)	X-linked recessive
Mucopolysaccariodoses VI (MPS VI)	Arylsulphatase B (N-acetyl Galactosamine 4-sulphatase)	Dermatan sulphate, Chondroitin sulphate	Galsulphase (Naglazyme®)	Autosomal recessive

replaced by recombinant FIX, and this remained the mainstay in the treatment of haemophilia B. Recombinant factor IX was fused with the Fc domain of human immunoglobulin G1 to form a fusion protein rFIXFc, the first bioengineered clotting factor. The fusion protein was expressed in human embryonic kidney (HEK) 293H cells. It is commercially available as Alprolix®. rFIXFc has a prolonged half-life, improved safety and efficacy, etc. (Peters et al. 2010). But the major disadvantage of Factor IX infusion is the development of antibodies in some patients.

2.7.3 Recombinant Activated Factor VIIa

Despite the advantages of infusion therapy with clotting factors, the major challenge encountered is the development of alloantibodies against clotting factors FVIII/FIX. The antibodies developed act as inhibitors of infusion therapy and demand bypassing agents. Inhibitor development is more common in haemophilia A (30%) compared to haemophilia B (1–6%). Infusion therapy by replacing F VIII/IX is unsuccessful in patients with inhibitors, thereby increasing the bleeding risk and mortality. Thus there is a reduction of quality of life in haemophilia patients with inhibitors compared to patients without inhibitors (Coppola et al. 2010). Treatments with bypassing agents are also practised in patients with inhibitors. Recombinant activated factor VII (rFVIIa) is a bypassing agent to manage bleeding disorders in patients with inhibitors. It is a serine protease that can bypass FVIII and FIX in clotting (Hoffman and Dargaud 2012). Recombinant FVIIa was licenced for on-demand treatment of bleeding complications in patients with inhibitors. In 2007 it was approved by the European Medicines Agency. AryoSeven™ and NovoSeven® are two commercially available forms of rFVIIa.

2.8 Other Therapeutic Enzymes

There are many therapeutic enzymes which do not fit the previously discussed categories. A few of them with divergent properties are described here.

2.8.1 Serrapeptase

Serrapeptase (SP) is an exciting therapeutic enzyme with a wide variety of clinical applications. It is an enzyme produced by the bacteria *Serratia* E-15 isolated from the intestines of the silkworm *Bombyx mori*. It is also known as serratiopeptidase or serralysin. It is zinc metalloprotease and comprises 470 amino acids with a molecular weight of approximately 50 KDa. Serrapeptase was later identified from various species of *Serratia*, *Pseudomonas aeruginosa* and *Aspergillus oryzae.* It has been used in Japan since the 1960s for reducing inflammation. It is administered orally as a microencapsulated formulation to protect from gastric acid and has high bioavailability (Moriya et al. 1994). Reduction of inflammation is achieved through thinning and decreasing the amount of fluids accumulated in tissues and also by helping

to drain out the fluids. It also breaks down dead tissue around an injury and thus aids in the healing process. This anti-inflammatory property makes the enzyme suitable for treatment of breast engorgement, reduction in swelling due to sports injuries, treatment of chronic sinusitis, reduction of post-surgery inflammation, etc. Another property of serrapeptase is its analgesic effect by inhibiting pain inducing bradykinin like amines in inflamed tissues and its potential in clot lysis (Bhagat et al. 2013). So far no major side effects have been reported with SP usage. Non-steroidal anti-inflammatory agents (NSAIDs) used to treat rheumatoid arthritis and osteoarthritis could be very well replaced by serrapeptase (Fig. 2.12).

2.8.2 Superoxide Dismutase

Superoxide dismutase (SOD) is an enzyme which is used for prevention of oxygen toxicity. Reactive oxygen species (ROS) is formed from oxygen which causes oxidative stress and cellular damage. ROS can be converted to hydrogen peroxide by SOD and is essential for protection from damage by oxygen free radicals because of its antioxidant potential. Most organisms produce SOD. SOD is used therapeutically as a powerful antioxidant and also as an anti-inflammatory agent. SOD has established its efficacy in the management of inflammatory diseases such as rheumatoid arthritis and osteoarthritis. It is also effective for elimination of side effects associated with chemo- and radiation therapy (Flohe 1988). SOD is commercially available as Orgotein (bovine CuZnSOD). However, immune response associated with orgotein has pushed it back from the market. SODm or superoxide dismutase mimics are currently under development which are similar to endogenous SOD while they are a synthetic small molecule (Fig. 2.13).

2.8.3 Adenosine Deaminase

Severe combined immunodeficiency disorders (SCID) are due to deficiency in lymphocyte functions (T, B and NK lymphocytes). Adenosine deaminase deficiency is one of the major reasons for SCID. It is an enzyme which breaks down adenosine and deoxyadenosine into inosine and deoxyinosine respectively. Accumulation of nucleotides and other metabolites blocks lymphocyte maturation. This makes SCID patients susceptible to infections due to lack of defence mechanisms. Enzyme replacement therapy with adenosine deaminase is one of the major ways to manage the disease and therapeutic application began in 1986. Bovine adenosine deaminase with polyethylene glycol (PEG) is commercially available as Adagen® (pegadamase bovine) and was one of the first to receive approval by the FDA in 1990 under the Orphan Drug Act (Fig. 2.14).

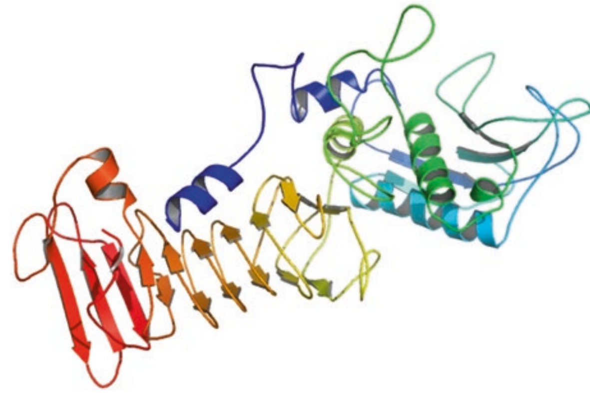


Fig. 2.12 Crystal structure of serrapeptase from *Serratia* sp. E-15 (pdb id: 1SRP)

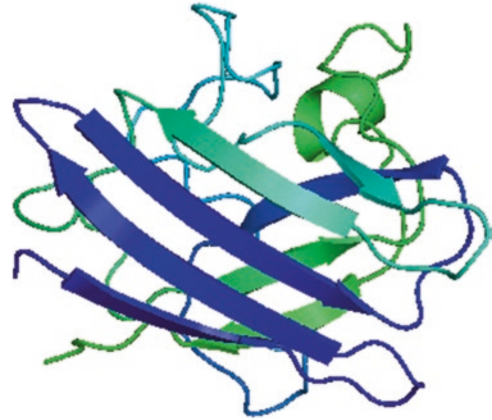


Fig. 2.13 Crystal structure of bovine superoxide dismutase chain A (bovine CuZnSOD) (pdb id: 1SXA)

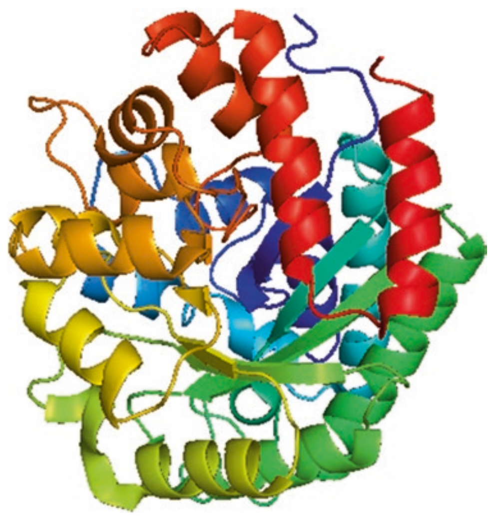


Fig. 2.14 Crystal structure of bovine adenosine deaminase (pdb id: 1KRM)

2.8.4 Phenylalanine Ammonia Lyase (PAL)

Phenylalanine hydroxylase (PAH) is an enzyme converting phenylalanine to tyrosine, and mutations in the PAH gene cause an autosomal recessive disorder called phenylketonuria. Disease control is made possible by methods such as dietary treatment, amino acid supplementation, tetrahydropterin treatment, enzyme therapy, cell therapy and gene therapy. Enzyme therapy is used to reduce the level of accumulated phenylalanine. Enzyme replacement therapy with phenylalanine hydroxylase (PAH) or enzyme substitution with phenylalanine ammonia-lyase (PAL) is practised, of which treatment with PAL is advantageous. PAL can be immunogenic upon injection and can be reduced by treating with PEG. PEG-PAL is less immunogenic, and PEG-PAL formulations with more stability are also in development for oral intake (Longo et al. 2014).

2.8.5 Dornase

Cystic fibrosis (CF) is an autosomal recessive disorder affecting exocrine glands. Pulmonary disease is the major reason for mortality in CF patients. Airway obstruction leading to lung inflammation and bronchiectasis occurs in CF. Sputum becomes thick due to dead neutrophils. Dornase alfa is a recombinant human deoxyribonuclease (DNase). It breaks down nucleic acid materials in the airways and thus reduces the viscosity of sputum. Dornase alfa is commercially available as Pulmozyme® and reduces airway obstruction by facilitating mucus clearance in lungs (Jones and Wallis 2010).

2.8.6 Rasburicase

Tumour lysis syndrome (TLS) occurs within 72 h after initiation of cancer therapy and is characterized by release of tumour cell contents in the circulatory system leading to hyperuricemia, metabolic acidosis, etc. TLS can be life-threatening and oliguria (acute kidney injury) may occur due to crystallization of uric acid and calcium phosphate in the renal tubules. Hyperuricemia can be overcome by degrading uric acid by urate oxidase. Rasburicase is a recombinant urate oxidase from *Aspergillus flavus* and is expressed in *Saccharomyces cerevisiae* (Bayol et al. 2002). The US Food and Drug Administration (FDA) approved rasburicase in 2009 and it is commercially available as Elitek/Fasturtec ®. It is effective in TLS management and the preferred choice in paediatric patients with acute leukaemia and lymphoma.

2.8.7 Pegloticase

Gout and inflammatory disease occur due to accumulation of monosodium urate crystals in joints. Hyperuricaemia is the key player here too. Urate oxidase enzymes can alleviate hyperurecaemia too. Though rasburicase (urate oxidase) is helpful in managing TLS, immunogenicity and a short half-life make it impractical for use in gout. As gout is intolerant to urate-lowering strategies, recombinant mammalian uricase was developed. Pegloticase is a PEGylated variant of recombinant porcine/ baboon urate oxidase expressed in *E. coli* (Ea and Richette 2012) and got US Food and Drug Administration (FDA) approval in 2010 for the treatment of chronic gout. Pegloticase is available commercially as Krystexxa®.

2.8.8 Glucarpidase

Methotrexate (MTX) and other antifolates administered for cancer therapy are normally eliminated through urine. High-dose methotrexate (HDMTX) is taken with precautions; however, improper elimination can lead to toxicities and renal failure. Carboxypeptidase enzymes hydrolyse MTX into non-toxic products. Glucarpidase is a recombinant *Pseudomonas* carboxypeptidase G2 expressed in *E. coli*. It readily cleaves MTX into glutamate and 2, 4- diamino-N10-methyl-pteroic acid and is excreted by the hepatic system (Sherwood et al. 1985).

2.8.9 Ocriplasmin

Ocriplasmin is the truncated recombinant mutant of plasmin while retaining its fibrinolytic potential and it is expressed in *Pichia pastoris* (Nagai et al. 2003). Vitreomacular adhesion occurs due to partial vitreous detachment characterized by attachment of a part of the vitreous to the macula. It may form macular holes while traction occurs. Vitrectomy is the only remedy for this condition. However, vitreous liquefaction and detachment from the retina can be treated with ocriplasmin for bypassing surgical protocols. Vitreomacular adhesion happens via proteoglycans including fibronectin and laminin, which are plasmin receptors. Proteolytic action of ocriplasmin against fibronectin and laminin helps in vitreomacular detachment. Thus ocriplasmin aids in release of traction and macular hole closure (Stalmans et al. 2012). It is commercially available as JETREA®.

2.9 Conclusions

Enzymes have great potency in therapeutic applications. Though therapeutic enzyme development is not a new approach, FDA approval has been received by only a few enzymes and available for clinical application while some of the therapeutic enzymes are in clinical trials. Success of therapeutic enzyme application has

been limited by a few shortcomings such as large size, antigenicity, stability and cost. Extensive research is ongoing for evolving newer therapeutic enzymes and also for improvement of the safety and potency of available drugs. Progression in the latest technology enables manufacturers to make safer and better therapeutic enzymes.

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Enzymes for Bioremediation and Biocontrol

3

Indu C. Nair and K. Jayachandran

Abstract

Forging of past, present and future technologies is the most required strategy in exploiting the functional diversity and specificity of enzymes. Production process and field application studies of enzymes are on the brink of becoming a reality everywhere. Considering green chemistry as the forerunner in all environmental management strategies, application of enzymes in bioremediation and biocontrol is an essential event for a sustainable environment. Bioremediation leads to the complete elimination of polluting molecules and biocontrol is required for the biological eradication of pathogenic insects and pests. The wide diversity, high specificity, high productivity and the need of ambient conditions make the enzymes the unique candidate for exploring the possibility of protecting and preserving nature. Bioremediation processes and biocontrol measures, irrespective of the type of organism, condition and methods adopted, hold enzyme action as the most active rate-controlling mechanism. The type of enzyme, source and mode of action in bioremediation and biocontrol need extensive analysis for exploiting these green processes for a better future.

Keywords

Enzymes • Diversity • Bioremediation • Biocontrol

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3.1 Introduction

The growing awareness of environmental conservation has brought out impacts of human interference on the environment. A huge extent of environmental pollution is due to industrialisation, which has introduced synthetic and toxic chemicals to the biosphere, imposing disastrous after effects. Population explosion demanded increased food production, which led to uncontrolled use of pesticides and herbicides. The increase in the rate of water, air and soil pollution is an index of uncontrolled population and industrialisation resulting in anthropogenic activities and unchecked release of toxic chemicals. This has ultimately contributed to the disruption of the delicate balance in the natural system.

Every form of life that has survived on earth must have adapted to the situations of its neighbouring ecosystems. High-quality environmental conditions should be restored for a healthy normal life on earth. Physico-chemical methods like high temperature, incineration, adsorption, absorption, UV oxidation, extraction, base-catalysed dechlorination and others have commonly been used for the removal of these chemicals. But these processes are complex, uneconomical, difficult to execute, not cost effective and invariably end up in the release of additional compounds as secondary pollutants into the environment (Atlas 1995).

The current efforts to reverse this situation mainly concentrate on biological methods which are not deleterious to the environment. Bioremediation and biocontrol are two promising areas of research which explore recent advances in environmental conservation.

Bioremediation make use of microorganisms that specifically target particular pollutants to alter or reduce the toxicity of those pollutants through different catabolic activities**.** It results in complete and cost-effective elimination of chemical pollutants through diverse microbial metabolic processes (Abdulsalam et al. 2011; Longoria et al. 2008). Biocontrol is another area similar to bioremediation where biological methods instead of chemical methods are being used for pest and insect control. Biological control or biocontrol is the use of specific organisms or their metabolic by-products to limit the harmful or negative impact of a plant pest. Biocontrol is an effective and nature-friendly alternative to increased use of agrochemicals and pesticides.

In both bioremediation and biocontrol processes the active principle involved is enzymes. Enzymes are instrumental in biodegrading a variety of organic compounds from the simple glucose molecule to the complex mixture of hydrocarbons and pesticides. Researchers the world over are intensively exploring the possibility of detoxifying complex xenobiotics with specific enzymes. Similarly, in the field of biocontrol new and promising approaches are being developed to eradicate pests and insects from the agriculture fields even at field application level. Application of enzyme-based novel technologies is increasing day by day. The most impotortant accelerating component in this context is the technological advancement as a result of which the industries are now in a position to isolate, purify and characterise enzymes from any source. The added advantage is that they can be easily produced

on a large scale using bioreactors facilitating extensive field trials and application studies.

Enzymes are mostly proteinaceous and hold immense structural integrity and offer much selectivity in their action. It is the diversity existing within the protein structure of the enzyme that gives way to novel activities in complex and stressed environments. The wide range of substrate selectivity distributed among the infinite domain of plant, animal and microbial enzymes makes them highly competent in acting as principal agents in most of the bioprocesses including bioremediation and biocontrol.

3.2 Enzymes in Bioremediation

The decomposition/mineralisation of materials through biological agents is referred to as biodegradation. Microorganisms are effective mediators of biodegradation because they have diversified metabolic capabilities. Biodegradation is unique and effective as it results in complete mineralisation of toxic chemicals or results in the generation of biogas (Providenti et al. 1993; Jain et al. 2005). Microorganisms support life on earth through their sole activity of biogeochemical cycling. During the course of evolution on earth, which covered both aerobic and anaerobic phases, microorganisms have been offering versatile biochemical pathways. This equipped them to utilise or degrade a given substance and hence hypothetically they are said to be infallible. Microorganisms can be considered as metabolic machines, dependent on gathering chemicals from their environment to obtain carbon, other elements and energy to compete favourably against other microbes.

Biodegradation is a triple corner process which is influenced by pollutants, different environments and type of organisms. Biodegradation involves the application of actinomycetes, yeast, fungi or bacteria (Strong and Burgess 2008; Ruiz-Duenas et al. 2009) and it helps in restoring the original nature of the environment. These approaches are mainly based on two principles, metabolism or absorption of xenobiotic by living organisms. Microorganisms have the potential to utilise a variety of compounds as their energy and carbon source and this often results in structural changes of the substrate (De Schrijver and De Mot 1999; Nair et al. 2009). Biological methods have gained importance because of their simple operation and as they generate very small amounts of secondary wastes besides bringing complete mineralisation (Alfonsin et al. 2015; Rajamohan et al. 2015).

In recent times, many microbial ecologists have identified varieties of microbial species that act as efficient degraders of toxic organic compounds (Nair et al. 2007, 2009; Joseph et al. 2010; Cherian and Jayachandran 2010). A single microorganism is typically incapable of degrading a mixture of toxic compounds. Hence a consortium or mixed culture designed in a proper way can degrade a wide range of toxic compounds present in a mixture very effectively (Al-Wasify and Hamed 2014; Vijayana et al. 2014; Dhanya 2016). Development of syntropic bacterial consortia with specialised catabolic steps is a less controversial approach in the biodegradation of complex pollutants.

The bioremediation process can be *ex situ* and *in situ* (Hamzah et al. 2013) and is influenced by many external factors. The deciding factors may include physical, chemical, physiological and environmental factors such as extent of aeration, solubility of the substrate, toxicity of the substrate, pH, temperature, incubation time, agitation of the medium and growth rate of the selected organism (Atlas 1995; Al-Sulaimani et al. 2010; Adams et al. 2014).

3.3 Microbial Enzymes as Bioremediators

The use of microbial enzymes may represent a good alternative for the remediation of xenobiotic pollutants. Varieties of bacterial, fungal and phytoenzymes can be catalysts in the degradation of these pollutants of concern. It is observed that such enzymes have a broad specificity range. Enzymes are catalysts with either narrow or broad specificity and cause the complete conversion of toxic chemicals to inorganic end products. They can be applied under extreme conditions and are more mobile than microorganisms. All these make enzymes eco-friendly catalysts and the mechanisms an environment-friendly process.

Some protein superfamilies are specifically important in biodegradation and biocatalysis. Usually enzymes involved in biodegradation are found to exhibit a broad range of specificity; for example, naphthaline dioxygenase has 70 known substrates and toluene dehydrogenases has more than 100 substrates (Seo et al. 2010). The important classes of enzymes involved in the remediation of pollutants include: hydrolases, dehalogenases, transferases and oxidoreductases. Phosphotriesterases, amidases, proteases, cellulases, amylases, lipases, depolymerases, mono- or dioxygenases, reductases, cytochrome P450 monooxygenases, phenoloxidases, laccases, tyrosinases, lignin and manganese peroxidises are the main classes of enzymes involved in biodegradation of xenobiotic compounds (Table 3.1).

3.3.1 Oxidoreductases

Monooxygenases form an important group of degradative enzymes belonging to oxidoreductase. These enzymes are involved in cometabolism and transformation of xenobiotics. Microbial monooxygenases differ in their susceptibility with respect to the nature of compounds like aliphatic n-alkynes. Monooxygenases are critical in the conversion of aromatic compounds like phenol. The destruction of the resonant structure of benzene ring can be achieved by the enzyme action. As a convergent point in the decomposition of most aromatic compounds, catechol is formed by introducing a hydroxyl group to the aromatic ring. Catechol can be further cleaved by the ortho or meta pathway (Nair et al. 2008). In monoxygenase action, reductive dechlorination of compounds takes place under less oxygenated situations, and under high oxygen levels oxidative dehalogenation takes place.

Cytochrome P450 monooxygenase is a well-studied enzyme in bioremediation. The initial step of cDCE degradation was catalysed bycytochrome P450

Table 3.1 Enzymes in Bioremediation and its possible applications

Enzymes	References	Substrate	Applications
1: *Oxidoreductases*: Enzymes mediated the detoxification of pollutants through oxidative coupling. The oxidation-reduction reactions mediated by the enzymes finally oxidised the toxins to less harmful end products.			
Oxygenases: Enzymes incorporate oxygen atoms to the substrate utilising Flavin Adenine Dinucleotide (FAD)/Nicotinamide Adenine Dinucleotide(reduced) (NADH)/Nicotinamide Adenine Dinucleotide Phosphate(reduced) (NADPH) as cosubstrate and utilise the substrate as reducing agents leading to desulfurisation, dehalagenation, denitrification, ammonification and hydroxylation of substrates.			
Monooxygenases	Whyte et al. (2002)	Alkane, aromatic compounds, halogenated compounds, fatty acids	Protein engineering, synthetic chemistry, pharmaceutical industry, bioremediation, etc
	Jin et al. (2003)		
	Urlacher and Eiben (2006)		
	van Beilen et al. (2006)		
	van Beilen and Funhoff (2007)		
[1] Flavin-dependent monooxygenases	Morikawa (2010)		
	Redmond et al. (2010)		
	Tinberg et al. (2011)		
	Luesken et al. (2011)		
	Toda et al. (2012)		
[2] P450 monooxygenases	Urlacher and Girhard (2012)		
	Guengerich and Munro (2013)		
	Nishino et al. (2013)		
	Suttinun et al. (2013)		
	Taylor et al. (2013)		
Dioxygenases	van Beilen et al. (2003)	Aromatic, chlorinated, halogenated compounds	
	van Beilen et al. (2006)		
	Scott et al. (2008)		
	Seo et al. (2010)		
	Boyd et al. (2011)		
	Ji et al. (2013)		

(continued)

Table 3.1 (continued)

Enzymes	References	Substrate	Applications
2: *Laccases*: Enzymes resulted in the oxidation, decarboxylation, depolymerisation and demethylation of substrates. These enzymes occur in multiple isoenzyme forms. The substrate specificity and affinity of laccase can vary with changes in pH.			
Laccases	Clemente et al. (2001)	Dyes, azo dyes, phenols and their derivatives, polyamines, lignins, aryldiamines, benzopyrenes	Food industry, paper and pulp industry, textile industry, dye industry, cosmetics, bioremediation, etc
[1] intracellular laccases	Mayer and Staples (2002)		
[2] extracellular laccases	Kandelbauer et al. (2004)		
	Jimenez-Juarez et al. (2005)		
	Machado and Matheus (2006)		
	Tadesse and Luque (2011)		
	Tang et al. (2012)		
	Brandt et al. (2013)		
3: *Peroxidases*: are ubiquitous enzymes that oxidise the substrate at the expense of H_2O_2 in the presence of a mediator. The enzymes can be heam or nonheam proteins.			
Manganese peroxidases	Hofrichter et al. (1998)	Phenol, lignin, dyes	Food industry, paper and pulp industry, textile industry, pharmaceutical industry, dye industry, bioremediation, etc
	Hakala et al. (2006)		
	Dawkar et al. (2008)		
	Longoria et al. (2008)		
	Weber et al. (2008)		
	Ruiz-Duenas et al. (2009)		
	Ghasemi et al. (2010)		
	Hofrichter et al. (2010)		
Lignin peroxidases	Kordon et al. (2010)	Aromatic, polycyclic, halogenated phenolic compounds	Food industry, paper and pulp industry, textile industry, pharmaceutical industry, bioremediation, etc
	Lundell et al. (2010)		
	Mui et al. (2010)		
	Ng et al. (2010)		
	Chandra et al. (2011)		
	Krishnaveni and Kowsalya (2011)		

(continued)

Table 3.1 (continued)

Enzymes	References	Substrate	Applications
Versatile peroxidases eg: Chloro peroxidases	Huber and Carre (2012)	Methoxybenzenes, phenolic and nonphenolic aromatics	Paper and pulp industry, textile industry, pharmaceutical industry, bioremediation, Industrial biocatalyst and biosensors, etc
	Mamatha et al. (2012)		
	Marco-Urrea and Reddy (2012)		
4: *Hydrolases*: Hydrolytic enzymes disrupt major chemical bonds in the substrate like lipid, protein and cellulose and included hydrolysis, condensation and alcoholysis of the substrate.			
Lipases	Gibson and Parales (2000)	Aromatic hydrocarbons, oil spills, triacylglyceroles	Control of oil spills, detergent production, baking industry, paper and pulp industry, personal care products, bioremediation etc
	Kleeberg et al. (2005)		
	Sachelaru et al. (2005)		
	Furukawa (2006)		
	Gohel et al. (2006)		
	Loffler and Edwards (2006)		
	Whiteley and Lee (2006)		
	Kim et al. (2007)		
	Field and Sierra-Alvarez (2008)		
	Park et al. (2009)		
	Adrangi et al. (2010)		
	Chang et al. (2010)		
	Jankiewicz et al. (2012)		
Cellulases	Suma and Podile (2013)	Cellulosic substance	Textile manufacturing. Detergent production, paper and pulp industry, bioremediation, etc
[1] endoglucanases			
[2] exoglucanases			
[3] β glucanases			
Proteases		Proteins	Leather, laundry, pharmaceutical industries, biocatalyst, bioremediation, etc
[1] endopeptidases			
[2] exopeptidases			

monooxygenase (Nishino et al. 2013). The phylogenetic studies of the cytochrome P450 gene proposed that there is a progenitor proficient to decompose 1,2-dichloroethane, and the studies on neighbouring genes support this. The progenitor may involve ancestral forms of the cytochrome P450 monooxygenase gene from the same or related pathways. Characterisation of P450 enzymes and manifestation of P450 enzymes in a wide variety of reactions were reviewed by Guengerich and Munro (2013).

N-alkanes undergo aerobic degradation and usually result in complete mineralisation. The initial step of this degradation involves introduction of the hydroxyl group and is mediated by the action of alkane hydroxylases. Monooxygenases such as methane monooxygenase, propane monooxygenase, butane monooxygenase along with cytochrome p450 alkane hydroxylases and long-chain alkane monooxygenases (LadA) are also involved in the process. For industrial applications long-chain monooxygenases are selectively used as they often act as rate-limiting step in the whole process of biodegradation (Ji et al. 2013).

Arene dioxygenases participate in the cis-dihydroxylation of meta-substituted phenols (Boyd et al. 2011) and the reaction catalysed by this enzyme resulted in the formation of cyclohexenone cis-diol metabolites leading to the effective degradation of *m*-phenol substrates. *Candidatus Methylomirabilis oxyfera* played an important role in anaerobic waste water treatment and indicated the participation of dioxygenase, and the enzyme perform nitrite-dependent anaerobic methane oxidation (Luesken et al. 2011).

3.3.2 Laccases

Laccases are multicopper enzymes and uses molecular oxygen as the final electron acceptor. The enzyme action is much affected by pH and is usually associated with the degradative removal of many specific pollutants such as polyamines, phenols, alkenes, diamines aromatic amines, thiols and pesticides. Hence these enzymes are extensively used in industrial applications such as dye decolourisation, pulp bleaching, effluent detoxification, biosensors and bioremediation (Canas et al. 2007).

The most important application of laccases is in the field of textile industry effluent treatment for bringing decolourisation (Casieri et al. 2008). This is achieved by the selective decomposition of specific dyes such as malachite green, Azure B, Bromophenol Blue and Brilliant Blue (Tauber et al. 2008; Guo et al. 2008; Huang et al. 2007; Camarero et al. 2005).

3.3.3 Peroxidases

Peroxidases have a potential role in the biodegradation of phenols, cresols and chlorinated phenols, paper industry, textile-dye degradation, rhodamine dyes, lignin, dimethoxybenzene, amines, aromatic alcohols, dioxins, polychlorinated biphenyls, petroleum hydrocarbons, endocrine disruptive chemicals, herbicides, pesticides and

other xenobiotics (Hofrichter et al. 2010; Lundell et al. 2010; Chandra et al. 2011; Huber and Carre 2012; Marco-Urrea and Reddy 2012; Bansal and Kanwar 2013) and have been recognised and recently reviewed.

Lignin peroxidase was isolated from a range of fungi. Versatile peroxidases are very effective in the degradative transformation of compounds and they show extraordinarily broad specificity. The peroxidase enzyme produces less soluble free radicals and the precipitates can be removed by centrifugation and thus suggested for decolorisation purposes. Peroxidases are seen in all groups of organisms with various well-studied functions. In higher mammals they are involved in hormonal regulation and defence activities. In plants they have roles in lignification, auxin metabolism, etc. Microbial peroxidises are classified in to three types, viz., lignin peroxidise, manganese peroxidise and versatile peroxidases.

3.3.4 Hydrolases

Hydrolases can reduce toxicity of compounds by degrading them. They bring about condensation and alcoholysis. Their advantages include lack of stereospecificity, easy availability and tolerance to addition of solvents. Polyaromatic hydrocarbons are reported to be disrupted by these enzymes from a consortium (Balaji et al. 2014).

Chitinases are hydrolases and are considered as ideal candidates for acting as biocontrol agents. Ihrmark et al. (2010) isolated and characterised chitinases from *Trichoderma harzianum*, *T. virens*, *T. atroviride* and *T. asperellum.* Chitinases are also produced by many bacterial species. Bacteria produces chitinases for utilising chitin as a growth substrate. The recent increase in the occurrence of pathogenic fungus has resulted in the accelerated search for novel antifungal agents like chitinases (Davies and Henrissat 1995; Bourne and Henrissat 2001). There are several reports indicating the biocontrol activity of chitinase enzymes, which includes the report on chitinase by *Bacillus subtilis* NPU 001 (Chang et al. 2010) They reported that the purified chitinase inhibited hyphal extension of the fungus *Fusarium oxysporum* and showed potential activity against plant insects. Chitinase enzyme produced by a rhizosphere strain of *Stenotrophomonas maltophilia* strain MUJ showed considerable thermal stability during 2 h incubation at 45 °C and inhibited the growth of many fungal phytopathogens (Jankiewicz et al. 2012).

A purified chitinase chiIO8 from *Bacillus cereus* exhibited activity against *Botrytis cinerea* in an in vivo assay and the result was reported by Hammami et al. (2013). *Bacillus subtilis* JD- 09 was reported to restrict the growth of hyphae of *Fusarium* oxysporum. The isolated enzyme was also active against the fungus and exhibited high rates of chitin degradation (Velusamy and Das 2014). A 42-kDa extracellular chitinase which is active at 40 °C and pH 4.6 was reported from a entomopathogenic fungus *Verticillium lecanii* by Yu et al. (2015). The isolated enzyme was examined for antagonistic activity against different phytopathogens and could effectively resist their growth. The activity was observed to be strongly influenced by the presence of Mg^{2+}ions. Chitinases in the *Trichoderma* genus have

been investigated intensively and extensively for their widespread application in field biocontrol.

Carboxylesterases are enzymes with very broad substrate specificity and are remarkable candidates in the decomposition of organophosphate pesticides. Pyrethroids and malathion are examples of this. This group of enzymes can catalyse the breakage of ester, amide and thioester linkages. Singh et al. (2012a, b) carried out genus-level identification of some bacteria which are able to degrade malathion effectively in liquid culture with the enzyme carboxylesterase. *Lysinibacillus* sp. KB1, *Brevibacillus* sp. KB2 and *Bacillus cereus* PU are these strains and *Lysinibacillus* sp. KB1 could degrade 20% malathion and 47% malaoxon. For malaoxon 72.20% and for malathion 36.22% were utilised by *Brevibacillus* sp. strain KB2. *Bacillus cereus* PU showed the highest degree of degradation with 87.40% of malaoxon and 49.31% of malathion. The incubation time was 7 days for the experiments. Wu et al. (2006) reported that *Klebsiella* sp. strain ZD112 could produce an esterase-degrading pyrethroid (EstP) and cloned the same gene in *E. coli*. They found that the purified EstP could hydrolyse malathion, pesticides of the pyrethroid group and rho-nitrophenyl esters of some fatty acids. Cloning of pyrethroid-hydrolysing carboxylesterases gene was also reported by Wang et al. (2009) and Zhai et al. (2012) from *Sphingobium* sp. strain JZ-1 and *Ochrobactrum anthropi* YZ-1.

3.4 Enzymes in Biocontrol

Accelerated use of agrochemicals has adversely affected nature and has resulted in the emergence of a more acceptable and environment-friendly approach such as biocontrol strategy (Harman et al. 2010). Biocontrol agents include actinomycetes, bacteria and fungi, and may act on pathogens through antagonism, parasitism or antimicrobial compounds or by the induction of systemic resistance representing an environment-friendly approach to reduce losses due to pests and diseases or as an alternative to agrochemicals (Lugtenberg et al. 2002; Whipps 2001).

In the natural ecosystem antagonistic microorganisms have critical roles in keeping the balance of microbial habitats. This antagonistic property can be exploited for biological control (Henis and Chet 1975). Fungal pathogens are effectively restricted by degrading their cell walls containing chitin and β-1,3-glucans (Lam and Gaffney 1993). There are reports about members of bacteria and fungi that host enzymes that can hydrolyse glucans, protein, chitin, etc. Chitinases, glucanases and proteases are the major hydrolytic enzymes involved in biological control mechanisms. Abraham (2015) reported that even the tough structures like zoospores, conidia, chlamydospores, etc. could be destabilised by enzymes like chitinases. Extracellular proteases and ß-1,3-glucanases were also able to degrade spores.

3.4.1 Chitinases

Being composed of β-1,4-linked N-acetylglucosamine units, chitin is a modified polysaccharide abundantly present in fungal cell walls. The β-1,4 linkage is broken by the enzyme (Poly {1, 4-N-acetyl D-glucosaminide} glucano hydrolase) commonly designated as chitinase. The cleavage site of chitinase is the bond between C and C of two consecutive N-acetylglucosamine monomers (Flach et al. 1992). The chitinase enzyme has the potential to degrade the chitin-containing cell wall of pathogens. The exoskeleton of insect pests is also affected by chitinases. Thus in controlling the insect and fungal pathogens chitinases are powerful biological tools. As enzymes are not persistent, the pathogens fail in developing resistance against them.

Chitinases form a diverse group of enzymes and are divided majorly into two classes based on the mode of action. The International Union of Biochemistry and Molecular Biology recommends them as exochitinases and endochitinases. Endochitinases (EC 3.2.1.14) cleave randomly at internal sites, generating low-molecular mass multimers of N-acetylglucosamine (NAG) such as chitotriose and chitotetraose. Exochitinases are further divided into two subcategories, β-(1,4) N-acetyl hexosaminidases (EC 3.2.1.52) and chitobiosidases (EC 3.2.1.30). Chitobiosidases catalyse the release of diacetyl chitobiose from the nonreducing end of chitin monofibril. The oligomers obtained by endochitinases and chitobiosidases are cleaved by β-(1,4) N-acetyl glucosaminidases to monomers of N-acetyl glucosamine (Dahiya et al. 2006). Some chitinases also show lysozyme activity and in the cell wall of bacteria they can lyse the β-(1,4) linkage of the peptidoglycan layer (Gokul et al. 2000).

Henrissat and Bairoch (1993) report three families of chitin-degrading enzymes which have related sequences of amino acids and they are the glycosyl hydrolase families of 18, 19 and 20. In a family, the members show differences in amino acid sequences, three-dimensional structure, domain fold, reaction mechanisms and specificity of substrate. In family 18, which is multifaceted, animal, plant, bacterial, fungal and viral chitinases are represented. Family 19 consists of chitinases from *Streptomyces* and some members of plant chitinases. Certain chitinases of human origin and streptomyces origin belong to family 20. The reactions at the active site of chitinases of family 18 suggested a substrate-assisted catalysis mode (Tews et al. 1997). Eight α-helices and eight β-strands are oriented to form the active site of this family. This provides a (β/α) barrel appearance (Perrakis et al. 1994). Family 19 chitinases exhibit an acid base mechanism during catalysis (Hart et al. 1995) and their active site domains are composed of α- helica structures (Hart et al. 1995; Monzingo et al. 1996).

Table 3.2 Chitinases producing biocontrol agents against plant pathogens

Sl. No.	Biocontrol agents	Pathogens	References
1.	*Streptomyces lydicus*	*Pythium* and *Aphanomyces*	Mahadevan and Crawford (1997)
2.	*Trichoderma harzianum*	*Crinipellis perniciosa*	De Macro et al. (2000)
3.	*Serratia marcescens*	*Sclerotium rolfsii*	Ordentlich et al. (1988)
4.	*Serratia plymuthica*	*Bothrytis cinerea* and *Sclerotinia sclerotiorum*	Kamensky et al. (2003)
5.	*Pseudomonas* sp.	*Colletotrichum falcatum*	Viswanathan et al. (2003)
6.	*Aeromonas caviae*	*Rhizoctonia solani* and *Fusarium oxysporum* and *Sclerotium rolfsii*	Inbar and Chet (1991)
7.	*Arthrobacter* sp.	*Fusarium oxysporum*	Sneh (1981)
8.	*Bacillus cereus*	*Rhizoctonia solani*	Pleban et al. (1997)
9.	*Enterobacter agglomerans*	*Rhizoctonia solani*	Chernin et al. (1995)
10.	*Paenibacillus illinoisensi*	*Rhizoctonia solani*	Jung et al. (2003)

Chitinases can be further classified into six different classes (Iseli et al. 1996). The grouping is based on N-terminal sequence, inducers, signal peptide, isoelectric point and the enzyme location. Class I chitinases are located in vacuoles. Signal peptides show high valine content and N terminal has high cysteine content (Flach et al. 1992). This class has two subdivisions, basic (Ia) and acidic (Ib) chitinases, and both are reported from plants with an intracellular action. The class II chitinases are of plant, fungal and bacterial origin and secreted extracellularly. Sequence similarity is observed between Class I and Class II chitinases but class II lack the cysteine-rich N- terminal. Class III do not show any sequence similarity with class I and class II. Class IV chitinases are smaller in size (Collinge et al. 1993). A few examples of classes V and VI are reported (Iseli et al. 1996). The various chitinase-producing biocontrol agents and the pathogens affected are detailed in Table 3.2.

3.4.2 β-Glucanase

β-glucans, made up of β-linked glucose residues, are important structural components in the fungal cell walls and contribute to rigidity and offer protection (Stone and Clarke 1992). Enzymes such as β-1,3-glucanase act against β-1,3-glucosidic linkages and hence can disrupt the fungal cell wall. β-1,3-glucanase production has

Table 3.3 β-glucanases producing biocontrol agents against plant pathogens

Sl. No.	Biocontrol agents	Pathogens	References
1.	*Streptomyces* sp.	*Phytophthora fragariae*	Valois et al. (1996)
2.	*Actinoplanes philipinensis* and *Micromonospora chalcea*	*Pythium aphanidermatum*	El-Tarabily (2006)
3.	*Streptomyces cavourensis*	*Colletotrichum gloeosporioides*	Lee et al. (2012)
4.	*Pseudomonas stutzeri*	*Fusarium solani*	Lim and Kim (1995)
5.	*Trichoderma harzianum*	*Sclerotium rolfsii*	El-Katatny et al. (2001)
6.	*Paenibacillus* sp.	*Fusarium oxysporum*	Singh et al. (1999)
7.	*Pseudomonas cepacia*	*Rhizoctonia solani, Sclerotium rolfsii* and *Pythium ultimum*	Fridlender et al. (1993)
8.	*Bacillus amyloliquefaciens*	*Phytophthora meadii*	Abraham et al. (2013)
9.	*Lysobacter enzymogenes*	*Pythium* sp.	Palumbo et al. (2005)
10.	*Pichia anomala*	*Botrytis cinerea*	Grevesse et al. (2003)

been reported from fungi, bacteria, actinomycetes, algae, molluscs and higher plants (Pitson et al. 1993) and many of these organisms are being exploited as efficient biocontrol agents. Depending upon the mode of hydrolysis the β-1,3-glucanases can be classified as exo-β-1,3-glucanase (EC3.2.1.58) and endo-β-1,3-glucanase (EC 3.2.1.39). Endohydrolases act randomly on β-1,3-D-glucan generating small oligosaccharides (Reese and Mandels 1959) whereas exo-β-1,3-glucanases remove glucose units from terminal β-1,3-D linkages generating D-glucose as the sole product (Pitson et al. 1993). Along with this, based on the folding properties, amino acid sequences and structural properties of glucanases can be further classified into many groups. The various biocontrol agents offering beta glucanase activity and the pathogens affected are listed in Table 3.3.

3.4.3 Proteases

Besides chitin and glucan, the skeleton of filamentous fungal cell walls contains proteins. Proteases may therefore play a significant role in the cell wall lysis that occurs during pathogen – biocontrol agent interactions.

Proteases are subdivided into two major groups based on the position of the peptide bond cleavage: exopeptidases and endopeptidases. The exopeptidases act only near the ends of polypeptide chains. Exopeptidases cleave the peptide bond proximal to the amino or carboxy terminal of the substrate. Based on their site of action at the N or C terminus, they are classified as aminopeptidases and carboxypeptidases, respectively. The former act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide or a tripeptide while the latter act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Endopeptidases cleave peptide bonds distant from the termini (Barrett and McDonald 1986). The peculiar characteristic of endopeptidases is their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini.

Based on the pH optimal for their functioning, proteolytic enzymes can be classified as alkaline, neutral or acidic proteases.

3.4.3.1 Acid Proteases

Acid proteases are proteases which are active in the pH range of 2–6 (Rao et al. 1998) and are mainly fungal in origin (Aguilar et al. 2008). Common examples in this subclass include aspartic proteases of the pepsin family. Some of the metalloprotease and cystein proteases are also categorised as acidic proteases.

3.4.3.2 Neutral Proteases

Neutral proteases are highly stable enzymes with broad substrate specificity. They cleave peptide bonds of hydrophobic amino acids from the aminoterminal end. The enzyme works best at neutral pH and is activated in the presence of calcium, magnesium and manganese ions. The main sources are plant, fungi and bacteria (Aguilar et al. 2008).

3.4.3.3 Alkaline Proteases

Alkaline proteases are an enzymatic group that is exploited in the industrial field. They can also be used to restrict some pests and pathogens. They show maximum activity at a pH of 8–13. Halophilic bacteria as well as alkaline bacteria are dependable sources of alkaline proteases. The presence of this hydrolytic enzyme make these organisms suitable for exploiting the biocontrol property. In most cases the active site consists of a serine residue, though some alkaline proteases may have other amino acid residue in their active site (Rao et al. 1998).

Four main groups of proteases are observed. They are: (1) serine proteases, (2) aspartic proteases, (3) cysteine proteases and (4) metalloproteases. This division is based on the mechanism of catalysis and the nature of the functional group present at the active site.

3.4.3.4 Serine Proteases

Serine proteases are identified as biocontrol agents and isolated from both prokaryotes and eukayotes. Viruses also exhibit their presence. The antinematode activity

of serine proteases is well studied. The serine residue is a characteristic in the active site of this enzyme group. Serine proteases are the most widely distributed group of proteolytic enzymes. They are tolerant to high alkaline pH though the optimum is at 7–11. The diverse serine proteases are classified into 20 families. Barett (1994) reports that there are six clans and that they are supposed to have common ancestors. The sequence analysis of the four clans, chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC) and Escherichia D-Ala–D-Ala peptidase A (SE), shows wide differences among them, leading to the conclusion that they are descended from various origins.

The compound diisopropyl fluorophosphate (DFP) can inhibit the enzyme activity. Tosyl-L-lysine chloromethyl ketone and phenyl methyl sulphonyl fluoride are also found to inhibit serine proteases. Serine proteases hydrolyse an amide bond at the carboxyl end of the polypeptide which has tyrosine, phenylalanine or leucine residues.

3.4.3.5 Aspartic Proteases

Aspartic proteases can exhibit biorestriction of insect pests. They are mostly active at a pH of 3–4. Many reports on them show a fungal origin, though bacterial and protozoan candidates are present. In the active site they possess two aspartate residues which are conserved.

3.4.3.6 Cysteine/Thiol Proteases

A promising group of enzymes in biocontrol is Cysteine/thiol proteases which have occurrence in plants, animals and prokaryotes. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine (Barrett and McDonald 1986). From different sources, they exhibit different pH optima. They can function at acidic or alkaline pH. For activity, they need the proximity of Hydrogen Cyanide (HCN) or cysteine-like reducing agents. A classical example of cysteine protease is papain and it has best activity around pH 7. But lysosomal proteases exhibit maximum activity at acidic pH. Cysteine proteases are affected by sulfhydryl agents, but it is reported that EDTA or DFP does not impair activity.

3.4.3.7 Metalloproteases

Metalloproteases possess metal ions, often $Zn^{2+,}$ $Co^{2+,}$ Mn^{2+}, and are affected by EDTA because it can chelate metal ions, and are insensitive to sulphydryl agents. The reactivation is enabled by providing the required metal ions. They are maximally active at a pH of 5–9. They were isolated and characterised from bacteria and fungi. The stability of domain structure is maintained by calcium. The list of various protease-producing biocontrol agents and the pathogens affected by tyhem are given in Table 3.4.

Table 3.4 Proteases producing biocontrol agents against plant pathogens

Sl No.	Biocontrol agents	Pathogens	References
1.	*Streptomyces griseorubens*	*Rhizoctonia solani*	Al-Askar et al. (2015)
2.	*Streptomyces griseus*	*Fusarium solani*	El-Khonezy et al. (2015)
3.	*Stenotrophomonas maltophila*	*Pythium ultimum*	Dunne et al. (1997b)
4.	*Trichoderma harzianum*	*Fusarium oxysporum*	Sivan and Chet (1989)
5.	*Trichoderma harzianum*	*Botrytis cinerea*	Elad and Kapat (1999)
6.	*Trichoderma virens*	*Rhizoctonia solani*	Pozo et al. (2004)
7.	*Bacillus amyloliquefaciens*	*Phytophthora capsici*, *Fusarium solani*	Kota et al. (2015)
8.	*Talaromyces flavus*	*Botrytis fabae*	Haggag et al. (2006)
9.	*Pseudomonas putida*	*Phytophthora drechsleri*	Tabarraei et al. (2011)
10.	*Pseudomonas* sp.	*Pyricularia oryzae*	Sharifi-Noori et al. (2015)

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Enzymes as Molecular Tools

4

Gayathri Valsala and Shiburaj Sugathan

Abstract

Development of molecular techniques has led to revolutionary advancements in various fields of biosciences. Enzymes that modify nucleic acids are of paramount importance in these techniques. A wide range of enzymes involved in the catalysis of polymerization, ligation, cleavage, and other manipulations of DNA and RNA are currently available. Novel enzymes with improved properties are being developed by manufacturers. Molecular cloning is now a regular laboratory technique with a vast array of enzymes available from various commercial sources and to select an enzyme apt for a particular application has become a tedious task. This chapter aims to provide details on the different commercial enzymes available for routine molecular biology work, in addition to reviewing the important classes of enzymes that are used in molecular biology.

Keywords

Polymerases • Nucleases • Ligases • Enzymes • Molecular biology

4.1 Introduction

Enzymes are biomolecules capable of acting as catalysts and help accelerate biochemical reactions. They are responsible for the regulation of biochemical pathways in all living organisms. The catalytic ability of enzymes is highly specific and is more efficient in accelerating the rate of a chemical reaction than any

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human-made artificial systems. Almost all enzymes are proteins with the exception of ribozymes or RNAzymes, which are RNA molecules that are capable of catalyzing specific biological reactions. Molecular biology has benefited more from enzymes than any other field in biosciences. Of various enzymes used in molecular biology, restriction endonucleases are of utmost significance. These bacterial enzymes serve as defense against bacteriophages by cleaving the phage DNA while the host DNA is protected due to methylation. Type II restriction enzymes are the most important among restriction enzymes, because of their capability to cut DNA molecules at specific sites known as restriction sites. The discovery of restriction endonucleases laid the foundation stone for rDNA technology. A vast number of restriction enzymes from varying sources of bacteria are now available commercially. Danna and Nathans (1971) carried out the pioneer work in demonstrating the possibilities of restriction enzymes in molecular biology, which paved the way for the modern field of molecular biology as we see it today. Molecular biology currently employs a wide array of enzymes that are involved in the manipulation of DNA, RNA, and protein molecules. In this current scenario, one must be familiar with the specific properties and utilities of each enzyme to choose the best one for application in a particular technique. Particulars regarding the most preferred enzymes and their characteristics are discussed here.

4.2 DNA-Dependent DNA Polymerases

DNA polymerases are enzymes that catalyze the synthesis of DNA molecules from their monomer units, deoxyribonucleotides. These enzymes are essential for DNA replication and usually work in pairs to create two identical DNA strands from one original DNA molecule. They are responsible for duplication and passing of genetic data over generations. During cell division, DNA polymerases carry out semi-conservative replication of DNA, where a daughter DNA strand is synthesized from a single-stranded parent DNA template using four deoxyribonucleotides triphosphate monomers (dTTP, dCTP, dGTP, dATP) as precursors. DNA polymerases are unable to carry out de novo synthesis of a polynucleotide molecule and can only incorporate nucleotides to an existing 3′-OH end of a primer. DNA polymerization occurs only in the 5′ → 3′ direction and each deoxynucleotide added are complementary to the one present on the template strand and thus the genetic information is passed through generations. In addition, many DNA polymerases show proofreading via 3′ → 5′ exonuclease activity. If an error occurs during DNA synthesis, the misincorporated nucleotide is excised out as a monophosphate, and the correct base is introduced by the polymerase. Another catalytic property of DNA polymerases is its 5′ → 3′ exonuclease, nick translation activity which is required for the elimination of RNA primers during replication. Some DNA polymerases possess terminal transferase activity by which a single nucleotide (usually adenine) is added to the 3′ termini of PCR products.

The type and number of DNA polymerases vary among different organisms. They are all grouped together into A, B, C, X, and Y families based on amino acid

sequences of catalytic region (Burgers et al. 2001). They all share similar structural configuration and have conserved regions in their catalytic site (Franklin et al. 2001; Steitz 1998). DNA polymerases used in molecular biology belong to families A and B. They are used for DNA manipulations like terminal or whole sequence labeling, modification of DNA termini, production of double-stranded cDNA, DNA sequencing, extension of synthetic DNA sequences for gene synthesis, site-directed mutagenesis, and amplification of a particular portion of DNA for analysis.

DNA polymerase I of *Escherichia coli* was discovered by Arthur Kornberg in 1956 (Kornberg 1957). Sanger et al. (1977) used it for developing dideoxy sequencing of DNA. Kary Mullis in 1985 developed the PCR technique (Cetus Corporation, California, USA) and was awarded the Nobel Prize in Chemistry in 1993 (Mullis et al. 1986). Initially, PCR was carried out with the Klenow fragment of DNA polymerase I from *E. coli*, but being sensitive to the high temperature used for denaturation step, it had to be replenished after each cycle. This was resolved by the use of a thermostable DNA polymerase from *Thermus aquaticus*, a thermophilic bacterium isolated from geysers in Yellowstone National Park, Montana, USA (Saiki et al. 1988). Since then, polymerases from a number of thermophilic microorganisms like *Pfu* polymerase from *Pyrococcus furiosus*, Wind or Tli polymerase or Vent polymerase from *Thermococcus litoralis*, and Tth polymerase from *Thermus thermophilus* have been developed for use in molecular biology. Initially, the amplification capacity of PCR was limited to a short stretch of DNA of up to 10 kb size. With the development of recombinant polymerases, it became possible to amplify fragments up to 70 kb size (Blanco et al. 1989).

A wide selection of DNA polymerases with unique properties is currently available with different rates of extension and proofreading activity (Table 4.1). The choice of polymerase is based on the technique used and the type and size of template. PCR technique has revolutionized medical and forensic science and is currently used for the detection of hereditary diseases, gene cloning, DNA fingerprinting, parentage testing, and in the detection of infectious diseases like AIDS.

4.3 Terminal Deoxynucleotidyl Transferases

Terminal deoxyribonucleotidyl transferase (TdT), also known as DNA nucleotidylexotransferase (DNTT) is a template-independent DNA polymerase first identified in calf thymus. It is expressed in mammalian lymphocytes and is currently manufactured commercially by the over expression of the bovine gene DNA nucleotidylexotransferase (DNTT) in *E. coli*. This enzyme catalyzes the incorporation of deoxynucleotides to the 3′-OH termini of DNA without the help of a template. It can efficiently act on 3′ protruding ends, while addition of nucleotides to blunt and 3′-recessed ends of DNA is of low efficiency. TdT lacks 3′ → 5′ and 5′ → 3′ exonuclease activities (Chang and Bollum 1986). Cobalt is an essential cofactor required for the functioning of this enzyme.

Table 4.1 Commercially important DNA polymerases

Name of enzyme and description	Special characteristics	Source	Applications	References
DNA Polymerase I: A103 kDa single-polypeptide protein coded by *polA* gene. It was first commercially produced by the cloning of the *polA* gene into a lysogenic strain of bacteriophage λ.	3′ → 5′ and 5′ → 3′ exonuclease activity.	*E. coli*	DNA labeling by nick translation	Kornberg (1957)
	5′ → 3′ RNA-dependent DNA polymerase activity with low efficiency.		Second strand cDNA synthesis	
			Conversion of staggered DNA ends into blunt end.	
The Klenow Fragment of DNA Polymerase I: The larger of the two fragments (76 kDa) of DNA polymerase I is enzymatically cleaved with protease. Used by Sanger for DNA sequencing by dideoxy chain-terminating method.	5′ → 3′ polymerase activity and 3′ → 5′ exonuclease activity for proofreading.	*E. coli*	Modified enzyme lacking 3′ → 5′ exonuclease activity is used in DNA labeling	Klenow and Henningsen (1970)
			Filling of 3′ DNA ends with 5′ extensions.	
T4 DNA Polymerase: 104-kDa single polypeptide protein coded by *gene 43* of bacteriophage T4. Production either from phage-infected *E. coli* cells or by cloning and over expression of the specific gene in *E. coli*.	3′ → 5′ exonuclease activity 200-fold stronger than DNA pol I which gives it a high level of fidelity	Bacteriophage T4	5′ DNA overhangs are filled by polymerase activity and 3′ overhangs are cleaved by exonuclease activity to create blunt ends.	Nossal (1984)
	It does not displace oligonucleotides hybridized to DNA		DNA labeling of 3′ ends and for the detection of stable DNA lesions	
			Site-specific mutagenesis by primer extension	
Native T7 DNA Polymerase: It is synthesized by *E. coli* infected with bacteriophage T7. The enzyme has two subunits, an 84 kDa protein coded by T7 *gene 5* and 12 kDa thioredoxin of *E. coli*. Thioredoxin is a small redox protein that helps to stabilize binding of the protein-to-the primer-template to improve processivity by more than 100-fold.	High processivity	Bacteriophage T7	PCR, strand extensions in site-directed mutagenesis and second strand synthesis of cDNA	Huber et al. (1987) and Tabor et al. (1987)
	3′ → 5′ exonuclease activity		T7 polymerase can be used for the synthesis of long stretches of DNA template due to its high fidelity and rapid extension rate	

Sequenase: Modified T7 DNA polymerase with low 3′ → 5′ exonuclease activity created by an iron-catalyzed oxidation reaction. A mutant with nil exonuclease activity but enhanced polymerase activity has led to version 2 of Sequenase.	Exonuclease activity is greatly reduced.	Bacteriophage T7	Sanger DNA sequencing.	Tabor and Richardson (1989)
***Taq*DNA Polymerase:** Most well known among DNA polymerases and is responsible for transforming PCR into an indispensable tool for molecular biology. This 94-kDa polypeptide has a 5′ → 3′ structure-dependent nuclease activity in addition to its 5′ → 3′ polymerase activity.	Nontemplate-dependent terminal transferase activity Lacks 3′ → 5′ exonuclease activity	*Thermus aquaticus*	PCR, TA cloning which uses the complementarity between a single 3′-T overhanging of a linearized ‘T-vector’ with PCR products having 3′-A overhangs	Brock and Freeze (1969), Zhou and Gomez-Sanchez (2000)
***Pfu*DNA Polymerase:** A 90 kDa thermostable DNA polymerase isolated from a hyperthermophilic archaebacteria. It exhibits a long half-life and higher stability than *Taq* polymerase.	3′ → 5′ exonuclease activity and a high proofreading efficiency	*Pyrococcus furiosus*	DNA polymerization reactions requiring high-fidelity synthesis such as gene cloning, gene expression, or mutation analysis	Angers et al. (2001)
Vent DNA Polymerase: Also known as *Tli* DNA polymerase, it is a 93 kDa high-fidelity thermostable DNA polymerase from a hyperthermophilic archaebacteria.	3′ → 5′ proofreading exonuclease activity	*Thermococcus litoralis*	PCR	Belkin and Jannasch (1985)
	High thermostability with a half-life of 8 h at 95 °C and about 2 h at 100 °C		Primer extension	
Deep Vent DNA Polymerase: High-fidelity thermophilic DNA polymerase from a *Pyrococcus* sp. isolated from a submarine thermal vent at 2010 m with temperatures as high as 104 °C.	3′ → 5′ proofreading exonuclease activity	*Pyrococcus* sp. GB-D	PCR	Jannasch et al. (1992)
	High stability with half-life of about 24 h at 95 °C.		Primer extension	

(continued)

Table 4.1 (continued)

Name of enzyme and description	Special characteristics	Source	Applications	References
KOD DNA Polymerase: Isolated from an extreme thermophile. Three different versions of KOD Pol are commercially available (KOD HiFi, KOD Hot Start. and KOD XL).	High extension rate and high fidelity	*Thermococcus kodakaraensis* KOD1	KOD HiFi: amplifies DNA targets up to 6 kb	Takagi et al. (1997)
			KOD Hot Start: KOD HiFi DNA polymerase with two monoclonal antibodies that inhibit the DNA polymerase and 3′ → 5′ exonuclease activities at ambient temperatures	
			KOD XL: Blend of KOD HiFi DNA polymerase and a mutant form of KOD HiFi deficient in 3′ to 5′ exonuclease activity. It can amplify longer (up to 30 kb) and more complex GC-rich targets.	
ULTMA DNA Polymerase: The first commercial DNA polymerase from a hyperthermophilic bacterium.	Low 3′ → 5′ exonuclease proofreading activity	*Thermotoga maritima*	It was not a commercial success because of its poor fidelity	Diaz and Sabino (1998)
***Bst*DNA Polymerase:** A 75-kDa moderately thermostable DNA polymerase. A recombinant *Bst* DNA lacking the 5′ → 3′ exonuclease domain is also available.	5′ → 3′ exonuclease activity	*Bacillus stearothermophilus*	Synthesis of difficult DNA regions like repetitive sequences, GC-rich regions, and problematic secondary structures like hairpin loops. It is also ideal for DNA synthesis reactions requiring strand displacement.	Stenesh and Roe (1972)
	Temperature optimum of 60–65 °C			
	Strand displacement property			
phi29 DNA Polymerase: A 68-kDa DNA polymerase that preferentially acts on single-stranded DNA, isolated from *Bacillus subtilis* bacteriophage phi29. The main demerit is its half-life of only 10 min at 65 °C.	Highly accurate DNA synthesis due to its 3′ → 5′ exonuclease activity	Bacteriophage phi29	Whole genome amplification, rolling circle amplification, multiple displacement amplification, protein-primed DNA amplification, and in situ genotyping with padlock probes.	Blanco et al. (1989)
	Synthesis of DNA fragments of more than 70 kb			Alsmadi et al. (2009)

TdT is widely used in molecular biology for applications such as labeling of 3′ends of DNA with modified nucleotides for primer extension DNA sequencing, generation of DNA blunt ends, in TUNEL (TdT dUTP Nick End Labeling) assay for detecting apoptosis. Another use of this enzyme is in the addition of complementary homopolymeric tails to linearized plasmid DNA with G's and the cDNA with C's which when incubated together anneal together and can be then transformed into *E. coli* (Rittié and Perbal 2008).

4.4 DNA-Dependent RNA Polymerases

DNA-dependent RNA polymerases catalyze the transcription process, which is the 5′ → 3′ synthesis of RNA using a DNA template. Similar to DNA polymerases lacking exonuclease activity, RNA polymerases are capable of adding an extra base to the end of a transcript. RNA polymerase was discovered independently by Charles Loe, Audrey Stevens, and Jerard Hurwitz in 1960. They are ubiquitously found in all living organisms and also in many viruses. Applications of RNA polymerases in molecular biology include *in vitro* synthesis of antisense RNA transcripts, labeling of RNA probes, and for RNase protection assay (Melton et al. 1984; Zinn et al. 1983).

DNA-dependent RNA polymerases that are used in *in vitro* transcription include *E. coli* RNA polymerase and bacteriophage RNA polymerases T7, T3, and SP6 (Struhl 1997). The bacteriophage RNA polymerases are coded by *gene 1* present in a family of related bacteriophages. They are capable of catalyzing high-yield transcription of DNA sequences present downstream from their promoter, with tenfold higher elongation rate than that of *E. coli* RNA polymerase. In addition, their initiation is extremely specific for the individual promoter sequence.

4.4.1 *E. coli* RNA Polymerase (Holoenzyme)

E. coli RNA polymerase holoenzyme (~480 kDa) consists of the core enzyme complex composed of 5 subunits ($\alpha_2\beta\beta'\omega$) along with sigma factor 70 (σ^{70}). RNA synthesis by this enzyme can be initiated by σ^{70} specific bacterial and phage promoters on recognition of the −10 and −35 sequences in the promoter region and terminate at terminator sequences. Initial studies on *in vitro* transcription were carried out using *E. coli* RNA polymerase (Chamberlin and Berg 1962). Transcription efficiency is influenced by DNA template quality, promoter strength, and terminator sequences, in addition to the composition of reaction mixture. *E. coli* RNA polymerase holoenzyme can also be used to identify cloned DNA segments containing promoter sequences required for expression in *E. coli* cells. *E. coli* RNA polymerase core enzyme is directly obtained from *E. coli* cells, while the sigma factor 70 is obtained after cloning, expression, and purification of the

protein in *E. coli*. Bacteriophage RNA polymerases have replaced this enzyme for most applications due to the following demerits: detachment of subunits leading to premature termination of *in vitro* and incapability in synthesizing uniform RNA population (Chamberlin and Ryan 1982). *E. coli* RNA polymerase holoenzyme is still in use for synthesizing transcripts for cloning into vectors lacking phage RNA polymerase promoter. The core enzyme devoid of the σ subunit can synthesize uniform short transcripts from DNA template under conditions of high concentrations of random primers and low concentrations of NTP (nucleoside triphosphate).

4.4.2 T7 RNA Polymerase

T7 RNA polymerase is a polypeptide of 98 kDa size isolated from T7 bacteriophage (Stahl and Zinn 1981). It has high promoter specificity and can carry out transcription of DNA present downstream from a T7 promoter. RNA synthesis by this enzyme requires a double-stranded DNA template in addition to Mg^{2+} as cofactor (Chamberlin et al. 1970). T7 RNA polymerase is usually stimulated by BSA or spermidine. It has a very low error rate and can transcribe through poly(A) regions, which helps in multiple transcriptions of circular DNA template without dissociation. T7 RNA polymerase is usually employed in the transcription of DNA cloned into vectors containing two different phage promoters in opposite orientations. RNA synthesis can be carried out selectively from either of the inserted DNA strands using different polymerases. Commercial production is from *E. coli* cloned and expressed with T7 gene I. The major applications of this enzyme include generation of homogeneously labeled single-stranded RNA, nonisotopic labeling, RNA vaccine synthesis and production of antisense RNA for gene expression experiments.

4.4.3 T3 RNA Polymerase

E. coli bacteriophage T3 RNA polymerase is a 99 kDa enzyme that catalyzes *in vitro* RNA synthesis from a cloned DNA sequence under the T3 promoter. It is coded by gene I of T3 phage. The amino acid composition of this polymerase shows 82% identity to T7 RNA polymerase. Promoter sequence for the T3 RNA polymerase is present 12 base pairs away from the stop codon of structural gene. This enzyme can be used to generate huge quantities of particular RNA transcripts from cloned vectors containing T3 promoter. Such RNA transcripts have application in Northern and Southern blotting as probes, studies on *in vitro* translation and RNA processing, manufacture of RNA vaccines, and for exon-intron mapping of genomic DNA (McGraw et al. 1985). It can polymerize both radioactively labeled and hapten-labeled nucleoside triphosphates.

4.4.4 SP6 RNA Polymerase

SP6 RNA polymerase was isolated from SP6 bacteriophage-infected *Salmonella typhimurium* LT2. It is of 96 kDa size and requires Mg^{2+} for its activity. This enzyme has been found to be activated by spermidine and serum albumine (Butler and Chamberlin 1982). SP6 shows high promoter specificity and can only transcribe DNA segments inserted downstream to their specific promoter. It also has the ability to transcribe through poly(A) stretches (Melton et al. 1984). SP6 RNA polymerase can be used for all techniques mentioned for T7 RNA polymerase. SP6 polymerase requires a complete double-stranded DNA template for its activity, in contrast to T7 RNA polymerase, which has high efficiency when 18 base promoter regions are double-stranded. RNA yield is much higher for SP6 polymerase in comparison to T7 polymerase, and on scale up of the reaction, even milligram quantities of RNA can be obtained.

4.4.5 Thermus RNA Polymerase

Thermus RNA polymerase is isolated from *Thermus aquaticus*, a thermophile, and is the only commercially available RNA polymerase that can remain stable and function at temperatures higher than 65 °C. It is structurally similar to *E. coli* RNA polymerase and has the same promoter requirements. However, Thermus RNA polymerase is resistant to several inhibitors of the *E. coli* enzyme, including fidaxomicin and rifampin. It has low transcription termination efficiently and has high rate of intrinsic transcript cleavage. Commercial production of this enzyme is by cloning and over expression of Thermus RNA polymerase gene in *E. coli* (Minakhin et al. 2001).

4.5 RNA-Dependent RNA Polymerases

RNA-dependent RNA polymerases (RdRp) are enzymes that catalyze RNA strand synthesis complementary to a given RNA template. They are encoded by all viruses with negative sense RNA as its genetic material. These enzymes transcribe the RNA into messenger RNA with the help of viral and host factors. A number of eukaryotes have also been found to produce putative RdRps that are considered to be involved in RNA-triggered sequence-specific gene silencing.

4.5.1 phi6 RNA Polymerase

An RNA-dependent RNA polymerase from *Pseudomonas* bacteriophage phi6, it has high processivity and ability to act on single- or double-stranded RNA template. Even though it does not require a primer for initiation, the sequence 5′-NNUUUUUUUUCC-3′ is essential on the template strand for optimal initiation

(Makeyev and Bamford 2000). The phi6 RNA polymerase can generate labeled RNA that can be used as a probe in hybridization and in the synthesis of dsRNA used in RNA interference (RNAi) for *in vivo* gene silencing. Another application is in primer-independent RNA sequencing using chain terminators, (RdRP sequencing) where 3′ proximal or even complete sequence of target RNA molecules can be rapidly deduced without prior sequence information. This has applications in RNA virus research and diagnostics (Makeyev and Bamford 2001).

4.6 Template-Independent RNA Polymerases

Majority of the RNA molecules in cells are synthesized from a DNA template. In some cases, like poly(A) tailing of messenger RNA, RNA synthesis occurs without a template.

4.6.1 Poly(A) Polymerase

It is a template-independent polymerase that synthesizes the poly(A) (adenine) tail at the 3′ end of messenger RNA. By using ATP as precursor, poly(A) polymerase catalyzes the addition of AMP residues to the free 3′-hydroxyl terminus of RNA (Edmonds 1982). It highly prefers ATP when compared to other NTPs. Varying lengths of poly(A) tail can be synthesized by adjusting the time of incubation, enzyme quantity, and available number of 3′ termini for addition. Poly(A) polymerase from *E. coli* and yeast are used in molecular biology for RNA tailing and 3′ end labeling. Poly(A)-tailed RNA molecules can be PCR amplified using oligo(dT) primers by reverse transcription, and the resultant cDNA can be used in cloning. Since poly(A) polymerase can incorporate modified nucleotides like biotin-N6-ATP and digoxigenin-11-UTP with high efficiency, it can be used to synthesize RNA molecules with radioactive or fluorescent label for using as probes in various applications (Martin and Keller 1998).

4.7 Reverse Transcriptases

They are RNA-dependent DNA polymerases that catalyze the synthesis of a DNA molecule complementary to a given RNA template. Reverse transcriptases are encoded by retroviruses and are essential for their life cycle. The viral RNA genome is converted into DNA via reverse transcriptase and the synthesized proviral dsDNA molecule integrates into host cells during infection. Under *in vitro* conditions, reverse transcriptase can synthesize DNA using either ssRNA or ssDNA templates with the help of a primer. Reverse transcriptases have lower fidelity than DNA-dependent DNA polymerases since they lack $3' \rightarrow 5'$ exonuclease proofreading activity.

In addition to the DNA polymerase activity, reverse transcriptases also show RNase H activity involved in the degradation of RNA in an RNA-DNA hybrid, such as those obtained during reverse transcription using an RNA template. It has both endonuclease and exonuclease activities. Reverse transcriptase has become an essential component of molecular biology with the introduction of reverse transcription polymerase chain reaction (RT-PCR) used for the synthesis of single-stranded "complementary" cDNA of a RNA template.

4.7.1 AMV/MAV Reverse Transcriptase

It is the most widely used reverse transcriptase in molecular biology and is isolated from Avian Myeoloblastosis Virus (AMV), an alpha retrovirus that causes acute myeloblastic leukemia (AML) in chicken (Baluda et al. 1983). Being a deficient virus, AMV requires a helper virus, Myeloblastosis Associated Virus (MAV) for the synthesis of envelope and RT proteins. The actual source of RT in AMV life cycle is MAV (Perbal 2008). The AMV/MAV RT is composed of two structurally similar subunits, α of 65 kDa and β of 95 kDa, which assemble to form a holoenzyme, the active reverse transcriptase. The α subunit contributes to the RT and RNase H activities, the latter of which is conferred by a 24 kDa fragment of the α subunit by which RNA strands can be specifically degraded from RNA-DNA hybrids in both directions. AMV/MAV RT is extensively used in molecular cloning for the synthesis of cDNA from mRNA using poly dT or random primers, for techniques such as quantitative PCR, rapid amplification of cDNA ends (RACE), and nucleotide sequencing.

4.7.2 M-MuLV Reverse Transcriptase

This reverse transcriptase is isolated from Moloney Murine Leukemia Virus (M-MuLV), is devoid of DNA endonuclease activity and $3' \rightarrow 5'$ exonuclease activity. RNAse H activity of this enzyme is comparatively much lower than AMV/MAV RT (Moelling 1974). It has four times less efficiency and lower stability than AMV/MAV RT. The main advantage of M-MuLV RT is its ability to synthesize transcripts of higher length than AMV/MAV RT (Houts et al. 1979). A recombinant M-MuLV reverse transcriptase with lower RNase H activity and higher thermostability has been produced under the name ProtoScript II Reverse Transcriptase (New England Biolabs), which is active up to 48 °C, shows higher specificity and cDNA yield, and can synthesize cDNA of up to 12 kb size.

4.7.3 Tth DNA Polymerase

This thermostable DNA polymerase of ~94 kDa size, isolated from *Thermus thermophilus* strain HB8, can efficiently carry out reverse transcription of RNA in a

reaction mixture containing $MnCl_2$. Its half-life is 20 min at 95 °C and can synthesize DNA at 74 °C. It also lacks RNase H activity. This enzyme can be used to amplify DNA from RNA templates containing GC-rich sequences or secondary structures since it stays active at elevated temperatures used to denature such template RNA and increase primer hybridization specificity. Reverse transcription and PCR amplification is possible with a single enzyme, eliminating the chance of cross-contaminations that occurs frequently in two-steps RT-PCR. Tth pol has wide applications in detection, quantization, and cloning of RNA due to its ability to catalyze both reverse transcription and DNA amplification. Tth DNA polymerase is apt for use in RT-PCR of RNA up to 1000 bp, PCR amplification of single-stranded DNA, primer extension reactions at elevated temperatures, and labeling of DNA fragments with radionucleotides, digoxigenin, or biotin (Myers and Gelfand 1991).

4.7.4 *C. therm* Polymerase Klenow Fragment

The Klenow fragment of polymerase from *Carboxydothermus hydrogenoformans* (*C. therm.*) has reverse transcriptase activity in the presence of Mg^{2+} and is found to be active up to 72 °C. It can catalyze the reverse transcription of mRNA, polyadenylated RNA, and viral RNA at high temperatures, which helps to reduce the effect of secondary structures in mRNA and increases primer annealing specificity. In addition to this, the enzyme also has $3' \rightarrow 5'$ exonuclease proofreading activity.

4.8 Nucleases

Nucleases are enzymes that cleave the phosphodiester bonds in the nucleic acid backbone. Deoxyribonucleases cleave DNA, while ribonucleases cleave RNA. Nucleases are classified into exonucleases and endonucleases according to their site of action. Exonucleases cleave end portion of nucleic acid molecules while endonucleases cut internal sites in nucleic acids. Some enzymes show both exonuclease and endonuclease activities. Nucleases, especially restriction endonucleases are of extreme importance in molecular biology for the manipulation of nucleic acids for techniques like cloning.

Nucleases are ubiquitous in living organisms and are responsible for partial or complete digestion of nucleic acids. They are involved in nucleic acid degradation, senescence, apoptosis, replication, recombination, and repair.

4.8.1 Deoxyribonuclease

Deoxyribonucleases (DNase) catalyzes the hydrolysis of phosphodiester bonds in DNA backbone, thus cleaving it. They are categorized into exo- and endonucleases on the basis of their requirement for a free terminus for cleavage. Exonucleases can cleave either 3′ or 5′ end of a DNA molecule, while endonucleases cleave internal phosphodiester bonds either randomly or specifically depending on the enzyme.

4.8.1.1 Deoxyribonuclease I

Deoxyribonuclease I (DNase I) obtained from bovine pancreas was the first DNase to be isolated. This endonuclease cuts DNA nonspecifically and generates di-, tri-, and oligonucleotides with 5′-phosphate and 3′-hydroxyl ends as products. Earlier, this enzyme was obtained directly from pancreatic tissues, but now it is produced by over expression in *E. coli* containing the bovine pancreatic DNase I gene. This 31 kDa glycoprotein cleaves ssDNA, dsDNA, chromatin, and RNA- DNA hybrids. However, lysis of ssDNA requires high concentration of enzyme (Vanecko and Laskowski 1961). DNase I has been commercially available for more than 25 years, and is used for the complete degradation of unwanted DNA in protein and RNA samples. RNA samples can be treated with this enzyme prior to RT-PCR to remove any contaminant DNA. It is also used in partial hydrolysis of DNA molecule for nick translation, random fragmentation of DNA for dideoxy sequencing, in DNA-protein interaction analysis for DNase foot printing and to eliminate the DNA template after *in vitro* transcription for mRNA production. The cleavage action of this enzyme requires Ca^{2+}, and its activation needs Mg^{2+} or Mn^{2+} ions. DNase I cleaves each strand of dsDNA separately and randomly when Mg^{2+} is present, while both DNA strands are cleaved roughly at the same site in the presence of Mn^{2+}. DNase I can be eliminated from a reaction mixture, by heating the mixture with 5 mM EGTA (Ethylene Glycol Tetraacetic Acid) to 75 °C for 5 min (Huang et al. 1996).

4.8.1.2 Endonuclease IV, *E. coli*

Endonuclease IV isolated from *E. coli* is a 30 kDa Zn^{2+}-dependent endonuclease that identifies apurinic/apyrimidinic (AP) or abasic sites in dsDNA and hydrolyze phosphate group present 5′ to the abasic site generating a 3′-OH on one terminus and a deoxyribose 5′-phosphate at the 5′ end. It also has 3′ → 5′ exonuclease activity and 3′-diesterase activity that can generate 3′-phosphoglycolate or 3′-phosphate from the 3′ end of dsDNA (Levin et al. 1988). Endonuclease IV is produced by over expression in *E. coli* cloned with *nfo* gene. It has applications in DNA damage and repair studies, comet assay, analysis of DNA structure, SNP genotyping, and in *in vivo* studies on the effects of antitumor drugs on nucleic acids (Hosfield et al. 1999).

4.8.1.3 Endonuclease V, *T. maritima*

Endonuclease V obtained from the thermophilic bacterium *Thermotoga maritima,* is a thermostable endonuclease involved in DNA repair. It can cleave the phosphodiester bond present on the 3′-side, 1 nucleotide away from a deaminated base lesion and remove deaminated bases from defective DNA. Inosine (I), a product of adenosine deamination, xanthosine and oxanosine derived from guanosine deamination, and uridine from cytidine deamination act as substrates for endonuclease V (Feng et al. 2006). This 24.9 kDa enzyme has an optimal temperature range from 65 to 70 °C and is used in mutation research, DNA repair studies, mismatch cleavage, and genotyping.

4.8.1.4 Exonuclease I, *E. coli*

Exonuclease I of *E. coli* is a 3′ → 5′ exonuclease that acts specifically on single-stranded DNA releasing deoxyribonucleoside 5′-monophosphates (dNMP) sequentially, leaving 5′-terminal dinucleotides unaltered. This enzyme, coded by SbcB

gene, requires magnesium and a free 3′-hydroxyl terminus for its action. It cannot act on DNA strands with terminal 3′-OH containing phosphoryl or acetyl groups. Inactivation of this enzyme is done by heating it to 80 °C for 15 min. It is mainly used for the removal of linear ssDNA and oligonucleotides from a heterogeneous mixture of nucleic acids (Lehman and Nussbaum 1964).

4.8.1.5 Exonuclease III, *E. coli*

Exonuclease III, from *E. coli* strain BE 257/pSGR3 is a 28 kDa monomeric enzyme that has both endonuclease and exonuclease activity on ds DNA. Its endonuclease activity can create a gap at abasic sites in ds DNA, while as an exonuclease it acts on blunt end or recessed 3′- recessed termini of ds DNA in 3′ → 5′ direction creating ssDNA segments on the opposite strand. The enzyme is not active on single-stranded DNA, and hence they cannot act on 3′-extensions.

This enzyme also has RNase H activity that degrades RNA strands of DNA-RNA hybrids (Keller and Crouch 1972) and phosphatase activity that dephosphorylates DNA strands with 3′- phosphate terminus (Rogers and Weiss 1980). It is relatively specific for double-stranded DNA and displays sequence dependence (C > A = T > G) (Demple and Harrison 1994).

Exonuclease III can be used in combination with *E. coli* Klenow fragment for the synthesis of strand-specific labeled probes. It can be used along with S1 nuclease in a step-by-step manner to shorten the length of dsDNA. Other applications include site-directed mutagenesis and synthesis of ssDNA for dideoxy sequencing.

4.8.1.6 Exonuclease VII, *E. coli*

Exonuclease VII is an 88-kDa polypeptide, obtained from *E. coli* K12 strain. It catalyzes the degradation of ssDNA, but cannot act on RNA or DNA-RNA hybrids (Chase and Richardson 1974). It can degrade ssDNA in both directions and release 5′-phosphomononucleotides in an ATP-independent fashion and hence can be used to remove single-stranded protruding ends in dsDNA and generate blunt ends. It is the only ssDNA-specific exonuclease that has bi-directional activity. The degradation occurs progressively with initial release of large, acid-insoluble oligonucleotides, which are subsequently degraded into acid-soluble oligonucleotides.

Exonuclease VII does not require divalent cations for their action and are hence active even in the presence of chelating agents like EDTA (Ethylenediaminetetraacetic acid). It can be used in nested PCR after the first PCR reaction to remove single-stranded oligonucleotide primers, prior to the second PCR reaction using another set of primers. Another application is in exon-intron mapping of genomic DNA.

4.8.1.7 Lambda Exonuclease

Lambda exonuclease is produced by *E. coli* infected with lambda bacteriophage. It is an exonuclease that selectively acts on 5′-phosphorylated strand of dsDNA in 5′ → 3′ direction. The products of its degradation are 5′-mononucleotides and nonhydrolyzed complementary single-stranded DNA (ssDNA). It has only low digestive activity on ssDNA and nonphosphorylated DNA. Lambda exonuclease is used to convert dsDNA into single-stranded form for DNA sequencing and single-strand conformation polymorphism (SSCP). It is used in *in-situ* 3′-tailing reaction (ISTR)

for generating single-stranded 3′-ends, on which terminal deoxynucleotidyl transferase (TdT) can act (Little 1981).

4.8.2 Restriction Endonucleases

Restriction endonucleases are enzymes that hydrolyze the phosphodiester backbone of dsDNA at or near specific nucleotide sequences called restriction sites. Few restriction enzymes have been found to be active on ssDNA, but with only low efficiency. Majority of the restriction enzymes have been isolated from bacteria and archaea, where they provide defense against bacteriophages. Phage DNA is digested by restriction enzymes present in a prokaryote, but its own genomic DNA is protected by methylation of target sequence by DNA methyltransferase.

Restriction sites are usually between 4 and 8 bases long and many of them are *inverted repeat* palindrome, where the base sequence on one strand when read forward is the same as the sequence on the complementary strand when read backward. Restriction enzymes make two cuts in the sugar-phosphate backbone of dsDNA, one in each strand (Pray 2008). They are categorized into four types based on their differences in structure, cofactors, restriction sequence specificity, and position of cleavage from the restriction site.

Type I restriction enzymes cleave DNA nonspecifically at positions far from their recognition sequences and hence do not have much practical application. They have both restriction and methylase activities and require ATP and S-adenosyl-L-methionine.

Type II restriction enzymes form homodimers that cleave DNA within its restriction site or at specific positions near it. They lack methylase activity. Most enzymes in this class are ATP independent, but require Mg^{2+} as a cofactor. Majority of the commercially available restriction enzymes belong to this category and are routinely used for DNA analysis and molecular cloning. Of the 3500 different Type II restriction enzymes that have been discovered, more than 600 are commercially available.

Type III restriction enzymes are hetero-oligomeric proteins that cleaves DNA at about 20–30 base pairs away from the recognition site. They require two different nonpalindromic recognition sequences that are reversely oriented within the same DNA molecule to carry out cleavage. AdoMet and ATP are essential for DNA methylation and restriction digestion activities respectively.

Type IV restriction enzymes act preferentially on DNA molecules with modifications like methylation, hydroxymethylation, and glucosyl-hydroxymethylation (Williams 2003).

4.8.2.1 Nomenclature

Restriction enzymes are usually named after their source of origin. The first three letters of the name are abbreviations of the genus and species names of the organism. The fourth letter comes from the bacterial strain designation, and the Roman

numerals indicate the order in which the restriction enzyme was discovered in a particular strain. For example, *Eco*RI was the first restriction enzyme to be isolated from *Escherichia coli* strain RY13.

4.8.2.2 Patterns of DNA Cutting

Type II restriction enzymes that cleave at a position inside their recognition sites can generate either a blunt end or a staggered end with single-stranded overhangs.

Blunt Ends When restriction enzymes cleave dsDNA at the exact position on the recognition sites of two strands, a blunt end without single-stranded overhangs will be generated.

E.g. ***Sma*I restriction site**
5′-C C C↓G G G-3′
3′-G G G↑C C C-5′

Sticky Ends In a staggered cut, the enzyme cuts unsymmetrically within the restriction site resulting in single-stranded overlapping ends called sticky ends or cohesive ends, because they can readily anneal with DNA ends having a single-stranded complementary sequence. Cleavage by some enzymes like *Bam*HI results in 5′ overhangs while some other restriction enzymes like *Kpn*I results in 3′ overhangs.

***BamHI* restriction site**	***Kpn*I restriction site**
5′-G↓G A T C C-3′	5′-G G T A C↓C-3′
3′-C C T A G↑G-5′	3′-C↑C T A G G-5′

4.8.2.3 Isoschizomers, Neoschizomers, and Isocaudomers

Isoschizomers are different restriction endonucleases that identify the same recognition site and cleave at the same position. *Sac*I and *Sst*I are examples of isoschizomers.

Restriction site of *Sac*I and *Sst*I
5′-G A G C T↓C-3′
3′-C↑T C G A G-5′

Neoschizomers are restriction enzymes that recognize the same restriction site, but cut at different positions on it. *Sma*I and *Xma*I are neoschizomers of each other.

Restriction site of *Sma*I	**Restriction site of *Xma*I**
5′-C C C↓G G G-3′	5′-C↓C C G G G-3′
3′-G G G↑C C C-5′	3′-G G G C C↑C-5′

Isocaudomers are restriction enzymes that slightly differ in their restriction sites, but generate identical cohesive ends after cleavage. *Mbo*I and *Bam*HI are isocaudomers.

MboI **restriction site**	***BamHI*** **restriction site**
5′-↓G A T C-3′	5′-G↓G A T C C-3′
3′-C T A G↑-5′	3′-C C T A G↑G-5′

4.8.2.4 Star Activity

Some restriction enzymes, when under reaction conditions highly different from those optimal for the enzyme, cleave at restriction sites which are similar, but non-identical, to their restriction sites. This relaxation or alteration in the specificity of restriction enzyme is termed as “star activity” (Williams 2003).

4.8.3 Ribonucleases

Ribonucleases (RNases) are a group of enzymes heterogeneous in structure and function, that catalyzes the cleavage of phosphodiester bonds in RNA, either inside the polynucleotide chain or at its ends. A number of endoribonucleases that can cleave at specific recognition sequences on single-stranded RNA have been identified (Saïda et al. 2004). Ribonucleases are ubiquitously found in all living organisms and are involved in maturation of all RNA molecules and degradation of unwanted cellular RNA. They also serve as a first line of defense against RNA viruses.

4.8.3.1 Ribonuclease A

Ribonuclease A (Rnase A) is an endoribonuclease that cleaves the phosphodiester bond of ssRNA at the 3′ end of pyrimidine residues. The cleavage occurs in two steps, first of which involves the generation of 2′, 3′-cyclic phosphodiester intermediate by the cleavage of 3′,5′-phosphodiester bond, which is hydrolyzed in the second step to the corresponding 3′-nucleoside phosphate. The first step is nonspecific to nitrogenous base while the second step is highly specific for pyrimidine nucleotides with terminal 2′,3′-cyclic phosphates (Anfinsen et al. 1961). RNase A is isolated from ruminant pancreas and it is the first enzyme and third protein to be sequenced (Raines 1998). The crystalline enzyme usually contains a minute amount of RNase B, which is the glycosylated form of RNase A in them.

RNase A can be used to degrade RNA contaminants in DNA and recombinant protein samples. It is also used for mapping single-base mutations in DNA or RNA, because it can specifically cleave RNA in RNA-DNA hybrids at sites of single nucleotide mismatch. RNase A is highly stable under varying conditions and is difficult to inactivate.

4.8.3.2 Ribonuclease H

Ribonuclease H (RNase H) can specifically hydrolyze RNA in RNA-DNA duplex substrate with 3′-OH and 5′-P-terminated products. This endonuclease does not act on single-stranded and double-stranded DNA or RNA. RNases H was first isolated from calf thymus in 1969 (Stein and Hausen 1969). Commercially available RNase H is produced from *E. coli* strain that has been cloned and over expressed with RNase H gene (rnh) from *E. coli*. It is used in molecular biology to degrade RNA template from RNA-DNA hybrids produced after first-strand cDNA synthesis by reverse transcription, for poly(A) tail removal from mRNA linked to oligo(dT), for selective degradation of noncoding RNA inside or outside the living cell and also in procedures like nuclease protection assays. RNase H requires Mg^{2+} ions or Mn^{2+} ions and hence can be inhibited using a chelator like EDTA (Goodwin and Rottman 1992).

4.8.3.3 Ribonuclease T_1

Ribonuclease T_1 (RNase T_1) is a 11 kDa monomeric endoribonuclease that specifically cleaves the phosphodiester bond in ssRNA (or deaminated RNA) after guanine residues. It acts between the 3′ end of guanine residues and 5′-hydroxy residue of adjacent nucleotides, producing corresponding 2′,3′-cyclic phosphate as an intermediate (Takahashi 1966). It does not require any metal ions for its activity. RNase T_1 was first discovered in a commercial enzyme mixture from the mold *Aspergillus oryzae* called Takadiastase. The RNase T1 is now commercially synthesized by over expression in *E. coli* strain cloned with Ribonuclease T1 gene of *A. oryzae* (Fujimura et al. 1990). Due to the specificity of RNase T_1 for guanine, it is used to digest denatured RNA prior to sequencing, in RNA mapping and RNA structure studies. It can also be used for removing RNA from DNA samples or recombinant protein preparations, in the synthesis of nucleoside 2′,3′-cyclic phosphates and oligonucleotides (Mohr and Thach 1969).

4.8.4 Sugar Nonspecific Nucleases

As the name implies, they have the ability to hydrolyze nucleic acids with ribose (RNA) or deoxyribose (DNA) sugars. They do not act on specific sequences, but can recognize different nucleic acid structures. This property has been utilized in studying different nucleic acid structures and their interactions with intercalating molecules. Of about 30 sugar nonspecific nucleases isolated so far, many show multiple activities and metal ion requirement.

4.8.4.1 S1 Nuclease

S1 nuclease is a single-strand endonuclease of 32 kDa size, isolated from *Aspergillus oryzae*. It specifically hydrolyzes ssRNA or ssDNA into 5′-phosphoryl mono- or oligonucleotides, but does not act on double-stranded nucleic acids (dsDNA, dsRNA, or RNA-DNA hybrids). This enzyme is stable at 65 °C, and its activity requires Zn^{2+} as a cofactor. Zn^{2+} can be replaced by Co^{2+} and Hg^{2+}, which show only

low efficiency. S1 nuclease remains active in the presence of denaturing agents but is sensitive to chelating agents like EDTA (Hofstetter et al. 1976). S1 nuclease is widely used in molecular biology for the specific removal of single-stranded regions in double-stranded nucleic acid molecules. It is used in DNA-DNA or DNA-RNA hybridization studies, S1 transcript mapping for finding transcriptional initiation sites, removal of single-stranded overhangs in sticky ends prior to DNA ligation and degradation of hairpin loops. Other applications include studies on DNA-binding molecules, isolation of inserts from plasmid DNA, DNA palindromic analysis, heteroduplex analysis of PCR products, and structural studies on tRNAs and rRNAs (Rittié and Perbal 2008).

4.8.4.2 P1 Nuclease

P1 nuclease is a zinc-dependent single-strand specific endonuclease isolated from *Penicillium citrinum* that has a molecular weight of 42–50 kDa. It has phosphodiesterase and monoesterase activities and can completely hydrolyze single-stranded DNA and RNA to yield mononucleoside 5′-monophosphates as the product. The optimum temperature for this enzyme is about 70 °C, but it is preferable to use below 60 °C for reactions having long incubation time. P1 nuclease does not cleave double-stranded DNA or RNA-DNA hybrids in native state. It is used to remove single-stranded overhanging at the end of double-stranded DNA, in eukaryotic mRNA cap isolation, in the analysis of nucleic acids base composition, and for nucleic acid degradation during protein purification (Desai and Shankar 2003).

4.8.4.3 Benzonase

Serratia nuclease, commercially known as “Benzonase,” is a nonspecific endonuclease isolated from *S. marcescens* which hydrolyzes both single- and double-stranded DNA and RNA. It is a highly processive enzyme which requires Mg^{2+} for the cleavage of phosphodiester bond, releasing 5′-phosphorylated oligonucleotides (Benedik and Strych 1998). Since it lacks proteolytic activity, it is an ideal tool for removing nucleic acid contamination from purified proteins. It is also used for the reduction of bacterial lysate viscosity for downstream steps enhancement.

4.8.4.4 BAL 31 Nuclease

BAL 31 nuclease was first isolated from a marine bacterium which was originally classified as *Pseudomonas* BAL 31 and later renamed as genus *Alteromonas espejiana* Bal 31. Two different molecular types of nucleases have been obtained from this strain, named as fast (F) and slow (S) Bal 31 nucleases. Both of these enzymes show exonuclease and endonuclease activities. As an exonuclease, it degrades double-stranded DNA and RNA from both 5′-phosphate and 3′-hydroxyl termini. It shows endonuclease activity on single-stranded DNA and RNA and cleaves at nicks and gaps. F- and S-Bal 31 enzymes show identical activity on ssDNA, but on dsDNA, their reaction speed differs. F-Bal 31 has 20 times faster hydrolytic activity on dsDNA when compared to S-Bal 31, and this reaction rate is dependent on the C/G content of the substrate DNA (Talmadge et al. 1980; Kilpatrick et al. 1983). Ca^{2+} is an essential cofactor for its exonuclease and endonuclease activities. This enzyme

has high resistance to inactivation and denaturation by detergents, urea, or high concentrations of electrolyte, and is stable upon extended storage in the cold. F-Bal 31 nuclease is used in restriction site mapping, progressive deletion of sequences in double-stranded DNA fragments from both termini, mapping B-Z DNA junctions, and reducing the length of RNA molecules. The S-Bal 31 is used only for restriction mapping and the controlled length reduction of linear duplex DNA.

4.8.4.5 Mung Bean Nuclease

This 39 kDa single-strand specific endonuclease can act on ssDNA and ssRNA, but has no activity on dsDNA, dsRNA, or DNA-RNA hybrids under normal conditions**.** However, high concentrations of enzyme can degrade double-stranded nucleic acids when incubated for long duration. It is isolated from sprouts of mung bean *Vigna radiata*. Zn^{2+} is essential for the action of this enzyme while EDTA or SDS can cause its irreversible inactivation (Johnson and Laskowski 1970). This enzyme is ideal for the removal of 3′ and 5′ single-stranded overhangs of DNA and RNA molecules producing ligatable blunt ends, for the digestion of hairpin loops and in transcript mapping.

4.9 Polynucleotide Ligases

Polynucleotide ligases or ligases are enzymes that catalyze the joining of nucleic acid molecules by the formation of phosphodiester bonds between them. Ligases have been reported in various organisms including bacteria, phage-infected bacteria, yeasts, amphibians, and mammals, where they catalyze many important cellular processes like DNA replication and repair of damaged DNA. Ligases are essential for many techniques in recombinant DNA technology like cloning of cDNA or genomic fragments for library construction, mapping, sequencing, or used as probes. They are classified into DNA ligases and RNA ligases on the basis of their nucleic acid specificity.

4.9.1 DNA Ligases

They catalyze the covalent joining of nicks in dsDNA by phosphodiester bond formation between a 3′-hydroxyl and a 5′-phosphate group, thereby joining the DNA fragments (Lehman 1974). Inside a cell, they are involved in the linking of Okazaki fragments during replication and in DNA repair. Bacterial DNA ligases require NAD^+ as a cofactor while those in eukaryotes and viruses use ATP (Doherty and Wigley 1999). The phosphodiester formation by all DNA ligases occurs in three different catalytic steps. At first, ligase is activated through the formation of a covalent DNA ligase-AMP intermediate. Second step involves the transfer of AMP moiety to the 5′-phosphate terminus of nicked DNA strand and in the final step, the DNA-AMP joins with the 3′-hydroxyl of the DNA break site, forming a

phosphodiester bond with the release of free AMP. DNA ligases are routinely used in molecular biology for joining DNA fragments produced by restriction digestion, for the addition of linkers or adaptors to DNA, or to repair nicks in DNA. They can join both blunt and sticky ends of DNA. Many thermostable DNA ligases have been isolated from thermophilic microbes, which have application in techniques like ligase chain reaction (LCR) used for identifying single-base mutations.

4.9.1.1 *E. coli* DNA Ligase

It is a monomeric NAD^+-dependent 77 kDa ligase enzyme that prefers sticky dsDNA ends, but can also join blunt ends in the presence of Ficoll or polyethylene glycol. *E. coli* DNA ligase can be used for reactions where double-stranded sticky end DNA ligation is required along with prevention of blunt end ligation (Panasenko et al. 1978). It has applications in molecular cloning of dsDNA with cohesive ends, cDNA cloning of second strand cDNA synthesis product, and DNA cloning by replacement synthesis where its inability to ligate RNA to DNA is an advantage.

4.9.1.2 T4 DNA Ligase

It is the most widely used DNA ligase in molecular biology. T4 DNA ligase is an elongated monomeric enzyme of 68 kDa size. It was isolated from *E. coli* infected with T4 bacteriophage. This enzyme requires Mg^{2+} and ATP as cofactors for its activity. It can be used for joining both blunt and sticky ends and can repair single-stranded nicks in dsDNA, dsRNA, or DNA-RNA hybrids. Ligation of blunt-ended DNA is slower, but the rate can be increased by the addition of 150–200 mM NaCl and low concentration of PEG. The enzyme also behaving as an AMP-dependent endonuclease, producing nicked DNA which along with the ligase activity result in complete relaxation of supercoiled DNA (Murray et al. 1979). T4 DNA ligase is mainly used in the cloning of restriction digestion fragments and connecting synthetic linkers and adapters to DNA blunt ends.

4.9.1.3 T3 DNA Ligase

T3 DNA ligase, isolated from T3 bacteriophage is an ATP-dependent dsDNA ligase that can ligate sticky ends and blunt ends and also repair nicks in dsDNA. As with T4 DNA ligase, its efficiency in blunt-ended ligation is low, but can be enhanced using PEG 6000. It has high tolerance for NaCl and KCl when compared to T4 DNA ligase. T3 DNA ligase is higher than five times more efficient than T4 DNA ligase in ligation of sticky DNA ends. It is more phylogenetically similar to T7 DNA ligase than T4 DNA ligase (Cai et al. 2004). T3 DNA ligase is used in DNA cloning, joining of linkers or adapters, circularization of linear DNA and in nick sealing.

4.9.1.4 T7 DNA Ligase

It is a 41 kDa ATP-dependent dsDNA ligase isolated from *E. coli* infected with T7 bacteriophage. It catalyzes the ligation of sticky ends of duplex DNA and single-stranded nick sealing (Doherty et al. 1996). It cannot efficiently ligate blunt ends under typical reaction conditions, but the addition of high concentrations of PEG 6000 can improve its activity. T7 DNA ligase is used in techniques that require selective ligation

of only cohesive ends of DNA in a mixture containing both cohesive and blunt ends. Applications include cloning of DNA fragments, joining of linkers or adapters, circularization of linear DNA, nick sealing, and in site-directed mutagenesis.

4.9.1.5 PBCV-1 DNA Ligase

PBCV-1 DNA ligase or *Chlorella* virus DNA Ligase, commercially known as SplintR ligase is an ATP-dependent DNA ligase from *Paramecium bursaria Chlorella* virus PBCV-1 (Ho et al. 1997). It catalyzes the ligation of two nicked ssDNAs that are splinted by a complementary RNA strand, which has applications in characterization of miRNAs and mRNAs. This enzyme is suitable for next-generation sequencing and molecular diagnostics. Its affinity for RNA-splinted DNA substrates can be utilized for the detection of subnanomolar quantities of unique RNA species within a complex mixture (Lohman et al. 2014).

4.9.1.6 Taq DNA Ligase

It is a thermostable DNA ligase isolated from *Thermus aquaticus*, which can ligate cohesive ends in dsDNA with the help of NAD^+ as a cofactor. It can remain active at temperatures as high as 45–65 °C and hence is used in techniques that require this property (Housby et al. 2000). This enzyme is used in the detection of single-nucleotide polymorphism (SNP) using ligase detection reaction and ligase chain reaction and mutagenesis by insertion of a phosphorylated oligonucleotide during primer extension amplification.

4.9.1.7 Pfu DNA Ligase

Pfu DNA ligase is a thermostable ligase isolated from a hyperthermophilic marine archaebacterium *Pyrococcus furiosus*. It catalyzes ligation of dsDNA at high temperatures ranging from 45 to 80 °C. Its optimum temperature for nick sealing is 70 °C. This enzyme has high ligation specificity and higher thermostability with a half-life of more than 1 h at 95 °C (Günther et al. 2002). It is used in ligase chain reactions (LCR) for higher reliability by permitting higher melting temperatures.

4.9.1.8 Tth DNA Ligase

Tth DNA ligase is a thermostable ligase isolated from *Thermus thermophilus*, which can join dsDNA fragments and also repair single-stranded nicks in dsDNA. It catalyzes the formation of a phosphodiester bond between adjacent nucleotides hybridized to a complementary target DNA. This reaction will occur only if the two nucleotides are perfectly paired to a complementary target DNA without any gaps. This property can be applied for the detection of even a single-base substitution. The high fidelity and thermostability of this enzyme makes it an excellent choice for ligase chain reaction (LCR) and ligase detection reaction (LDR) to identify point mutations associated with genetic disorders and pathogens (Luo et al. 1996).

4.9.2 RNA Ligases

They are ATP-dependent enzymes that can catalyze the ligation of RNA molecules via phosphodiester bonds. RNA ligases have been found in organisms of all major phyla, where they serve important cellular functions like RNA splicing, editing, and repair. RNA ligation occurs by the joining of 3′-hydroxyl RNA termini of one fragment to the 5′-phosphate termini of the adjacent fragment, through three nucleotidyl transfer steps. The initial step involves the reaction between RNA ligase and ATP to produce an intermediate covalent ligase-(lysyl-*N*)-AMP and pyrophosphate as products. In the next step, the bound AMP is transferred from ligase-(lysyl-*N*)-AMP to the 5′-phosphate termini of RNA to form an RNA-adenylate intermediate (AppRNA) and in the final step, RNA ligase catalyze a nucleophilic attack on AppRNA end by 3′-OH of the other RNA strand, releasing AMP and forming a phosphodiester bond that joins the two RNA strands (Ho et al. 2004).

4.9.2.1 T4 RNA Ligase

T4 RNA ligase, isolated from *E. coli* cells infected with T4 phage, is the most widely used and the best characterized of RNA ligases in molecular biology. This 48 kDa monomeric enzyme requires ATP as a cofactor to catalyze the ligation of ssRNA, ssDNA, and polynucleotides to RNA molecules. T4 RNA ligase is used in oligonucleotide synthesis to ligate oligonucleotide adaptors to cDNA, 5′ nucleotide modifications of nucleic acids, primer extension for PCR, and for circularizing RNA and DNA molecules. It can also be used for linking oligonucleotide adaptors to 5′ termini of mRNA for techniques like RLM-RACE. Another RNA ligase has now been identified in bacteriophage T4 which has been named T4 RNA ligase 2 or T4 Rnl-2. It has the ability to catalyze both intramolecular and intermolecular RNA strand ligations. T4 RNA ligase 2 has higher efficiency in nick sealing of dsRNA than joining ssRNA fragment ends (Ho et al. 2004). A truncated form of T4 RNA ligase 2 composed of only first 249 amino acids is ATP independent and lacks 5′ termini adenylation activity. It requires a pre-adenylated substrate for ligation reaction and is used in cloning of small RNAs because it restricts the background formation of circles and multimers of RNAs.

4.9.2.2 Thermostable RNA Ligases

Thermostable RNA ligases are sought after in molecular biology for use in techniques where the secondary structure of RNA becomes a constraint in ligation experiments, which can be resolved by applying higher temperature. Such enzymes have been isolated from a thermophilic archaebacteria, *Methanobacterium thermoautotrophicum*, and from two thermophilic bacteriophages TS2126 and RM378 that infect the thermophilic eubacteria *Thermus scotoductus* and *Rhodothermus marinus*, respectively (Blondal et al. 2003, 2005).

A mutant form of *M. thermoautotrophicum* RNA ligase has been developed that lack de-adenylation activity and is ATP independent. The enzyme retains its activity of ligating RNA or ssDNA to a pre-adenylated linker and hence is an excellent choice for NextGen RNA sequencing. Use of RNA ligases with de-adenylation activity can lead to activation of RNA and background production of ligation products. Since the mutant lack self-adenylation property, it does not have this problem and there is also the added advantage of being functional at 65 °C, which minimizes the constraints of RNA secondary structure in RNA ligation (Zhelkovsky and McReynolds 2012).

4.10 Phosphatases and Kinases

Phosphatases are enzymes that remove a phosphate group from a substrate while kinases are involved in the transfer of a phosphate group to a substrate. Phosphatase catalyzes the hydrolytic cleavage of phosphate group from a substrate in an irreversible reaction. On the basis of catalytic activity, phosphatases have been classified into two, that is, acid phosphatase and alkaline phosphatase of which the latter is more common.

4.10.1 Alkaline Phosphatases

Alkaline phosphatase are homodimeric enzyme that catalyzes the hydrolysis of 5′-phosphate groups from nucleic acids and are most active at alkaline pH of about 10. They contain two Zn^{2+} ions and one Mg^{2+} ion. Alkaline phosphatases have been isolated from different sources like microorganisms, tissues of invertebrates, fish, and mammals, but have not been isolated from higher plants, of which the most commonly used are those from calf intestinal mucosa and from *E. coli*. Alkaline phosphatases are used in molecular biology for dephosphorylation of 5′ end of DNA or RNA to prevent self-ligation. This is usually carried out in vectors like plasmid or bacteriophage after restriction digestion to prevent self-ligation, thereby increasing the ligation of desired insert. Another application is in dephosphorylation of nucleic acid termini, enabling subsequent *in vitro* modification like labeling with radioactive phosphate using T4 polynucleotide kinase which can be used for DNA or RNA sequencing and fragment mapping.

4.10.1.1 Bacterial Alkaline Phosphatase

Bacterial alkaline phosphatase (BAP) is an 80 kDa homodimer isolated from *E. coli* that shows optimum activity at 65 °C. It is a zinc and magnesium-containing protein and is hence sensitive to chelating agents like EGTA, and low concentrations of inorganic phosphate (Halford 1971).

4.10.1.2 Calf Intestinal Alkaline Phosphatase

Calf intestinal alkaline phosphatase (CIAP, CIP, or CAP) is a glycoprotein comprising of two identical subunits isolated from calf intestine, which catalyzes dephosphorylation of 5′ termini of DNA and RNA. This 140 kDa enzyme also contains

zinc and magnesium ions and is inactivated by chelating agents (Mössner et al. 1980).

4.10.1.3 Shrimp Alkaline Phosphatase

It is a highly specific, heat-sensitive phosphatase enzyme isolated from arctic shrimp (*Pandalus borealis*). Its catalytic action is similar to BAP and CIP, but being heat sensitive, it can be irreversibly inactivated by heat treatment at 65 °C for 15mins. It is used for 5′ dephosphorylation of DNA or RNA for subsequent labeling of the ends, to prevent self-ligation, and for the inactivation of unused dNTPs from PCR product prior to sequencing.

4.10.2 Acid Phosphatases

Acid phosphatases have optimal pH below 7.0. It is commercially produced from wheat germ. Three isozymes of acid phosphatase have been isolated, namely EI, EII, and EIII with similar molecular weights (55 kDa ± 5 kDa) and having optimum pH's 5.5, 4.5, and 4.0, respectively (Verjee 1969).

4.10.2.1 Tobacco Acid Pyrophosphatase

Tobacco acid pyrophosphatase (TAP) catalyzes the hydrolysis of various pyrophosphate bonds, including the triphosphate bridge of 5′-terminal methylated guanine nucleotide cap of mRNA, generating a 5′-phosphate terminus which can be dephosphorylated with alkaline phosphatase, and labeled with T4 polynucleotide kinase for radiolabeled probe production or ligated with oligonucleotide adaptors for RACE. EpiCentre, the only manufacturer of TAP, has discontinued its production. TAP was produced directly from tobacco callus tissue due to difficulties in making a recombinant enzyme. Decapping Pyrophosphohydrolase from tebu-bio and Cap-Clip™ Acid Pyrophosphatase from CellScript are the available replacements for TAP of which the latter was found to have better catalytic activity.

4.10.3 T4 Polynucleotide Kinase

T4 polynucleotide kinase, isolated from T4 bacteriophage infected *E. coli,* is the most widely used polynucleotide kinase in molecular biology. It is a homotetramer of 33-kDa monomers encoded by *pse* T gene. T4 polynucleotide kinase has phosphatase activity at 3′ end and kinase activity at 5′ end. It catalyzes the transfer of a phosphate group from ATP molecule to the 5′ end of double-stranded and single-stranded DNA or RNA or nucleoside 3′-monophosphates. It can also catalyze the removal of 3′-phosphoryl groups from 3′-phosphoryl polynucleotides. Applications of T4 polynucleotide kinase include labeling DNA or RNA ends with radiolabeled ATP as a phosphate donor for probe preparation and base-specific sequencing, mapping of restriction sites, removal of 3′-phosphoryl groups, and synthesis of substrates for DNA or RNA ligase by the addition of 5′-phosphates (Wang et al. 2002).

4.11 Conclusions

Enzymes have a pivotal role in molecular biology and a wide array of enzymes is now commercially available for cleaving, ligating, synthesis, and modification of nucleic acids. A majority of these enzymes are produced as recombinant proteins and expressed in heterologous hosts by DNA cloning. Enzymes can be even modified to suit specific needs by altering coding sequences to enhance its activity or to remove a particular catalytic activity. Thus, a good understanding of the various enzymes available along with their catalytic characteristics and optimum conditions is essential to select the best one for a particular requirement.

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Biotransformation Enzymes

5

Sudheesh Sudhakaran, T.M. Archana, and C.N. Aguilar

Abstract

Biocatalysis, which was an area of least concern during the past years, has now a remarkable space in the field of chemistry. Biotransformation is the process through which the functional groups of organic compounds are modified by living cells to a chemically different product. The process explores the specific properties of biological catalysts, which include stereospecificity and region specificity and their capability to withstand reactions at no extreme temperatures and pH values. It may involve the use of plant cells, animal cells or microbial cells or purified enzymes as catalysts to bring about specified transformations of complex substrates. Biotransformation enzymes help to produce "nature-like" biodegradable compounds, and are gaining importance in this aspect. This chapter focuses on biotransformation enzymes, which catalyse highly reaction-specific and stereo-specific reactions to synthesize compounds that cannot be produced by chemical means. Moreover, these enzymes help meet the increasing demand of society for eco-friendly compounds by producing the bulk of biodegradable 'green' products. This chapter discusses the recent advances and applications in biotransformation.

Keywords

Biotransformation • Enzymes • Biocatalysis • Natural products • Xenobiotics

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5.1 Introduction

Biotransformation or biocatalysis entails the use of the catalytic part of the biological systems such as plant cells, animal cells or microbial cells or purified enzymes for the biosynthesis of novel compounds. Biotransformation has a high prospective to bring about novel products and to improve known products more effectively. It differs from biosynthesis and biodegradation in that it involves interconversion of molecules by living systems, whereas biosynthesis is the combining of simpler substrates to form complex products and biodegradation is the decomposition of complex substances to simpler ones.

Biotransformation commonly occurs in human, plant and microbial cells as part of metabolizing xenobiotics. Also, it serves as an efficient, specific, and eco-friendly process for the production of industrial products, thereby replacing the toxic chemical transformation reactions. In many instances, chemical modification of compounds by biotransformation alters their biological effects, either making them useful to the organism or ending up with toxic effects. But in most cases, biotransformation terminated with the production of propitious products.

Recent reviews suggest that biocatalysis also forms a sustainable way in environmental fields to mop up environmental pollutants. It helps harness the catabolic activity of living systems to degrade and transform a wide range of compounds to chirally pure compounds, thereby offering several advantages over the use of chemicals and microorganisms. Thus, biotransforming enzymes are gaining importance.

Properties of biotransforming enzymes are as follows:

- Biocatalysis, which is normally performed in an aqueous environment but can, in many cases, also be conducted in solvent mixtures, liquid–liquid two-phase systems, and even in pure organic solvents. A relevant practical example is the use of esterases and lipases to catalyse esterifications in organic solvents such as vinyl acetate.
- They require mild reaction conditions. Therefore, biocatalysis offers great chances and advantages for successful applications (also in cases where either the substrates or the products of the reaction are chemically labile).
- There is no, or only limited, use of protecting groups, for example, for the chemo-enzymatic synthesis of complex carbohydrates and glycoconjugates.
- They have high chemo-, regio- and stereoselectivities (Oreste Ghisalba et al. 2010).

A wide variety of biotransforming enzymes exist and are highly substrate specific. Some of the biotransforming enzymes are similar in plants, animals and in humans but there are a few species specific ones. In this context, we can classify the biotransformation enzymes into three as occurring in humans, plants and microbial cells.

5.2 Biotransforming Enzymes in Humans

In the case of humans, all those molecules which the body does not recognize as its own are considered as 'foreign' or *xenobiotics*. This section explains the biotransformation of xenobiotics by respective enzymes, thus lessening their toxic effects and easily removing them from the body.

Today all living beings of the earth are constantly and unavoidably exposed to xenobiotics, which include both manufactured and naturally occurring chemicals such as industrial chemicals, pesticides, drugs, pollutants, alkaloids, secondary plant metabolites, and toxic substances. Xenobiotics are readily absorbed by the human body because of their lipophilic property. It is due to this physical property that the homeostasis becomes altered. Consequently, there comes the role of biotransformation enzymes, which aid in the elimination of xenobiotics by converting them to hydrophilic compounds. The process of biotransformation ends up with the change in properties of foreign molecules, i.e. from those preferring absorption (lipophilicity) to those which are easily eliminated either through urine or faeces (hydrophilicity).

Having a broad substrate specificity, the biotransforming enzymes metabolize a wide range of endogenous compounds such as acetone, ethanol, steroid hormones, bilirubin, bile acids, fatty acids, eicosanoids, vitamins A and D, etc. Some of these enzymes are either expressed constitutively or are induced by the xenobiotic itself. The rates of xenobiotic biotransformation among individuals may vary depending upon the amino acid sequence, i.e. the structure of a given biotransforming enzyme. In general, a variant form of a xenobiotic biotransforming enzyme (*allelic variant* or an *allelozyme*) has diminished enzymatic activity compared with that of the wild-type enzyme, although this is not always the case. However, the impact of amino acid substitution on the catalytic activity of a xenobiotic biotransforming enzyme is usually substrate-dependent (Parkinson et al. 2013). The reactions catalysed by xenobiotic biotransforming enzymes are generally divided into two groups: Phase I and Phase II (Table 5.1).

5.2.1 Phase I Reactions

Phase I reactions mainly involve three reactions – oxidation, reduction and hydrolysis. These reactions introduce a functional group (−OH, −SH, $-NH_2$ or –COOH), and usually end up in the formation of slightly hydrophilic compounds. The functional groups added during phase I biotransformation form the site for phase II biotransformation.

Oxidative Reactions Oxidation is the most prevalent and an important way of metabolizing xenobiotics. It includes withdrawal of an electron, followed by addition of oxygen into the molecule. Molecular oxygen most often forms the source and in some cases the oxygen is obtained from water. Most organic compounds undergo single-electron or double-electron redox reactions. Some undergo a 4e-

Table 5.1 Xenobiotic metabolizing enzymes

Reaction	Enzyme	Localization
Phase I		
Oxidation	Cytochrome P450	Microsomes
	Flavin monooxygenases	Microsomes
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Cytosol, mitochondria
	Xanthine oxidase	Cytosol
Hydrolysis	Carboxyl esterase	Cytosol, microsomes, lysosomes, blood
	Peptidase	Lysosomes, blood
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro-reduction	Microsomes, cytosol, microflora
	Carbonyl reduction	Microsomes, cytosol, blood
	Quinone reduction	Microsomes, cytosol
Phase II		
	Glucuronide conjugation	Microsomes
	Sulfate conjugation	Cytosol
	Glutathione conjugation	Microsomes, cytosol
	Amino acid conjugation	Microsomes, mitochondria
	Acylation	Mitochondria, cytosol
	Methylation	Microsomes, cytosol, blood

oxidation. The most common electron acceptor is molecular oxygen. This can undergo a 2e- reduction to yield H_2O_2 or a 4e- reduction to generate water.

Common oxidative reaction includes

- Dehydrogenation (involves a hydride abstraction)
- Hydroxylation of aromatic carbons
- Hydroxylation of aliphatic carbons
- Oxidations involving carbon-heteroatom systems (Amin M. Kamel 2007).

The main enzymes involved in the oxidative reactions include xanthine oxidase (XO), monoamine oxidases (MAOs), diamine oxidases (DAOs), flavin-containing monooxygenases (FMOs), cytochrome P450s (P450s), etc. (Amin M. Kamel 2007).

Cytochrome P450s Cytochrome P450 enzymes (CYPs or P450s) belong to the super family of heme-dependent proteins that are synthesized by different mammalian tissues such as liver, small intestine, lungs, kidneys, brain and placenta. In the present scenario 57 different P450 isoforms have been identified in man. Based on the protein sequence, the enzymes have been classified into 18 families and 43 subfamilies. Of these 18 families, only the first three (CYP1, CYP2, CYP3) are

involved in xenobiotic metabolism and they form approximately 70% of the total CYP content in human liver.

The heme is ferric (Fe^{3+}) containing porphyrin cofactor with cysteine as the fifth ligand and the sixth coordination site to bind and activate molecular oxygen; NADPH is used as electron source and an NADPH reductase system to recycle $NADP^+$ to NADPH.

The liver microsomal P450 enzymes play a very important role in determining the intensity and duration of action of drugs, and they also play a key role in the detoxification of xenobiotics. Cytochrome P450 catalyses the basic reaction of monooxygenation, where one atom of oxygen is incorporated into a substrate (RH), with the subsequent reduction of other to water with reducing equivalents derived from NADPH (Amin M. Kamel 2007).

$$\text{Substrate}\,(\text{RH}) + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{Product}\,(\text{ROH}) + \text{H}_2\text{O} + \text{NADP}$$

The reactions catalysed by cytochrome P450 include hydroxylation of an aliphatic or aromatic carbon, an epoxidation of a double bond, heteroatom (S-,N-,I-) oxygenation, an N-hydroxylation, heteroatom (O-,S-,N-) dealkylation, oxidative group transfer, cleavage of esters and dehydrogenation. It can also catalyse the reduction of azo and nitro compounds and cause reductive dehalogenation. Catalysis of these reactions makes cytochrome P450 system a unique one in the overall metabolism of pollutants and the xenobiotics. The protein part of the enzyme determines the substrate specificity. The cytochrome P450 enzymes are now abbreviated in codes as CYP followed by a number to indicate the family and a letter to specify the subfamily and number, which codes further the enzymatic activity of that particular protein. For example, CYP1-3 families have significance in the oxidation of pollutants and drugs (Osmo Hanninen 2009).

Alcohol Dehydrogenases and Aldehyde Dehydrogenases Alcohol dehydrogenases are the zinc-containing cytosolic enzymes present in the highest level in liver and in kidney, lungs, gastric mucosa, etc. Human ADH is a dimeric protein with two subunits of 40 kDa. The subunits may be α, β, γ, π, χ, σ or μ and are encoded by six different gene loci. Based on the different subunits present, ADH may be of various types, given in Table 5.2.

Aldehyde dehydrogenases (ALDH) play a major role in xenobiotic metabolism. They are enzymes which oxidize aldehyde to carboxylic acid using NAD^+ as cofactor. The enzymes also exhibit esterase activity. Twelve ALDH genes were found in humans and they may differ in their primary amino acid sequence and the quarternary structure.

Flavin Monooxygenases FMOs are microsomal enzymes which need NADPH and O_2, and catalyse reactions similar to those catalysed by cytochrome P450. In mammals, the FMO gene family comprises five enzymes, FMO1 to FMO5, with about 550 amino acid residues each. The amino acid sequence shows 50–58% identity across the species lines. The highly conserved glycine-rich region (residues 4 to 32)

Table 5.2 Types of alcohol dehydrogenases (ADH)

Class	Enzyme	Function	Location
Class I	α-ADH	Oxidation of ethanol and other small aliphatic alcohols.	Liver, adrenal glands, lower levels in kidney, lungs, blood vessels.
	β-ADH		
	γ-ADH		
Class II	π-ADH	Oxidation of large aliphatic and aromatic alcohols.	Liver, lower levels in stomach.
Class III	χ-ADH	Oxidation of long-chain alcohols and aromatic alcohols.	All tissues, including brain.
Class IV	σ/μ-ADH	Conversion of ethanol to acetaldehyde and oxidation of retinol.	Stomach and other areas of gastrointestinal tract.

of each FMO enzyme non-covalently binds one mole of FAD present near the active site; adjacent to this is a second highly conserved glycine-rich region (residues 186 to 213) that binds NADPH. FMO is heat-labile and can be deactivated in the absence of NADPH by heating microsomes to a temperature of 50 °C for 1 min in contrast to cytochrome P450, which is deactivated with a non-ionic detergent, such as 1% Emulgen 911. The pH optimum for FMO-catalysed reactions is 8 to 10, which is slightly higher than that for most P450 reactions (pH 7 to 8).

The reactions catalysed by FMO include the oxidation of nucleophilic tertiary amines to *N*-oxides, secondary amines to hydroxylamines and nitrones, and primary amines to hydroxylamines and oximes. It also oxidizes several sulfur-containing xenobiotics (such as thiols, thioethers, thiones, and thiocarbamates) and phosphines to *S*- and *P*-oxides, respectively. With few exceptions, FMO acts as an electrophilic oxygenating catalyst, which distinguishes it from most other flavoprotein oxidases and monooxygenases. During the oxygenation of xenobiotics, the 4a-hydroperoxyflavin is converted to 4a-hydroxyflavin with the transfer of the flavin peroxide oxygen to the substrate. The final rate-limiting step in the catalytic cycle involves dehydration of 4a-hydroxyflavin (which restores FAD to its resting, oxidized state) and release of $NADP^+$. Also this step determines the upper limit of the rate of substrate oxidation. Binding of $NADP^+$ to FMO during catalysis is important because it prevents the reduction of oxygen to H_2O_2. In the absence of bound NADP, FMO would function as an NADPH-oxidase that would consume NADPH and cause oxidative stress through excessive production of H_2O_2.

In humans, FMO plays a major role in the biotransformation of several drugs (e.g. benzydamine, cimetidine, clozapine, guanethidine, methimazole, olanzapine, sulindac sulfide, tamoxifen and various dimethylaminoalkyl phenothiazine derivatives such as chlorpromazine and imipramine), xenobiotics (e.g. cocaine, methamphetamine, nicotine, tyramine) and endogenous substrates (e.g. trimethylamine, cysteamine). The major flavin monooxygenase in human liver microsomes, FMO3, is predominantly involved in the conversion of (*S*) nicotine to (*S*)-nicotine *N*-1-oxide, excreted in the urine of cigarette smokers or individuals wearing a nicotine patch. Therefore, the presence of *trans*-(*S*)-nicotine *N*-1-oxide in urine is clinically used as an in vivo probe of FMO3 activity in humans. FMO3 is also the principal

enzyme involved in the *S*-oxygenation of cimetidine, an H_2-antagonist widely used in the treatment of gastric ulcers and other acid-related disorders.

Monoamine Oxidase, Diamine Oxidase and Polyamine Oxidase Monoamine oxidase (MAO), diamine oxidase (DAO) and polyamine oxidase (PAO) – all these three enzymes are principally involved in the oxidative deamination of primary, secondary and tertiary amines. Many of the naturally occurring amines, such as the monoamine serotonin (5-hydroxytryptamine), the diamine putrescine and histamine, and monoacetylated derivatives of the polyamines spermine and spermidine form the substrate for these enzymes. Of the three enzymes, MAO is particularly involved in xenobiotic metabolism. Oxidative deamination of a primary amine produces ammonia and an aldehyde, whereas oxidative deamination of a secondary amine produces a primary amine and an aldehyde. The products of the former reaction, an aldehyde and ammonia, are those produced during the reductive biotransformation of certain oximes by aldehyde oxidase. The two forms of monoamine oxidase are MAO-A and MAO-B. The genetical information suggests that the genes coding MAO-A and -B are of common ancestral origin but encoded by two distinct genes, both localized on the X chromosome and both comprising 15 exons with a similar intron–exon organization. The amino acid sequence of MAO-A (Mr 59.7 kDa) is 70% identical to that of MAO-B (Mr 58.0 kDa).The mechanism of catalysis by monoamine oxidase is illustrated below:

$$RCH_2NH_2 + FAD \rightarrow RCH = NH + FADH_2$$

$$RCH = NH + H_2O \rightarrow RCHO + NH_3$$

$$FADH_2 + O_2 \rightarrow FAD + H_2O_2$$

The substrate is oxidized by the enzyme, which itself is reduced (FAD → $FADH_2$) (Parkinson et al. 2013). The oxygen incorporated into the substrate is derived from water, not molecular oxygen; hence the enzyme functions as a true oxidase. The catalytic cycle is completed by reoxidation of the reduced enzyme ($FADH_2$ n FAD) by oxygen, which generates hydrogen peroxide (which may be a cause of oxidative stress). The initial step in the catalytic cycle appears to be abstraction of hydrogen from the α-carbon adjacent to the nitrogen atom; hence, the oxidative deamination of xenobiotics by MAO is generally blocked by substitution of the α-carbon.

Although not present in mitochondria, PAO resembles MAO in its cofactor requirement and basic mechanism of action. Both enzymes use oxygen as an electron acceptor, which results in the production of hydrogen peroxide.

Diamine oxidase is a cytosolic, copper-containing pyridoxalphosphate-dependent enzyme present in liver, kidney, intestine, and placenta. Its substrates include histamine and simple alkyldiamines with a chain length of four (putrescine) or five (cadaverine) carbon atoms. Diamines with more than nine carbon atoms are not substrates for DAO, although they can be oxidized by MAO.

Xanthine Dehydrogenase–Xanthine Oxidase Xanthine dehydrogenase (XD) and xanthine oxidase (XO) are two forms of the same enzyme that differ in the electron acceptor used in the final step of catalysis. In the case of XD, the final electron acceptor is NAD^+ (dehydrogenase activity), whereas in the case of XO it is oxygen (oxidase activity). XD is converted to XO by reversible oxidation of cysteine residues (Cys993 and Cys1326 of the human enzyme) and/or by proteolytic cleavage. Under normal physiologic conditions, XD is the predominant form of the enzyme found in vivo (Parkinson et al. 2013). However, during tissue processing, XO or a combination of XO and XD is involved. The induction of XD and/or the conversion of XD to XO in vivo are thought to play a crucial role in ischemia-reperfusion injury, lip polysaccharide (LPS)-mediated tissue injury, and alcohol-induced hepatotoxicity. During ischemia, XO levels increase because hypoxia induces XD/XO gene transcription and because XD is converted to XO. During reperfusion, XO contributes to oxidative stress and lipid peroxidation because the oxidase activity of XO involves the reduction of molecular oxygen, which results in the generation of reactive oxygen species.

5.2.2 Hydrolytic Enzymes

Primary hydrolytic enzymes are carboxylesterases, peptidases and epoxide hydrolase. Other enzymes are cholinesterases and paraoxonases. Hydrolysis involves cleavage of ester or amide bonds, resulting in carboxylic acids, mainly in the presence of water. Generally, esters are hydrolysed much faster than amides (Fig. 5.1).

Fig. 5.1 Hydrolysis of esters and amides

Epoxide Hydrolase
H_2O
OH
OH
H_2O
OH
OH
H_2O
OH
H
OH
H
Epoxides produced by CYP450's are generally converted to phenols
The formation of aromatic diol indicates that the epoxide is stable and can be released from the active site.

Fig. 5.2 Hydration of epoxides

Hydrolysis of epoxides involves addition of water to alkene epoxides and arene oxide to form the corresponding diols (Fig. 5.2).

Carboxylesterases Carboxylesterases are ~60-kDa glycoproteins that are present in a wide variety of tissues, including serum. Most of the carboxyl esterase activity in liver is associated with the endoplasmic reticulum, although remarkable carboxylesterase activity is present in lysosomes and cytosol (Parkinson et al. 2013). The enzyme plays a significant role in producing pharmacologically active metabolites. For example, the pharmacologically active metabolite lovastatin hydroxy acid, which inhibits HMG-CoA reductase and lowers plasma cholesterol levels, is produced by converting lovastatin to lovastatin hydroxy acid by liver carboxylesterases.

Carboxylesterases in serum and tissues and serum cholinesterase collectively determine the site and duration of action of certain drugs. For example, procaine, a carboxylic acid ester, is rapidly hydrolyzed; hence, this drug is used mainly as a local anesthetic. In contrast, procainamide, the amide analogue of procaine, is hydrolyzed much more slowly, because of which the drug reaches the systematic circulation and is useful in the treatment of cardiac arrhythmia. In general, enzymatic hydrolysis of amides occurs more slowly than that of esters (Parkinson et al. 2013).

Peptidases Peptidases are enzymes which cleave peptides in the blood and tissues. They include amino peptidases, carboxypeptidases and endopeptidases. Amino peptidases hydrolyze amino acids at the *N*-terminus and carboxypeptidases at the *C*-terminus. Endopeptidases hydrolyse peptides at specific internal sites; for exam-

ple, trypsin cleaves peptides on the *C*-terminal side of arginine or lysine residues. Peptidases also function as amidases since they cleave the amide linkage between adjacent amino acids.

Epoxide Hydrolase The *trans* addition of water to alkene epoxides and arene oxides (oxiranes) is catalysed by epoxide hydrolase. Epoxide hydrolase is present in almost all tissues, including the liver, lung, kidney, skin, intestine, colon, testis, ovary, spleen, thymus, brain and heart. The five distinct forms of epoxide hydrolase present in mammals include microsomal epoxide hydrolase (mEH), soluble epoxide hydrolase (sEH), cholesterol epoxide hydrolase, LTA4 hydrolase and hepoxilin hydrolase. As their names imply, cholesterol epoxide hydrolase, LTA4 hydrolase, and hepoxilin hydrolase exclusively hydrolyze endogenous epoxides, but have no particular role in the detoxification of xenobiotic oxides. LTA4 hydrolase is distinct from the other epoxide hydrolases in that it is a bifunctional zinc metalloenzyme that has both epoxide hydrolase and peptidase activities as well as because of the two hydroxyl groups introduced during the conversion of LTA4 to LTB4. Many epoxides and oxides are intermediates formed during the cytochrome P450-dependent oxidation of aromatic and unsaturated aliphatic xenobiotics. These metabolites which may otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations are removed by epoxide hydrolases, particularly mEH and sEH. They rapidly convert the potentially toxic metabolites to the corresponding dihydrodiols, which are less reactive and easier to excrete. Because of these activities epoxide hydrolases are widely considered as a group of detoxification enzymes (Parkinson et al. 2013).

5.2.3 Reductive Reactions

Certain metals (e.g. pentavalent arsenic) and xenobiotics with an aldehyde, ketone, disulfide, sulfoxide, quinone, *N*-oxide, alkene, azo or nitro group often undergo reduction reaction, although it is sometimes difficult to ascertain whether the reaction proceeds enzymatically or non-enzymatically by interaction with reducing agents (such as the reduced forms of glutathione, FAD, FMN and NAD(P)) (Parkinson et al. 2013).

Azo- and Nitro-Reductions During azo-reduction, the nitrogen–nitrogen double bond is sequentially reduced and cleaved to produce two primary amines, using four reducing equivalents. Nitro-reduction requires six reducing equivalents, which are consumed in three sequential reactions, for the transformation of nitrobenzene to aniline. Azo- and nitro-reductions can be catalysed by enzymes of intestinal flora (Fig. 5.3), two liver enzymes cytochrome P450 (has the capacity to reduce xenobiotics under low oxygen or anaerobic conditions) and NAD(P)H-quinone oxidoreductase (a cytosolic flavoprotein, also known as DT-diaphorase), and interactions with reducing agents (reduced forms of glutathione, NADP, etc.). Under certain circumstances, a third liver enzyme, aldehyde oxidase, may also catalyse azo- and nitro-reduction reactions (Parkinson et al. 2013).

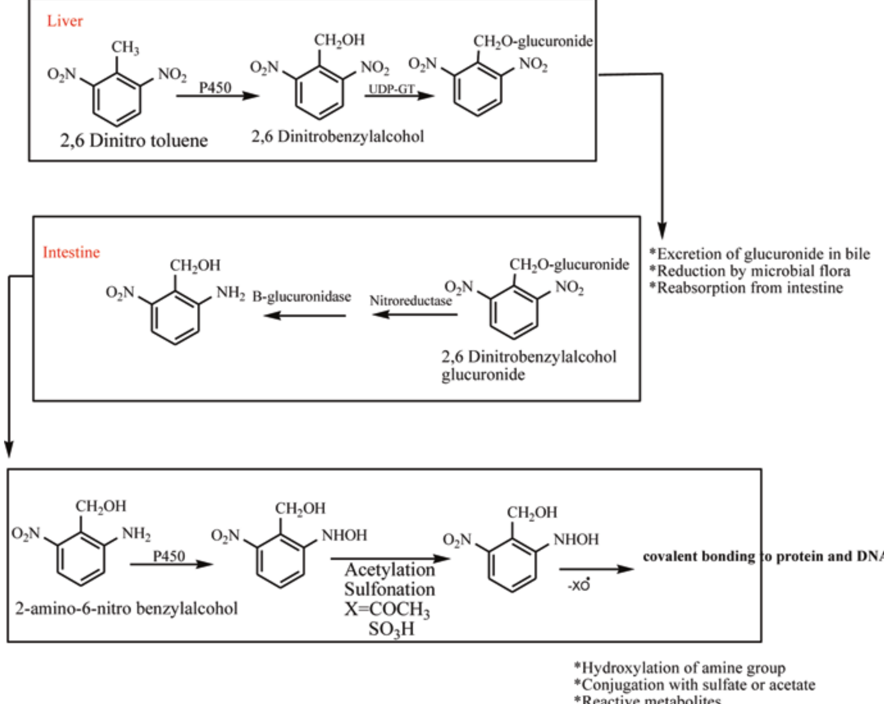

Fig. 5.3 Reduction – role of intestinal microbial flora in biotransformation

Carbonyl Reduction Carbonyl reductases are a class of enzymes which catalyse the reduction of certain aldehydes to primary alcohols and of ketones to secondary alcohols alcohols. These enzymes are monomeric, NADPH-dependent and present in blood and the cytosolic fraction of the liver, kidney, brain and other tissues and structurally belong to the short-chain dehydrogenase/reductase (SDR) super family which includes certain hydroxysteroid dehydrogenases and prostaglandin dehydrogenases. The major circulating metabolite of the antipsychotic drug haloperidol is a secondary alcohol formed by carbonyl reductases in the blood and liver. The enzyme shows a high degree of stereoselectivity in the reduction of ketones to secondary alcohols.

Quinone Reduction The cytosolic flavoprotein NAD(P)H-quinone oxidoreductase, also known as DT-diaphorase, catalyses the two-electron reduction of quinones to stable hydroquinone with stoichiometric oxidation of NAD[P]H without oxygen consumption. The reaction can also be catalysed by carbonyl reductase, especially in humans. Although there are exceptions, this pathway of quinone reduction is essentially nontoxic as it is not associated with oxidative stress, unlike the one-electron reduction of quinones by NADPH-cytochrome P450 reductase (Fig. 5.4). The other substrates for DTdiaphorase include a variety of potentially toxic compounds, including quinone epoxides, quinone imines, azo dyes and *C*-nitroso deriv-

Fig. 5.4 Reduction of quinones

atives of arylamines. The second pathway of quinone reduction is catalysed by NADPH-cytochrome P450 reductase (a microsomal flavoprotein) and results in the formation of a semiquinone free radical by a one-electron reduction of the quinone.

Semiquinones are readily auto-oxidizable, which leads to nonstoichiometric oxidation of NADPH with oxygen consumption. The auto-oxidation of a semiquinone free radical produces superoxide anion, hydrogen peroxide and other active oxygen species, which can be extremely cytotoxic and result in oxidative stress. DT-diaphorase levels are often elevated in tumour cells, since agents that are biotransformed by DT-diaphorase have implications over chemotherapy.

5.2.4 Phase II Reactions

Phase II enzymes are another class of biotransformation enzymes which catalyse biotransformation of endogenous compounds and xenobiotics mainly by conjugating reactions. The products formed thereby are of easily excretable forms as well as act as a metabolic inhibitor of pharmacologically active substances. These include glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. The cofactors involved in these reactions react with functional groups that are either present on the xenobiotics or are introduced during phase I biotransformation. The main consequence of phase II biotransformation

Fig. 5.5 Conjugation with glucuronic acid (glucuronidation)

reactions is to excrete out the foreign chemicals by increasing their hydrophilicity to a larger extent.

Glucuronidation Glucuronidation plays a major role in the biotransformation of xenobiotics in almost all mammals. Glucuronidation requires the cofactor uridine diphosphate-glucuronic acid (UDP glucuronic acid), and the reaction is catalysed by UDP glucuronosyl transferases (UGTs), localized in the endoplasmic reticulum of the liver and other tissues, such as the kidney, intestine, skin, brain, spleen and nasal mucosa. The site of glucuronidation is generally an electron-rich nucleophilic heteroatom (O, N or S) (Fig. 5.5). Therefore, substrates for glucuronidation contain such functional groups as aliphatic alcohols and phenols (which form *O*-glucuronide ethers), carboxylic acids (which form *O*-glucuronide esters), primary and secondary aromatic and aliphatic amines (which form *N*-glucuronides), and free sulfhydryl groups (which form *S*-glucuronides). Coumarin and certain other carbonyl-containing compounds are glucuronidated to form arylenol-glucuronides. Other than xenobiot-

ics, substrates for glucuronidation include several endogenous compounds, such as bilirubin, steroid hormones and thyroid hormones (Parkinson et al. 2013). Xenobiotics and endogenous compounds on glucuronidation become polar, water-soluble conjugates that are eliminated from the body through urine or bile, which depends on the size of the parent compound or phase I metabolite.

Sulfation Sulfation is a main conjugation pathway for phenols which can also occur for alcohols, arylamines, N-hydroxy compounds and, to some extent, thiols. As with sugar conjugation, the active donor is 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (Fig. 5.6). Sulfation results from the interaction of the drug with PAPS in the presence of the cytosolic enzyme sulfotransferase. Sulfate conjugation occurs less frequently than does glucuronidation presumably because PAPS cellular concentration is considerably lower (75 mM) than Uridine 5′- diphosphoglucuronic acid (UDPGA) (350 mM). Hence the capacity of sulfation is low and only fewer numbers of functional groups undergo sulfate conjugation. Functional groups that can be sulfated are phenols: Ar-OH, alcohols: R-OH, arylamines: Ar-NH2, and N-hydroxy compounds: R-NH-OH (Amin M. Kamel 2007).

Methylation Though methylation reaction is common, it is considered as a minor pathway of xenobiotic biotransformation (Fig. 5.7). In this reaction, the methyl group donor SAM (*S*-adenosylmethionine) is converted to *S*-adenosylhomocysteine. Methylation reactions include endogenous compounds such as melatonin, histamine, serotonin, dopamine, etc. Compared to conjugation reactions, methylation reduces the polar and hydrophilic nature of the substrates, thereby deactivating their biological activities.

Acetylation N-Acetylation is a major route of biotransformation for xenobiotics containing an aromatic amine ($R–NH_2$) or a hydrazine group ($R–NH–NH_2$), which are converted to aromatic amides ($R–NH–COCH_3$) and hydrazides ($R–NH–NH–COCH_3$), respectively (Evans 1992). The enzyme which catalyses the *N*-acetylation of xenobiotics is N-acetyltransferases. The enzyme requires acetyl-coenzyme A (acetyl-CoA) as the coenzyme (Fig. 5.8). Except for cystein conjugates, which are transformed to mercapturic acids by N-acetylation, primary aliphatic amines are rarely its substrates. Like methylation, N-acetylated metabolites are less polar than their parent compounds since N-acetylation masks an amine with a nonionizable group.

Conjugation with Amino Acids There are two principal pathways by which xenobiotics are conjugated with amino acids: one which conjugates with the amino group of the amino acid and the other with the carboxylic group. The first pathway involves conjugation of xenobiotics containing a carboxylic acid group with the amino group of amino acids such as glycine, glutamine and taurine. This pathway involves activation of the xenobiotic by conjugation with CoA, which produces an acyl-CoA thioether that reacts with the *amino group* of an amino acid to form an amide linkage. The second pathway involves conjugation of xenobiotics containing an aromatic hydroxylamine (*N*-hydroxy aromatic amine) with the *carboxylic acid group* of such

Fig. 5.6 Sulfation

Fig. 5.7 Methylation

Fig. 5.8 Acetylation

amino acids as serine and proline. This pathway involves activation of an amino acid by aminoacyl-tRNA-synthetase, which reacts with an aromatic hydroxylamine to form a reactive *N*-ester (Kato and Yamazoe 1994).Carboxylic acids, particularly aromatic acids and aryl acetic acids, are conjugated with polar endogenous amino acids. The quantity of amino acid conjugation is minute because of the limited availability of amino acids in the body and competition with glucuronidation for carboxylic acid substrates. Amino acids conjugation of carboxylic acids leads to amide bond forma-

Fig. 5.9 Conjugation with amino acids (glycine conjugation with benzoic acid)

Fig. 5.10 Conjugation with glutathione

tion. Glycine conjugates are the most common amino acid conjugates in animals (Fig. 5.9). Conjugation with L-glutamine is most common in humans and other primates, but it seems to occur to a lesser extent in non-primates. Taurine, arginine, asparagine, histidine, lysine, glutamate, aspartate, alanine and serine are the other amino acid conjugates found in mammals (Parkinson et al. 2013).

Conjugation with Glutathione The tripeptide glutathione (GSH) is found in virtually all mammalian tissues (Fig. 5.10). The presence of a potent nucleophilic thiol group helps glutathione function as a scavenger of harmful electrophilic compounds ingested or produced by metabolism. Xenobiotics conjugated with glutathione are either highly electrophilic as such or are first metabolized to an electrophilic product prior to conjugation. Conjugation with glutathione reduces drug toxicity that results from the reaction of cellular nucleophiles with electrophilic metabolites. The glutathione S-transferases (GSTs) are a family of enzymes that catalyse the conju-

gation of tripeptide glutathione with xenobiotic compounds forming thioether conjugates. To a larger extent, a number of endogenous compounds such as prostaglandins and steroids are also metabolized via glutathione conjugation (Parkinson et al. 2013).

5.3 Biotransforming Enzymes in Plants

Plants are permanently exposed to various xenobiotics and pose potential danger. Like other living beings, plants also include a variety of xenobiotic-metabolizing enzymes for detoxification of compounds (Table 5.3). Though some of them show similarities with those in humans and animals, there are several plant-specific ones. Plant cell suspension cultures serve as tools for the biotransformation of xenobiotics. Our environment is contaminated by unregulated contaminants, which include personal care products, pesticides, herbicides and industrial compounds that are thought to have long-term adverse effects on ecosystems. But according to Powles and Yu (2010), in the case of plants, the increased activity of xenobiotic-metabolizing enzymes results in faster deactivation of xenobiotics, which may be manifested as improved detoxification of environmental contaminants but also as a resistance to herbicides.

Veterinary drugs are used worldwide to treat diseases and protect animal health. However, veterinary drugs are also unwantedly introduced into the environment mostly via animal excrements, which persist in the environment for a long time and may impact on the non-target organisms. Plants are able to uptake the veterinary drugs, transform them to non- or less-toxic compounds and store them in the vacuoles and cell walls. This ability may protect not only the plants themselves but also other organisms, predominantly invertebrates and wild herbivores (Bartikova et al.

Table 5.3 Xenobiotic-metabolizing enzymes in plants

Reaction	Xenobiotic-metabolizing enzymes
Biotransformation phase I	Cytochromes P450
	Peroxides
	Ascorbate peroxidases
	Peroxides III-class
	Alcohol dehydrogenases
	Aldehyde dehydrogenases
	Short-chain dehydrogenases/reductases
	Aldo-ketoreductases
	Carboxylesterases
	Epoxide hydrolases
Biotransformation phase II	Glutathione-S-transferases
	Glycosyltransferases
	Malonyltransferases
	Sulfotransferases
	Methyltransferases

2015). Although antibiotics belong to the most important veterinary drugs, information about their biotransformation in plants is only limited. Migliore and his group (2003) studied phytotoxicity, uptake, and biotransformation of enrofloxacin in crop plants such as *Cucumis sativus*, *Lactuca sativa*, *Phaseolus vulgaris* and *Raphanus sativus*. Their results showed that plants were able to metabolize enrofloxacin into ciprofloxacin via deethylation reaction. The other example includes the biotransformation of the NSAID diclofenac. NSAID diclofenac is found in the environment worldwide due to its intensive use and poor elimination during wastewater treatment processes. In order to test phytoremediation as a tool for the removal of this drug from wastewater, the uptake of the compound into plant tissues and its metabolic pathway was addressed using barley (*Hordeum vulgare*) and a hairy root cell culture of horseradish (*Armoracia rusticana*) as model species. Diclofenac was taken up by plants and underwent rapid biotransformation. A phase I reaction resulted in the hydroxylated metabolite 40OH-diclofenac, which was conjugated subsequently in phase II to a glucopyranoside, a typical plant-specific metabolite (Huber et al. 2012).

5.4 Biotransforming Enzymes in Microbes

The recognition of microbial biotransformation has created a boom in the chemical and pharmaceutical industries. In recent years, microbial biotransformation has become an inevitable process in green chemistry. The wide variety of microbial strains and enzymes with their selective biotransformation potential has resulted in the bioconversion of a myriad of different substances into desired products.

It is only after the nineteenth century, targeted application of microbial transformations came into emergence. The fusion of two sciences, namely, organic chemistry and microbiology, has resulted in the tremendous growth of this field. The technology of microbial transformation deals with the isolation of microbial enzymes to catalyse bioconversions of organic compounds.

Microbial transformation offers the advantages of operating at non-extreme pH, near room temperature and reduced levels of toxic waste products with high selectivity. This metabolic flexibility of the microorganisms is exploited in the production of different enzymes for different reactions. However, the syntheses of these enzymes are regulated according to the physiological needs of the cells. The practice of biotransformation with recombinant microbial enzymes is gaining importance; the applications may include the production of hormones, antibiotics and special chemicals. Microbial transformation might be useful in cases where there is no chemical solution for chemical transformations.

Microbial transformations make use of enzyme-catalysed reactions within living cells, typically exploiting single chemical reactions like oxidation, reduction, hydrolysis and degradation, formation of C-C or C-hetero atom bonds (Gopishetty et al. 2007) (Table 5.4).

Economically, microbial biotransformations can be used in the manufacture of alkaloids, antibiotics, vitamins, amino acids, fermented beverages and fermented foods. Another feature of microbial transformation is its ability to imitate

Table 5.4 Reactions catalysed by microorganisms

Microbial strain	Reaction catalysed	Examples
Gliocladium roseum	Hydroxylation	Hydroxylation of unsubstituted coumarin to 7-hydroxy coumarin
	Glycosylation	Glycosylation of 7-hydroxy coumarin to 6,7-dihydroxy-coumarin-6-glycoside
Candida tropicalis	Alkylation	*O*-alkylation of 7-hydroxy coumarin to 7-methoxy coumarin
Streptomyces griseus	Oxidation	*O*-deethylation of 7-ethoxy coumarin to 7-hydroxy coumarin
	O-, *N*-dealkylation	Hydroxylation of 7-hydroxy coumarin to 6,7-dihydroxy coumarin
	Aromatic hydroxylationEpoxidation	Methylation of 6,7-dihydroxy coumarin to 7-hydroxy-6-methyl coumarin
	Carbon-carbon fission	
Aerobacter aerogenes	Microorganisms with mercury-metabolizing capability	
Bacillus megaterium		
Clostridium cochlearium		
Desulfovibrio desulfuricans		
Desulfovibrio vulgaris		
Desulfobulbus propionicus		
Enterobacter aerogenes		
Escherichia coli		

mammalian metabolism of drugs, which enables structure determination of drug metabolites for use in preclinical trials, toxicity studies and regulatory process.

5.5 Industrially Significant Biotransformations

Selected milestones of industrially relevant biotransformation and biocatalytic processes are summarized in Table 5.5.

Table 5.5 Some selected milestones of industrially relevant biotransformation and biocatalytic processes

Year	Process
5000 BC	Vinegar production
800 BC	Casein hydrolysis with chymosin for cheese production
1670	"Orlean" process for the industrial bio-oxidation of ethanol to acetic acid
1680	Anton van Leeuwenhoek first to see microorganisms with his microscope
1897	E. Buchner discovers yeast enzymes converting sugar into alcohol
1934	Regioselective bio-oxidation of sorbite to sorbose for the Reichstein vitamin C synthesis
1940	Sucrose inversion using an invertase
1950	Bioconversion of steroids
1970	Hydrolysis of pencillin to 6-aminopencillanic acid
1973	First successful genetic engineering experiments
1974	Glucose to fructose isomerization with immobilized glucose isomerase
1985	Enzymatic process for the production of acrylamide
1990	Hydrolysis of protease (trypsin) of porcine insulin to human insulin
1995	3000 ton pa plant for the biotransformation of nicotinonitrile to nicotinamide

High Fructose Syrups Production of high-fructose glucose syrups, by glucose isomerase, was one of the earliest biotransformation processes done by immobilized enzyme technology, and it has been operating now since 1972. It is used in the soft drinks and confectionary industries as it has health advantages over the conventional sugar, sucrose, i.e. fructose is twice as sweet, lower in calories and not absorbed into the bloodstream as quickly as sucrose (Collins and Kennedy 1999).

Antibiotics Many antibiotics synthesized from their precursors are produced by the biotransformation process catalysed by the respective enzymes. Some examples include semi-synthetic penicillin produced from its precursor 6-amino-penicillanic acid. This is done by using the immobilized penicillin acylase (*amidase*), which has been operational since 1973.

Vitamins From the economic point of view, microbial biotransformations are widely used to produce vitamin B_{12}, riboflavin, ascorbic acid and p-carotene. For example, oxidation of sorbitol by *Acetobacter suboxydans* or *Acetobacter xylinum* yields ascorbic acid (vitamin C).

$$\text{D-Glucose} \rightarrow \text{D-Sorbitol} \rightarrow \text{L-Sorbose} \rightarrow \text{L-Ascorbic acid (vitamin C)}.$$

$$\boxed{Acetobacter\ \ suboxydans}$$

Flavours and Fragrances The role of biotransformation technologies in the flavours and fragrances industry is becoming more prominent for its ability to produce single enantiomers. For example, the (L) enantiomer of carvone tastes of spearmint, whereas its (D) enantiomer tastes of dill/caraway. Also only (L) form of monoso-

dium glutamate has taste-enhancing properties, and out of eight isomers of menthol only (L)-menthol has the desired combination of mint taste and cooling sensation (Collins and Kennedy 1999).

Food Industries There is an increasing demand by consumers for natural, environmentally friendly and healthy products made from natural, renewable sources, for use in both food ingredients and personal care products. Biotransformation enzymes play a prominent role in the production of various food products like cheese, wine, food additives, etc. The advantages of using biotransformation for these types of preparations are the ability to operate under mild conditions, hence retaining the traditional properties of the food products.

5.6 Conclusions

Biotransformation is a process by which organic compounds are transformed from one form to another, aided by organisms such as bacteria, fungi and enzymes. It is used as a valuable strategy to build molecules similar to parent drugs in the drug discovery programme. It can also be used to synthesize compounds or materials. Microbial biotransformation or microbial biotechnology is extensively used to generate metabolites in bulk amounts. Biotransformation approaches and synthetic methods in tandem provide a source for generating compounds around core structures, which can be screened for various biological activity studies. These studies help in screening and advancing compounds through various stages of the drug discovery programme. Hence, biotransformation experiments can be effectively utilized to synthesize more compounds. Identification of the tentative structure of metabolites helps to design and synthesize new molecules similar to the parent compound. The synthesized metabolites can be a compound or material whose properties might be similar to those of the parent drug, and can serve as an ideal backup compound for parent drug in clinical trials (Ravindran et al. 2012).

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6 Enzyme Engineering

Soorej M. Basheer and Sreeja Chellappan

Abstract

Enzymes, also called biocatalysts, are widely used in various industrial applications, especially in the manufacturing of bulk chemicals and pharmaceuticals. Enzyme engineering is the process of improving the efficiency of an already available enzyme or the formulation of an advanced enzyme activity by altering its amino acid sequence. This technology has developed as a potential tool to overcome the disadvantages of native enzymes as biocatalysts. Rational design and directed (molecular) evolution are the two general approaches in enzyme engineering. Genetic engineering techniques are widely used to improve enzyme efficiency. For exploring enzyme sequences and for creating new and efficient biocatalysts, the combination of directed evolution and rational protein design using computational tools is becoming increasingly relevant. Various other strategies such as enzyme immobilization, de novo enzyme design, peptidomimetics, flow cytometry, and designed divergent evolution help in creating a tailor-made enzyme for a given process. A better awareness of how the structure of an enzyme influences its properties and a more crucial interpretation of the many engineering aspects are necessary to make rapid advance in the field of enzyme engineering.

Keywords

Enzymes • Engineering • Rational design • Directed evolution

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6.1 Introduction

Enzymes are widely used in novel and diverse applications in the food, feed, agriculture, paper, leather, and textile industries producing significant decline in their manufacturing cost. Currently, rapid technological advancements are stimulating the chemistry and pharma industries to adopt enzyme technology, a trend that is strengthened by the concerns occurring in the areas of health, energy, raw materials, and the environment. In the case of industrial enzymes, the global market approached nearly \$4.9 billion in 2015 and is anticipated to reach \$6.3 billion by 2021 (BCC Research 2017). Novo Nordisk is one of the biggest enzyme producers, which manufactures genetically engineered (GE) and non-GE enzymes. Enzymes are abundantly used in the large-scale production of chemicals and pharmaceuticals, and more than 300 processes have been already established (Schmid et al. 2001; Schoemaker et al. 2003). The microbial kingdom represents an enormous and still underexplored reservoir for enzymes with specific properties, and hence biocatalysts of microbial origin are used in a majority of industrial processes.

6.2 Major Classification of Enzymes

According to the International Enzyme Commission constituted by the International Union of Biochemistry (1956), enzymes are divided into six major classes depending on the nature of reaction catalysed (Table 6.1).

Table 6.1 Major classes of enzymes

Class	Reaction catalysed	Examples	General scheme of reaction
1. Oxidoreductase	Redox	Dehydrogenase, oxidase	Oxidation ↔ Reduction $AH_2 + B \leftrightarrow A + BH_2$
2. Transferase	Group transfer between two substrates	Kinase, transaminase	Group transfer $A - X + B \leftrightarrow A + B - X$
3. Hydrolase	Hydrolysis of a substrate	Esterases, digestive enzymes	Hydrolysis $A - B + H_2O \leftrightarrow AH + BOH$
4. Lyase	Non-hydrolytic bond cleavage and generation of double bonds	Aldolase, histidase	Addition ↔ Elimination $A - B + X - Y \leftrightarrow AX - BY$
5. Isomerase	Intramolecular rearrangement of groups	Triose phosphate isomerase, phosphohexose isomerase	Interconversion of isomers $A \leftrightarrow A'$
6. Ligase (Synthetase)	Bond formation at the expense of ATP	Glutamine synthetase, succinate thiokinase	Condensation (dependent on ATP) A + B ↔ A – B (ATP → ADP + Pi)

Most of the enzymes in its natural form have diverse applications. But a serious problem arises due to the reason that their availability becomes scarce on account of the cost effectiveness in its industrial production. The process of enzyme engineering depends upon the modification or production of novel enzymes with maximum efficiency and high yield.

6.3 Enzyme Engineering

Enzyme engineering is the process by which one can improve the activity and efficiency of an existing enzyme or create a new enzyme activity by making relevant changes in its amino acid sequence. The term protein engineering is used when this similar approach is applied to alter the properties of any protein, whether enzyme or non-enzyme. Recombinant DNA technology is utilized in the process of enzyme engineering to introduce appropriate changes in the amino acid sequences of enzymes. It is regarded as a valuable technology that helps to enhance our basic knowledge on various enzyme functions and their evolution. It is also an important procedure for reconstructing enzyme properties for various industrial uses in pharmaceuticals, biofuels, and green chemistry.

Recombinant DNA technology enables the modification of amino acid sequences by gene alteration: genes from less active microbes can be transferred to higher active ones, and multiple genes can be moved and expressed in a single organism. The past few decades saw the enhancement in a number of potentially valuable biocatalysts due to the development of metagenomics approach (Ferrer et al. 2009), which aids in the discovery of new enzymes from various microbial sources.

The modification of amino acids of enzymes is the major principle behind enzyme engineering, which results in an enzyme with altered properties. The variations thus arising due to such modifications are mostly apparent in the primary structure of enzymes or proteins coded by amino acids. An enzyme's property can be modified by introducing changes only in the specific domains of the amino acid sequence. Hence, it becomes essential that the gene coding for such regions be determined and then altered as required. Kinetic properties; allosteric regulation; specificity; effect of factors like temperature, solvents, pH; etc., are the different properties which are normally addressed for advancements by enzyme engineering (Fig. 6.1).

Since enzymes are proteins, enzyme engineering is regarded as a component of the larger activity of protein engineering. Generally, the objective of protein engineering is to modify the protein sequence and thus its three-dimensional structure in order to design enzymes with enhanced functional properties, such as stability, without product inhibition, high specific activity, and selectivity towards non-natural substrates (Singh et al. 2013).

In the last decade, the utilization of genetic engineering to enzyme technology has been a most exciting development. There are a huge number of characteristics which may be altered or improved by genetic engineering technology including the yield and kinetics of the enzyme, the ease of downstream processing, and various

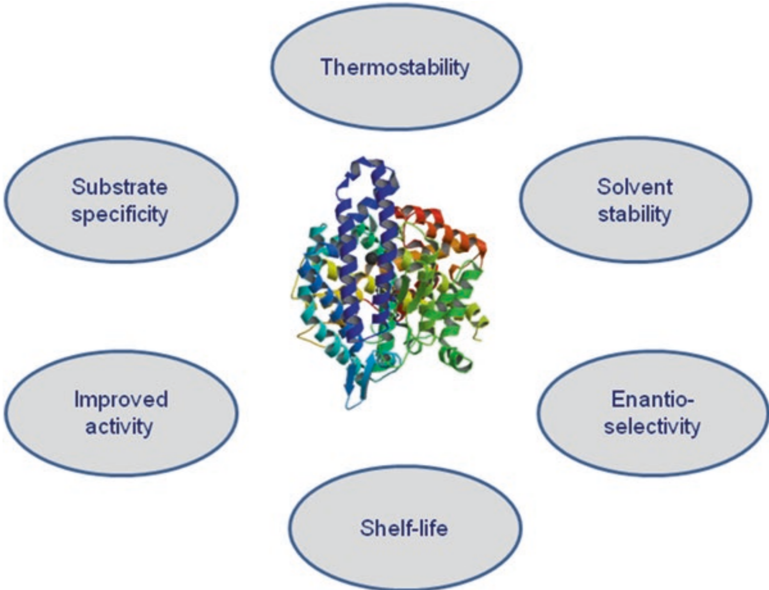

Fig. 6.1 Enzyme properties to be modified during enzyme engineering process

safety aspects. Enzymes from hazardous microorganisms and from slow growing or limited plant or animal tissue may be cloned into safe high-production microorganisms. Eventually, enzymes may be improved to suit various industrial processes.

6.3.1 Objectives and Principles

The principal objective of enzyme engineering is in the production of an enzyme that is more beneficial for industrial and/or other applications. The various characteristics of an enzyme that may be altered to accomplish this objective are to improve kinetic properties, to eliminate allosteric regulation, to enhance substrate and reaction specificity, to increase thermostability, to make it suitable for use in organic solvents, to increase or decrease optimal temperature and pH, to enhance stability towards oxidizing agents and heavy metals, to be resistant to proteolytic degradation, and to fuse two or more enzymes to create bi- and poly-functional enzymes. Stability to heavy metals can be improved by replacing Cys and Met residues, and by removing surface carboxyl groups. Similarly, stability to oxidizing agents can be increased by replacing easily oxidizable residues, e.g., Cys, Trp, Met, by sterically similar but non-oxidizable amino acids, e.g., Ser, Phe, Glu, respectively. Increased stability at non-physiological temperatures, pH, etc., can be achieved by introduction of disulphide bridges in the enzyme molecule and by increasing the number of salt bridges or that of internal hydrogen bonds.

Generally, the primary structure, i.e., the amino acid sequence, determines the structure and function of an enzyme molecule or of any protein molecule. It is important to understand about the critical regions of the molecule for the various activities of an enzyme and thereby enable to predict the effect of change in specific amino acids in these areas on different functions. Still the current information about the relationships between amino acid sequence and the structure and properties of enzymes obtained from the available database is only partially operative. Enzyme engineering provides an explanation of the alterations in the structure and function on the basis of the modifications in amino acid sequence, but it does not provide a dependable prediction of the influences of specific amino acid changes on the structure and function of enzymes. It might be expected that as more improved softwares and elaborate databases become available, the structural and functional modifications in enzymes produced by the specified alterations in their amino acid sequences can be predicted with far greater confidence. Thereby the capability of enzyme engineering will be enormously enhanced and may have an amazing influence on enzyme technology.

6.3.2 Steps in Engineering

Protein engineering generally involves three steps: choosing a change in the protein (engineering strategies, such as rational design or randomization), accomplishing those modifications (mutagenesis), and evaluating the protein variants for enhanced properties (screening or selection) (Kazlauskas and Bornscheuer 2009). Selection of different strategies can lead to a variety of advantages or disadvantages in each of these steps. The selection of the outstanding method is driven by limitations such as structural information, various tools and techniques for mutagenesis, and methods for screening or selection. Many different strategies have been used by protein engineers, and most of those strategies will ultimately yield an improved protein. Increasing the thermostability of an enzyme is one of the common protein engineering goals. Structure-based approaches, like crystallographic and nuclear magnetic resonance (NMR) studies, presume that a more rigid enzyme will be more stable at high temperatures. The structure of an enzyme derived by X-ray crystallography can be employed to design specific stabilizing interactions such as disulphide bonds or salt bridges, stabilize the loop regions by removing glycine or introducing proline, or find the most flexible region of the target proteins from the experimental factors and focus mutagenesis at those regions. An evolution- and bioinformatics-based approach has the underlying belief that the stability of protein is contributed by conserved amino acids. The engineering strategy is to analyse similar sequences and engineer the target protein to simulate the consensus sequence.

Protein engineering generally utilizes two different approaches: rational design and directed evolution. To create appropriate modifications via site-directed mutagenesis, in rational design, the structure, function, and catalytic mechanism of the protein must be well understood. However, such acquaintance is absent for most proteins of interest. According to Bloom and Arnold (2009), even though

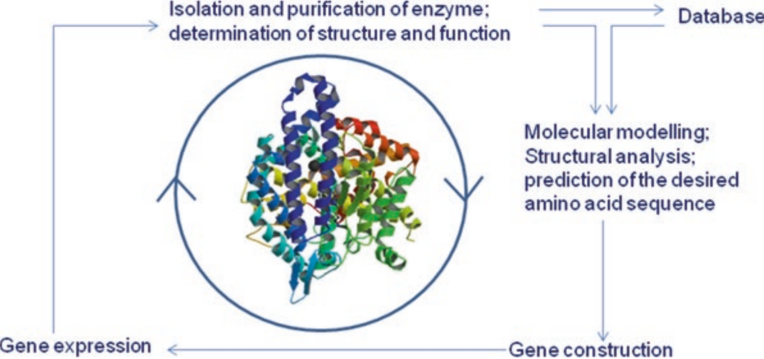

Fig. 6.2 Various steps involved in enzyme engineering

computational protein design algorithms were developed to predict optimal mutations at specific residue positions in the protein, only limited success has been demonstrated, whereas only the knowledge of protein sequence is required in the case of directed evolution approach. This approach involves repeated cycles of random mutagenesis and/or gene recombination followed by screening or selection for positive mutants (Zhao and Tan 2015). The method of directed evolution mimics natural evolution and normally produces better results to rational design. The directed evolution has been widely applied with the aim of developing highly efficient biocatalysts (Adamczak and Krishna 2004; Bloom and Arnold 2009; Rubin-Pitel and Zhao 2006; Turner 2009). The methodology or the approach that permits one to attain the goal with least effort can be considered as the best protein engineering strategy. This criterion makes it improbable that a purely rational design or purely random mutagenesis approach will be best.

The various steps involved in enzyme engineering are briefly described in Fig. 6.2.

6.4 Procedure for Engineering Enzymes

1. *Study of enzymes*: The first step consists of isolation of the concerned enzyme and determination of its structure and properties. Factors influencing enzyme features like structure are extensively studied comprising the primary, secondary, and tertiary structures. Information on three-dimensional structures is usually obtained from X-ray diffraction, nuclear magnetic resonance (NMR), etc.
2. *Molecular modelling*: The data so obtained are scrutinized along with the database of known and putative structural effects of amino acid substitutions on enzyme structure and function. All the available information are gathered together, and molecular modelling is executed to resolve the possible change in

amino acid sequence for the desired improvement in function/structure of the enzyme.

3. *Site-directed mutagenesis*: The next step consists of constructing a gene that will encode the specific amino acid sequence. This is best achieved by isolation and cloning of the natural gene encoding the concerned enzyme and using this gene for site-directed mutagenesis. In the case of site-directed mutagenesis, specified changes in the base sequence are introduced at specified sites of genes. The genes for the specific amino acid are first isolated, modified appropriately by site-directed mutagenesis, and cloned into an expression vector to obtain a gene construct. Once the appropriate gene is constructed, it is introduced and expressed in a suitable host, e.g., *Escherichia coli*. The recombinant or mutant enzyme so produced is isolated, purified, and used for the determination of its structure and properties. The alterations made in the amino acid sequence will be reflected in the characteristics of enzymes produced by recombinant DNA, which can be compared with that of the native enzymes. The information so obtained is added to the database. If the enzyme structure and function are not altered as desired, the next cycle of experimentation is undertaken.

6.4.1 Examples of Enzyme Engineering

Even though enzyme engineering strategies have been widely employed for studying the relationship between amino acid sequences and the structures and functions of various enzymes, commercial examples are few. Some of the enzymes studied are tyrosyl-tRNA synthetase, *β*-lactamase, dihydrofolate reductase, subtilisin, lysozyme, alcohol dehydrogenase, and lactate dehydrogenase. Various studies have also focused on alterations of industrial enzymes to augment their efficiency. The results from these studies have helped to exhibit the strength of the technique, to generate highly beneficial data on sequence–structure–function relationships, and also to reveal the limitations of our knowledge.

Subtilisin Subtilisin (EC 3.4.21.62) is a non-specific protease initially obtained from *Bacillus subtilis*. The advantage of enzyme engineering can be illustrated by utilizing subtilisin (produced from *B. amyloliquefaciens*), the principal enzyme in the detergent enzyme preparation, Alcalase (Ottesen and Svendsen 1970). In order to enhance the enzyme activity in detergents, it is important to improve the enzyme stability at higher temperatures, pH, and oxidant (bleach) strengths. The P_1 cleft of this enzyme possesses the amino acid residue on C-terminal side of the targeted peptide bond. Substitutions of amino acid in the P_1 cleft help in enhancing the specificity of the enzyme for specific peptide bonds, while specificity for others is diminished. This alteration can be predicted reasonably precise. For some applications, the increase in relative specificities may be beneficial. Subtilisin is exceptional in that it has a large hydrophobic site, which is fairly non-specific in activity that can be made more specific quite easily (e.g. by reducing its size). The effect of a substitution in the P_1 cleft on the relative specific activity between substrates may be

reasonably correctly predicted even though the complete effects of such modifications remain less successful. Many substitutions have been found to increase the specificity of the enzyme for particular peptide links, whilst reducing it for others, particularly for the glycine residue at the bottom of the P_1 cleft (Gly_{166}).

The oxygen produced by bleaches can inactivate subtilisin; this inactivation is due to the oxidation of the methionine residue at position 222 (Met_{222}) to its sulphoxide. Met_{222} lies adjacent to the active site Ser residue, which is necessary for catalysis. Oxidation of Met_{222} most likely prevents substrate access to the active site. Engineered subtilisin in which the Met_{222} residue was substituted with serine or alanine was much less sensitive to oxygen than the native enzyme; however, these enzymes demonstrated much reduced specific activities (only 53% and 35% specific activities, respectively, in comparison to their wild-type counterparts) (Bradshaw and Purton 2012).

Lactate Dehydrogenase Lactate dehydrogenase, LDH (EC 1.1.1.27), catalyses the transformation of lactate to pyruvic acid and back, as it converts NAD^+ to NADH and back. The lactate dehydrogenase produced by *Bacillus stearothermophilus* was used for enzyme engineering (Holmberg et al. 1999). The specificity of this enzyme can be shifted from lactate to maleate by modifying (engineering) this enzyme. This specificity was found to be associated with the existence of glutamine at position 102 (Gln_{102}). Replacement of Gln_{102} with arginine resulted in change in specificity from lactate to maleate. The engineered enzyme became specific to maleate to the same magnitude to which the native enzymes have specificity for lactate. Meng et al. (2016) reported that an NADH-dependent dehydrogenase (D-LDH) from *Lactobacillus delbrueckii* 11842 can be rationally engineered to efficiently use both NADH and NADPH as cofactors. These examples clearly demonstrate that enzyme engineering may be capable to create potential enzymes with novel substrate specificities.

α-Amylase The enzyme α-amylase (1,4-α-D-glucan glucanohydrolase, EC. 3.2.1.1) catalyses the hydrolysis of α-(1,4) glycosidic linkages in starch and related polysaccharides to yield malto-oligosaccharides, such as maltotriose (G3), maltotetraose (G4), maltopentaose (G5), and maltohexaose (G6). The production of malto-oligosaccharides has attracted attention in food industry as they contribute desirable changes to enhance flavour and physicochemical characteristics of food in addition to many beneficial properties to human health. They are highly useful in beverages, bakery products, confectionery, infant milk powders, yogurts, and dairy desserts. However, the price of pure malto-oligosaccharides is exceptionally huge as the chemical structure of malto-oligosaccharides is bigger than maltotriose and because of its difficulty in production (Subramanian et al. 2012).

The enzyme AmyUS100 produced by *Bacillus stearothermophilus* has a primary amino acid sequence identical to that of another enzyme AmyS produced by a different strain (*B. stearothermophilus* strain DN1792) of the same bacterium. Among the 516 residues, only three residues varied in the mature protein, one of which was in the terminal region that is not involved in catalysis. Both the amylases, AmyUS100 and AmyS, demonstrated identical optimum pH and temperature but differed in their starch hydrolysis profile. Thus, it was implicated that the two amino acids

(Asn_{315} and Val_{450}) were involved in the catalysis (Ben Ali et al. 2006). Site-directed mutagenesis helped in the production of three mutants from AmyUS100: AmyUS100-D (with mutation N315D), AmyUS100-G (with mutation V450G), and AmyUS100-D/G (with double mutation N315D/V450G). Among the three, the V450G mutation did not change the starch hydrolysis profile, whereas the introduction of both substitutions strongly affected the hydrolysis profile, and the main end products shifted from G6/G5 to G3/G2 (Ben Ali et al. 2006). The α-amylase from *B. amyloliquefaciens* was engineered by site-directed mutagenesis, and a modified optimum pH activity profile was exhibited by the variants thus produced. The variants improved the volume of the bread as compared to the parent bacterial α-amylase (Danielsen and Lundqvist 2008).

Xylanase The enzyme xylanase (EC 3.2.1.8) degrades hemicellulose, one of the major components of plant cell walls by breaking the linear polysaccharide β-1,4-xylan into xylose. One of the best stabilizations ever achieved by means of directed evolution strategy of enzyme engineering (increase in melt temperature, Tm, of more than 30 °C) was reported by Palackal et al. (2004). Xylanase was obtained by screening 50,000 plaques from a complex environmental DNA library derived from fresh bovine manure. The application of site-saturation mutagenesis and screening of nearly 70,000 clones helped in the identification of nine interesting mutations, which, when combined, increased the Tm by 34.2 °C (Palackal et al. 2004).

Enzyme immobilization can be considered as a physical method to improve the efficiency of enzymes, which are mainly used in industrial applications, e.g., in food and beverage industry (Mohamad et al. 2015; Ribeiro et al. 2010; Sharma et al. 2017).

Naringinase Naringinase, an enzymatic heterodimeric complex composed of two subunits, α-L-rhamnosidase (EC. 3.2.1.40) and β-D-glucosidase (EC. 3.2.1.21), is generally acquired from fungi. The use of naringinase provides a valuable choice for the removal of excess bitter taste of some citrus fruit juices, such as grapefruit, in the beverage industry. Naringin (a flavonoid that gives such flavour to the juice) is hydrolysed by naringinase to naringenin, glucose, and rhamnose. Despite its usefulness, the huge manufacturing cost of naringinase restricts its use on an industrial scale. Busto et al. (2007) obtained biocatalytically active beads of naringinase from *Aspergillus niger* by entrapping the enzyme in a polymeric matrix consisting of poly (vinyl alcohol) hydrogel, which was cryostructured in liquid nitrogen. Due to this strategy, the optimum temperature of naringinase derivative raised from 60 up to 70 °C. This experiment proves that increased operational stability of an enzyme can be provided by the stabilization of its tertiary structure (Busto et al. 2007).

Puri and co-workers used wood chips, an unusual immobilization support, to describe an exceptional rate of hydrolysis using naringinase (but still with inhibition by-products) (Puri et al. 2005). Wood chips, activated with glutaraldehyde, was used to covalently immobilize the naringinase obtained from *Penicillium* sp. Studies showed that when a small amount (10 UI) of the immobilized enzyme (with 120% of recovered activity) was incubated with freshly prepared kinnow mandarin juice (50 mL), a maximum of 76% hydrolysis of naringin was obtained in 1 h. The enzyme preparation also exhibited a more acidic optimum pH (from 4.5 to 3), which

is favourable for this purpose because the juices to be treated with the enzyme are quite acidic (Puri et al. 2005). Lei et al. (2011) reported another example of improved enzyme activity due to an immobilization method. They demonstrated the immobilization of naringinase obtained from *P. decumbens* on mesoporous silica MCM-41 activated with glutaraldehyde used to debitter white grapefruit, which yielded a naringin conversion on the juice of up to 95%.

β-Glucosidase β-Glucosidases (EC 3.2.1.21) are enzymes that catalyse the hydrolysis of the glycosidic bonds to terminal non-reducing residues in beta-D-glucosides and oligosaccharides, converting them to glucose. Increasing aroma represents the most valuable application of glucosidases (Romo-Sánchez et al. 2014). β-Glucosidases are generally used to enhance the aroma of tea. Among more than 500 kinds of tea aroma constituents, the floral aroma of tea is largely contributed by monoterpene alcohols (i.e. linalool and geraniol) and aromatic alcohols (i.e. benzyl alcohol and 2-phenylethanol) (Wang et al. 2001). These aroma compounds are present in fresh leaves of tea plants as flavourless monosaccharide or disaccharide glycoside precursors (β-D-glucopyranosides), which can be hydrolysed by endogenous enzymes, such as β-D-glycosidase to release free aroma constituents (Ogawa et al. 1995; Su et al. 2010). However, the limitations of glycosidases from tea plant exhibit low activity under natural conditions, and they can be destroyed to a huge degree during the tea manufacturing process. Hence, most of the glycoside precursors will remain in the final tea products, and there are big potentialities to improve the aroma quality of tea by exogenous glycosidases. The cross-linking–entrapment–cross-linking immobilization on an alginate of a commercial *β*-glucosidase resulted in a stable enzyme preparation, which was successfully used to treat the total amount of essential oil in green tea, oolong tea, and black tea increasing it by 20.7%, 10.3%, and 6.8%, respectively. This engineered biocatalyst has been repeatedly used 50 times, maintaining a final residual activity of about 93.6% compared to its initial activity (Su et al. 2010).

Lipases Lipases (EC 3.1.1.-) are enzymes that catalyse the hydrolysis of fats and lipids. Lipases are extensively employed for a plethora of applications in food and feed industries (Hasan et al. 2006; Jaeger and Eggert 2002). Among them, two of the most valuable examples include the preparation of functional ingredients in general and omega-3 fatty acids in particular. The omega-3 fatty acids derived from fish oils, mainly consisting of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are highly beneficial to health (Swanson et al. 2012). DHA is required in large amounts in the brain and retina, in the early stages of life, as a physiologically essential nutrient to provide optimal neuronal functioning (learning ability and mental development) and visual acuity (Heird 2001), while EPA is considered to have beneficial effects in the prevention of cardiovascular diseases in adults (Saremi and Arora 2009). Considering these facts, the preparation of triglycerides enriched in both of the omega-3 acids (DHA and EPA) or even in only one of them could be very attractive. The first crucial step for the production of triglycerides of omega-3 is the rapid and selective release of PUFAs from fish oils. Lipases immobilized on porous supports have been extensively described for the hydrolysis of fish oils

(DiCosimo et al. 2013). However, as a main interference, immobilized lipases cannot undergo interfacial activation via interaction with oil or solvent interfaces because oil drops are unable to penetrate inside the porous structure of the catalyst. The lack of interfacial activation can be compensated by promoting the activation of lipase during its immobilization. An innovative methodology was reported by Filice and Marciello (2013), expecting the hyperactivation of *Rhizomucor miehei* lipase (RML) through different concentrations of different detergents and the subsequent immobilization of the most active open form via intense multipoint anion exchange. RML immobilized inside porous supports by means of this strategy becomes highly stable, very active, and selective for the mild hydrolysis of fish oils with EPA production without interaction with interfaces and of aggregation phenomena. In this way, interfacial activation of soluble lipases on oil drops is not really necessary because a similar activation can be achieved via a careful non-natural hyperactivation of the immobilized enzyme.

Another strategy to enhance the production level of an enzyme is by presenting more copies of the relevant gene into the concerned organism. For instance, the penicillin-G-oxidase-encoding gene from *E. coli* was integrated into the vector pBR322 and then the recombinant plasmid was introduced into *E. coli*. The recombinant strain produced considerably higher quantities of the enzyme than their original/parent strain, thereby reducing the production costs and also enhancing the purity of the enzyme. The applications of recombinant DNA technology are considered to be much more advantageous as well as diverse than that for modifying amino acid sequences of enzymes. Such enhanced yields are economically significant due to enhanced volumetric productivity and reduced downstream processing costs, and also due to the fact that the crude enzymes are more pure. Robas et al. (1993) used the same principle to enhance the activity of penicillin-G-amidase in *E. coli*.

Many of the commercially available enzymes are produced by genetically modified microorganisms (GMMs) (Pedersen et al. 1995), which provide enzymes with higher specificity and purity. Because of an efficient separation process, the GMMs are removed completely from the final enzyme product. Novozymes uses the strategy of GMMs for marketing a wide range of enzymes for various industrial purposes. Several enzyme preparations made by GMMs are currently used in the beverage industry. NovoShape™, containing a pure pectin esterase, helps retain the original shape and structure of individual fruit pieces during processing and thereby offers a finished product that is more appealing (http://www.novozymes.com). Pectinex® SMASH, containing a variety of different pectinases, is used for treating apple and pear mash for higher yield and capacity (http://www.novozymes.com).

6.5 Challenges in Enzyme Engineering

Enzyme engineering has developed as an essential mechanism to overcome the constraints of using native enzymes as biocatalysts. Latest advances in enzyme engineering have mainly aimed at applying directed evolution to enzymes, especially important for organic synthesis, such as monooxygenases, ketoreductases, lipases, or aldolases in order to improve their activity, enantioselectivity, and stability.

Protein engineering via directed evolution or rational design has developed as a very powerful mechanism to design and modify the properties of enzymes. The use of computational tools in combination with directed evolution and rational protein design is becoming more and more important in order to explore enzyme sequence space and to develop modified or unique enzymes. The advancements in these areas should support in expanding the application of microbial enzymes in industry.

This engineering technology rapidly found its use for a wide range of proteins, especially in the field of biocatalysis. The application of directed evolution has been made more easy and feasible with the development of a diverse set of molecular biology tools to create well-balanced mutant libraries as well as suitable high-throughput screening methods. Directed evolution has developed as a standard methodology in protein engineering, which can be used complementarily or in combination with rational protein design to meet the various demands for industrially applicable biocatalysts.

The technology of enzyme engineering has enormous, virtually limitless, potential. But the realization of this potential is limited by several factors. An awareness of the three-dimensional structure of an enzyme provides the basis for deciding the changes to be made in the amino acid sequence of the enzyme. Data on this aspect of many enzymes are not available at present; this limits the application of enzyme engineering to such enzymes. At present, the knowledge of the exact interactions, viz., hydrogen bonding, electrostatic interactions, and hydrophobic interactions, between the active site of an enzyme and the substrate of this enzyme is, at best, incomplete. This knowledge is essential for the prediction of the effect of a change in a specific amino acid of the active site of an enzyme. Various factors that determine the stability of proteins are not well known. But salt bridges and other electrostatic interactions are known to confer thermostability. Therefore, many enzyme engineering experiments are designed to generate these data, which are then used to plan further experiments.

As the basis of protein engineering is established well, eventually protein engineering will shift towards rational design. Identification of the best protein engineering strategy will help in the advancement of protein engineering more quickly. If we compare different strategies, solutions can be attained with less effort. It is unlikely that one strategy will prevail, as each problem differs in its objectives, amount of information available, and specificities of the protein. Comparison of different strategies will also help in establishing fundamentals of protein engineering and enhancing our knowledge of enzyme mechanism. This knowledge will make rational design more reliable and further speed up the path to solutions.

Regardless of the advances in the field of enzyme engineering, major challenges still prevail to harnessing the benefits of biocatalysis completely. Even though the field of enzyme engineering has advanced much rapid than a decade ago, mutating 30–40 amino acids and screening tens of thousands of samples still need an enormous research team. Most of the engineering strategies yield improved variants and help to find them quicker, but decision on the selection of better strategies still remains unclear. Direct comparison of different strategies for similar problems and examination of assumptions behind different strategies will help in identifying the most powerful or productive ones.

Even though a large database is available for sequence–structure correlations, which grows expeditiously along with advanced softwares, currently it is inadequate to predict three-dimensional changes arising as a result of various substitutions. The major hurdle in engineering is in evaluating the overall effects on the new structure. Hence, protein engineering is currently considered a random process which may be utilized with only little realistic possibility for immediate success. Probably quite small alteration in sequence may bring about considerable conformational changes and even affect the rate-determining step in the enzymatic catalysis. Nevertheless, it is reasonable to expect that, if an adequately accurate database plus appropriate software are provided, the relative possibility of success will boost in the future, and the products of protein engineering will provide a major impact on enzyme technology.

Considerable effort has been spent on engineering more thermophilic enzymes. The enzymes from thermophiles are usually only 20–30 kJ more stable than their mesophilic counterparts. The thermophilic property can be attained by the inclusion of few extra hydrogen bonds, an internal salt link, or extra internal hydrophobic residues, giving a slightly more hydrophobic core. All of these modifications are less enough to be accomplished by protein engineering. The secondary structure of the enzyme must be conserved, in order to establish a more predictable outcome. Wherever allowable, small increases in the interior hydrophobicity may also increase the thermostability by substituting interior glycine or serine residues with alanine. It should be understood that creating an enzyme more thermostable reduces its overall flexibility and, hence, it is possible that the artificial enzyme thus produced will have decreased catalytic potential.

In the food and feed industry, the integration of enzymes has been a strong approach, but it is clear that dedicated research efforts should be constantly provided to make this application of biological agents more effective and/or diversified. In spite of the success obtained in the field of enzyme engineering, there is a deficiency of a set of universally applicable rules, including both technical and economic requirements. However, it can be anticipated that attempts will be made towards developing novel immobilized enzymes with relevant physical, chemical, and geometric features that can be used in various reactor configurations and that respond to the economic requirements for large-scale utilization.

According to Behe and Snoke (2004), when the desired modification involves a concurrent alteration in several amino acids, it is not expected to be achieved by the random approach. In several cases, the better option is a combination of approaches to develop the necessary structure or function and its improvement by random techniques. Park et al. (2006) modified a metallohydrolase through designed deletion and insertion of various structural loops in the active site to form a novel enzyme with a diverse catalytic function and then applied random techniques to enhance the designed activity. Bloom et al. (2006) established that the stability of the protein scaffold enhances its evolvability. Specifically, when the stability of a protein scaffold is increased, it may be desirable to make mutations that create a truly new property. This approach can be very hopeful in finding new enzymatic solutions that otherwise can be missed in the functional screening.

6.6 Conclusions

The era of 'protein engineering' started in the early 1980s. The period of 35 years of protein engineering has culminated in a remarkable collection of genetic engineering and computational tools as well as several concrete outcomes in altering and improving the characteristics of enzymes. On the one hand, we have learned to recognize the phenomenon of functional proteins in the sequence space and, on the other hand, the large diverse potential of biotechnologically relevant protein functions. We only need to develop strategies to excavate them out. We know superficially in what way or manner a huge number of life-like proteins look like; nonetheless, we are still far from understanding how life-like proteins are designed.

In spite of the advancements in the field of enzyme engineering, we still lack a general theory on how a sequence generates a specific structure and how a structure resolves its function. Enzyme engineering has an important function in the three main fields of chemical biotechnology: metabolic engineering, single-step biocatalysis, and enzymatic cascades. It not only represents a mechanism for process optimization but is also essential for establishing successful strategies for developing modified enzymes. It has helped in realizing new and efficient pathways and opened up entry to a variety of diverse products. Enzyme engineering has a great influence in biocatalysis and will remain to do so in the future as it promotes the catalysis of novel non-physiological reactions, the design of innovative pathways, the synthesis of novel products, and the optimization of the required processes. Furthermore, supplementary methods like the de novo design of enzymes and the utilization of catalytic promiscuity will help to fight against one of the greatest defect of directed evolution approaches: the missing initial activity. Even though previously reported de novo enzymes exhibited very limited catalytic features, Hilvert and co-workers demonstrated that their activities could be greatly enhanced through enzyme engineering (Hilvert 2001). The impact of enzyme engineering and de novo design, particularly in combination, will be responsible for the progress of unique reactions and will establish new chemoenzymatic and biosynthetic strategies.

Major advances in the field of DNA technologies and in bioinformatics over the past decade have contributed critical assistance to the field of biocatalysis. The exploration of novel enzymes in natural resources was promoted by these tools, and they have also extensively accelerated the redesign of the existing biocatalysts. Next-generation DNA sequencing technology (NGS) has allowed sequence analysis on an enormous scale and at greatly lowered cost. Isolation of genomic DNA has become the first step for protein engineering due to the low-cost in DNA synthesis. Whole-genome DNA synthesis favours the codons to be optimized for the host organism and gene structures such as promoters, terminators, enhancers, and restriction sites to be inserted at convenient sites.

Bioinformatics tools have developed into an indispensable part of modern protein engineering accompanied with the experimental advances (Bornscheuer et al. 2012). Genes with similar catalytic activities have been identified using multiple sequence alignments across immense enzyme families and homology searches, leading to novel, potent biocatalysts (Hohne et al. 2010). Multiple sequence alignments help in

identifying the consensus sequences and amino acid substitutions that generate stable enzymes. These data can be used to design small libraries with a huge percentage of catalytically active variants, which have been used to discover enzymes with highly efficient biocatalytic properties (Jochens and Bornscheuer 2010).

Multiple amino acid substitutions are usually required for large changes in enzyme properties. More and more powerful screening could be considered as the simplest solution to this problem. High-throughput methods like fluorescence-activated cell sorting, which are capable of screening tens of millions of variants in a short time, can be used to monitor the alterations in substrate specificity (Becker et al. 2008; Fernandez-Alvaro et al. 2011).

As of now, the perfect way to create multiple mutations is to add them simultaneously, but to limit the choices using statistical or bioinformatics methods. Jochens and Bornscheuer (2010) used this method to enhance the enantioselectivity of a *Pseudomonas fluorescens* esterase. Weinreich and co-workers (2006) studied the effectiveness of cooperative interactions (mutations) in the development of a β-lactamase with advanced properties. In this study, the reaction rate was enhanced by mutation A, but this mutation destabilized the β-lactamase. The overall effect was slightly advantageous, whereas the reaction rate remained unaffected by mutation B but stabilized the β-lactamase; by itself, it had no effect. In combination, mutations A and B were greatly advantageous because the β-lactamase showed higher reaction rate and preserved its stability, but addition of mutations stepwise will most probably miss these kinds of synergism.

The integration of enzymes with nanomaterials and in complex multi-enzyme assemblies holds potentials for the future (McDonald et al. 2007). Enzyme immobilization strategy was in use since the early days of biocatalysis, but it may be more efficient when the biocatalyst's surface orientation is controlled. Future protein engineering has to focus on the challenges that emerge through the interfacing of individual biocatalysts with other proteins in a metabolic pathway or support matrices.

'Peptidomimetics' is another essential approach that finds utilization in protein engineering and is considered as an important method for medical and bioorganic chemistry. It involves imitating or hindering the activity of enzymes or natural peptides upon design and synthesis of peptide analogues that are metabolically stable. Peptidomimetics is considered as an important approach for medical and bioorganic chemistry. A variety of synthesis methods such as the use of a common intermediate, solid-phase synthesis, and combinatorial approaches are used in peptidomimetics (Trabocchi and Guarna 2014). Protein engineering also uses another technique called 'flow cytometry', which is an effective approach for single-cell analysis (Mattanovich and Borth 2006; Wittrup 2001).

Another valuable protein engineering approach which is utilized in remodelling enzyme properties and function is the 'designed divergent evolution', whose methodology works based on the theories of divergent molecular evolution. According to the theory of divergent evolution, initially enzymes with more specific functions have evolved from those enzymes with promiscuous functions. Secondly, this process is driven by a few amino acid substitutions; and finally, the effects of double/multiple mutations are usually additive. Thus, this approach permits the selection of

combinations of mutations that would provide the introduction of desired functions into the enzymes (Yoshikuni and Keasling 2007; Yoshikuni et al. 2006).

Less stability and inferior activity towards unusual substrates were considered as the weaknesses of biocatalysts; these issues can be solved by efficient utilization of various protein engineering methods. Previously, in order to compensate for less activity, large amounts of protein were used, which caused emulsions that hindered reaction and reduced the yield. Highly active enzymes solve this problem because emulsions do not form using smaller amounts of protein. Modified enzymes with extended shelf life, stability in organic solvents, and good activity should promote biocatalysis to spread further into industrial laboratories. Recent advancement in protein engineering has attained the equivalent of converting mouse proteins into human proteins. The amino acid sequences of similar proteins in mice and human typically differ by approximately 13% (Consortium 2002). Modern advanced protein engineering methods create similar changes in converting a wild-type enzyme into an enzyme suitable for chemical process applications.

In recent years, the catalytic properties of the enzymes have enhanced quantitatively by factors of thousands to millions, and the engineered enzymes have the capability to act in unusually harsh conditions. In order to contribute more expeditious advancement in the field of protein engineering, excellent knowledge of how protein structure influences protein properties and a crucial evaluation of the many protein engineering approaches are needed.

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Part II

Anti Cancer and Anti-inflammatory Molecules

Anti Cancer Agents from Microbes

7

Vipin Mohan Dan and Rahul Sanawar

Abstract

Microbial diversity has a pivotal role in discovering antibiotics and anticancer agents. Anticancer drug discovery has seen a lot of technological development in the last few years. Screening for cancer drugs has moved forward from the traditional cell-based screening that looks for antiproliferative effects to specific approach to scan for molecules that can target prominent proteins or pathways in cancer. These employed technologies will help to find molecules selective for cancer cells while avoiding normal cells, thus improving efficacy and selectivity in cancer therapy. Microbial diversity has a lot to offer in terms of drug discovery. Among the different groups of microorganisms, members of actinomycetes have provided more number of anticancer compounds and other bioactive metabolites. Symbiotic microorganisms associated with higher marine organisms are also identified as a potential source of novel anticancer lead compounds. Metagenomic approaches to screen uncultivable microorganisms also offer a novel source for the invention of new therapeutic metabolites.

Keywords

Cancer • Microbes • Streptomyces • Anticancer

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7.1 Introduction

Microbes form the largest community of living organisms on the face of earth. They are present in a wide range of places, from the gastrointestinal tract of animals to the deepest sea vents, where complex life forms cease to exist. Presently, most of the well-known commercial antibiotics, anticancer drugs, and industrial enzymes are from microbes. Microorganisms have a genetic makeup that encodes a wide variety of structurally diverse secondary metabolites which has led to the discovery of many compounds of therapeutic potential. Work on microbes has fascinated researchers for decades, and this fascination has only intensified in the recent years, with evolving modern technologies taking the front seat in uncovering novel microbes and their attributes. Microbes are omnipresent and can survive in a wide range of environmental conditions and also give a helping hand to other organisms to adapt to the stress in an environment in which they establish. This belief has surged the interest in search for novel microbes in every corner on the face of earth and the positive outcomes of such research studies have boosted this expedition to continue for the many decades that are in store. Microbes are known to evolve more efficiently than any other organism according to the demands of the environment or in sync with the environmental stress factors. The evolution of modern medical world and bio-based industries has a close association with the unicellular dynasty. History stands proof that microbes have played a vital role in eradicating and controlling many human ailments and, at the same time, touching other arenas of agricultural and industrial importance. Biosynthesis of numerous valuable pharmaceutical molecules, ranging from antibiotics; antitumor, anticancer, antiviral, antihypertensive, antiparasitic, antidiabetic, antioxidant, and immunological agents; enzyme inhibitors; and hormones, now aids microbes as the smallest unit of factories for mass production (Jeandet et al. 2013).

The search for drugs began late in the nineteenth century with the growing acceptance of the germ theory of disease. This has resulted in scientists putting a lot of time and effort into searching for drugs that would kill pathogenic bacteria. Alexander Fleming pioneered drug discovery from microbes way back in 1928, when his Petri plate with *Staphylococcus aureus* was contaminated with a mold leading to the inhibition of growth of the bacteria (Fleming 1929). The mold responsible for the action was *Penicillium notatum* which produced the component penicillin, which was later isolated, produced commercially, and was used as an antibacterial agent during World War II. This work generated interest in naturally occurring substances, and research focused on finding more novel molecules was initiated. In the early phase of microbial secondary-metabolite research, much focus was put on to discover antimicrobials and enzymes. Later on, research organizations and pharmaceutical industries extended their search beyond antifungal, antibacterial, and antiviral agents and looked for therapeutic compounds that have value in other clinically relevant diseases (Cardenas et al. 1998; Schwartsmann et al. 2002). The horizon of microbial research spread out in search of immunosuppressants, anticancer drugs, enzyme inhibitors, insecticides, antiparasitics, and other pharmacologically relevant agents.

Cancer is a complex disease that has roots from different causes put together. To understand the biochemistry of cancer involves studying various factors starting from the external environment to the intracellular network of biochemical pathways involved. The disease has shown an increasing incidence with changing lifestyle, food habits, and environmental threats including global warming. The battle against cancer is weakening with the lack of number of potential drugs in clinical use. Many of the drugs that are in clinical use either have varying degree of side effects or are ineffective due to the surging problem of multidrug resistance property possessed by the cancer. Natural products obtained from either plant or microbes have been known to aid cancer treatment in the past decades.

Most of the anticancer drugs drive cancer cells into one of the modes of programmed cell death (PCD) namely apoptosis, autophagy, necroptosis, or a programmed mode of necrosis. It is necessary to understand the mechanism employed by the anticancer drug in order to analyze the outcomes and possible side effects caused. Apoptosis is a cascade of enzymatic events which leads to programmed cell suicide. Apoptosis is also involved in normal development and aging, and it maintains cell population in tissues by selectively removing damaged or stressed cells. This mode of cell death is also employed as a defense mechanism in the event of cell damage by disease or other toxic agents (Norbury and Hickson 2001). Cancer cells equip themselves with highly complex mechanism to avoid PCD and thus evade apoptosis by down-regulating or up-regulating expression of proteins required for the cancer-cell sustainment and proliferation. Induction of apoptosis can be initiated by a range of stimuli or combination of physiological and pathological conditions. Most of the anticancer drug treatment leads to DNA damage, based on the type of cell being treated; apoptosis; or other forms of cell death. Apoptosis involves a group of enzymes called caspases that carry out the cell death process. Apoptosis leads to series of morphological changes, during the initial phase, and the caspase-dependent mechanism puts the last nail on the coffin of the cancer cell.

Autophagy is another well-defined process that employs a self-degradative mechanism that plays important role in the event of development and nutrient stress. Autophagy also helps in the eviction of misfolded or damaged proteins, damaged organelles, and removal of intracellular pathogens. Autophagy is considered a cell-survival mechanism, but it is also linked to nonapoptotic cell death. There are around 32 different autophagy-related genes identified through genetic screening in yeast, and most of these genes are conserved in plants and mammals (Nakatogawa et al. 2009). Necrosis, which was initially believed by researchers to be a passive form of cell death, was later researched and identified to be programmed mechanism of cell death (Degterev et al. 2005). Some of the cellular protein molecules like TNFα, FasL, and TRAIL are known to be involved in induction of apoptosis and are also linked to necrosis.

7.2 Anticancer Drugs from Marine Microorganisms

Drug discovery for cancer has been vastly based on hunting potential molecules from terrestrial organisms, from microbes to plants. Many of these compounds and their derived forms from terrestrial origin are in clinical use or undergoing clinical

trials or in preclinical stage. A clear look into the list of these compounds shows an alarming fact that the marine compounds are largely missing in the present pharmacopoeia. The ongoing research has increased confidence in scientists that marine environment can hold a treasure of compounds that can replace the terrestrial compounds used in clinics. As marine environments occupy 95% of the biosphere, it gives a clear indication of the treasure of compounds waiting to be discovered (Jimeno et al. 2004). Even though the marine world has an enormous microbial diversity, it is surprising that nearly 60% of the antibiotics in commercial production are from cultivable soil microorganisms. Most of the marine microbes are not friendly to traditional methods of culturing, and the few cultivable ones have laborious culturing methods, restricting the study of marine microbes.

During the evolution history of 3.5 billion years, of microorganisms, a wide array of biosynthesis mechanisms has evolved and given rise to diversity of compounds in terrestrial organisms. The same diversity can be expected from the marine ecosystem. Marine organisms have adapted to extreme environmental conditions of salinity, pressure, and temperature. These extreme conditions in the marine environment enable the organism to explore novel pathways to produce unique compounds with varying structural and functional characteristics (Kathiresan et al. 2008). The future of these marine-based compounds as therapeutic agents is still in the cradle stage, as they require further validation through clinical studies to reach clinics. There are also problems related to the collection and processing of marine samples which have led to retardation of work in this area. The marine microbial flora encompasses a large diversity of bacteria, actinobacteria, cyanobacteria, and fungi. The marine area in the form of ocean covers 71% of the earth, and the rich biodiversity has more than 90% contributed by microflora and microalgae with respective to ocean biomass (Kathiresan and Duraiswamy 2005). In the late 1960s, researchers began exploring the marine ecosystem in search of novel compounds of therapeutic importance. A systemic approach to deal with isolation of marine compounds came into being in the mid-1970s. The years between 1977 and 1978 saw a rise in discovery of novel compounds with therapeutic potential from marine flora, with the discovery of over 2500 new compounds with varying structures and functions.

Bryostatins, sarcodictyin, discodermolide, and eleutherobin are some of the anticancer compounds derived from marine bacteria. Marine animal phyla are known to produce toxins, and research has revealed that symbiotic bacteria living in association with these animals are responsible for toxin production (Simidu et al. 1990; Kodama et al. 1988). *Noctiluca scintillans* has association of a symbiotic bacteria that produces the metabolite, macrolactin-A. This compound has the capability to suppress B16-F10 murine melanoma cancer. Macrolactin-A also protects T lymphocytes against the attack and reproduction of human immunodeficiency virus (HIV) (Carte 1966). Kahalalide F (KF) is a potent antitumor agent isolated from marine organism, namely Hawaiian herbivorous marine mollusk *Elysia rufescens* and alga *Bryopsis*. *E. rufescens* uses this compound as a deterrent against fish predators (Becerro et al. 2001). Initial studies investigating the possible symbiotic bacteria responsible for the production of KF have brought forward two probable candidates, two strains of *Vibrio* species. Liquid chromatography-mass

spectroscopy and nuclear magnetic resonance (NMR) confirmed the production of the compound by these bacterial strains (Hill et al. 2007). Research studies to analyze the production in vitro from cultured strains of *Vibrio* sp. failed to give result. Thus more research is yet to be done to finalize the bacterial symbiont that harbors the synthesis pathway for production of KF. The mechanism of action of the compound remains to be elucidated.

Bryostatins are cyclic polyketides produced by the bacterial symbiont that lives in association with the bryozoan *Bugula neritina* (Hale et al. 2002). All compounds in the bryostatin family have macrolactone core complexes with three tetrahydropyran rings. The bryostatins can be differentiated among themselves based on the substituents at positions C-7 and C-20 and depending on the presence of a γ-lactone ring linked to C-19 and C-23 tetrahydropyran ring. The initial discovery of byrostatins was based on an anticancer-based bioassay fractionation. Among the bryostatins, bryostatin 1 is the most scientifically investigated and have been in clinical trials. Bryostatin 1 is significant for the presence of a hydrophobic alkyl chain at C-20. The compound associates with protein kinase C (PKC) at the regulatory domain by specifically binding to the binding site of diacylglycerol (Nelson and Alkon 2009). Short-time duration treatment by the compound leads to activation of PKC, while long-time exposure induces prominent down regulation of PKC. In vitro study in a range of cancer cell lines has shown to block proliferation leading to apoptosis. Preclinical studies have revealed that bryostatin 1 has the property of efficiently increasing the effects of chemotherapy. The compound has produced significant results when combined with standard chemotherapy thereby showing promise as a candidate for further study in cancer treatment (Kortmansky and Schwartz 2003). Research studies have shown that the compound can enhance the sensitivity of cancer cells to cisplatin. Cisplatin is a major antitumor drug that is employed as a chemotherapeutic agent in the treatment of various cancers, including cervical, ovarian, and small-cell lung cancers (Alberts et al. 1991). Cisplatin therapy is affected by tumor cell resistance, and PKC plays a significant role in cisplastin sensitivity. It is at this juncture where bryostatin can influence the action of PKC (Basu and Lazo 1992).

Actinomycetes placed under the phylum actinobacteria are known for their filamentous nature and comes under the gram-positive bacteria (Ventura et al. 2007). Actinomycetes follow a complex life cycle and remains well adapted across various ecosystems. Nearly 23,000 bioactive secondary metabolites from microbes were discovered by research investigations, and among these, 10,000 are sourced from actinomycetes alone, accounting to 45% of all the secondary metabolites of microbial origin (Berdy 2005). Thus, this group forms a treasure house of bioactive compounds. The group has members that produce well-known anticancer drugs like aclarubicin, doxorubicin, bleomycin, actinomycin D, pentostatin, mitomycins, and many others (Olano et al. 2009; Newman and Cragg 2007). Polyketide-gene-derived secondary metabolites with antitumor activity are well known within the *Streptomyces* species.The polyketide gene family has a array of enzymes like ketoreductase, dehydratase and enoylreductase that works on the polyketide carbon skeleton resulting in the end product secondary metabolite (Gokhale et al. 2007).

Arenicolide is a type I polyketide produced by the marine actinomycetes *Salinispora arenicola*. The microbe was isolated from marine sediment samples collected from the island of Guam. At an IC 50 touching 30ug/mL the compound arenicolide A showed cytotoxicity toward human colon adenocarcinoma cell line HCT-116 (Williams et al. 2007). Saliniketal A and B are two other polyketides sourced from the same strain of actinomycetes. Saliniketal inhibits the expression of ornithine decarboxylase (ODC). ODC is highly expressed in many cancers and remains as a major focus for chemoprevention of cancer (Gerner and Meyskens 2004). Chalcomycin is a macrolide with antitumor potential produced by *Streptomyces* sp. M491 isolated from samples collected from Qingdao, coastal region of China. Compounds similar to Chalcomycin were isolated from another *Streptomyces* strain B7064 found from mangrove sediments in Hawaii (Asolkar et al. 2002).

7.3 Antitumor Agents and Mechanism of Action

Knowledge of the mechanism undertaken by an agent to kill cancer cells will help in getting a picture of the possible events that will undergo after the therapy is started. It will also help in allowing structural modification of the drug, thereby increasing specificity, efficiency, and safety. The mechanism of action employed by the drug will pave way for understanding the reasons as to why a particular side effect has occurred in response to a drug. Combinational chemotherapies will be successful when two drugs can complement each other's drawbacks, but for a successful combination the action-mechanism of both drugs should be well researched to avoid any adverse effects.

Actinomycin D was the first and oldest microbial metabolite to be approved for treatment of cancer. Actinomycin A is a close relative of actinomycin D; it was isolated from *Streptomyces antibioticus.* Actinomycin has a cyclic polypeptide structure that helps it to selectively bind DNA and block transcription (Reich and Goldberg 1964; Goldberg et al. 1962). This transcription-inhibiting property helps by interfering with the function of RNA polymerase. In particular, transcription at ribosomal level is found to be most sensitive to the action of actinomycin (Perry 1963). The initial x-ray crystallography study of actinomycin-deoxyguanosine complex stated a possible three-dimensional structure of actinomycin binding to DNA. Based on this study, it was highly believed that the phenoxazone ring present in actinomycin positions into the space between adjacent base pairs and the pentapeptide chain in the B-helix narrow groove. The formation of hydrogen bonds takes place with guanine residues in the opposite chain (Sobell et al. 1971). It was later proposed by the same group (Sobell and group) with some modifications to the initial three-dimensional structure that actinomycin complexes with a premelted β-DNA conformation that is present at the transcriptional complex. The complex is not aptly twofold symmetric, and due to this, the compound binds firmly to a particular guanine molecule thereby staying more firmly to one chain when compared to the opposite chain. Thus the new proposed modification clarifies that actinomycin binds to β-DNA in a completely different structural form but not with B-DNA. The author clarified that β-DNA behaves as an imperious structural intermediate that is metastable in DNA melting. This understanding of β-DNA has concreted the action mechanism of actinomycin.

Perylenequinones are placed under second generation of cellular photosensitizer, which are highly valued as potential therapeutic agents in cancer treatment. This class of compounds possesses a wide range of attributes like good solubility, dark inactivation, accessible to chemicals modifications, and various others, making it a good candidate for safe clinical use (Beck et al. 1999; Diwu and Lown 1993). Calphostin C is a PKC-inhibiting lipophilic perylenequinone isolated from the fungus *Cladosporium cladosporioides*. Calphostin C specifically and selectively abrogates the function of PKC in a photodependant manner (Bruns et al. 1991). Lamin B, a component of nuclear lamina, the fibrous region lining the inner areas of nuclear envelope, is also a target of calphostin C. In the event of illumination, calphostin travels to cytoplasm and concentrates mainly in the endoplasmic reticulum and Golgi apparatus leading to stress (Kaul and Maltese 2009). The activated calphostin, owing to its lipophilicity (ability to dissolve in fats), gets accumulated in the endoplasmic-reticulum-derived cell nuclear envelope but does not gain a direct entry into nucleus. Investigations have revealed that in the first initial 30 min of illumination, the compound in its active form triggers a progressive decline in lamin B, which from thereon will completely disappear during the subsequent incubation in dark for 30 min. In the 30 min of illumination, the activated calphostin in the cytoplasm will lead to decrease in activity of different cytoplasmic PKC forms (Dal Pra et al. 2000; Chiarini et al. 2006). At this stage, there will be no initiation of apoptogenesis due to caspase activation or exit of cytochrome c from mitochondria. In the event of transfer of cells into dark, the destruction of lamin B1 proceeds to end. The light-restrained activities of cytoplasmic PKC will regain its functional activity in the dark. This resurrection of PKC activities will initiate the first steps of apoptogenesis, leading to mitochondrial cytochrome c release. Caspase activation will lead to full cellular action, leading to PCD, and roughly more than 90% of the cells die within duration of 3.5 h after the starting of dark phase. These results ensure that the light-activated calphostin is necessary for PKC-related actions to take place in dark and further ensuing in PCD (Chiarini et al. 2006). The presence of activated calphostin in the cytoplasm leads to intranuclear aggregation of aqueous peroxidase that causes the selective degradation of nuclear envelope (lamin B1).

Target of rapamycin (TOR) is a combination of two structurally separate serine/threonine kinase enzyme termed TOR complex 1 and TOR complex 2. This enzyme complex is evolutionary conserved from yeast to mammals, and the mammalian counterpart is called mammalian target of rapamycin (mTOR) (Wullschleger et al. 2006). The components of mTOR complex have distinct functions with mTORC1 assisting in cell growth through control of protein anabolism, nucleotide biosynthesis, autophagy, glycolysis, and lipogenesis. The mTORC2 is involved in actin cytoskeleton arrangement, glucose metabolism, and lipogenesis (Betz and Hall 2013). The TOR complex came to light on elucidating the mechanism of action of TOR inhibitor called rapamycin. Rapamycin is a complex macrolide which is known for its wide range of roles as antitumor agent, immunosuppressant, and fungicide with its high specificity to mTOR. Rapamycin was isolated from *S. hygroscopicus* from the soil samples collected from Easter Island. It is a white crystalline substance with less solubility in water and high solubility in organic solvents. Among the two components of mTOR complex, mTORC1 is the primary target of rapamycin. Rapamycin

is known to associate with the protein FKBP12 thereby forming a complex that binds to mTOR complex (Choi et al. 1996). An activated mTORC1 will activate its two downstream effectors that evoke a signal leading to translational initiation. In the mammalian system, translation is regulated via the ribosomal S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4EBP1) which are under the direct phosphorylation control of mTORC1 (Hay and Sonenberg 2004). In the protein synthesis machinery, the assembly of small ribosomal units to mRNA requires the presence of eukaryotic translational initiation factor 4F (eIF4F) complex at the 5′ end of mRNA. The eIF4F complex constitutes three components, namely eIF4E, eIF4G, and eIF4A (Pestova et al. 2001; Gebauer and Hentze 2004). The binding of eIF4E to 5′cap of mRNA leads to further binding of other components of the eIF4F complex. The association of eIF4E to other component of eIF4F will be inhibited by binding of 4E-BP1to eIF4E, thus causing blockage in translation initiation. Signal transduction-mediated activation of mTORC1 will lead to phosphorylation of 4E-BP1 that leads to its dissociation from eIF4E (Gingras et al. 1999). The phosphorylation of 4E-BP1 is regulated by a complex mechanism of events. 4E-BP1 requires phosphorylation at four sites for its dissociation from eIF4E, namely, Thr-37, Thr-46, Ser-65, and Thr-70. Phosphorylation of Thr-37 and Thr-46 is the initiating event that causes conformation change in the protein leading to exposure of the other two sites for phosphorylation (Thoreen, et al. 2009). The phosphorylation sites Thr-37 and Thr-46 are rapamycin insensitive while the sites Ser-65 and Thr-70 are rapamycin sensitive (Fingar et al. 2004). Rapamycin, at the same time, leads to complete inhibition of p70s6k. Rapamycin has no effect on the action of mTORC2, so the functions of this protein remains stable while the functions of mTORC1 are partially inhibited (Fig. 7.1).

Pentostatin is produced by *S. antibioticus* and is widely used in treatment of hairy cell leukemia (HCL). It was used as purine analogue in treatment for HCL in the early 1980s. The compound is an effective inhibitor of adenosine deaminase (Grever and Lozanski 2011). In case of HCL, it was observed that on inhibition of adenosine deaminase by pentostatin leads to accumulation of deoxyadenosine triphosphate at the intracellular level, thus causing cytotoxicity. This accumulation further causes blockage of the repair system that repairs DNA breaks. All these events lead to the activation of p53 and further cyctochome c release from mitochondria, thus driving the cell to apoptosis (Johnston 2011).

Manumycin is a compound of marine origin from the bacteria *S. parvulus*. The compound is known to inhibit Ras farnesyltransferase inhibitor from the initial microbial screening. Ras proteins are major molecules within cells that play vital role in many signal transduction pathways. The protein is involved in controlling pathways that are linked with proliferation, cytoskeletal integrity, and differentiation. Farnesyltransferase (FTase) inhibitors are potential anticancer drugs that works by inhibiting the farnesylation required for function of several cellular proteins. Manumycin functions as a competitive inhibitor of protein FTase competing with farnesylpyrophosphate and at the same time play as a noncompetitive inhibitor in relation to Ras protein (Hara et al. 1993; Yang et al. 1997). FTase is involved in the enzymatic reaction that is important for posttranslational modification of Ras and

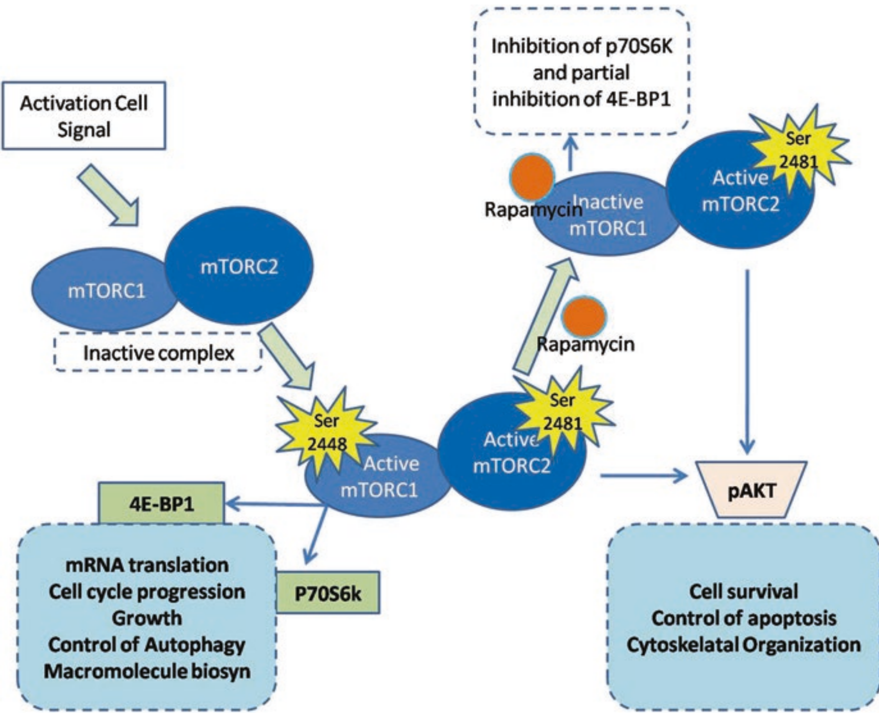

Fig. 7.1 mTOR pathway and mechanism of rapamycin action

other related cellular proteins. The reaction involves the shift of farnesyl isoprenoid group from farnesylpyrophosphate to the terminal cysteine present on the carboxyl end of destined proteins. This transfer of the franesyl isoprenoid group is important for activating the functional properties and cellular localization of the target proteins (Cox and Der 1997). The fully active and functional Ras protein transfers mitogenic signals from various cellular locations to the nucleus. Mutation in the Ras oncogene will lead to permanent activation of Ras proteins thereby leading to uncontrolled proliferation and growth, eventually leading to cancer. Ras oncogene is found to be in a mutated status in almost 20% of cancers. So blocking the process of Ras activation by FTase with FTase inhibitors like manumycin A is a promising treatment option in cancer therapy. Manumycin A has been found to have varying effects on the cell cycle based on the origin of cancer cells. Cell cycle arrest at G2/M phase was observed in case of treatment of the compound to hepatocellular carcinoma cells (HepG2) and breast cancer cells (MDAMB-231 and MCF7) (Zhou et al. 2003; Haddadin 2010). Whereas in case of colon adenocarcinoma cells (HT-29), cell cycle arrest was observed in the S phase of cell cycle (Sačková et al. 2011). The compound can trigger cancer cells to undergo apoptosis. The agent blocks Ras farnesylation of p21, thus inducing apoptosis in a time as well as concentration-dependant manner (Wang and Macaulay 1999). In HepG2 cells, the treatment leads to activation of caspases which in turn leads to cleavage of poly (ADP-ribose)

polymerase PARP, destruction of lamin B, and also fragmentation of genomic DNA (Zhou et al. 2003). Intrinsic mode of apoptosis with role played by p38 MAPK was found in ARO and KAT-4 cells (She et al. 2006).

7.4 Metagenomics: A Tool to Uncover Novel Anticancer Drugs

Microbial dynasty has been a never-ending treasure house of various biologically active compounds. Even as researchers try to understand more about microbes and their various attributes, the horizon of microbial dynasty seem to be nowhere in sight. Microbes have always been the reservoir for discovery for many types of anticancer drugs that went onto gain clinical and commercial importance. In the past decades, the contribution made by cultured microbes to the list of anticancer therapeutic agents has been enormous and many of them have found way into clinical use. Some of these that were effective as cancer chemotherapeutic drugs are doxorubicin hydrochloride, bleomycin, daunorubicin, and mitomycin (Pettit 2004). On the other hand, there are new problems in cancer therapy like multidrug resistance cancers, outbreaks of new diseases, and increasing cases of cancer incidences, both in developing and developed countries. The above-mentioned scenario places an enormous weight on the scientific community to explore newer techniques to discover novel potential anticancer molecules that could be the solution to various problems related to cancer treatment.

In this respect, research has come out with a molecular biology tool that helps in exploring the treasure of secondary metabolites of uncultured microbes through metagenomic approach. Research across globe came to the convincing conclusion that the uncultured microbial counterpart weighed more in number than the cultivable ones (Pace et al. 1985). Metagenomics involve isolation of DNA from the source sample of any origin and then delivery of the DNA into host bacteria with the help of an appropriate vector. These libraries can be screened for presence of specific antibiotic or enzyme-encoding genes (Schloss and Handelsman 2003). The tool will help in uncovering therapeutic compounds that will be more efficient than the compounds that are in clinical use presently. Polyketides are the most well-known cluster of genes that encode for production of a variety of secondary metabolites, especially antibiotics and those with antitumor properties. Due to the importance of polyketide-gene family in drug discovery from microbes, metagenomic searches has mostly concentrated on hunting natural compounds in metagenomic libraries from these gene clusters. Polyketides offers a diverse group of secondary metabolites, showcasing diversity both in terms of their structure and function. Polyketide-based compounds are known to possess pharmacologically diverse group of functions that include antimicrobial, antifungal, antiparasitic, antitumor, and agrochemical properties. The large spectrum of functional use of polyketide family of compounds keeps them as economically important, clinically significant, and of high commercial value in Industrial aspect (Cheng et al. 2003).

The discovery of new antibiotics, Turbomycin and its variants from a soil metagenomic library, showed that the potential lies with the tool in discovering

novel compounds with diverse functions, including antitumor molecules (Gillespie et al. 2002). Metagenomic libraries were effective in bringing forward pigments with antibacterial potential like violacein, indigo, and cyclic peptides like nocardamine. Pederin, an antitumor agent, was initially known to be produced from the beetle *Paederus fuscipes*. A metagenomic library developed using cosmid vectors lead to the new fact that the gene encoding the molecule pederin was from an uncultured symbiotic bacteria *Pseudomonad* (Piel et al. 2004).

Metagenomics as a tool has helped to identify some of the marine microbes that live in association with higher organisms, and these symbiotic microbes are responsible for the production of compounds that are of therapeutic value. Bryostatin, dolastatins, didemnins, and ectenaiscidins are high clinical potential anticancer compounds from marine organisms. Phylum porifera has treasured many highly potential anticancer compounds like arabinosyl cytosine, halichondrin B, and spongistatin. Recent research has brought forward that most of these marine macroorganisms provide an optimum environment for growth of selected microbial community. Metagenomic analysis has revealed that these microbial symbionts are those which encode the genes for synthesis of so-called marine compounds from higher organisms like sponge and porifera. The marine biochemical diversity is the right field for employing metagenomic tools to hunt for natural products as most of the marine microbial symbionts won’t grow in laboratory conditions.

Metagenomics has lead to the identification of novel antitumor polyketides from microbial symbionts of sponges *Theonella swinhoei*, *Pseudoceratina clavata*, and *Discodermia dissolute* (Ferrer et al. 2009). Scientific studies with help of metagenomic tools have brought up parts of the putative pederin, bryostatin, and onnamide biosynthesis genes from clones of metagenomic libraries of a beetle, bryozoan, and a sponge, respectively (Schirmer et al. 2005). The metagenomic library produced from the marine sponge *Didemnidae* helped in the discovery of a new cytotoxic cyclic peptide named patellamide. Study by Piel and others (2004) helped in isolating the gene clusters that code for production of pederin-related compounds onnamide and psymberin from bacterial symbionts of *Demospongiae* sponges. Later, Zimmermann and others (2009), studied the use of recombinant O-methyltransferase, PedO, derived from pederin biosynthetic gene cluster to site-specifically methylate mycalamide A giving the production of a derivative that showed more potential anticancer activity.

7.5 Conclusions

Research in anticancer discovery will be a never waning field as the need for novel drugs with specificity for targeted therapy is the need of modern era. Natural product research regained attention recently with the Nobel Prize in Physiology or Medicine (2015), honoring researchers who discovered drugs from plant/microbes that served the common man. Advanced methodologies and techniques have helped in sequencing bacterial genomes that has opened the gates of diverse compounds with novel structures and functional properties encoded within the basic four letters

(A,T,G,C) of life. The answer to many human aliments lie within these microbial genes; what is required is a well-planned and structured interplay between researchers from various fields like genetics, bioinformatics, chemistry, and molecular biology to tap the potential from this genomic information available.

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Cancer Combating Biomolecules From Plants

8

Anu Augustine and Geetha S. Pillai

Abstract

Nature has been a source of medicinal products for many years, with many useful drugs developed from plant sources. Plant-based systems continue to play an essential role in healthcare, and their use by different cultures has been extensively documented. Several secondary metabolites from plant sources have proved to be an excellent reservoir of new medical compounds. Many anti-cancer agents have been isolated from various plant sources. Attempts to explore new anti-cancer and other medical compounds from natural sources are progressing in various laboratories. This chapter outlines the process of carcinogenesis potential anti-cancer agents, ayurvedic concept of carcinogenesis, the 'thridoshas', the correction methods, databases of naturally occurring anti-cancer agents, chemotherapeutic and chemoprotective activities of the compounds and their molecular targets. Curcuminoids, boswellic acid, polyphenols like catechin, procyanidins, camptothecin, cannabinoids, resveratrol, diallyl disulphide, combrestatin, ashwagandha, tanshinones, polygala, and ayurvedic formulations are among the ones discussed.

Keywords

Carcinogenesis • Ayurveda • Plant-based anti-cancer compounds

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8.1 Introduction

Carcinogenesis is the conversion of normal cells to cancerous cells through many stages, which happen over many years or even decades. In the initiation stage of carcinogenesis the carcinogens react with the DNA of the cells, and blocking this stage (onset) of cancer is an important approach in cancer prevention or treatment. Promotion, the second stage of cancer, may arise slowly over a long period of time, ranging from several months to years. The third stage is the progressive stage, involving the spread of the cancer. During the initiation and promotion stages of cancer, a change in lifestyle and diet could possibly prevent development of cancer. During the progressive stage, protective factors such as diet or lifestyle do not have much impact (Reddy et al. 2003). Garlic, ginger, soya, curcumin, onion, tomatoes, cruciferous vegetables, chillies, and green tea provide protection against cancer.

According to the International Agency for Research on Cancer (IARC), in 2012 "there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) worldwide. 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions. In India, 1.02 million new cancer cases, 0.7 million cancer deaths and 1.8 million people living with cancer (within 5 years of diagnosis) in 2012" (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx).

In spite of the amount of money in billions being spent on cancer research and the availability of the best health care in the world, there is high incidence of cancer in the United States. Lifestyle seems to be one major contributing factor to cancer, evident from the high incidence of cancer among immigrants from the East to the West (Kolonel et al. 2004a).

Carcinogenesis begins with cellular transformation, progresses to hyperproliferation and inflammatory processes, and finally leads to angiogenesis and metastasis. It is a three-phase process, which includes initiation, promotion, and progression of the tumour (Berenblum 1982). Oxidative damage to DNA, proteins, and lipids, resulting from an increase in oxidative stress, is considered to be one of the most important mechanisms contributing to the development of cancer. Since cancer is a multi-step process, the preventative action of phytochemicals may result from their additive or synergistic effects. A number of mechanisms exist by which phytochemicals aid in the prevention of cancer and they may include:

(i) Anti-oxidant and free radical scavenging activity
(ii) Induction of apoptosis
(iii) Anti-proliferative activity
(iv) Cell-cycle arresting activity
(v) Enzyme inhibition
(vi) Gene regulation (Liu 2004)

The search for potential anti-cancer agents from natural products dates back to 1550 BC. Scientific research reports only started emerging in the 1960s, with

Table 1 Some anti-cancer/cytotoxic drugs developed from plant sources

Therapeutic agent/Chemical compound	Plant source	Type of cancer	References
Vinblastine, vincristine (alkaloids)	*Catharanthus roseus*	Hodgkin's disease	Mans et al. (2000)
Etoposide, teniposide (epipodophyllotoxin)	*Podophyllum peltatum*	Testicular cancer, and small cell lung carcinoma, leukemias, Lymphomas	Lee (1999) Mans et al. (2000)
Paclitaxel, docetaxel (taxanes)	*Taxus brevifolia*	Ovarian and breast carcinoma	Mans et al. (2000)
Irinotecan, topotecan, 9-aminocampothecin, 9-nitrocampothecin (alkaloids)	*Camptotheca acuminata*	Advanced colorectal cancer, also active in lung, cervix and ovarian cancer	Srivastava et al. (2005)
Homoharingtonine (alkaloids)	*Harringtonia cephalotaxus*	Various leukemias	Mans et al. (2000)
4-Ipomeanol (Pneumotoxic furan derivative)	*Ipomoea batatas*	Lung cancer	Mans et al. (2000)
Salograviolide A (sesquiterpene)	*Centaurea ainetensis*	Colon cancer	Salla et al. (2013)
Iso-eco-tanapartholide (sesquiterpene)	*Achillea falcata*	Colon cancer	Salla et al. (2013)
Betulinic acid (tri-terpene)	*Ziziphus mauritiana.*	Human melanoma	Pisha et al. (1995)
Pervilleine A (tropane alkaloid)	*Erythroxylum pervillei*	Oral cancer	Silva et al. (2001)
Sylvesterol	*Aglaia foveolata* Pannell	Prostrate cancer	Hwang et al. (2004)

investigations by Hartwell and colleagues (Pettit 1995), on the anti-cancer effect of podophyllotoxin and its derivatives. The biological and chemical diversity among natural resources promotes the discovery of novel compounds. Administration of these compounds, especially in combination with synthetic agents leads to the management and cure of human cancer. Nearly 75% of the medications being prescribed for use in cancer treatment are sourced from plants (Table 1) and approximately 74% of these were discovered from the traditional claims (Shishodia and Aggarwal 2004).

The phytochemicals that offer protection against cancer are "curcumin, genistein, resveratrol, diallyl sulfide, (S)-allyl cystein, allicin, lycopene, ellagic acid, ursolic acid, catechins, eugenol, isoeugenol, isoflavones, protease inhibitors, saponins, phytosterols, vitamin C, lutein, folic acid, beta carotene, vitamin E and flavonoids, to name but a few" (Reddy et al. 2003).

Modern day medicine uses few plant products in cancer therapy, taxol and vinca alkaloids, to name a few. Out of the 121 drugs currently in vogue for cancer therapy 90 are plant derived (Craig 1997, 1999). According to Newman et al. (2003) 48 out of 65 drugs approved for cancer treatment during a period between 1981 and 2002, were based on natural products, or mimicked natural products in one form or another. These phytochemicals, which combat disease by preventing inflammatory response, are commonly called chemotherapeutic or chemo-preventive agents.

8.2 Ayurvedic Concept of Carcinogenesis

The balanced condition of *Vata*, *Pitta*, and *Kapha* ('Thridoshas- three humors') in body, mind, and consciousness is the ayurvedic concept of health. Ayurvedic treatment restores the balance between these three systems. Charaka and Sushruta, in their compilations or "samhitas", use the terms *Granthi* and *Arbuda* for benign tumour and malignant tumour respectively (Charaka 700 BC; Susrutha 700 BC). Tumours become malignant when all three "doshas" lose mutual co-ordination, causing morbidity (Singh 2002). Several reports suggest that ayurvedic plants and their constituents have modulating effects on several therapeutic targets. However, ayurvedic drugs are yet to be validated by current scientific procedures (Aggarwal et al. 2006).

The ayurvedic approach to treatment of cancer including *Sodhana chikitsa* (detoxification) is the prime method for medical management of cancer. Internal and external purification processes include five ways of treatment, collectively named *Panchakarma chikitsa*. *Samana chikitsa* (palliative treatment) is to rectify the dosha and to cure the disease. Rejuvenative therapies restore and strengthen the patient and remove any ill effects that may have occured due to purification or cleansing. This is the step prior to therapy specific to the disease (Balachandran and Govindarajan 2005).

There are numerous pre-clinical studies with individual herbs and their derivatives and a few reports on complex herbal formulations like *Rasagenthi lehyam, Brahma rasayana*, *Semecarpus lehyam*, and *Triphala*, etc. (Joseph et al. 1999; Rekha et al. 2001; Jena et al. 2003; Naik et al. 2005).

Cancer is a highly complex disease developed over a period of 20–30 years or more, before it can be detected. Interruption of a cell-signalling pathway has been the method of cancer treatment in most cases, but multi-targeted therapy may have better chances of success. Current treatment methods for cancer concentrate at the molecular level rather than organism level (Reductionist approach). On the other hand, ayurvedic treatment for cancer is holistic, which may be preferred (Garodia et al. 2007).

8.3 Anti-tumour Phytoconstituents

The anti-tumour activity of curcumin is manifold and research evidence accumulated over the last 50 years indicates that curcumin prevents and cures cancer. The anti-cancer property of curcumin is via its ability to suppress the proliferation of a variety of tumours. Curcumin inhibits carcinogenesis of the breast, colon, liver, lung, skin, stomach, etc. and the proliferation of a wide variety of malignant cells in culture. It also promotes apoptosis by way of caspase-9 activation, cytochrome c release, caspase-3 activation, inhibition of IkappaBalpha kinase, and so on (Mukhopadhyay et al. 2001; Anto et al. 2002; Aggarwal et al. 2003, Siwak et al. 2005; Yan et al. 2005; Aggarwal et al. 2005, Bachmeier et al. 2008, 2010). John et al. (2002) found copper complexes and its derivatives to be better anti-cancer agents than the original compounds. Karikar et al. (2007) reported the cancer-related application of “nanocurcumin” (<100 nm) on pancreatic cell lines. Pre-clinical studies on the anti-cancer property of liposome-bound curcumin formulation when compared to oxaliplatin (a standard chemotherapeutic agent for colorectal cancer) showed significant apoptotic effects in vitro and in vivo (Li et al. 2007).

Jančinová et al. (2011) found that curcumin (diferuloylmethane) not only suppressed mechanisms leading to inflammation, but also resolved inflammation by apoptosis of neutrophils. Curcumin decreased phagocytotic potential in neutrophils, both in vitro and in vivo when orally administered.

Boswellic acid, the active component of *Boswellia serrata*, inhibited 5-LOX and leukocyte elastase, thereby reducing inflammation (Safayhi et al. 1992, 1994, 1995; Ammon et al. 1993; Kapil and Moza 1992). Acetyl-keto-beta-boswellic acid (AKBA), an active principle from *B. serrata*, was found to combat inflammatory diseases, including cancer. AKBA inhibits cancers of brain, colon, liver, pancreas, blood, etc. (Shao et al. 1998; Glaser et al. 1999; Jing et al. 1999; Huang et al. 2000; Winking et al. 2000; Liu et al. 2002; Zhao et al. 2003; Park et al. 2011). Neeta and Dureja (2014) highlighted the modalities of treatment, the structure, and the toxicological profiles of the different *Boswellia* species. Yadav et al. (2012) reports boswellic acid analogue to prevent proliferation and spread of colorectal cancer of humans in vivo using nude mice models.

Molecular targets of biomolecules from ayurvedic plants include nuclear factor kB acted upon by a wide range of plant-derived molecules like those from *Curcuma longa* (more than 32), *Withania somnifera*, *Boswellia serrata*, *Zingiber zerumbet*, etc.; transcription activators (STAT) -3, Nrf-2; targeted by *C. longa*, *Indigofera tinctoria*, *Vitis vinifera*; Growth factors like EGF transforming growth factor β, vascular endothelial growth factor; inflammatory cytokines, protein linase, etc. were acted upon by *C. longa* (Garodia et al. 2007).

8.4 Dietary Polyphenols as Anti-cancer Agents

Reactive oxygen (ROS) and nitrogen species are produced during metabolism, and activities of the immune system and mitochondria. These are kept under check by detoxification mechanisms (Hansen et al. 2006). Polyphenols have antioxidant as well as specific biological activity against different types of cancer. These include phenolics like catechin, procyanidins (B1 & B2), phloridzin, etc. from apples and apple juices shown to exert anti-cancer activity against cell lines HT-29 (colon) and MKN45 (stomach); phloretin, quercetin, etc. against Caco-2 (colon); ellagic acid, quercetin derivatives, kaemferol 3- glucoside, cyanidin 3-glucoside, pelargonidin 3- glucoside, etc. from black berry against HL-60 (leukemia) and A549 (lung); tannic acid from black sesame against HT 29 (colon), and other phenolic derivatives active against a wide range of cell lines. Extracts of bean, cocoa, coffee, grape seeds, onion, honey, olive oil, and potato also show anti-proliferative activity against several cell lines (Roleira et al. 2015).

Massi et al. (2012) in their review stressed upon the importance of cannabidiol (CBD) in modulating the stages of tumourigenesis in several types of cancer and the need to look into CBD/CBD analogues as alternative therapeutic agents. Manju Sharma et al. (2014) described the use of non-tetrahydrocannabinol plant cannabinoids with no psychotropic effects for the management of prostate cancer. Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin, the active principle found in red wine and grape skins. It is found in compound formulations like *Triphala ghrita*, *Khadirarista*, *Madhusnuhi rasayana*, *Maha triphaladya ghrita*, and *Panchatikta guggulu ghrita* and indicated in the ayurvedic texts for management of cancer/tumour. Aluyen et al. (2012) found that resveratrol's chemoprotective effect is dose and duration dependent. They also report synergistic activity of resveratrol with other cancer drugs. Tsubura et al. (2011) reported the inhibitory activity of garlic and its derivatives on breast cancer cell lines and the increased efficiency of oil-soluble fraction containing diallyl disulfide . Curcumin and resveratrol showed a synergistic cancer effect on colon/colorectal cancer (Majumdar et al. 2009; Patel et al. 2010). Du et al. (2013) suggested the combination treatment of these to be a promising novel anti-cancer strategy against liver cancer. Mangal et al. (2013) have developed a database, Naturally Occurring Plant-based Anti-cancer Compound-Activity-Target (NPACT, http://crdd.osdd.net/raghava/npact/), with 1574 compounds that provides information on plant-based anti-cancer compounds, accessed by key word search and other advanced options. Vetrivel et al. (2009) developed another database for compounds from Indian Plants (InPACdb), providing details on the type and target of the cancer, 3D image, etc. for each compound. Greenwell and Rahman (2015) gave an insight into the use of medicinal plant compound formulations like *Triphala ghrita*, *Khadirarista*, *Madhusnuhi rasayana*, *Maha triphaladya ghrita*, and *Panchatikta guggulu ghrita*.

Alvaradoin E, and its 10 (R) isomer, alvaradoin F isolated from the leaves of *Alvaradoa haitiensis* Urb. (Picramniaceae) was found to be toxic to the KB cell line by Phifer et al. *(*2007). Alvaradoins E and F also showed inhibition of KB, LNCaP, and Col2 cells when administered intraperitonially (Mi et al. 2005).

Quassinoids found in Simaroubaceae members and a novel one 2′-(R)-O-acetylglaucarubinone isolated from *Odyendyea gabonensis* showed potent cytotoxicity against human cancer cell lines like prostate (DU145), lung (A549), and oral epidermoid carcinoma (KB) cells (Usami et al. 2010). Tanshinone I, tanshinone IIA, and cryptotanshinone exhibited significant in vitro cytotoxicity against cell lines of breast cancer, cervical cancer, etc.

In the early 1960s, the anti-cancer property of camptothecin (from *Camptotheca acuminata)*, a drug-inhibiting DNA topoisomerase1, was discovered and this revolutionized the field of chemotherapy (Wall et al. 1966; Wall 1998), also inhibiting colon and pancreatic cancer cells (Redinbo et al. 1998; Staker et al. 2002) and cancer types like breast, liver, prostate, etc.

Combretastatins are anti-cancer agents isolated from the bark of the South African tree *Combretum caffrum* (Pettit et al. 1987). Combretastatin A-4, a simple Stilbene, was found to inhibit the polymerization of brain tubulin by binding to the colchicine site (Hamel and Lin 1983). It is also cytotoxic to human cancer cell lines like MDR. CA-4 could serve as a lead molecule for drug development against cancer (McGowan and Fox 1990; El-Zayat et al. 1993). CA-4 induces apoptosis and mitotic catastrophe there by eradicating bladder cancer (Shen et al. 2010). Considering the potent activity of CA-4 for the treatment of tumours, many synthetic analogues of CA-4 have been synthesized to improve upon its cytotoxic activity and inhibition of tubulin polymerization (Ohsumi et al. 1998, Nam 2003; Tron et al. 2006). Combretastatin A4 phosphate (CA4P; fosbretabulin), a tubulin-binding vascular disrupting agent, displays potent and selective toxicity towards tumour vasculature (Tozer et al. 1999). Shen et al. (2010) described the scope for using CA-4 for intravesical therapy, as it inhibited cell migration in vitro.

Ayurveda, the traditional Indian system of medicine, is a potential treasure chest for chemicals useful in the prevention and treatment of cancer (Devi 1996). *Cedrus deodara, Berberis aristata, Picrorhiza kurroa*, and *Piper longum* L. were shown to have anti-cancer activity against cell lines (Gaidhani et al. 2013). The anti-cancer value of *Withania somnifera* (ashwagandha) documented over four decades ago is attributed to withaferin A, a crystalline steroidal compound isolated from its leaves (Shohat et al. 1976). *W. somnifera* contains steroidal lactones collectively referred to as withanolides isolated from the root or leaf (Jayaprakasam et al. 2003; Ichikawa et al. 2006). Withaferin A is found to be the most effective among these, proved by in vivo pre-clinical studies on rodent systems. It is clear that Withaferin A targets multiple molecules/pathways that may be cell line-specific (Vyas and Singh 2014). Recent studies indicate Withaferin A to be a possible chemotherapeutic drug candidate for human oral cancer (Yang et al. 2015).

Tanshinones, isolated from *Salvia miltiorrhiza*, showed significant in vitro cytotoxicity against several human carcinoma cell lines such as breast cancer cells, cervical cancer cells, prostate cancer growth, etc. (Zhang et al. 2012).

Couroupita guianensis, commonly known as Nagalinga pushpam in Tamil, was found to have anti-cancer activity against cancer cell lines, viz., Caco2, MCF-7, A-431, and HeLa, using MTT assay. In vitro, antioxidant activities against free

radicals, viz., 2,2-diphenyl-1–picrylhydrazyl (DPPH)-radical, Nitric oxide, Hydroxyl radical, etc., were also found (Ramalakshmi et al. 2014).

Plant-derived sesquiterpene lactones salograviolide A (Sal A) and iso-seco-tanapartholide (TNP) showed synergistic anti-cancer activities as reported by Mohamed Salla et al. (2013). They found increased activity when these were used together than alone. Sal A or TNP at low doses when used individually did not have an effect on cell viability, but in combination at the same concentrations they initiated apoptosis. Apotopsis caused by the combined treatment is due to the production of ROS, which induces apoptosis. The mitogen-activated protein kinase (MAPK) pathway plays a vital role in signalling apoptotis, which in turn is triggered by toxic stimuli or stress (Benhar et al. 2002, Zhang et al. 2005). Three known MAPKs, the extracellular signal-regulated kinase (ERK1/2), the c-Jun N-terminal kinase/stress-activated protein kinase, and p38, when activated induce cell death (Zhang et al. 2005). ROS accumulation also causes JNK and p38 activation, in turn leading to cell death (Guyton et al. 1996; Cho et al. 2005; Kamata et al. 2005).

Anti-cancer property has been reported for *Polygala* sp. by Alagammal et al. (2013), *Nigella sativa* by Soumya et al. (2011), and *Bauhinia variegata by* Amita Mishra et al. (2013).

Garlic contains quite a few biomolecules that have antioxidant and anti-carcinogenic properties. Some of them are flavonoids like quercetin and cyaniding, ajoene, a sulphur-containing compound inhibiting mutagenesis, selenium, which is an anti-oxidant, and diallyl sulphides, all contributing anti-carcinogenic properties to it (Sakarkar and Deshmukh 2011).

Actinidia chinensis root is used against cancer in Chinese medicine. (*The wealth of India: A Dictionary of Indian Raw Materials and Industrial Products Vol –I (A-B)*1985, pp. 29.) Aloe-emodin in *Aloe vera* fights cancer and inhibits metastasis by activating macrophages and immune cells (Pecere et al. 2000). *Camellia sinensis* (tea) contains polyphenolics (catechins and gallates) with anti-mutagenic and anti-cancer activity, which gives protection against cancers of liver, oesophagus, stomach, intestine, and lung (Kim et al. 1995; Dreosti 1996).

Ginkgolide-B *from Ginkgo biloba* prevents cancer proliferation by controlling the activity of the platelet-activating factor and also protects DNA from damage induced by nuclear radiation (Kleijnen and Knipschild 1992a; Tyler 1994).

Soya bean is found to induce differentiation in cancer cells by converting them to normal cells, by virtue of isoflavones. Genisten, an isoflavone found in soy, induces apoptosis in cancerous cells. It also prevents the spread of cancer by preventing platelet aggregation in turn by inhibiting the tyrosine kinase inhibitor enzyme, and also by blocking angiogenesis (Kleijnen and Knipschild 1992b).

The glycoside glycyrrhizinin in liquorice shows anti-cancer activity in animal systems (Ambasta 2000a). Gossypol from *Gossypium barbadense* has shown selective toxicity towards cancerous cells (Ambasta 2000b). Plant lignans in flax seed when converted to lignans enterolactone and enterodiol (mammalian lignans) by bacterial fermentation in the colon are anti-carcinogenic. These are structurally similar to estrogens and can bind to estrogen receptors there by inhibiting the growth of estrogen-stimulated breast cancer (Serraino and Thompson 1991, 1992).

Anti-carcinogenic activity is also shown by monoterpene compounds in *Mentha piperita* oil (Dorman et al. 2003, Romero-Jimenez et al. 2005). *Zingiber officinalis* (ginger) rhizomes contain gingerols with pronounced anti-inflammatory activity against various cancers (Katiyar et al. 1996, Kikuzaki and Nakatani 1993).

Gaidhani et al. (2013) evaluated *Taxus baccata* L and compound formulations like *Triphala ghrita*, *Khadirarista*, *Madhusnuhi rasayana*, *Maha triphaladya ghrita*, and *Panchatikta guggulu ghrita* indicated in ayurvedic texts for management of cancer/tumour. *Cedrus deodara* (Roxb.) ex Lamb. and *Berberis aristata* (Roxb.) ex DC. showed maximum anti-cancer activity (against 3 cell lines) as compared to *Withania somnifera* Dunal. (against two cell lines) and *Picrorhiza kurroa* and *Piper longum* L. (against one cell line).

8.5 Conclusions

The chapter gives a new perspective on cancer prevention and cure using biomolecules from plants/ayurvedic sources. Natural compounds tend to have an undeniable role in cancer prevention and cure. Drug discovery from medicinal plants is time consuming and cumbersome. Techniques such as nuclear magnetic resonance spectroscopy and mass spectroscopy could facilitate compound isolation from medicinal plants. Though it is challenging, medicinal plants still remain a major source/reservoir of novel drug candidates for cancer. In the near future, plant-derived compounds could hold a major share in the array of cancer medicines available for therapy.

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Anti-inflammatory Molecules: Enzyme Inhibitors

9

C.S. Sharanya and Madathilkovilakath Haridas

Abstract

Structure/receptor-based drug design, a new approach of drug discovery, has become a fascinating area of drug discovery/development. This rational approach of drug discovery may lead to the production of many drugs, and some products developed based on this approach are presently available in the market. Examples of some of these drugs include saquinavir for the treatment of AIDS, captopril against hypertension, dorzolamide for glaucoma and Zanamivir against influenza. Many molecules are designed based on their structure or receptors and are under different stages of clinical trials. For applying rational approach, we have to first identify the pathway of pathogenesis and the target proteins related to the specific disease. After the identification of the specific protein, the compound which inhibits the target protein may be designed to fit into the binding site of the protein most appropriately. Compounds from natural sources are tested first, taking cues from various kinds of information. Such compounds may form a basic structure which could be appropriated to the binding site of the target protein. This chapter has been devoted to the inhibitors of the enzymes in the arachidonic acid pathway which may serve as anti-inflammatory compounds. Target enzymes of inflammatory pathway include isozymes of phospholipase A_2 (PLA_2), cyclooxygenase (COX) and lipoxygenase (LOX).

Keywords

Inflammation • Phospholipase A_2 inhibitors • Cyclooxygenase inhibitors • Lipoxygenase inhibitors

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9.1 Introduction

The most general and fundamental indicator of all pathological conditions initiates with inflammation, a general form of resistance broadly defined as a non-specific response to tissue malfunction, and it is employed by immune systems to combat pathogenic invaders (Ashley 2012).The Roman encyclopaedist Aulus Cornelius Celsus described four principal effects of inflammation which were rubor, tumor, calor and dolor and finally loss of function which was added by Virchow. Inflammation is initiated by the presence of physical agents, chemicals, microorganisms, inappropriate immunological responses and tissue death. An important stimulus of inflammation is caused by the endotoxins of bacteria and viruses. Viruses mediate inflammation by entering and destroying the cells of the body. Loss of blood flow causes tissue death due to lack of oxygen or nutrients, which also results in inflammation. Inflammation is a major problem associated with diseases like rheumatoid arthritis, cancer, obesity, neurodegenerative diseases, type 2 diabetes, cardiovascular diseases and ageing.

Inflammatory processes are generally of two kinds, acute and chronic. Acute inflammatory response will rapidly set in and last briefly. It may be accompanied by systemic reaction known as acute-phase response. It will be characterized by a rapid change in several plasma proteins levels. Chronic inflammation develops when an antigen persists for a long time and is characterized by macrophage accumulation. Chronically activated macrophages release cytokines which stimulate fibroblast proliferation and collagen production.

9.1.1 Mechanism of Inflammation

Cytokines which regulate inflammation also regulate a series of immunological, physiological and behavioural processes. Initial step of inflammatory cascade is the identification of infection or damage. Pathogen-associated molecular patterns (PAMPs), essential for pathogen survival, are primarily detected by Alarmins and also recognized by the innate immune system. Innate immune system lacks the ability to identify the different strains of pathogen than the adaptive system (Ashley et al. 2012). Other receptors which identify damage signals are Toll-like receptors (TLRs) and intracellular nucleotide-binding domain and NOD-like receptors or NLRs (Medzhitov 2008). Once the ligands recognition occurs, NF-κB becomes activated to TLR. NF-κB is released from IκB during transduction of signal and translocated to the nucleus, where transcription is upregulated through binding to target genes (Ashley et al. 2012). New protein synthesis is not required for NF-κB elicitation and permits a rapid response. After transcription and translation, expression of interleukin-1-beta (IL- 1β), IL-6, tumour necrosis factor-alpha (TNF-α), the major pro-inflammatory cytokines, occurs. Then these molecules recruit monocytes and neutrophils at the site of infection. Rapid release of chemicals by neutrophils occurs and this process requires the consumption of both glucose and oxygen, known as the respiratory burst (Ashley et al. 2012) (Fig. 9.1).

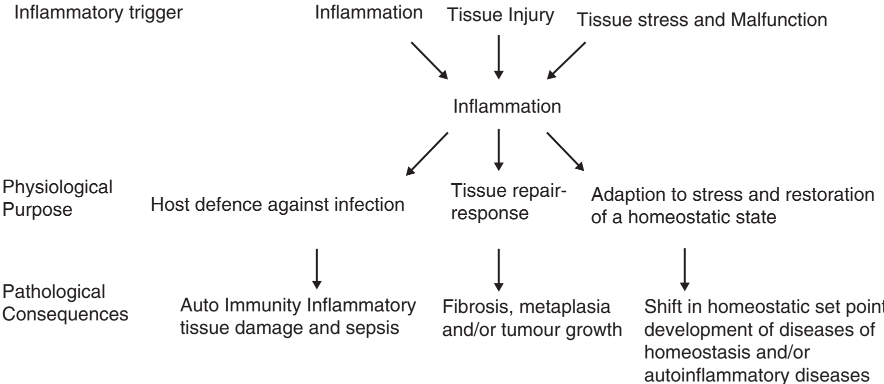

Fig. 9.1 An overview of inflammation showing causes, physiological and pathological outcomes

9.1.2 Mediators of Inflammation

Coordinated action of a large number of mediators initiates inflammatory responses. Inflammatory responses are initiated by inducers, and they activate specialized sensors. These specialized sensors then activate mediators altering the functional state of tissues and cells for withstanding the environment created by specific inflammatory inducers. Specifically we may say that inducers – sensors – mediators and effectors together resulted in the inflammatory pathway. Inducers are either endogenous or endogenous in nature. Exogenous inducers include both microbial and non-microbial ones. Microbial inducers are virulence factors and have pathogen-associated molecular patterns (PAMPs). Non-microbial exogenous inducers include allergens, toxic compounds, foreign bodies and irritants. Signals from damaged tissue are endogenous inducers, which initiate the production of mediators from plasma proteins. Based on the biochemical properties mediators can be classified into different categories, including lipid mediators, fragments of complement components, cytokines, vasoactive amines, vasoactive peptides, chemokines and proteolytic enzymes (Medzhitov 2008). Lipid mediators include eicosanoids and platelet-activating factors which are produced through the coordinated action of phospholipase A_2, cyclooxygenase and lipoxygenase on membrane phospholipids. Membrane phospholipids will get converted to arachidonic acid by phospholipase A_2 and further metabolized to prostanoids by cyclooxygenase and leukotrienes with the help of lipoxygenase.

9.1.2.1 Prostaglandins

Prostaglandins are important inflammatory mediators found in a variety of human tissues, animals and also in coelenterates. They exert specific biological reactions via G protein-coupled receptors. Significant functions of prostaglandins include immune responses, nerve growth and development, wound healing (Dhanjal et al. 2015), blood clotting, ovulation, initiation of labour, bone metabolism and blood vessel tone Prostaglandins are created by cells and then act only in the surrounding

area before they are broken down. The two main classes of prostaglandin receptors are nuclear PPAR receptor class (i.e. PPARα, PPARγ and $PPAR_{\delta}$) and G-coupled cytoplasmic receptor class (i.e. EP1–4 for PGE_2) (Simmons et al. 2004).

9.1.2.2 Thromboxane

Thromboxane is known to be the key mediator of platelet activation and aggregation, and an important mediator of platelet-induced coronary artery constriction. Cyclooxygenase produces thromboxane A_2 via sequential oxygenation of arachidonic acid, and its properties include vasoconstriction and platelet aggregation. Thromboxane A_2 which is either a potent platelet agonist or a weak agonist has a significant role in amplifying the response of platelets to more potent agonists. It exerts its actions via specific G protein-coupled receptors.

9.1.2.3 Leukotrienes

Leukotrienes (LTs) are arachidonic acid derived pro-inflammatory mediators formed via 5-LOX and contribute to pathophysiological conditions in asthma and many other disease conditions. They include cysteinyl LTs (LTC_4, LTD_4 and LTE_4) and LTB_4 (Montuschi 2007). $CysLT_1$ and $CysLT_2$ are the two receptor subtypes. Microvascular permeability increases airway and smoothens muscle activity and airway mucus secretion in asthma by the stimulation of $CysLT_1$ receptor (Montuschi et al. 2007). The second family of leukotrienes includes LTB_4 which acts in inflammatory conditions of cystic fibrosis, psoriasis and inflammatory bowel disease. Nowadays drugs are designed based on both cysteinyl-leukotriene receptor antagonists and leukotriene synthesis inhibitors. Leukotriene formation can be inhibited by direct 5- LOX inhibitors or through FLAP inhibitor or via receptor antagonist mechanism.

9.1.3 Anti-inflammatory Mechanism

An important anti-inflammatory mechanism is the inhibition of enzymes producing eicosanoids. By the action of phospholipase A_2 (PLA_2) arachidonic acid is released from membrane phospholipids through its cleavage, and further, a signal cascade leads to the production of prostaglandins (PGs) and thromboxane A_2 (TXA_2) by cyclooxygenase (COX) pathway and hydroperoxyeicosatetraenoic acids (HpETEs), hydroxyeicosatetraenoicacids (HETEs) and leukotrienes via lipoxygenase (LOX) pathway. Mainly two forms of cyclooxygenase COX 1 and COX 2 are available, and another variant form COX 3 is also discovered recently. COX 1 is responsible for normal homeostasis while COX 2 is inducible and expressed during inflammatory conditions. Leukotrienes are produced by lipoxygenase and different isoforms of LOX are present. But only 5 and 12-LOX are involved in the inflammatory response.

9.2 Anti-inflammatory Molecules-Enzyme Inhibitors

9.2.1 Phospholipase A_2 Inhibitors

During tissue injury, membrane phospholipids get hydrolysed to produce fatty acids and lysophospholipid through phospholipase A_2 (Farooqui 2000). Hydrolysis follows in the ester linkage of sn-2 position of phospholipids. These metabolites serve as the precursor for platelet-activating factor (PAF) and eicosanoids, the main inflammatory mediators, and the reactions represent the rate-limiting step in the eicosanoid production. More than 19 different isoforms of PLA_2 have been identified in mammalian system. They have been shown to participate in physiological events related to cell injury, inflammation and apoptosis. PLA_2 was purified first from cobra venom and later from rattlesnake venom. During 1970, as the protein-sequencing methodologies advanced, sequence was identified and these enzymes indicated the presence of more cysteine (> 10% of the amino acids) residues. Mammalian and porcine pancreatic PLA_2s were identified during the same period and were found to have the same disulphide bond pattern as that of cobra venom.

In 1988, Jeffery J. Seilhamer and Lorin K. Johnson from California Biotechnology Inc. and Ruth M. Kramer from Biogen Research Corporation (Dennis et al. 2011) independently purified and sequenced new human non-pancreatic PLA_2 from synovial fluid and found its disulphide pattern similar to that in rattle snake venom named as secretory PLA_2. After that $sPLA_2$ was isolated and purified from different cells and designated as $sPLA_2$ GI, GII, GIII, GV, GIX, GX, GXI, GXII, GXIII and GXIV. Molecular weights of these enzymes were found to be within 13–15 kDa, and their catalytic residue contains His/Asp. Later cytosolic PLA_2 was isolated and purified but its sequences were unrelated to $sPLA_2$ and reported as GIV having a molecular weight of 60–224 kDa and catalytic residues include Ser/Asp. Another cytosolic PLA_2 was identified which is Ca^{2+} independent and designated as GVI found to be 84–90 kDa in molecular weight. A new PLA_2 was isolated from human plasma that hydrolyses platelet-activating factor and termed as lipoprotein-associated PLA_2 (Dennis et al. 2011). These enzymes have specificity for a short acyl chain on *sn-2* position, and these are labelled as GVII and GVIII. These enzymes are 20–45 kDa in molecular weight, and their catalytic residue contains Ser/His/Asp. A new type of PLA_2 was identified from lysosome (GXV) and adipose tissue (GXVI) with 45 and 18 kDa in molecular weight, and their catalytic site contains Ser/His/Asp and His/Cys, respectively (Burke and Dennis 2009).

9.2.1.1 Structural Characterization of Phospholipase A_2

Secretory PLA_2 is found to contain a catalytic site and a highly conserved Ca^{2+}binding loop. All forms of these enzymes share a common protein fold with a slight difference. They have same catalytic His/Asp dyad and contain three long α-helices and two β-sheets also (Dennis et al. 2011). Cobra venom PLA_2 is the best studied PLA_2, and structural characterization of PLA_2 was explained based on this enzyme. This enzyme has six conserved disulphide bonds with an additional bond between 11 and 71 residues. Calcium ions bind with carbonyl oxygen of tyrosine and glycine

from calcium-binding loop as well as conserved aspartic acid residues. Calcium is required for the hydrolysis of the sn-2 carbonyl oxygen and coordinates with a catalytic water molecule. Crystal structures of PLA_2 reveal that only about 9–10 carbon of sn-2 acyl chain interacts with enzyme and rest of the chains are buried within the lipid–water interface. The hydrophobic residues like Leu2, Phe5, Trp19, Tyr52 and Tyr69 wrap around the acyl chain of the lipid substrate (Gelb et al. 1994).

PLA_2 activity is based on the interaction of the protein with large lipid aggregates. The presence of aromatic residues, mainly the tryptophan residue, contributes to the interfacial binding surface which assists the hydrolysis of zwitterionic phospholipids. As suggested, catalytic mechanism of PLA_2 is introduced by His48/Asp99/calcium complex within the active site. Bridging of the second water molecule by His48 is the basis of nucleophilicity. The basicity of His48 is enhanced through hydrogen bonding with Asp99. Since the asparagine amide group can function to lower the pKa of the bridging water molecule, substitution of asparagine with His48 can maintain wild-type activity. Calcium-coordinated oxy-anion composed of tetrahedral intermediate degradation is the rate-limiting stage. Relatively small cations like nickel or cobalt may duplicate the role of calcium.

9.2.1.2 Disease Implication of PLA_2

The $sPLA_2$s have significant role in several inflammatory diseases. Patients suffering from osteoarthritis, rheumatoid arthritis, crystal-associated arthritis, acute pancreatitis (Dennis et al. 2011), septic shock, Crohn's diseases, adult respiratory distress syndrome (ARDS), inflammatory bowel disease, atherosclerosis and ulcerative colitis showed an increased amount of GII $sPLA_2$ in their synovial fluid. PLA_2 also has a role in tumourigenesis and increased expression found in prostate cancer, breast cancer and in neoplastic prostatic and gastric adenocarcinomas (Dennis et al. 2011).

9.2.1.3 Synthetic Inhibitors of PLA_2

Many PLA_2 inhibitors have been obtained and described from natural and synthetic sources. Initially, phospholipid analogues were synthesized and used as inhibitors in studies and here we describe a series of synthetic compounds under investigation. 1-Stearyl-2-stearoylaminodeoxy phosphatidylcholine (Fig. 9.2a) was studied and found to be a reversible inhibitor of PLA_2 from cobra venom (*Naja naja*) (Davidson et al. 1986). At the same time, long chain difluoro ketone analogues were also studied. In the series of phosphatidyl ethanolamine modifications, a thioether amide of PE was found to be a potent inhibitor (Fig. 9.2b). An acyl amino analogue (Fig. 9.2c) of phospholipids was developed at the same time as an inhibitor of porcine pancreatic PLA_2 (De Haas et al. 1989). A class of phosphonate analogue of phospholipid was found to be a tight inhibitor of the same enzyme while acyl amino analogue of phospholipid was developed as an inhibitor of porcine PLA_2.

Dicarboxylic acid derivatives (BMS-181162) (Tramposch et al. 1992) are found to be specific inhibitors of 14 KDa PLA_2 to block arachidonic acid release and biosynthesis of LTB_4 and PAF in calcium ionophore-stimulated human PMNs with an IC_{50} of 10 μM (Dennis et al. 2011). Another derivative BMS 188184 (Tramposch et al. 1994) has better stability and inhibits human non-pancreatic PLA_2 with an IC_{50} of 17 μM and reduces mouse ear oedema with an $ED_{50} = 9.37$ μg/ear (Fig. 9.3).

Fig. 9.2 Phospholipid analogues

Fig. 9.3 Dicarboxylic acid derivatives

Fig. 9.4 Biaryl acid inhibitors

Fig. 9.5 Sulphonamide derivatives

In the case of biaryldiacid inhibitors, biarylacetic acid derivatives were more potent inhibitors than biaryl acid or biarylpropanoic acid (Springer et al. 2000) (Fig. 9.4). These compounds have an IC_{50} value of 8 μM and 4 μM, respectively, and reduce mouse ear oedema with an ED_{50} = 32 and 73 μg/ear. Another series of benzene sulphonamides were prepared and they significantly inhibited membrane-bound PLA_2 (Oinuma et al. 1991) (Fig. 9.5).

Computer-aided drug design and chemical modifications also lead to the development of novel indole derivatives as PLA_2 inhibitors. Indole-3-acetamides (Fig. 9.6) (Dillard et al. 1996) interact with active site residues and inhibit PLA_2.

Another series of indole-3- glyoxamide derivatives were developed which have higher potency than acetamide (Snyder et al. 1999).

Another class of inhibitors was developed based on the derivatization of D-tyrosine. Structure of benzyl derivative of tyrosine co-crystallized with human PLA_2 revealed the presence of hydrogen bond through amide NH group to His48, multiple hydrophobic interaction and a T-shaped aromatic group His interaction along with metal-chelating carboxylate and amide oxygen atom (Hansford et al. 2003).

9.2.1.4 Natural Inhibitors of PLA_2

PLA_2 inhibitors were isolated from plants, microorganisms and marine-associated organisms. *Aristolochia sp.*, used in traditional medicines for snakebites, contains Aristolochic acid which significantly inhibited PLA_2. Other naturally derived flavonoid compounds, including rutin and quercetin, also have inhibitory effect. Coumarin derivatives isolated from *Eclipta alba*, Ellagic acid, 3,3′-di-O-methyl ellagic acid, 3′-O-methyl ellagic acid, 3-O-methyl-3,4′-methylenedioxy ellagic acid from *Casearia sylvestris* SW (Flacourtiaceae), 2- hydroxy −4-methoxy benzoic acid from *Hemidesmus indicus* root extract and Rosmarinic acid from *Cordia verbenacea* were found to be inhibitors of PLA_2. Marine sources are also potent inhibitors of PLA_2. The first isolated compound manolide (IC_{50}–7.5 μm) (Mayer 1989) and other compounds including ircinin (Cholbi et al. 1996), variabilin (IC_{50}–6.9 μM) (Escrig et al. 1997) and petrosaspongiolide M (Garcia-Pastor et al. 1999) inhibited PLA_2.

An isoquinoline alkaloid berberine (Chandra et al. 2011) from *Cardiospermum halicacabum* when complexed with Russell Viper venom phospholipase A_2 gave crystal diffracting at a resolution of 1.93 Å showed in two hydrogen bonds by Gly30 and His48, one direct and the other water mediated which were formed between berberine and the enzyme (Chandra et al. 2011). Hydrophobic interaction was found between the hydrophobic surfaces of berberine and hydrophobic contacts with side chains of neighbouring amino acids. The bio-transformed berberine also has a better PLA_2 inhibition and its co-crystal structure has a resolution of 2.4 Å. Structural details proved that insertion of dihydroxy-berberine in an identical orientation to that of berberine causes an immediate increase in negative electron density. This proves that dihydroxy-berberine binds in an inverted orientation with respect to native berberine. This is corroborative to the results of in silico molecular docking studies conducted earlier (Fig. 9.7).

Another study revealed that catechol, (1,2-dihydroxybenzene) naturally occurring polyphenol, binds at the active site cleft opening (Dileep et al. 2011) (Fig. 9.8). This inhibition nature of catechol can be explored for its use as an anti-inflammatory agent. The enzyme kinetics study of n-hexadecanoic acid (Aparna et al. 2012) proved that it can also inhibit PLA_2 in a competitive manner. Crystal structure of n-hexadecanoic acid with PLA_2 obtained at 2.5 Å resolution is shown in Fig. 9.8. The binding constant and binding energy were calculated, and the results from the structural and kinetics studies reported that the fatty acid, n-hexadecanoic acid, is an inhibitor of PLA_2(Aparna et al. 2012). The presence of this anti-inflammatory

Fig. 9.6 Indole derivatives

R1	R2	IC_{50} (μM)
COOH	CH_3	13.6 ± 4.2
$CONH_2$	CH_3	0.84 ± 0.17
$CONH_2$	CH_2CH_3	0.26 ± 0.11

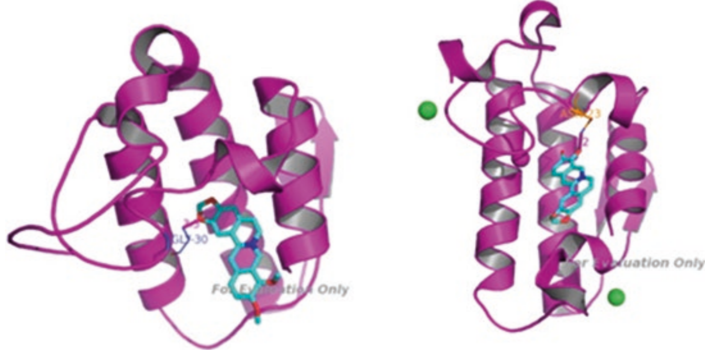

Fig. 9.7 PLA_2 in complex with Berberine (2QVD) and dihydroxyberberine (3 L30)

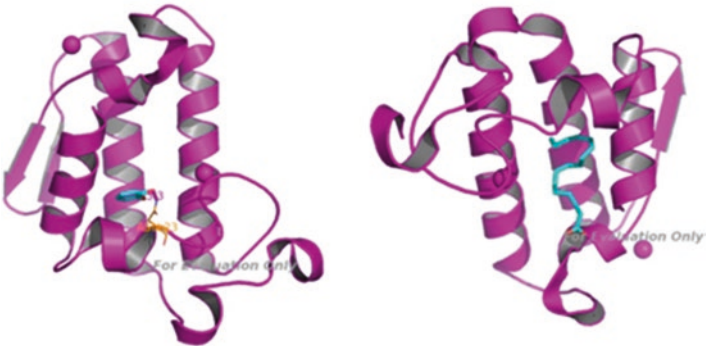

Fig. 9.8 PLA_2 in complex with catechol (3O4M) and n-hexadecanoic acid (3QLM)

compound supports the rigorous use of medicated oils rich in n-hexadecanoic acid and similar fatty acids for the treatment of rheumatic symptoms in the traditional medical system of India, Ayurveda. Inhibition studies of curcumin with PLA_2 also gave remarkable results. Crystal structure of photodegraded product of curcumin

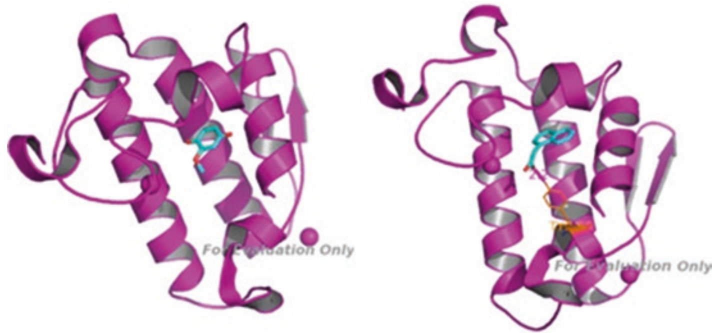

Fig. 9.9 PLA_2 in complex with 2-methoxycyclohexa-2-5-diene-1,4-dione (3HSW) and naphthalene acetic acid (4O1Y)

Fig. 9.10 PLA_2 in complex dibutyl benzene-1,2-dicarboxylate dibutyl phthalate (4G5I)

2-methoxycyclohexa-2-5-diene-1,4-dione with PLA_2 has been described by Dileep et al. (2013) at a resolution of 2.5 Å. 1-Naphthalene acetic acid and dibutyl benzene-1,2-dicarboxylate dibutyl phthalate also binds at the entry of active site and inhibits the entry of substrate, thus it may act as a good anti-inflammatory lead molecule (Figs. 9.9 and 9.10).

9.2.2 Cyclooxygenase Inhibitors

Cyclooxygenase is the enzyme which primarily transforms Arachidonic acid into unstable prostaglandin G_2 (PGG_2) via the oxygenase function, then to the more stable prostaglandin H_2 (PGH_2) via the peroxidase function, and is therefore known as prostaglandin H synthase (PGHS) (Meirer et al. 2014). This is a

membrane-bound enzyme and bifunctional in nature. History of cyclooxygenase was revealed in the late 1970s and an isoform of COX was reported in 1984, from the laboratory of Herschman and colleagues, who studied phorbol-ester-induced genes in Swiss 3 T3 cells and discovered a novel cDNA with a predicted structure similar to COX 1 (Flower 2003). Both COX 1 and COX 2 have two separate but linked active sites, and differ in substrate and inhibitor selectivity in their intracellular locations. These enzymes' action is explained through mutagenesis, kinetics and also through crystallographic studies. COX 1, responsible for the basal homeostatic prostaglandin synthesis, is constitutively expressed, COX 2 the inducible form is stimulated by growth factors, bacterial lipopolysaccharides (LPS), pro-inflammatory cytokines and by tumour-promoting agents (Meirer et al. 2014). Its variant, COX 3, was also identified. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX and play an important role in treating inflammation, fever and pain.

9.2.2.1 Structure of Cyclooxygenase

Both COX 1 and COX 2 are located in the lumen of the nuclear envelope and endoplasmic reticulum. Protein sequence experiments revealed that cyclooxygenase enzymes are homodimers of 576 and 581 amino acids, respectively, for COX 1 and COX 2. They are rich in mannose oligosaccharides (three) which facilitate protein folding. Another specialty for COX 2 is the presence of a fourth oligosaccharide, which regulates its degradation. Due to the structural similarity, it is not a surprise that they are nearly superimposable. Each subunit of the dimer consists of three domains, the epidermal growth factor domain (residues 34–72), the membrane-binding domain (residues 73–116) and the catalytic domain that contains peroxidase and cyclooxygenase active sites on either side of the heme group (Fig. 9.11) (Rouzer and Marnett 2009).

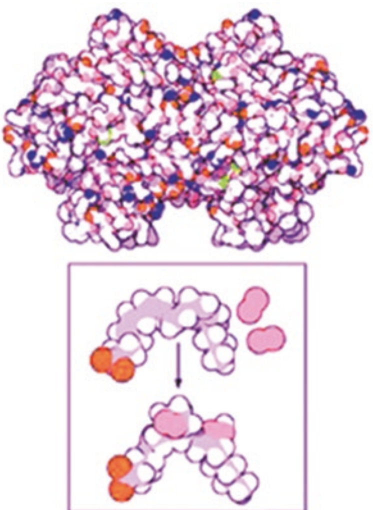

Fig. 9.11 Structure of cyclooxygenase enzyme

Dimerization Domain Hydrophobic interactions, salt bridges and hydrogen bondings hold the dimers of both COX 1 and COX 2 together. Heterodimerization of these subunits does not occur. Approximately 50 amino acids are encoded by the dimerization domain near the amino terminus of the functional protein. Three disulphide bonds hold this domain together. A fourth disulphide bond links the dimerization domain with the catalytic domain which are required for maintaining oxidizing environment. Membrane Binding Domain creates a hydrophobic surface through sequence of four amphipathic helices that spear into the upper portion of the luminal side of lipid bilayer. Helices are encoded by about 50 amino acids which are found close, after carboxy-terminal to the dimerization domain. About 80% of protein comes under the Catalytic Domain and have two distinct active sites. Carboxy-terminal of membrane binding domain constitutes the catalytic domain. The two distinct active sites include Peroxidase active site and Cyclooxygenase active site (Simmons et al. 2004).

Peroxidase active site has two separate interlinking lobes which create a shallow cleft on upper surface of enzyme where heme is bound and an iron-histidine (His388) coordination is involved. Other important interactions identified include those between the protoporphyrin and coordination of PGG_2 with specific amino acids. The geometry of heme binding leaves a large portion of one side of the heme exposed in the open cleft of the peroxidase active site. This facilitates its interaction with PGG_2 and other lipid peroxides.

Active site of cyclooxygenase is a long, narrow, dead-end channel of largely hydrophobic character whose entrance is bordered by the four amphipathic helices of the membrane-binding domain. The globular catalytic domain has an extent of 25 Å with an average width of about 8 Å. However, the channel narrowing is observed where Arg120, one of the two ionic residues, found in the COX active site, protrudes into the channel to form a hydrogen-bonded network with Glu524 and Tyr355. Arg120 is essential for binding substrates and carboxylate-containing NSAIDs in COX 1. Major difference in the structure of COX 1 and COX 2 is a substitution of Ile523 in COX 1 for Val523 in COX 2 (Fig.9.12). This structural difference makes COX 2 drugs accessible for the active site. Other differences in the structure make changes which result in slightly enlarged active site of COX 2 than COX 1 (Simmons et al. 2004).

COX 3, the variant form of cyclooxygenase, is present in brain, and it is having a structure similar to COX 1 and COX 2 but have an array of additional 30 amino acids. COX 3 shows a property of unusual drug sensitivity. Simmons et al. (2004) experiments revealed that COX3 was more sensitive to paracetamol, diclofenac, ibuprofen and aspirin than COX1 or COX2. COX 3 was also demonstrated to be sensitive to drugs like antipyrine, aminopyrine, dipyrone and phenacetin, which are not inhibitors to other COX preparations.

9.2.2.2 Mechanism of the Cyclooxygenase Reaction

The conversion of PGG_2 from arachidonic acid includes the abstraction of pro-S hydrogen from carbon-13. For the catalytic action to occur cyclooxygenase must activate the process dependent on peroxidase activity. These reactions (electron

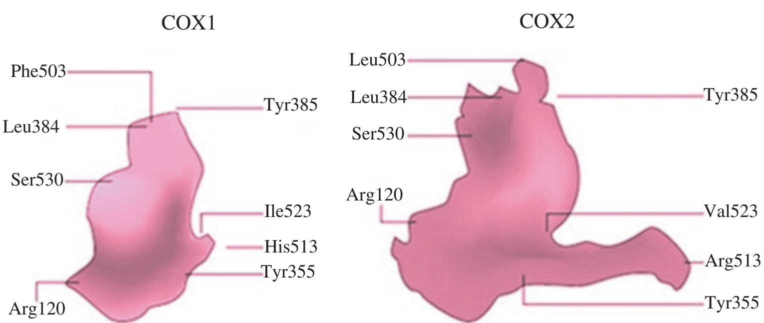

Fig. 9.12 Structural difference between active site residues of COX 1 AND COX 2 (Flower 2003)

transfer from heme to Tyr385) generate tyrosyl radical in the enzyme active site. Process of pro-*S* hydrogen abstraction from arachidonic acid takes place with the help of these radicals and initiates cyclooxygenase reaction. At last, PGG2 formed by the reduction of peroxyl radical to hydroperoxide regenerate tyrosyl radical. Activated COX can carry out multiple turnovers without the activation step.

9.2.2.3 Inhibitors of Cyclooxygenase

Aspirin, the world's most favourable drug, was discovered in 1898 but its therapeutic mechanism of prostaglandin inhibition was invented only in the late 1970s. A series of cyclooxygenase inhibitor drugs were developed from 1940 to 1980 which include phenylbutazone, fenamatesin, indomethacin, proprionates and oxicams. But the discovery of COX isozyme in 1990 lead to a great breakthrough in the development of new drugs better than the older drugs evolved. Isozyme of COX was defined in 1991 and a new class of inhibitors were developed by several researchers and industrialists. The most extensively represented class of cyclooxygenase inhibitors includes acidic sulphonamides, diarylheterocycles, zomepirac analogues, indomethacin analogues and di-tertiary-butylphenols. All these compounds are slow and mediate tight binding inhibition.

NSAIDs show three different modes of binding. (i) low affinity fast reversible binding followed by, higher affinity, slow reversible binding (fluorbiprofen), (ii) reversible binding (ibuprofen) and (iii) fast reversible binding followed by a covalent modification of the enzyme (aspirin). A protein conformational change occurs when the enzyme inhibitor is formed. Aspirin, the golden drug, covalently modifies the protein by acetylating Ser530, which is compared to Arg120, and it is more active against COX 1 than COX 2. Acetoxyphenyl heptynylsulphide an aspirin-like molecule was developed which exhibits 20-fold selectivity for COX 2 and acetylates only Ser530 (Rao and Knaus 2008).

DuP697 (IC_{50}–0.8 μM for COX 1 and 0.01 μM for COX 2) (Habeeb et al. 2000) drug developed in 1990 has shown in vitro COX inhibitory effect more on rat brain prostanoid synthesis. This compound is safe from gastrointestinal toxicity. Other compounds with similar properties include NS398 (IC_{50} – ˃100 μM for COX 1 and 0.1 μM for COX 2 (Leone et al. 2007), flosulide and CGP28238. A Similar in vitro

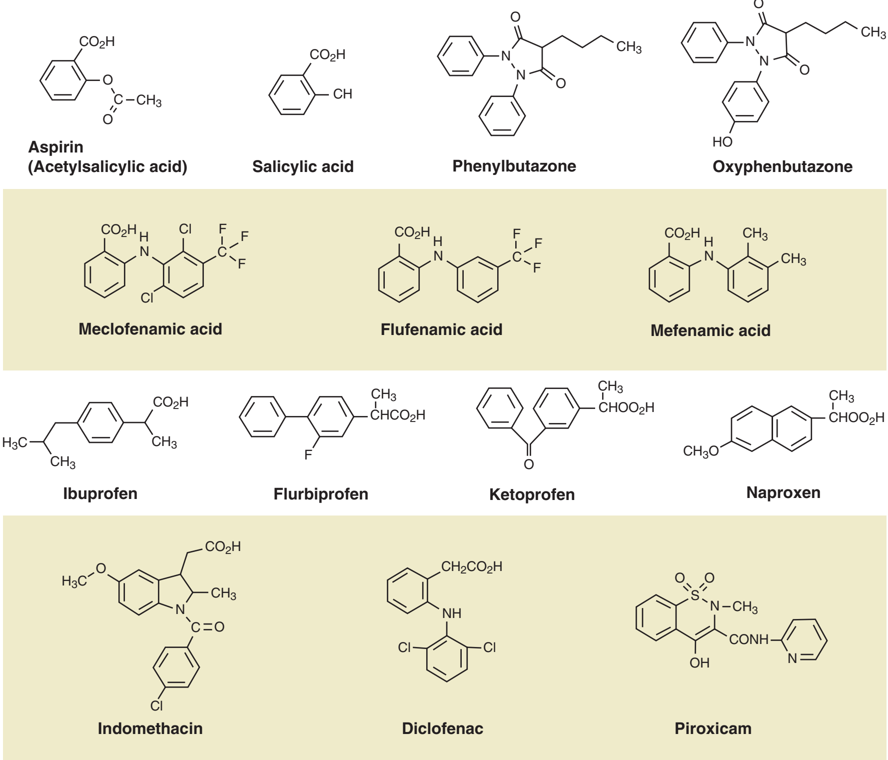

Fig. 9.13 Structure of classical NSAIDs and related compounds

model inhibition was shown by selective inhibitors like SC58125 (IC_{50} – ˃1000 μM for COX 1 and 0.1 μM for COX 2) and SC558 (a celecoxib prototype; IC_{50}–17.7 μM for COX 1 and 0.0093 μM for COX 2) which exhibited good efficacy in rodent models of inflammation, fever and pain (Figs. 9.13 and 9.14) (DeWitt 1999).

9.2.2.4 Structural Diversity of Cyclooxygenase Inhibitors

NSAIDs inhibit cyclooxygenase in production of prostaglandins in the treatment of inflammatory musculoskeletal conditions. Most of the NSAIDs inhibit COX 1 and COX 2. NSAIDs are known to be aspirin-like drugs because they follow a structural diversity with a carboxylic acid functional group which follows the anti-inflammatory, analgesic and antipyretic action. Further they exhibit the characteristic side effects, including suppression of blood clotting via inhibitory action on platelet function (Flower 2003) and gastric intolerance.

Indomethacin analogue's selectivity is not at the side of the cyclooxygenase active site while substitutions take place at the top. 4-Bromobenzyl indomethacin complex with COX 2 indicates that 4-bromobenzyl group makes van der Waals contact with Leu503 at the terminal of the COX 2 active site. Phe503 in COX1 cannot easily replace as leucine by bromobenzyl group and the COX 1 active site thus

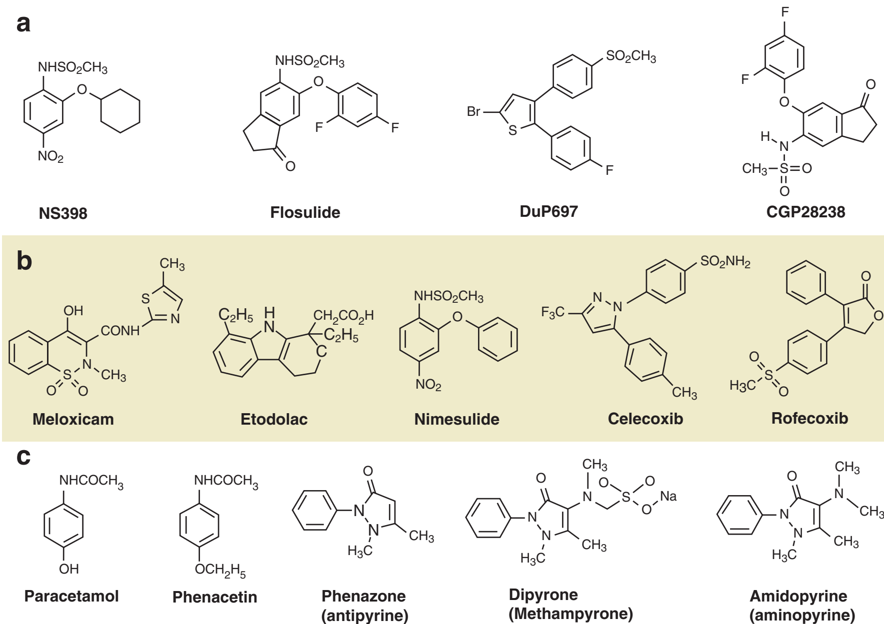

Fig. 9.14 Structure of NSAIDs: (**a**) Structures of DuP697, NS398 (**b**) isoform inhibitors (celecoxib and rofecoxib) and COX 2 selective inhibitors (meloxicam, etodolac and nimesulide) (**c**) Effective COX-3 inhibitors

becomes less flexible at the top which may reduce the affinity of the protein for the indomethacin analogue, thereby accounting for its COX 2 selectivity. All structures will not give an outlook into the mechanism of selectivity. Sulphonamide group of NS-398 pairs ionically to Arg120. Similarly the crystallographic data of zomepirac-derived COX 2 selective inhibitors do not provide a justification for their selective COX 2 inhibition. The sulphonamide moiety of the inhibitor forms hydrogen bonds with Arg120, Glu524 and Tyr355 in COX 2.

Crystal structure of COX was elucidated and new inhibitors were developed. Based on the crystal structure, the active site binding and mode of inhibition were studied in detail. COX1 structure was explained in 1994 and COX2 in 1996. Several co-crystal compounds were reported in PDB these days which can be developed into anti-inflammatory drugs. The crystal structure of sheep COX 1 complexed with an anti-inflammatory drug celecoxib was determined at 2.75 Å (Fig.9.15). Residues Gln192 and Leu352 made hydrogen bonds with proteins and ligands. A large number of van der Waal's contacts are established with the residues His590, Ser516, Leu352, Ser353, Ile523, Phe518, Tyr355, Van349, Ala527 and Met522. This structure is deposited in PDB and available with the ID 3KK6 (Rimon et al. 2011).

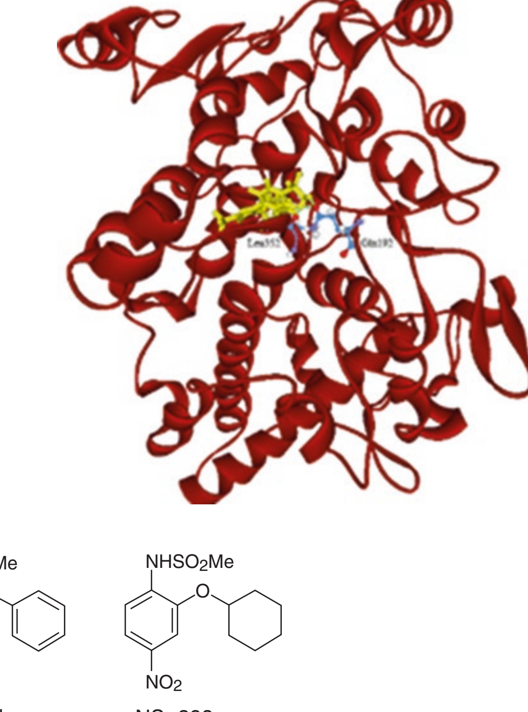

Fig. 9.15 Crystal structure of COX 1 in complex with celecoxib. (PDB ID 3KK6)

NHSO2Me NHSO2Me
O O
NO2 NO2
Nimesulide NS- 398

Fig. 9.16 Methanesulphonanilide inhibitors

9.2.2.5 Synthetic Inhibitors of Cyclooxygenase

Methane Sulphonanilide Inhibitors These are alkylsulphonanilide derivatives and Nimesulide is the best example under this category. Their structural analogues have also better inhibitory potential. Nimesulide has IC_{50} of 10 μM for COX 1 and 1.9 μM for COX 2 (Leone et al. 2007) (Fig. 9.16).

Diarylheterocycles-Derived Inhibitors Most of the COX 2 inhibitors come under these groups and DuP-697 is the first member in this group. This compound has a thiophene 5-membered ring in the tricyclic group and 1,2- diaryl substitution in that thiophene ring. -SO_2Me, or a -SO_2NH_2 group at *para* position of phenyl ring increases the inhibitory effect. A *para*-F- substitution at non-sulphonyl vicinal phenyl ring also increases the activity. Due to long unusual plasma half-life this drug become clinically unfit and removed. Diphenyl cyclopentenes and cyclopentenone derivatives of DuP-697 were developed and SC57666 is the first compound in this category which does not have any gastric complications. -SO_2NH_2 and -SO_2Me at *para* position lead to another molecule with increased oral bioactivity (Fig. 9.17).

Diarylheterocycles Have Central 5-Membered Pyrazole Ring 1,5-Diarylpyrazole group of compounds were found to be significant cyclooxygenase inhibitors. In that

Fig. 9.17 Chemical structure of COX inhibitors

Fig. 9.18 Chemical structure of COX inhibitors with a central 5-membered pyrazole ring

first compound (Fig. 9.18a) was synthesized and has an IC_{50} = 0.24 μM for COX 1 and IC_{50} > 100 μM for COX 2 (Talley 1999). So many lead compounds were thus developed including SC58125 (IC_{50} – ˃1000 μM for COX 1 and 0.1 μM for COX 2) (Zimmermann et al. 1998) (Fig. 9.18b). Another designed compound (Fig. 9.18c) which contains an azide group has a potent inhibitory activity (COX 2 IC_{50} = 1.55 μM, COX 1 IC_{50} > 100 μM) (Habeeb et al. 2001). Celecoxib is an extensively studied drug molecule that has an IC_{50} of 0.04 μM for COX 2 and 13 μM for COX 1 (Fig. 9.18d).

Diarylheterocycles with a Central 5-Membered Isoxazole Ring Isoxazole significantly inhibits cyclooxygeanse (COX 2 IC_{50} = 0.18 μM, COX 1 IC_{50} > 1000 μM) (Talley 1999) and has a better in vivo activity also. Its derivatives also showed better inhibition (Valdecoxib (a) COX 2 IC_{50} = 0.005 μM, COX 1 IC_{50} = 140 μM) (Habeeb et al. 2001). Valdecoxib is a second-generation COX 2 selective drug with anti-inflammatory and analgesic properties but withdrawn from market due to severe side effects. Other two isoxazole regio isomers were synthesized and are selective inhibitors of COX 2 [Compound (b): COX 2 IC_{50} < 0.005 μM, COX 1 IC_{50} > 500 μM; Compound (c) COX 2 IC_{50} = 0.23 μM, COX 1; IC_{50} = 256 μM] (Habeeb et al. 2001) (Fig. 9.19).

Diarylheterocycles Possess a Central 5-Membered Furanone Ring These compounds also possess COX-2 inhibitory activity with an IC_{50} of 0.01 μM for COX 2 and 4.7 μM for COX 1 (a) (Habeeb et al. 2001). *Para*-SO_2NH_2(b) substituent of the first compound resulted in decreased activity. Rofecoxib (c) comes under this

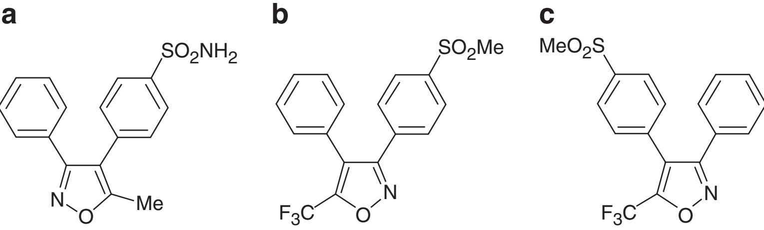

Fig. 9.19 Chemical structure of COX inhibitors with a central 5-membered isoxazole ring

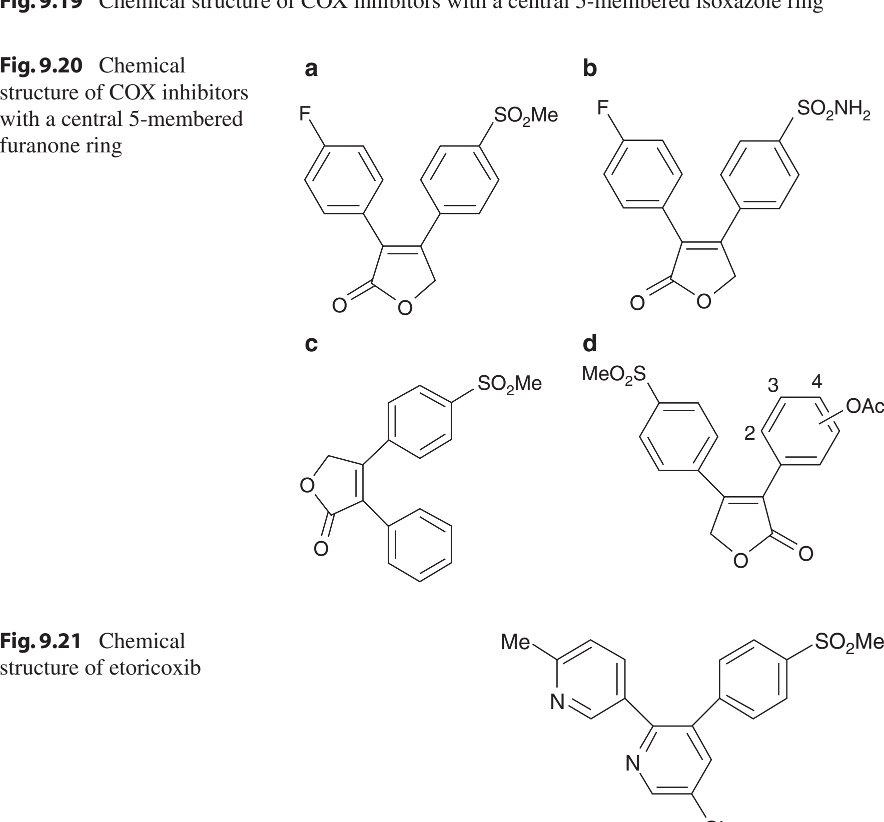

Fig. 9.20 Chemical structure of COX inhibitors with a central 5-membered furanone ring

Fig. 9.21 Chemical structure of etoricoxib

category and has significant anti-inflammatory and analgesic properties with reduced gastrointestinal toxicity. Rofecoxib analogues were prepared by substituting 2-, 3- or 4-acetoxy moiety on the C-3 phenyl group with increased IC_{50} value (c) (Fig. 9.20).

Diarylheterocycles Have a Central 6-Membered Pyridine Ring Etoricoxib belongs to this category with good anti-inflammatory activity but causes severe risk to cardiovascular system (COX 2 $IC_{50} = 0.08$ μM and COX 1 $IC_{50} = 12$ μM) (Chauret et al. 2001) (Fig. 9.21).

Fig. 9.22 Chemical structure of COX inhibitors with a central 6-membered pyridazinone ring

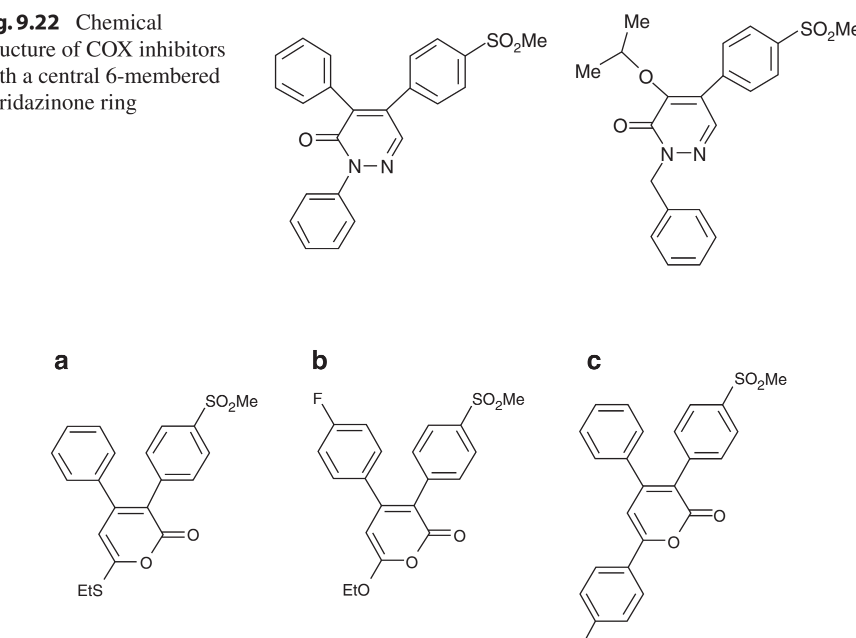

Fig. 9.23 Chemical structure of COX inhibitors with a central 6-membered pyranone ring

Diarylheterocycles with a Central 6-Membered Pyridazinone Ring Pyridazinone ring-based designed compounds are excellent COX 2 inhibitors (Li et al. 2003) (Fig. 9.22).

COX 2 IC_{50}–0.08 μM; COX 1 IC_{50} > 10 μM; COX 2 IC_{50}–0.02 μM; COX 1 IC_{50} > 10 μM.

Central 6-Membered Pyranone Ring in Diarylheterocycles Central 6-membered lactone on tricyclic class serves as an excellent template for COX 2 inhibitors. Other designed 3,4-diphenylpyran-2-ones with a central six-membered lactone ring has an IC_{50} of 3 nM for COX 2 and 386 μM for COX 1 (a) (Rao et al. 2003). But this compound has lesser anti-inflammatory activity, hence a new compound was designed with increased anti-inflammatory potential in vitro and in vivo (COX 2 IC_{50} = 0.10 μM; COX 1 IC_{50} = 288 μM) (b) (Rao et al. 2003). Regioisomeric molecule with 3,4,6-triphenylpyrones substituents were designed and exhibited increased potency (COX 2 IC_{50} = 0.02 μM; COX 1 IC_{50} > 100 μM) (c) (Rao et al. 2004). This compound is more potent than celecoxib and rofecoxib (Fig. 9.23).

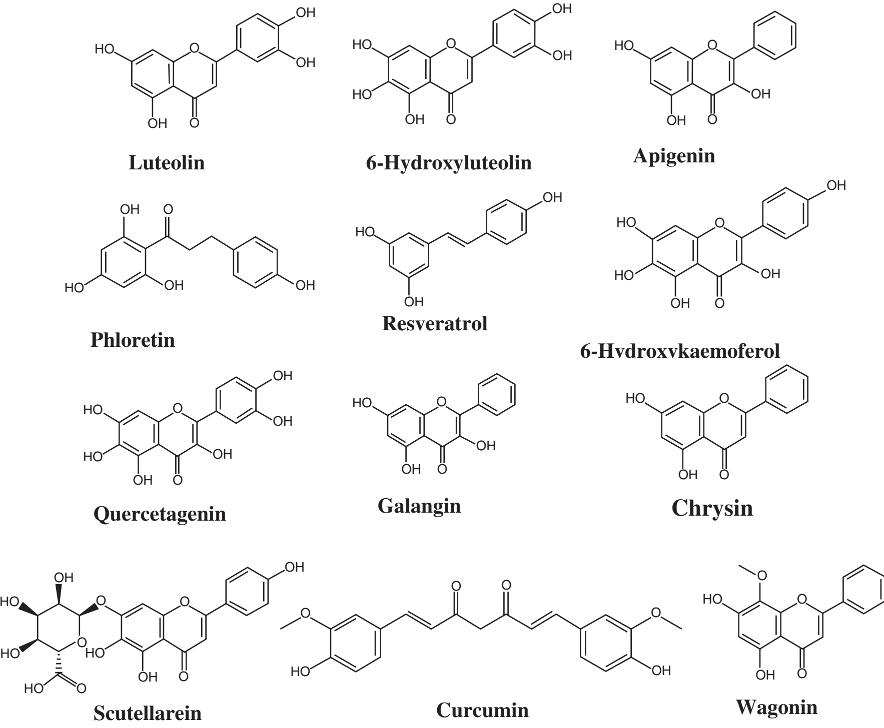

Fig. 9.24 Natural flavonoids as cyclooxygenase inhibitors

9.2.2.6 Structure-Activity Relationships of Biological Compounds with COX Enzyme

Polyphenols and flavonoids are plant secondary metabolites which are antioxidants and protect our body from harmful effects. There are different classes of flavonoids based on their structural modifications. Polyphenols may be classified into four groups: flavonoids, stilbenes, lignans and phenolic acids. Polyphenols found in diet, such as galangin and luteolin, inhibit AA peroxidation. Flavones, including apigenein, chrysin and phloretin, inhibit platelet aggregation and showed depressed COX activity. Flavonoids from *Tanacetum parthenium*, 6-hydroxykaempferol, quercetagenin, 6-hydroxyluteolin and scutellarein from *T. vulgaris* (Zhang et al. 2006) were found to inhibit COX activity in leukocytes. Resveratrol a stilbene compound was found suppress carrageenan-induced pedal oedema via the inhibition of COX activity. The golden compound curcumin showed significant inhibitory effects on peroxidase activity of COX 1 than that of COX 2. Chinese herbs, *Scutellaria baicalensis* Georgi and *S. barbata* D. Don, contain wogonin flavonoid which inhibited 12-myristate 13-acetate (PMA)-induced COX 2 expression. Quercetin also inhibted leukocyte infiltration in mice, therby inhibting cycloxygenase action (Kim et al. 2013) (Fig. 9.24).

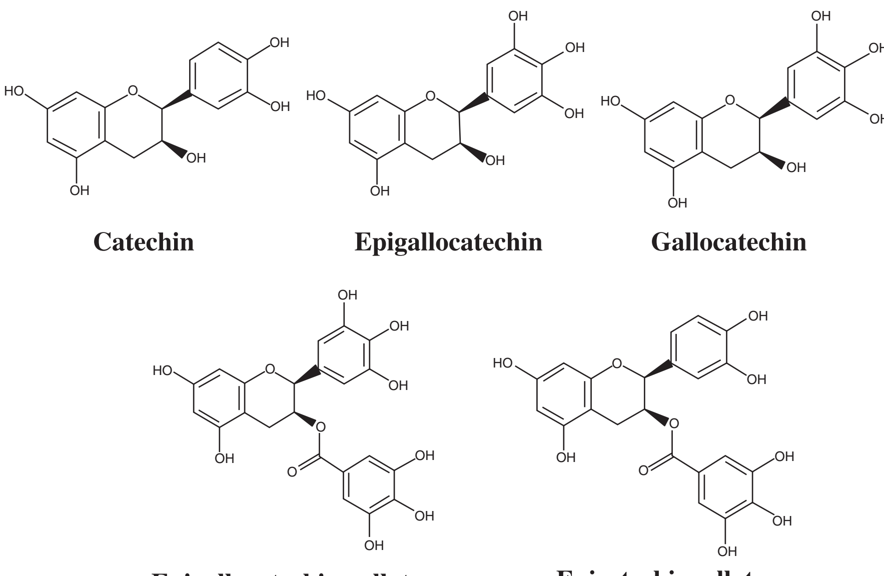

Fig. 9.25 Natural proanthocyanidins as cyclooxygenase inhibitor

Structure-activity relationship studies with different proanthocyanidins exposed that the galloyl moiety of proanthocyanidins is significant for their inhibitory actions. Furthermore, (−)- epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-gallocatechin (GC), (−)-epicatechin gallate (ECG) and (−)-catechin gallate (CG) have been revealed to have an inhibitory effect on COX 1/COX 2 enzymes in human and mouse cell lines (Fig. 9.25).

Prenylated flavonoids are typical flavonoids with isoprenyl, geranyl, 1,1-dimethylallyl and/or a lavandulyl moiety as part of their flavonoid backbone structure. Among these morusin, kuwanon C, sanggenon D and kazinol B inhibit COX 2 (IC_{50} = 73–100 μM), but less inhibitory than that of NS-398 (IC_{50} = 2.9 μM). Other C-8 lavandulyl moiety contains compounds kuraridin, kuwanon C and sophoraisoflavanone A which inhibit COX-1 with high efficiency (IC_{50} = 0.1 to 1 μM) comparable to that of indomethacin (IC_{50} = 0.7 μM) (Chi et al. 2001) (Fig. 9.26).

COX-1 selective inhibition was revealed by acetyl-alpha-boswellic acid, beta-boswellic acid, acetyl-11-keto-beta-boswellic acid, acetyl-beta-boswellic acid and betulinic acid with IC_{50} values of approximately 10 μM. COX 2 inhibitory effect was also given by Senkyunolide O and cryptotanshinone with IC_{50} values of 5 μM and 22 μM, respectively. Phenethyl-trans-ferulate (31 ± 15 μm) and roburic acid (9 ± 3 μm) (Fig. 9.27) also have COX 2 inhibitory effects (Cao et al. 2010).

Ginger constituents and their synthetic derivatives are found to be inhibiting COX enzyme with an IC_{50} value ranging from 1 to 25 μM. The constituents include

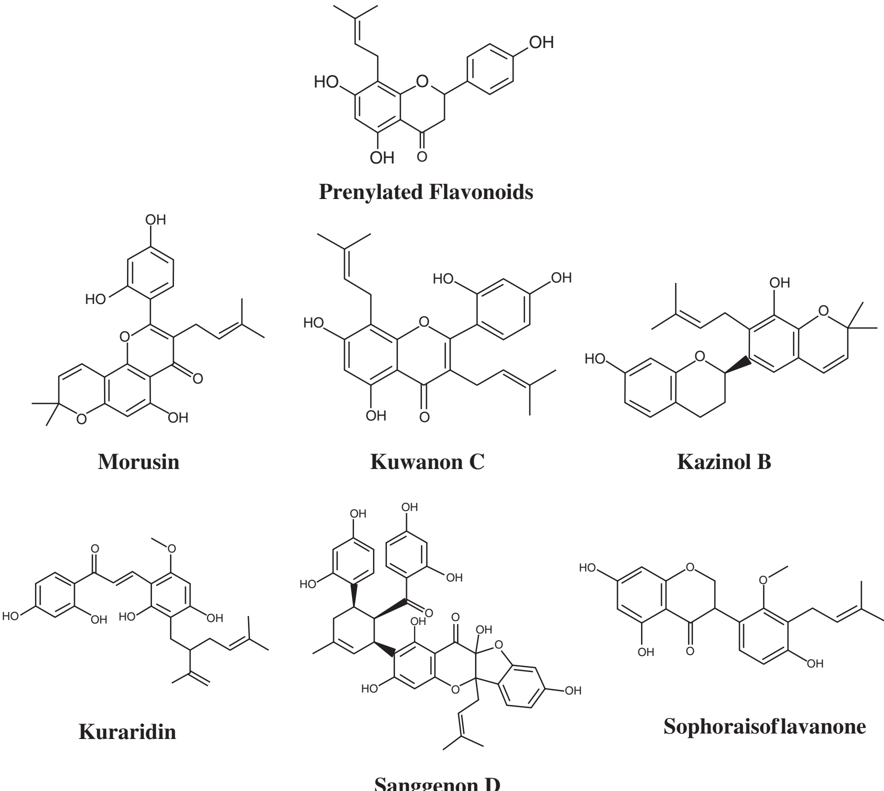

Fig. 9.26 Natural prenylated flavonoids as cyclooxygenase inhibitor

[8]-paradol and [8]-shogaol, 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) decane and 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl) dodecane. 6-Shogaol (IC_{50}–2.1 μm), 10-Gingerol (IC_{50}–32.0 ± 1.5 μM), 6-Gingerol (IC_{50} > 50 μm), 8-Gingerol (IC_{50}–10 μm) also have same inhibitory effect on Cycloxygenase (Van Breemen et al. 2011) (Fig. 9.28).

9.2.3 Lipoxygenase Inhibitors

Arachidonic acid metabolites like leukotrienes are produced via lipoxygenase pathway. Mammalian LOX are generally classified into five types based on the oxidation of arachidonic acid, and they are 5-LOX, 8-LOX, 11-LOX, 12-LOX and 15-LOX. Arachidonic acid and linoleic acid are the natural substrates of LOX. Linoleic acid is the standard substrate for plant LOX, whereas arachidonic acid is considered as the substrate for mammalian LOX. Hydroperoxides of these substrate are the major products.

Fig. 9.27 Natural cyclooxygenase inhibitors

Acetyl-11-keto-beta-boswellic acid

Acetyl alpha- -boswellic acid

Beta –boswellic acid

Acetyl beta- boswellic acid

Betulinic Acid

Senkyunolide O

Cryptotanshinone

Roburic Acid

Phenethyl-Trans-Ferulate

Mammalian 15-LOX oxidizes linoleic acid and plant LOX oxidizes arachidonic acid. In this aspect we can conclude that soyabean lipoxygenase is similar to mammalian 15-LOX in many aspects, hence LOX inhibitors from plants may be beneficial for the development of new drugs to manage diseases related to arachidonic acid metabolism.

10-Gingerol **8-Gingerol**

6-Gingerol **6-shogaol**

8-shogaol **8-paradol**

3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) decane **5-hydroxy-1-(4-hydroxy-3-methoxyphenyl) dodecane**

Fig. 9.28 Cyclooxygenase inhibitors from ginger

5-LOX-derived arachidonic acid metabolites are considered to represent potent mediators of inflammatory reactions (Kang et al. 2015). Considerable evidence has shown that the 5-LOX pathway plays a role in developing allergic diseases such as asthma and many inflammatory disorders such as rheumatoid arthritis and cardiovascular disease. Lipoxygenase pathway has a role in tumourigenesis, especially in the development of certain forms of leukaemia (Steinhilber and Hofmann 2014). The combined action of 5-LOX and 12-LOX or 15-LOX can lead to lipoxins (LXs) (Werz and Steinhilber 2006), which are bioactive trihydroxytetraene-containing lipid mediators. They appear to function as stop signals for inflammatory responses and to promote repair and wound healing (Batt 1992).

9.2.3.1 Structure of Lipoxygenase

Lipoxygenase is a single ~ 75–80 kDa polypeptide in animals and ~94–104 kDa in plants. The proteins have a larger catalytic domain containing a single atom of non-heme iron and N terminal β-barrel domain structure. The metal is liganded to the

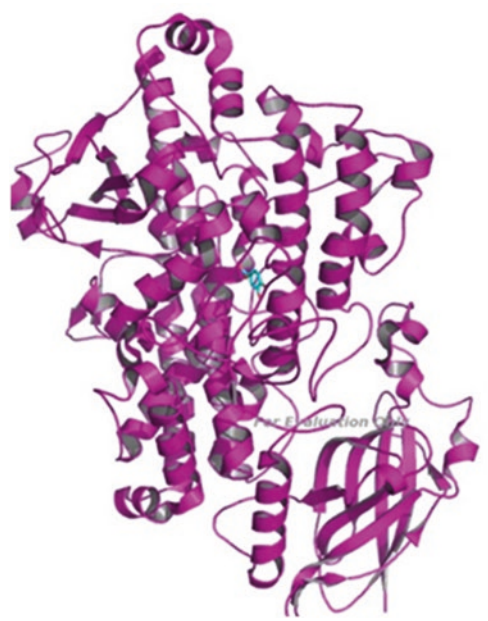

Fig. 9.29 Structure of lipoxygenase (PDB ID: 1N8Q)

carboxyl group of a conserved isoleucine at the C terminus of the protein and conserved histidines. The active site non-heme iron of catalytic domain serves as both oxidizing and reducing agents. During catalysis the inactive ferrous form of enzyme is converted to active ferric form. This is favoured by lipid hydroperoxides. The octahedral arrangement shown by the amino acids close to iron is at the corners of a tiltedbipyramid (Brash 1999).

Soybean lipoxygenase-1 is a 95 kDa polypeptide. The 839 residues of this enzyme were organized in N-terminal domain (domain I, 146 residue) and C-terminal domain (domain II, 693 residues). The structure of lipoxygenase-3, another soybean lipoxygenase isozyme, is very similar with a huge difference in sequence. Soybean seed isoenzymes are 94–97 kDa monomeric proteins with distinct isoelectric points found within 5.7–6.4. They may be distinguished by optimum pH, substrate specificity, product formation and stability (Fig. 9.29).

LOX Inhibitors Starting from the early 1980s until today, there are reports on the suppression of biosynthesis of LOX products by plant extracts or specific bioactive compounds present in the plant extracts. Experiments in isolated cells from human, rat or mice sources were carried out for analysing the plant-derived compounds having the ability to block the LT synthesis. Nordihydroguaiaretic acid (NDGA) a polyphenol from Mexican dessert plant *Larrea divaricata* was the first plant-derived 5-LOX inhibitor. Caffeic acid, eupatilin and 4-demethyl eupatilin from *Artemisia rubris* inhibited 5-LOX activity in a cell-free assay as well as the formation of LTC4 and D4 in ionophore-stimulated mastocytoma cells. In parallel, coumarin like esculetin, fraxetin, daphnetin and other coumarin derivatives also identified as 5-LOX

inhibitors. Another group of flavonoids including cirsiliol, sideritoflavone, hypolaetin glucoside, hypolaetin, oroxindin, quercetagetin glucoside, hibifolin, gossypetin and gossypin are identified as in vitro inhibitors. It is assumed that the 5-LOX inhibition by these compounds is due to the catechol moiety present in them, which has combined antioxidant and iron-chelating properties. Plant-derived compounds which mimic fatty (arachidonic) acid structures also inhibit 5-LOX by binding and/or competing at the substrate-binding site. It may also be proposed as the fatty acid-binding cleft of 5-LOX. Thus these compounds are found to be good 5-LOX inhibitors in vitro and in vivo.

9.2.3.2 Synthetic Inhibitors of 5-LOX

5-LOX inhibitors are grouped into three main categories: (i) Redox active compounds, (ii) Iron-ligand inhibitor with weak redox property and (iii) non-redox-type inhibitors (Werz and Steinhilber 2005). Rational inhibitor development led to a number of potent and orally active 5-LOX inhibitors with weak redox properties, such as AA-861, BW755C or ICI-207968 (Werz 2004). However, these compounds were more or less nonspecific and did not enter the market due to severe side effects. Iron-ligand inhibitors that chelate the active site iron are potent 5-LOX inhibitors. BW-A4C is a selective inhibitor of 5-LOX coming under the hydroxamic acid member, with an IC_{50} of 40 nM in stimulated granulocytes. However, fast inactivation and the formation of toxic nitroxide radicals make the search for alternative ligand groups essential (Steinhilber and Hofmann 2014).

This led to the development of the hydrolytic, stable N-hydroxy urea derivative A-64077 (zileuton), which is available in the USA for the treatment of asthma. In a panel of cells from animals (rat, mouse, guinea pig and monkey) or humans, challenged by a variety of stimuli, zileuton gave consistently IC_{50} values of 0.5–1 μM (Rossi et al. 2010). Clinical studies reveal its potency in asthmatic conditions and low effectiveness in other inflammatory disorders. Also attempts for increasing its potency and oral half-life led to the development of new drug ABT-761 which inhibits LOX with an IC_{50} of 23 nM. This new drug is also found to be fivefold more potent than zileuton in bronchospasm with an oral half-life of 16 hrs (Rossi et al. 2010).

Non-redox-type inhibitors are compound-specific and depend on the assay conditions. The efficacy of these type inhibitors (ZM 230487, L-739,010) in cells relay on the stimuli, peroxide level and phosphorylation status of 5-LOX enzyme (Werz 2004). LOX inhibitors, linoleylhydroxamic acid (LHA) is a simple chemical derivative of the naturally occurring LA, inhibit LOXs at micromolar concentrations. A seleno-organic compound, Ebselen, also exhibits anti-inflammatory activity.

Most of the synthetic inhibitors are designed by pharmacological companies and are patented. The molecules include sulphonamide (a) derivatives with an IC_{50} ranging from 100 to 500 nM. Diaryl-pyrimidines (b) inhibit LTB_4 production in intact rat neutrophils with IC_{50} values ranging from 0.32 to 0.97 μM. 3-O-acetyl-11-keto--beta-boswellic acid (AKBA) analogues (c) are also found to be good inhibitors. Carboxyl group in 3-O-acetyl-11-keto-beta-boswellic acid was replaced by a methylcarbamoyl moiety and showed in vitro and in vivo inhibition. Another derivative

Fig. 9.30 Synthetic 5-LOX inhibitors

(d) of 3-O-acetyl-11-keto-beta-boswellic acid showed 16.3% paw oedema inhibition. 1-Indolizine derivatives and 3-indolizine derivatives (e, f) showed $IC_{50} < 0.3$ μMin a human leukocyte assay. Benzothiophene compounds inhibited LTB_4 and cysteinyl LT production with an $IC_{50} < 0.3$ μM. Hydroxamic acid-derived 5-LOX inhibitor with sulphonamide linker was also reported to be a better inhibitor (h). Cimiracemate A and their derivatives are undergoing further in-depth study. Cimiracemate A inhibited potato 5-LOX with $IC_{50} \sim 70$ μM (i) (Fig. 9.30) (Hofmann and Steinhilber 2013).

9.2.3.3 Current Status of Lipoxygeanse Drugs

FDA-approved 5-LOX inhibitory drug was Zileuton, but it exhibits liver toxicity and has a shorter half-life. Most advanced N-hydroxyurea derivative (Atreleuton; VIA-2291) drugs are under phase II trials for the treatment of cardiovascular diseases and vascular inflammation (Hofmann and Steinhilber 2013). Setileuton (MK0633) amino oxadiazole substituted coumarin and completed phase II trials for asthma, atherosclerosis and COPD. Selective non-redox-type inhibitor PF4191834 (Pfizer) completed phase II trial for asthma and terminated for use in knee arthritis (Steinhilber and Hofmann 2014). FDA-regulated drug flavocoxid rich in baicalin and catechin inhibiting COX-2/LOX-5 is under trial for the treatment of knee arthritis. Drugs which are under phase II trials are indicated in Fig. 9.31 (Steinhilber and Hofmann 2014). Drugs which are under phase III clinical trials include Licofelon and Veliflapon (DG-031) for knee arthritis and myocardial infarction and stroke, respectively, which inhibit FLAP (Fig. 9.32).

Zileuton

Atreleuton

Setileuton

Baicalin

Fig. 9.31 5-LOX inhibitors under Phase II trials

Fig. 9.32 FLAP inhibitors under Phase III clinical trials

Licofelone

Veliflapon

9.2.4 Status of Traditional Medicines as Anti-inflammatory Agents

Most of the diseases primarily progress with inflammatory process and currently available anti-inflammatory therapies are futile due to intolerable side effects. Thus new pharmacological approaches are needed and plant-based therapies are now under consideration. Traditional systems of medicines are now well in practice, and their scientific validations are required. Different traditional systems include Indian Ayurveda, European-based plant collections, Kampo system and Chinese traditional medicine. In most of the developing countries, people rely on these medicines to meet their primary health care. They cannot afford the price of the available drugs. By the nineteenth century we came to know that the chemical compounds in the plants are responsible for the pharmacological action and can be isolated and used as single agents. Prominent plant-derived compounds include vincristine, vinblastine, galantamine, paclitaxel, capsaicin, morphine, quinine, colchicine, atropine and pilocarpine.

Curcumin, an important constituent of *Curcuma longa*, has been used for years for curing inflammatory disorders in Ayurveda. Curcumin has a wide variety of

pharmacological action and work is going on for its clinical application and is under phase trials. Colchicine, another well-studied compound from *Colchicum autumnale*, and FDA approved this as a drug for treatment of familial Mediterranean fever as well as for the treatment and prevention of acute gout flares. Resveratrol, a stilbene derivative and phytoalexin are found in many plants and in grape fruits. Similar to Curcumin, resveratrol also has high pharmacological activities and studies are ongoing to manage its poor bioavailability. Quercetin is another herbal compound which is also under phase II trial for chronic obstructive pulmonary disease (COPD) and diabetes (Furst and Zundorf 2014).

Most of the ayurvedic formulations like *asava* and *arishtas* are developed from these plants and are rich source of bioactive molecules. Combined effect of these molecules leads to the cure of diseases. Ayurvedic formulations are prepared through fermentation which also leads to the structural modifications in bioactive molecules and can lead to better remedial power. Fermentation is a method in Ayurveda for the preparation of medicines but an authentic reason for the preference of this method is not yet explored. These fermentative steps are similar to the digestive transformation and metabolic transformation as described in classical texts of the science of Ayurveda (Chandra et al. 2015). Traditional fermentation in ayurvedic preparation takes place with the help of Woodfordia flowers rich in yeast. Our earlier reports suggest that the fermentation transforms the compounds present in the materials used in different medicinal preparations (Chandra et al. 2015). Such transformed products have significantly increased pharmacological activity than normal compounds. Berberine, an isoquinoline alkaloid found in various plants (Chandra et al. 2015) and has so many biological properties, was found inhibiting PLA_2 and its microbially transformed (Woodfordia as starter culture) product dihydroxyberberine has increased inhibition on PLA_2 (Naveen et al. 2013). Cuminaldehyde isolated from cumin seeds is a good 5- LOX inhibitor (Tomy et al. 2014a) and benzoic acid, isolated from polyherbal preparations, also has higher inhibitory potential for lipoxygenase enzymes. in vitro and in silico validations of these enzymes are a good outcome for developing new drugs from polyherbals. Black pepper, used in many ayurvedic formulations, and its constituent piperine have inhibitory activity against COX 1 and LOX. Bio-transformed derivatives of piperine showed better inhibition than piperine for lipoxygenase enzyme (Tomy et al. 2014b) and further related studies will explain their use as better anti-inflammatory agents.

9.3 Conclusions

Inflammation is the initial step in most of the diseases and also has an important role in homeostasis. There are many mediators and their inhibitions are important in anti-inflammatory mechanism. Mainly, studies are based on the specific enzymes that come under the inflammatory pathways (PLA_2, COX and LOX) and their inhibition by molecules leading to the drug design process for inflammation. Rational drug design, the new method in drug development, produces many synthetic lead molecules suitable for these drug targets. Traditional herbs also contain a large consortium of bioactive molecules and they may also be explored to develop novel drug leads.

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Anti-inflammatory Molecules: Immune System Mediators

10

B.C. Bhavya and Madathilkovilakath Haridas

Abstract

Inflammation typifies the chief defensive mechanism against infection or injury. However, its obnoxious activation often elicits exaggerated immune response and the onset of clinically relevant detrimental responses. Suppression of inflammatory response by means of anti-inflammatory molecules is therefore weighed as a pivotal therapeutic solution to a wide range of diseases. The quest for newer and vital anti-inflammatory molecules has been considered as an active research vista for the development of potential drug candidates for a variety of pathological conditions. Inflammation and immune responses are inseparably linked in many instances, where the persisting inflammatory response serves as the key trigger for immune disorders. The immune system comprises immune cells and immunoglobulins, which are the major mediators of defensive response against obnoxious stimuli. Several anti-inflammatory molecules have been found to ameliorate immune reaction underlying many pathogenesis, via directly regulating production/function/activation of the prime components of the immune system and limit tissue damage. This chapter provides an insight into the ability of such anti-inflammatory molecules which target components of the immune system. The interplay of adaptive immunity and inflammatory mechanism in terms of chronic inflammatory disease pathogenesis is critically evaluated and demonstrated. An effort has been made to explain how this approach of inhibiting inflammatory targets qualifies as a potent therapeutic solution for immune disorders and transplant rejection. The emphasis is on identifying plausible anti-inflammatory agents targeting immune system mediators as a logical strategy for novel and efficient therapeutic interventions.

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Inflammation • Immune response • Immune system mediators • Anti-inflammatory agents • Chronic inflammatory diseases

10.1 Introduction

Inflammation entails the immediate defensive response to infection or injury. However, persistent inflammatory response often stimulates undesired immune responses resulting in detrimental effects. Exaggerated immune response promotes the onset of autoimmune, hypersensitive, and inflammatory disease pathogenesis. Inappropriate immune response is demonstrated in transplant rejections as well. Unregulated inflammatory response-initiated immune cell activation has been identified as a prominent contributor to metabolic, neurological, and cardiovascular disorders as well as cancer malignancies. Ongoing immune responses may eventually lead to detrimental effects causing irreparable tissue damage and loss of function. Anti-inflammatory agents with potential caliber to modulate the components of immune system may offer a competent treatment option against inflammation-linked immune disorders.

10.1.1 Immune Response and Mediators

Immunity refers to the capacity of the body to avail protection against infectious diseases and is classified broadly into innate and adaptive immune responses. Innate immunity is evolutionarily the older form of defense and facilitates the first line of protection against infection mediated via a variety of cells: neutrophils, polymorphonuclear leukocytes, eosinophils, basophils, mast cells, megakaryocytes, and platelets. Innate defensive strategy also involves barriers such as anatomic (skin and mucosal membrane), physiologic (pH, temperature, chemical mediators), phagocytic, endocytic, and inflammatory responses. Inflammation is an innate immune response to infection or injury and mediated through complement activation and phagocytosis. Innate immune response can integrate with adaptive immunity by antigen presentation to specific T lymphocytes via macrophages. Adaptive immunity is activated in response to an antigenic challenge to the organism. T lymphocytes and immunoglobulins are the major executors. Regulation of immune response can be brought about by various mechanisms. Antigen-presenting cells (APC) costimulate T cells by promoting cytokine and chemokine synthesis. T cells regulate immune response through subsets such as TH1, TH2, and TH17. Regulatory T cells belong to CD4 or CD8 subpopulations and inhibit immune response via anti-inflammatory cytokines interleukin (IL)-10 and TGF-β. Neuroendocrine (corticosteroids) and genetic factors (MHC) can also regulate immune response. Cytokines constitute major inflammatory mediators with direct impact on immune regulation. The immune system requires both cellular and humoral factors for its induction and

execution. Several of these cellular components serve as the major mediators of both immune response and inflammation. Cytokines, a prominent inflammatory mediator class, regulate differentiation of various cellular components of innate and adaptive immunities.

10.1.2 Inflammation and Its Players

Inflammatory response upon pathogenic encounter is evoked when the host cells recognize specific, conserved molecules termed as PAMPs (pathogen-associated molecular pattern) present on the surface of the pathogen and express PRRs (pattern recognition receptors), which belong to the Toll-like family of receptors (TLRs). The TLRs recognize the lipopolysaccharide-like PAMP present on the surface of microorganisms. On the other hand, injured tissue following mechanical or chemical exposure expresses damage-associated molecular patterns (DAMPs) that can be recognized by the TLRs (El-Asrar 2012). This would initiate the release of inflammatory mediators to recruit more immune cells to the site in order to re-establish homeostasis via remodeling or elimination of damaged cells or pathogens by complement activation and phagocytosis respectively.

Inflammatory response is broadly divided into acute and chronic on account of its duration. Acute inflammation sets off once the irritant has been removed and the body is adequately protected. However, chronic inflammation occurs when the acute response fails to remove the noxious agent or if the tissue homeostasis is not re-established, eventually leading to undesired immune reactions which may impair tissue or organ function. Persistent inflammatory responses act as catalysts for the development of many pathological manifestations. The link between inflammation and the development of various pathogeneses is shown in Fig. 10.1.

Acute inflammation proceeds through vascular and cellular events. Vascular events are the initial response to inflammatory stimuli and are induced by the plasma-derived factors released by the injured tissue. Histamine and bradykinin are the important mediators of vascular events. During vasodilation, volume of blood in the area near the injury increases, resulting in redness (erythema). Vascular permeabilization facilitates influx of fluid and cells from the dilated capillaries into the site of injury, resulting in edema. Endothelial gap formation facilitates neutrophil leakage, extravasation to the site of injury for phagocytosis, and clearance of the pathogen. Exudation of fluid activates kinin, clotting, and fibrinolytic systems which facilitate the cellular events associated with acute inflammation. Cellular events encompass the influx of leukocytes from the capillaries into the site of injury (leukocyte extravasation) facilitated by chemokines. They could accomplish phagocytotic clearance of the foreign component or damaged cells. However, in case of chronic inflammatory response, either of the immune responses or both are activated in an attempt to counteract the cause of inflammation. Inflammation is initiated by the release of a vast array of mediators or effectors from injured tissues or invading pathogens and immunologically activated cells.

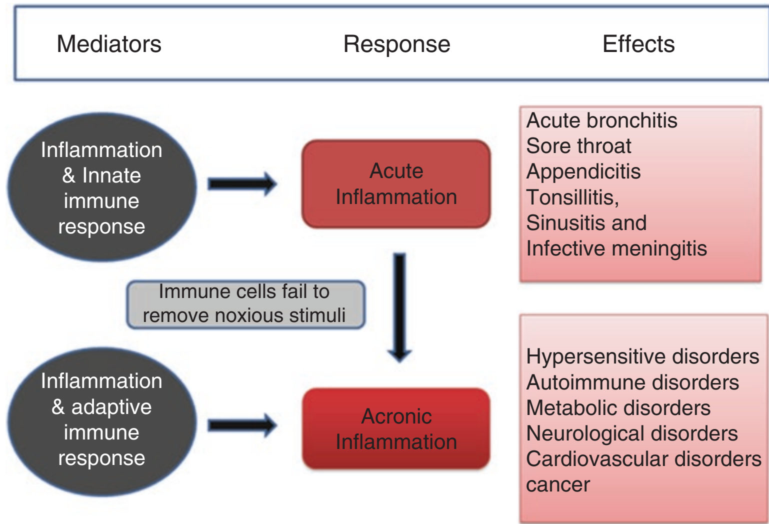

Fig. 10.1 *Acute and Chronic inflammation in the development of diseases:* Acute inflammation usually renders protection; however, severe response elicits certain pathogenic conditions triggered by innate and inflammatory mediators. When acute inflammation fails to protect, adaptive components of the immune response will be activated, which leads to various disease pathogeneses, as has been shown

Table 10.1 Plasma-derived inflammatory mediators and their functions

Name	Examples	Action
Hageman factor/factor 12a		Activate all four systems below
Kinin system	Bradykinin	Bradykinin production, bronchoconstriction
Clotting system	Thrombin	VD, chemotactic for WBCs
Fibrinolytic system		Formation of plasma, VD, conversion of C3a to C5a
Complement system	C3a, C5a	Phagocytosis, leukocyte activation, stimulation of histamine release, LOX activation

Inflammatory mediators include cell-derived and plasma-derived components. Plasma-derived factors involve four interlinked systems such as kinin, clotting, fibrinolytic, and complement systems. Major cell-derived factors include histamine, cytokines, prostaglandins, and leukotrienes. Cellular players of innate immune response are the major mediators of inflammation. Table 10.1 describes the different types and the respective functions of each plasma derived mediators of inflammation (Renné et al. 2012; Bas et al. 2007; Mosesson et al. 2001; Sharma et al. 2011).

10.2 Inflammatory Mediators with Immune Modulation

The existence of crosstalk between innate and adaptive immunities is well established. The interdependence of innate and adaptive immunity is found to be mediated by various inflammatory mediators. The major proinflammatory mediators

Table 10.2 Important cellular inflammatory mediators with their functions

Name	Examples	Source	Action
Vasoactive amines	1. Histamine	Mast cells, basophils	Vasodilation, increase vascular permeability, activation of endothelial cell
	2. Serotonin	Platelets	Vasodilation, stimulate vascular permeability
Prostaglandins	1. PGD2	Endothelial cells,	Vasodilation, edema,
	2. PGI2		Inhibit platelet aggregation,
	3. TXA2	Platelets	Vascular constriction, stimulate platelet aggregation
Leukotrienes	1. LTB4	Neutrophils, macrophages, mast cells	Chemotactic agent for neutrophils
	2. LTC4, LTD4, LTE4		Vascular constriction bronchospasm
	3. LTG4		Endogenous antagonists of leukotrienes
Platelet activating factor (PAF)		Leukocytes and endothelial cells	Vascular constriction, bronchoconstriction
Cytokines	TNF and interleukins, chemokines	Leukocytes, macrophages, lymphocytes	Inflammation and immunomodulation
ROS		Macrophages, neutrophils	Cytokine synthesis, phagocytosis, necrosis
NO		Neurons, macrophages, endothelial cells	Vasodilation, antimicrobial property, WBC recruitment
Lysosomal enzymes	Acid protease	WBCs	Phagocytosis
	Neutral peptides		

with potential immune-modulating effects include, cytokines, histamines, leukotrienes, and complement components, of which cytokines are considered as the prominent immune-modulators (Abramson et al. 1981; Tesch and König 1979; Soter and Austen 1976). These inflammatory molecules can either up- or down regulate immune response by positively or negatively influencing immune system mediators. Table 10.2 explains the major cellular mediators of inflammation, their origin and activities, which are elaborately explained further in later sections of the chapter.

10.2.1 Complement System

Complement system is the highly regulated humoral component of immune or inflammatory response and primarily involved in eliminating cellular debris and immune complexes. Phagocytosis and opsonization are the two elite functions of the complement system by which it inhibits pathogen invasion. Complement system

is one of the crucial entities linking innate and adaptive immune responses and thus imparts immunomodulatory activities in both branches of immune response. Complement components could mediate several immune activities, including stimulation of T cells and antibody activities, removal of self-reactive B cells, and augmentation of immunologic memory. Components of the complement system are synthesized in pro form and later activated on demand via three diverse pathways. The antigen–antibody complex (usually IgM or IgG) initiates the classical pathway and C5 split product is generated from C5 by C5 convertase in the classical pathway. Alternative pathway is activated by microbial surfaces and complex polysaccharides (yeast cell walls, endotoxins, or viral particles), and C3 is converted into C3a and C3b by the C3 convertases. These split products C5b, C6, C7, C8, and C9 associate to form a macromolecular membrane attack complex (MAC) and directly lyze the target cell by opsonization. Complement split products can carry out a number of crucial functions such as disintegrating bacteria, damaged cells, and immune complexes from circulation, accelerating the phagocytosis process via opsonization, and eliciting the release of immunoregulatory molecules. Components of the complement can be produced from many cells such as hepatocytes, monocytes, macrophages, etc. The three anaphylatoxins—C3a, C4a, and C5a—released during the cascade of enzymatic cleavage are potential inflammatory mediators. The anaphylatoxins mediate smooth-muscle contraction and increase vascular permeability and aid in histamine release. Extravasation and chemotaxis of leukocytes at the inflammatory site is promoted by C3a, C5a, C5b, C3a, C5a, and C5b6. Activation of the complement system results in influxes of fluid that carries antibodies and phagocytic cells to the site of antigen entry. Some of the active split products of complement regulate immune response by directly or indirectly acting on the effectors of immune response. C3a molecules suppress immune response by acting through macrophages. C3b inhibits leukocyte extravasation, thereby directly modulating immune reactions. On the other hand, C5a augments antibody production and also indirectly activates T-cell proliferation. The production of LPS-induced IL-6 is found to be regulated via C5a. Similarly, complement activation is inherently regulated by interfering in the dissociation or association of C3 convertase. The key endogenous proteins involved in the regulation of complement include factor 1, factor H, CR1 (complement receptor 1), CR2 (complement receptor 2), MCP (membrane cofactor protein), DAF (decay acceleration factor), etc. (Sarma and Ward 2011).

10.2.2 Histamine and Its Receptors

Histamine is a potent vasoactive amine which plays a crucial function in early stages of inflammation. It is produced by basophils and mast cells via decarboxylation of the amino acid histidine. Its primary function is vasodilation, and thus regulates many physiological processes involving dilation of vessels and muscles, such as

blood pressure regulation, gastric acid release. Histamine gets activated by binding with specific receptors of seven transmembrane-spanning receptor families. Four types of histamine receptors are identified, viz., H_1R, H_2R, H_3R, and H_4R. The cellular response triggered by histamine depends on the type of receptor it binds with. Recent findings indicate their role in immune regulation as well.

Histamine binding with HR1 promotes allergic reaction accompanied by the production and secretion of mucus. Histamine triggers hypersensitive reaction. It elicits enhanced expression of P-selectin. It plays a major role in immune system disorders as it can regulate many cellular immune system mediators, such as NK cells, T and B lymphocytes. Monocytes and dendritic cells express all four known histamine receptors. Histamine activates TH2 cells and immunoglobulin E and thus augments adaptive immune response. H1R and H2R play an active role in immune regulation. The antigen-presenting ability of the dendritic cells is potentiated by histamine binding with H1R and H3R. H1R is found to positively modulate TH1 and TH2 functions and thus amplify immune response. Histamine acting through receptor HR2 enhances the peripheral antigen tolerance induced by T regulatory (TReg) cells and thus plays a direct role in regulating cell-mediated immune response.

Histamine docking with H1R contributes to sustained chronic inflammation leading to rhinitis and asthma. Histamine has been shown to suppress the release of IL-12 while stimulating that of IL-10 and increasing TH2 response. Moreover, H1R activation could lead to the release of many proinflammatory mediators, viz., colony-stimulating factor, IL-1, IL-6, IL-8, IL-10, IL-11, eotaxin, RANTES, and tumor necrosis factor (TNF) α, through stimulation of many immunologically active cells. Histamine via H1R triggers the increased expression of cellular adhesion molecules, including intracellular adhesion molecule (ICAM-1), E-selectin, and leukocyte-associated antigen, thereby facilitating neutrophil extravasation to the site of inflammation that further shoots up immune response.

10.2.3 Eicosanoids

These are active fatty acid-based signaling molecules identified with a vital role in inflammation and immunity and the major eicosanoids include prostaglandins, prostacyclins, thromboxanes, leukotrienes, and lipoxins (Khanapure et al. 2007). The members may either augment or reduce inflammatory response. Eicosanoids are considered as second mediators in the process of inflammation, as their biosynthesis requires prior activation of other mediators like histamine. The release of phospholipase A2-mediated arachidonic acid (AA) from membrane phospholipids is the initial step in eicosanoid production. AA undergoes oxidative reactions by enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX) pathways to generate prostaglandins and leukotrienes respectively. Eicosanoid production is tremendously activated during inflammation.

10.2.4 Leukotrienes

Leukotrienes (LTs) are a class of eicosanoids which have inflammatory and immunomodulatory actions by stimulating the infiltration of immune cells. LTs are synthesized by the oxidation of arachidonic acid by arachidate-5-lipoxygenase from leukocytes. They have a prominent role in allergic hypersensitive reactions and facilitate mucus production. Leukotrienes are activated by coupling with specific receptors falling under G protein-coupled receptor families. Once activated, they enhance leukocyte accumulation, phagocytosis, and microbicidal activity. Leukotrienes facilitate immunomodulatory functions as well and regulate both innate and adaptive immune responses. LTs with inflammatory functions are divided into two classes, namely, LTB4, which carries hydroxyl moieties, and the cysteinyl LTs (LTC4, LTD4, and LTE4), which carry a cysteine amino acid moiety. Leukotriene B4 causes adhesion and chemotactic properties. It aids in superoxide generation in neutrophils. The cysteinyl leukotrienes are potent bronchoconstrictors and promote vascular permeability and mucus secretion. Thus, they are regarded as the principal components playing pathophysiological role in immediate hypersensitivity reactions. Among these, LTB4 is considered as the most potential inflammatory mediator. Agents inhibiting the biosynthesis of leukotrienes, such as LOX inhibitors, act as potential anti-inflammatory agents.

Chemically, leukotrienes are lipid molecules with hydroxyl or amino acid moieties. The presence of three conjugated double bonds is the common feature of leukotrienes. Based on the affinity toward different types of leukotrienes, the receptors are categorized into LTB4 receptor and cysteinyl LT receptors (cysLT1 and cysLT2). LTB4 receptors facilitate the binding of LTB4 and thereby facilitate leukocyte infiltration and promote release of cytokines and lysosomal enzymes from immune cells. Two receptors for the cysLTs, termed type 1 and type 2 cysLT receptors, have been identified so far (Chagnon et al. 1985; Parker 1982). These receptors are also extensively exploited as potential anti-inflammatory targets. LTD4 is the preferred binding agonist of the cysLT1 receptor, and LTC4 is the preferred binding partner of the cysLT2 receptor. This triggers bronchoconstriction, migration of eosinophils into the airways, and mucus secretion. CysLTs and their receptors are thus considered as the chief participants in asthmatic and allergic reactions.

10.2.5 Cytokines and Sub-families

These low-molecular-weight regulatory glycoproteins propagate many vital cellular events like immune response modulation, hematopoiesis, and inflammation. They are primarily secreted by various cells such as macrophages and T lymphocytes in response to a variety of immunologic or nonimmunologic stimuli. Cytokines are categorized on the basis of their biological response and effect on immunocytes and comprise both pro- and anti-inflammatory cytokines. Major proinflammatory cytokines include lymphokines, interferon, monokines, interleukin, colony-stimulating factor, and tumor necrosis factor (TNF) α and β. Cytokines regulate inflammatory

response and possess redundant and pleiotropic effect. Cytokines IL-1 (α and β) and TNF-α are involved in acute inflammation. Cytokines that mediate chronic inflammation are classified as follows: (a) those mediating humoral responses, such as IL-4, IL-5, IL-6, IL-7, and IL-13; and (b) those mediating cellular responses, such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons, transforming growth factor α, and tumor necrosis factor α and β (Cook 2004; Feghali and Wright 1997). IL-1 can significantly contribute to both acute and chronic inflammation. The activation of cytokine occurs by binding to the respective receptors on the surface of target cells, which would induce a cascade of signaling events comprising the alteration at the gene level triggering the production and release of cytokines. Cytokines exhibit autocrine, paracrine, and endocrine mode of actions. Certain subsets of cytokines possess immunomodulatory action as well by regulating T lymphocytes and macrophages. Some cytokines can in turn inhibit or activate other cytokines. Cytokines produced by leukocytes are interleukins, and so far about 25 of them have been identified. Certain cytokines influence the generation of subpopulations of helper T cells TH1 and TH2. Cytokine overproduction and activation has been found to associate with a number of pathological manifestations. The high concentrations of TNF-α secreted by mast cells may contribute to shock in systemic anaphylaxis. TNF-α and IL-1 are found to be overproduced in response to bacterial endotoxins and superantigens and trigger septic shock. Their activity is implicated in many lymphoid and myeloid cancers as well as in autoimmune diseases and diabetes.

Cytokines possess potential immune response-regulating activity and are mainly synthesized by immune cells. They regulate immunity by directly influencing synthesis/differentiation of different subsets of T lymphocytes and activation of a variety of immune cells such as macrophages, cytotoxic T cells, and NK cells to eliminate intracellular pathogens (Handa and Kowdley 2013; Jang et al. 2006). They also enhance humoral immunity by activating TH2 cells, stimulating the generation of immunoglobulin isotypes, and augmenting IgE-mediated immune responses. Interleukins (IL), a prominent subclass of cytokines, are bestowed with a potential role in immune cell functional regulation. IL-1 enhances lymphocyte function and inflammatory response. IL-2 activates T cells and NK cells, IL-5 and IL-6 regulate T-cell synthesis and B-cell proliferation, and macrophage activation. IL-10 stimulates mast cell replication but negatively regulate cellular immune response.

Molecular mass of all types of cytokines falls below 30 kDa. Irrespective of the variations in the amino acid sequences of these family members, all possess a similar degree of α-helical structure and little or no β sheet structure. The molecules share a similar polypeptide fold, with four α-helical regions in which each pair of helices run parallel to one another and are connected by loops (Rickert et al. 2005; Trivella et al. 2010). Activation of cytokine is mediated via binding with the respective receptors expressed by many types of cells. Biochemical characterization of cytokine receptors revealed the existence of five structurally diverse families of receptors such as immunoglobulin superfamily receptors, class I cytokine receptor family, class II cytokine receptor family, TNF receptor family, and chemokine receptor family. Class I cytokine receptor family consists of cytokine-binding

receptors with immune- and hematopoietic-related functions. The members of this receptor family possess four conserved cysteine residues and sequence motif comprising tryptophan–serine–(any amino acid)–tryptophan–serine residues in the extracellular domain. The class II cytokine receptors possess the conserved CCCC motifs, but lack the WSXWS motif present in class I cytokine receptors (class I cytokine) and lack tyrosine kinase domain for signaling activity. IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, and IL-12 are the ligands for class I receptors, and IFN-α and IL-10 are the ligands for class II receptor family. TNF receptor family allows the binding of ligands such as TNF-α and TNF-β, CD 40, and nerve growth factor. Chemokine receptor family has the affinity toward IL-8, RANETS, MIP1 (macrophage inflammatory protein), PF4 (platelet factor), etc.

Binding of a cytokine to the respective receptors induces dimerization of the receptor subunits, which leads to the activation of Janus kinase (JAK) by reciprocal phosphorylation. Activated JAK phosphorylates various tyrosine residues of the downstream signaling kinases, such as signal transducer and activator of transcription (STATs), their dimerization, and translocation to the nucleus, where they activate transcription of specific genes important in inflammation or immunomodulation. The secretion of cytokines and chemokines from cells is a fundamental response to injury and infection in the body. A wide range of cell types secrete cytokines including epithelial and endothelial cells. Innate immune cells such as macrophages/monocytes, dendritic cells (DCs), natural killer (NK) cells, mast cells, eosinophils, and neutrophils release a range of cytokines and regulate the immune response to injury or infection. T lymphocytes are the principal immune cells that secrete inflammatory and immunomodulatory cytokines in the adaptive immune response. Cytokine secretion integrates innate and adaptive immunities and thus regulates the overall immune response (Banyer et al. 2000).

Chemokines are an important class of cytokines with multiple potential. They are obsessed with chemotactic ability. Broadly chemokines are classified on the basis of their specificity toward receptors. The elevated levels of chemokines are often associated with a variety of tumor malignancies and many autoinflammatory and immune disorders as well. Chemokine receptors are considered as potent therapeutic targets in several immune disorders with inflammatory background, such as rheumatoid arthritis (RA), inflammatory bowel disorders (IBD), allergic diseases, and cancer. The elevated levels of chemokines are often associated with a variety of tumor malignancies and many autoinflammatory and immune disorders as well.

10.3 Chronic Inflammation and Onset of Immune-Related Malignancies

Chronic inflammation is an extended low-grade inflammatory response, often initiated when the acute inflammatory response fails to eliminate the invader or re-establish homeostasis. The shift from acute to chronic inflammation involves the participation of many components of the immune system with the commencement of adaptive immune response. Thus, chronic inflammatory response can act as a

bridge between innate and adaptive immunities. The activation of macrophages and lymphocytes in adaptive immune response would further augment the release of inflammatory mediators resulting in prolonged inflammation. The prolonged activation and accumulation of macrophages stimulate undesired immune reactions resulting in host cell damage accompanied by fibrosis. The damage and perturbation of homeostasis of the host system is regarded as a prime hallmark of chronic inflammation. The major cellular components in chronic inflammatory response are macrophages, lymphocytes, plasma cells, and fibroblasts. The chief chemical mediators involved in the development of chronic inflammatory responses include IFN-γ, TNF-α, and interleukins. TNF-α is a prominent cytokine found to play a crucial role in the development and progression of many immune-mediated diseases in which the ongoing chronic inflammatory response serves as a major contributing factor. Diseases such as rheumatoid arthritis, inflammatory bowel disorder, asthma, psoriasis, psoriatic arthritis, and ankylosing spondylitis are some with direct link to altered TNF activity. Controlling TNF activation by various means to reduce the inflammatory response toward ameliorating disease symptoms has been viewed as a potent therapeutic approach. TH1 cells, NK cells, and TC cells release IFN, while activated macrophages secrete TNF-α. IFN-γ can activate macrophages, and these activated macrophages overexpress class II MHC molecules. This leads to increased cytokine production and bactericidal activity with the release of more hydrolytic enzymes, ROS, and nitrogen intermediates which could eventually cause in-depth damage to tissues, thus aggravating the process of inflammation. TNF-α contributes to much of the tissue wasting associated with chronic inflammation. Activation of macrophages by TNF-α promotes its own transactivation and production. Moreover, TNF-α can act synergistically with IFN-γ to initiate chronic inflammation while inducing increased expression of ICAM-1 (intercellular adhesion molecule 1), E-selectin, and class I MHC molecules. This further facilitates the hyperinflux of cells to the site of inflammation and promotes tissue damage. This perpetual accumulation of immune cells at the site leads to fibrosis and granuloma formation, which are the major hallmarks of chronic inflammation. The persistent activation of macrophages leads to the formation of high endothelial venules (HEV) along the vasculature in the tertiary extralymphoid site of chronic infection or damage. This HEV-like region acts as the hub to recruit more leukocytes into the site, thus aggravating inflammatory response with the destruction of the normal cell as well. The extended inflammatory response consequently worsens the physiological and immunological balance of the host and consequences are manifested as pathogenic conditions. This HEV-like region has been observed in many chronic inflammatory diseases, including RA, Crohn's disease, ulcerative colitis, Graves' disease, ankylosing spondylitis, Hashimoto's thyroiditis, diabetes mellitus (Kadioglu and Sheldon 1996). Many disease conditions like hypertension, fibromyalgia, depression, etc., are reported to have inflammatory connections. Chronic inflammation is also associated with many autoimmune, hypersensitive, and neuroinflammatory diseases. The inadequately regulated chronic inflammation is a major risk factor for the development of many types of cancer.

10.3.1 Auto-inflammatory Diseases

Ongoing inflammatory response may act as the primary trigger for the development of certain diseases. It includes inflammatory, hypersensitive, autoimmune, and neurodegenerative diseases. Inflammatory reactions play a primary role in the pathophysiological conditions such as allergy, Crohn's disease and colitis, Hashimoto's thyroiditis, and Graves' disease. However, in some cases, the chronic inflammatory response may function as secondary trigger to enhancing the chance of initiation, progression, or regression of certain diseases. Erroneous activation of many inflammatory mediators has been found to directly aggravate immune responses, contributing to the symptomatic manifestations of a variety of diseases. Overactivation of interleukin 12 (IL-12) and IFN-γ is correlated with Crohn's disease, and IL-13 production is associated with the pathogenesis of colitis (Medzhitov 2008; Navab et al. 2008). TNF-α is the critical mediator of IBD pathogenesis. Moreover, TNF also substantially influences intestinal epithelial cell growth and thus supports bacteria-induced granulomas through the induction of MCP-1 production by endothelial cells. Inhibition of proinflammatory cytokines appears as a promising choice of therapy.

Atheroma formation in atherosclerosis is facilitated following the hyperinflammatory signals from leukotrienes and proinflammatory cytokines like IL-1, IL-6, and TNF-α. Inhibitors of leukotriene and phospholipase A_2 are suggested as the plausible targets of therapy. *Mycobacterium tuberculosis* infection provokes chronic inflammatory response, leading to granuloma formation and tissue damage. Proinflammatory cytokines such as TNF-α and IL-1 and chemokines from PMN and monocytes are overtly implicated in its pathogenesis. Proinflammatory responses have been actively related with the onset of several neurodegenerative disorders. However, the precise implications of the inflammatory response for neurodegenerative diseases have not been elucidated yet. IL-1b, TNF-α, and IL-6 are the major mediators involved in the pathophysiology of Alzheimer's disease. The overexpression of chemokines and its receptor CCR1 is observed in the brain tissues of Alzheimer's patients, suggesting their direct impact on neuronal cell damage. The inappropriate inflammatory response contributes severely to nerve cell damage in the central nervous system in other neuroinflammatory diseases such as Parkinson's disease and multiple sclerosis.

Inflammatory response has been inseparably linked with the pathogenesis of literally all types of cancers. Cytokine signaling can contribute to the progression of tumors either by stimulating proliferation, aiding in metastasis, promoting angiogenesis, or by conferring resistance to apoptosis (Grivennikov et al. 2010). Several leukocyte subpopulations appear to establish an inflammatory microenvironment in the epithelial-originated tumors. Cytokines (IL1, 6, 8, and 10, TNF-α, TGF-β, and colony-stimulating factors) have been reported in many instances with direct regulatory role in different stages of carcinogenesis.

Chemokines play crucial functions in facilitating cancer metastasis. NFκB (nuclear factor of kappa light polypeptide) and AP1 (activating protein 1) activated by inflammatory stimuli are the widely studied proteins and their role in the

development, progression, and regression of many types of aggressive cancers have been thoroughly established. Eicosanoid prostaglandin synthesizing enzyme, COX2, has been found to boost neoplastic transformation, angiogenesis, invasion, and metastasis (Greene et al. 2011). A potent COX2-specific anti-inflammatory drug, coxibs, has proved to be effective in suppressing inflammatory complications and the treatment of cancer.

Prevailing chronic inflammatory response in obese individuals results in deregulated immune system priming the initiation of type 2 diabetes as a result of devastating alterations in adipose tissue, liver, and pancreatic islets. The infiltration of immune cells occurs at the site of inflammation in the adipose tissue with the release of proinflammatory mediators such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1β), and interleukin-6 (IL-6) (El-Asrar 2012). This persistent inflammation promotes fibrosis and the permanent loss of tissues and organ failure that embody diabetic complications. Many proinflammatory cytokines and acute-phase reactants are involved in multiple metabolic pathways regulating insulin resistance and adipocyte function. Inflammatory enzymes such as COX, LOX, and cytochrome P450 enzymes are involved in the pathogenesis of type 1 and type 2 diabetes by interfering with pancreatic β-cell function and its destruction.

10.3.2 Allergic and Autoimmune Disorders

Asthma is an airway hypersensitivity disorder, initiated upon contraction with allergens, and results in hyperinflammatory response characterized by red and swollen bronchial tubes. The pathogenesis of allergic asthma is complex and grouped under chronic inflammatory disorder. Many inflammatory cells (mast cells, eosinophils, neutrophils, T lymphocytes, epithelial cells, and macrophages) are involved in this anaphylaxis with the release of a wide array of inflammatory mediators, viz., histamine, leukotrienes, NO (nitric oxide), and various cytokines. IgE (Immunoglobulin E) is the antibody primarily involved in the activation of allergic reactions. Once contracted with the antigen, IgE attaches to the cells expressing high affinity to IgE receptor (mast cell, basophils, dendritic cells, and lymphocytes) and triggers degranulation of the mast cell, aiding the release of inflammatory mediator histamine. The important cellular components mediating the asthmatic reaction include mast cells, eosinophils, neutrophils, dendritic cells, macrophages, and epithelial cells. However, among these, TH 2 lymphocytes are more significant as they are directly involved in IgE synthesis and also release a variety of cytokines such as IL-5, IL-4, IL-13 1 L-1β, and TNF-α. Although many proinflammatory mediators are strongly associated with asthma pathogenesis, histamines and leukotrienes are considered as the factors with the most devastating effects leading to the airway smooth-muscle constriction and mucus production. These reactions finally symptomatically appear as edema, cough, wheezing, and shortness of breath, characterizing asthmatic manifestation. Antihistamine- and corticosteroid-based therapeutics are widely employed for asthma treatment. However, leukotriene inhibitors are also emerging as a preferred treatment option.

Erroneous activation of many inflammatory mediators is found associated with many autoimmune disease manifestations, suggesting the inflammatory connection in the pathophysiology of autoimmune disease development. Autoimmunity is classified as type III hypersensitivity reactions in which the autoantibody binds to self-antigens present in tissue or in circulation. The resulting freely circulating complexes may eventually become heavy and insoluble over time, resulting in their accumulation in the tissues and blood vessels, which ultimately triggers the activation of complement and T cells, leading to uncontrolled inflammatory reactions. As a direct consequence, localized cell death occurs, and the function of the tissue or even the organ would be lost due to blockage of blood vessels by clot formation (e.g. vasculitis in systemic lupus erythematosus). T cells sensitized to self-antigens may cause tissue damage through the release of lymphokines (e.g., TNF), which either directly damage the tissues or attract inflammatory cells to the site. These events initiated by self-reactive T cells result in the type of pathology typically associated with type IV hypersensitivity. Current therapies for autoimmune diseases include treatment with immune-compromising drugs, thymectomy, and plasmapheresis, and for diseases involving immune complexes, T-cells-specific vaccination for a given autoantigen, administration of synthetic blocking peptides that compete with autoantigen and, treatment with monoclonal antibodies that react with some component specifically involved in an autoimmune/inflammatory reaction. Autoimmune disorders are either mediated by autoantibodies or activated TH1 cells. Goodpasture's disease is caused by the autoantibodies activated against basement membrane antigens of the kidney glomeruli and lung alveoli followed by the activation of complement split products leading to progressive nephropathy and pulmonary hemorrhage. Etiologically, type 2 diabetes is also categorized as an autoimmune disease in which the autoimmune attack on pancreatic β cells via activated cytotoxic T cells and autoantibodies leads to clinical complications of insulin deficiency. Local production of many cytokines such as IFN-γ, TNF-α, and IL-1 are increased during its pathogenesis, thereby aggravating the disease complications. The systemic autoimmune diseases are directed against a broad spectrum of tissues and have manifestations in a variety of organs resulting from cell-mediated responses and cellular damage caused by autoantibodies or immune complexes. A number of autoimmune diseases stem from circulating complexes of antibody with self-proteins, with glycoproteins, or even with DNA such as in systemic lupus erythematosus. Rheumatoid arthritis (RA) is a chronic, multiple, invasive autoimmune disease with two chief clinical manifestations, arthrosynovitis and exarthrosis, and is a long-lasting autoimmune disorder that primarily affects joints. TNF-α and interleukins such as IL-1, IL-6, and IL-8 are the important cytokines implicated in the RA pathophysiology. Hyperinflammation via increased activation of these cytokines triggers hyperproliferation of synovial tissues and infiltration of blood-derived cells, resulting in the erosion of cartilage and bones. Inhibition of TNF-α has been attributed as a promising approach toward its treatment. Other diseases that may present similar attributes include systemic lupus, psoriatic arthritis, and fibromyalgia. Rheumatoid arthritis is featured by the generation of rheumatoid factors which are reactive against the Fc region of immunoglobulin. Autoantibodies bind to circulating IgG and form IgM.IgG complexe

which get accumulated at the joints. These immune complexes can further activate the complement split products, resulting in a type III hypersensitive reaction, which ultimately causes chronic inflammation of the joint. RA pathogenesis is multifactorial and no specific treatment is resolved yet. However, NSAIDs are used more frequently. TNF-α blockers and complement inhibitors are other promising therapeutic options. Infliximab (Remicade) is a human monoclonal chimeric antibody against TNF-α approved clinically for treating RA as well as for Crohn's disease.

Multiple sclerosis is a neurologic disability afflicting multiple parts of the body characterized by an ongoing autoimmune response against the central nervous system. Hyperactivated autoreactive T cells form inflammatory lesions, with severe damage on the myelin sheath of nerve fibers; however, the inflammatory aspect is not well established. IFNs (IFN 1α and β) are the major cytokines with a beneficial role in suppressing autoimmune response in MS (Owens 2003). Aberrant TNF activation has also been linked with the development of MS. Steroids are recommended for acute episodes as anti-inflammatory therapy for Multiple Sclerosis (MS). However, Avonex and Rebif with IFN 1α- and β-protein-expressing drugs are found effective in treating progressive MS conditions.

10.4 Inflammatory Mediators in Disease Pathogenesis

10.4.1 Complement in Pathogenesis

Inappropriate complement activation participates in the pathogenesis of many immune disorders such as inflammatory and autoimmune diseases. Apart from these, complement hyperactivation is reported in neurodegenerative, infectious, and metabolic disorders. Elevated levels of complement by-products are found in plasma samples of RA and systemic lupus erythematosus patients. Complement fragments at higher levels are reported in the serological samples of patients suffering from Crohn's disease, psoriasis, asthma, age-related macular degenerating lupus erythematosus, multiple sclerosis, myocardial infarction, atypical hemolytic uremic syndrome, and stroke. Complements are also involved in ischemia, reperfusion-mediated kidney injury, transplant rejections, and carcinogenesis. Complement inhibition is thus recognized as a possible solution to ameliorate the severity of these diseases. Complements play a vital function in organ transplantation, especially in graft rejection associated with kidney transplantation, by triggering adverse effects on the graft.

10.4.2 Histamine in Pathogenesis

The role of histamine in disease pathogenesis is determined by the receptor subtype it gets to interact with. Histamine is primarily involved in allergic manifestations. H1R receptor is involved in smooth-muscle constriction and mucus production, resulting in dysmenorrhea and respiratory discomfort. H2R affects gastric acid

secretion and cyclic AMP level, plays a role in smooth-muscle relaxation and vasodilation and is the prime inducer of peptic ulcer. H3R possesses central nervous system regulatory potential and H4R has a role in asthma and allergic reactions. The two important histamine receptors of pharmacological significance are HR1 and HR2. H1R–histamine complex provokes type 1 hypersensitive conditions like rhinitis and asthma. Antihistamines are the best medicines suggested for allergic rhinitis. These drugs act by binding to the histamine receptors on target cells and thus prevent the binding of histamine. The H1 receptors could be blocked by the classical antihistamines and the H2 receptors by a newer class of antihistamines.

10.4.3 Leukotriene in Pathogenesis

The undesirable production and activation of leukotrienes LTB4 and CysLTs have been implicated in a wide variety of inflammatory disorders. CysLTs are more frequently implicated in the pathogenesis of asthma and allergic rhinitis (Hedqvist et al. 1985). Macrophages are the major source of LTB4. The accumulation of macrophages at atherosclerotic sites is a classic hallmark of this condition, thus accelerating LTB4 production and proinflammatory activities in cardiovascular diseases.

10.4.4 Cytokines in Pathogenesis

Plasma levels of various cytokines are found to be elevated in a number of inflammatory and autoimmune disorders. Thus they are best exploited for diagnostic and prognosis assessment of many diseases, including RA. TNF-α production is found altered in obese individuals; as a result macrophage population in adipose tissue would be increased. Moreover, the development of many chronic inflammatory diseases is associated with the increased production of proinflammatory cytokines. Proinflammatory cytokines such as TNF-α impair insulin action and contribute directly to insulin resistance, and IL-6 regulates insulin sensitivity. Elevation of IL-1β, IL-6, IL-8, and TNF-α have been found in patients with uncontrolled diabetes. Altered levels of TNF-α and IL-6 have been associated with arthritic diseases such as gouty, rheumatoid, and psoriatic arthritis. Elevated levels of proinflammatory cytokines cause osteoporosis. Adverse effects of cytokines have been linked to many disease conditions ranging from depression to cancer.

10.5 Anti-inflammatory Agents Modulating Immune Response

The purpose of inflammation is to protect the body from harmful invaders and stressful endogenous and exogenous signals via mediators of the immune system. Inhibiting major clinically relevant inflammatory targets like cytokine, leukotriene, and complements may reduce or augment immune response depending on their

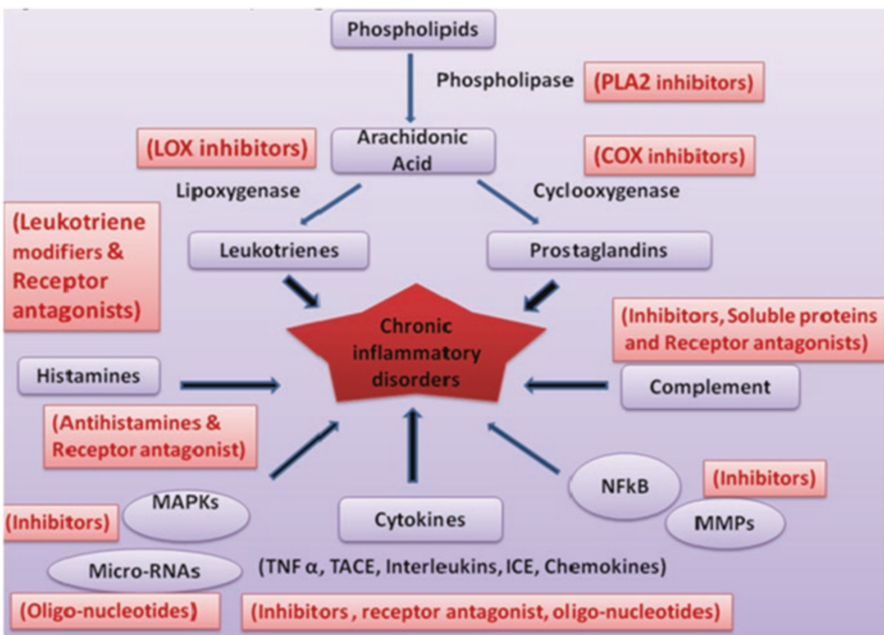

Fig. 10.2 Anti-inflammatory targets: Major inflammatory targets for anti-inflammatory strategy are shown

impact on the function/production of various cellular components of the immune system. Therefore, therapeutically significant response can be achieved by a careful selection of inflammatory targets with positive immune regulatory action. Thus, anti-inflammatory strategies have been developed as an attractive therapeutic rationale toward the treatment of many immune disorders with inflammatory background with the hope to ameliorate the hyperimmune response underlying these disease conditions. The major pharmacologically important inflammatory targets are shown in Fig. 10.2. Majority of the anti-inflammatory agents approved clinically as drug candidates fall into steroidal or nonsteroidal (NSAID) groups according to their nature. Majority of NSAIDs are either COX2 or LOX inhibitors. Biosynthesis inhibitors of inflammatory mediators such as prostaglandin and leukotrienes are considered as an attractive target. However, the discovery of many potential anti-inflammatory targets has boosted the development of diverse anti-inflammatory agents with promising efficacy with fewer side effects. Inflammatory mediators like vasoamine, complement components, cytokines and their corresponding receptors, as well as signaling molecules involved in leukocyte extravasation, cytokine release, and biosynthesis could serve as vital anti-inflammatory therapeutic targets. Antibody therapies against various adhesion molecules like integrin, LFA-1 (lymphocyte function associated antigen 1), and ICAM are being developed to limit inflammatory response by reducing neutrophil extravasation. Immunotherapy against cytokine receptor TNF-α is another potent treatment modality against RA. Corticosteroids

like glucocorticoids are powerful steroid-based anti-inflammatory molecules with a prominent role as immunosuppressants. The corticosteroids have been shown to induce increased transcription of the NFκB inhibitor, which reduces NFκB activation, further inhibiting cytokine processing and release by T lymphocytes through inhibition of transactivation of p38 kinase, c-Jun N-terminal kinase MAP kinase, NFkB, and matrix metalloproteinases. Cortcosteriods reduce phagocytic and killing ability of macrophages and neutrophils and thereby bring about anti-inflammatory activity. Antisense- and soluble-protein approaches are under consideration to be developed as anti-inflammatory agents. Interestingly, many dietary components from fruits, vegetables, grain, and fishes are found to exhibit potential anti-inflammatory property and serve as natural anti-inflammatory agents.

10.6 Natural Anti-inflammatory Molecules

Several naturally derived potent anti-inflammatory molecules have been identified with direct implications in regulating immune response. Dietary pattern has been shown to influence the likely chance of development of inflammatory pathologies. Fats from fish, nuts, and grains, and flavonoids and antioxidants from fruit and vegetables have proved to play a profound role in inhibiting inflammation. Both polyunsaturated fatty acid such as omega-3 fatty acid and monounsaturated fatty acids possess anti-inflammatory activities. Many flavonoids like quercetin have long been demonstrated to exhibit anti-inflammatory properties by disrupting signaling events leading to the biosynthesis of many proinflammatory mediators such as eicosanoids, leukotrienes, and cytokines. Curcumin from *Curcuma longa* is an active anti-inflammatory agent with powerful inhibitory potential against 5-lipoxygenase and cyclooxygenase enzymes (Furst and Zundorf 2014). Similarly, boswellia, the gum resin of the boswellia tree, and bromelain have been shown to inhibit the 5-lipoxygenase enzyme in white blood cells. Flavonoids are a large class of naturally occurring compounds widely present in fruits, vegetables, and beverages. Many flavonoids inhibit the biosynthesis of proinflammatory mediators, adhesion molecules, and C-reactive proteins. Certain flavonoids inhibit transcription factors such as NFκB and activating protein-1 (AP-1), as well as activation of nuclear factor-erythroid 2-related factor 2 (NFE2L2), both of which play active proinflammatory actions.

10.7 Eicosanoids as Agents for Anti-inflammatory Therapy

10.7.1 COX Inhibitors

Cyclooxygenase (COX) inhibitors constitute the most relevant nonsteroidal anti-inflammatory drugs (NSAIDs). They are one of the most widely used drugs for a range of diseases with inflammatory background, which include acute and chronic

pain, dysmenorrhea, and fever. COX is a microsomal enzyme that converts arachidonic acid into the prostaglandin. It is a common molecular target of several anti-inflammatory drugs like aspirin, indomethacin, and ibuprofen. Three members of COX enzymes are identified: COX1, COX2, and COX3 (Rouzer and Marnett 2009). COX1 is constitutively expressed in most of the tissues and produces anti-inflammatory prostaglandins protecting the gastrointestinal tract, kidneys, and liver. Therefore, COX1 inhibitors may adversely affect these organs. COX2 enzyme emerged as the potential therapeutic target with lesser gastrointestinal side effects. COX2 is responsible for the formation of proinflammatory prostanoids and is the prominent enzyme with proinflammatory function. The traditional NSAIDs are known to inhibit both isoforms of COX (1 and 2), which imparts grave side effects on the gastrointestinal tract and cardiovascular system. However, the development of selective COX2 inhibitors like celecoxib 3 (Celebrex) and rofecoxib 4 (Vioxx®) has enabled more safety and potential anti-inflammatory activity. Treatment with COX2 inhibitors has been implemented to ameliorate hyperimmune response underlying many autoimmune, inflammatory, and hypersensitive diseases. They are found effective in managing pain and inflammation associated with rheumatoid arthritis and osteoarthritis. However, drugs based on inhibition of COX2 exhibit many side effects, including cardiovascular complications that limit their use in ailments. Therefore, the development of potentially effective, safe, and economical therapy for treating inflammatory conditions is on high demand. COX2 induction appears to be mediated by cytokines (IL-1, TNF-α) and growth factors (FGF, PDGF, EGF). Some of the COX2 inhibitors are found to modulate immune response by regulating a variety of cytokines such as IFN-γ and IL-10 production (Ni et al. 2007) and modulating TH1 and TH2 activity (Wu et al. 2008). Many COX2 inhibitors are therefore considered for treating autoimmune disorders like systemic lupus erythematosus (Zhang et al. 2007).

10.7.2 PLA_2 Inhibitors

Enzymes PLA2 are involved in biosynthesis of arachidonic acid from membrane-bound phospholipids, and their inhibitors could perform as potential anti-inflammatory agents. PLA_2 is involved in the biosynthesis of the potent inflammatory mediator platelet-derived factor, and promotes the production and release of a variety of cytokines and hence is implicated in many inflammatory diseases and in cancer (Lam et al. 1988). Selective PLA_2 enzyme inhibitor or its receptor antagonists have been proved to have promising anti-inflammatory properties against inflammatory diseases, including rheumatoid arthritis, lung inflammation, multiple sclerosis (MS), atherosclerosis, and cancer as well, where the apparent undesired and hyperimmune response acts as the major obstacle for effective treatment (See Chap. 9).

10.7.3 Leukotriene Inhibitors

Leukotrienes are fatty acid-derived proinflammatory molecules released in response to inflammatory stimuli from mast cells, basophils, and eosinophils. They trigger airway constriction, mucus production, and swelling and inflammation in the lungs, and mediate allergic and asthmatic complications, and also play immune-modulating functions. Thus, leukotriene modifiers or blockers are used to inactivate leukotriene-mediated proinflammatory activities and thereby ameliorate the hyperimmune reactions associated with hypersensitive and autoimmune basis of development and progression (Chagnon et al. 1985). Leukotriene inhibitors are effective in treating allergic and asthmatic complications. So leukotriene blocking has been regarded as an effective therapeutic strategy against allergic reaction (Garcia-Marcos and Schuster 2001; Kemp 2003). This can be achieved by two approaches: either targeting leukotriene receptors or by inhibiting the biosynthesis of leukotriene such as 5-lipoxygenase inhibitor. Leukotriene modifiers are usually used to treat and prevent chronic asthma and allergic rhinitis along with corticosteroids for the best result. Leukotriene receptor antagonists (LTRAs) are a class of nonsteroidal oral medication. LTRAs are considered as anti-inflammatory agents that prevent bronchoconstriction, thereby inhibiting inflammation in the airways (Garcia-Marcos and Schuster 2001; Montuschi et al. 2006). Leukotriene inhibitors can potentially reduce hyperimmune response following allergic reactions and thus implemented for treating atopic dermatitis, aspirin-induced asthma, and chronic idiopathic urticaria.

Leukotriene inhibitors approved for clinical use are Montelukast, Zafirlukast, and Zileuton. Montelukast and Zafirlukast are leukotriene receptor antagonists and Zileuton is a 5-LOX inhibitor. Montelukast is used for treating seasonal allergy by inhibiting LTC4, LTD4, and LTE4 (Garcia-Marcos and Schuster 2001; Kemp 2003). Zafirlukast is a peptide inhibitor against leukotriene receptor and can prevent smooth-muscle constriction. It is generally prescribed to treat different levels of asthma and allergic rhinitis in patients who are above seven years old. Other leukotriene receptor antagonists developed include Verlukast and Pranlukast, which are under investigation and development. Zileuton is a 5-LOX inhibitor that inhibits the synthesis of LTA4, LTC4, LTD4, and LTE4 and is used to treat chronic asthma in adolescents (Piatti et al. 2003). Other 5-LOX inhibitors include ZD2138 and A-79175. Compared to the effectiveness of these inhibitors against asthma, leukotrienes are less effective over the inhaled corticosteroids. Leukotriene inhibitors are thus prescribed along with inhaled corticosteroids for control of mild to moderate, persistent asthma for better efficacy. Leukotriene antagonists help when used with β2 agonist drugs and are found promising in aspirin-sensitive asthma patients. Leukotriene inhibitors are also found promising for esophagitis. Montelukast has been used to successfully treat a chronic inflammation of the bladder as well. Leukotriene antagonists are the only class of asthma drugs with bronchoconstrictive and anti-inflammatory potential. Some of the clinically relevant leukotriene-modifying drugs are summarized in Table 10.3.

Table 10.3 Clinically relevant leukotriene modifiers

Name	Structure	Site of action	Type	Side effects	Treatment
Montelukast		LTC4, LTD4, and LTE4	Leukotriene receptor antagonist	Headache, gastrointestinal disturbances, hypersensitivity, and mild neuropsychiatric effect	Allergic rhinitis Asthma, urticaria, Primary dysmenorrhea
Zafirlukast		LTC4, LTD4, and LTE4	Leukotriene receptor antagonist	Liver toxicity, abdominal pain, psychotic effects, hypersensitivity reactions, loss of appetite	Exercise-induced and chronic asthma
Zileuton		Cys LT1	5-LOX inhibitor	Sinusitis, nausea	Exercise-induced and chronic asthma

10.7.4 Histamine Inhibitors in Immune Disorders

Histamine blockers mostly fall into the class of histamine receptor antagonists. H1 antagonists prevent binding of histamine to H1 receptors and help to ameliorate inflammatory and immune reactions associated with allergy and associated complications. Histamine plays a critical function in type I hypersensitive allergic reaction. The allergen interacts with IgE antibody and gets crosslinked with the cells expressing IgE receptors, such as mast cells, basophils, and triggers a series of complex reactions, eventually leading to cell degranulation and the release of histamine and other inflammatory chemical mediators. The released histamine interacts with respective cells expressing histamine receptors and imparts a variety of biological responses implicated in inflammation and immune regulation. The role of histamines and their receptors in the development of different chronic disease conditions has been previously described. Agents blocking histamine activity (antihistamines) are considered as likely therapeutic solution against a number of manifestations. Antihistamines that target H1 receptor antagonists are used in the treatment of allergic hay fever and rhinitis. Many herbal molecules and antioxidants are shown to possess antihistaminic action. Vitamin C and quercetin can decrease the production of histamine. Fennel, cardamom, ginger, and mangosteen extract have been proved as potential antihistamines. Histamine, acting on H1 receptors, produces a variety of effects, including pruritus, vasodilation, hypotension, flushing, bronchoconstriction, etc. Antihistamines that antagonize H2 receptors inhibit histamine-induced stomach acid secretion and are thus clinically exploited for treating peptic ulcer. H3 antagonists are in clinical trials against complications pertaining to obesity and central nervous system disorders (memory, learning deficits, and epilepsy). H4 receptor

antagonists are emerging as potential therapeutic agents against allergic rhinitis and atopic dermatitis (Jablonowski et al. 2004; Kiss and Keseru 2012, 2014; Martinel Lamas et al. 2015). Pharmacological targeting of the H4 receptor, either alone or in combination with H1 receptor antagonists, was proved effective for treating both allergy and asthma.

Histamine receptor 1 antagonists are generally known as antihistamines. They are broadly divided into six classes and then categorized according to the modifications over time into first, second, and third generation (Krystal et al. 2013). First-generation antihistamines are one of the most extensively used drugs with symptomatic relief from allergies and the common cold, but they have potent sedative effect on patients. This paved the way for the development of second-generation antihistamines without the CNS side effects leading to sedation; these include terfenadine, astemizole, loratadine, and cetirizine. However, terfenadine and astemizole were found to cause potentially serious arrhythmia when plasma concentrations became elevated subsequent to impaired metabolism. Thus, second-generation drugs were further modified to eliminate cardiac toxicity effect. Fexofenadine (the active metabolite of terfenadine) is the first drug marketed in this category. Third-generation antihistamines, such as norastemizole and descarboethoxy loratadine, astemizole and loratadine metabolites, respectively, are under clinical validation and development for the treatment of allergic rhinitis and chronic urticaria (Du Buske 1996). Clinically used H1R antagonists-based drugs are presented in Table 10.4.

10.7.5 Complement Inhibitors

Complement is a key player in innate immune response and inflammation. Complement activation during an inflammatory reaction contributes to inflammation-driven tissue injury, especially in ischemia/reperfusion (I/R), nephritis, and arthritis. This persistent complement activation elicits sustained immune response which ultimately culminates in tissue injury or organ failure. C1q component product is found to be overactivated in lupus and RA and Alzheimer's disease. C5a seems to play a major role in psoriasis and complement-mediated asthma as well. Thus the central role played by complement in the origin of certain inflammation-based diseases makes it an attractive therapeutic target. Logically, the therapeutic inhibition of complement in these contexts may ameliorate the associated complications by decreasing the underlying hyperimmune reactions. Blocking complement activation can be achieved by a number of ways. The potential of inherent protein regulators of complement activation such as CR1, CR2, etc., has been exploited for the development of complement activation blockers by generating modified soluble version of the recombinant human CR1, CR2, etc. Soluble CR1 (sCR1) is synthesized by removing the transmembrane and cytoplasmic domain of the endogenous CR1 protein as recombinant protein product (Ballanti et al. 2011). Trials with sCR1 reduced the complement-induced tissue injury, lung injury, vascular injury,

Table 10.4 Currently used H1R-based antihistamines

Name	Structure	Treatment in	Side effects
First-generation			
Chlorpheniramine		Rhinitis, urticaria	CNS effect
Clemastine		Allergic rhinitis	CNS effect
Diphenhydramine		Allergy, common cold, insomnia, motion sickness	Cardiac effect, ataxia and photophobia, dry mouth and throat
Hydroxyzine		Allergy, urticaria, atopic or contact dermatoses, pruritus, used as a tranquilizer in dentistry	CNS effect, hypotension, gastrointestinal effect
Promethazine		Allergic reactions, cough, motion sickness, used as sedative	CNS effect, respiratory discomfort, tardive dyskinesia
Second-generation			
Acrivastine		Allergy, hay fever	Dry mouth, loss of appetite, dizziness
Cetirizine		Allergy, hay fever, urticaria, angioedema, rhinovirus infection, Kimura's disease	Headache, drowsiness, dry mouth, and fatigue
Desloratadine		Allergic rhinitis and nasal congestion	Headache, dry mouth, fatigue and gastrointestinal effects
Ebastine		Rhinitis, chronic idiopathic urticaria	Mild headache, dry mouth, drowsiness, inflammation of pharynx/nose/sinus, abdominal pain
Fexofenadine		Seasonal allergy, hay fever, nasal congestion, chronic urticaria	Headache, drowsiness, back pain, anxiety insomnia, miosis
Loratadine		Allergy, hay fever, urticaria	Sedation, psychomotor retardation
Mizolastine		Allergy, rhinoconjuctivitis, chronic urticaria	Dry mouth, diarrhea, constipation, drowsiness in some patients
Levocetirizine		Allergic reactions	Headache, drowsiness, dry mouth, palpitation, blurred vision

Table 10.5 Synthetic complement inhibitors

Inhibitor types	Chemical structure	Site of action
Peptide analogs and derivatives	C5a	C5aR antagonist
	C5a, C-terminal octapeptides	C5aR antagonist
	C5a, His ^{67} modified C terminal octapeptide analogs	C5aR antagonist
		C5aR antagonist
	C5a	C5aR antagonist
	C5a hexapeptide	C5aR antagonist
Peptide with aromatic substistutions (C089)	C5aR	C5aR antagonist
	C3a terminus	C3aR antagonist
PR226 peptide	C3b based phage display screening	C3
	CH2 domain of human IgG	
	C1q	C3 convertase
	Factor B related hexapeptides	Factor D
	C1q B chain helical region	C1q
	C3	C3
CBP2 peptide	K76	Factor D
	K76	Factor D
K76 analogs and derivatives	K76	Factor D
BCX-1470 (analog)	Nafamstat mesilate	Classical and alternate pathway
TKIXc (derivative)	Oligodeoxy ribonucleotide	Classical and alternate pathway
K 76 COOH		
FUT-175		
PS-oligo		

glomerulonephritis, and allergic and asthmatic complications and protected from xenograft rejections. Phase I clinical trial with this agent in myocardial infarction and in severe burn-induced respiratory discomfort were found promising. A mutant version of sCR1 lacking LHR-A sCR1 was then developed to selectively inhibit alternative complement pathway. However, selective inhibition of classical pathway was found more effective in facilitating protection from myocardial ischemic injury. Many protein inhibitors have been developed in this line and are illustrated in Table 10.5.

Soluble protein inhibitors possess the disadvantage of inhibiting a range of complement split products as they are not very selective against individual members. Therefore, anti-C5 and anti-C3 monoclonal antibodies were developed against individual complement components for immunotherapy-mediated complement inhibition to reduce tissue injury (Mollnes and Kirschfink 2006; Perricone et al. 2011; Petering et al. 2000; Scully et al. 2010). Many synthetic peptides and their analogs have been developed against the most active components in the complement system such as C5a, C3a, and C3b and to their receptors (Ehrnthaller et al. 2011; Perricone et al. 2011; Petering et al. 2000; Scully et al. 2010). L156602 is a cyclic peptide isolated from *Streptomyces* against C5aR. But the clinical trial has been called off

because of the potent toxicity. Other cyclic peptide antagonists include cyclohexylalanine, Trp5, and CD53. Among these, CD53 and its analogs are found to be more efficient in inhibiting C5a and C5a-mediated neutrophil chemotaxis and cytokine production from macrophages in vitro (Perricone et al. 2011; Scully et al. 2010). CD53 stably binds at the transmembrane region of the C5aR near the extracellular interface through various residues.

Many organic molecules also have been developed as complement inhibitors such as a fungal metabolite K76 against factor D. Similarly, many nonpeptide ligands with potential complement protein or its receptor binding ability have been characterized. WW54011 and NDTP952492 are such ligands which interact at the similar region where CD53 binds; however, they all fail to form a stable interaction with the receptor (Mollnes and Kirschfink 2006; Perricone et al. 2011). Many complement inhibitors have proved to be effective in preventing chronic rejections of xenograft transplant. Antibodies directed against C5 and C8 complement products are used to prevent hyperacute rejections in transplantation. Several naturally occurring compounds that could potentially block complement components have been identified. Heparin and its glycosaminoglycan compounds and derivatives are the epitome of natural complement inhibitors (Mollnes and Kirschfink 2006). Heparin blocks the interaction between C1q and complement activators and inhibits C3 convertase. Heparin-coated extracorporeal circuits are used to inhibit complement during cardiac surgery (Holers 2016; Kirschfink 2001); however, the success rates are very low.

A number of promising inhibitors were developed, out of which only one complement inhibitor has been so far approved by FDA for clinical use. Eculizumab is the first complement-targeting drug approved for treatment (Keating 2013; Rathbone et al. 2013). It is a monoclonal antibody against C5 protein and is used to treat paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome (Haspel and Hillmen 2008). This drug has been actually developed for chronic inflammatory (autoimmune) conditions. Results from ongoing clinical studies are awaited in patients suffering from autoimmune syndromes. However, some complement inhibitors are either in clinical trial or later steps of preclinical development (Mollnes and Kirschfink 2006).

10.7.6 Cytokine and Their Receptors as Anti-inflammatory Targets

Proinflammatory cytokines and their importance in the pathophysiology of many inflammatory response linked disease conditions were discussed in the previous section. IL-1 along with IL-6 and TNF-α are some with potential pharmacological significance. Cytokines prominently activate immune cells and thus amplify immune response leading to deleterious pathological effects. Proinflammatory cytokines exert their biological response by interacting with respective receptors. Cytokine–receptor complex would then activate a series of downstream signaling events involving MAP kinases. MAPKs such as p38, ERK, and SAPK/JNK activate

a variety of transcription factors like ATF-2, Elk1, NFκB, TRAF6, and STAT involved in the synthesis and release of many proinflammatory cytokines, which further accelerate the inflammatory cascade. Thus the inhibition of these cytokines can be achieved via three basic strategies: antibodies or small-molecule ligands against cytokines or to their specific receptors, small-molecule inhibitors which either target regulatory site on protein or active site of enzyme or the receptor, and targeting the molecules involved in cytokine synthesis and signaling pathways (interleukin or TNF-converting enzymes, MAPKs) (Arend 2002).

10.7.6.1 IL-1 Inhibitor

Interleukin-1 (IL-1) inhibitors possess anti-inflammatory and immunomodulatory actions. They are found promising in managing refractory gout or for patients who are unable to tolerate conventional NSAIDs, colchicine, or glucocorticoids. IL-1 inhibitors are used against a variety of autoinflammatory diseases. The IL-1 inhibitors used in gout include anakinra, canakinumab, and rilonacept (Camp et al. 2005; Morton et al. 2005; Somm et al. 2006). Anakinra is such peptidyl drug synthesized as a recombinant protein of IL-1 receptor antagonists for treating RA. Anakinra inhibits the binding of IL-1 to both IL-1RI and IL-1RII with high affinity competitively thus preventing the IL-1from binding with the receptors. Interleukin inhibitors can also serve as immunosuppressive agents (Clark et al. 2004; de La Mata et al. 2007; Waugh and Perry 2005).

10.7.6.2 IL-6 Inhibitors

IL-6 has been found to play a crucial role in the pathogenesis of RA and a viable target for autoimmune diseases. Inhibitors of IL-6 were found promising in in vivo models of autoimmune disease. Tocilizumab, a humanized monoclonal antibody specific for the IL-6 receptor, has been developed for treating RA (Patel and Moreland 2010; Van Snick 1990).

10.7.6.3 IL-17 Inhibitor

IL-17 overproduction and hyperactivity is directly correlated with the pathophysiology of many autoimmune and inflammatory disorders such as plaque psoriasis. IL-17 blocker seems to regulate immune response underlying psoriasis. Cosentyx is the first IL-17 inhibitor approved for different levels of psoriasis (Leonardi et al. 2012; Moseley et al. 2003).

10.7.6.4 ICE Inhibitors

IL1α and IL1β represent the isoforms of IL-1. IL1β is expressed in inactive form and IL1β converting enzyme (caspase 1) converts it into its active form by inducing proteolytic cleavage at Asp116-Ala117. This step is rate limiting and hence ICE is regarded as a potent target for therapeutic exploitation to limit interleukin-mediated inflammation. Many ICE inhibitors have been developed as anti-inflammatory or immune response-regulating agents based on this concept. Several peptides and nonpeptide ICE inhibitors were synthesized (Okamoto et al. 1999; Shahripour et al. 2002; Vezzani et al. 2010). The peptidomimetic ICE inhibitors were developed from

bicyclic/tricyclic pyrrolopyrimidinone and peptidyl 2,2-difluoro-4-phenylbutyl ketone (Milligan et al. 1995; Okamoto et al. 1999; Semple et al. 1998). The nonpeptidyl ICE inhibitors were synthesized from a range of chemical backbones including pyridone aldehydes, phenylalkyl ketones, and activated ketones (Randle et al. 2001). A series of pyridazine derivatives possess ICE inhibitory action. Inhibition of ICE is thus regarded as a potential approach for the treatment of inflammation and arthritis. Fused bicyclic peptidomimetic compounds were synthesized as potent IL1β converting enzyme inhibitors (Soper et al. 2006). Similarly, many synthetic ICE inhibitors were developed including unsaturated caprolactams, pyrrolopyrimidinones, pyridone, ICEBERG, sulfonamide derivatives, etc. (Harter et al. 2004; Wagner and Laufer 2006). Several of these are presently validated in in vivo murine models for sepsis and polyarthritis. Inhibition of IL-1β and IL-18 production by ICE inhibitors is a promising strategy in managing many inflammatory diseases.

10.7.6.5 Chemokine Inhibitors

Chemokines play an important role in leukocyte infiltration. Their deregulation is associated with the development and pathophysiology of many inflammatory diseases. The critical role of chemokines in the pathophysiology of many autoimmune diseases and in metastasis and progression of many cancer types is well established. Therefore, chemokines and their receptors could serve as vital targets for anti-inflammatory therapies. Broad-spectrum chemokine inhibitors are regarded as better choice because of the complexity and redundancy of chemokine system (Castro Villegas Mdel et al. 2012; D'Ambrosio et al. 2003; Donnelly and Barnes 2006; Feldmann and Maini 2001; Hampel et al. 2013). Chemokine receptor antagonists are recently recognized as anti-inflammatory agents against autoimmune diseases and are under clinical trials. Several CCR1 antagonists were found as potential anti-inflammatory and immunosuppressive agents. CCR1 receptors are found to be associated with the progression of many inflammatory disorders (rheumatoid arthritis, multiple sclerosis) and organ transplant rejection. BX-471 Berlex is a nonpeptide CCR1 receptor antagonist developed for the treatment of autoimmune diseases (Elices 2002). CCR2 is another class of chemokine receptors with potential implications in the pathological development of multiple therapeutic agents against autoimmune diseases, atherosclerosis, pain, and metabolic diseases. Maraviroc is a CCR5 antagonist approved by FDA to prevent HIV infection, and is now acclaimed for its role as an immune system modulator in autoimmune disorders (Capetti et al. 2010; Francisci et al. 2014). However, clinical trials with single chemokine receptors were not found effective for chronic inflammatory diseases. Thus multitargeting inhibitors have been developed. TAK-779 is a small-molecule antagonist developed against mouse CCR2, CCR5, and CXCR3. Scheme 527123, another CXCR2 receptor inhibitor which is chemically derived from 3,4-diaminocyclobut-3-ene-1,2-dione, is under clinical evaluation (Russo et al. 2014). CXCR4 antagonist is the second drug approved by FDA and is employed to mobilize hematopoietic stem cells. CCR9 inhibitor for treating Crohn's disease is in phase III clinical trial (Proudfoot et al. 2010). These are some of the chemokine receptor antagonists developed for clinical applications.

10.7.6.6 TNF-α Inhibitor

TNF-α, a proinflammatory cytokine, is synthesized by activated macrophages and T cells in human body. Elevated levels of TNF-α and its activation is the root cause of the development of certain diseases with inflammatory and deregulated immune response background. Its implication in chronic inflammation and inflammatory manifestations is previously described. Therefore, inhibition of TNF activation is a promising therapeutic option (Castro Villegas Mdel et al. 2012). Many approaches have been developed to inhibit or reduce TNF activity in these disease settings (Feldmann and Maini 2001). Monoclonal antibodies directed against TNF or its receptors can inhibit the binding with its receptors and thereby limit TNF-initiated inflammatory cascade and ameliorate the immune response. Adalimumab, Infliximab, and Golimumab are anti-TNF-α monoclonal antibody-based drugs, and etanecept is a soluble TNF-α receptor antagonist (Bell and Kamm 2000; Martorana et al. 2001; Present et al. 1999). Certolizumab pegol is another TNF-α inhibitor made of humanized Fab fragment combined with polyethylene glycol (2009; Schreiber 2007; Smith et al. 2010). However, immunotherapy often suffers from the potential disadvantage of the necessity of administration at the site of action, wide range of action, and cost. In addition to protein-based drugs, many natural or synthetic inhibitors of TNF-α are under development. Several biologic drugs have been developed for treating RA. Another way of inhibiting TNF activity is by inhibiting the enzyme which forms the mature TNF-α. TNF-converting enzyme plays a pivotal role in active TNF formation from its inactive form. Active TNF is formed by the proteolytic cleavage of its inactive form by TNF-α-converting enzyme and so the enzyme can be another therapeutic target for treatment of RA. TACE inhibitors have exhibited side effects in clinical trials and so more selective TACE inhibitors have to be developed. The search for identification of new macrocyclic hydroximates as selective TACE inhibitors is in progress. Screening for better TACE inhibitors from natural resources is another sector which needs attention.

10.8 Novel Anti-inflammatory Strategies

New approaches for safer and economic anti-inflammatory drug development are highly recommended over the currently available drugs which often possess potential side effects. Therefore, novel target identification and validation are mandatory. Target proteins can be either involved in the biosynthetic regulation of inflammatory mediators or the proteins involved in the signaling events of inflammation. Many biologic drugs have been developed for treatment so far, and are an extensive area of active research. MAPKs (mitogen-activated protein kinases) are a family of serine/threonine protein kinases which mediate biological activities triggered by stress signals. They are involved in the biosynthesis of many inflammatory mediators, especially in the synthesis of proinflammatory cytokines. Inhibitors targeting p38 MAPK and JNK pathways have been developed as anti-inflammatory agents, and in clinical trials in treating chronic inflammatory diseases. The kinase inhibitors of these proteins can inactivate the downstream events and can also be considered for

the development of anti-inflammatory compounds. The natural or synthetic structural analogs of their kinase substrate have been shown to inhibit inflammatory response in vivo. Since most of these inhibitors are small molecules, they hold potential pharmacological significance. Enzymes like phosphodiesterase, aldose reductase, and cyclin-dependent kinase and pro-resolving mediators like endogenous autacoids like lipoxins and their receptors are recently getting attention as anti-inflammatory therapeutic targets. Similarly, NFκB, involved in the biosynthetic pathway of proinflammatory cytokines, is also considered as a target of anti-inflammatory drug development. In addition, adhesion molecules which are involved in leukocyte extravasation, could serve as attractive anti-inflammatory drug targets. The search for more selective drugs against many inflammation-based diseases such as rheumatoid arthritis, inflammatory bowel disease, allergies, multiple sclerosis, asthma, atherosclerosis, etc., has suggested that ICAMs could function as better therapeutic agents. Integrins are the principal proteins involved in immune-cell extravasations. Inflammatory reactions can be reduced via interfering with the signaling events preventing extravasation of leukocytes via integrins. Toll like receptor 5 (TLK5) is recognised as a potential antiinflammatory target. Microgliosis is an important feature of Alzheimer's disease (AD) etiology. Amyloid β peptide is hypothesized to act as a stimulus for microglia followed by nonreceptor tyrosine kinases and secretion of proinflammatory cytokines. Therefore, the signaling pathways mediating microglial activation can be considered for pharmacological exploitation in search of anti-inflammatory therapy for AD. The significance of vascular endothelial cells (VECs) revealed their role in leukocyte recruitment, cytokine production, and angiogenesis. Therefore, drugs targeting VECs may yield great therapeutic benefit against many (chronic) inflammatory disorders. The potential role of miRNAs in the regulation of basic inflammation and in the pathogenesis of many autoimmune and inflammatory diseases has been unraveled. miRNAs, miR-126, miR-132, miR-146, miR-155, and miR-221, are strongly implicated with proinflammatory responses (Wang et al. 2010). Strategies can be adopted to control inflammation at the genetic and epigenetic level as the next-generation therapeutics. Gene therapy via synthetic oligonucleotides against micro-RNAs and proinflammatory genes can be envisaged as future therapeutic perspective against autoimmune and hypersensitive diseases where genetic factors serve as a significant contributing factor.

10.9 Conclusions

Undesired and persistent inflammatory response is a major hallmark of immune disorders and cause of transplant rejections. Therapeutic amelioration of many autoimmune and hypersensitive diseases still extends limited efficacy. Anti-inflammatory molecules with potential ability to reduce hyperimmune response underlying such manifestations offer a promising therapeutic strategy. Antagonists targeting cytokines, eicosanoids, histamines and the components of complements have shown to ameliorate inflammatory and immune responses and thus, these

agents are considered as potential drug candidates aganist a variety of clinical manifestations. As there is a greater scope for exploration of our vast bioresources in screening out efficient anti-inflammatory molecules, it has become a prime agenda for the global pharmaceutical industry.

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Part III

Antimicrobials

Antimicrobial Agents from Plants

11

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Abstract

Plants represent an inexhaustible source of novel molecules for recent drug discovery studies. Plant extracts and their active constituents have long been used in traditional medicine for the treatment of several diseases. The emergence of antibiotic-resistant microbial strains and the resurgence of newer and more deadly diseases have called for the need to discover novel antimicrobials. The entire natural resources are being screened in a rapid pace to identify potential drug leads. Recent advances in the development of techniques for isolation, characterization and pharmacological evaluation have led to an interest in plant secondary metabolites as a source of new drugs. Natural product research is continuously exploring the chemical diversity of several lead molecules, which can be used as templates for new drug discovery. This chapter will focus on the significance of plant derived compounds for the development of novel antimicrobial agents.

Keywords

Natural products • Antimicrobials • Phytochemicals

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11.1 Introduction

Plants act as an attractive source of antimicrobial agents. The study of medicinal plants as antimicrobial agents is indispensable for gaining insight into the real value of medicinal flora. The traditional use of medicinal plants is useful as a guideline in the search for new drugs. Moreover, plants are a source of novel lead compounds which would normally not have been synthesized. Isolation of these biologically active lead compounds is a slow and expensive process. The therapeutic potential of these compounds should be periodically reviewed, and it must be clinically proven in order to accelerate the drug discovery process. Likewise, research in this area should be carried on till the agent responsible for the activity has been identified.

Pharmaceutical sector is looking for unique drug candidates to manage microbial infections. Many phytochemicals and plant extracts are already reported as potent antimicrobials when used alone or as synergists of other antibacterial agents. Infections caused by multidrug-resistant bacteria raise an alarming situation due to the shortage of newly developed promising antibiotic candidates. Traditional methods employed for the discovery of antibiotics are futile to keep pace with the rate of evolution of drug resistance. The problem of drug resistance necessitates the search for novel lead molecules that target vital proteins in the prokaryotic system. Secondary metabolites in plants form the defence system which fights against predators like herbivores, insects and microorganisms. Plant secondary metabolites act by different mechanisms than conventional antibiotics, and thus it can be utilized in the treatment of resistant bacteria. Pharmacokinetic parameters such as bioavailability and capacity to bind plasma protein are also important characteristics of plant secondary metabolites. New drug targets in the bacterial structure are also important in beating the resistant strains as it will be more effective if a drug has several modes of action.

11.2 Antimicrobial Compounds

The beneficial medicinal properties of plants characteristically result from the combinations of several compounds present in them. In plants, these compounds are mainly secondary metabolites such as alkaloids, tannins and other phenolic compounds, which are localized in specific parts or in all parts of the plant. Mostly, these phytochemicals serve as plant defence mechanisms against predators and microbes. Some, like quinones and tannins, give pigmentation to plants, others, like terpenoids, impart plants in their odours, and some other compounds are associated with flavour and aroma. Figure 11.1 shows the chemical structures of some important classes of antimicrobial plant metabolites.

Detailed information on the chemical nature and pharmacological activity of some of these classes are given below.

Fig. 11.1 Chemical structure of some antimicrobial phytochemicals

11.2.1 Alkaloids

Alkaloids are generally heterocyclic nitrogen compounds, and amino acids are the most common precursors of alkaloids. They are often colourless in nature, optically active and most are crystalline and a few like nicotine exist in liquid form at room temperature. Morphine isolated from *Papaver somniferum* was the first medically useful alkaloid. Antimicrobial properties were shown by many of the diterpene alkaloids from plants belonging to Ranunculaceae group (Atta-ur-Rahman and Choudhary 1999). Berberine is an important alkaloid with a wide range of activity against bacteria, fungi, viruses, trypanosomes and plasmodia (Kim et al. 2002; Freiburghaus et al. 1996; Omulokoli et al. 1997). It is effective against different microbes which target RNA polymerase, topoisomerase IV, gyrase as well as nucleic acid (Yi et al. 2007). The aromatic alkaloids like berberine and harmane possess DNA intercalating activity also (Phillipson and O'Neill 1987). Berberine also targets FtsZ protein, which is involved in the bacterial cell division machinery and perturbs cytokinesis (Domadia et al. 2008; Boberek et al. 2010).

Carpaine is a major antimicrobial alkaloid obtained from the leaves and seeds of *Carica papaya* L. In addition to carpaine, *C. papaya* contains many other biologically important compounds of which the alkaloid is a significant group (Giordani et al. 1991). It was found that sanguinarine, a benzophenanthridine alkaloid from *Sanquinaria canadensis*, induced filamentation in Gram-positive as well as Gram-negative bacteria and inhibited bacterial cell division. It also perturbed the cytokinetic Z-ring formation in *Escherichia coli* (Beuria et al. 2005). Developments of drug leads based on plant-derived alkaloids are scientifically relevant and attract the attention of more and more researchers and entrepreneurs. Countries like India where the diversity of living things is huge should focus more on in-depth studies for the sustainable utilization of medicinal herbs by way of identifying and characterizing novel antimicrobial agents.

11.2.2 Phenolic Compounds

Phenols and phenolic acids are best considered together since they are usually identified together during plant analysis. Free phenols are quite rare in plants. Phenolic compounds not only possess anti-infective properties but also have anti-oxidative properties. They are a diverse group of aromatic compounds consisting of simple phenols and phenolic acids, flavones, flavanols and flavonoids with one carbonyl group; quinones with two carbonyl groups; tannins and coumarins.

Phenolics in plants are mainly synthesized from the phenylpropanoid pathway. In vitro antimicrobial activities of the phenylpropanoid pathway intermediates, including *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid, and pathway derivatives, including flavonoid aglycones and glycosides, have been demonstrated experimentally (Barber et al. 2000). Phenolic compounds with less complex structures, such as catechol and coumarin, have also shown to exhibit bactericidal and fungicidal activities (Cowan 1999). Syringic acid, caffeic acid and 4-hydroxybenzoic acid were reported to have inhibitory effects on *Ganoderma boninense* (Chong et al. 2009). Pyrogallol and catechol are hydroxylated phenols which also show toxicity to microorganisms.

Phenolic compounds from plants are principally modifiers of membrane protein function. Five widely consumed phenols – curcumin (from turmeric), capsaicin (from chilli peppers), epigallocatechin gallate (from green tea), resveratrol (from grapes) and genistein (from soya beans) – modify lipid bilayer properties and alter the function of diverse membrane proteins. These phytochemicals modify bilayer properties by localizing at the bilayer/solution interface (Ingólfsson et al. 2014), which results in their antimicrobial activity.

Curcumin, a polyphenolic compound, possesses potent antibacterial activity against several pathogenic bacteria, including *Enterococcus*, *Staphylococcus aureus* and *S. epidermidis*. It was found that curcumin induced filamentation in *Bacillus subtilis* 168, suggesting that it inhibits bacterial cytokinesis. The assembly and stability of FtsZ protofilaments have been shown to play a critical role in bacterial cytokinesis. Further, curcumin strongly inhibited the assembly of FtsZ protofilaments and also increased the GTPase activity of FtsZ. Recent developments in the topic suggest that FtsZ may be considered as an important antibacterial drug target where the phenolics can give better results (Dipti Rai et al. 2008a, b).

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a type of natural phenolic compound and a phytoalexin produced by many plants in response to injury or attack by pathogens like bacteria (Frémont 2000). It possesses antimicrobial properties against bacteria, fungi and yeasts (Paulo et al. 2011). Resveratrol is reported to inhibit the virulence factor expression in bacteria like *Proteus mirabilis*, which is an important pathogen infecting the urinary tract, and inhibits urease activity in the carcinogenic strain of *Helicobacter pylori* (Paulo et al. 2011). Due to the action of resveratrol, FtsZ expression and Z-ring formation are suppressed, which in turn inhibits bacterial cell growth (Hwang and Lim 2015). The major source of resveratrol is grapes and grape-derived products, including aged wine.

11.2.3 Flavonoids

Flavonoids are aromatic compounds with phenolic structures which are widely distributed in the plant kingdom. Structurally they are derived from the parent substance flavone. They are classified into several classes, including flavonols, flavones, isoflavones, glycoflavones, anthocyanins. Some flavonoids occur as glycosides which are hydrolysed in the human gut to aglycones. Flavonoids also exist as monomers, dimers or oligomers (Cook and Sammam 1996; Cushnie and Lamb 2005) and are the most abundant polyphenols in our diets (Jin Dai and Russell 2010). They are abundantly found in many natural sources like fruits, vegetables, nuts, seeds, tea, flowers, honey and propolis and therefore form part of the normal diet of humans (Cook and Sammam 1996). Screening of various isoflavonoids isolated from *Erythrina variegata* (Leguminosae) for antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) indicated that erycristagallin (11a-dehydropterocarpan) and orientanol B (9-hydroxy-3-methoxy-2-γ,γ-dimethylallylpterocarpan) showed significant anti-MRSA activity (3·13–6·25 μg ml^{-1}) (Tanaka et al. 2002).

Many reports claim the effectiveness of flavonoids in medical conditions, including antimicrobial, anti-inflammatory, oestrogenic, antioxidant and chelating, vascular and antitumour activities (Cook and Sammam 1996; Cushnie and Lamb 2005). More lipophilic flavones and their derivatives can cause disruption of microbial membranes (Tsuchiya et al. 1996). The catechins present in green tea exhibited antibacterial activity against *Vibrio cholerae* (Borris 1996), *Streptococcus mutans* (Sakanaka et al. 1989) and many other microbes (Tsuchiya et al. 1996; Vijaya et al. 1995). Their activity may be attributed to their ability to complex with extracellular as well as soluble proteins (Cowan 1999). The flavonoids (6-hydroxy7-methoxyluteolin) and the xanthones (8-carboxymethyl 1,5,6 trihydroxy-3-methoxy xanthone) extracted from the leaves of *Leiothrix spiralis*, a plant belonging to the Eriocaulaceae family, revealed a considerable activity against *Pseudomonas aeruginosa and E. coli* (Araujo et al. 2011). Recent reports even indicated significant antibacterial activity of some flavonoids against *Mycobacterium tuberculosis* (García et al. 2012).

Various studies have examined the relationship between the structure of flavonoids and their antibacterial activity, and these are in close agreement with what have been already reported. The antibacterial mechanism of action of selected flavonoids has been elucidated. For example, the activity of quercetin has been partially attributed to DNA gyrase inhibition. It has also been proposed that (−)-epigallocatechin gallate and sophoraflavone G inhibit cytoplasmic membrane function and the licochalcones A and C inhibit energy metabolism. The mechanism of action of the flavonoids robinetin, myricetin, apigenin, rutin, galangin and lonchocarpol A has also been investigated and identified, and these compounds represent novel drug leads. Future studies may lead to the development of pharmacologically acceptable antimicrobial agents from these lead compounds (Cushnie and Lamb 2005).

11.2.4 Quinones/Naphthoquinones

Quinones are aromatic rings with two ketone substitutions. Quinones are coloured and contain the same basic chromophore, that of benzoquinone, which is composed of two carbonyl groups in conjugation with two carbon–carbon double bonds. For their identification, quinones are divided into four groups: anthraquinones, benzoquinones, naphthaquinones and isoprenoid quinones. They are highly reactive and are ubiquitous in nature. Quinones are known to form complexes with nucleophilic amino acids in proteins (Stern et al. 1996) resulting in loss of function. Cell wall polypeptides, surface-exposed adhesins and membrane-bound enzymes are the possible targets of quinones in the microbial cell. It may also render substrates unavailable to microorganisms (Cowan 1999). Hypericin, an anthraquinone from *Hypericum perforatum*, a popular antidepressant, also possesses antimicrobial property (Duke 1985; Kazmi et al. 1994).

11.2.5 Tannins

Tannins are polyphenols found in almost all plant parts showing antibacterial activity due to the inactivation of bacterial adhesins, transport proteins and vital enzymes (Savoia 2012). The different possible modes of antimicrobial activity of tannins include protein binding, enzyme inhibition and substrate deprivation (Scalbert 1991; Haslam 1996).

The antimicrobial activity of tannins can be attributed to the following properties: (1) enzyme inhibition and substrate deprivation, (2) metal ion deprivation and (3) action on the membranes of the microorganisms (Chung et al. 1998a). This is evident from the fact that many microbial enzymes in raw culture filtrates as well as in purified forms are inhibited when mixed with tannins. Gallotannin-rich plant extracts showed bacterial inhibition which is attributable to the strong affinity for iron and to the inactivation of membrane-bound proteins (De Pasquale et al. 1995).

Tannic acid, which is present in many foods like tea, grapes and strawberry, was found to be inhibitory to the growth of intestinal bacteria such as *Escherichia coli*, *Clostridium perfringens*, *Enterobacter cloacae* and *Bacteroides fragilis*. Chung et al. (1998b) found that the inhibitory effect of tannic acid on the growth of intestinal bacteria may be due to its strong iron-binding capacity. However, many microbes have evolved to resist greater concentration of tannins. Fungi like *Penicillium* and *Aspergillus*, which produce tannases, are good examples of tannin-resistant microorganisms (Scalbert 1991).

11.2.6 Coumarins

Coumarins are fragrant organic chemical compounds in the benzopyrone chemical class, which are colourless crystalline substances in its standard state. A natural substance found in many plants, coumarins are also phenolic compounds consisting of fused benzene and α-pyrone ring. At low concentrations (between 16 and 125 μg/mL), coumarins showed significant antibacterial activity against both Gram-negative and Gram-positive bacteria (Adriana Basile et al. 2009). The coumarins, agasyllin and aegelinol from the roots of *Ferulago campestris* (Apiaceae), showed significant antibacterial activity. Warfarin, a coumarin, possesses antiviral effects (Berkada 1978; Liedtke and Rathbun 2009). Coumarin was also found to inhibit *Candida albicans* (Thornes 1997). It has been also used to avoid cold sore recurrences due to HSV-1 in humans. Phytoalexins, which are hydroxylated derivatives of coumarins, are produced in carrots in response to fungal infection and seem to have antifungal activity (Hoult and Payá 1996). Hydroxycinnamic acids, related to coumarins, are inhibitory to Gram-positive bacteria (Fernández et al. 1996).

11.2.7 Polypeptides and Lectins

Plant antimicrobial peptides (AMPs) are a component of the barrier defence system of plants. They have been isolated from roots, seeds, flowers, stems and leaves of a wide variety of species and have activities towards phytopathogens, as well as against bacteria pathogenic to humans. Peptides are often positively charged and contain disulphide bonds (Zhang and Lewis 1997). Their mode of action may be the formation of ion channels in the microbial membrane, which results in a state of disrupted membrane transport (Terras et al. 1993; Zhang and Lewis 1997). Barley and wheat comprise of peptides called thionins, which are toxic to bacteria as well as yeasts (Fernandez de Caleya et al. 1972). The antibacterial and antifungal activities of plants like *Amaranthus* have already been identified (De Bolle et al. 1996). Recent research has been focused on studying anti-HIV peptides and lectins.

11.2.8 Terpenes and Terpenoids

The terpene compounds are also referred to as isoprenoids and have the general chemical structure $C_{10}H_{16}$. They occur as diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), sesquiterpenes (C_{15}) and hemiterpenes (C_5). Terpenoids are derivatives of terpenes containing additional elements, usually oxygen.

Antimicrobial activity of terpenes has been well studied. Totarol, a diterpenoid phenol, has been reported to inhibit the proliferation of numerous pathogenic Gram-positive bacteria like *Mycobacterium tuberculosis*. Totarol inhibits bacterial proliferation by targeting FtsZ and suppresses the GTPase activity of MtbFtsZ. It may be beneficial as a lead compound to develop an effective antitubercular drug (Jaiswal et al. 2007). Terpenes or terpenoids possess a wide range of activity against bacteria

(Amaral et al. 1998), fungi (Ayafor et al. 1994), viruses (Fujioka et al. 1994) and protozoa (Ghoshal et al. 1996). Artemisinin, a sesquiterpenoid, and its derivative α-arteether find their use as antimalarials (Vishwakarma, 2004). Trichorabdal A, a diterpene from a Japanese herb, is reported to inhibit *Helicobacter pylori* (Kadota et al. 1997).

Two pungent compounds in capsicum species (capsaicin and dihydrocapsaicin) were studied for their antimicrobial activities. The plain and heated extracts showed inhibition of *Bacillus subtilis, B. cereus, Clostridium tetani, C. sporogenes* and *Streptococcus pyogenes* (Cichewicz and Thorpe 1996). Capsaicin, a terpenoid constituent, is bactericidal to *H. pylori* (Jones et al. 1997). Another hot-tasting diterpene, aframodial, from a Cameroonian spice, shows broad-spectrum antifungal activity also (Ayafor et al. 1994)

11.2.9 Essential Oils as Antimicrobials

Essential oils are complex mixtures of volatile secondary metabolites isolated from plants by steam or hydrodistillation. Essential oils contain a wide variety of secondary metabolites that are capable of inhibiting or slowing the growth of bacteria, yeasts and moulds. The major constituents of essential oils like monoterpenes, sesquiterpenes and phenylpropanoids are responsible for the fragrance and biological properties of aromatic medicinal plants (Reichling 1999).

Different concentrations of specific compounds can affect the antimicrobial potential of essential oils. For example, higher concentrations of eugenol, cinnamaldehyde or citral confer antimicrobial properties to essential oils (Lis-Balchin et al. 1998; Davidson 2001). Eugenol, a phenylpropene, was found to reduce the viability and resulted in the inactivation of *Salmonella typhi*. The observed high antibacterial activity at alkaline pH favours the fact that the compound can work more efficiently when given *in vivo*. Eugenol-induced deformation of macromolecules in the membrane and the subsequent antibacterial activity is due to the interaction of eugenol on bacterial cell membrane (Devi et al. 2010).

The mechanism of action of essential oil depends on the chemical composition of the bioactive compounds, and their antimicrobial activity is never based on a unique mechanism but is instead a cascade of reactions encompassing the entire bacteria (Burt 2004). Essential oil constituents have a variety of targets, especially the membrane and cytoplasm, and in some cases, they completely alter the cell morphology (Nazzaro et al. 2013). In general, essential oils act to inhibit bacterial growth as well as the production of toxic bacterial metabolites. Essential oils possess better activity on Gram-positive bacteria than Gram-negative strains, and this effect is mostly attributed to the difference in the cell wall compositions (Chorianopoulos et al. 2008; Gutierrez et al. 2008; Marino et al. 1999).

The cell membrane integrity is essential for the survival of bacteria because it is a vital factor for the basic biological activities taking place within the cells. In some cases, essential oils perturb the permeability of membrane by destroying the electron transport system (Tassou et al. 2000). Some of the components of the essential oils such as carvone, carvacrol and thymol, lead to an increase in the intracellular

concentration of ATP, which is related to the destruction of the microbial membrane (Helander et al. 1997). The alteration of permeability of cell membrane and the defects in the transport of molecules and ions result in an imbalance within the microbial cell. This further results in the coagulation of cytoplasm, the denaturation of several proteins and the loss of ions and metabolites (Burt and Reinders 2003).

The essential oil of cinnamon bark is about 98% cinnamaldehyde, which has shown antimicrobial activity against a broad range of microbes. This natural product from spices is also reported to inhibit cell separation in *Bacillus cereus.* Cinnamaldehyde decreases the in vitro assembly reaction and bundling of the bacterial cell division protein, FtsZ. Studies show that cinnamaldehyde binds FtsZ, perturbs the cytokinetic Z-ring formation and inhibits its assembly dynamics. This suggests that cinnamaldehyde, a small molecule of plant origin, is a potential lead compound that can be developed as an anti-FtsZ agent towards drug design (Domadia et al. 2007).

11.3 Biofilm Inhibition

Biofilm, which is an assemblage of microbial cells that are irreversibly linked with a surface and enclosed in an extracellular polymeric substance matrix, develops on a wide variety of surfaces, including living tissues, medical devices, natural aquatic systems (Donlan 2002). Biofilms play a significant role in certain infectious diseases and are also associated with a variety of device-related infections. The development of a biofilm may also cause the aggregate cell colony to be more prone to antibiotic resistance. Quorum sensing or cell-cell communication has been involved in the formation of biofilm in many bacterial species. Novel and effective biofilm control strategies have great importance as far as public health is concerned.

Biofilm-based infections cause harm to millions of people annually. The difficulty of eradicating biofilm bacteria with standard antibiotic treatment is a major concern in medicine. *Propionibacterium acnes*, a biofilm-forming microorganism responsible for acne vulgaris, showed susceptibility to plant extracts containing resveratrol, icariin and salidroside compounds which showed the inhibition of biofilm formation. The antivirulent property of the plant *Melia dubia* was studied, and a few compounds antagonizing the quorum-sensing systems of uropathogenic *E. coli* were identified (Ravichandiran et al. 2012). Four compounds isolated from the aerial parts and roots of *Krameria lappacea*, *Aesculus hippocastanum*, *Chelidonium majus* and *Macleaya cordata* containing many alkaloids and flavonoids revealed significant activity against Staphylococci (Artini et al. 2012), which are a clinically significant bacterial strain. Two compounds, proanthocyanidin A2-phosphatidylcholine (proAc) isolated from *A. hippocastanum* and chelerythrine (CH) purified from *Macleaya cordata*, revealed an inhibition of de novo biofilm formation without having bactericidal activity. These alkaloids downregulate some vital proteins involved in different pathways and inhibit biofilm formation. Sanguinarine and chelerythrine also act on some elements of bacterial cytoskeleton, which is a potential target for antibacterial therapy. The inhibitors of cytoskeletal proteins may serve as lead compounds for the development of novel antimicrobial agents.

A polyphenolic compound, hamamelitannin, extracted from the bark of *Hamamelis virginiana* significantly reduces biofilm activity of various microorganisms (Cobrado et al. 2012). Similarly, a monoterpenic phenol, carvacrol, had an effect on the biofilms formed by *Staphylococcus aureus* and *Salmonella enterica* serovar *Typhimurium* (Knowles et al. 2005). This compound, together with thymol, is the principal phenolic component that contributes to the antimicrobial property of oregano oil on staphylococci (Nostro et al. 2007). These molecules, possessing a hydrophobic nature, interact with the lipid bilayer of cytoplasmic membrane causing alterations in its structural and functional properties and loss of integrity of bacterial cell. Furthermore, these compounds may diffuse through the polysaccharide matrix of the biofilm resulting in its destabilization.

11.4 Synergistic Activity of Plant Compounds

Several plant compounds can be used along with known antibiotics to increase their potency and to avoid undesirable side effects. Knowledge on the synergistic activity of various phytochemicals could further boost the usage of medicinal plants, extracts or natural products, either alone, combined with each other or along with antibiotics. The effect of ethyl gallate on β-lactam susceptibility in methicillin-resistant and methicillin-sensitive strains of *Staphylococcus aureus* was studied by Shibata et al. (2005) and found that it enhanced the activity of the antibiotic. This synergistic effect of the alkyl gallates is specific for β-lactam antibiotics, and it does not cause any change in the effectiveness of other classes of antibiotics tested.

Another remarkable study was conducted on the effect of 5-methoxyhydnocarpin, a compound isolated from chaulmoogra oil, on the activity of berberine (Stermitz et al. 2000), and the results indicated that 5-methoxyhydnocarpin enhanced the action of berberine against *Staphylococcus aureus* even though it did not show any antimicrobial activity on its own. It is observed that the accumulation level of berberine in the cells increased sharply in the presence of 5-methoxyhydnocarpin, allowing this natural product to deactivate the mechanism of bacterial resistance against berberine. In the absence of 5-methoxyhydnocarpin, berberine is easily extruded by the multidrug resistance pumps of *Staphylococcus aureus*. This indicates the potential use of a weak antimicrobial natural compound along with another compound to intensify its activity (Ríos and Recio 2005). Table 11.1 gives a combination of natural products and synthetic drugs used to combat fungal infections.

11.5 Antiviral Compounds

Plants have enormous biosynthetic capacity, and the complexity of their bioactive constituents seems to provide a rich source of natural compounds that may serve as excellent phytotherapeutic agents (Schmidt et al. 2008). The development of antimicrobial drugs that are plant derived has not progressed, especially, in the case of antiviral chemotherapy as compared to antifungal and antibacterial due to the

Table 11.1 Combination of natural products and synthetic drugs to combat fungal infections

Natural source	Antibiotics	Sensitive fungal species	References
Allium sativum	Ketoconazole	*Trichophyton rubrum, T. erinacei* and *T. soudanense*	Pyun and Shin (2006)
Essential oil fraction of *Pelargonium graveolens* and its main components, geraniol and citronellol	Ketoconazole	*Aspergillus niger* and *A. flavus*	Shin (2003)
Essential oil from *Agastache rugosa* and its main component, estragole	Ketoconazole	*T. erinacei, T. mentagrophytes, T. rubrum, T. schoenleinii* and *T. soudanense*	Shin and Kang (2003)
Euphorbia characias latex	Ketoconazole	*Candida albicans*	Giordani et al. (2001)
Santolina oil	Clotrimazole	*Candida albicans*	Suresh et al. (1997)
Essential oil from *Thymus vulgaris* (thymol chemotype)	Amphotericin B	*Candida albicans*	Giordani et al. (2001)

problem of selectivity (Kinchington et al. 1995). There are many viral diseases that we continue to fight relentlessly, yet they prove to be infectious and fatal. While some can be curtailed by the use of antiviral drugs, some are developing resistance to these drugs over a period of time. Plant-derived compounds like ellagitannins have potent antiviral activity, and they inhibit even highly resistant strains of the herpes simplex virus. Such plant-derived antiviral agents are to be commercialized at low costs (Mishra et al. 2013).

Many plants have been reported to contain antiviral components such as terpenoids, polyphenols, coumarins, proteins, alkaloids, tannins and biflavonoids, which can inhibit viral life cycle at different stages (Chinsembu and Hedimbi 2009). The (+) -calanolide A, isolated from *Calophyllum lanigerum*, is a non-nucleoside inhibitor of HIV-1 reverse transcriptase, and it acts through a complex mechanism which involves possible binding at two sites (Kashman et al. 1992; Chinsembu and Hedimbi 2009). This natural compound had shown positive results in in vitro and animal studies. PA-334B, which is a khellactone coumarin (Kothari 2007), and PA-457, a derivative of betulinic acid (Li et al. 2003), are also plant-derived antiviral compounds that have been under clinical trials. Anti-HSV-2 activity has been shown by extracts from *Rhus javanica* (Nakano et al. 1998), which contains moronic acid, a simple triterpenoid keto acid, and it has been effective against wild-type HSV (both type 1 and type 2) oral infection in mice (Kurokawa et al. 1999).

The plant-based antiviral compounds would show only minimum side effects in the long run and may also be used in combination with other known effective antiviral molecules and drugs to cure vicious infections such as HIV, which has a tendency to undergo rapid mutation to develop into drug-resistant forms. Table 11.2 shows the details of some of the plant-derived antiviral compounds.

Table 11.2 Plant-derived antiviral compounds

Sl. no.	Plant and compounds	Family	Chemical class	Antiviral activity against	References
1.	*Curcuma longa* – curcumin	Zingiberaceae	Polyphenol	HIV	Mazumder et al. (1995)
2.	*Hypericum* sp. – hypericin	Hypericaceae	Anthraquinone	HIV	Hudson et al. (1993a)
3.	*Chrysanthemum morifolium* – chrysin	Asteraceae	Flavone	HIV	Critchfield et al. (1996)
4.	*Ancistrocladus korupensis* – michellamine B	*Ancistrocladaceae*	Alkaloid	HIV	McMahon et al. (1995)
5.	*Prunella* sp. – prunellin	*Lamiaceae*	Polysaccharide	HIV	Yao et al. (1992)
6.	*Schumanniophyton magnificum* – schumannificine 1	Rubiaceae	Alkaloid	HIV	Houghton et al. (1994)
7.	*Quercus rubra* – quercetin	*Fagaceae*	Flavonoid	HIV	Fesen et al. (1993)
8.	*Glycyrrhiza glabra* – glycyrrhizin	Fabaceae	Flavonoid	HIV	Watanbe et al. (1996)
9.	*Tripterygium wilfordii* – salaspermic acid	Celastraceae	Flavonoid	HIV	Chen et al. (1992)
10.	*Camellia sinensis* – catechin, theaflavin, Epicatechin gallate epigallocatechin gallate	Theaceae	Polyphenol	HIV	Liu et al. (2005) and Naik and Juvekar (2003)
11.	*Calophyllum lanigerum* – calanolide A	Calophyllaceae	Dipyrano-coumarin	HIV	Chinsembu and Hedimbi (2009)
12.	*Coriandrum sativum* – coriandrin	Apiaceae	Coumarin	HIV	Hudson et al. (1993b)
13.	*Lomatium suksdorfii* – suksdorfin	*Apiaceae*	Coumarins	HIV	Lai et al. (1990)
14.	*Schisandra sphaerandra* – nigranoic acid	Schisandraceae	Terpenoids	HIV	Sun et al. (1996)
15.	*Syzygium claviflorum* – betulinic acid, platanic acid	Myrtaceae	Terpenoids	HIV	Fujioka et al. (1994)
16.	*Rosmarinus officinalis* – carnosolic acid	Lamiaceae	Terpenoid	HIV	Paris et al. (1993)

11.6 Antimalarial Agents

Many plants are used in traditional medicine for the treatment of malaria. It is therefore worthy to study such plants, which have been used over the centuries for medicinal purposes. The newly developed techniques in the area of isolation, characterization and pharmacological testing have led to an increased interest in plants as a source of new drugs. Several classes of the secondary plant substances are responsible for antimalarial activity, but the most important and diverse biopotency has been observed in alkaloids, quassinoids and sesquiterpene lactones (Saxena et al. 2003). Quinine, an alkaloid isolated from the bark of *Cinchona* species (Rubiaceae), was the first antimalarial drug. It is one of the most important antimalarial drugs that are still used today. The discovery of artemisinin, the most potent antimalarial drug, and the success of quinine, both from plant sources, have led to the exploration of plants as antimalarial agents. Artemisinin (*qinghao su*) is isolated from the plant *Artemisia annua* (White 1997). Now several research groups are working to develop new antimalarial drugs in order to combat chloroquinone-resistant malarial parasite especially *Plasmodium falciparum*.

Some of the active constituents isolated from the root bark of *Zanthoxylum gilletii* (Rutaceae), the tubers of *Cyperus rotundus* (Cyperaceae) and the root bark of *Margaritaria discoidea* (Euphorbiaceae) were *a*-cyperone, N-isobutyldeca-2,4-dienamide and securinine, respectively. The significant antimalarial activity of these compounds was attributed to the presence of *a*,*b*-unsaturated carbonyl moiety. This moiety was supposed to undergo a Michael reaction with nucleophilic sites in the DNA molecule of the malarial parasite and inhibit the growth of *P. falciparum* (Weenen et al. 1990; Achenbach et al. 1992). Several plant-based compounds can thus be utilized for the successful treatment of malaria, which is one of the most prevalent insect-borne diseases.

11.7 Methods of Extraction

A systematic methodology is inevitable for the scientific analysis of plant components. Active components may be present in any parts of the plant. Plants are collected either randomly or based on the knowledge from classical texts like those of Ayurveda and traditional healers. Usually crude aqueous or alcohol extracts are used for initial screening of the plants for antimicrobial activities, and it can be followed by different organic extraction strategies. Since most of the compounds from plants are aromatic or saturated organic constituents, they are mostly obtained through the initial extraction using ethanol or methanol. For taking alcoholic extracts, the plant parts are dried, ground to fine powder and then soaked in ethanol or methanol for extended periods. The slurry is filtered and washed, then it may be

dried under reduced pressure and redissolved in the alcohol to a specific concentration. Soxhlet apparatus can be used for the extraction process.

A variety of solvents were examined by Eloff (1998) for their ability to solubilize antimicrobials from plants. The focus of the study was to provide a more standardized extraction method for the various researchers working in diverse areas. Although it is not one of the more frequently used extracts in studies published to date, acetone received the highest overall rating. Generally, most of the studies avoid the use of aqueous fractionation altogether. Some exceptional water-soluble compounds, such as polysaccharides (e.g. starch) and polypeptides, including fabatin and various lectins, are commonly more effective as inhibitors of pathogens and would not be detected in the screening techniques commonly used. Occasionally, terpenoids and tannins will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Cowan 1999).

The active chemicals can be purified from the crude extracts using different methods of separation and purification developed by natural products chemists who mainly include chromatography techniques. Successive extractions of the dried plant parts using different solvent systems like hexane, chloroform and methanol are also performed in certain cases.

Chemical analysis of the purified material can be done using techniques like chromatography, bioautography, radioimmunoassay, mass spectrometry, high-performance liquid chromatography, capillary zone electrophoresis, nuclear magnetic resonance spectroscopy and X-ray crystallography (Borris 1996). The products obtained can then be used for testing antimicrobial studies using disc diffusion and broth dilution assays. Table 11.3 sums up the different solvent systems used for the extraction of plant metabolites.

Table 11.3 Different solvent systems used for the extraction of plant metabolites

Solvent	Active components
Methanol	Anthocyanins, flavones (Sato et al. 1996), lactones (Rao et al. 1993), polyphenols (Vijaya et al. 1995), saponins, tannins (Taylor et al. 1996), terpenoids (Taylor et al. 1996), totarol (Kubo et al. 1992), quassinoids (Kitagawa et al. 1996)
Ethanol	Alkaloids (Ivanovska et al. 1996), flavonol (Brandão et al. 1997; Hufford et al. 1993), terpenoids (Habtemariam et al. 1993), polyphenols (Nakahara et al. 1993), polyacetylenes (Brandão et al. 1997), sterols (De Pasquale et al. 1995)
Dichloromethanol	Terpenoids (Mendoza et al. 1997)
Acetone	Flavonols (Afolayan and Meyer 1997)
Chloroform	Flavonoids (Perrett et al. 1995), terpenoids (Ayafor et al. 1994)
Ether	Alkaloids, coumarins, fatty acids, terpenoids
Water	Anthocyanins (Kaul et al. 1985), tannins (Scalbert 1991), saponins, (De Pasquale et al. 1995), terpenoids, lectins

11.8 Conclusions

Plants and plant-derived products have been in use for centuries for the treatment of infections even before the active constituents in the plant products could be revealed through the advances in science and technology. Chemotherapeutic treatment of infections using phytochemical principles is badly needed in the present era due to the microbial drug resistance. The ethnopharmacological approach used in the search for new antimicrobial compounds from plants appears to be predictive compared to the random screening strategies.

Scientists from divergent fields have found literally thousands of plant-derived antimicrobials which inhibit all types of microorganisms *in vitro*. Many of these compounds are to be subjected to animal and human studies to determine their effectiveness in whole-organism systems, and toxicity studies are to be conducted. It would be beneficial to standardize the procedures of extraction and *in vitro* testing so that the studies could be more organized for proper interpretation of results. The lead compounds developed by high-throughput method could produce safe, inexpensive and effective drugs which efficiently mitigate bacterial, fungal as well as viral diseases.

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Antimicrobials from Microbes

12

Dileep Francis

Abstract

Microorganisms are potential sources of antimicrobial substances, including antiseptics, disinfectants and antibiotics, used to prevent the growth and spread of harmful microbes. Most of the antimicrobials produced by microorganisms are secondary metabolites. The antimicrobials produced by microorganisms fall under chemical classes like Polyketides, terpenes, shikimates, peptides and alkaloids. Microorganisms differ in their potential to produce antimicrobial substances. Bacteria that belong to the genus *Streptomyces, Bacillus* and *Pseudomonas* are prolific producers of antimicrobials. Myxobacteria and filamentous fungi are also producers of important antimicrobials among other prokaryotes. The development of drug-resistant bacteria has resulted in the search for novel antimicrobials from hitherto untapped microbial sources such as uncultured bacteria.

Keywords

Antimicrobials • Secondary metabolites • Antimicrobial resistance • Antibiotics • Lantibiotics • Pyocins • Polyketides • Teixobactin

12.1 Introduction

Microorganisms have survived the course of natural selection since life originated on earth with their simple cellular organization and miniature form. They successfully inhabited every possible surface on the earth including extremes of

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temperature and pH normally inaccessible to advanced higher organisms. Human beings are also included in the natural habitat of microorganisms. They inhabit the skin, mucous layers, and the gastrointestinal tract of humans. Most of the human microflora are nonpathogenic symbionts, and many of them endow the host with numerous benefits, including a defense mechanism against the invasion by foreign microbes. These microorganisms have coexisted with the human system for thousands of years and have learned to settle amicably inside it. But many microbes do not render this favor to human beings. They access and survive in the human system by causing serious damages to the host and are collectively called infectious microorganisms. Yet another group called the opportunistic pathogens normally inhabits humans asymptomatically but causes diseases when an opportunity arises. The fight against infective microorganisms had always been a primary goal of the biologists. With the advent of modern science, systematic control measures were sought against microorganisms. Synthetic azo dyes were discovered to have potential to inhibit the growth of microorganisms. The discovery of penicillin in 1928 opened up an entirely new arena in the fight against microorganisms. Incidentally, the antimicrobial substance penicillin was discovered from another microorganism, a fungus called *Penicillium notatum*. This discovery initiated the search for antimicrobial substances from natural sources including microorganisms. These substances find applications not only against microorganisms infecting human beings but also against those infecting domesticated and economically important animals and plants. Advances in the science of microbiology, fermentation technology and combinatorial chemistry greatly facilitated the search for novel antimicrobial compound from microorganisms. Initially, during the so-called golden era of antibiotics, it appeared that humans have won the fight against infective microorganisms. But it did not take much for microbial pathogens to counteract the antimicrobials used against them by developing mutated molecules and processes that offered them resistance to antimicrobials. Moreover, the discovery of antimicrobials from microorganisms reached a level of saturation in the 1960s. Now the focus has shifted to identify hitherto unknown classes of microbial metabolites that target novel molecules or pathways in microorganisms that do not provide them opportunity to develop resistance. For instance, antimicrobials targeting lipids involved in cell wall synthesis are well sought for targets as lipids do not develop mutations the way proteins do. Untapped but potential sources such as uncultured microorganisms are now targeted for novel antimicrobials. This chapter explores the important antimicrobial molecules isolated from microorganisms.

12.2 Antimicrobials

Antimicrobials can be defined as any substance of natural, semisynthetic or synthetic origin that inhibits the growth of microorganisms. The term *antimicrobial* covers different classes of substances including disinfectants, antiseptics and antibiotics. Disinfectants are antimicrobials that are applied to nonliving surfaces to inhibit the growth of microorganisms. Antiseptics are applied topically on living

tissues to prevent the growth and spread of microorganisms and thereby prevent infection. Antibiotics are low-molecular-weight substances of natural origin that at low concentrations inhibit the growth of microorganisms. They are the most important and widely used group of antimicrobial. Although by definition antibiotics are natural compounds, now the term has come to include semisynthetic and synthetic variants too. It is important to note that all antibiotics are antimicrobials but all antimicrobials are not antibiotics. Antimicrobials produce the inhibitory effect on the microorganism either transiently or permanently. When the inhibition due to an antimicrobial persists only for the duration it exists in the medium, the effect is called 'static' (e.g., bacteriostatic antimicrobials). If the antimicrobial inhibition is irreversible, the effect is called 'cidal' (e.g., bactericidal and fungicidal antimicrobials). Antimicrobial agents act against a wide range of microorganisms, including bacteria (antibacterial antimicrobial), fungus (antifungal antimicrobial), protozoa (antiprotozoal antimicrobial), and virus (antiviral antimicrobials).

Antimicrobials can be classified based on their spectrum of activity, chemical structure or mode of action. Antimicrobials are said to have a narrow spectrum of activity when they inhibit only a limited number of organisms. For example, glycopeptides and bacitracin inhibit only gram-positive bacteria, whereas polymyxins act against gram-negative bacteria. While amino glycosides and sulfonamides act primarily against aerobic organisms, nitroimidazoles inhibit only anaerobic microbes. Antimicrobials are said to have a broad spectrum of activity when they are able to inhibit a wide range of organism across groups. For instance, tetracycline and fluoroquinolones are effective against both gram-positive and gram-negative bacteria.

12.2.1 Classes of Antimicrobials

12.2.1.1 Disinfectants and Antiseptics

These are biocidal antimicrobials that are extensively used for surface sterilization in hospitals, healthcare and household settings and play an important role in preventing infection. In general, these agents have a wider spectrum of activity compared to antibiotics and are nonspecific in terms of the intracellular targets that they attack to kill the microorganism. Disinfectants are agents used to prevent, control or destroy microorganisms present on nonliving objects or surfaces, while antiseptics are antimicrobial agents used for inhibition of microorganisms present on living surfaces. Certain chemical agents like tincture of iodine, iodophore and alcohol are used as both disinfectants and antiseptics. Three levels of disinfectants are normally identified. High-level disinfectants kill some bacterial spores, fungi, many viruses, vegetative bacteria and mycobacteria. Examples of this group of disinfectants are 8% formaldehyde, 2% gluteraldehyde, 100% ethylene oxide gas, etc. Intermediate disinfectants kill all of the above agents except bacterial spores. Examples are 70–90% alcohol and 0.5% chlorine. Low-level disinfectants like quaternary ammonium compounds can only act on vegetative bacteria, fungi and lipid viruses.

12.2.1.2 Antibiotics

Antibiotics are low-molecular-weight metabolites produced by microorganisms that at low concentrations interfere with the growth of other microorganisms. Antibiotics generally fall within the size limit of about a few thousand Daltons at the maximum. Microbial products of higher molecular weight like lysozyme and other complex proteins do inhibit the growth of other microorganisms but are not considered as antibiotics. The concentration at which an antibiotic acts is an important part of the definition. At high concentrations, even amino acids like glycine and products of anaerobic fermentation like ethanol or butanol can have a detrimental effect on the growth of microorganisms but are not considered antibiotics. Although by definition all antibiotics are strictly natural products, now the term encompasses semisynthetic and completely synthetic variants of natural products too. Antibiotics are chemically heterogeneous in nature. Based on the chemical structures, several families of antibiotics are identified. β-Lactam antibiotics are the first group to be discovered and are characterized by a four-member ring closed by an amide bond. They are subdivided into classes like penicillins, cephalosporins, carbapenems and monobactams. The aminoglycoside group of antibiotics contains an alicyclic six-member ring with hydroxyl and amino substitutes called the aminocyclitol ring along with a few sugars or aminosugars. The tetracyclines contain four linearly condensed rings and act by inhibiting protein synthesis, while the anthracyclines having the same structure act at the DNA level. Macrolides with antibacterial activity are characterized by a large lactone ring, while those with antifungal activity contain a few conjugated double bonds in the lactone ring. Rifamycin group of antibiotics is made of aromatic rings spanned by an aliphatic chain closed by an amide bond.

12.2.2 Modes of Action

Antimicrobials especially antibiotics exert their inhibitory potential by many different mechanisms essentially targeting vital steps in the survival or reproduction of the microorganisms (Fig. 12.1). These mechanisms include inhibition of bacterial or fungal cell wall assembly, blocking the synthesis of proteins, inhibition of nucleic acid replication and inhibition of metabolism and alteration of plasma membrane permeability.

12.2.3 Resistance to Antimicrobials

The clinical use of antimicrobials for the treatment of infections has been in practice since the 1940s. The wonder drug penicillin was introduced into clinical practice around this time. It did not take much for microorganisms to evolve strategies to combat antimicrobials. By the mid-1940s penicillin-resistant *Staphylococcus aureus* was reported. These strains of *S. aureus* produced a plasmid-encoded enzyme called penicillinase (β-lactamase) that cleaved the β-lactam ring of penicillin. In an attempt to combat antimicrobial resistance, the structure of penicillin was modified

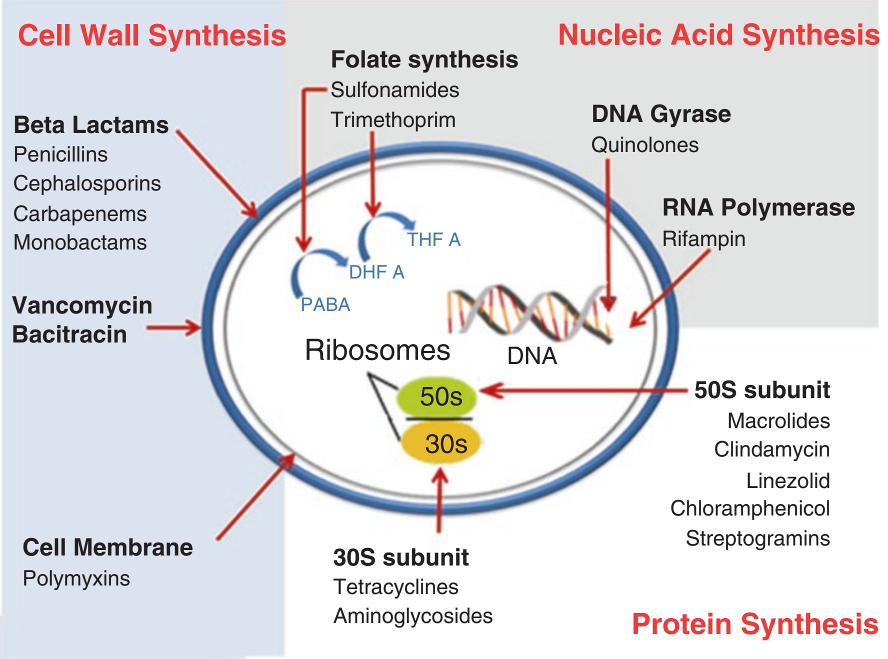

Fig. 12.1 Mechanisms by which antibiotics exert their inhibitory action on microorganisms

to produce penicillin-stable antibiotics like methicillin that inhibited penicillinase-producing staphylococci. But by early 1960s, methicillin-resistant *S. aureus* (MRSA) was reported. MRSA exhibits resistance to all β-lactam antibiotics by virtue of a modified penicillin-binding protein (PBP) called penicillin-binding protein 2A that has reduced affinity to β-lactam antibiotics. Today the global burden of methicillin-resistant *S. aureus* is steadily increasing both in hospital and in community settings.

Vancomycin, a glycopeptide antibiotic, was introduced to combat methicillin-resistant *S. aureus* in the mid-1950s. With ever-increasing use of this antibiotic, vancomycin-resistant *S. aureus* (VRSA) emerged in clinical settings. Evolution of resistance to antimicrobials is not restricted to *S. aureus*. *Escherichia coli* has steadily developed resistance against fluoroquinolones, broad-spectrum penicillins and cephalosporins. *Klebsiella pneumoniae* has developed resistance against broad-spectrum penicillins like ampicillin and amoxicillin and carbapenems. The organism has also developed resistance against oral antibiotics like cotrimoxazole and fluoroquinolones leaving few options for oral treatment of Klebsiella infections worldwide. *Streptococcus pneumoniae* is the leading cause of community-acquired pneumonia worldwide. The bacterium has developed resistance to penicillin antibiotics by acquisition of mutations in the penicillin-binding proteins (PBPs). Several clones of multidrug-resistant salmonella were reported during the late 1990s and early 2000s. *Salmonella enterica* serotype *Typhimurium* that carries a mobile genetic element

rendering it resistant to five antimicrobials, namely, ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline, has been reported. Shigella, the causative agent of shigellosis, has developed resistance against cotrimoxazole. *Neisseria gonorrhoeae*, responsible for gonorrhea, has been reported to evolve reduced susceptibilities to third-generation cephalosporins and such strains were found to be resistant to almost all antibiotics relevant for treatment. They are classified as multidrug-resistant gonococci. Microorganisms develop resistance to antimicrobials by four general mechanisms: inactivation or modification of the antibiotic, structural modification of the antibiotic target so as to reduce the binding affinity of the antibiotic, modification of the metabolic pathways to prevent the action of antibiotics and reduction in intracellular accumulation of antimicrobials by decreasing the permeability of membranes or enabling efflux mechanisms to prevent entry of antibiotics.

12.3 Antimicrobial Secondary Metabolites

Secondary metabolites represent the microbial metabolites found as specially differentiated molecules restricted to specific taxonomic groups and are not essential for cellular metabolism. The term was introduced by Bu'Lock in the early 1960s to differentiate such molecules from the primary essential metabolites including sugars, amino acids and nucleic acids produced by all organisms. Secondary metabolites are generally low-molecular-weight microbial products synthesized by specific microbes as part of the biochemical differentiation of the producer. They do not have any specific role in the growth or survival of the producer in cultures, and mutant strains that do not produce a specific metabolite remain unaffected in terms of cellular growth and metabolism. They are most often produced as families of similar structure and activity. Polyketides, terpenes, shikimates, peptides and alkaloids represent various classes of secondary metabolites produced by microorganism classified based on biosynthetic origin. In microorganisms grown in cultures, secondary metabolite production is at the lowest during lag and logarithmic phases of growth and is at the maximum during the stationary phase. Most of the pharmaceutically important bioactive molecules produced by microorganisms are secondary metabolites. The ability to produce bioactive secondary metabolites is highly varied among microorganisms (Fig. 12.2). Among unicellular prokaryotic bacteria order Actinomycetales, genus *Bacillus* and genus *Pseudomonas* represent the most prolific producers of bioactive metabolites. Recently *Myxobacteria* and *Cyanobacteria* joined the group with large number of bioactive compounds being reported. *Mycobacteria* and Mycoplasmatales represent a relatively dull group of producers with a share of only around 3800 metabolites (17%). The filamentous Actinomycetales are the largest group of producers with more than 10,000 (around 45% of all microbial secondary metabolites) bioactive metabolites identified. About 7600 of them are produced by the genus *Streptomyces* and around 2500 are produced by rare actinomycetes. Among the microscopic fungi ascomycetes, basidiomycetes and other filamentous endophytic fungi are the most significant producers. Yeasts, phycomycetes and slime molds are weak producers of bioactive secondary

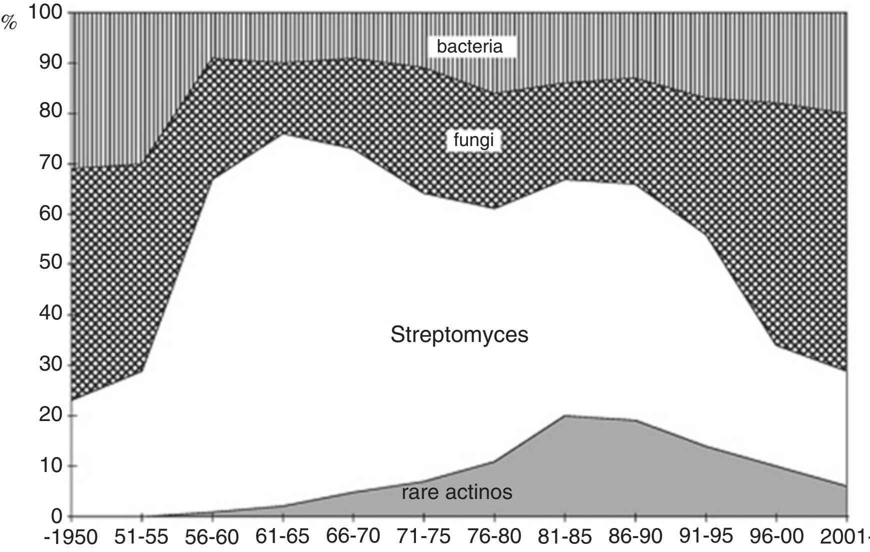

Fig. 12.2 Comparison of antimicrobials discovered from different groups of microorganisms (Source: Bérdy 2005)

metabolites. The total number of bioactive metabolites produced by fungi is approximately 8600, representing 38% of the total metabolites. Microscopic algae, including seaweeds, dinoflagellates and diatoms, produce around 1300 bioactive metabolites. It is important to note that a large fraction of the secondary metabolites produced by microorganisms lack any biological activity. Further, only a small fraction of the biologically active molecules is used in direct practical applications in humans, in animals or in agricultural practice. From around 22,500 molecules known as biologically active metabolites from microbes, only about 150 are translated to useful products (Bérdy 2005).

12.4 Biology and Activity of Antimicrobials

12.4.1 Genus *Streptomyces*

The genus *Streptomyces* is a group of chemoorganotrophic, aerobic, gram-positive bacteria with a filamentous morphology superficially similar to fungus. It is the largest genus of the phylum Actinobacteria and belongs to the family Streptomycetacea. More than 500 species of the genus *Streptomyces* have been described. The members of the genus are characterized by the high G+C content ranging from 69 to 78% of their genome. They produce filamentous aerial hyphae that differentiate into spores that are formed by the fragmentation of the filament and appear in straight, wavy or helical chains. *Streptomyces* spp. produce geosmin,

which gives their cultures a characteristic soil-like odor. They are nonmotile, non-acid–alcohol-fast, and coagulase positive; reduce nitrates to nitrites; degrade adenine, casein, gelatin hypoxanthine, starch and L-tyrosine; and produce pigments.

12.4.1.1 Antimicrobials from *Streptomyces*

Streptomyces is a leading producer of biologically active and industrially important secondary metabolites such as antibiotics, anticancer drugs, herbicides, antihelminthic drugs, vitamins and immune modulators. The period from 1942 to 1960 was marked by the rapid discovery of antibiotics from *Streptomyces*. More than 75% of antibiotics in clinical use today are derived from *Streptomyces* (Watve et al. 2001). The history of antibiotic discovery from *Streptomyces* initiated with the discovery of streptothricin in 1942. This was followed by the isolation of streptomycin in 1943. In 1949, the first antifungal antibiotic nystatin was discovered from *Streptomyces noursei*. As the incidence of antimicrobial resistance has grown in alarming proportions, the search for novel antibiotics from *Streptomyces* spp. has intensified in the recent years. The genomes of many species of *Streptomyces* have been completely elucidated in the recent years, starting with *S. coelicolor* in 2001. The availability of genome information enables a more rapid and highly targeted screening for antibiotics.

Aminoglycosides The active structure of aminoglycosides contains one or more aminated sugars joined by glycosidic linkages to a dibasic cyclitol. Amino glycosides are bactericidal in action and acts mainly by binding to prokaryotic ribosomes, thereby inhibiting protein biosynthesis. The first amino glycoside antibiotic streptomycin was discovered from soil samples containing *S. griseus*. Streptomycin was the first clinically effective drug against tuberculosis. Following streptomycin many more aminoglycosides were discovered form *Streptomyces* including neomycin, kanamycin, tobramycin and paromomycin. Neomycin was first discovered in 1949 from *S. fradiae*. Neomycin is used in topical preparations like antimicrobial creams and ointments. Kanamycin is isolated from *S. kanamyceticus*. Tobramycin is produced by *S. tenebrarius*. It is used mainly in the treatment of pseudomonas infections and possesses excellent activity against gram-negative bacilli. Paromomycin was first isolated in the 1950s from *S. krestomuceticus*.

Tetracyclines and Chloramphenicol In 1948, Benjamin Duggar discovered the first tetracycline, chlorotetracycline, also known by the trade name Aureomycin, from *S. aureofaciens* isolated from soil samples collected from Missouri. In 1950, oxytetracycline was isolated from *S. rimosus*, and is sold under the trade name Terramycin. These compounds are very similar in structure and biological activity. Tetracycline was derived from chlorotetracycline by means of catalytic dehalogenation. Later, high yielding induced auxotrophs of *S. viridifaceans* were selected for the production of tetracycline. Tetracyclines are bacteriostatic in action. They bind reversibly to the prokaryotic 30s ribosome and inhibit protein synthesis. Tetracyclines have a broad spectrum of activity and are used for treatment of a variety of infections

including Rocky Mountain spotted fever and Lyme disease. Chloramphenicol was first isolated in 1949 from *S. venezuelae* and was sold under the trade name Chloromycetin. Later the antibiotic was manufactured in large amounts synthetically. Chloramphenicol is particularly effective in treatment of typhoid fever, meningitis, plague and cholera.

Macrolides The prototype of the macrolide class of antibiotics is erythromycin. This was the first antibiotic of the macrolide group and was isolated from *S.s erythreus* in 1952. A 12–16-membered lactone group is the characteristic structural feature of macrolides. Erythromycin binds to the 50s prokaryotic ribosome and inhibits RNA-dependent protein synthesis. Erythromycin is highly active against many gram-positive bacteria and a few gram-negative bacteria especially those isolated from the respiratory tract. Erythromycin is usually the drug of choice when a legionella infection is suspected. Erythromycin is also used for the treatment of chlamydial infections in pregnancy. Spiramycin is a macrolide antibiotic isolated from *S. ambofaciens* with antimicrobial activity against gram-positive bacteria and Neisseria. Carbomycin is another macrolide produced by *S. halstedii* with antimicrobial activity against gram-positive bacteria and certain mycoplasma strains.

Glycopeptides Soon after the discovery and clinical introduction of penicillins and cephalosporins, antibiotic-resistant strains of microorganisms began to emerge. This intensified the search for novel antibiotics, particularly penicillinase-resistant antibiotics. In 1956, the first glycopeptide antibiotic with penicillinase resistance was isolated from *S. orientalis* obtained from soil samples collected from India and Indonesia and was named vancomycin. Initially the spread of penicillin-resistant *S. aureus* resulted in wide spread clinical use of vancomycin but reports of side effects like ototoxicity and nephrotoxicity and the development of better penicillin derivatives resulted in a gradual decline of vancomycin use. Today highly purified formulations of vancomycin are available in contrast to impure earlier versions and have thus resulted in decreased side effects. Vancomycin exerts its antibacterial action by inhibiting the synthesis of peptidoglycan which is essential for the formation of bacterial cell wall. Vancomycin is used in the treatment of gram-positive aerobic and anaerobic bacteria. Methicillin-resistant *S. aureus* (MRSA) and most strains of coagulase-negative staphylococcus are susceptible to vancomycin. Streptococci and pneumococci are also susceptible. Glycopeptide (vancomycin) intermediate resistant *S. aureus* (GISA) was reported among clinical isolates in Japan and the United States. There are three phenotypes of glycopeptides resistance, namely, Van A, Van B and Van C. The organisms showing the Van A-resistant phenotype shows resistance to both vancomycin and teicoplanin, while Van B and Van C phenotypes are resistant only to vancomycin.

Polyene Mycotics The genus *Streptomyces* also produce antifungal antibiotics structurally belonging to the polyene group. Polyenes are polyunsaturated organic molecules characterized by the presence alternating carbon–carbon single and dou-

Fig. 12.3 2D structures of some polyene and peptide antimicrobials produced by members of the genus *Streptomyces*

ble bonds. These drugs interact with ergo sterols of fungal cell membrane to produce pores that make them leaky. The polyene class of mycotics includes Amphotericin B, nystatin and primaricin (Natamycin). Amphotericin B (Fig. 12.3a) is naturally produced by *S. nodosus* and is the mainstay antifungal agent in clinical use for the treatment of life-threatening mycoses. It is a broad-spectrum antifungal agent that is active against most of the medically important fungi and yeasts, including many dimorphic pathogens like *Coccidioides immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis*.

It is the drug of choice in treating infections caused by opportunistic fungal pathogens like *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans* and

zygomycetes. The drug is intravenously administered and may show side effects like phlebitis at the infusion site, chills and renal toxicity that may be severe. An understanding that the renal toxicity of the drug involved a tubuloglomerular feedback has led to the use of sodium chloride along with the drug to reduce the suppression of glomerular filtration.

Nystatin is an antifungal polyene produced by *Streptomyces noursei* and was the first clinically successful antimycotic antibiotic to be developed. Nystatin has a broad spectrum of activity, but its usefulness is limited by serious side effects. The drug is usually administered as a topical antifungal agent. Pimaricin (Fig. 12.3b), also known as Natamycin, is another antifungal antibiotic produced by *Streptomyces*. It is produced during the fermentation of *S. natalensis* commonly found in the soil micro flora. It is commonly used for topical treatment of superficial eye infections.

Peptide Antibiotics *Streptomyces* also produce cyclic and noncyclic peptide antibiotics. Methylsulfomycin was isolated from the fermentation broth of a *Streptomyces* sp. HIL Y-9420704. This cyclopeptide shows antimicrobial activity against a wide range of gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA) and vancomycin, and teicoplanin-resistant strains. Zelkovamycin (Fig. 12.3c), another cyclic peptide, was isolated from the fermentation broth of *Streptomyces* sp. K96-0670. It shows antimicrobial activity against *Xanthomonas oryzae*, *Acholeplasma laidlawii*, *Pyricularia oryzae* and *S. aureus*. Diperamycin (Fig. 12.3d) is a cyclic hexadepsipeptide antibiotic isolated from the fermentation broth of *Streptomyces griseoaurantiacus* MK393-AF2. It shows antimicrobial activity against gram-positive bacteria, including *Enterococcus seriolicida* and methicillin-resistant *S. aureus*. Arylomycins A and B are biaryl-bridged lipopeptide antibiotics isolated from culture filtrate and mycelial extracts of *Streptomyces* sp. Tu6075 with antibacterial activity against gram-positive bacteria. Streptocidins A–D represent a group of cyclic decapeptide antibiotics isolated from *Streptomyces* sp. Tu6075 with activity against gram-positive bacteria.

Other Antibiotics *Streptomyces* produces many more antibiotics apart from the structural classes of antibiotics outlined above. Clavulanic acid is a broad spectrum antibiotic produced by *S. clavuliegerus*. It is a β-lactamase inhibitor marketed in combination with amoxicillin, a β-lactam, under the trade name Augmentin. Imipenam, the first member of the carbapenem class of antibiotics, was derived from thienamycin produced by *S. cattleya*. Thienamycin had a broad spectrum of activity and was penicillinase resistant but was extremely unstable in aqueous solutions. This led to the development of the more stable version called imipenem. Lincomycin belonging to the lincosamide group of antibiotics was isolated from *S. lincolnensis*. Lincomycin is a narrow-spectrum antibiotic with activity limited to gram-positive bacteria, including pathogenic streptococci, staphylococci and mycoplasma. Clindamycin is a synthetic variant derived from lincomycin by 7(S) chloro-substitution of the 7(R) hydroxyl group. Rifamycin is a broad-spectrum antibiotic produced by *S. mediterranei*. It is active against many gram-positive and some gram-negative bacteria including *Mycobacterium tuberculosis*. Rifampicin is

a synthetic derivative of rifamycin that has largely replaced isoniazid in treatment of tuberculosis especially when isoniazid resistance is indicated. Rifamycin exerts its effect by binding to prokaryotic RNA polymerases and prevents the synthesis of mRNA in target organisms. The broad-spectrum antibiotic phosphomycin was originally isolated from *S. fradiae* although now it is produced by chemical synthesis. The antibiotic originally known as phosphonomycin prevents cell wall formation in target organisms by binding and inactivating the enzyme UDP-N-acetylglucosamine-3-enolpyruvyltransferase, also known as MurA which is essential for the biosynthesis of peptidoglycan. Cycloserine, an unusual amino acid derivative effective against *Mycobacterium tuberculosis* sold under the trade name Seromycin, was originally isolated from *S. orchidaccus*. Cycloserine acts by inhibiting cell wall biosynthesis and is known to have neurological side effects as the drug penetrates the central nervous system. Caboxamycin (Fig. 12.3e), a benzoxazole antibiotic, was isolated from a marine strain *Streptomyces* sp. NTK 937. Unlike other benzoxazoles isolated from *Streptomyces*, Caboxamycin exhibited a broad spectrum of antimicrobial activity. It was effective against gram-positive bacteria like *Bacillus subtilis*, *Staphylococcus lentus*, *S. epidermidis* and the yeast *Candida glabrata.* It shows weak inhibition of biofilms formed by *Staphylococcus xylosus* and inhibits phytopathogenic bacteria *Xanthomonas campestris* and *Ralstonia solanacearum* (Hohmann et al. 2009). Meonomycins, the only group of antibiotics that directly inhibits bacterial peptidoglycan glycosyltransferases, are produced by at least four *Streptomyces* species like *S. ghanaensis, S. bambergiensis, S. ederensis* and *S. geysiriensis.* They show antimicrobial activity against gram-positive bacteria (Ostash and Walker 2010). Essramycin (Fig. 12.3f), a triazolpyramidine antibiotic with a broad spectrum of activity, was isolated from *Streptomyces* sp., isolate Merv8102. It shows antimicrobial action against both gram-positive and gram-negative bacteria with significant activity against *Pseudomonas aeruginosa* (El-Gendy et al. 2008).

12.4.2 Genus *Bacillus*

The genus *Bacillus* comprises of gram-positive rod-shaped bacteria belonging to the family Bacillaceae of the phylum Firmicutes. The bacteria are mostly obligate aerobes, while some are facultative anaerobes. They are widely distributed in the environment with both free-living and parasitic species. Many of the members are mesophiles, but some are extremophiles. They are characterized by the ability to form endospores under stressful environmental conditions. They are mobile by virtue of the presence of peritrichous flagella. The bacteria are chemoorganotrophs that rely on organic substances for energy.

12.4.2.1 Antimicrobials from *Bacillus*

Bacteria of the genus *Bacillus* are well-known producers of secondary metabolites like antibiotics and siderophores (Table 12.1). Most of the antibiotics produced by *Bacillus* spp. are polypeptides of low molecular weight that are synthesized either

Table 12.1 Antimicrobial secondary metabolites produced by the genus *Bacillus*

Structural Class	Compounds	Producer	Activity
Bacteriocin	Lichenin	*B. licheniformis*	Bactericidal and bacteriolytic
	Megacin	*B. megaterium*	
	Coagulin	*B. coagulans*	
	Polyfermenticin	*B. polyfermenticus*	
	Cerein	*B. cereus*	
	Thuricins	*B. thuringiensis*	Bactericidal and bacteriolytic
	Tochicin		Bactericidal
	Kurstakin		Fungicidal
	Entomocin		Bactericidal
	Bacthuricin		Fungicidal
Lantibiotic	Subtilin	*B. subtilis*	Antibacterial
	Ericin		
	Mersacidin		
	Sublancin		
	Subtilolysin		
Dipeptide	Bacilysin	*B. subtilis, B. amyloliquefaciens, B. pumilus*	Antifungal and antibacterial
Phospholipid	Bacilysocin	*B. subtilis*	Fungicidal and antibacterial
Cyclic lipoheptapeptide	Mycosubtilin	*B. subtilis*	Fungicidal
	Iturin	*B. subtilis, B. amyloliquefaciens*	Antifungal
	Fengycin		
	Bacillomycin		
Polyketides	Difficidin	*B. amyloliquefaciens*	
	Bacillaene		
	Macrolactin		
Aminopolyol	Zwittermicin	*B. thuringiensis, B. cereus*	Antibiotic and antifungal

ribosomally or nonribosomally. Ribosomal peptides are gene encoded and are synthesized by the ribosome using a messenger RNA in the form of precursor peptides which are then posttranslationally modified. Nonribosomal peptides are secondary metabolites synthesized by nonribosomal peptide synthetases (NRPS) without relying on a messenger RNA. The antimicrobials from bacillus are used in clinical applications, in control of food microbes, and in control of plant diseases.

Bacteriocins Bacteriocins are a diverse group of ribosomally synthesized peptides or proteins with microbicidal activity against species or strains that are closely related to the bacteria that produced it. Bacteriocins often differ in molecular weights, spectrum of activity, biochemical characteristics and mechanism of action. Several of the antimicrobial substances produced by *Bacillus* fall under the class I bacteriocins. They include lantibiotics which are small molecular weight, gene-encoded peptides characterized by the presence of polycyclic thioether amino acids, lanthionone and/or methyl lanthionine involved in the formation of a ring structure through intramolecular posttranslational modifications. They are also

characterized by the presence of the unusual amino acids didehydroalanine (Dha) and didehydrobutyrine (Dhb).

Lantibiotics are classified into Group A and Group B based on their molecular weight, structure and biological activity. Type A lantibiotics (2.1–3.5 KDa, 21–38 amino acid residues) generally have a more linear secondary structure and are active at nanomolar concentrations against gram-positive bacteria. They kill their targets by forming pores in the cytoplasmic membrane. Type A lantibiotics produced by *Bacillus* species include Subtilin (3320 Da) (Fig. 12.4a), which is a cationic, 32 amino acid pentacyclic lantibiotic produced by *B. subtilis* strain ATCC 6633. This antimicrobial peptide is similar in its structure and mechanism of action to nisin, a bacteriocin globally used as a food preservative (Gálvez et al. 2007). The peptide is resistant to high acid and temperatures. It has a broad spectrum of activity against many gram-positive bacteria, including *Propionibacterium acnes* (causative of skin acne), *Streptococci*, *Staphylococci* and Clostridia. Subtilin binds to the cell wall precursors lipid II and undecaprenyl pyrophosphate to form pores in the cell membrane and thereby cause antimicrobial activity (Parisot et al. 2008). Subtilin is biosynthesized from the structural gene SpaS which is a part of an operon-like structure containing about ten genes involved in activation, regulation and cellular transport of the gene product. Ericin S (3442 Da) and Ericin A (2986 Da) are two lantibiotic-like peptides obtained from *B. subtilis* A1/3 produced from a gene cluster with high degree of homology and occupying the same locus as the subtilisin gene cluster in *B. subtilis* 6633. These peptides are active against many gram-positive bacteria, including *Clavibacter michiganensis*, the causative organism for tomato bacterial canker. Type B lantibiotics include globular and uncharged peptides. Mersacidin (1825 Da) (Fig. 12.4b) produced by *Bacillus* sp. strain HIL Y-8554728 is a tetracyclic peptide lantibiotic belonging to this group. Mersacidin exhibits a globular structure due to the presence of four intermolecular thioether bridges. They are shown to have better protease resistance compared to other bacillus lantibiotics owing to its structure. mersacidin" is active against methicillin-resistant *S. aureus* (MRSA) and is similar to the glycopeptide vancomycin in its activity. It is also active against vancomycin-resistant enterococci. Unlike type A lantibiotics, mersacidin inhibits cell wall biosynthesis instead of creating pores in the cell wall to bring about its action. The biosynthetic gene cluster of mersacidin (12.3 kb) contains 10 open reading frames in addition to the mersacidin structural gene mrsA. Apart from the structural genes, the cluster contains genes for precursor modification enzymes (MrsM and MrsD) and a transport protein (MrsT). Screening of genome sequence based on homology for lantibiotics resulted in the identification of a broad-spectrum two-peptide mersacidin-like lantibiotic called as lichenicidin (3020 Da) from *B. licheniformis* ATCC 14580 and another peptide (3250 Da) with similar properties from *B. licheniformis* DSM 13. These peptides exhibit antimicrobial activity against methicillin-resistant *S. aureus*, *Listeria monocytogenes* and vancomycin-resistant enterococci.

Subtilosin (Fig. 12.4c) and Sublancin 168 are peptides of *Bacillus* origin classified in a distinct group of lantibiotics due to their deviant structures. Subtilosin is an anionic macrocyclic peptide antibiotic produced by *B. subtilis* and *B.*

(a) SUBTILIN

(b) MERSACIDIN

(C) SUBTILOSIN

Fig. 12.4 2D structures of some lantibiotics produced by *Bacillus* spp

amyloliquefacians with a molecular weight of 3398.9 Da composed of 32 amino acids along with some non-amino-acid residues. The structure contains a posttranslational linkage between a thiol group and the alpha carbon of an amino acid residue. They do not contain lanthionine and methyl lanthionine residues. Subtilosin shows strong antibacterial activity against *Listeria monocytogenes* even at high temperatures and in a wide range of pH conditions. Subalancin 168 (3887.8 Da) is a member of a group of glycosylated antimicrobial peptides called glycosins produced by *B. subtilis* 168. Sublancin contains two disulfide bridges and a single lanthionine bridge. It shows antimicrobial activity against *S. aureus*, *Streptococcus pyogenes* and *Bacillus cereus.*

Class II bacteriocins are small-molecular-weight peptides which do not contain modified residues or linkages other than disulfide bridges. Coagulin (4612 Da), a pediocin-like protease-sensitive antibacterial peptide produced by *B. coagulans*, belongs to this group. Coagulin shows antimicrobial activity against bacteria such as *Enterococcus*, *Leuconostoc*, *Listeria* and *Pediococcus*. Coagulin is active at temperatures up to 60 °C and within 4–8 pH range.

Antimicrobial proteins or peptides that are not well characterized but are similar to bacteriocins in activity are called bacteriocin-like inhibitory substances. Cerein 7A & 7B, MXRI & 8A are peptide antibiotics from *B. cereus*. They have a broad spectrum of antibacterial activity against gram-positive pathogens and are used as natural food biopreservatives. Tochicin (10.5 kDa) and a family of Thuricins are bacteriocin-like inhibitory substances identified from *B. thuringiensis*, a soil bacillus phylogenetically similar to *B. cereus.* Thuricin S is a well-characterized thuricin with a molecular weight less than 10 KDa and activity against *Listeria monocytogenes*, *Salmonella enterica* and *Pseudomonas aeruginosa*. Thuricin H and Thuricin 17 are other members of the thuricin group with a broad spectrum of activity. Other bacteriocins produced by genus *Bacillus* include Lichenin produced by *B. licheniformis* 26–103 RA strain, Megacin produced by *B. megaterium* and polyfermenticin SCD produced by *B. polyfermenticus*. Bacteriocins are used as preservatives in food systems, agents of biocontrol of phytopathogens and precursors of antibiotics.

Nonribosomally Biosynthesized Peptides Nonribosomal peptides include a group of peptide antibiotics synthesized by nonribosomal peptide synthetases independent of mRNA. The genus *Bacillus* produces several peptides using the nonribosomal mechanism, including iturins (cyclopeptides), fengycins and surfactins (macrolactones). Iturins (Fig. 12.5a) consist of a group of lipopeptides isolated from the culture media of various strains of *B. subtilis* and *B. amyloliquefacians*. They are amphiphilic in nature and contain a characteristic ring of seven amino acid residues including an invariable D-Tyr-2, with the constant chiral sequence LDDLLDL closed by a C14–C17 aliphatic β-amino acid. They exhibit antimicrobial activities against a wide variety of pathogenic yeasts and fungi but antibacterial activities are limited to few species like *Micrococcus luteus*. The antifungal properties of iturins are due to its interaction with the cell membranes of target cells and the formation of ion-conducting pores resulting in increased permeability of ions. The iturin group of antimicrobials includes A, C, D and E isoforms, Bacillomycin D, F and L and Mycosubtilin. Iturin A is antagonistic to *Fusarium oxysporum*, the phytopathogen causing potato disease. Iturins that inhibit fungal plant pathogens are also produced by *B. amyloliquefaciens* strains B94 and FZB42. Bacillomycin D produced by *B. amyloliquefaciens* strain FZB42 was shown to suppress the *Fusarium oxysporum*. The iturin A operon is 38–40 kb in size and consists of four reading frames ItuA, ItuB, ItuC and ItuD.

The fengycin (Fig. 12.5b) class of lipopeptides, including plipastatin, is produced by several species of *Bacillus* like *B. subtilis, B. cereus*, *B. amyloliquefaciens* and *B. globijii*. These bioactive molecules are lipodecapeptides comprised of a

(a) ITURIN C

(c) SURFACTIN

(b) FENGYCIN

Fig. 12.5 2D structures of some nonribosomally synthesized antimicrobials from the genus *Bacillus*

chain of 10 amino acid residues attached to a β-hydorxy fatty acid chain which can either be saturated or unsaturated and contains a lactone ring. The number of carbon atoms in the fatty acid chain can vary between C-14 and C-17 giving rise to different homologs. Fengycins are classified as fengycin A and fengycin B based on the amino acid at the sixth position in the peptide ring. Fengycin A contains alanine at the sixth position, whereas fengycin B contains valine. Fengycins are promising candidates for biocontrol of plant diseases as they are active against a wide range of plant fungal pathogens and postharvest pathogens.

Surfactins (Fig. 12.5c) are a group of cyclic lipoheptapeptides with a chain of seven amino acid residues attached to a β-hydorxy fatty acid with a chain length of 12–16 carbon atoms and contains a lactone ring. They are produced by *B. subtilis*, *B. licheniformis*, *B. natto* and *B. pumilus* strains. A variety of surfactin isoforms have been described under different names such as bacircine, halo- and isohalobacillin, lichenysin A/G, daitocidin and pumilacidin.

They exert an action similar to detergents on biological membranes. Surfactins can vary based on the sequence of the seven amino acids and the length of the fatty acid chain. These molecules are powerful biosurfactants used in biomedical and pharmaceutical industry as antimicrobial, antimycoplasma and antiviral agents. They are not antifungal in nature but show some synergetic action with iturin A against fungus. Bacilysin is a small nonribosomal peptide secreted by *B. subtilis.* This dipeptide consists of an L-alanyl (N-terminus) and L-anticapsin (C-terminus) units linked by a peptide bond. It shows antimicrobial activity against a wide range of bacteria including *S. aureus* and certain fungi, including *Candida albicans*. Bacilysin binds and inhibits the enzyme glucosamine synthase that is required for the synthesis of nucleotides, amino acids and coenzymes in microbial systems and thereby bring about cell death. Chlorotetaine is a chlorinated derivative of bacilysin with similar activity produced by certain strains of *B. subtilis.* Bacitracin (1486 Da) is a mixture of related nonribosomal cyclic peptides secreted by certain strains of *B. licheniformis and B. subtilis*. These peptides can disrupt the cell walls of both gram-positive and gram-negative bacteria and are therefore used in topical applications as an antimicrobial agent. Bacitracin is used together with polymixin and neomycin in medicinal preparations to treat skin and eye infections. Polymixins are a group of positively charged cyclic peptides isolated from cultures of *Paenibacillus (Bacillus) polymyxa* strains and are used against gram-negative bacteria (Landman et al. 2008). The long fatty acid chains in the structure of polymixins show high affinity to lipopolysaccharides of gram-negative cell membranes and hence have a disruptive effect on membrane integrity.

Polyketides In addition to peptides, the genus *Bacillus* also produces polyketides with important biological activities. They are synthesized from acyl-CoA precursors by decarboxylative Claisen condensations. *B. amyloliquefacians* FZB42 and GA1 strains produce difficidin (Fig. 12.6a), bacillaene (Fig. 12.6b) and macrolactin. Difficidin, oxydifficidin and bacillaene are also isolated from fermentation broths of *B. subtilis* strains. Difficidin and oxydifficidin are highly unsaturated 22-membered macrolide phosphates with broad-spectrum antibacterial activity against both aerobic and anaerobic organisms. Difficidin was shown to be effective against *Erwinia amylovara*, an important plant pathogen responsible for necrotrophic fire blight disease of apple and other members of rosaceous plants. Difficidin exerts its bactericidal activity by inhibiting protein synthesis.

Bacillaene (580 Da) is a bacteriostatic polyene antibiotic with a broad-spectrum activity which acts by inhibiting protein synthesis. Macrolactins and their derivatives succinyl or glycosylated macrolactins are polyketides with a macrolide-like structure containing three separated diene structure elements in a 24-membered lactone ring. At least 18 macrolactins were described from *Bacillus* spp., including seven compounds with a molecular mass of 402 Da. They are shown to have antibacterial activity against gram-positive bacteria like *S. aureus.* Macrolactin A (Fig. 12.6c) exhibits antiviral and cytotoxic activities. Macrolactin W, a recently identified macrolactin, shows activity against both gram-positive and gram-negative bacteria (Mondol et al. 2011).

a DIFFICIDIN b BACILLAENE c MACROLACTIN A

d AMICOUMACIN A e ZWITTERMICIN A f BACILYSOCIN

Fig. 12.6 2D structures of some antimicrobial metabolites produced by the genus *Bacillus*

Other Antibiotics Amicoumacin is an antibiotic complex isolated from the culture broth of *B. pumilus* BN-103. The antibiotic complex is composed of Amicoumacin A (423 Da), Amicoumacin B (424 Da) and Amicoumacin C (406 Da). Amicoumacin A shows activity against gram-positive bacteria, *Salmonella* spp. and *Shigella* spp. Amicoumacin C and D do not exhibit significant antibacterial activity compared to Amicoumacin A. Amicoumacin A (Fig. 12.6d) inhibits protein synthesis by binding to the ribosome and stabilizing the mRNA–ribosome interaction to prevent translocation (Polikanov et al. 2014). Zwittermicin A (Fig. 12.6e) is a linear aminopolyol antibiotic first isolated from *B. cereus* strain UW85. It represents a novel class of antibiotic with the biosynthetic pathway being a hybrid of polyketide pathway and nonribosomal peptide synthesis pathway (Luo et al. 2011). It shows a broad spectrum of antimicrobial activity inhibiting gram-positive, gram-negative and some eukaryotic organisms. Bacilysocin (Fig. 12.6f) is a phospholipid antibiotic that accumulates in the cells of *B. subtilis* 168. The structure of bacilysocin is elucidated as 1-(12-methyltetradecanoyl)-3-phosphoglyceroglycerol. Bacilysocin (lysophosphatidylglycerol) may be derived from phosphatidylglycerol through acyl ester hydrolysis. It shows antimicrobial activity, especially against certain fungi.

12.4.3 Genus *Pseudomonas*

Pseudomonas is a genus containing strictly aerobic, gram-negative bacteria belonging to the family *Pseudomonadaceae*. They are ubiquitous in nature with relatively low virulence. Approximately 191 valid species of Pseudomonas have been identified. *Pseudomonas* species inhabits soil, water, plants and animals. In fact, more than 50% of humans harbor *P. aeruginosa* (the most common species of *Pseudomonas*) asymptomatically. Pseudomonas is clinically significant as some species are opportunistic pathogens with the ability to cause nosocomial infections.

Apart from being causatives of some serious life-threatening infections, they rapidly develop resistance to antimicrobials used for therapy.

12.4.4 Antimicrobials from *Pseudomonas*

Pyocins Pyocins are high-molecular-weight bacteriocins produced by *P.s aeruginosa* as defense molecules against other strains of the same species. Besides being antimicrobial against strains of *P. aeruginosa,* they are reported to have limited antibacterial activity against other bacteria. Pyogenecity or the ability to produce pyocins is widely distributed in the strains of *P. aeruginosa,* but the level of spontaneous production of pyocin is very low because only a fraction of cells in a culture take part in the production of pyocins. The production of pyocins is enhanced by mutagenesis either using ultraviolet irradiation or addition of mitomycin C to actively growing cultures. Pyocins insert themselves into target cell walls after binding to specific receptors on the cell surface and disrupt the membrane potential of the target cells causing cell death. A single pyocin molecule is capable of producing such bactericidal effects. Three types of pyocins are described. R-type pyocins resemble the rod-like, contractile tail structure of microviridiae bacteriophages with no head structure and DNA content. They are protease and acid resistant. Examples of R-type pyocins include pyocins R1-R5, pyocin C9, 21 and 430 c. R-type pyocins are active against *Neisseria gonorrhoeae*, *N. meningitidis*, *Haemophilus ducreyi*, and *H. influenzae*. F-type pyocins are particulate, flexuous pyocins similar to the tail structure of noncontractile bacteriophages like λ phage. F-type pyocins include pyocin 28, pyocin F1 and F2 and 430 f. The third group of pyocins is called S-type pyocins. They are soluble pyocins that include pyocin S1–S5. Unlike bacteriocins, which are plasmid encoded, the structural genes for pyocins are chromosomally located. The synthesis of pyocins is a regulated genetic event with inducible regulator genes located near the structural genes (Michel-Briand and Baysse 2002).

Siderophores Siderophores are ferric (Fe^{3+})-ion-binding, low-molecular-weight molecular chelators (>1500 Da) that facilitate the transport of iron into microbial cells under conditions of low iron stress. Siderophores are used as biocontrol agents due to their ability to sequester and render iron unavailable to microbial pathogens along with other applications. Siderophores are used as agents for selective drug delivery in multidrug-resistant bacteria. The transportation abilities of siderophores are exploited for this by the production of drug-siderophore conjugates. Fluorescent *Pseudomonas* spp. produce two important classes of siderophores, namely, pyoverdines and pyochelins. Pyoverdines contain a dihydroquinoline-like chromophore attached to a peptide. At least 60 different pyoverdines are described with variable peptide lengths and sequences. Under specific conditions, pyoverdines function as a diffusible bacteriostatic or fungistatic antibiotic. Pyochelins are sparingly water-

soluble low-molecular-weight thiazoline derivatives containing a 2-hdroxyphenyl-thiazolinyl-thiazolidine scaffold. Pyoverdines and pyochelines are reported to enhance antibacterial activity of antimicrobial drugs if administered in the form of a complex. Pyoverdin-ampicillin conjugates and pyochelin-gallium conjugates were shown to have better antimicrobial activity against *Pseudomonas aeruginosa* when compared to activity of the drugs alone.

Polyketides Pseudomonas produces many secondary metabolites of biological importance using the polyketide pathway with the help of the enzyme polyketide synthase. Mupirocin, pyoluteorin and 2,4-diacetylphloroglucinol are examples of polyketide antimicrobials produced by *Pseudomonas*. Pseudomonic acids are a group of closely related antibiotics with a 17-carbon skeleton in their structure. Pseudomonic acid A–E are produced by *P.s fluorescens.* Pseudomonic acid A is also called mupirocin (Fig. 12.7a), which shows activity against gram-positive organisms, especially against superficial infections caused by *S. aureus*. The antibiotic is also active against some gram-negative bacteria like *Haemophilus influenzae* and *Neisseria gonorrhoeae* but is much less active against most gram-negative bacilli anaerobes. Mupirocin shows bacteriostatic effect at low concentrations and is bactericidal at high concentrations. Mupirocin exerts its antibacterial activity by binding to the isoleucine tRNA synthetase thereby inhibiting protein synthesis.
Pyoluteorin (Fig. 12.7b) is an antifungal compound containing a biochlorinated pyrrole linked to a resorcinol moiety produced by certain strains of *P. flurescens*. Pylouteorin can effectively inhibit phytopathogenic fungi, including the plant pathogen *Pythium ultimum*, and suppress plant diseases caused by this fungus. 2, 4-Diacetylphloroglucinol (DAPG) is a polyketide produced by *P. fluorescens* both in vitro and in the rhizosphere of wheat. Apart from its activity as a biocontrol agent, DAPG shows broad-spectrum antifungal and antibacterial effects. It causes membrane damage to the phytopathogenic *Pythium* spp. and is particularly inhibitive of the zoospores of these fungi (de Souza et al. 2003).

Modified Lipopeptides Lipodepsinonapeptides are a class of phytotoxins which are composed of a polar head and a hydrophobic 3-hydroxy fatty acid chain. Syringomycin (Fig. 12.7c) is a representative compound of this group produced by most strains of *P. syringae*. Three types of syringomycins are produced that differ only by the length of the fatty acid moiety: decanoic acid (syringomycin A1), dodecanoic acid (syringomycin E) and tetradecanoic acid (syringomycin G). Syringotoxins and syringostatins are lipodepsinonapeptides produced by *P. syringae* isolated from citrus and lilac hosts, respectively. Pseudomycin is isolated from saprophytic *P. syringae* inhabiting barley. Syringomycin and other lipodepsinonapeptides show fungicidal activity against a broad spectrum of filamentous fungi such as *Geotrichum candidum* and yeasts such as *Rhodotorula pilimanae*. The antifungal properties of syringomycins were sought to be exploited against clinically relevant fungi like *Candida* and postharvest fungal pathogens of citrus fruit such as

a MUPIROCIN

b PYOLUTEORIN

c SYRINGOMYCIN

d TABTOXIN

e PYRROLNITRIN

Fig. 12.7 Some antimicrobials produced by the genus *Pseudomonas*

Penicillium digitatum but the strong hemolytic activity offers obstacles in the path of commercial development. Besides being strong antifungal agents amphipathic syringomycins are strong biosurfactants. Lipodepsipeptides are another class of modified lipopeptides produced by *Pseudomonas* spp. Syringopeptins are lipodepsipeptides containing either 22 or 25 amino acid residues attached at the N-terminus to 3-hydroxy-decanoic acid or 3-hyroxydodecanoic acid depending on the strain

they are isolated from. Syringopeptins are produced by different strains of *P. syringae.* Syringopeptin shows antimicrobial activity against some gram-positive bacteria and fungi. It is highly active against *Botrytis cinerea* and *Bacillus megaterium*. The antimicrobial properties of syringomycin and syringopeptides can be attributed to their ability to produce pores in the plasma membranes of target cells. Corpeptins are another group of lipodepsipeptides produced in the cultures of *Pseudomonas corrugata,* the causal agent of tomato pith necrosis, with surfactant, antimicrobial and phytotoxic activities. Fuscopeptins A and B are produced by the plant pathogen *P. fuscovaginae*, the causative agent of 'sheath brown rot' of cultivated and wild gramineae. They form pores in the membrane to bring about their antimicrobial activity (Coraiola et al. 2008).

Tolaasin is a 1985-Da lipodepsipeptide secreted in the culture medium of *P. tolaasi,* a saprophytic bacterium infecting edible mushrooms. Tolaasins are reported to have detergent-like action at high concentrations with the ability to dissolve eukaryotic membranes. Tolaasins are active against many basidiomycetes and gram-positive bacteria but inactive against gram-negative bacteria (Bassarello et al. 2004). Viscosin is a lipopeptide biosurfactant produced by *P. flourescens* that is active against the plant pathogen *pythium*. Viscosinamide is a cyclic depsipeptide isolated from *P. flourescens* with antibiotic and biosurfactant properties. It is active against the plant pathogens *Pythium ultimum* and *Rhizoctnoia solani* both *in vitro* and *in planta* (Haas and Défago 2005).

Other Antimicrobials Tabtoxin (Fig. 12.7d) is a dipeptide biotoxin belonging to the monocyclic β-lactam class of antibiotics produced by *Pseudomonas syringae* pv. tabaci, *P. syringae* pv. coronafaciens and *P. syringae* pv. garca. It consists of a Tabtoxin β-lactam (TβL) linked to threonine by a peptide bond. Tabtoxin is also known as the wildfire toxin due to its role in wildfire disease in tobacco. It is the precursor to the antibiotic tabtoxinine β-lactam, which unlike other β-lactam antibiotics exerts its effect by inhibiting glutamine synthetase. Tabtoxin shows strong antimicrobial activity. Phaseolotoxins are biotoxins produced by *P. syringae* pv. phaseolicola and *P. syringae* pv. actinidiae that cause halo blight in legumes and bacterial canker on Kiwi fruit respectively. The structure consists of a tripeptide made of ornithine, alanine and homoarginine linked to a sulfodiaminophosphinyl moiety. Phaseolotoxins exert their antimicrobial activity by inhibiting ornithine carbonyl transferase responsible for the conversion of ornithine and carbamoyl phosphate to citrulline in the urea cycle.

Pyrrolnitrin (Fig. 12.7e) is an antifungal antibiotic produced by certain species of pseudomonas like *Pseudomonas pyrrocinia*, *P. chlororaphis* and *P. cepacia*. Pyrrolnitrin is active against superficial and systemic mycoses involving *Candida albicans, Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Sporotrichum schenckii*, and *Histoplasma capsulatum.* It antagonizes the growth of plant fungi like *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Pyrolnitrin prevents fungal growth by inhibiting the respiratory electron transport system.

12.4.5 Myxobacteria

Myxobacteria are a group of proteobacteria that predominantly inhabit the soil and are characterized by the ability to sporulate within fruiting bodies. They are social microbes with the ability to form cellular associations that form swarms that move by axonal cellular motion called gliding. Such ripple-like movement is used to accumulate particulate organic matter they feed on. When these waves of myxobacterium collide, they form cellular aggregates that can accommodate up to 10^5 cells and form fruiting bodies. Cells within these aggregates form myxospores that germinate when nutrients are available. Events like sporulation and germination are regulated by intracellular and extracellular signaling mediated by proteins and small metabolites.

12.4.5.1 Antimicrobials from Myxobacteria

The myxobacterial metabolome is rich in diverse and biologically active metabolites (Schäberle et al. 2014). Up to 40% of biologically active metabolites described from myxobacteria represent novel structural classes of antimicrobials. They form nonglycosylated metabolites unlike actinomycetes and inhibit novel and unique targets. The diversity and novelty in the bioactives produced by myxobacteria have been argued to confer competitive advantage in the soil ecosystem, which enable effective cell–cell communication in population and in predation.

12.4.5.2 Inhibitors of Bacterial RNA Polymerases

DNA-dependent RNA polymerases are well-known targets for antimicrobials. They are essential for the survival of bacteria and are highly conserved. Four antimicrobials and their derivatives with the ability to inhibit bacterial RNA polymerase are identified from myxobacteria namely corallopyronin A, myxopyronin A, ripostatin A and sorangicin A. Corallopyronins are produced by a strain of *Corallococcus coralloides.* They contain a core pyrone ring attached to two conformationally flexible chains called the western and eastern chains. The western chain is lipophilic with three methyl groups, a hydroxyl group and a diene element. The eastern chain contains one methyl group, an enamide functional group and a carbamate moiety. Three analogues, namely, carollopyronin A (Fig. 12.8a), B and C are also known. Myxopyronin A and B are antibiotics that are structurally similar to carollopyronin isolated form *Myxococcus fulvus* strain. Myxopyronin A and Myxopyronin B share the pyrone ring and western chains of carollopyronin but have a shorter and unhydroxylated eastern ring terminated at carbon C-24 and carbon C-25 of corallopyronins, respectively. Carollopyronin A shows antimicrobial activity against *S. aureus* and *Bacillus meagaterium*. Other carollopyronins show weak activity against these organisms. Carollopyronin A shows weak activity against *Mycobacterium smegmatis* and relatively better activity against *M. bovis*. Carollopyronin A is also active *in vivo* against *Wolbachia* species, intracellular bacteria of nematodes. This offers the possibility of developing a drug for filariasis without the risk of developing cross-resistance to *mycobacterium*. Myxopyronins shows activity against *S. aureus*

and *Bacillus megaterium*. None of these antibiotics are active against gram-negative organisms.

Ripostatins are a group of RNAP inhibitor antibiotic isolated from myxobacteria. Ripostatin A, B and C were isolated from *Sorangium cellulosum* Soce377. Ripostatin A and B are 14-membered macrolide antibiotics with double bonds at positions 2, 5 and 8. Ripostatin C is noncyclic in structure, and all the three ripostatins share a terminal phenyl ring. Ripostatin A and B are active against *S. aureus* and *E. coli* tolC. Ripostatin C does not show antimicrobial activity. Ripostatin B (Fig. 12.8b) showed minor activity against fungi like *Nadsonia fulvescens* and *Debaryomyces hansenii*. Sorangicins are another group of antimicrobials isolated from the sporangium genus of myxobacteria. Sorangicin A, Sorangicin B and Sorangiosides were isolated by activity-based screening of the fermentation broth of *Sporangium cellulosum* So ce12. The structure contains a core macrocyclic hydrolacton with seven carbon–carbon double bonds. The macrocycle consists of three pyran rings, one being a trisubstituted dihydropyran ring and the other two tetrasubstituted tetrahydro pyran rings. Sorangicin A and B are highly active against gram-positive bacteria, including *Mycobacterium phlei* and *Nocardia corallina*. Gram-negative bacteria were only inhibited at high concentrations. Etnangien characterized by a 22-membered polyhydroxylated macrolide ring is also isolated from strains of *Sorangium cellulosum* (Irschik et al. 2007). It shows a broad spectrum of activity against gram-positive bacteria, including mycobacteria.

12.4.5.3 Inhibitors of Protein Synthesis

Some of the antimicrobial molecules derived from myxobacteria act by inhibiting protein synthesis in target cells. Althiomycin (Fig. 12.8c) is a sulfur-containing antibiotic originally isolated from *Streptomyces althioticus*. It is also produced by the genera cystobacter and myxococcus of myxobacteria as well as the insect pathogen *Serratia marcescens*. The chemical structure of althiomycin is comprised of an oxime group, a thiazole, a thiazoline and a methoxypyrrolinone ring. Althiomycin shows a broad spectrum of antimicrobial activity against both gram-positive and gram-negative bacteria, including *Klebsiella pneumoniae*, *S. aureus*, and *Corynebacterium diphtheriae*. Angiolam A is a lactam-lactone antibiotic containing a 19-member macrocyclic ring with methyl, carbonyl and hydroxyl groups and a single carbon–carbon double bond. It is isolated from *Angiococcus disciformis* An d30. Angiolam is a bacteriostatic antibiotic with a narrow spectrum of activity limited to few members of gram-positive Bacillaceae, including *Clostridium perfringens*. Myxovalargin A and its derivatives myxovalargin B and C are peptide antibiotics produced by *Myxococcus fulvus* strain Mx f65. They are composed of a linear peptide chain made of 14 amino acids, including nonproteogenic amino acids like 3-methylbutyric acid, α,β-dehydrovaline, α,β-dehydroleucine and (S)-β-tyrosine. Myxovalargins are active against gram-positive bacteria like *Micrococcus luteus* and *Corynebacterium mediolanum*.

a CORALLOPYRONIN A

b RIPOSTATIN B

c ALTHIOMYCIN

d AURACHIN A

e CAROLACTON

f MYXOVIRESCIN A

Fig. 12.8 Some antimicrobials produced by Myxobacteria

12.4.5.4 Inhibitors of Respiration

Three metabolites of the isoprenoid quinoline alkaloid group, namely, aurachin A (Fig. 12.8d), B and C are produced by *Stigmatella aurantiaca* and members of *Rhodococcus* species. Aurachins D and E are also isolated as minor products. All aurachins share the quinoline nucleus substituted with a sesquiterpene. Aurachins show a narrow spectrum of activity directed toward gram-positive bacteria and have no effect on gram-negative bacteria. Aurachins also exhibit antiplasmodial activity by inhibiting *Plasmodium falsiparum* in vitro. They show weak antifungal activity against fungi like *Debaryomyces hansenii* and *Saccharomyces cerevisiae.* They share structural similarities with the respiratory chain inhibitor 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) and is able to prevent NADPH oxidation at very low concentrations compared to HQNO.

Thuggacins are a group of macrolides containing a thiazol group produced by *Sorangium cellulosum* strain So ce895. Four thuggacins are identified and named as thuggacin A, B, C and methyl thuggacin A. In addition to the thiazole ring, thuggacins contain a diene moiety, an α, β unsaturated lactone with an *n*-hexyl side chain attached at C-2 and an additional side chain at C-16 containing three hydroxyl and a diene function. More thuggacins were isolated later from *Chondromyces crocatus* strain Cm c5. Thuggacins exhibit antibacterial activity against gram-positive bacteria, including *Micrococcus luteus.* They also show activity against mycobacteria like *M. luteus*, *M. phlei*, *M. chitae* and *M. tuberculosis*. Thuggacins bring about their antibiotic effects by inhibiting the terminal stages of respiration thereby cutting down the energy supply to the target cells (Steinmetz et al. 2007).

12.4.5.5 Inhibitors of Biofilm Formation

Biofilms offer increased resistance to antibiotics compared to free-living bacteria because of the impermeable barrier created by biofilms to antibiotic penetration. The search of antimicrobials that can disrupt biofilm structures is active. Carolacton (Fig. 12.8e) is a macrolide antibiotic isolated from *Sporangium cellulosum* strain So ce960 that show biofilm disruptive activity. The structure contains a 12-membered lactone ring with two secondary hydroxyl groups at C-17 and C-18 and a terminal carboxyl group on the side chain. Carolacton shows inhibitory activity against the gram-negative bacterial strain *E. coli* tolC along with a disruptive action on bacterial biofilms. It showed promising activity against the formation of dental caries and endocarditis caused by *Streptococcus mutans* (Kunze et al. 2010). *Streptococcus mutans* forms biofilms that are sensitive to carolacton. Carolacton shows promising results as an additive in dental filling material. The biofilm inhibition of carolacton is comparable with that of triclosan and chlorhexidine. Carolacton also shows antifungal activity against *Aspergillus niger*, *Pythium debaryanum* and *Sclerotina sclerotiorum.*

12.4.5.6 Inhibitors of Lipoprotein Processing

Lipoprotein signal peptidase A (LspA) is a type II signal peptidase involved in the processing of lipoproteins, essential components for the survival of gram-negative bacteria and to a certain extent for gram-positive bacteria. Lipoproteins are

components of the outer membrane that play a role in cell viability and pathogenesis. LspA offers a novel target for antibiotics. Myxovirescins (Fig. 12.8f) represent a class of LspA binding antibiotics isolated from myxobacterial strains. The first antibiotic of this type was isolated from *Myxococcus virescens* strain Mx v48. The basic structure contains a 28-member ring with lactam and lactone functionality. Myxovirescins are active against gram-negative bacteria like *E. coli* and *P. aerugenosa* and gram-positive bacteria like *S. aureus* and *B. megaterium*. Myxovirescins bind to the type II signal LspA to bring about its bactericidal effect. Binding to LspA results in a toxic accumulation of lipopolysaccharides inside the cells, leading to a lethal cross-linking of the cell wall and the inner membrane and results in the improper localization of lipoproteins on the outer membrane.

12.4.5.7 Other Antibiotics

Myxobacteria produce a number of antimicrobials of which the mode of action is not yet determined. Chondrochlorens are produced by *Chondromyces crocatus* strain Cmc5. They are chlorohydroxy styryl amides with the styrene moiety linked with an aliphatic side chain by an amide bond. Chondrochlorens A and B are described. Chondrochlorens A shows weak inhibitory activity against *Micrococcus luteus* and *Schizosaccharomyces bombe*. Chondrochlorens B is active against *M. luteus and B. subtilis* (Jansen et al. 2003). Indiacens A and B are 3-formylindol derivatives isolated from *Sandaracinus amylolyticus* strain NOSO-4 T, belonging to a recently characterized myxobacterial genus. Indiacin B is the chlorinated derivative of Indiacin A. The compound shows moderate activity against gram-positive and gram-negative bacteria (Steinmetz et al. 2012).

Maracin A and maracen A are molecules with antimycobacterial activity isolated from *Sorangium cellulosum* strain Soce 880 and Soce 1128, respectively. Maracin A is characterized by the presence of an unusual ethynyl-trans-vinyl ether moiety which is replaced by an α-chlorovinyl group in maracen A. Nannochelins are a group of citrate hydroxamate siderophores isolated from *Nannocystis exedens* strain Na e485. Nannochelins show antibacterial activity against gram-positive organisms, including *Bacillus* spp. and *Brevibacterium ammoniagenes* along with weak antifungal activity. Roimatacene is a chemically unstable polyenic carboxylic acid isolated from *Cystobacter ferrugineus* Cb G35. The molecule shows selective activity against gram-negative bacteria unlike other antibiotics derived from myxobacteria (Zander et al. 2011). Sorangiadenosine isolated from *Sorangium cellulosum* strain KM1003 contains a nucleoside substituted with a sesquiterpene. It is a moderate inhibitor of gram-positive bacteria (Ahn et al. 2008). Sulfangolids are sulfate ester containing secondary metabolites isolated from *S. cellulosum* strains. They are structurally composed of macrolides conjugated to a triene or tetraene (Zander et al. 2012). Kulkenon is another metabolite structurally identical to sulfangolids isolated from *S. cellulosum* (Symkenberg and Kalesse 2014). Salimyxin B and enhygrolide A were isolated from the marine myxobacterium *Enhygromyxa salina*, and they show antimicrobial activity against *Arthrobacter cristallopoietes* (Felder et al. 2013).

12.4.6 Filamentous Fungi

Fungi have been historically recognized as the food of gods. Many fungi are considered as sources of highly nutritious and palatable sources of food materials since ancient times. Many cultures, particularly the Asian culture, had an understanding of the curative properties of fungi and were sometimes referred to as an elixir of life. Today, fungi are being explored as potential sources of pharmaceutically important molecules. The macroscopic fungi represented by edible morels, mushrooms, puffballs and cultivated agarics sold as food form only a tiny fraction of the fungal kingdom. The microscopic fungi represented by molds and yeasts form the majority of economically important plant parasites, allergenic species and opportunistic pathogens of humans and animals. The microscopic fungi are characterized by filamentous vegetative outgrowths called as the hyphae. A mass of such hyphae forms the vegetative body of the fungus called the thallus composed of mycelium. The hyphae of fungi can be aseptate as seen in phylogenetically primitive molds like water molds, bread molds and other sporangial forms. They produce coenocytic filaments composed of multinucleate cells without cross walls. Septated hyphae are produced by advanced fungi in which the filaments are separated into uninucleate or multinucleate compartments by the formation of the septum. The septa help in intercytoplasmic communication. Certain fungi like the yeast do not form filamentous hyphae. They exist as unicellular organisms that reproduce by vegetative budding. Some fungi, especially opportunistic pathogens like *Candida albicans*, are dimorphic in nature with the ability to grow as hyphal forms outside the host and as vegetative reproducing yeast inside the host system. Fungi are heterotrophic in nature and can either be saprophytic or parasitic. Saprophytic fungi rely on nonliving organic matter as the source of carbon, while parasitic fungi utilize living organic matter for carbon source. Micromolecules like simple sugars and amino acids enter the fungal cell by simple diffusion after accumulating around the hyphae. Macromolecules are digested in the extracellular atmosphere before being taken up into the cell by secreted proteolytic, glycolytic and lipolytic enzymes.

12.4.6.1 Antimicrobials from Fungi

The fungi belonging to basidiomycota and ascomycota are valuable reservoirs of biologically active molecules. Several biomedically significant secondary metabolites have been isolated from the fungal fruiting bodies, mycelia and culture broth of submerged cultivations. Basidiomycota and ascomycota represent the most diverse groups in the fungal kingdom. They produce secondary metabolites with wide range of biological activity including antibacterial, antiviral, antifungal, antitumor, anti-inflammatory and immunomodulatory effects along with an impact on cardiovascular disorders, hypercholesterolemia and diabetes. Some of the recently identified antimicrobial lead compounds from fungi are enlisted in Table 12.2.

12.4.6.2 Antibacterial Antibiotics

The first antibiotic ever isolated from a natural source came from a fungus belonging to the phylum ascomycota. Penicillin was discovered in 1928 by the Scottish

Table 12.2 Some of the recently identified antimicrobial secondary metabolites from fungi

Species	Source	Preparation	Biological activity/Targets
Ganoderm alucidum	Mycelium	Ganoderan	Antiviral
Ganoderma pfeifferi	Mycelium	Ganomycin	Antibacterial
Grifola frondosa	Fruit bodies, mycelium and culture liquid	β-(1,3)-D-glucan	Antiviral and antimicrobial activity
Pleurotus ostreatus	Mycelium	Ethanolic extract	Antibacterial, antifungal
Lentinus edodes	Fruit bodies and mycelium	Lentinan	Antiviral and antibacterial (*Streptococcus*, *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp. and *Porphyromonas* spp. of bacteria)
	Culture liquid	Cortinelin and Eritadenin	Antibiotic activity
Inonotus hispidus	Fruit bodies	Hispolon and Hispidin	Antiviral activity
Coprinus spp	Culture fluid	Coprinol	Multidrug-resistant gram-positive bacteria
Pleurotus eryngii		Eryngin (Antifungal peptide)	*Fusarium oxysporum* and *Mycosphaerella arachidicola*
Monascus spp.	Ethanol: *n*-hexane (1:10) extract	Monascidin A	*B. subtilis, P. aeruginosa* and *E. coli*
			Aspergillus, Penicillium, Mucor,
		Rubropunctatin and monascorubin(Pigments)	*Fusarium, Alternaria* and *Botrytis*
		Citrinin	Gram-positive and gram-negative bacteria
Pleurotus sajor-caju		12 kDa ribonuclease	*F. oxysporun*, *M. arachidicola*, *P. aeruginosa* and *S. aureus*

bacteriologist Alexander Fleming from *Penicillium chrysogenum*, a common mold found in indoor environments such as dust, damp air and building materials. *P. chrysogenum* was earlier known as *P. notatum*. Penicillin was the first antibiotic of the β-lactam class of antibiotics that contain a characteristic β-lactam ring in their molecular structure. Other members of this group are produced by modification of the β-lactam ring. Methicillin was the first modified antibiotic of this group. Modifications to the β-lactam ring were performed to change the properties of the original molecule to produce new classes of penicillins like the aminopenicillins, carboxypenicillins and ureidopenicillins. These antibiotics have a broad spectrum

of activity and generally act by inhibition of cell wall synthesis. Cephalosporins are a subgroup of β-lactam antibiotics with structural and functional similarity to penicillins. The first cephalosporin antibiotic was discovered from *Cephalosporium acremonium*, saprotrophic fungi found in soil, plant debris and decaying mushrooms. The natural cephalosporin was then modified chemically to produce the modern-day versions of the antibiotic.

12.4.6.3 Antifungal Antibiotics

Griseofulvin (Fig. 12.9a) is an orally administered antifungal agent isolated from the fungus *Penicillium griseofulvum*. Griseofulvin is used to treat fungal dermatological infections in humans. It is a fungistatic agent that disrupts the mitotic spindle in fungal cells to arrest them in metaphase of the cell cycle. Strobilurins are a group of agricultural fungicides derived from natural fungicidal derivatives of β-methoxy acrylic acid produced by a range of basidiomycete wood-rotting fungi (Balba 2007). Strobilurin A (Fig. 12.9b) and Oudemanzin A are produced by *Strobilurus tenacellus* and *Oudemansiella mucida* respectively. They are respiratory inhibitors that act by inhibiting mitochondrial respiration by binding to cytochrome b. This binding prevents the electron transfer between cyctochrome b and cytochrome c1 and prevents the fungal cell from producing ATP. These naturally occurring molecules were modified to produce commercially used strobilurins including azoxystrobin, the world's largest agricultural fungicidal agent.

Echinocandins are a class of antifungal drugs that act by the inhibition of β-D glucans in the fungal cell wall. They are characterized by low toxicity and a wide antifungal spectrum including most isolates of *Candida* spp., *Aspergillus spp*. and *Pneumocystis carinii.* The first commercially licensed echinocandin is caspofungin acetate sold by Merck. Inc. under the trade name Cancidas for the treatment of invasive aspergillosis. Echinocandins are chemically modified lipopeptides originally obtained from fermentation broths of various fungi. Aculeasin A was isolated from *Aspergillus aculeatus*, echinocandin B (Fig. 12.9c) from *Aspergillus rugulovalvus*, pneumocandin B from *Zalerion arboricola*, enfumafungin from a hormonema-like fungus and the papulacandin from *Papularia sphaerosperma.* Sordarins are derivatives of sordarin (Fig. 12.9d), originally isolated from fermentation broths of terrestrial ascomycete *Sordaria araneosa*. They are recognized as potential antifungal agents with a broad spectrum of activity against many fungal pathogens infecting immune-compromised individuals. They are active against *Candida* spp., *Cryptococcus neoformans*, *Pneumocystis carinii*, as well as dermatophytes. These molecules exert their activity by inhibiting the elongation step of bacterial protein synthesis in the target fungi (Liang 2008). This mechanism of action is unique among typical antifungal agents that act by inhibiting cell wall biosynthesis.

12.4.6.4 Alkaloids and Polyketides

Ergot alkaloids are mycotoxins produced by the genus *Claviceps* belonging to the phylum ascomycota. Two alkaloids belonging to this group, agroclavine (Fig. 12.9e) and festuclavine, show bacteriostatic activity against *E. coli, S. aureus, P. aeurogenosa* and other bacteria (Matuschek et al. 2011). Diketopiperazine alkaloids are

a GRISEOFULVIN

b STROBILURIN A

c ECHINOCANDIN B

d SORDARIN

e AGROCLAVINE

f TERRAIN

Fig. 12.9 Some antimicrobial compounds produced by fungi

produced by the genus *Penicillium*, including rugulosuvines, brevianamides and fellutanine A–E; isofellutanine B and C are also reported to have antibiotic potentials. Viridicatin and Viridicatol are quinoline alkaloids produced by the *Penicillium* fungi with antimicrobial activity against *Mycobacterium tuberculosis*. Quinocitrinins are another group of secondary metabolites produced by the *Penicillium* group with known activity against gram-positive and gram-negative bacteria, yeast and fungi.

Other metabolites from the genus *Penicillium* like roquefortin and meleagrin are also known to have antimicrobial activities. Terrein (Fig. 12.9f) is a small antifungal polyketide produced by *Aspergillus terreus*. Terrein was later found to be an inhibitor of breast cancer, with additional activity against pancreatic and liver cancer cell lines. Terracyclic acid A is a sesquiterpene antifungal isolated from the same species. Brefeldin A is another small molecular weight polyketide with antifungal and antitumor activities isolated from *Penicillium brefeldianum*. Gliotoxin is a diketopiperazine alkaloid produced by *Aspergillus fumigates* with known antifungal and antimicrobial activities. The ophiobolins are sesterterpenes isolated mainly from the genus *Bipolaris* but also produced by *Aspergillus, Sarocladium and Drechslera* with antifungal activities.

3-*O*-methylfunicone (*Talaromyces pinophilus*), chaetomugilin family of azaphilones (*Chaetomium globosum*), Xanthocillin X (*Dichotomomyces albus, P. chrysogenum*), emestrin A (*Emericella striata*), wortmannin (*T. wortmannii*), cytochalanins and chaetoglobosins (Aspergillus spp., zygosporium, etc.), macrophorin A (*Talaromyces purpurogenus*), botryodiplodin (*T. stipitatus*) and the atpenins (*P. atramentosum*) are examples of fungal metabolites that exhibit antifungal activity along with antitumor activity (Bladt et al. 2013).

12.4.7 Antimicrobials from Other Prokaryotic Sources

Many bacteria other than the major producers discussed above produce antimicrobials. *Micromonospora* a genus of gram-positive, aerobic, saprophytic bacteria belonging to Micromonosporacea family are sources of aminoglycoside, macrolide and other antibiotics normally designated with names ending in '–micin' to differentiate them from antibiotics produced by *Streptomyces*. These include gentamycin produced by *Micromonospora purpurea*, mutamicin and netilmicin produced by *M. inyonensis*, everninomicins produced by *M. carbonacea*, citreamicins produced by *M. citrea*, sisomicin produced by *M. inositola* and rosaramicin from *M. Rosaria* and megalomicin produced by *M. megalomicea.* Teicoplanins and Ramoplanins are broad-spectrum antibiotics produced by the bacterial genus *Actinoplanes*.

Uncultured bacteria represent 99% of all microbial diversity and are untapped bioresources for anitbacterial discovery. Development of methods to culture bacteria in their natural environments based on cultivation in diffusion chambers has enabled identification of novel antimicrobial from previously uncultured bacteria. Lassomycin is a highly basic ribosomally encoded cyclic peptide with an unusual structural fold that only partially resembles that of other lassopeptides. Lassomycin exhibit potential bactericidal activity against both growing and dormant mycobacteria, including drug-resistant forms of *M. tuberculosis*, but little activity against other bacteria or mammalian cells. Lassomycin exerts its antibacterial activity by uncoupling ATPase from its proteolytic activity (Gavrish et al. 2014). Teixobactin (Fig. 12.10), a novel antibiotic from a β-proteobacteria provisionally named

Fig. 12.10 The structure of Teixobactin

Eleftheria terrae, was identified using a multichannel device called the ichip that enables the growth of bacteria in their natural environment.

The antibiotic belongs to a novel antibiotic class exerting its effects by binding to a highly conserved domain of lipid II (precursor of peptidoglycan) and lipid III (precursor of cell wall teichoic acid), thereby inhibiting cell wall biosynthesis. Teixobactin shows activity against gram-positive pathogens like S. aureus and *Mycobacterium tuberculosis*. Teixobactin was exceptionally active against *Clostridium difficile* and *Bacillus anthracis*. Importantly no mutants with resistance to these strains were isolated from cultures inoculated with the antibiotic (Ling et al. 2015).

12.5 Conclusions

Microorganisms are a highly potent source of therapeutically important metabolites. The development of resistance to antimicrobials poses the greatest threat to chemotherapeutic intervention of human diseases. New lead compounds that target novel pathways in the growth and survival of microorganisms with the lowest probability of developing resistance are a need of the hour. Without the advent of such molecules the incidence and spread of multidrug-resistant pathogens will remain unchecked.

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Novel Sources of Antimicrobials

13

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Abstract

The battle between humanity and pathogenic microorganisms started since the appearance of human race on earth. The challenges posed by pathogenic microbes by their antibiotic resistance forced the scientific community to think out of the box and look for unusual natural sources of antimicrobials. Nature has always been a great source of bioactive compounds. Both marine and terrestrial organisms are being explored for novel compounds as the secondary metabolites produced by them could have antimicrobial properties. Terrestrial plants, their symbiotic associations, animals and their products, and soil microorganisms produce various compounds that have antimicrobial activity. Several thousands of bioactive compounds have been identified from different types of marine organisms including invertebrates and their symbiotic associations, algae, fungi, and higher plants. Due to the unique metabolic requirements and ecological adaptations, organisms from extreme habitats grab great attention as the source of novel bioactive compounds. Deep-sea sediments have been proved to be another rich source of microorganisms that produce antimicrobial compounds which are active even against drug-resistant bacteria. Right use of new technologies in the systematic exploration of the vast fauna and flora has been providing novel antimicrobial leads and new drugs. The current chapter highlights several novel sources of antimicrobials which are being explored.

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13.1 Introduction

13.1.1 Pathogens and Antimicrobials

Microorganisms are the most abundant forms of life and components of every ecosystem on the earth. "Microorganisms" is a general term used to represent a vast variety of minuscule organisms consisting of bacteria, protozoa, yeasts, molds, etc. They range in complexity from single to multicellular organisms. Magnification techniques are invariably required to observe and study them. Despite the fact that viruses cannot live and reproduce on their own, they are also classified under this group. From an anthropocentric perspective, microorganisms are classified as beneficial and harmful. Though the beneficial species evidentially outnumber the harmful ones, man has a tendency to relate the term "microorganisms" only to pathogenic (disease-causing) species. This may be due to the lingering dreadful memories of deadly epidemics he witnessed that wreaked havoc to humanity like the Black Death (bubonic plague), tuberculosis, influenza, smallpox, cholera, malaria, etc. During early days he was left clueless about the causes, and measures for prevention and control of those diseases. Man, being an ever-exploring species, with his innate quest for knowledge and the advent of technologies, was able to identify the causes of these diseases and discover methods to treat them.

Antimicrobial substance is any chemical compound or physical agent that kills or inhibits growth of microorganisms even at very low concentrations but causes little or no damage to the host. This is a general term used to represent all agents that act against various types of microorganisms – bacteria (antibacterial), viruses (antiviral), fungi (antifungal), and protozoa (antiprotozoal). These substances are of natural, synthetic, or semisynthetic origin. The term "antimicrobial" was derived from the Greek word *anti* (against), *mikros* (little), and *bios* (life). This term is largely used interchangeably with "antibiotic." By strict definition, the word antibiotics refers to substances produced by microorganisms that act against another microorganism. This term is derived from the Greek words *anti* (against) and *biotikos* (concerning life) (Michael et al. 2014).

13.2 Brief History of Antimicrobials

Contrary to popular belief that the use of antimicrobials began in the mid-twentieth century, research revealed the use of antibiotics by ancient populations. Traces of tetracycline were found in the ancient Sudanese human skeletal remains that date back to fourth to sixth century AD (Bassett et al. 1980, Nelson et al. 2010). Interestingly, it is inferred that the rate of infectious diseases documented in this

population was low. Another independent study revealed the presence of tetracycline in the late Roman period skeletons of Egypt (Armelagos 1969, Cook et al. 1989). These observations underline the fact that ancient civilizations used materials containing tetracycline as part of their diet.

Alexander Fleming's serendipitous-yet-miraculous discovery of Penicillin in 1928 revolutionized the method of treatment. Although molds were used to combat infectious diseases by different civilizations during ancient times, Fleming's persistent pursuance on the topic and the purification methods perfected by Howard Florey and Ernst Chain led to mass production and clinical use of Penicillin (Chain et al. 1940). This molecule was rightly nicknamed as "wonder drug" following its ability to cure deadly diseases such as syphilis, gangrene, and tuberculosis. Much before the discovery of Penicillin and its first clinical use in 1940s, the foundation stone for "antibacterial chemotherapy" had already been laid by Paul Ehrlich. In 1909 he discovered Salvarsan, a synthetically derived compound which was used to treat syphilis (Ehrlich and Hata 1910). This event is remembered not only for the discovery of an antimicrobial compound but also for the introduction of a systematic screening procedure for drug discovery, that resulted in the identification of thousands of drugs and their subsequent clinical use. Following Ehrlich's methodology, Gerhard Domagk tested Prontosil, a sulfa drug synthesized by Josef Klarer and Fritz Mietzsch, in 1935 and found that it is effective against many important bacterial infections in mice (Chain et al. 1940). The discovery of Penicillin, Salvarsan, and Prontosil inspired the scientific community, which resulted in the discovery of many antimicrobials and clinical use of some of them.

13.3 Need of Novel Sources

As man was broadening his arsenal with more and more antimicrobial agents and inching toward victory over pathogens by complete eradication of infectious diseases, the microbes were in preparation of a silent revolt – antimicrobial resistance. WHO defines antimicrobial resistance as the "resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it." Various reasons are attributed to microbial drug resistance that include selective pressure, mutation, horizontal gene transfer (HGT), etc. Due to the development of resistance of pathogens to various drugs, the treatment regimen was redefined to include more than one drug in order to kill those pathogens which could be resistant to one (or more) of the drugs in the cocktail. The sigh of relief did not last longer as microbes decided to win the race against man by developing worse strategies of drug resistance of various degrees. Emergence of multidrug-resistant (MDR) pathogens such as *Mycobacterium tuberculosis*, *Campylobacter jejuni*, *Clostridium difficile*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Serratia* spp., *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pneumoniae*, etc. collapsed treatment plans (Davies and Davies 2010), and extremely drug resistant (XDR) and totally drug resistant (TDR) strains of *M. tuberculosis* made treatments practically

impossible (Shah et al. 2007). Research on novel antimicrobials becomes an absolute necessity.

13.4 Search for Novel Antimicrobials

Finding novel compounds that are effective against infectious agents including those that are resistant to the available drugs became the need of the hour. Investigation for novel, natural compounds from untapped sources would be a preferred approach over the production of (semi-) synthetic compounds. The structural diversity of Parvome (small molecules produced by living organisms) and the higher degrees of affinity and specificity for their targets that they gained due to millions of years of evolution give the former approach an edge. The challenges would be accessing the difficult-to-explore sources, designing methods to isolate bioactive compounds from noncultivable microbes, screening of compounds and successful completion of clinical trial, and finally, finding strategies for economical production of drugs.

Natural remedies were sought by ancient men for thousands of years to treat such diseases; plants being the chief source. Such traditional medicines are in use even today in countries like India and China (Dan et al. 1993). Tribal groups from various parts of the world have identified certain plants that are used against various diseases ranging from common colds to massive wounds (Isaacs 2002; Mazid et al. 2012). Centuries-long experience and intellect of human race lying latent in the traditional knowledge was cleverly clubbed with modern knowledge on chemistry and automation that paved the way for development of modern day antimicrobials. Scientific discoveries of modern times on antimicrobial drug development followed the cues provided by traditional knowledge and provided meaningful therapeutic solutions. Natural products have always been the mainstay of modern drug development, which is evident from the fact that about 78% of antibacterial agents are based on them (Concepcion et al. 2001).

Search for novel antimicrobials led researchers to untapped natural sources. During the process, new organisms are also discovered. Both terrestrial and marine sources are explored and the latest technologies are used for the identification and characterization of novel products isolated from them (Harvey 2000). Organisms living in extreme habitats are also studied in great detail as they are potent sources of novel bioactive molecules. They are thought to produce such compounds due to their unique metabolic requirements as well as ecological adaptations (Clardy and Walsh 2004).

13.5 Terrestrial Sources

Significant progress is observed in the discovery of novel antimicrobials from natural terrestrial sources in recent times. Soil microorganisms are the primary sources of antibacterial agents including many commercially available drugs such as

penicillins, cephalosporins, tetracyclins, etc. Plants and their preparations have also been considered as rich sources of bioactive compounds as they produce many therapeutically important primary as well as secondary metabolites. Animals of different phyla are proved to be great sources of compounds with antimicrobial properties. Extracts of various body parts, secretions, and other animal products such as venoms are also explored for such compounds.

13.5.1 Plants

Traditional medicinal preparations, indigenous to different cultures that stood the test of time, invariably used plants as source of antimicrobial chemotypes. They have proved effective against infectious diseases and at the same time exhibit very little or no side effects. Considering their enormous therapeutic potential, plants need to be studied in greater detail (Tagboto and Townson 2001). Only 10–15% of higher plants have been investigated and among them only approximately 6% have been screened for biological activity. This underscores the need for a detailed study on plant-derived compounds and their role as potential antimicrobials (Borris 1996; Lucas et al. 2010; Osman et al. 2012). Plants produce a plethora of bioactive compounds called secondary metabolites. Many of these compounds are used in the development of antimicrobial drugs. These compounds exist in biologically active or inactive forms in plants. Pathogen attack or tissue damage triggers the biologically inactive molecules and they become active in order to protect the plant from such adverse conditions (Cowan 1999; Rauha et al. 2000). Three families of phytochemicals are largely known: phenolics, terpenes, and alkaloids; the former being the largest group of secondary metabolites having exhibited antimicrobial activity. Phenols, phenolic acids, flavones, flavonoids, coumarins, etc. are examples of subclasses in this group. Some of these molecules are used as drugs while some others are used as drug precursors, templates for synthetic modification, and pharmacological probes (Salim et al. 2008).

The potential role of phenolic compounds to be used as antimicrobials could be inferred from the understanding that they are known to protect plants from microbial infections (Saleem et al. 2010). These compounds exhibit different modes of action for combating microbes. Flavones disrupt bacterial envelopes and catechins form complexes with bacterial cell wall. The ability of phenolic compounds such as quinones, anthraquinones, etc. to bind with and cause loss of function of microbial proteins underlines their therapeutic potential (Kurek et al. 2011; Saleem et al. 2010). Another group of phenolic compounds, tannins, are active against microbes by their ability to inactivate bacterial adhesins, enzymes, and membrane-bound proteins (Engels et al. 2011). Flavonoids – which are commonly found in plant parts such as stem, flowers, fruits, and seeds, and plant derivatives such as wine, honey, etc. – and their derivatives also show excellent antimicrobial activity. Their ability to bind to form complexes with both extracellular and soluble proteins renders the bacteria inactive. Detection of anticandidal and antibacterial activity of plant-derived flavonoids like angusticornin B and bartericin A (Kuete 2010) makes

flavonoids suitable candidate molecules for drug development. Flavonoids from the leaf extract of *Leiothrix spiralis* are shown to be effective against *E. coli* and *P. aeruginosa* (Araujo et al. 2011). Alkaloids, on the other hand, attack bacteria by acting as a DNA intercalating agent or by inhibiting enzymes such as RNA polymerase, DNA gyrase, and topoisomerase IV (Yi et al. 2007).

Essential oils from plants are a great source of antimicrobials. Antibacterial, antifungal, and antiviral properties of essential oils containing volatile substances such as monoterpenes, sesquiterpenes, and/or phenylproanoids are reported (Prabuseenivasan et al. 2006). Both phenolic and nonphenolic compounds of essential oils are found effective against various pathogens. Phenolic compounds of essential oils such as oleuropein (olive oil), terpenoids (tea-tree oil), etc. have broader antimicrobial effects. Nonphenolic oil compounds present in lemongrass, rosemary, garlic, clove, cinnamon, etc. show promising antibacterial activity on both Gram positive and Gram negative organisms (Gutierrez et al. 2008, Holley and Patel 2005; Mandalari et al. 2007). Other nonphenolic constituents of essential oils such as allyl thiocyanate and allyl isothiocyanate are proved to be effective against Gram negative bacteria and many fungi (Nielsen and Rios 2000; Yin and Cheng 2003). Other secondary metabolites such as terpenes, steroids, polyketides, peptides, etc. are also considered to be brilliant candidates for antimicrobial therapeutic development with their proven ability to act even against multidrug-resistant *M. tuberculosis* (Garcia et al. 2012). Natural resins derived from plants belonging to various families including *Euphorbiaceae*, *Apiaceae*, *Burseraceae*, *Fabaceae*, etc. and their specific extracts and compounds isolated from them are suggested to have potential antimicrobial activities. They are found to be active against different types of microorganisms including bacteria, fungi, and protozoan parasites (Termentzi et al. 2011). Since such plants have been used successfully in traditional medicine for centuries, it is never too far from getting therapeutically active compounds isolated from them. Technological advancements and focused screening programs conducted in this field would function together toward this goal (Savoia 2012).

Endophytes are organisms that live inside (*Greek: Endo*) a plant (*Greek: Phyte*) for at least a part of their life (Wilson 1995). Since the endophytes are nonpathogenic fungi and bacteria, they are rightly considered endosymbionts. Ecomycins, pseudomycins, munumbicins, and xiamycins are examples of endophyte-derived compounds having antimicrobial effects (Christina et al. 2013). Among them, ecomycins and pseudomycins are two families of peptide antimycotics which are proved to be very active against *Cryptococcus neoformans* and *Candida albicans* (Harrison et al. 1991). The other two classes of antibiotics are derived from *Streptomyces* spp.; the former is a wide-spectrum antibiotic and the latter is shown to have anti-HIV activity (Castillo et al. 2002; Ding et al. 2010).

Antimicrobial peptides (AMPs) or host defence peptides (HDPs) are small peptides (6–50 amino acids) having net positive charge (Bradshaw 2003; Hancock and Patrzykat 2002) that are part of the innate immune response. AMPs derived from plants is yet another interesting class of molecules having antimicrobial activities. They are shown to be active against plant pathogens as well as bacteria pathogenic to humans (Montesions and Bardaji 2008). AMPs are diverse peptides and they

exhibit broad spectrum antimicrobial activities. These peptides differ in their amino acid composition and are classified into a number of families based on amino acid sequence homology. The main AMP families are defensins, thionins, lipid transfer proteins, cyclotides, snakins, and hevein-like proteins. Certain plants synthesize hundreds of different AMPs. Due to their broad spectrum activity and efficiency, AMPs would be a suitable alternative to less-efficient drugs currently in use (Pinheiro Da Silva and MacHado 2012).

13.5.2 Animals

A number of bioactive compounds have been isolated from various types of animals, mainly insects and reptiles. Biomolecules with antimicrobial potential are believed to be produced by such animals by virtue of the physiological and morphological adaptations they acquire for survival in local ecological conditions (Latifi et al. 2015). An example is the identification of small molecules that suppress environmental pathogens produced by bacterial symbionts of insect-agricultural systems (Ramadhar et al. 2014). Potential antimicrobial antibiotic properties from brain extracts of cockroaches and locusts that inhabit the environment prone to bacterial infection are also identified (Khan et al. 2008; Lee et al. 2011). There are other instances where peptides and proteins with antimicrobial properties are identified from hemolymph of insects (Moreno-Garcia et al. 2013) that are experimentally challenged with live bacteria (Qu et al. 1982). Cecropin, insect defensins, attacin-like (glycinerich) proteins, proline-rich peptides, and lysozymes are the major groups of antimicrobial peptides identified from insects so far (Hultmark et al. 1983). All these findings, and more, point fingers toward the need of active research in this field for identification and commercial availability of novel antimicrobials.

Interestingly, peptides or proteins of animal venom, including that of wasps, honeybees, spiders, scorpions, and snakes, are also shown to have antimicrobial effect (Dani et al. 2003; Benli and Yigit 2008; Fennell et al. 1967; Perumal samy et al. 2007). Venoms of different species of rattle snake, viper, cobra, and krait show high antimicrobial activity in vitro which are as effective as commercially available antibiotics such as chloramphenicol and ceftazidime (Perumal Samy et al. 2006). Crude venom of wasp (*Vespa orientalis*) is effective against both Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative (*E. coli* and *K. pneumonia*) bacteria (Jalaei et al. 2014). Venom of funnel web spider (*Agelena labyrinthica*) is shown to be active against common human bacterial pathogens by causing loss of bacterial cytoplasm and eventually its death. A number of antimicrobial peptides were isolated from venoms of different animal species and their mechanisms of action are being identified by various research groups across the globe, keeping the expectations of the world high for better antimicrobials that can tackle issues associated with drug resistance.

Antimicrobial peptides (AMPs) of animal origin are a diverse group of small peptides that are essential components of nonspecific defence mechanisms of host

organism. They exhibit broad spectrum (Gram positive bacteria, Gram negative bacteria, fungi, and virus) antimicrobial activities (Saeed et al. 2013). Pleurocidin, lactoferrin, defensins, and protamine are some of the AMPs with potential antimicrobial properties. Most of the AMPs rapidly kill microbes by targeting their membranes and reduce the possibility of mutation and eventual drug resistance (Tiwari et al. 2009). These antimicrobial peptides are isolated from various sources such as mucous membranes of fish (pleurocidin) (Cole et al. 2000), epithelial cells of chicken and turkeys (defensins) (Tiwari et al. 2009), and milk (lactoperoxidase) (Seifu et al. 2005). In addition to peptides, certain polysaccharides and lipids of animal origin are also reported to have broad spectrum antimicrobial properties. They are found to be active against Gram positive and Gram negative bacteria and fungi. Chitosan – a natural polysaccharide obtained from the exoskeletons of certain arthropods – and milk lipids are examples of such molecules (Saeed et al. 2013).

13.6 Extreme Habitats

As the name implies, extremophiles live in extreme habitats and require unusual mechanisms to adapt to such conditions and they produce diverse molecules in their body in order to aid them in the process (Sanchez et al. 2009). Resources untapped thus far were explored for identification of natural compounds that could possess antimicrobial activities (Zengler et al. 2005). Microorganisms living in extreme habitats produce unique molecules that help them adapt to the unusual life conditions. Therapeutic potential of many such compounds have been identified (Sanchez et al. 2009). Extremophilic microorganisms were recognized to be promising sources of therapeutically important compounds after the identification of antimicrobial activities of such molecules (Ravot et al. 2006). Exploration did not leave extreme habitats such as caves, as cave biofilms were relatively unstudied ecosystems (Boston et al. 2001). These were later found to be excellent sources of new antimicrobial compounds (Maria de Lurdes and Dapkevicius 2013). Last two decades witnessed discovery of a number of new species from gold mines and caves from different parts of the world that produce useful secondary metabolites (Cheng et al. 2013; Kay et al. 2013; Lee 2006, Margesin et al. 2004; Maria de Lurdes and Dapkevicius 2013; Rule and Cheeptham 2013). Various molecular and culture-independent methods are being employed to identify organisms from volcanic caves and lava tubes (Rule and Cheeptham 2013). Such studies are the need of the hour as they are directed toward identification and characterization of novel compounds and qualify them to be used in clinical space.

Hot springs, sulfurous thermal springs, stromatolytes, etc. are examples of terrestrial extreme habitats. Recent studies show that extremophilic microbes are another probable source of novel biomolecules due to their adaptive ability to unusual habitats. Early in this century a compound called pyochelin was isolated from extracts of a novel thermophilic species of *Pseudomonas*, which was found to have antifungal activity (Combie et al. 2001). Later, investigations were successful in finding microcin-like compounds (proteinaceous toxins produced by bacteria to

inhibit closely related strains) from psychrophilic organisms, which showed broad spectrum antibacterial activity (Sanchez et al. 2009, 2010). Various other extreme habitats including abandoned minewaste disposal sites are being explored in search of novel organisms and bioactive compounds that could aid in antimicrobial chemotherapy (Garcia-Moyano et al. 2015; Milshteyn et al. 2014).

13.7 Marine Sources

Although only partly explored – due to vastness and complexity – marine sources of antimicrobials have already become interesting topics of research and clinical trials. Bioactive compounds from marine flora and fauna have extensive past and present use in the treatment of many diseases and serve as compounds of interest both in their natural form and as templates for synthetic modification. The multitude of life forms (oceans host about 87% of life on earth) such as microorganisms, their symbiotic associations, plants, invertebrate animals, etc. offer excellent opportunity for discovery of more and more novel compounds each day. It is estimated that at least 30,000 compounds have been isolated from marine organisms within a short span of 15–20 years of active research. Approximately 16,000 marine bioactive compounds have been identified from various types of marine organisms. Major contributors are sponges, coelenterates, and microorganisms followed by other sources including algae, echinoderms, and tunicates. Rich diversity of marine life is reflected in the plethora of antimicrobials isolated from them. Some of the important compounds are aplasmomycin, himalomycins, pelagiomycins (bacteria), Ara-A, Ara-C, Variabillin (sponges), asperidol, eunicin (coelenterates), cycloeudesmol, prepacifenol (algae), marinomycins C & D (actinomycetes), etc. (Doshi et al. 2011). Path toward identification of new generation antimicrobials and making them available for therapeutic use is quite promising despite discontinuation of some of the compounds due to their inefficiency and/or toxicity (Schuster et al. 2005).

13.7.1 Marine Flora

Marine flora is composed of unicellular as well as multicellular organisms ranging from bacteria to flowering plants (Sithranga Boopathy and Kathiresan 2011). Metabolites and other compounds produced by thousands of species of marine flora could be utilized for discovery of novel drugs.

Marine actinobacteria in general and the genus *Streptomyces* in particular are the best known sources of a variety of compounds of therapeutic interest (Pathom-aree et al. 2006). Their importance is underscored by the fact that 70% of all the bacterial secondary metabolites (more than 22,000) are produced by them (Subramani and Aalbersberg 2012). These organisms proved to have the ability to produce novel

metabolites that make them ideal candidates in drug-screening programs (Manivasagan et al. 2013). Extensive research on marine flora led to the discovery of novel genera of actinomycetes (Claverias et al. 2015), adding to the diversity of potential bioactive compounds. Efforts are being taken to cultivate marine flora (Joint et al. 2010) which would certainly make the screening process easy due to the easy availability of samples.

Wide array of secondary metabolites produced by the cyanobacteria (blue green algae) are interesting candidates of pharmaceutical importance due to their selective activity against various types of organisms including bacteria, fungi, and viruses (Lopes et al. 2010). Cyanobactins are small cyclic peptides produced by cyanobacteria. Some of the cyanobactins are reported to have antimalarial activity (Sivonen et al. 2010). Another metabolite Viridamide A, obtained from a blue green algae of genus *Oscillatoria*, showed antitrypanosomal and antileishmanial activity with IC 50 as low as <2 μM in both cases (Simmons et al. 2008b).

Due to their abundance, great species diversity, and rich secondary metabolites, marine fungi hold an important position as a source of antimicrobial compounds (Swathi et al. 2013; Xu et al. 2015). Sansalvamide A isolated from *Fusarium sp*. showed inhibitory effect on topoisomerase enzyme. This compound was found to be active against pox virus *Molluscum contagiosum* that causes pink rashes in humans (Hwang et al. 1999). Zofimarin and griseofulvin are antifungal agents isolated from marine fungi *Zopfiella marina* and *Penicillium waksmanii*, respectively (Mayer et al. 2013; Pietra 1997). Compounds isolated from marine fungi are diverse. They have not only antifungal and antiviral properties but antibacterial (e.g., pestalone and sumiki's acid), antihelminthic (e.g., nafuredin), and antimalarial (e.g., aigialomycin D and halorosellinic acid) activities too (Bugni and Ireland 2004; Overy et al. 2014). Zopfiellamides A and B are two compounds identified to have antimicrobial activities during the early 2000s (Daferner et al. 2002). A number of novel compounds are still being identified, e.g., fumigaclavine (antifungal) from *Penicillium viridicatum*, 3-phenyl-2-propenoic acid, cyclo-(Phe-Pro) and cyclo-(Val-Pro) (antibacterial) from *Cladosporium* sp., 6-methoxyspirotryprostatin B (antiparasitic) from *Aspergillus fumigatus*, and nodulisporacid A (antiplasmodial) from *Nodulisporium* sp., etc. (Swathi 2013).

Marine algae comprise a diverse group of organisms classified into two major categories based on their size – microalgae and macroalgae. Microalgae are small photosynthetic organisms that could be seen only with the help of a microscope. These tiny organisms are shown to have antibiotic, antimycotic, and antiviral properties (Patterson et al. 1994). Macroalgae, commonly called seaweeds, include members of red, green, and brown algae. Crude preparations of marine algae have traditionally been used against diseases due to iodine deficiency, intestinal disorders, etc. (El Gamal 2010). A polyunsaturated fatty acid, eicosapentaenoic acid (EPA), isolated from a diatom (a type of microalga) was effective against a range of both Gram positive and Gram negative bacteria including methycillin-resistant *Staphylococcus aureus* (MRSA) (Desbois et al. 2009). The viral infections that microalgae are active against include viral hemorrhagic septicaemia virus (VHSV) and African swine fever virus (ASFV) (Fabregas et al. 1999). A number of

macroalgae also showed antimicrobial activity. The red alga *Sphaerococcus coronopifolius* and the green alga *Codium iyengarii* showed antibacterial activity, while another green alga, *Ulva fasciata*, showed antiviral activity by virtue of a novel sphingosine it produced (Ali et al. 2002). Antiviral properties of polysaccharides extracted from seaweeds were documented in late 1950s. It was observed that algae-derived polysaccharides were able to inhibit mumps and influenza B viruses (Gerber et al. 1958). The next two decades were eventful and more polysaccharide fractions from red algae were reported to have activities against herpes simplex virus (HSV) and other viruses (Deig et al. 1974). Compounds such as dictyota diterpenes isolated from brown alga *Dictyota menstrualis*, griffithsin from the red alga *Griffithsin* sp., and sulfated polymannuroguluronate (SPMG) from the brown alga *Laminaria aponica* showed inhibition or deactivation of Human Immunodeficiency Virus (HIV) (Meiyu et al. 2003; Pereira et al. 2004; De Souza et al. 2005). The mechanism of action of SPMG is identified; it binds to the trans-activating protein of HIV (Tat) and thereby interferes with the entry of the virus to host T lymphocytes. This compound is under the phase II clinical trials in China (Wu et al. 2011). Various other alga-derived compounds show antifungal and antiparasitic activities (Torres et al. 2014; Yu et al. 2014).

Sea grasses are marine flowering plants growing anchored in the sand or mud of shallow areas of the sea. There are about 60 species of sea grasses identified so far. The secondary metabolites they produce are believed to be part of their defense mechanism. Many of these compounds have therapeutic importance (Athiperumalsamii et al. 2008). *Cymodocea* spp. is used to treat diseases like cough and malaria, while *Halophila* spp. is found to be used to cure malaria and certain skin diseases. Latter is effective to treat leprosy in the early stages (Yuvaraj et al. 2012). The true therapeutic potential of compounds from sea grasses is only being discovered.

13.7.2 Marine Fauna

Bioactive compounds from marine fauna have been used as antimicrobials from olden times. Therapeutically important compounds are isolated mainly from invertebrates such as sponges, cnidarians, annelids, arthopods, molluscs, etc. Major classes of marine animal derived compounds are steroids, terpenoids, isoprenoids, nonisoprenoids, quinones, brominated compounds, nitrogen heterocyclics, nitrogen sulfur heterocyclics, etc. They are used as natural molecules or as synthetically modified compounds. These compounds are used against various pathogenic microorganisms including bacteria, virus, fungi, protozoan, helminthic parasites, etc. (Bhakuni and Rawat 2006).

Sponges are very simple, cellular grade (no organized tissues) multicellular organisms found in aquatic habitats. They are the richest source of bioactive compounds in the marine environment. These compounds are generally the secondary metabolites originated from sponges and their symbionts by the action of functional enzyme clusters (Laport et al. 2009; Sagar et al. 2010). Sponges alone produce more

than 3300 antibiotics and other bioactive compounds. Marine sponges have always been organisms of antimicrobial research interest since the discovery of pharmaceutically important nucleosides spongothymidine and spongouridine in the early 1950s (Heider et al. 2010; Min et al. 1998). In addition to the nucleoside molecules, various types of compounds isolated form marine sponges have also shown to have therapeutic properties. These include fatty acids, sterols, alkaloids, peptides, amino acid derivatives, etc. (Laport et al. 2009; Sagar et al. 2010). A myriad of compounds of therapeutic interest is isolated from sponges that are active against both bacteria and fungi. These fall under various chemical groups such as alkaloids, terpenes, cyclical compounds, etc. (Perdicaris et al. 2013). Alkaloids isolated from marine sponges *Agelas mauritiana* and *Lotrochota purpurea* (halogenated) exhibited antibacterial and antifugal properties (Shen et al. 2012; Yang et al. 2012), while cyclic compounds isolated from the *Haliclona sp*. showed antibacterial activity against Gram positive strains (Lee et al. 2012). Diterpenes and pentacyclic ingamine alkaloids isolated from various marine sponges were found to have antimalarial activity (Chanthathamrongsiri et al. 2012; Ilias et al. 2012). One of the psammaplysin derivatives (19-hydroxypsammaplysin E) identified from the Indonesian marine sponge *Aplysinella strongylata* also showed promising antimalarial activity (Mudianta et al. 2012). Research was focused more toward control of HIV and compounds such as clathsterol, dehydrofurodendin, crambescidin, mirabamides, etc. isolated from various marine sponges were found to be very effective anti-HIV molecules. Clathsterol and dehydrofurodendin act against HIV by inhibiting the reverse transcriptase enzyme while crambescidin and mirabamides act by inhibiting HIV-1 envelop fusion with host cells (Chang et al. 2003; Shoshana et al. 1999; Plaza et al. 2007; Rudi et al. 2001). Microspinosamide, petrosins, and neamphamide were also proved to be active against this deadly virus (Oku et al. 2004; Rashid et al. 2001; Venkateshwar Goud et al. 2003).

Cnidaria is another phylum which needs to be explored well for antimicrobial compounds. As this group of organisms are very diverse and known to have developed defense mechanisms to combat pathogenic microbes in the sea, the possibility of them producing therapeutically important compounds would be high. Efforts for identification of compounds from cnidarians began after the discovery of prostaglandins from corals in the late 1960s. Soft corals are promising sources of bioactive compounds as they rely on chemical substances for defending themselves from microbial attack, while the bony corals seem to have developed other mechanisms. Ceramideas from *Lobophytum crassum* (Vanisree and Subbaraju 2002) and desoxyhavannahine from *Xenia macrospiculata* (Kelman et al. 2006) are examples of antimicrobial compounds isolated from soft corals.

Molluscs are another interesting group of organisms under the radar of scientists in search of novel antimicrobials. Development of potential antibacterial drugs were suggested from the findings in the late 1990s that the egg capsule extract of the gastropod *Rapana rapiformis* exhibited significant activity against bacterial pathogens (Amruthalakshmi and Yogamoorthi 2015). Recent findings of broad spectrum antibacterial effect of whole body extracts of *Nerita albicilla* and *N. oryzarum* and egg mass extracts of *Chicoreus virgineus* and *C. ramosus* underscore the importance

of molluscs as source of antimicrobial compounds (Ramasamy and Murugan 2005). Shell extracts of *Donax faba* and gut methanol extract of *Perna viridis* also exhibit antibacterial activities (Giftson and Patterson 2014). Further studies down the road are required for identification, isolation, and commercialization of bioactive molecules having antimicrobial activity from marine molluscs.

Arthropods have been speculated to produce antimicrobial molecules after the observation of bactericidal activities of the plasma of lobster *Homarus americanus* as early as 1972 (Stewart and Zwicker 1972). Crustacean species are known to produce antimicrobial peptides, e.g., penaeidins isolated from the hemolymph of the shrimp *Penaeus vannamei* (Destoumieux et al. 1997). This molecule was shown to have both antifungal and antibacterial activities following its large-scale production employing recombinant DNA technologies. Penaeidins are found to be active against filamentous fungi and Gram positive bacteria (Destoumieux et al. 1999). Several extracts of crustaceans which showed antibacterial activity are being tested for bioactive compounds that would aid in the development of new antibiotics (Kiran et al. 2014).

Other invertebrates such as annelids are also found to produce antimicrobial substances. Organisms from all the three classes of phylum Annelida (Polychaeta, Oligochaeta, and Hirudinea) are studied for their ability to produce antimicrobial peptides (AMPs). Hedistin, perinerin, and arenicin are the main AMPs isolated from different species of polycheates. The AMPs isolated from oligochaetes include a peptide having 62 amino acids, lumbricin-1, its derivative lumbricin-1 (6-34) having 29 amino acids and 3 very short peptides, F-1, F-2, and OEP3121, having only 5 amino acids. Theromacin, theomyzin, and Perptide B are examples of AMPs islotated from organisms of class hirudinea (Tasiemski 2008).

13.7.3 Marine Extremophiles

The search for organisms that produce unique metabolites led researchers to explore extreme habitats. The extreme conditions in the marine environment include high as well as low temperatures, elevated hydrostatic pressure, and hypersalinity. Hypoxic areas, oil-contaminated sites, and deep-sea sediments are also considered extreme habitats. The main challenges of exploring these habitats are the extremely difficult logistics procedure of collecting samples, unstable conditions at the sites for recollection of samples, and culturing the extremeophilic organisms in laboratory conditions. However, search for antimicrobials from deep-sea hydrothermal vents yielded fruits in the form of antimicrobial peptide (AMP) alvinellacin from polychaetous annelid *Alvinella pompejana* that inhabits active deep-sea hydrothermal vents (Tasiemski et al. 2014) along with other bioactive compounds isolated from various bacterial species (Branco et al. 2008). Though hypersaline environments are usually highly alkaline and hypoxic they are inhabited by microorganisms including actinomycetes (Phillips et al. 2012; Swan et al. 2010; Tang et al. 2009; Ventosa 2006). A recent finding that microorganisms of hypersaline microbial mat can produce antimicrobial and quorum sensing inhibitory compounds (Abed et al. 2013) points the

finger toward the need for further exploration of such habitats. Mangroves also produce a wide array of medicinally important natural products. These are mainly active metabolites belonging to chemical classes such as alkaloids, phenol, steroids, terpenoids, and tannins. Two compounds, innophyllums B and P, isolated from a mangrove-associated plant species *Calophyllum inophyllum* could inhibit HIV reverse transcriptase and were active against HIV-1 in cell culture (Patil et al. 1993).

Another least explored natural resource is the deep-sea sediments. Though the deep-sea area is geographically vast, man has very little knowledge about the organisms found there. Recent studies show that microorganisms including new actinomycetes found in the deep-sea sediments would serve as sources of novel antimicrobials (Das et al. 2006). Marinispora is one such actinomycete, which produces macrolides called marinomycins, which are active against drug-resistant bacteria (Kwon et al. 2008).

13.8 Symbiotic Associations

Among different types of symbiotic associations (the living together of unlike organisms), mutualism is considered the "true symbiosis." This is the faithful and fruitful interaction between different biological species, where survival of one species requires involvement of another. Recent investigations suggest that the true source of bioactive compounds isolated from marine algae and other eukaryotes would be their symbionts (Bhatnagar and Kim 2010). Symbiotic microorganisms including prokaryotic bacteria, archaea, blue green algae (cyanobacteria), and fungi are found in association with most marine eukaryotes. Terrestrial and marine bacteria are similar in many aspects, but since the adaptation required by a marine species, due to unique chemical and physical environments, differs significantly, ability to produce novel compounds is thought to aid their survival in marine environment. Symbiont of marine sponge *Xestospongia* sp., *Micrococcus luteus* is found to have antimicrobial activity (Bultel-Ponce et al. 1999). Other examples of antimicrobials isolated from symbionts of marine species include quinolones and phosphatidyl glyceride isolated from a *Pseudomonas* sp. which are associated with *Homophymia* sp. (sponge) (Bultel-Ponce et al. 1999).

13.9 Novel Technologies in Antimicrobial Discovery

Advances in technology have positively contributed to identification of novel antimicrobials from a variety of sources. Understanding the chemical structure, mechanism of drug-host interactions, and the possibilities of intelligent modifications that enhance efficacy are equally important as discovery of a novel compound. Thanks to the branches of science such as metagenomics, nanotechnology, metabolomics, synthetic biology, bioinformatics, and sociomicrobiology and relatively newer techniques such as next-generation DNA sequencing, novel microbial culture methods, mass spectrometry, imaging mass spectrometry (IMS), NMR spectroscopy, and

crystallographic techniques that play crucial roles along with genomics, molecular biology, and proteomics in the discovery and clinical availability of novel antimicrobial compounds. Reduction in turnaround times and improvements in data quality can be achieved by advancements in automation and employment of computer-assisted data analysis.

Metagenomics is a culture-independent approach used largely to identify microbes and get detailed information about the diversity of them from environmental samples. In metagenomics the results are obtained by DNA sequence analysis (de Castro et al. 2014). Next-generation deep sequencing and data analysis methods raise metagenomics to a new plain by providing accurate and detailed sequence information for understanding the compounds produced by microbes and mechanisms of antibiotic resistance in the in situ microbial community (Forsberg et al. 2012; McGarvey et al. 2012).

Genome sequencing of various microorganisms resulted in identification of gene clusters that are believed to direct the production of unknown metabolites. Prediction of role of a gene or a gene cluster in production of previously unknown natural compounds is made possible by a novel technique called microbial genome mining (Davies 2011). Identification of a novel peptide, coelichelin, from *Streptomyces coelicolor* can be cited as an example of fruitful culmination of genome mining (Challis and Ravel 2000). Possibility of the presence of numerous bioactive compounds that are yet to be identified and characterized is evident from such findings.

Imaging mass spectrometry is an immensely useful technique in the field of natural product research (Esquenazi et al. 2009). IMS enables researchers to visualize the spatial distribution of chemical compositions, e.g., compounds, metabolites, and peptides on a substrate such as a plant part. This method helps researchers to identify symbiotic microorganisms as the true producers of secondary metabolites (Esquenazi et al. 2009; Simmons et al. 2008a). A slight modification of IMS, called thin layer agar natural product matrix-assisted laser desorption/ionization-time of fligh (MALDI-TOF) imaging, brought in possibility of analyzing a complete set of metabolites produced by microorganisms under various culturing conditions. These microorganisms are cultured on thin agar film on a MALDI plate followed by application of matrix and analysis by MALDI (Yang et al. 2009).

Isolation chip (iChip) is a novel high-throughput platform for parallel cultivation and isolation of previously uncultivable microbial species in situ within their natural environments. Teixobactin is the new compound identified by employing this method (Wright 2015). The bacterium that produces teixobactin is a hitherto undescribed microorganism, which was provisionally named as *Eleftheria terrae* (Piddock 2015). Teixobactin was able to cure mice of various bacterial infections including pneumonia and blood infections but is yet to undergo human trials. This compound inhibits cell wall synthesis of bacteria in a unique way – by inhibiting peptidoglycan biosynthesis by binding to a highly conserved motif of precursors of peptidoglycan (lipid II) and teichoic acid (lipid III) – that is likely to avoid development of drug resistance (ling et al. 2015). The scientific world is optimistic about discovery of new potential antibiotics with the help of iChip technology.

13.10 Conclusions

Our current arsenal of antibiotics is inadequate to combat many microorganisms especially the drug-resistant ones (MDR, XDR, and TDR). As humanity is drifting toward a vulnerable state equivalent to preantibiotic era, scientists are turning to unusual sources for novel compounds. They are leaving no stones unturned in the quest for finding effective drugs that can combat microorganisms and save humanity from infectious diseases. Thousands of compounds have been identified from various sources that await further investigation and clinical trials. The efforts will continue until man has the last laugh on pathogenic microorganisms.

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14 Quorum Quenching Compounds from Natural Sources

G. Seghal Kiran, Saqib Hassan, Arya Sajayan, and Joseph Selvin

Abstract

Quorum sensing is a process of bacterial communication system wherein the production and secretion of small signaling molecules known as autoinducers enables the bacteria to express specific genes at particular population densities. Quorum quenching (QQ) can be used as an alternative approach to regulate pathogenicity. Well-established QQ strategies include amide bond hydrolysis, lactone hydrolysis, paraoxonase enzymes, and QQ modification of acyl chain. Plants in general lack advanced immune systems, and may have evolved to produce QQ compounds to combat with plant invading pathogens. Most common sources of QQ compounds in marine environment are bacteria, fungi, algae, bryozoan, corals, and sponges. Marine cyanobacteria have become one among the best source for obtaining biologically active and structurally unique QQ natural products. QQ compounds are being innovated as alternatives of antibiotics to treat pathogenic infections. Marine ecosystem is a unique and unexplored hotspot for the development of new derivatives of potential QQ compounds.

Keywords

Quorum Quenching • Biofilm • Motility • Virulence • Signalling molecules

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14.1 Introduction

Quorum sensing (QS) comprises of a bacterial communication system wherein the production and secretion of small signaling molecules known as autoinducers occurs and these molecules get accumulated in the extracellular environment and increase in concentration as a function of cell density (Romero et al. 2011). This process assists bacteria in communication, regulating gene expression, and synchronizing phenotypic expression of biofilm development, motility, bioluminescence, and virulence factor production such as phospholipase, hemolysin, protease, etc. (Tang and Zhang 2014). Quorum sensing system was first mentioned in bioluminescent *Vibrio fischeri*, and the term "quorum sensing" was introduced by E.P. Greenburg (Nealson and Hastings 1979; Davis 2004). As quorum sensing plays a vital role in the pathogenesis of human, animal, and plant, the identification of mechanisms that can cause disruption of quorum sensing molecules in such pathogenic bacteria has become an interesting area of research in microbiology and many compounds have been found to be able to inhibit this signaling pathway in various pathogenic organisms and the trials are ongoing (Defoirdt et al. 2013). Identification of signaling molecules, their receptor and target site, and the mechanism of signaling are the important aspects of cell-cell communication in bacteria.

Quorum sensing bacteria access the cell population density using autoinducers (Henke and Bassler 2004). This enables the bacterial group to express specific genes only at particular population densities. The discovery of species specific as well as cosmopolitan intracellular signaling molecules unfolds that the bacteria interact with one another using astonishingly sophisticated mechanisms of communication. In recent past, bacteria were considered to be living a rather solitary life. But, new research has revealed that in fact bacteria have a comprehensive chemical signaling system that enables them to communicate intraspecifically as well as interspecifically. When the population of bacteria increases, the individual organisms produce and secrete the autoinducers into the extracellular environment. Quorum Sensing is shown by both Gram-positive and Gram-negative bacteria. Examples of well-studied quorum sensing molecules include acylhomoserine lactones, autoinducer 2, and peptide signals, but many other signals, such as indole, cholera autoinducer also exist (Nazzaro et al. 2013). In addition to signals, signal synthases, signal receptors, signal response regulators, and regulated genes (Quorum Sensing regulon) are key components of any quorum sensing system.

Based on signaling molecules and sensing mechanism, there are three major classes of QS systems:

1. Gram-negative LuxI/LuxR-like quorum sensing system that uses AHLs as signaling molecules (Fuqua et al. 1994).
2. Gram-negative *V. harveyi*–like two-component signaling circuits that recognize three different signaling molecules, AHLs, FBD, and an uncharacterized Cal −1 molecule (Bassler et al. 1993, 1994; Henke and Bassler 2004).
3. Gram-positive two-component signaling systems that use modified oligopeptides as autoinducers (Lazazzera and Grossman 1998).

14.2 Quorum Quenching

The term "Quorum Quenching" was coined to describe all the processes that interfere with quorum sensing. Quorum quenching has been recommended as a promising innovative tool for the development of novel therapeutics to control microbial infections. The application of quorum quenching compounds to fight bacterial pathogenicity is particularly alluring with the emergence of multidrug resistant bacteria (Adak et al. 2011). One bacterial species may have advantage over the other in its ability to disrupt quorum sensing; this is mostly found in niches where the bacterial populations compete for limited resources. Similarly, the host's ability to step-in bacterial cell-cell communication may play an essential role in preventing colonization by pathogenic bacteria that use quorum sensing to coordinate virulence. Thus, quorum quenching has evolved as a mechanism to interfere with bacterial cell-cell communication (Waters and Bassler 2005; Adak et al. 2011).

Some of the well-known examples of quorum quenching compounds include acylases/lactones that are involved in the degradation of N-(3-oxoctanoyl)-homoserine lactone (HSL) autoinducers, synthase inhibitors like analogues of anthranilic acid (LaSarre and Federle 2013; Defoirdt et al. 2013) acting as quorum quenchers and obstructing the synthesis of quinolone signals (Lesic et al. 2007; García-Contreras et al. 2013). Some receptor inhibitors also act as quorum quenchers, such as brominated furanones (Defoirdt et al. 2007; García-Contreras et al. 2013). In addition to this some of the antibiotics like azithromycin, ceftazidime, and ciprofloxacin in low concentrations have been found to inhibit quorum sensing in *Pseudomonas aeruginosa* (Skindersoe et al. 2008; García-Contreras et al. 2013). Niclosamide, the antihelminthic drug is also a quorum quenching compound which reduces biofilm formation, surface motility, and the production of the secreted virulence factors like elastase, pyocyanin, and rhamnolipids (Imperi et al. 2013). *Stenotrophomonas maltophilia* produces *cis*-9-octadecenoic acid, which is a quorum quenching compound and it involves in the reduction of violacein production by *Chromobacterium violaceum* and also affects the biofilm formation in *P. aeruginosa* (Singh et al. 2013; García-Contreras et al. 2013). Quorum sensing dependent characters, such as elastase activity, protease activity, and the production of pyocyanin, was inhibited by a cyclic dipeptide 2,5-piperazinedione; which act as a quorum quencher (Musthafa et al. 2012; García-Contreras et al. 2013). Some of the quorum quenching bacteria was isolated from healthy coral species (Golberg et al. 2013; García-Contreras et al. 2013). The presence of anti-QS compounds in corals may possibly provide the clues about the competitive interactions of coral-associated bacteria. Quorum quenching compounds are secreted from the dominant communities to minify undesirable marine biofouling.

14.3 Quorum Quenching Strategies

Some of the quorum quenching strategies are described below:

(a) *Amide bond hydrolysis*: AHL acylases are the enzymes required for the complete and irreversible degradation of AHLs. They undergo hydrolysis and releases homoserine lactone and the relevant fatty acid. This enzymatic activity was described in both prokaryotes and eukaryotes. Leadbetter and Greenburgin (2000) first demonstrated this type of enzymatic activity in *Variovorax paradoxus* (Leadbetter and Greenberg 2000; Uroz et al. 2009). Six genes that encode AHL-acylases have been characterized and all of these AHL-acylases degrade long chain AHLs more expeditiously than the short chain forms. Enzymes such as Aac, AhIM, PvdQ, QuiP are unable to degrade AHL that has an acyl chain composed of less than eight (8) carbons (Park et al. 2005; Uroz et al. 2009). Porcine kidney acylase which was previously known for its ability to hydrolyze a variety of N-acyl-L-amino acids has been shown to degrade AHLs with an acyl chain that had four (4) to eight (8) carbons (Xu et al. 2003; Uroz et al. 2009).

(b) *Lactone hydrolysis*: In this case AHL lactonases induce the hydrolysis of the homoserine lactone ring of the AHLs which in turn leads to the generation of acyl homoserine. This reaction resembles the pH-mediated lactonolysis, and thus it can be reversed by the acidification of the medium. Lactonase activities have been identified in several bacterial genera and also in eukaryotic cells. AHL-lactonase activity was first demonstrated in *Bacillus* sp. (Dong et al. 2001; Uroz et al. 2009). This lactonase has been found to hydrolyze short and longer (C4- and C14-HSL), with or without a substitution at the C3 position (e.g., *Acidobacteria*). Crystal structure of AHL lactonase shows that the enzyme has two zinc (Zn^{+}) ions in its active site and thus is a metalloprotein. Those residues which are involved in metal coordination directly are completely conserved in all AHL-lactonases. The catalytic mechanism that occurs in AHL-lactonase, described as follows: It starts with the attack of the substrate's carbonyl carbon by a nucleophilic water/hydroxide that is bridging the two (Zn^{+}) ions. Second step involves the formation of negatively charged intermediate. Finally an open ring product is formed by breaking of C-O bond of lactone ring of AHL inactivation enzymes.

(c) *Paraoxonase enzymes and Quorum Quenching*: AHL inactivation by paraoxonase enzyme was performed in human epithelial cells. Later, this AHL inactivation ability was found to be widely conserved in the sera of 6 mammalian species – human, bovine, sheep, horse, mouse, and rabbit. As far as the characteristics of these AHL inactivation enzymes are concerned they are dependent on Ca^{2+} ion and lactonase like activity. Physiologically important hydrolytic activities are carried out by these paraoxonase enzymes (PONs). These paraoxonase enzymes include PON1, PON2, and PON3. These enzymes play a vital role in drug metabolism and organophosphate detoxification. PON enzymes, PON1 particularly, are known to catalyze the hydrolysis of many synthetic chemicals which include organophosphate-based insecticides, nerve agents aromatic carboxylic acid esters, cyclic carbonate esters, aromatic lactones, and alkyl lactone.

(d) *Modification of acyl chain*: AHL oxidase and reductase: Acyl chain modification was reported only in bacteria. The first AHL oxido-reductase activity was reported in a strain of *Rhodocuccus erythropolis* (Uroz et al. 2005; Tay and Yew 2013). P450 monooxygenase is an AHLase obtained from *Bacillus megaterium*, which oxidizes fatty acids and *N*-fatty acyl amino acids. Here the AHL molecules are not degraded, instead there occurs a modification in the structure of the signal and this modification might play a role in the regulation of QS-related functions (Chowdhary et al. 2007; Uroz et al. 2009).

14.4 Sources of Quorum Quenching Inhibitors

14.4.1 Natural Quorum Sensing Inhibitors

Numerous substances have been evaluated for their ability to interfere with QS. Quorum sensing inhibitors which are isolated from natural products are excellent resources for developing powerful antivirulence drugs. Plants and fungi have coexisted with QS bacteria for millions of years and some of them are expected to produce quorum quenching compounds. *Penicillium* sp. found to produce secondary metabolites with quorum quenching ability. Two of these compounds which have been identified are patulin and penicillic acid (PA) which target Rh1R and Las R quorum sensing regulators, respectively. Algal-derived brominated furanones especially from the *Delisea pulchra* have been found to exhibit quorum quenching activity. Their quorum sensing effect may be attributed to conformational changes which occur due to the binding of blockers to the receptors which ultimately leads to destabilization of the receptor. By intervening with QS-controlled motility they prevent bacterial colonization and macrofouling. *Serratia liquefaciens* MG1 is one such example of inhibition of swarming motility by halogenated furanones (Adak et al. 2011; Kjelleberg et al. 1997).

14.4.1.1 Quorum Quenching Compounds from Terrestrial Sources

Natural products, especially plants, have been explored mostly for their therapeutic uses in traditional medicine; plant-derived biologically active constituents have led to the discovery of new drugs which have been used for the treatment of various ailments (Hanson 2003; Koh et al. 2013), but now the research has shifted from this to their ecological role in regulating interactions between the microorganisms. Plants as we know lack of advanced immune systems like humans and other mammals are possessing, so, instead of relying on biochemical and cellular defense systems, plants may have evolved to produce quorum quenching compounds (antiquorum sensing compounds) that can be employed to overcome quorum sensing pathogens that invade plants (Koh et al. 2013). Some plants have been found to produce chemicals which are capable of interfering with bacterial quorum sensing. These plants include *Daucus carota subsp. sativus* (carrot), *Solanum lycopersicum* (tomato), *Glycine max* (soyabean), *Capsicum chinense* (chili), *Nymphaea* (water lily), *Pisum sativum* (pea), and *Allium sativum* (garlic) (Adak et al. 2011). Eukaryotes have evolved a more efficient way to manipulate bacterial quorum sensing system

to keep pathogenic organisms at bay (González and Keshavan 2006; Koh et al. 2013). A variety of phenolic compounds have shown inhibitory activity against AHL-dependent quorum sensing. Flavonoids have been well studied and have displayed many pharmacological activities apart from presenting their structural divergence (Havsteen 2002; Tang and Zhang 2014). The first identified flavonoid compound flaven-3-ol catechin was isolated from *Combretum albiflorum* bark, which is capable of cutting down the production of virulence factors in *Pseudomonas aeruginosa* PAO1 by interfering with Rh1R (Vandeputte et al. 2010; Tang and Zhang 2014). Now, many other flavonoids inhibit quorum sensing in bacteria (Vikram et al. 2010; Vandeputte et al. 2011; Mansson et al. 2011; Tang and Zhang 2014). Honey and propolis have been found to be capable of interfering with quorum sensing in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*, respectively, which may be because of the presence of high quantity of flavonoids (Havsteen 2002; Tang and Zhang 2014). Another group of phenolic compounds called hydrolysable tannins from various plants have shown quorum sensing inhibition against *Chromobacterium violaceum* and *Pseudomonas aeruginosa* (Adonizio et al. 2008; Giménez-Bastida et al. 2012; Tang and Zhang 2014). One more phenolic compound hamamelitannin is capable of blocking quorum sensing in *Staphylococcus* spp. (Kiran et al. 2008; Li et al. 2011; Mansson et al. 2011; Tang and Zhang 2014). *Candida albicans,* an opportunistic pathogen, has been found to use farnesol (a common sesquiterpene) as a quorumsensing signal that inhibits the Pseudomonas quinolone signal (PQS) circuit of *Pseudomonas aeruginosa* by promoting nonproductive interaction between PqsR and the pqsA promoter (Cugini et al. 2007; Amaya et al. 2012; Tang and Zhang 2014). Cugini et al. (2007) in their studies reported that farnesol may probably play a role in interkingdom communications. Apart from farnesol two more sesquiterpene derivatives, drimendiol obtained from *Drimys winteri* and sesquiterpene lactones obtained from *Centratherum punctatum*, have been identified as AHL-dependent quorumsensing inhibitors (Jakobsen et al. 2012; Paza et al. 2013; Tang and Zhang 2014). Cinnamaldehyde was used as a flavoring substance (Brackman et al. 2011; Tang and Zhang 2014). It has been found that low concentrations of Cinnamaldehyde are effective in inhibiting QS process in *Vibrio harveyi* (Niu et al. 2006; Tang and Zhang 2014).

14.4.1.2 Quorum Quenching Compounds from Marine Sources

Most common sources for quorum quenching compounds in marine environment are bacteria, fungi, algae, bryozoan, corals, and sponges. Marine cyanobacteria have become one among the best source for obtaining biologically active and structurally unique natural products. Quorum sensing inhibitors that are AHL-dependent have been found in various species of marine cyanobacteria. From *Blennothrix cantharidosmum,* tumonoic acids (E, F, G, and H) are extracted which inhibit the bioluminescence *of Vibrio harveyi* BB120 without affecting the bacterial growth. Among these tumonoic acids, tumonoic acid F has been found to be more potent (Clark et al. 2008; Tang and Zhang 2014). One more cyanobacteria *Lyngbya majuscula* produces four distinct compounds including malyngolide (MAL),

8-epi-malyngamide C, lyngbyoic acid, and lyngbic acid which inhibit LasR of *Pseudomonas aeruginosa* responding to exogenous 3OC12-HSL (Dobretsov et al. 2010; Tang and Zhang 2014). Compounds known as Honaucins A-C, isolated from *Leptolyngbya crossbyana*, are found to have dual activity, that is, they act both as anti-inflammatory as well as anti-quorum sensing compounds. Such compounds can be of tremendous use to produce multifunctional drugs. Marine cyanobacteria produces various kinds of AHL-dependent quorum sensing inhibitors, and have most likely evolved strategy to check the associated microbial communities by intervening with their cell-to-cell communication thereby disrupting the communication process (Koh et al. 2013). Some of the other marine microorganisms have also been found to produce quorum quenching compounds. *Halobacillus salinus,* a Gram-positive bacterium, produces a couple of compounds including *N*-(2-Phenylethyl)-isobutyramide and 3-methyl-*N*-(2-Phenylethyl)-butyramide which have the capability of inhibiting violacein biosynthesis of *Chromobacterium violaceum* CV026 in the presence of exogenous AHLs (Teasdale et al. 2009; Tang and Zhang 2014). Both Gram-positive *Bacillus cereus* and Gram-negative *Marinobacter* sp. SK-3 produce diketopiperazines (DKPs) which inhibit AHL dependent Quorum Sensing (Teasdale et al. 2009; Abed et al. 2013; Tang and Zhang 2014). Piericidin isolated from marine actinobacteria inhibits violacein biosynthesis in *Chromobacterium violaceum* CV026 (Ooka et al. 2013; Duncan et al. 2014; Tang and Zhang 2014). The protective features of halogenated furanones in brine shrimp, rotifers, and rainbow trout against pathogenic Vibrio species has been well reported by various researchers (Rasch et al. Rasch et al. 2004; Defoirdt et al. 2006; Tinh et al. 2007; Tang and Zhang 2014). These marine-derived furanones have also been found to be effective against lung infections caused by *Pseudomonas aeruginosa* in mice (Wu et al. 2004; Tang and Zhang 2014). Some furanones have also been found to be toxic to rainbow trout, rotifers, and human fibroblasts (Rasch et al. 2004; Tinh et al. 2007; Tang and Zhang 2014). So, the need of the hour is to produce less toxic and more effective furanone derivatives commercially that may promote their therapeutic value.

14.4.1.3 Quorum Quenching in Marine Environment – a Resource to Be Explored

As we all realize that marine environment is one of the exceedingly assorted ecosystem, microorganisms with abilities of producing QS inhibitors remain to be discovered. Scientists have proposed that quorum quenching is liable to be more common in marine bacteria because a high ratio of quorum quenching bacteria has been reported (Romero et al. 2012; Tang and Zhang 2014); in addition to this a high incidence of quorum quenching genes has been discovered in marine metagenomes (Romero et al. 2011; Tang and Zhang 2014; Hentzer and Givskov 2003). Therefore, majority of quorum quenching bacteria may still be undiscovered and the occurrence of quorum quenching enzymes in marine microbes is also expected to be on the higher side. Thus, an intensified attempt is needed in the invention of new natural quorum quenching compounds from marine microorganisms as it may prove to be a boon in the field of developing quorum quenching compounds from natural environments.

14.4.1.4 Synthetic Quorum Sensing Inhibitors

There are three ways to block QS using synthetic quorum sensing Inhibitors:

1. Substitution of acyl side chain without any change in the lactone ring inhibits quorum sensing in an effective manner.
2. Substitutions and alterations in the lactone ring with unchanged acyl side chain.
3. Extensive modifications in both the acyl side chain and the lactone ring.

Several quorum sensing inhibitor compounds with structures unrelated to the signal molecules, including 4-nitro-pyridine-N-oxide (4-NPO), indole, p-benzoquinone, 2,4,5-tribromoimidazole, indole, and 3-nitrobenzene sulphone amide, have also been found to exert similar quorum sensing inhibitory effects (Adak et al. 2011).

14.5 Applications of Quorum Quenching

Most of the applications have been already discussed and some specific applications are discussed in the following sections

(a) *Quorum Quenching in periodontal bacteria*

Oral bacteria like *Porphyromonas gingivalis*, *Actinobacillus, Actinomycetum commitans, Streptococcus* species, etc. communicate and coordinate their pathogenic behavior via quorum sensing (Yada et al. 2015). Thus, disruption of quorum sensing in such pathogens will be effective in controlling periodontal diseases (Defoirdt et al. 2013).

(b) *Quorum Sensing Inhibitors for animal breeding*

It has been found that at least half of the dairy cattle are affected by uterine infection causing infertility by disrupting uterine and ovarian function. *Trueperella pyrogenes*, *Escherichia coli, Bacillus* sp., and *Streptococcus uberis* are mostly involved in this disease. Most of these bacteria are biofilm forming and for the formation of biofilms they use QS. Bacterial behavior within biofilms is regulated by the phenomenon of QS (Bhargava et al. 2010; Romero et al. 2012). Disrupting the bacterial Quorum Sensing process using various Quorum Quenching agents will hamper the biofilm formation which may pave the way to combat many diseases.

(c) *Control of membrane biofouling*

Membrane biofouling is a most frequent and an adverse event in wastewater treatment resulting in significant loss of treatment efficiency as well as the economy. Inactivation of AHL molecules has been proved to be a promising approach to check membrane biofouling (Xu et al. 2003; Lade et al. 2014). Two quorum

quenching compounds, namely, AHL-acylase and AHL-lactonase have been found to be of great potential to control biofouling via disrupting the quorum sensing process in *Aeromonas hydrophila* mostly involved in biofilm formation in waste water treatment plants (Lade et al. 2014).

(d) *Quorum Quenching and multidrug resistance*

It has also been envisioned that some of the compounds from plant and microbes can control the AHL-mediated biofilm formation without affecting bacterial growth thus reducing the virulence and risk of multidrug resistance (Lade et al. 2014).

(e) *Quorum Quenching and biological control*

Quorum quenching can be used as an alternative approach to regulate pathogenicity. The strategy involves direct introduction of a gene coding for AHL Synthase to the plant cells or the use of AHL disrupting bacteria to protect plants and heterologous expression of the genes encoding AHL-degrading enzymes in pathogenic cells or plant tissue. Thus, bacteria misapprehend the size of the population and this leads to the production of virulence factors before the pathogen population is significant enough to continue infection (Anderson et al. 2006; Moghaddam 2014). Various studies have shown that the application of bacterial cells having the ability to produce AHL-degrading enzymes can be employed as natural control agents for controlling bacterial diseases in plants. Dong et al. (2007) have reported first such an example in transgenic plants *Solanum tuberosum* (potato) and *Nicotiana tabacum* (tobacco) expressing the gene encoding AiiA lactonase showing strong resistance against *Pectobacterium cartovorum* infection. Heterologous expression of AiiA gene encoding the AiiA lactonase from *Bacillus* sp. in plant pathogen cells *Pectobacterium cartovorum*, *Burkholderia thilandensis,* and *Erwinia amylovorai* showed disease symptom development. Hence, the quorum sensing inhibitors together with AHL-degrading enzymes can be applied successfully to disrupt bacterial quorum sensing and to reduce bacterial colonization (Dong et al. 2007; Moghaddam 2014).

(f) *Quorum Quenching and immunotherapy*

The process that how quorum sensing inhibitors can be used as defense molecules via immuno-pharmacotherapeutic approach for the attenuation of AHL-dependent quorum sensing in the human pathogen *Pseudomonas aeruginosa* was first reported by Kaufmann and group (Kaufmann et al. 2006, 2008). 3-oxo-C_{12}-HSL does not help in regulating the expression of virulence factors but it also plays a pivotal role in pathogenesis via exerting cytotoxicity on host cells. A similar approach was reported in *Staphylococcus aureus* by Park et al. (2007). Thus, these findings provide a strong basis for further research in this area to develop immunotherapy to treat bacterial infections in those cases where quorum sensing controls the expression of virulence factors.

14.6 Limitations of Quorum Quenching

1. Quorum quenching might have a detrimental role on nonpathogenic or beneficial microbes (Uroz et al. 2009)
2. Quorum quenching compounds can be easily proteolyzed and majority of the quorum quenching compounds are heat sensitive (Tang and Zhang 2014)
3. Some of the bacteria have evolved resistance mechanisms against quorum quenchers (García-Contreras et al. 2013)
4. Lacking of appropriate delivery system of quorum quenching compounds for developing antivirulence drugs (Tang and Zhang 2014)

14.7 Competitive Environment to Produce Quorum Quenching Compounds

We are aware that different organisms develop survival strategies to compete with each other for space, nutrition, and even their ecological niches. Quorum sensing has been employed by various organisms for coordination and for other social engagements between microorganisms. The competition between microorganisms may force one species to produce anti-quorum sensing compounds against the other species, which will indirectly control the proliferation of that species whose quorum sensing process has been disrupted (Tang and Zhang 2014).

14.8 Conclusions

Mechanism of bacterial communication has been studied in vast detail, since the last decade, but we need to explore more resources for obtaining quorum quenching compounds in order to provide alternatives for developing antivirulence therapy against the pathogenic organisms. Marine ecosystem is so vast and it may contain a lot of hidden compounds which need to be explored. So, the need of the hour is to put increased efforts in obtaining such molecules/compounds which can help us to achieve success in eradicating the multidrug resistant pathogens and various other environmental concerns like membrane biofouling. Apart from the use of this process in medicine, we can look for microorganisms that will help in production of biochemical, microbial biosensors, etc.

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Antimycobacterial Peptides

15

Seema Madhumal Thayil and Anup Kumar Kesavan

Abstract

One third of the world population is infected latently with tuberculosis, a bacterial disease caused by *Mycobacterium tuberculosis* (MTB). Ten percent of this latent infection may lead to active TB. With the advent of drug resistance against conventional antibiotics, there is an urgent need for new therapeutics that can target and treat tuberculosis, especially multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis. Antimycobacterial peptides, having low immunogenicity, high affinity to bacterial cell envelopes and diverse mode of action, have shown to be promising candidates in this regard. Some of the challenges faced by these peptides include difficulty in delivery to the target sites traversing the unique mycobacterial cell wall, stability of the peptides at physiological conditions and degradation by cytoplasmic proteases. This chapter will discuss the structure, composition, isolation and mode of synthesis of potential antimycobacterial peptides isolated from various sources as well as the methods used to overcome some of the challenges in delivery and bioavailability.

Keywords

Antimycobacterial Peptides • AMPs • Multidrug-resistant tuberculosis

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15.1 Introduction

Antimicrobial peptides (AMPs) are cationic, amphipathic short stretches of amino acid residues that are genetically encoded and have been demonstrated to play an important role in host innate immunity. These naturally occurring peptides have been attributed with an array of activities, including broad-spectrum antibacterial, antiviral, antifungal and antiparasitic activities (Wang et al. 2000). In addition, the AMPs have properties such as immune modulation, chemotaxis, wound healing and anticancerous activity. LAMP is a free database (Zhao et al. 2013), available at: http://biotechlab.fudan.edu.cn/database/lamp, which details over 5000 naturally occurring as well as synthetic peptides along with the class, structure and major functions attributed to these peptides. The secondary structures taken up contribute to the antimicrobial potentials of these peptides.

The AMPs are classified into four classes based on their secondary structures (Wang 2015):

1. α-Helix
2. β-Hairpin structure
3. Antiparallel β-sheet
4. Linear peptides

Antimicrobial peptides have a unique structure and diverse mechanism of action (Waghu et al. 2014). Some peptides act on the membrane of the bacteria through electrostatic interactions, some act on the ion channels thereby changing the internal pH of the organism, other peptides in addition to targeting the membrane can also act on intracytoplasmic molecules like lipid II, and certain peptides can traverse the membrane and accumulate inside the cytoplasm, thereby reducing the viability of the microbe.

The main models explaining membrane disruption include Carpet model, where peptide aligns parallel to the surface of the membrane, increasing the surface tension of the membrane leading to its disruption. In Barrel-stave model, the hydrophobic end of peptide inserts into the membrane and the hydrophilic ends jutting out, thus forming pores in the membrane. In “toroidal pore” model, the peptides have extensive lattice-like conformation with membrane lipids forming pores (Brogden et al. 2003; Yeaman and Yount 2003; Duplantier and van Hoek 2013). Disruption of cytochrome C and cell wall synthesis machinery (Wenzel et al. 2014), molecular electroporation model (Miteva et al. 1999) and sinking raft mechanism (Pokorny and Almeida 2005) are some of the other models explaining membrane disruption by AMPs. *Mycobacterium tuberculosis* complex (MTB complex) consists of *M. tuberculosis*, *M. africanum* and *M. bovis* and the *Bacillus Calmette-Guérin* strain, *M. microti*, *M. canetti*, *M. caprae*, *M. pinnipedii*, *M. suricattae* and *M. mungi*, which are related species of acid fast bacteria that cause tuberculosis in humans (Frothingham et al. 1994). Tuberculosis infects one third of the world population,

with an estimated two million deaths every year (WHO 2015). The five commonly used anti-TB drugs are rifampicin, isoniazid, ethambutol, pyrazinamide and streptomycin (WHO 2013). Resistance to all these anti-TB drugs has been reported over time. Latest estimate puts the multidrug-resistant (MDR) TB burden to be at an estimated 0.48 million, among which around 9% are extensively drug-resistant (XDR) TB cases (WHO 2015). The only new anti-TB drug approved in the last 40 years is bedaquiline (http://www.accessdata. fda.gov/drugsatfda_docs/label/2012/204384s000lbl.pdf) with an FDA approval for its use only in MDR and XDR patients.

The sturdy multilayered cell wall of *Mycobacterium* consists of an inner peptidoglycan layer, surrounded by a layer of mycolic acid linked to arabinogalactan and the outermost layer of glycolipids and lipoglycans (Brennan 2003). This unique nature of mycobacterial cell makes it impenetrable to most common surfactants and antibacterial agents. The ability of bacteria to thrive in acidic pH inside the macrophage gives mycobacteria additional survival advantages. Mycobacteria have recently been shown to produce biofilms (Ojha et al. 2008). Thus in addition to resistance toward existing drugs, all the aforementioned factors make a compelling case for accelerated research to identify novel antimycobacterial candidates. One such area of drug discovery research with high potential is antimicrobial peptides. AMPs have low immunogenicity and a wide range of activity, thus making them desirable antimicrobial candidates. A wide range of peptides are derived from all life forms with target sites ranging from the cell membrane to intracellular sites. In this chapter, we will discuss the origin, synthesis and isolation techniques of various antimycobacterial peptides as well as their mechanism of action.

15.2 Cell Membrane Inhibitors

15.2.1 E50-52A

Bacteriocins are cationic antimicrobial peptides produced by bacteria that are ribosomally synthesized and can permeate membranes (Cotter et al. 2005). E50–52A is a class IIa bacteriocin isolated from *Enterococcus faecalis* (Sosunov et al. 2007). It has a molecular weight of ~4 kDa and an isoelectric point of 8.0. The antimicrobial activity of E50–52A ranges from the inhibition of genus *Campylobacter*, *Yersinia*, *Escherichia* and *Salmonella*. Its inhibition of *M. tuberculosis* H37Rv is at par with rifampicin. The major drawback with the use of E50–52 peptide is its cytotoxicity at higher levels as well as its inability to penetrate the host membrane at physiologically tolerable concentrations. In a macrophage model of infection, the peptide was conjugated with phosphatidylcholine–cardiolipin. This E50–liposome complex could enter macrophages and contain mycobacterial growth without lysis of macrophages. Similarly, in a mouse model of acute TB infection, E50–liposome complex inhibited mycobacterial growth in vivo and increased the lifespan of infected mice (Sosunov et al. 2007).

15.2.2 Purification of Bacteriocin E50-52A

Sosunov et al. (2007) isolated E50–52A from *E. faecalis by* growing the bacteria in modified Kugler broth media. The protein was precipitated with ammonium sulfate from centrifuged culture supernatant. After dialysis, this crude antimicrobial preparation (CAP) was subjected to gel filtration on Superose 12HR 16/50 columns (Pharmacia, Uppsala, Sweden), and eluted fractions were tested for antimicrobial activity against *Campylobacter jejuni* strains. The bioactive fractions were purified on 20 cm Mono Q HR 5/5 columns and on 20 cm CM-Sepharose columns (Pharmacia). The molecular weights of bioactive fractions were determined by electrophoresis and confirmed by matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry. Edman degradation was used to determine the amino acid sequence of the peptide, and a highly conserved motif "YGNGV" was found to be present in all class IIa bacteriocins as the amino-terminal sequence.

15.2.3 Ub2

Ub2 is a peptide derived from ubiquitin identified in lysosome (Kieffer et al. 2003). Immune response kills mycobacteria by trafficking of the bacterium to the lysosome – a phenomenon called autophagy. Alonso et al. (2007) found that solubilized lysosomes isolated from resting bone marrow–derived macrophages were bactericidal toward both *M. tuberculosis* and *M. smegmatis*. These solubilized lysosomal materials were fractionated by HPLC. The bioactive fraction on analysis by mass spectrometry revealed the bactericidal activity to be associated with ubiquitin (Ub) peptides. Purified ubiquitin lacked bactericidal activity, but on digestion with cathepsin protease, they found few peptides that exhibited bactericidal activity. Similarly, they found the de novo synthesized peptide Ub2 with amino acid sequence STLHLVLRLRGG to be bactericidal against mycobacterial species (Foss et al. 2012). The bactericidal activity of ubiquitin was confirmed by Purdy et al. (2009). When lysosomal extract was treated with polyclonal antibodies against ubiquitin, immunodepletion of ubiquitin led to diminished activity of the extract. Since the lysosomal lysates from autophagic and activated macrophages have increased levels of ubiquitin, these macrophages exhibit increased microbicidal activity when compared to resting macrophages. Further bactericidal experiments by Purdy et al. (2009) using synthesized Ub2 at different pH against mycobacterial cultures grown in 7H9 media confirmed that Ub2 exhibited maximum bactericidal activity between pH 5.5 and 6. These experiments further confirm the effective role of Ub peptides at relevant physiological pH, as the internal pH of phagosomes varies from 5.5 to 6.2 depending on its maturity. The β-sheet in Ub2 is primarily responsible for the membrane targeting of the molecule (Foss et al. 2012).

15.2.4 Granulysin

Granulysin is a cationic protein and a member of the saponin-like protein family (Pena and Krensky 1997; Pitabut et al. 2013). Granulysin has an important role in cytotoxic T cell response to intracellular bacterial pathogens (Mueller et al. 2011). The effect of granulysin on the viability of virulent *M. tuberculosis* was examined by culturing *M. tuberculosis* in 7H9 media in the presence of various concentrations of granulysin (Stenger et al. 1998). These experiments showed that the granulysin killed 90% of *M. tuberculosis* in vitro within 72 h in a dose-dependent manner. However, this antimycobacterial activity was not detected ex vivo, when granulysin was added to *M. tuberculosis*–infected macrophages.

Okada et al. (2011) have shown promising results against both MDR-TB and XDR-TB in mice and a monkey model of infection using a DNA vaccine expressing mycobacterial heat shock protein 65 and IL-12 as well as a granulysin vaccine. Granulysin-derived synthetic peptides GranF2 and G13 have been shown to inhibit in vitro growth of clinical isolates of MDR tuberculosis strains. Toro et al. (2006) have demonstrated that GranF2 (Andreu et al. 1999) and G13 (Wang et al. 2000) inhibited multidrug-resistant *M. tuberculosis* to a higher extent when compared to inhibition of drug-susceptible strains. GranF2 in combination with first-line drug ethambutol enhanced its antibacterial effect. In combination with streptolysin O, GranF2 efficiently reduced the intracellular growth of multidrug-resistant *M. tuberculosis*. The difference in the susceptibility of drug-resistant versus sensitive strain of MTB was attributed to the difference in cell wall architecture. The drug-sensitive strains exhibit a robust cell wall and membrane and the resistant strains have reduced fitness and growth. Andreu et al. (1999) identified a 22-residue alpha helical sequence and a loop to be the site with antimycobacterial activity in NK-lysin and granulysin.

15.2.5 HNP-1

Human neutrophil peptides 1, 2 and 3 collectively called "defensins" were first purified by Ganz et al. (1985) from neutrophils and exhibited a broad range of antimicrobial activity (Liu and Modlin 2008). Sharma et al. (2000) have shown an inhibition of *M. tuberculosis* H37Rv growth both in vitro and ex vivo in the presence of HNP-1. During mycobacterial infection, HNP-1 modulates the innate response by mediating the secretion of TNF alpha required for monocyte migration to the site of infection. Purification of human defensins HNP-1, HNP-2 and HNP-3 were done from polymorphonuclear leukocyte (PMN) cells by homogenization and differential centrifugation. This homogenate was subjected to acetic acid extraction to obtain the granule protein, which was fractionated by gel filtration using Bio-Gel

P-10 column. Next, ion exchange, gel filtration and reverse-phase high-performance liquid chromatography (RP-HPLC) led to the extraction of pure HNP-1, HNP-2 and HNP-3 peptides. These peptides were reduced and alkylated and subjected to hydrolysis in order to determine the amino acid composition. The reduced peptides were digested with proteases, and the resulting amino acids were quantitated as phenylthiocarbamyl (PTC) derivatives.

HBD-2 a, β-defensin, has been found to augment the antimycobacterial activity of first-line anti-TB drugs isoniazid and rifampicin (Kalita et al. 2004; Sharma et al. 2001). Kisich et al. (2001) showed that on treatment with HBD-2 mRNA (MIC of approximately 2 μg/mL), MTB-infected macrophages show a concentration-dependent inhibition of mycobacterial growth, and with a single HBD-2 mRNA treatment, the antimycobacterial effect lasted for at least 7 days. Administration of L-isoleucine in drug-sensitive H37Rv strain as well as in MDR-TB-infected mice showed an increase in the induction of beta-defensins 3 and 4 (Pazgier et al. 2006) and a significant decrease in bacillary load.

Sequence determination: The S-carboxamidomethylated HNP-1, HNP-2 and HNP-3 were subjected to gas-phase Edman degradation. Phenylthiohydantoin (PTH) amino acids were identified by RP-HPLC. The carboxyl-terminal segment of each defensin was labeled with tritium at its carboxyl terminus and then digested with trypsin. The digestion fragments were purified by RP-HPLC, and the tritium-containing fragments were characterized by amino acid analysis. The human peptides are composed of 29 or 30 amino acids. The sequence of HNP-1 was determined as "ACYCRIPACIAGERRYGTCIYQGRLWAFCC."

The human defensins possess a net positive charge at pH 7.0. Six cysteine residues are conserved among human defensins, and it has been proposed that the molecular conformation afforded by these residues plays a major role in immunity against microbes.

15.2.6 PR39

PR39 is a neutrophil-derived peptide with antimicrobial activity requiring the 26 residues of amino-terminus (Shi et al. 1996). Its mechanism of action was proposed to be the inhibition of DNA and protein synthesis (6). It was first isolated from porcine intestine and later from human neutrophils (Agerberth et al. 1991; Shi et al. 1994). PR39 peptide with amino acid sequence "RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP" has been successfully synthesized, purified and tested for its antibacterial activity against *E. coli and Salmonella* sp. (Shi et al. 1996). Linde et al. (2001) demonstrated that the proline–arginine-rich porcine peptide PR39 inhibited *M. tuberculosis* H37Rv to significant levels in a concentration-dependent manner in vitro.

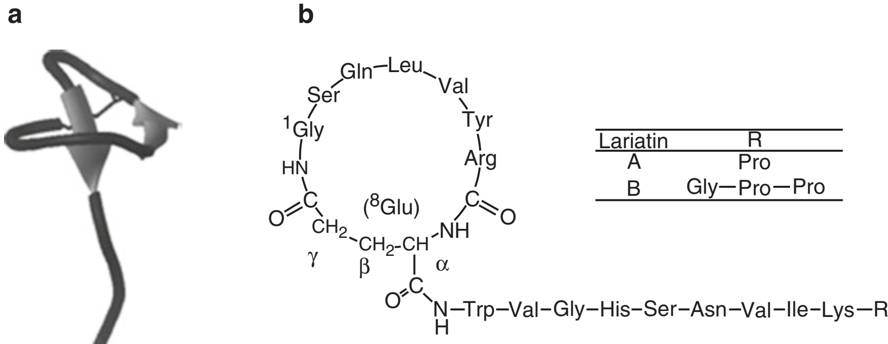

Fig. 15.1 (**a**) Lasso structure of lariatin A (Courtesy of Hiroshi Tomoda). (**b**) Structures of lariatins A and B (Courtesy of Hiroshi Tomoda)

15.2.7 Lariatins A and B

Lariatins A and B are antimycobacterial peptides isolated from cultures of *Rhodococcus jostii* (Iwatsuki et al. 2007). Both the peptides have a lasso structure (Fig. 15.1a) consisting of 18 and 20 amino acid residues, with the γ-carboxyl group of Glu8 and the α-amino group of Gly1 forming an internal linkage giving the peptide its ring and tail appearance as depicted in Fig. 15.1b. Lariatins A and B were isolated by the fermentation of a broth culture of *R. jostii*. The maximum production of lariatin A was observed at day 10, whereas peak production of lariatin B was observed by day 5. After fermentation, the broth was centrifuged to remove fungal mycelia followed by sequential passage of supernatant and resulting eluates through a series of columns. The active component was finally subjected to a preparative HPLC with C18 column to obtain lariatin A at retention time of 44 min and lariatin B at 56 min. The molecular formula of lariatin A is $C_{94}H_{143}N_{27}O_{25}$ and B is $C_{101}H_{153}N_{29}O_{27}$. Both lariatins inhibited the growth of *M. smegmatis*. In addition, lariatin A also exhibited antimycobacterial activity against the virulent strain *M. tuberculosis*, at ranges comparable to the first-line drug isoniazid, and the mechanism of action was postulated to be the inhibition of cell wall synthesis.

15.2.8 Lacticin 3147

Bacteriocin is a broad-range AMP with antimycobacterial activity isolated from *Lactococcus lactis* subsp. *lactis*. Lacticin 3147 is a thermostable compound active at pH 7.4. It is encoded by a 60.2 kDa plasmid. Lacticin 3147 forms pores in the cell membrane of the target organism, thereby disrupting the membrane potential and the pH gradient (Cotter et al. 2005; Carroll et al. 2012).

15.2.9 Preparation of Lacticin 3147

McAuliffe et al. (1998) purified the bacteriocin by growing *L. lactis* subsp. *lactis* DPC3147 in tryptone–yeast broth. The culture was centrifuged, and culture supernatant was passed through XAD-16 bead. The bioactive bacteriocin was applied to activate C18 reverse-phase column. The column was washed with 30% ethanol, and the active bacteriocin was eluted in 70% isopropanol–10 mM acetic acid (pH 2). Active fractions thus obtained were concentrated and subjected to a fast protein liquid chromatography (FPLC). Extracellular ATP levels, transmembrane electric potentials, and intra- and extracellular K^+ concentration were used to determine the bacteriocin potential.

15.2.10 LLKK and II-D

Two unnatural D-amino acid synthetic peptides, LLKK and II-D, have shown promising results against both MDR and susceptible clinical isolates in vitro within 4 days of treatment. The peptides were resistant to proteases. Live-cell imaging and confocal microscopy revealed that the peptide could transverse the mycobacterial membrane and accumulate in the cytoplasm (Khara et al. 2016).

15.3 LPS Inhibitors

15.3.1 LL37

LL37 is the C-terminal domain of human cathelicidin known as human cationic antimicrobial protein (hCAP18) (Mendez-Samperio 2010). Human cathelicidin exhibits various functions like immunomodulation and chemotaxic activity in addition to microbial inhibition (Bowdish et al. 2006). Cathelicidin-mediated antimycobacterial effect of vitamin D was demonstrated by Liu et al. (2007). In this experiment, the human monocytic cell line THP1 cell was infected with *Mycobacterium* H37Ra and stimulated with 1,25-dihydroxyvitamin D3. The inhibition of mycobacterial growth was recorded in the presence of cathelicidins; upon knocking down cathelicidin production using siRNA, the antimycobacterial effect of vitamin D was lost. This led to the conclusion that the induction and expression of cathelicidins were important for the antimycobacterial effect of vitamin D ex vivo. Martineau et al. (2007) observed an iron-dependent antimycobacterial effect of LL37. On prolonged exposure of LL37 (5 g/mL), they found a 1.8-fold reduction in mycobacterial colony forming units (CFU) in iron-depleted cultures when compared to cultures that were not exposed to LL37. The antimicrobial potency of LL37 peptide was increased by substituting Q22, N30 and D26 residues with positively charged lysine residues forming LLKKK18 peptide. Silver nanoparticles coated with LLKKK18 have shown promising antimycobacterial activity. Recently, the use of self-assembling hyaluronic acid (HA) nanogels for the delivery

of AMP LLKKK18 has been explored. These nanogels were found to be less cytotoxic as well as more resistant to degradation by host proteases, thereby increasing the potential for its use as an antimycobacterial agent (Silva et al. 2016).

Another analog of LL37 peptide called D5 was designed by Jiang et al. (2011). In D5, three alanine residues of LL37 were substituted with more hydrophobic leucine residues, and a valine at position 16 is substituted with lysine.

Five peptide analogs, D1–D5, were synthesized de novo by Jiang et al. (2011). The peptides were synthesized using *t*-butyloxy-carbonyl (*t*-Boc) chemistry and 4-methylbenzhydrylamine resin by solid-phase synthesis methodology. The peptides were cleaved from the resins and purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Zorbax 300 SB-C8 column. A gradient of 0.2% aqueous trifluoroacetic acid (TFA), pH 2, and 0.2% TFA in acetonitrile was used as the mobile phase. The purity of the peptides was verified by analytical RP-HPLC, and the peptides were characterized by mass spectrometry (LC/MS) and amino acid analysis. Characterization of helical structure was done using circular dichroism (CD) spectroscopy, and peptide concentrations were determined by amino acid analysis.

Antimycobacterial activity of peptides was determined by adding known concentrations of peptides to fresh mycobacterial suspension. After initial incubation for a week, these suspensions were added to 7H11 solid plates, and colony-forming units were enumerated after 3 weeks of growth. Peptide D5 (Iwatsuki et al. 2007) had the highest antimycobacterial activity compared to LL37 and all its derivatives. The aforementioned substitutions also resulted in decreased hydrophobicity, amphipathicity, helicity, self-association ability and hemolytic activity of the peptides. The mechanism of action of D5 includes membrane disruption and biofilm disassembly (McGrath et al. 2013; Carmona et al. 2013; Kolodkin-Gal et al. 2010).

15.4 Envelope Inhibitors

15.4.1 RNase 3

RNase 3 was first identified as an eosinophil secretion product and named eosinophil cationic protein (ECP) (Ahlstedt 1995). Activated eosinophils secrete ECP during inflammation, and its levels in biological fluids are used as a marker for the diagnosis of allergy and eosinophilia disorders. RNase 3 is a small cationic protein belonging to RNase A superfamily. It shows potent antibacterial and antiparasitic activities (Venge et al. 1999; Bystrom et al. 2011). It was shown that eosinophils can mediate their antibacterial effect through the release of cationic granule proteins (Linch et al. 2009). Antibacterial action of ECP was shown by binding to anionic sites, i.e., LPS on bacterial membranes (Boix et al. 2012). In addition to its antibacterial activity, ECP also has immunomodulatory activity; it regulates fibroblasts, induces mucous secretions and exerts cytotoxic and neurotoxic activities in mammalian cells (de Oliveira et al. 2012). It was shown that in combination with a

defensin, it kills *M. bovis-BCG* by degranulation. It exhibits potent antimycobacterial activity at micromolar concentrations.

15.4.2 RNase 7

RNase 7 also belongs to RNase A superfamily, which participates in the host immune response against pathogen infection. They are considered to be one of the main components of first-line protection of the innate immunity at the epithelial level. Harder and Schroder (2002), while analyzing healthy human skin for the presence of endogenous antimicrobial peptides, were able to identify a novel 14.5 kDa peptide and named it RNase 7. They demonstrated that RNase 7 was able to exhibit potent in vitro antimicrobial activity against various bacterial species and certain yeast species. Later Zhang et al. (2003) identified and cloned the corresponding RNase7 gene in *Escherichia coli*. They demonstrated that low micromolar concentration of RNase 7 inhibited bacterial growth. RNase 7 has high ribonuclease activity, which is not required for microbial inhibition. Huang et al. (2007) mutated the histidines and lysines to alanine at its catalytic site, to generate H15A, H123A and K38A recombinant RNase 7 peptide. The recombinant peptides were devoid of ribonuclease activity without loss of their antimicrobial activity against *Pseudomonas aeruginosa*. A similar experiment by Koten et al. (2009) using H123A peptide demonstrated its antimicrobial activity against *Enterococcus faecium* and *E. coli*. Pulido et al. (2013) have shown that RNase 7 works independently of its ribonuclease activity and exhibits mycobacterial growth inhibition at micromolar ranges. They also identified the active domain in RNase 3 and RNase 7 to be the N-terminal region of the peptide. The mechanism of action was due to change in the membrane polarity of the microbe by the AMP, leading to loss of membrane integrity and cell death.

15.5 Inhibitors of Cytoplasmic Components

15.5.1 F_1-F_0 ATPase Inhibitors

Magainins (Zasloff 1987) are peptides with broad-spectrum antimicrobial activity isolated from the skin of *Xenopus laevis*. The peptide was demonstrated to have (Morvan et al. 1994) antibacterial and antiparasitic activities. Magainins do not lyse non dividing mammalian cells. Magainin-I analog peptide (MIAP) is a de novo synthesized antimycobacterial peptide, which showed two-fold higher antimycobacterial activity than its natural analog magnanin-1. The peptide was found to act on F_1F_0-ATPase of *Mycobacterium* with magainin-1 inhibiting the ATPase activity to 30% and the de novo peptide MIAP completely abolishing the F_1F_0-ATPase. The antimycobacterial action of the peptide is brought about by a change in the internal pH of the organism (Santos et al. 2012).

15.6 Recent Major Developments

15.6.1 PK34

PK34 is derived from *Mycobacterium* phage D29. The precursor of PK34 is a hypothetical protein D29p63. D29p63 is a highly conserved small protein family, found in several mycobacteriophages. PK34 is a peptide composed of 34 amino acids that include eight basic and five acidic amino acids with a predicted isoelectric point (pI) of 10.25 (Wei et al. 2013). PK34 inhibits the growth of *M. tuberculosis* H37Rv with an MIC of 50 μg/mL. In addition to antimycobacterial activity, PK34 inhibits various proinflammatory cytokines, leading to reduced inflammation and granuloma formation in a mouse model of infection.

15.6.2 HCL2

HCL2 peptide derived from human mitochondrial protein COX3 was isolated by Samuchiwal et al. (2014). The targets for HCL2 are ESAT-6 and CFP10. Secreted antigenic ESAT-6 and CFP10 are virulence factors that play an important role in the pathogenesis of *M. tuberculosis* and are secreted into the extracellular matrix during broth culture of *M. tuberculosis*. Coexpression of HCL2 along with ESAT6 and CFP10 in a bacterial three-hybrid system leads to disruption of binding association of ESAT6 and CFP10. HCL2 could disrupt the heterodimeric interaction between ESAT-6 and CFP10 in vivo. A constitutive expression of HCL2 in *M. tuberculosis* H37Rv significantly inhibits the growth of the bacterium. Electron microscopic studies of these cells revealed feeble cells with porous membranes. In vivo studies with THP1 cells showed that HCL2-expressing *M. tuberculosis* showed reduced survival compared to the wild type. An antigen-specific hyperproliferative response was observed in mice infected with H37Rv expressing HCL2. The potential for use of HCL2 also lies in its target specificity, as it was found to be nontoxic to other bacteria as well as mice.

15.6.3 Temporins

Mohanram and Bhattacharjya (2016) have demonstrated the nonhemolytic and broad-spectrum antibacterial properties of LG21, an LPS–temporin B hybrid. The LG21 hybrid has a helical conformation forming a lollipop-like shape. The hydrophilic “head” consists of compacted bulky aromatic/cationic side chain/side chain packing and the N-terminal end constitutes the thinner “stick” which is hydrophobic in nature.

15.6.4 Inhalable Microparticles

Another promising antimycobacterial is the inhalable microparticles (MP). Lawlor et al. (2016) have developed poly(lactic-co-glycolic acid) (PLGA) MPs that reduced bacillary counts in H37Rv-infected THP1 cells ex vivo through an NFκB-mediated autophagy (Krensky and Clayberger 2009). These MPs can act as vectors to transport anti-TB agents. The advantage of using MPs is the ease of penetrating the human alveolar macrophages.

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Part IV

Emerging Trends

Probiotics

16

T.S. Swapna, A.S. Ninawe, and Sabu Abdulhameed

Abstract

Naturally occurring microorganisms in the human body have a very fragile balance in the immune system and the gastrointestinal tract. Probiotics are living beneficial microbes that can change the microbial ecology of the intestine by inducing antimicrobial metabolites and thereby enhancing the immune response of the body. Probiotic bacteria are normally chosen for their potential health benefits in the human body without causing negative impact. Dairy products can be considered as a vehicle for the effective delivery of probiotics, but they also need to survive and resist enzymes in the gastrointestinal tract. Nearly 60 species of *Lactobacillus* (lactobacilli) and *Bifidobacterium* (bifidobacteria) are found in fermented foods, including yoghurt, kefir, meso, aged cheese, kimchi, and tosa. Probiotic microflora are also associated with various therapeutic or prophylactic properties such as improved lactose digestion, better mucosal and immune function, decreased diarrhea, and fewer adenomas, colon cancers, and polyps. Probiotics could be a magic bullet in the era of antibiotic-resistant pathogenic bacteria. At the same time, ascertaining the safety or content of these supplements, especially for people with immune system disorders, pregnant women, children, and elderly people, is critical. Even though probiotics have long been

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used to maintain the homeostasis of enteric microbiota and for prevention of related diseases, the efficiency of this type of treatment is still in dispute, and interindividual variation is a major challenge. Probiotics constitute a major challenging and promising area for providing better health as well as for revenue generation.

Keywords
Probiotics • Beneficial microbes • Gut microflora • Intestinal microflora • Fermented products

16.1 Introduction

Microbes are tiny organisms that are prevalent in most parts of the body, living in the nose, mouth, intestinal tract, pharynx, and on the skin, etc., and thus are significant for body health in that an imbalance of microbes in the human body results in many diseases. More than ten times as many microorganisms are present in the human body as compared to the total number of cells in the body but constitute less than 3% of total body mass (Willis et al. 1999).

The Human Microbiome Project, sponsored by the National Institute of Health (NIH), has developed resources to study the microbial communities that live in our bodies and their role in human health and diseases. Probiotics have been with us from our first breath but may be new to the food and supplement industry. A baby picks up many bacteria, such as *Bifidobacterium* spp., *Escherichia coli*, and *Lactobacillus* spp., from the birth canal at the time of delivery.

The stomach flora mainly consists of lactobacilli, which are able to colonize heavily because of the low pH (Holzapfel et al. 1998). Some colonization in the stomach, such as *Helicobacter pylori*, is endemic for certain geographic locations. The concept of beneficial bacteria protecting organisms from harmful bacteria leads to probiotics. The concept of probiotics was first introduced by the Nobel Prize-winning scientist Elie Metchnikoff in 1900. He noticed that Bulgarian peasants have long and healthy lives and proposed it as being the result of their regular intake of milk and fermented milk products. Later, he hypothesized that the organisms in yogurt can protect the intestine from harmful bacteria. The term “probiotic” was probably introduced by Elie Metchnikoff and is considered to be derived from the Latin word “pro” and the Greek word “bios,” meaning “for life.” He suggested that live microbes might provide beneficial effects on human health when taken into the body. The definition of probiotics comprises the intake of concentrated bacterial supplementation in the diet and food fermented by a beneficial bacterium (Castellazzi et al. 2013). After 1930 the first clinical trials on the effect of probiotics on constipation were performed. A probiotic product for the treatment of scour in pigs as a drug was first licensed by the U.S. Department of Agriculture in the 1950s (Orrhage et al. 1994). Different microorganisms have been identified for their ability to prevent infection and cure diseases, leading to the blooming of

probiotics throughout the past century (Lidbeck et al. 1992; Lee et al. 1999). A new research area was opened up after findings about reduced blood serum cholesterol in people who drink fermented yogurt with wild strains of *Lactobacillus* sp. (Mann and Spoerig 1974).

In 2001, a panel of experts from the United Nations and the World Health Organization provided a definition for probiotics emphasizing the significance of their applications and demanding documentation of their benefits at the clinical level (FAO/WHO 2001). This definition is "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). The successive and joint guidelines published by these international organizations (FAO/WHO 2002) endorsed by the International Scientific Association for Probiotics and Prebiotics (Reid et al. 2003) indicated that scientific, manufacturing, and clinical standards should be met for products to be known as "probiotic."

16.2 Prebiotic

Another word commonly used with probiotics is prebiotic. Maintaining the appropriate balance between helpful and harmful bacteria is essential for good health. Many factors such as food, genetics, and age may influence the composition of microbiota in the body. Dysbiosis, which means imbalance of microbiota, may lead to many disorders. The synergistic action of prebiotics and probiotics is called synbiotics. Nondigestible carbohydrate acts as a prebiotic food for the probiotics and microbiota in the digestive tract. The growth and activities of microbes are selectively stimulated by prebiotics, which is beneficial for host health. Health benefits may include a cure for complications in the gastrointestinal tract such as enteritis, irritable bowel syndrome, constipation, allergic inflammations, cancer, and immunodeficiency diseases. In adolescents, and in postmenopausal women, bone calcium accretion and increased bone mineral density together with increased calcium absorption are attributable to the probiotic effect. From human trials and other experimental models, it was assumed that particular food products with prebiotics influence energy homeostasis and body weight, which has a beneficial effect in type II diabetics and obesity.

Oligosaccharides form major prebiotics, and generally they cannot be digested by the enzymes in the upper gastrointestinal (GI) tract. In the lower colon, they become fermented and are converted to short-chain fatty acids that will be nourishment for beneficial gut microbiota. Oligosaccharides are generally obtained from natural sources such as banana, barley, chicory, onion, garlic, honey, sugarcane juice, tomato, lentils, milk, mustards, rye, soybean, and wheat. Research is ongoing to evaluate the health benefits from these oligosaccharides (Van Loo et al. 1995).

Probiotics are live microbial cultures such as bacteria similar to natural microbes in the human GI tract consumed to provide a basic nutritional component and to maintain balance in the immune system and intestinal tract for health benefits (Agerholm-Larsen et al. 2000). Many oral probiotic products are available in the market as dietary supplements, yogurts, tablets, powders, and creams. The possible

mechanism of beneficial effect includes reducing harmful microbes in the intestine or by stimulating the body's immune response and production of antimicrobial metabolites.

16.3 Sources

Probiotic bacteria are generally known safe species that found in the human GI tract. They are isolated, multiplied, concentrated, and preserved. These probiotic bacteria could be used in food or dairy products as concentrated cultures at appropriate levels, or used as dietary supplements such as capsules or powder (Vemuri et al. 2014).

The selected probiotic strain must have potential for health benefits without any adverse effects in humans. For the application of probiotics as a dietary supplement, they must survive in the GI tract where they should be active. Probiotics must be able to resist salivary enzymes, stomach acids, intestinal enzymes, bile, and pH changes together with other food and beverages in the tract. Competition will occur between already existing microbes and probiotics. Viability, genomic stability and large-scale culturability are desirable for probiotic bacteria to be successfully utilized as per the technological requirements. Thus, intensive research is needed for the identification and isolation of suitable probiotic strains, which is a very complex process.

Probiotic organisms have a major role in our digestive tract where there is a healthy balance between many types of bacteria. Stress, lack of sleep, and drug overusage together with environmental factors may alter the microbiota, giving way to harmful bacteria.

Clostridia spp., *Escherichia coli*, enterococci, and lactobacilli were identified before the 1960s. Later, many bacteria were identified using modern technologies. A healthy digestive system filters and eliminates matter such as other bacteria that have negative impact on growth, toxic metabolites, and waste accumulated after digestion, which makes the body unhealthy. The healthy bacteria also assist in maintenance of gut barrier function and are reported to have benefits against infectious diarrhea, abdominal pain and bloating, gut transit, ulcer, enterocolitis necrosis, etc. (Katharina et al. 2007). Research data shows that prebiotics increase calcium, magnesium, and iron absorption (Katharina et al. 2007). Animal studies have shown that immune response to a vaccine can be enhanced by certain probiotic strains and that they reduce the risk of subsequent infection. Specialized Paneth cells in the small intestine produce antibacterial defensins, and this action could be enhanced by probiotics. As per *in vitro* analysis, probiotics and prebiotics can enhance the resistance of tight junctions, possibly by production of occludins and claudins in tight junctions of epithelium. Tight junction protein genes have been shown to enhance the expression by a specific *Lactobacillus* strain (Yahg et al. 2015)

Probiotics have a major function in the action of the immune system. Malfunction of the immune system results in allergic reactions and autoimmune disorders and systemic diseases such as diabetics and obesity. Selected natural strains can be referred to as first-generation probiotics, whereas in the next-generation

therapeutics, genetically engineered microbes such as *Lactobacillus* with genes for antiviral compounds are inserted (Hibberd and Davidson 2008).

16.4 Mechanism of Action

Several mechanisms about the mode of action of probiotics for therapeutic effects have been postulated. Various *in vivo* studies and probiotic clinical trials suggested beneficial effects for the relief of many disorders such as irritable bowel syndrome. The mechanism of action of probiotic organisms could be derived from their stimulation of phagocytosis, production of antimicrobial compounds, resistance to colonization, antimutagenic effects, cytokine production, and effects on enzyme activity and enzyme delivery (Vemuri et al. 2014).

Microbes ferment carbohydrates (saccharolytic fermentation) for the source of energy, which help in food production. Many microbes metabolize carbohydrates and dietary fiber, including nonabsorbed sugars, polysaccharides, and polyols. The genera *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, and *Ruminococcus* in the colon ferment carbohydrates. This fermentation results in the production of the short-chain fatty acids and lactic acid together with gases such as CO_2, hydrogen, and methane, which regulate the microbiota. Increase in the presence of fermentable substrates in the gut results in flatulence and intestinal discomfort. Proteins can be metabolized by *Clostridia* and *Peptococcus* species, which are utilized for nitrogen and produce branched-chain fatty acids along with many nitrogenous compounds. Metabolites such as polyphenols and isoflavones may also produce readily absorbable components such as equol after digestion. High stool bulk results in shorter gut transit time and low risk of bowel discomfort and bowel cancer. Bacterial mass causes fermentation of dietary fibers and contributes significantly to the stool bulk. Intestinal microbiota produce microbial antigens that have an important role in disease resistance and the maturation of lymphoid tissue in the gut (Hardy et al. 2013).

Probiotics have protective functions by modulation of immune activity and epithelial function in the intestine by regulation of the nuclear factor-κB pathway or alteration in signal transduction pathways. Inhibition of tumor necrosis factor and occlusion in the epithelial tight junctions leads to prevention of cytokine-induced apoptosis (Vemuri et al. 2014).

16.5 Common Gut Flora

At the time of birth, the GI tract is sterile and the immune system is simple and natural. In the postnatal period a new microflora is formed in the human gut with an additional source from the mother's milk. Common bacteria in mother's milk include streptococci, *Corynebacterium*, lactobacilli, micrococci, and *Bifidobacterium*, which may originate from the nipple or from the milk ducts. Human breast milk is a relevant source of lactobacilli for newborns (Castellazzi

et al. 2013). After birth, the GI tract is colonized by numerous types of microorganisms within 1 week after birth. Human intestinal microflora involves *Lactobacillus, Bifidobacterium*, and *Clostridia*, with significant differences between breast-fed and bottle-fed newborns (Castellazzi et al. 2013). Variation in the microbiome of mother's milk may be associated with mode of delivery, lactation state, use of drugs, and maternal health. The presence of carbohydrates in the milk might also regulate the microbiota in milk (McGuire and Mcguire 2015). The immune system is immature at early developmental stages and requires stimuli from other microbes or from the environment to mature and for increasing humoral immunity, T-cell response, and cell-mediated immunity. Secretory IgA released into the intestinal lumen is crucial in mucosal immunity (Rachmilewitz et al. 2004; Iliev et al. 2008). Development of immune tolerance will be delayed, which may increase the risk of allergic diseases by low exposure to exogenous antigens in early life (Caffarelli et al. 2004). The presence of these bacteria is important for T-regulatory cells, which are related to the maturation regulatory dendritic cells (Holt et al. 1997; Aureli et al. 2011). According to the "hygiene hypothesis" of Guarner et al. (2006a, b), lack of exposure to microbes in the early stage of life could be partly related to increased rates of allergies. Nowadays probiotics are used randomly, but a strategic and systematic delivery of probiotics should be developed for improved health.

Alteration of intestinal microbiota results in harmful microbial action as per the Agence Francaise de Sécurité Sanitaire des Aliments/ French Food Safety Agency (AFSSA 2005). The primary microbial stimulation in the host's body occurs with the initiation of the gut microflora, and the presence of other microbes may act as an inducer of the immune system that prevents atopic diseases (Berg 1996; Björksten 1999). Innate and adaptive immunity as activated in the early postnatal period with the presence of microbiota and stimulus is required for the successful maturation of the gut immune system. Insufficient microbial stimulus results in alteration of mucosal enzyme patterns, abrogation of oral tolerance, and reduction in surface area and the mucosal barrier (Gaskins 1997; Cebra 1999). A low bifidobacteria to clostridia ratio was observed in gut microbes in atopic children (Kalliomäki et al. 2001; Björkstén et al. 2001). The positive effect of probiotics is mainly the result of the capacity of regulating the gut microecology, immunological gut barrier function, intestinal permeability, and up- and downregulating the production of cytokines. Numerous studies have evaluated the effect of probiotic bacteria in children with atopic dermatitis, with contrasting results.

John H. Stokes and Donald M. Pillsbury (1930) hypothesized that emotional stress may change the intestinal microflora and associated increase in intestinal permeability and inflammation. Prebiotics and probiotics were reported to reduce inflammation and oxidative stress when consumed (Schiffrin et al. 2007; Mikelsaar and Zilmer 2009).They can also regulate the release of inflammatory cytokines within the skin and be beneficial in acne (Hacini-Rachinel et al. 2009). Probiotics have been postulated to improve insulin sensitivity, thus linking the role of high glycemic diets in the exacerbation of acne. Regulation of glycemic control is an additional mechanism that might influence acne via probiotics. It has become increasingly evident from the reports that there is a link between low-fiber

Table 16.1 Most widely used probiotic organisms

Organisms
Bacillus subtilis
Bifidobacterium species: *B. adolescentis, B. animalis, B. bifidum, B. breve, B. infantis, B. lactis, B. licheniformis, B. longum, B. coagulans*
Enterococcus faecium
Escherichia coli (Kruis et al. 1997)
Lactobacillus species: *L. acidophilus, L. amylovorus, L. brevis, L. casei, L. casei* subsp. *rhamnosus, L. caucasicus, L. crispatus, L. delbrueckii* subsp. *bulgaricus, L. fermentum, L. gasseri, L. helveticus, L. johnsonii, L. lactis, L. leichmannii, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus, L. salivarius* (McFarland et al. 1994)
Lactococcus lactis
Leuconostoc mesenteroides
Pediococcus acidilactici
Saccharomyces boulardii (McFarland et al. 1994), *Saccharomyces cerevisiae*
Streptococcus species: *S. oralis, S. uberis, S. rattus, S. salivarius, S. zooepidemicus, S. oligofermentans, S. thermophilus*

Sanders (1999), Sanders et al. (2007) and Vemuri et al. (2014)

carbohydrates and the risk of acne (Bowe and Logan 2010). Research also indicated the connection between glucose tolerance and gut microflora (Kleerebezem and Vaughan 2009a, b).

The capability of probiotic bacteria for delivering functional proteins and enzymes was also recorded. People with lactose intolerance can be treated with probiotic-mediated delivery of the enzyme that converts lactose into glucose and galactose. Some such enzymes can help to digest food, thus creating beneficial end products. Braat et al. (2006) reported successful use of genetically engineered *Lactococcus* to treat Crohn's patients by delivering the cytokine IL-10 to reduce intestinal tract inflammation.

16.6 Diversity in Probiotic Organisms and Fermented Products

Species of *Lactobacillus* have been commonly used for treating a large variety of diseases; nearly 50 species of lactobacilli are found in fermented dietary supplements. These species include *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *L. gasseri*, *L. johnsonii*, *L. plantarum*, *L. rhamnosus*, *L. reuteri*, and *L. salivarius* in food supplements (Vemuri et al. 2014). The types of probiotics most widely used and studied are listed in Table 16.1.

Research shows many benefits linked to *Lactobacillus* and the treatment of irritable bowel syndrome, urinary tract infection, infectious and traveler's diarrhea, and bacterial vaginosis, and preventing respiratory infections and treating yeast infections, lactose intolerance, and skin disorders such as blisters and eczema.

Another most common healthy colon bacterial genus is *Bifidobacterium*, which appears within days after birth and is a marker for intestinal health. Some of the

bifidobacteria species used as probiotics are *Bifidobacterium breve*, *B. lactis*, *B. longum*, *B. pseudolongum*, *B. infantis*, and *B. thermophilum*. Many researchers reported that bifidobacteria can help with improving glucose tolerance and lipid levels in the blood. Bifidobacteria can also effectively improve GI discomfort, bloating, and digestive disorders (Jungersen et al. 2014).

Saccharomyces boulardii is a commonly used yeast probiotic and is effective in treating traveler's diarrhea. It has also been reported that *Streptococcus thermophilus* is effective in prevention of lactose intolerance because it produces a large amount of lactase enzyme. *Enterococcus faecium,* commonly found in the intestinal tract of humans by competing for binding sites and nutrients, inhibits pathogenic bacteria. Probiotic or diet supplement with *Enterococcus faecium* is used to treat tooth decay, infectious diarrhea, irritable bowel syndrome, and vaginal infections. *Leuconostoc mesenteroides* has been used extensively in food processing throughout human history. The species is beneficial for cytokinin production. *Leuconostoc mesenteroides* and *Bacillus subtilis* together showed a synergic effect for many beneficial properties (Malathi et al. 2014)

Historical evidence suggests use of dairy products with probiotic bacteria (Fuller 1993). Most such bacteria are found to be present in various parts of the human body, including the mouth and digestive system. These probiotic bacteria are generally colonizing and mainly associated with fermentation of milk to various dairy products. Probiotic-associated dairy products are an advantageous delivery vehicle of useful bacteria (Tamime et al. 2003), such as the presence of lactic acid bacteria in the food that provide good health conditions. Bacteria are included throughout the fermentation process. Fermentation of carbohydrates to organic acids and alcohols by enzymes produced by microbes reduces the speed of decomposition and thereby expands the period of storage of perishable foods.

Lactobacillus acidophilus and *L. bulgaricus* are generally present in yogurt and curd along with *Bifidobacterium bifidum* and *Streptococcus thermophilus*, which have a positive impact on microorganisms in the gut and thereby improve lactose digestion and reduce lactose intolerance. They are also beneficial for treatment of gastrointestinal diseases, type 2 diabetics, allergies, and cardiovascular and respiratory diseases, as well as to improve bone and dental strength. A well-established probiotic drink in the market is sweetened acidophilus milk fortified with *L. acidophilus* or a combination of *L. acidophilus* and *Bifidobacterium*.

Kefir with *Lactobacillus acidophilus*, *L. casei*, and *Bifidobacterium* is considered as the most ideal probiotic dairy product that could be used by diabetic patients. The hemoglobin A1c levels that show average glucose level for the past 2 months were reported to be notably lower in persons with daily intake of kefir milk. Kimchi is produced by fermentation of Chinese cabbage, radish, and green onion with garlic, red pepper powder, and ginger. Kimchi is found to be rich in beneficial bacteria such as *Lactobacillus lactis*, *L. brevis*, *L. sakei*, and *L. curvatus*, with *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Weissella confusa*, and *W. koreensis*. The health benefits of kimchi have been recently linked with improvement of immunity and brain and skin conditions, with factors for reduction of cholesterol, obesity, constipation, and enhancement of colon health, with antioxidant and anti-aging effects (Sanders 2003).

Many other fermented foods, without any elaborate research, were also used commonly such as aged cheese, sauerkraut, miso from soybean; sour bread and pickles; gundruk, sinki from radish; khalpi from cucumber; traditionally used in ziangsang, which is produced from fermented mustard leaves; soidonis from bamboo shoots, etc. (Parvez et al. 2006). Aged soft cheese can boost the immune system and act as a carrier for probiotics. Probiotic-filled miso contains more than 160 species of bacteria and is commonly used as a salty soup with low calories and high vitamin B and antioxidant content. Buttermilk, which is rich in *Lactobacillus acidophilus*, is also reported to be a good probiotic. Probiotic fermented oat yosa with a lot of oat fiber and *Bifidobacterium* has health benefits similar to those of fortified porridge with flavor enhancers (Blandino et al. 2003). Many types of vegetable- and fruit-based products, such as juices of orange, cranberry, cashew apple, beet root, carrot, tomato, and cabbage, were commonly produced using diverse probiotic bacteria, including various species of *Lactobacillus* and *Bifidobacterium*, such as *L. acidophilus*, *L. casei*, *L. fermentum*, *L. paracasei*, *L. rhamnosus*, *L. plantarum*, and *B. bifidum*. These products have been evaluated for consumer acceptance and convenience as carriers for probiotic bacteria (Luckow et al. 2006; Yoon et al. 2006; Pereira et al. 2011). Meat has been considered as an ideal carrier for probiotic organisms because of its structure and components. Meat also is protective and effective against the mortal effect of bile on lactic acid bacteria. Introduction of probiotic bacteria through dry fermented sausages is not much reported except for the use of *L. gasseri* for meat fermentation and enhancement of safety (Arihara et al. 1998). Probiotic organisms should be sheathed by the fat and protein for protection in probiotic-based food (Lucke 2000). Encapsulated *L. helveticus* and *B. longum* in a chocolate base were tested for effectiveness as a safe carrier for oral delivery (Possemiers et al. 2010). Protection of probiotic bacteria from external adverse conditions is successful with the chocolate coating, which also helps for optimal delivery.

16.7 Therapeutic Properties

The probiotic microflora is often associated with some special therapeutic properties and related prophylactic properties, such as necrotizing enterocolitis in neonates that can be reduced by *Bifidobacterium* species (Caplan and Jilling 2000), and reduced duration of gastroenteritis-related acute diarrhea (Marteau et al. 2001). A mixed probiotic application that includes *Lactobacillus* strains is also effective in the satisfactory treatment of pouchitis (Vanderhoof 2000), reducing symptoms of lactose intolerance and diarrhea (Marteau et al. 1990). Probiotics are used for interference therapy, which means the elimination of harmful microbes using nonpathogenic bacteria (Bengmark 2000). Improved mucosal immune function using probiotic microbes was reported by Schultz and Sartor (2000) and prevention of disease by MacFarlane and Cummings (2002). Fermented food fortified with *Lactobacillus acidophilus* can decrease the side effects of pelvic irradiation such as diarrhea in cancer patients (Marteau et al. 2002).

Consumption of unfermented milk such as yogurt shows fewer symptoms in lactose-intolerant individuals than nonfermented dairy products. Yogurt helps in the digestion of lactose because of the lactase produced by lactic acid bacteria (LAB) such as *L. acidophilus* and *Bifidobacterium* (Fernandez et al. 2003). Various species of *Lactobacillus* were reported to have pharmacological importance, such as *L. reuteri* for acute diarrhea (Shornikova et al. 1997a, 1997b) and gastroenteritis (Marteau et al. 2001), *L. salivarius* for eradication of *Helicobacter pylori* (Aiba et al. 1998), and *L. rhamnosus* for enhanced cellular immunity (Tomioka et al. 1992).

Prebiotics and probiotics can influence fermentation in the colon, thereby increasing bacterial mass and the water-binding capacity of stool. Stool frequency may be increased, resulting in reduced levels of polyamines and harmful metabolites such as indoles that cause colon cancer and decreased polyps, as reported by Gorbach et al. (1987). Probiotic microbiota can also prevent urogenital infection caused by pathogens such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Sanders and Klaenhammer 2001). Reduced serum cholesterol was reported by Ouwehand et al. (2002) with the apt use of probiotics.

Lactobacillus plantarum is reported to reduce inflammation in the bowel (Vanderhoof 2000) and pouchitis (Schultz and Sartor 2000). Probiotic-induced reduction in pain, constipation, and bloating was also reported in experimental tests (Nobaek et al. 2000). Vanderhoof (2000) reported reduced recurrence of *Clostridium difficile*-induced diarrhea in the presence of *Saccharomyces boulardii* (Pochapin 2000). The synergic effect of *Clostridium difficile* with *Klebsiella oxytoca* reduces antibiotic-associated diarrhea (Marteau et al. 2001)

The action of the immune system is extremely complex and involves both antibody- and cell-based reactions against pathogens. Cancer, AIDS, leukemia, etc., and normal conditions such as old age or pregnancy, can cause immunodeficiency symptoms or autoimmune diseases such as allergies and inflammatory bowel disease (Perdigon et al. 1995; Dugas et al. 1999). Probiotic microflora could target intestinal epithelial cells, lymphocytes, and blood leukocytes, and lactobacilli adhered to epithelial cells of the intestine activate cytokines, immunoglobulins, interferons, and macrophages.

Cells of the innate immune system provide early protection for the host by reacting rapidly to challenges by infectious agents, resulting in a cascade of events to eliminate the invading pathogenic agent that involves phagocytic cells such as neutrophils, macrophages, and monocytes. Consumption of probiotic products initiates a host response by interacting with intestinal enterocytes, and intestinal cells when stimulated by bacteria produce various immunomodulatory molecules. It was reported that some probiotic organisms can modulate the *in vitro* expression of pro- and antiinflammatory molecules in a strain-dependent manner. Probiotics such as *Lactobacillus sakei* and *L. johnsonii* induce the expression of interleukin and tumor necrosis factor, respectively, by interaction between leukocytes and epithelial cells (Haller et al. 2000).

Many probiotic strains can influence phagocytosis. *Lactobacillus* species such as *L. acidophilus* and *L. casei* can stimulate the immune system by increasing phagocytosis (Perdigon et al. 1988). *L. rhamnosus* in fermented food upregulates the

expression of important phagocytosis receptors and natural killer cell function (Pelto et al. 1998).

Probiotics when regularly consumed may enhance the innate immune and cytotoxic action of natural killer cells in aged persons, and this potential remain above baseline even when probiotic intake is stopped (Gill et al. 2001; Sheih et al. 2001). Some lactobacilli strains regulated the expression of intercellular adhesion molecule-1 on HT-29 cells by induction of interferon (IFN)-γ (Delneste et al. 1998). Exposure of *Shigella flexneri* reduced expression of Caco-2 cells and *L. casei* regulated expression of chemokines to attract macrophages as per the result of mRNA microarrays (Tien et al. 2006). Protein kinase B is an antiapoptotic molecule activated by *L. rhamnosus* that inhibits cytokine-induced apoptosis (Yan and Polk, 2002).

Probiotic strains such as *L. acidophilus* enhance intestinal humoral immunity by stimulation of B cells. Intake of fermented milk with *B. bifidum* followed by vaccination against *Salmonella typhi* causes enhancement of IgA serum concentration (Link-Amster et al. 1994). Rotavirus vaccination in children also enhances the number of IgA-secreting cells in the presence of *L. rhamnosus* (Isolauri et al. 1995) and also helps in acute rotavirus-induced diarrhea (Kaila et al. 1995; Majamaa et al. 1995). Probiotic organisms digest food and the resulting metabolites may have immunoregulatory effects (Del Giudice et al. 2006). *L. paracasei* induced the development of a T-cell subset that participates in the oral tolerance and maintenance of stability in the GI tract (von der Weid et al. 2001). Immunomodulation properties of probiotics are bacteria- and strain specific. Walker (2000) reported enhancement of immunity in children who are HIV positive.

Evidence indicated that intake of a diet with probiotics, prebiotics, antioxidants, and vitamins has a positive impact on the immune system. Probiotic microbes help to restore equilibrium in the intestinal microbiome and in activation of immune cells. This property of probiotic organisms is found to be useful for treatment of immune-related disorders such as allergy (Castellazzi et al. 2013). Oral tolerance suppresses immune response against intestinal microflora and common food allergens. Immunity will develop against foreign agents but will not develop against self-antigens. Such “specific immunological hyporesponsiveness” depends on the GI tract, which is immunologically active (Johansson et al. 2001; Dahan et al. 2007; Leonardi et al. 2007).The effective action of probiotics for allergic respiratory diseases is not fully studied, especially in the infant stage where respiratory allergies occur frequently where intestinal microbes are not established (Vliagoftis et al. 2008). Oral *Lactobacillus* supplementation in babies less than 2 years of age is found to be not effective against asthma or atopic dermatitis (Rose et al. 2010). Experiments and clinical trials give contradictory results about probiotic efficacy in therapeutics because of the heterogeneity of strains and doses. Not many data for treatment of respiratory allergies are available, in contrast to atopic dermatitis (Kuitunen 2013; Burks et al. 2013).

Lymphoid tissue in the GI tract will be activated by probiotic bacteria (Vemuri et al. 2014). Probiotic bacteria such as *Lactobacillus plantarum* ameliorate inflammation and enhance tolerance by increasing IL-10 synthesis and secretion in

macrophages (Pathmakanthan et al. 2004). They can also act on immunological as well as nonimmunological defense mechanism of the gut (Majamaa and Isolauri 1997). Bifidobacteria and lactobacilli can also initiate IgA response against harmful antigens and reduce the production of cytokines responsible for strong IgG and IgE responses and allergies.

Low dietary fiber can cause colon cancer and probiotics can reduce the risk. Results from animal and *in vitro* studies showed reduction in DNA changes and tumor formation. Probiotics can change enzyme activities in the gut and modify regulation of apoptosis. Metabolites produced by certain lactobacilli are reported to have a positive effect on adults with mild hypertension (Liong 2007). Proteolytic cleavage of casein by *L. helveticus* leads to formation of metabolites that can reduce blood pressure. Decline in lipid level and resistance to insulin resistance by probiotic organisms was also reported by Zohreh et al. (2013).

Lactobacilli can bind mutagenic heterocyclic amines, and tumor formation could be delayed by the reduced absorption of mutagenic compounds, as reported by Hayatsu and Hayatsu (1993). Thus, probiotic food intake can reduce cancer risk as per Praveen et al. (2010). *E. coli* and *Clostridium perfringens* produce nitro reductase and beta-glucuronidase enzymes and, by maintaining favorable pH and other conditions, phagocytes tend to destroy tumor cells (Wollowski et al. 2001).

Bifidobacteria inhibit *Clostridium perfringens* and pathogens such as *Salmonella* and *Shigella*, which can cause colon and rectal cancer. They also improve lipid metabolism, reduce nitrogen excretion, and enhance the absorption of essential minerals. Insufficient or preliminary evidence exists for probiotics against prevention of cancer, reduction in cholesterol, enhancement of oral microbiota, and therapy for ischemic heart-associated and autoimmune disorders as per de Vresse and Schrezenmeir (2008).

New approaches for managing the biofilm formation of infectious bacteria were well studied, and probiotics were proposed to manage antibiotic resistance and difficulty in controlling biofilm formation to eradicate infections. Probiotics were effective against disruption of biofilms or restoration of beneficial biofilms.

Intravaginal applications of lactobacilli have been reported as effective against bacterial vaginosis because they maintain a favorable vaginal pH in the acidic range and cause production of antimicrobial metabolites (Anukam et al. 2006; Falagas et al. 2007). Bacterial vaginosis is caused by displacement of lactobacilli to anaerobic pathogens. In human studies, *Gardnerella* species were displaced (Burton et al. 2003) and *Lactobacillus* strains can prevent *Candida albicans* biofilms and also can eliminate yeast in vitro (Kohler and Reid 2006).

When used in skin care, probiotics help to prevent accelerated skin aging at a deeper level to boost cellular function, reduce inflammation, and promote luminosity, firmness, and hydration (Huang and Tang 2015). Orally ingested probiotics affected the skin by mechanisms initiated in the gut by changing systemic immune responses such as stimulating receptors and modulating specific T cells (Krutmann 2012). Probiotics are reported to replenish resident microbes that were drastically reduced in the presence of dysbiosis-associated skin inflammation (Gao et al. 2008). Effects of probiotic microbes in the cosmetic industry are not well studied even

though many reports are available for its effects in gastrointestinal disorders. Oral health improvement by probiotics is also increasing in pace in research because of its safety and efficacy (Zarco et al. 2012).

Probiotics can also be used to enhance the quality of life of HIV-positive people in whom chronic diarrhea often causes death and to prolong the time for onset of type I diabetes (Calcinaro et al. 2005). Antiinflammatory effects of the probiotics could have a major effect in the modulation of immunity (Braat et al. 2006). Any change in diet can alter gut microbes, and antiinflammatory LAB could be related to protection against diabetes, which is yet to be clearly investigated.

Inflammatory bowel disease is related to malfunctioning of the immune system, which causes diarrhea and abdominal cramps. Ulcers may develop in the large and small intestine in Crohn's disease. Probiotics may help to treat this disease by reducing inflammation (Lescheid 2014) and delaying the next disease incident. Ulcerative colitis can be treated with probiotics when a mixture of strains such as *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* is used. Research is ongoing to identify better probiotic treatment for intestinal disorders and antibiotic resistance.

Prevention of food allergy by *Lactobacillus rhamnosus* was reported by Sutas et al. (1996), and the reduction of intestinal disorders and pouchitis by *L. rhamnosus* was studied by Kuisma et al. (2003). Probiotics could reduce the negative impact of antibiotics and *Helicobacter pylori* (Canducci et al. 2000), which is a low-cost solution to prevent *H. pylori* colonization (Pantoflickova et al. 2007).

Probiotic microorganisms should have some essential requirement such as documentation of positive effects on health as well as ideal dosage, and must be administered alive (FAO/WHO 2001). Probiotics involves live microorganisms, and dead microbes cannot provide beneficial effects. So, the probiotic industry must seriously think about storage conditions for maximum shelf life and activity. The purity and number of viable bacteria in every dosage should be strictly maintained.

16.8 Safety of Probiotic Organisms

Safety for human health is the major determinant for probiotics selection. Probiotic strains should also be characterized by their absence of virulent character and low resistance to antibiotics (Snydman 2008). Many characteristics such as adhesion and receptors for adhesion as well as removal of pathogens and microbial viability for beneficial effects need to be examined in detail for complete awareness of probiotic action.

16.8.1 Negative Impacts

Probiotic microbes should be alive, and thus dietary products should be clearly monitored and stored for desirable health benefits and safety for children, pregnant women, and patients with immune disorders. Absence of proper care about storage may increase the chances of people becoming sick or developing sepsis, especially

if the immune system is malfunctioning. In 2011 the Agency for Healthcare Research and Quality assessment studied the safety of probiotics and pointed out the lack of evidence for the negative side effects of probiotics. However, the data on long-term effects and other side effects are limited.

As per the evidence in the technology report by the Agency for Healthcare Research (2011), which studied the safety of probiotics, most of the research indicated an absence of adverse events. Further studies are necessary for impact of different strains, either singly or in groups. More research will provide data about the use of probiotics to treat disease and the long-term impact. Probiotics are not regulated by the FDA as are drugs and are treated as dietary supplements. The method of preparation and mode of action of various probiotic supplements vary among different brands and different preparation. The only negative effects recorded are temporary gas formation and bloating. Very rare cases of side effects such as allergic reactions and infections were also reported, which happens only with patients having severe health problems (Gasser 1994; Saxelin et al. 1996; Husni et al. 1997). Patients with a compromised immune system could be vulnerable to infection but evidence for infection after consumption of probiotics is lacking. Overgrowth of commensal lactobacilli may lead to lactic acidosis in patients with short bowel syndrome (Bongaerts et al. 1997). Strains that exclusively produce L-lactate cannot pose a problem for such patients (Vanderhoof et al. 1998).

Detailed analysis is needed for evaluating the host–microbial interaction, which is important for in-depth understanding of the mechanism of translocation, infection, and survival of microbes in the human system. More evidence is needed for the desirable effects of probiotics in the treatment of diseases. Better understanding of the mode of action of probiotic dietary supplements is highly significant in the present scenario. So, research should be focused for improved probiotic strains that can be used for targeted therapeutic approach or as therapeutic adjuncts together with improvement of probiotics for vaccine delivery. To ensure commonality, it is necessary to identify the optimal strain and mode of action with molecular characterization.

Some rare cases of negative impact of probiotics have been reported. Malkov et al. (2006) reported the death of cancer patients who had consumed a *Bacillus oligonitrophilus*-containing product from unexpected liver failure and pulmonary edema. Carlsson et al. (2009) reported adverse effects of *Lactobacillus* and *Lactococcus* in two dementia patients, and some other patients needed antibiotics after probiotic intervention (Mego et al. 2006; Reid et al. 2001).

The term probiotics is used worldwide without any legally recognized definition or any identifiable standard. So, probiotic-labeled products are not required to meet any quality standards that are unique to probiotics. Products currently available in the market that are labeled as probiotic are unfortunately not well defined or established with human trials.

Generally, probiotic-labeled products should follow the guidelines fixed by a working group of the FAO (FAO/WHO 2002), but surveys have suggested that most of these products do not meet these criteria (Drisko et al. 2005; Temmerman et al. 2003a, b; Yeung et al. 2002). Probiotic products must have labeling about the shelf

life periods, and optimal storage and handling conditions should be recommended. Manufacturing units as well as shop owners must ensure viable storage and handling of these products. The genus, species, and strain designation along with viable cell count must be clearly stated on the label in a responsible way. Consumers of probiotic diet supplements should also be knowledgeable about the quantity needed to be consumed for achieving the desirable health benefit.

16.9 Future of Probiotics

Currently, use of probiotics is considered as a unconventional method for regulating equilibrium in gut microbiota, but the efficiency of the method is controversial. For the success of probiotic dietary supplements, a network and cooperation of food, nutrition, and medical scientists with laypeople who are in need of probiotics for their healthcare as well as awareness of the current scenario and development of new ideas and strains for improvement is highly essential. Even though the current trend indicates a boom of probiotic products, survival of probiotic microbes in these products is debatable. Thus, in the probiotic industry, technology for development of a protective layer around the live microbes to survive unfavorable surroundings is receiving much attention. Microencapsulation is now an evolving technology effectively utilized by industries for maintaining a protective barrier around probiotic microbial cells.

Microencapsulation or entrapment provides a barrier around the cells from its environment that occurs naturally when the growth of the cells occurs where they can produce the needed exo-polysaccharides. Secretions from the cell itself could produce a capsule-like structure to entrap the cells and reduce the action and permeability of external harmful compounds. But, as for some lactic acid bacteria, their own exo-polysaccharides may not be enough to protect and entrap themselves completely (Shah 2002). Another issue needing proper attention is the development of encapsulation technology and its viable delivery to the human body (Vidyalakshmi et al. 2009). The technology of microencapsulation of live bacteria that can produce active compounds to their external surroundings is still not well developed.

Health benefits for a large population could be attained by combining more than one probiotic with commonly used food materials, and this can be utilized for the treatment of allergies, inflammatory diseases, and cancer (Vanderhoof 2001). The action of intestinal microbiota against colon cancer and other GI tract-related cancers needs intense evaluation. Several metabolically active species of bacteria such as *L. acidophilus* can prevent MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine)-induced DNA damage and prevent many other carcinogens that will inactivate tumor suppressor genes or trigger proto-oncogenes (Fearon and Vogelstein 1990). Evaluation of lyophilized cell fractions and peptidoglycans of probiotic bacteria indicated that ample quantities when supplied have a beneficial character even when they are in the nonviable or inactive form (Fearon and Vogelstein 1990). Probiotics can also be used for treatment of extraintestinal diseases such as allergic reactions, cystic fibrosis-associated pneumonia in children, or inflammation in rheumatoid

arthritis, but the identification of ideal organisms or genetic engineering of microorganisms is needed for the development of novel characters (Malin et al. 1996; Guarino 1998; Mack et al. 1999). Careful and intensive research together with documentation of the efficiency of each potential microbe is of high importance before the clinical trials. Strong scientific evidence and cautious attention are essential for addition of clinically proven probiotics to common food supplements for their convenient and effective use by consumers (Vanderhoof 2001).

The key components of assessing safety include identification up to strain level, antibiotic resistance profile, safe history, and scientifically validated toxicity assessments (Borriello et al. 2003). Even though there is enhanced intake of lactobacilli and *Bifidobacterium* in probiotic supplements, there is no reported increase in infections in consumers (Salminen et al. 2002). So, the negative effect or infection risk is unimportant, which may be because of the constant exposure of the human body to microorganisms. However, mutation or change in their cell structure may cause antibiotic resistance, which is a risk to be considered frequently especially if expression of an antibiotic resistance gene is transferable.

Full characterization and molecular profiling of common probiotics using techniques such as sequencing of 16S ribosome and strain identification using RAPD analysis as well as the latest techniques such as matrix-assisted laser ionization and analysis of mass spectra are also of prime importance. These techniques will help to clear doubt at the time of new probiotic strain development. The complete genome is known for many common probiotic bacteria, and improvement in functional genomics will be beneficial for recognizing new possible functions and qualities. Such experimental analysis and data could initiate a better understanding of regulatory mechanisms of bacterial growth, survival, and cell signaling. The design of genetically engineered microbes with therapeutic capacity or organisms that act as vehicle for targeted delivery of cytokines, epitopes, or vaccines is of utmost importance in the present commercial realm. Better understanding of the bioactive principles and method of action and impact on consumers should be analyzed by well-designed controlled experiments.

Competition in the market has strengthened and companies provide well-researched formulations and products, looking for unexplored probiotic strains with benefits surpassing those currently in commercial use. As per the reports, probiotic bacteria produce a variety of compounds with antimicrobial potential, namely, organic acids, hydrogen peroxide, diacetyl, and bacteriocin-like substances (Mishra and Lambert 1996; Ouwehand et al. 1999). Organic acids such as lactic and acetic acids regulate intestinal pH and thereby hinder the microbes (Mishra and Lambert 1996), H_2O_2 inhibits the metabolism of gram-positive and gram-negative bacteria (Hollang et al. 1987; Mishra and Lambert 1996); diacetyl binds arginine-binding proteins and hampers bacterial growth (Jay 1986).

Generally, probiotic microbes generate low molecular weight antimicrobial substances such as reuterin from *Lactobacillus reuteri* and bacteriocins, which are high molecular weight protein complexes that can kill related bacteria (Klaenhammer 1988; Ouwehand 1998). But mutation can stop the bacteriocin production in *L. salivarius*, as reported by Corr et al. (2007). Mack et al. (1999) reported mucin

production regulation in probiotic microbes. Degradation of intestinal mucin and stimulation of mucous secretion was reported by Kohler et al. (2003). This regulation of mucous secretion gives an insight to host bacterial interaction, signaling, and biofilm formation (Holzapfel et al. 1998). Quorum-sensing molecules of microbes contribute knowledge about modulation of growth and virulence expression (Shiner et al. 2005; Cate 2006; Mittal et al. 2006). Identification of prospective microbial signal molecules can help with prevention and treatment of infections as in *L. reuteri*, which produces a molecule with potential for downregulation of exotoxin of *Staphylococcus aureus* (Laughton et al. 2006). Paneth cells of intestinal epithelial cells are reported to be affected by *Bacteroides thetaiotaomicron* (Stappenbeck et al. 2002), and *Lactobacillus* can induce mucin production in the epithelial layer (Mack et al. 2003) and also reduce inflammatory responses (Kim et al. 2006).

Immunomodulation in the human system can be regulated with probiotic–enterocytes coordination. Probiotic microbes mainly act on the intestinal epithelium and the action depends on strain and immunological factors. Appropriate biomarkers for identification of gastrointestinal health and functioning of the immune system are essential, and their lack prevents the improvement of the probiotic industry and the assessment of the health aspect of functional foods such as prebiotics and probiotics. Commonly used markers of gastrointestinal health such as transit time and stool bulk cannot fully express the beneficial aspect of prebiotics and probiotics. Available markers are useful related to the immune system, but cytokine levels and antibody production cannot be substantiated or predicted in connection with probiotics and their effect in the immune system and general health. Variations between individuals form a major hindrance in such studies. This variability depends on a variety of factors such as genome level changes, age, nutritional factors in diet, microbiota, and lifestyle, but probiotics can be important in enhancing the health standards of human beings (Gill and Guarner 2004). The coming era of probiotics (Lee and Salminen 1995) is very auspicious for providing new opportunities for revenue generation and in industry for the production of diverse health supplements.

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17 Stilbenes and Their Derivatives in Traditional Medicine

Nimisha Vijayan, Madathilkovilakath Haridas, and Sabu Abdulhameed

Abstract

Stilbenes are a non-flavanoid class of polyphenols. They are important for their medicinal values. Stilbenes and their derivatives possess a vast number of applications. Many naturally occurring stilbene compounds like resveratrol have multi-functional effects such as antioxidant, anti-inflammatory, anti-cancer and cardio-protectant, and recent studies have focused on their positive effect on age-related infertility problems. They increase vasorelaxation, lower cholesterol, improve Red Blood Cell (RBC) deformability, activate potassium channels, have a positive effect on myocardial remodelling and inhibit platelet aggregation. They are abundantly present in wine. They are important for their antioxidant activity. These compounds are also known as phytoalexins and are found in a variety of plant species where they are produced against various stresses. Stilbenes and their derivatives are effective against various diseases, especially cardiovascular diseases, cancer, etc., and hence they play an important role in modern medicine. This chapter focuses on the presence and role of stilbene and its derivatives in traditional Indian medicines and their biological activities.

Keywords

Stilbenes • Polyphenols • Antioxidant activity • Traditional medicine

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17.1 Introduction

Phenolics are a diverse group of aromatic compounds. Among them stilbenoids are the important compounds displaying a wide variety of biological activities. Stilbenes are low molecular weight phenolics, occur naturally in various plants (Hart 1981). They protect plants from cold, heat, fungal and bacterial infections and the growth of moulds. Grapes and related products are considered as the important sources for stilbene (Goldberg 1995 and Mattavi et al. 1995).

Recently there is an increasing interest in the study of stilbenes because of their medicinal value. Several reports are available relating to the moderate consumption of wine and their beneficial effects on human health. Red wines are reported to contain stilbenes (Siemann and Creasy 1992; Mattavi 1995; Mattavi et al. 1995). Wine seems to be the most potent cardio-protective beverage (Klatsky et al. 1992). There is an inverse relationship between wine consumption and incidence of coronary disease, the so-called 'French Paradox' (Barnard and Linter 1992). It has been suggested that the phenolic substances in the red wine may be the reason for these beneficial effects because of its antioxidant properties. Wine polyphenols have also been reported to have anti-carcinogenic properties delaying the onset of tumour in transgenic mice (Clifford et al. 1996). Stilbenes protect lipoproteins from oxidative damage.

Stilbene derivatives are formed during fermentation. So they are found in fermented preparations along with the production of alcohol. Asavas and arishtas are fermented alcoholic preparations. The process is such that alcohol is self-generated to a particular level with maximum alcoholic content being 15%. Arishta is a sweet, relishing, carminative and emacinating tonic indicated in the treatment of several diseases like piles, loss of appetite, heart diseases, tumour, epistaxis and tuberculosis. Stilbenes and their derivatives are present in these ayurvedic medicines (Barnard et al. 1999). So stilbenes contribute to the medicinal value of these ayurvedic medicines to a great extent.

17.2 Sources of Stilbenes

Stilbenes are synthesized by a wide range of plant species, including *Dipterocarpaceae, Cyperaceae, Gnetaceae, Pinaceae, Leguminosae, Myrtaceae, Moraceae, Fagaceae, Liliaceae and Vitaceae*. They are commonly found in the roots, barks, rhizomes and leaves (Aedin et al. 2000). Major dietary sources of stilbene include grape, peanuts, soy and Itadori tea, which have long been used in Japan and China as a traditional remedy for heart diseases and strokes. Analysis of these sources revealed that peanuts, grape and Itadori tea mainly contain *trans*-resveratrol glucoside. Red wines are primarily a source of *cis*- and *trans*-resveratrol. Peanuts and grape contain less level of stilbenes, while red wine and Itadori tea contain large amount of resveratrol. Stilbenes can also occur in oligomeric and polymeric forms known as viniferins. They are induced by oxidative polymerization of monomer resveratrol through the activity of peroxidase (Jean et al. 2006). Many viniferins

have been reported such as epsilon viniferin and delta viniferin for dimeric resveratrol (Vitrac et al. 2005) and alpha viniferin for trimeric resveratrol. Stilbenoids such as ampelopsin A and hopeaphenol have also been identified (Jeandet et al. 2000; Guebaila et al. 2006).

17.3 Srilbene Derivatives

Many stilbene derivatives (stilbenoids) are naturally present in plants. These agents are derived from micro-organisms, terrestrial plants, marine organisms and animals (Newman and Cragg 2004; Schwartsmann et al. 2003). Diethylstilbestrol is a stilbene derivative, which is mainly used for the treatment of prostate and breast cancer and to prevent threatened abortions (Metzler 1984). *Trans*-astringin and its aglycon are active anti-leukemic agents (Mannila and Talvitie 1992; Mannila et al. 1993). *Trans*-piceatannol is an inhibitor of protein tyrosine kinase (Geahlen and McLaughlin 1989; Oliver et al. 1994). The most widely distributed and biologically active stilbenoids are resveratrol, combretastatins and pterostilbene. These compounds and their analogues possess a wide variety of biological activities.

Resveratrol (3, 4′, 5-trihydroxy *trans*-stilbene) is the major stilbene present in grape wines. It is a naturally occurring phytoalexin and found in more than 70 plant species including grapes, peanuts and pines. It can exist in two isomeric forms and both these forms are found in grape wines (Sun et al. 2003) due to photochemical isomerization of *trans*-form into *cis*-form (Jeandet et al. 1995; Roggero and Garcia 1995). Resveratrol was first described in 1940 as a phenolic component of the medicinal herb hellebore *Veratrum grandiflorum.* Little attention was given to this compound until its role in inhibiting growth of cancer cells in culture was discovered (Baur and Sinclair 2006). The most important source of resveratrol in wine is the berry skin. It is absent or low in the fruit flesh (Creasy and Coffee 1988). It has been assumed that in ripe berries, skin can be elicited to synthesize resveratrol (Jeandet et al. 1995) as a free compound or as a glucoside. These compounds are supposed to be extracted during alcoholic fermentation and degraded during malolactic fermentation by enzymatic activity of malolactic bacteria (Pezet and Cuenat 1996). *Cis*-resveratrol is a byproduct of fermentation and is rarely found in grapes (Jeandet et al. 1995 and Mattavi et al. 1995). It is getting worldwide attention from the scientific community because of its variety of biological activities such as antioxidant, radio-protective, phytoestrogen, anti-bacterial and anti-fungal (Aggarwal and Shishodia 2006; Kumar 2007; Rocha et al. 2008; Alonso et al. 2009). Its therapeutic potential includes the chemo-prevention of cancer, inflammation, ageing, obesity, cardiovascular diseases and neurodegeneration (Kovacic and Somanathan 2010).

Pterostilbene (3, 5-dimethoxy-4′-hydroxy *trans*-stilbene) is chemically related to resveratrol. It is mainly found in blueberries and grapes that exhibit anti-cancer, anti-hypercholesterolemia and anti-hypertriglyceridemia properties. It is more stable in vivo than resveratrol. It was also suggested that this compound has

anti-diabetic and anti-fungal properties. But studies on this topic are scarce. The inhibitory effect of pterostilbene on the induction of NO synthase (NOS) and cyclo-oxygenase 2 (COX 2) in murine RAW 264.7 cells activated with lipopolysaccharides has been studied (Pan et al. 2008).

17.4 Stilbenes in Traditional Medicine

Ayurveda is a traditional medicinal system. Ayurveda comprises various types of medicines including the fermented forms, namely arishtas (fermented decoctions) and asavas (fermented infusions). These are regarded as the valuable therapeutics in traditional medicine because of their efficacy (Shekar and Mariappan 2008). Although modern medicine is now well developed, the interest in traditional medicine based on herbal medicinal principles is still there. Drakshasava is a well-known Indian herbal preparation. Its main ingredient is *Vitis vinifera* L. This ayurvedic medicine is prescribed as a cardiotonic and is also given for other disorders. High Performance Liquid Chromatography analysis of this preparation revealed the presence of resveratrol and pterostilbene (Barnard et al. 1999). The presence of stilbenes may be the reason for its cardiotonic and health-promoting properties.

The use of orchids in traditional medicine is widely described throughout history. Traditional Chinese medicine widely utilizes orchids in medicines. Stilbeneoids are the major component in this plant (Majumder and Ghosal 1993). Stilbenoids isolated from this plant are reported to possess various biological properties such as anti-allergic, anti-inflammatory, anti-microbial, anti-platelet aggregations, cytotoxicity, spasmolytic, vasoreluctant effects and others (Gutiérrez 2010; Kovács et al. 2008; Xiao et al. 2008). Ashtavarga, a group of eight medicinal plants, is a part of ayurvedic formulations like chyawanprash. Among them four plants belong to the *Orchidaceae* family (Kalaiarasan et al. 2012). In a chemical investigation of medicinal plants, the ethanol extract of *Arundina graminifolia* was found to possess the function of regulating immunity. The ethanol extract was further fractionated into light petroleum, ethyl acetate, acetone and methanol parts. Further chemical investigation of the ethyl acetate part resulted in the isolation of a novel stilbenoid designated as arundinan. Its structure was established as 2-(p-hydroxybenzyl)-3-hydroxy-5-methoxybibenzyl from the following spectral and chemical evidence.

The resin from the tree *Vateria indica Linn* is well known in traditional systems of medicine (Ayurveda and Siddha) as Sarja rasa in Ayurveda and vellai kungiliyam in siddha. The research has shown that this resin possesses resveratrols having anti-tumour properties (Mishima et al. 2003). It is credited with tonic, carminative and expectorant properties and is used for the treatment of respiratory disorders like throat troubles, chronic bronchitis, boils, piles, diarrhoea, rheumatism, etc. Kungiliya parpam is a siddha preparation made from this resin that is very much effective in the treatment of urinary tract disorders. Various other phytocompounds present in the very same resin have been proved to show various healing properties.

Pterostilbene is a methoxylated analogue of resveratrol. It is gaining importance as a therapeutic drug because of its higher lyophilicity, bioavailability and biological activity than resveratrol (Jhankar and Saroj 2013). The presence of pterostilbene is reported in various plant sources like *Pterocarpus marsupium* (Sehadri 1984), *P. santalinus* (Maurya et al. 1984) and leaves of *V. vinifera* (Langcake et al. 1979). As per Ayurveda, *P. marsupium* is the most versatile medicinal plant with a wide spectrum of biological activities (Ibrahim and Mishra 2013). Barks of *P. marsupium* are used as an astringent, anti-diarrheal and antacid (Tiwari et al. 2004) and are also effective in β-cell regeneration (Chakravarthy et al. 1980). Its wood water has been reported to have hypoglycemic activity in diabetic patients (Rajasekharan and Tuli 1976). Its heart wood has been found to decrease plasma glucose level in newly diagnosed diabetic patients. In the ayurvedic medicine drakshasava, pterostilbene is the main phenolic compound (6.8 mg/l) (Paul et al. 1999). *P. marsupium* extract containing pterostilbene was found to selectively inhibit COX 2, an enzyme in the inflammatory cascade. Pterostilbene shows potent anti-inflammatory activity, mediated through the inhibition of pro-inflammatory enzymes and cytokines. Pterostilbene has also been reported to have hypolipidemic properties comparable to clinically used fibrate lipid-lowering drugs (Rimando et al. 2005).

Knotweed rhizomes and young sprouts are used in traditional Asian medicine. They contain large amount of stilbenes. Resveratrol, piceid and other stilbenes have been reported in rhizomes of Japanese knotweed (Vastano et al. 2000; Xiao et al. 2000; Yang et al. 2001; Chu et al. 2005). Rhaponticin, a major compound in the stilbene glucoside family, exists widely in the medicinal plant genus *Rheum L.*, such as *Rheum officinale* Baill and *Rheum undulatum* Linn (Zhang et al. 2007). It is recommended by health professionals in Asian countries for the treatment and prevention of allergies (Matsuda et al. 2001; Kim et al. 2000).

Smilax aspera is an evergreen, creeping, tough shrub that belongs to the *Liliaceae* family. It has been used in herbal medicine for muscle relaxation, skin ailments, rheumatic pain, depurative, diuretic, diaphoretic, antigout, dropsy, stimulant and also for its tonic properties (Longo and Vasapollo 2006). It has also been used traditionally for the treatment of syphilis, diabetes, rheumatism and as an antioxidant (Demo et al. 1998). It is well known in Chinese traditional medicine. Chemical analysis of this plant resulted in the isolation of stilbene *trans*-resveratrol.

17.5 Biological Activities of Stilbenes

Stilbenes represent a small class of plant secondary metabolites. These phytochemicals have an enormous diversity of effects on biological and cellular processes applicable to human health. In addition to their participation in plant defence mechanisms, several stilbenes display several important pharmacological properties. Pharmacological activities of stilbenes include cancer prevention, cholesterol-lowering effect, enhanced insulin sensitivity and increased life span (Rimando and Suh 2008) and in this respect the best studied one is resveratrol. Hundreds of studies have shown that resveratrol can prevent or slow the progression of a wide variety of

diseases including cancer and cardiovascular diseases, as well as extend the life span of various organisms (Baur and Sinclair 2006).

E and Z forms of stilbene elicit different pharmacological activities. It has been found that the Z form is more potent compared to the E form (Roupe et al. 2006). *Trans*-stilbene compounds are more potent than *cis*-stilbenes in inhibiting cyclooxygenase 1 (Teguo et al. 2001).

17.6 Anti-inflammatory Action of Stilbenes

The mechanism of anti-inflammatory effects of stilbenes includes inhibition of synthesis and release of pro-inflammatory mediators, modification of eicosanoid synthesis, inhibition of activated immune cells, and inhibition of inducible nitric oxide synthase, cyclooxygenase 1 or cyclooxygenase 2. Resveratrol and related stilbenes reduce inflammation by inhibiting prostaglandin production, cyclooxygenase 2 activity, NF κB activity or activator protein 1 (AP1) (Kundu et al. 2006; Shankar et al. 2007; Hougee et al. 2005). Resveratrol also inhibits the inflammatory process by regulating upstream protein kinases such as IκB kinase (Kundu et al. 2006; Subbaramaiah and Dannenberg 2001), c-Jun N-terminal kinase (JNK) (Subbaramaiah et al. 1998), mitogen-activated protein kinase (MAPK) (Kundu et al. 2004; Yu et al. 2001), protein kinase C (PKC) (Yu et al. 2001), Phosphatidylinositol-3- kinase (PI3K) PI3K-AKT (Pozo-Guisado et al. 2004; Das et al. 2006) and p38 (Das et al. 2006). Consequently, resveratrol and related stilbenes down regulate several key inflammatory markers such as COX-2, 5-LOX and iNOS, as well as inflammatory mediators such as TNFa,VEGF, IL-1, IL-6 and IL-8 (Aggarwal et al. 2006; Das et al. 2006; Shankar et al. 2007). Pterostilbene, a naturally occurring stilbene in blueberries, was shown to decrease expression of inflammatory genes, such as inducible nitric oxide synthase (iNOS) in the colonic crypts and Aberrant crypt foci (ACF) in rats, suggesting that the anti-inflammatory properties of stilbenes may be critical in the prevention of colon tumourogenesis (Suh et al. 2007). Resveratrol is cytotoxic to macrophages, impairs their ability to proliferate (Billack et al. 2008) and reduces the expression of inflammatory mediators (Kim et al. 2011). The benzene ring appears to play an essential role in the anti-inflammatory properties of resveratrol. Resveratrol has been shown to reduce edema.

17.7 Antioxidant Properties of Resveratrol

Wine polyphenols are potent dietary antioxidants. The antioxidant property of resveratrol is an important aspect of the physiological properties of resveratrol, particularly in relation to protection from oxidative injuries. The antioxidant property of resveratrol constitutes the major part of preventing tumour induction. The antioxidant effect of resveratrol was manifested in blood mononuclear cells by a significant reduction in malondialdehyde content, an indication of oxidative injury (Losa 2003). The effect of resveratrol on oxidative/nitrative stress by peroxynitrite, which

is a strong physiological oxidant and inflammatory mediator, was determined in human blood platelets (Olas et al. 2007). The findings on the antioxidant properties of resveratrol suggest its possible application in therapy of human diseases. Resveratrol is a useful drug for the protection of liver cells from oxidative stress-induced damage (Rubiolo et al. 2008). Resveratrol can quench reactive oxygen species and can prevent pro-oxidative damage. It has been revealed that the protection of cardiomycetes from injury by resveratrol occurs partly by suppression of superoxide levels via antioxidant action. Authors proposed that the antioxidant and anti-apoptotic effects, together with the anti-inflammatory actions, are responsible for the cardio-protective effects of resveratrol (Li et al. 2006). Resveratrol also possesses free radical scavenging capacity (Kovacic and Somanathan 2010).

17.8 Anti-ageing Properties of Resveratrol

The anti-ageing properties of resveratrol have gained much attention. Anti-ageing properties of resveratrol include cardiovascular benefits via increased NO production, down regulation of vasoactive peptides, lowered level of oxidized low-density lipo protein, cyclooxygenase inhibition, effects on neural tissues, phytohormonal actions, anti-cancer properties via modulation of signal transduction, anti-microbial effects, sirtuin activation, possible benefits in Alzheimer's disease and prevention of photo-ageing (Baxter 2008).

17.9 Resveratrol in Cell Signalling

Cell signalling is known to be involved in various aspects of biological functions including normal processes, therapeutic drug action and toxicology. Resveratrol impacts many components of the intracellular cell-signalling pathways including regulation of apoptosis and cell survival, tumour switches involved with kinases, and transcription factors and their regulators. Evidence indicates that stilbene derivatives target Protein tyrosine phosphatase 1B (PTP1B) to inhibit platelet-derived growth factor receptor nitrogenic signalling (Venkatesan et al. 2008). Resveratrol mediates its effects through modulation of many different pathways by binding to numerous cell-signalling molecules (Harikumar and Aggarwal 2008). It activates various transcription factors.

17.10 Other Biological Effects of Resveratrol

Resveratrol reduces nuclear DNA fragmentation (Burkhardt et al. 2001). Reports indicate that resveratrol can act as an anti-mutagenic/anti-carcinogenic agent by preventing DNA damage. Resveratrol blocks the expression of genes related to the NFκB family (Leiro et al. 2005). Through its phycoestrogenic properties resveratrol regulates hormone-dependent genes in breast cells and provides a protective effect

against several types of cancer including breast cancer (Le Corre et al. 2004). Resveratrol by inducing the expression of several genes such as SIRT 1, SIRT2, SIRT4, FoxO1 and FoxO3a prevents ageing-related decline in the cardiovascular system including cholesterol level and inflammatory response.

The demonstrated pharmacological properties of naturally occuring stilbenes have led to the synthesis of analogues to improve activity. Structural analogues of resveratrol possess some of the effects of resveratrol and potentially even more benefits. Pinosylvin is chemically related to resveratrol. Pinosylvin has been studied because of its anti-cancer, anti-fungal and anti-oxidative properties (Roupe et al. 2006). Removal of the hydroxyl group from resveratrol results in pinosylvin. By this change pinosylvin is more lipophilic than resveratrol (Perecko et al. 2008). Piceatannol is a naturally occuring stilbene present in sugarcane, berries, peanuts, red wines and the skin of grapes.

17.11 Conclusions

Stilbene deserves a lot of interest. It is very important in the field of medicine because of its activity against various diseases. It is widely present in grape wines. So moderate consumption of wine can decrease the incidence of cardiovascular diseases and cancer to a great extent. Stilbenes are gaining importance in clinical trials where they are used as therapeutic drugs. Numerous studies are being conducted on the topic of stilbenes nowadays. Studies are also being carried out to discover new stilbenes and their analogues. New discoveries about stilbenes are giving new openings for advanced research work. With the knowledge of their role in traditional medicines, researchers are trying to expose their various activities so that they will be more useful in traditional medicinal system. Although there are new discoveries in modern medicine, common people always depend on traditional medicine. So study of these stilbenes will be helpful to make advances in the field of traditional medicine.

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18 Novel Targets and Advancements in Drug Discovery: The Case of HIV-AIDS

Nihar Ranjan, Umesh Kumar, and Sunil K. Deshmukh

Abstract

Since the very first report of acquired immune deficiency syndrome (AIDS) in the early 1980s in the United States, a number of advancements have taken place both in the structural and functional aspects of the human immunodeficiency virus (HIV) life cycle as well as anti-HIV drug design. While new drugs have come to the market and combination therapies have increased life expectancy, resistance and viral mutations have mandated introduction of new drugs in the market. Apart from two main classes of HIV inhibitors (reverse transcriptase and protease), new inhibitors targeting fusion and integration processes have provided additional sites for therapy development. More recently inhibitors of maturation and capsid assembly as well as viral replication have been studied to provide novel anti-HIV drugs. In this chapter, we briefly discuss the HIV life cycle and describe a few of the recent endeavors made to develop new anti-HIV agents. For brevity, we provide a limited number of examples of discoveries made in the main target sites of current HIV drug design.

Keywords

Novel targets • HIV • Reverse transcriptase • Antiviral drug

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18.1 Introduction

18.1.1 Current State of HIV-AIDS

Despite the decline in the number of new HIV-infected population globally in recent years, HIV-AIDS remains one of the leading causes of disease-related death. According to the latest UNAIDS data (UNAIDS 2016), 36.7 million people are currently living with HIV-AIDS worldwide. In 2015, 1.1 million people died from AIDS related illness and 2.1 million new HIV infections have been reported. Several awareness efforts and regulatory practices have led to many positive outcomes that augur well for significant control over HIV spread in the coming decade. However, success of all concerted AIDS control efforts is centrally linked to therapeutic control of the HIV virus using multifaceted drug combinations. These drug combinations have consequentially helped to increase the life span of HIV-infected population, arresting the viral proliferation for several years and curbing the viral growth to nondetectable levels. Introduction of new and more potent drugs by the Food and Drug Administration (FDA) in recent years that bind to new HIV targets has greatly boosted the available therapeutic arsenal, albeit these inclusions have been simultaneously countered with viral mutations leading to ineffectiveness of few clinically used drugs. Therefore, drug design efforts that target new sites to stop viral function and growth are needed to augment currently used multidrug combination therapy.

18.1.2 Target Identification and Drug Discovery Process

Drug discovery is an arduous process that takes several years for a new drug to come to the market (Fig. 18.1). There are more than a thousand (1222) new chemical entities (NCEs) that have been approved by the FDA for use as pharmaceutical therapies since 1950. Among them, only 8% NCEs developed at the benches are eventually approved and enter the market after surviving a process of drug development that takes an average of 13.5 years (Paul et al. 2010).

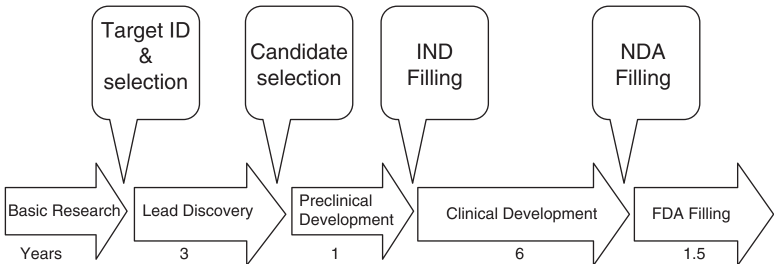

Fig. 18.1 Drug discovery process from target identification and validation through to filing of a compound and the approximate timescale for these processes. *FDA* Food and Drug Administration, *IND* investigational new drug, *NDA* new drug application (Reproduced from Hughes et al. 2010 with permission by Copyright 2010, John Wiley & Sons, Inc.)

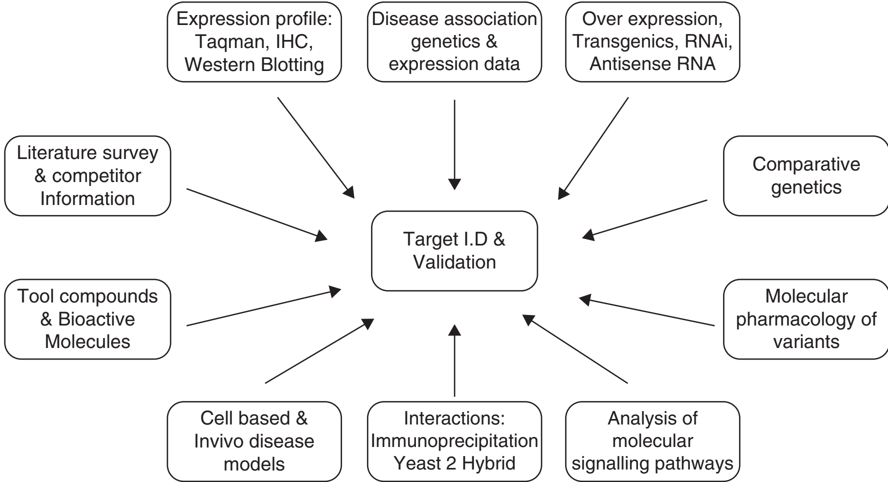

Fig. 18.2 Multifunctional process of target identification and validation. IHC: immunohistochemistry (Reproduced from Hughes et al. 2010 with permission by Copyright 2010, John Wiley & Sons, Inc.)

Despite the advancement in the techniques involved in genetics, proteomics, and high-throughput screening, there hasn't been any increase in the rate of NCEs gaining marketing approval. Therefore, there are increasing concerns about the viability of the current model of drug development (Munos 2009). The pharmaceutical industry is looking for new biological targets and innovative ways of generating NCEs and novel pharmacotherapy. One of the key steps in developing a new drug is target identification and its validation (Fig. 18.2). Target is a broad term for a range of biological entities which includes proteins, genes, and RNA. A good target should be efficacious, safe, meet clinical and commercial needs, and, above all, be 'druggable'. A 'druggable' target is something that is accessible to the potential drug molecule, which can be a small molecule or larger biomolecule, and upon binding elicit a measurable biological response both in vitro and in vivo. It is now well established that certain target classes are more suitable for small molecule drug discovery like G-protein-coupled receptors (GPCRs), whereas antibodies, which are good at blocking protein/protein interactions, are good for large molecule drug discovery (Hughes et al. 2010). Identification approaches also include examining mRNA/protein levels to determine whether they are upregulated or down-regulated with disease exacerbation or progression.

Once a target has been identified, drug discovery in recent decades has relied on extensive screening of chemical libraries (Table 18.1) to detect compounds with activity against the target. Commercial libraries including either combinatorial or natural products can now exceed one million different compounds. Furthermore, it has been estimated that there might be as many as 10^{40}–10^{100} possible small compounds that are potential drugs (Macarron 2006). Until now, major enzymes that have been targeted for drug discoveries included kinases, proteases, phosphatases, oxidoreductases, and transferases, whereas cellular targets include GPCRs, nuclear hormone receptors, and some ion channels. Further, it is interesting to note that

Table 18.1 Screening strategies for drug discovery (Reproduced from Hughes et al. 2010 with permission by Copyright 2010, John Wiley & Sons, Inc.)

Screening strategies	Description	Remarks
High throughput	Large numbers of samples or compounds can be analyzed in a single assay. Generally designed to run in plates of 384 wells and above	Large compound collections are often run by big pharmaceutical companies but small compound banks can also be run in either pharmaceutical companies or academia, which can help reduce costs. Companies are also now trying to provide coverage across a wide chemical space using computer-assisted analysis to reduce the numbers of compounds screened.
Focused screening	Compounds previously identified as hitting specific classes of targets (e.g. kinases) and compounds with similar structures	Provide a cheaper avenue to find a hit molecule but completely novel structures may not be discovered, and there may be difficulties in obtaining a patent position in a well-covered IP area
Fragment screening	Soak small compounds into crystals to obtain compounds with low millimolar activity which can then be used as building blocks for larger molecules	Can join selected fragments together to fit into the chemical space to increase potency. Requires a crystal structure to be available
Structure-aided drug design	Use of crystal structures to help design molecules	Often used as an adjunct to other screening strategies within big pharma companies. Here a compound is docked into the crystal structure and this is used to help predict where modifications could be added to provide increased potency or selectivity
Virtual screening	Docking models: interrogation of a virtual compound library with the X-ray structure of the protein or, if have a known ligand, as a base to develop further compounds	Can provide the starting structures for a focused screen without the need to use expensive large library screens. Can also be used to look for novel patent space around existing compound structures
Physiological screening	A tissue-based approach for determination of the effects of a drug at the tissue rather than the cellular or subcellular level, for example, muscle contractility	Bespoke screens of lower throughput. Aim to more closely mimic the complexity of tissue rather than just looking at single readouts, which helps to screen smaller number of compounds to give a more disease-relevant readout
NMR screening	Screen small compounds (fragments) by soaking into protein targets of known crystal or NMR structure to look for hits with low mM activity which can then be used as building blocks for larger molecules	NMR is used as a structure-determining tool
Integrated screen	Combined phenotypic screening of a directed small molecule library with competitive activity-based protein profiling to map and functionally characterize the targets of screening hits	Accelerate the identification and pharmacologic validation of new therapeutic targets

there are 20,000 genes and around 100,000 proteins in humans; among these, only 324 targets resulted in approved drugs (Mayr and Bojanic 2009). Of these, only 266 are human genome-derived proteins, the rest are microbial targets. Despite the potential profits and the extraordinary capacity of drug discovery technology, there is a scarcity of new drugs in the development pipeline, particularly for those medications that are likely to be highly profitable because they are used long term and by a large proportion of the population. Validation techniques range from in vitro tools through the use of whole animal models, to modulation of desired target in patients. While each approach is valid in its own right, confidence in the observed outcome is significantly increased by a multivalidation approach (Fig. 18.2).

Development of antiviral drugs is a very lengthy process as it involves many stages such as target identification and screening (Table 18.1), lead generation and optimization, preclinical and clinical studies, and final registration of the drug (Fig. 18.1). Increasing knowledge about viruses, mechanism of their infections, and the rapid evolvement of novel antiviral strategies and techniques will speed up the development of novel antiviral drugs. Here we emphasize one such advancement toward the development of HIV-AIDS therapeutics.

18.2 HIV Life Cycle in a Nutshell

18.2.1 Functions and Targets

In the last three decades, a number of efforts have led to better understanding of the virus life cycle and design of therapeutics for its control. The primary targets of the HIV virus are CD^+ T cells, monocytes, macrophages, and dendritic cells (Ramana et al. 2014). The decrease in the number of CD^+ T cells leads to failure of the immune response system, which eventually leads to fatality. From the point of entry into the human T cells, a number of processes are required for virus maturation. Each of the important stages of the viral maturation serves as a target for potential development of AIDS therapeutics. At present, there are about 30 FDA-approved (Table 18.2, Fig. 18.3) drugs of five different classes that are part of highly active antiretroviral therapy (HAART) (Zhan et al. 2016).

After the HIV infection, which is contracted via three major routes – sexual transmission, blood transfusion, and by passage from mother to child – seven key steps determine the life cycle of the virus, which are as follows: (1) binding, (2) fusion, (3) reverse transcription, (4) integration, (5) replication, (6) assembly, and (7) budding.

The HIV virus begins its life cycle by binding to CD4 receptor cells of the host. This binding is initiated by glycoprotein gp120 on the outer envelope of HIV and is promoted by chemokine receptors CCR5/CXCR4. The binding of gp120 to CD4 receptor leads to conformational changes in gp120, which promotes gp41-mediated fusion of the virus particle to the host cell (O'Hara and Olson 2002). The fusion is followed by release of HIV capsid which contains HIV RNA and three key enzymes, reverse transcriptase, integrase, and protease, that are essential to virus life and its proliferation. At this stage, within CD4 membrane, HIV capsid releases HIV RNA, which undergoes reverse transcription to make HIV DNA. The newly formed HIV

Table 18.2 Chronological discovery of anti-HIV drugs by class[a]

Class	Generic name	Role/function	Side effects	FDA approval date
Entry inhibitor	Maraviroc	Allosteric modulator of CCR5	Liver problems, skin reactions	August 6, 2007
Fusion inhibitor	Enfuvirtide	gp41binding	Insomnia, depression, anorexia	March 13, 2003
NRTI[b]	Zidovudine	NTP[c] mimic	Anemia, myopathy, neutropenia	March 19, 1987
NRTI[b]	Didanosine	NTP[c] mimic	Diarrhea, nausea, vomiting, abdominal pain	October 9, 1991
NRTI[b]	Stavudine	NTP[c] mimic	Headache, dizziness, abnormal thinking	June 24, 1994
NRTI[b]	Lamivudine	NTP[c] mimic	Headache, nausea, fatigue	November 17, 1995
NRTI[b]	Abacavir	NTP[c] mimic	Nausea, headache, fatigue, vomiting	December 17, 1998
NRTI[b]	Tenofovirdisoproxilfumerate	NTP[c] mimic	Diarrhea, nausea, vomiting,	October 26, 2001
NRTI[b]	Emtricitabine	NTP mimic	Diarrhea, nausea,	July 2, 2003
NNRTI[d]	Nevirapine	Binding to RT and blocking DNA- and RNA-dependent polymerase activity	Moderate rash	June 21, 1996
NNRTI[d]	Delavirdine	Blocking RT catalytic site	Rash, fatigue, nausea	April 4, 1997
NNRTI[d]	Efavirenz	Inhibiting viral DNA polymerase activity	Insomnia, confusion, memory loss	September 17, 1998
NNRTI[d]	Etravirine	Binding to RT and blocking DNA and RNA dependent polymerase activity	Skin rash and allergic reactions	January 18, 2008
NNRTI[c]	Rilpivirine	Binding to RT and blocking DNA and RNA dependent polymerase activity	Depression, headache, insomnia	May 20, 2011
Integrase inhibitor	Dolutegravir	Integrase-DNA complex binding	Insomnia and headache	August 13, 2003
Integrase inhibitor	Raltegravir	Integrase-DNA binding inhibition	Headache. Stomach pain,	October 12, 2007
Integrase inhibitor	Elvitegravir	Integrase-DNA binding inhibition	Diarrhea	September 24, 2014
Protease inhibitors	Saquinavir	Enzyme active site binding	Diarrhea, nausea	December 6, 1995

Protease inhibitors	Ritonavir	Enzyme active site binding	Diarrhea, nausea, vomiting	March 1, 1996
Protease inhibitors	Indinavir	Enzyme active site binding	Diarrhea, nausea, vomiting	March 13, 1996
Protease inhibitors	Nelfinavir	Enzyme binding	Flatulence, diarrhea, rash	March 14, 1997
Protease inhibitors	Atazanavir	Enzyme binding	Headache, nausea, rash, vomiting	June 20, 2003
Protease inhibitors	Fosamprenavir	Enzyme active site binding	Diarrhea, dizziness	October 20, 2003
Protease inhibitors	Tipranavir	Enzyme active site binding	Intracranial hemorrhage, hyperglycemia	June 22, 2005
Protease inhibitors	Darunavir	Enzyme active site binding	Diarrhea, headache, abdominal pain	June 23, 2006
Pharmacokinetic enhancers	Cobicistat	Inhibit functions of liver enzymes	Nausea, diarrhea, fatigue	September 24, 2014

[a]Partly adapted from aidsinfo.nih.gov factsheet (Retrieved May 30, 2016)
[b]Nucleotide reverse transcriptase (RT) inhibitors
[c]Nucleotide triphosphate
[d]Nonnucleotide reverse transcriptase (RT) inhibitors

Fig. 18.3 Chemical structures of FDA-approved HIV drugs

Indinavir

Atazanavir

Nelfinavir

Fosamprenavir

Tipranavir

Darunavir

Enfuvirtide

Fig. 18.3 (continued)

DNA then passes through the CD4 nuclear membrane where it undergoes integration with the host cell DNA facilitated by the enzyme integrase. Using this machinery, the proviral DNA hides itself within the host cell DNA and leads to the higher-latency period of the virus. Within the host nucleus, the proviral DNA undergoes routine steps of transcription and translation to make new HIV viral proteins. The final stage of the virus life cycle is assembly and maturation of the new HIV RNA where the RNA and proteins exit out of the host cell membrane during which they remain noninfectious. The long chains of polypeptides then get broken down into smaller fragments of proteins by proteases and finally get processed as infectious viral particles at the maturation stage.

All seven important steps of the HIV virus life cycle have been envisaged as potential targets for developing therapy. A number of advances toward inhibition of these processes have led to the development of HAART and other agents of chemotherapy which have curtailed virus proliferation and enhanced life expectancy. Here we list therapeutic advances made toward HIV therapy and highlight some of the current drug discovery approaches. Traditionally, discovery approaches have screened a large number of drug-like molecules, often using high-throughput assays to identify lead compounds and subsequently perform structure-based modifications to enhance their efficacy. But several new screening approaches have identified novel structural motifs for AIDS therapy. For example, fragment-based screening has led to the identification of molecules containing indole core as protease inhibitors. This work led to subsequent discovery of brominated benzoic and naphthoic acid derivatives as new protease inhibitors. These discoveries demonstrate that fragment-based design of new molecules can provide complementary approaches to established drug discovery screening essays. A number of other approaches such as privileged fragment-based reconstruction approach, dynamic ligation scattering, rapid diversity-oriented and in situ screening, and hierarchical virtual screening are in practice to supplement traditional methods of drug discovery (Ghosh et al. 2016).

18.3 Advances in the HIV Drug Development

Despite the progress in antiretroviral therapy, the significantly high mutation rate of the HIV virus has mandated newer alternatives to currently used drugs not just to have a different line of therapeutics but also to discover more efficacious drugs. Here we outline recent advances made toward inhibition of seven key steps required for virus life cycle.

18.3.1 Entry Inhibitors

The enveloped HIV virus undergoes cell surface fusion with the CD4 cells as the first step of its entry to the host cell. The virion is surrounded by a cell-surface glycoprotein gp120 and a transmembrane glycoprotein gp41, both of which can act as

Fig. 18.4 Chemical structures of a few recent small molecule inhibitors of HIV entry

targets of viral entry inhibition and are collectively known as entry inhibitors. The attachment of virus is believed to be facilitated by electrostatic interactions (nonspecific) between the positively charged domains of gp120 and negatively charged domains of proteoglycans of the host cell surface. However, certain specific interactions such as those between gp120 and integrin α4β7 are also known to mediate this process. Once attachment to the CD4 receptor is achieved, gp120 undergoes conformational changes. At this stage, chemokine receptors CCR5/CXCR4 help in gp120 binding, which is followed by further conformational changes in gp120.These events lead to conformational changes in the transmembrane glycoprotein gp41, which anchors fusion of viral and host cell membrane. Therefore, all these three stages serve as targets of therapeutic intervention at the entry stage.

Some of the earliest small molecule gp120-CD4 interaction inhibitors included BMS 378806 and BMS 448043, which were soon replaced by BMS 626529 and its prodrug BMS 663068. A line of small molecule inhibitors targeting coreceptor CCR5 were developed such as Aplaviroc, Vicriviroc, Cenicriviroc and Maraviroc. Of these, Maraviroc was granted FDA approval in 2007. Another set of small molecules aimed at blocking gp41-mediated membrane fusion were developed, of which Enfuvirtide (initially known as T-20, a 36-amino acid synthetic peptide based upon the heptad repeat sequence 2 of gp41) was granted FDA approval in 2003, and it remains the only FDA-approved fusion inhibitor to date. Several new molecules (Fig. 18.4), some of which are in advanced phases of clinical trials, have since been discovered and tested for their efficacy as entry inhibitors. These include Fostemsavir (Fig. 18.4(1)); NBD-556-based minimally toxic CD4 mimics (Fig. 18.4(2)) (Mizuguchi et al. 2016); nonnatural amino acids, pyrroloaryls, and pyrrolohetroaryls (Fig. 18.4(3)) (Patel and Park 2015); substituted tetrahydroisoquinolines (Fig. 18.4(4)) (Swidorski et al. 2016); small molecule sulfotyrosine mimics (Fig. 18.4(5)) (Dogo-Isonagie et al. 2016); and 1, 4 disubstituted piperazine derivatives (Fig. 18.4(6)) (Dong et al. 2012).

18.3.2 Reverse Transcriptase Inhibitors

Once the virus enters the cytoplasm of the host cell, the reverse transcriptase (RT) of the virus is activated. In addition to other assisting cellular factors, RT performs reverse transcription reactions using DNA polymerase and RNase H to make new copies of double-stranded DNA. The reverse transcription process begins with the binding of the tRNA primer to the 5′ end of viral RNA known as the primer binding site. The initiated minus-strand DNA synthesis is transferred to the 3′ end of either of the two copies of viral RNA, which is released from the capsid in the host cell cytoplasm. The minus-strand DNA synthesis is continued along with RNase H degradation. A polypurine tract in the RNA resists RNA degradation and ultimately serves as the template for plus-strand DNA synthesis. The plus-strand DNA synthesis also generates 18 nucleotides for the tRNA primer.

Inhibiting HIV RT activity has been one of the most sought after targets in HIV therapy. Of currently approved FDA drugs for HIV therapy, nearly half of them belong to the class of RT inhibitors. In fact, the very first anti-HIV drug, azidothymidine (AZT), belonged to the class of RT inhibitors. The RT inhibitors have been classified into two main classes (Table 18.2): nucleoside reverse transcriptase inhibitors (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs). NRTIs act as mimics of natural substrates of DNA synthesis. The sugar moiety of the nucleoside analogs lacks the 3′-OH functionality which is catalytically essential. Once these nucleoside mimics are incorporated inside the cells, they are converted as their triphosphate analogs by cellular enzymes (kinases) to be active. The triphosphate analogs thus produced are used by RT for making DNA, which, in turn, acts as chain terminators blocking the DNA synthesis. On the other hand, NNRTIs act by a non-competing mechanism where they induce allosteric conformational changes to the catalytic activity of the enzyme.

The success of AZT led to research focus on the development of NRTIs, which resulted in a few more FDA drugs such as Stavudine and Lamivudine by the mid-1990s which acted by mimicking natural nucleoside substrate thymidine triphosphate. But soon viral resistance necessitated alternatives, which resulted in the development of NNRTIs with Nevirapine being the first FDA-approved drug in this class. Several new molecules have been reported with excellent activity and promise (Fig. 18.5). From a large sample size (over 20,000 compounds) and using high-throughput assay, novel phenylaminopyridine derivatives (Fig. 18.5(7)) were identified as NNRTIs with low nanomolar inhibitory concentrations against RT activity (Kim et al. 2012). Using a molecular hybridization strategy, 6-substituted diarylpyridine derivatives (Fig. 18.5(8)) have been designed which show more potent activities than Nevirapine (Yang et al. 2016). RT-related RNase H activity has also been targeted recently with reasonable cell-based antiviral activity using hydroxypyridonecarboxylic acid derivatives (Fig. 18.5(9)) (Kankanala et al. 2016). Compounds containing the Imidazole-amide biaryl scaffold (Fig. 18.5(10)) have also shown good overall RT activity, which promises a new class of alternative compounds (Chong et al. 2012). Molecular simulation-based design of pyrimidine sulfonylacetanalides (Fig. 18.5(11)) has shown very good activity against clinically relevant

Fig. 18.5 Examples of some reverse transcriptase inhibitors

single- and double-mutant strains of HIV-1 (Wan et al. 2015). A new molecular scaffold containing N-hydroxyimide moiety (Fig. 18.5(12)) has been reported to have dual activity against both RT and integrase. Development of these dual binders as drug candidates could help in reducing drug dosage and the number of components in HAART therapy (Tang et al. 2011).

18.3.3 Integrase Inhibitors

Following reverse transcription, the viral DNA is transported into the nucleus where it undergoes integration with the host DNA using the enzyme integrase. The viral DNA undergoes highly specific 3′-processing (also known as dinucleotide processing) by the integrase enzyme, which removes two 3′-end nucleotides from both strands of the DNA. The 3′-OH on the viral DNA then attacks the phosphodiester bonds of the host DNA. The site of attack is separated by five nucleotides in the case of HIV. The resulting intermediate integration is repaired by cellular enzymes which conclude the integration process. Both the enzyme and the conserved DNA sequence that acts as primer for DNA synthesis of the provirus have been envisaged as potential targets for inhibiting integrase activity. Hydroxyisoquinolinediones (Fig. 18.6(12)) have recently been reported to have nanomolar affinity for integrase with good antiviral activity (Billamboz et al. 2016). A relatively weaker but new class of small molecule inhibitor of integrase has been reported recently. Based on molecular-docking simulations, naphthalene derivatives (Fig. 18.6(14)) have been designed (Gu et al. 2014). The potential of flavanoids (Fig. 18.6(15)) to act as metal chelates has been used to develop integrase activity. Furthermore, 8-hydroxyquinoline tetracyclic lactams (Fig. 18.6(16)) have also been identified as potent integrase strand transfer inhibitors (Velthuisen et al. 2016). Derivatives of 3-hydroxypyrimidine 2,4 diones (Fig. 18.6(17)) have recently been discovered to possess dual binding abilities where they have shown extremely potent activity against integrase and RT-associated

Fig. 18.6 Chemical structures of some recently discovered integrase inhibitors

RNase H (Wu et al. 2016). New agents based on naphthyridine moiety (Fig. 18.6(18)) have shown interstrand inhibitory effects with susceptibility to mutant integrases (Zhao et al. 2016).

18.4 Inhibitors of Tat-TAR Binding

The HIV life cycle is heavily reliant on the transcriptional and translational machineries of the host cell. The successful and fast transcription of the proviral DNA requires interaction of viral protein *tat* and the transactivating region (TAR) RNA with the human positive transcription elongation factor through a ternary complex formation. Prior to the ternary complex formation, the viral protein *tat* interacts with the TAR RNA (from the newly formed viral mRNA transcripts) in a highly specific manner with high affinity. TAR is a 59-nucleotide RNA structure that contains loops and bulges, a characteristic typical of the RNA structures. The three-nucleotide bulge (U23, C24, and U25) is crucial for *tat* binding where the arginine 52 residue of the *tat* protein binds. Therefore, inhibiting *tat*-TAR interaction has been a long sought after target as HIV provirus replication inhibitor despite the fact that none of the FDA-approved drugs belong to this class.

A number of these efforts have used TAR RNA binding by small molecules as a direct/allosteric competitor of *tat* binding. Based on the observations that arginine and arginine derivatives induced conformational changes in the TAR, ethidium-arginine conjugates were synthesized which showed micromolar anti-HIV activities (Peytou et al. 1999). Structure-guided peptidomimetic design led to β-hairpin inhibitors of *tat*-TAR interaction with nanomolar affinities (Athanassiou et al. 2004, 2007). Aminoglycosides, which are known to bind to various RNA structures including bulges, were investigated to assess their TAR RNA binding. Ribonuclease protection experiments showed that the binding site of neomycin (Fig. 18.7(19)), an aminoglycoside, on TAR was immediately below the three-nucleotide bulge UCU (Wang et al. 1998). Similarly, a DNA minor groove binder Hoechst 33258 (Fig. 18.7(20)) was

Fig. 18.7 Chemical structures of neomycin conjugates, the new inhibitors of *tat*-TAR interaction

found to bind TAR at unique GC-rich site GCUCU, unlike its natural preference for AT-rich sites in B-DNA duplexes (Dassonneville et al. 1997). Recently, conjugation approaches have been undertaken to enhance the affinity of TAR RNA binding drugs. Conjugation of neomycin to a Hoechst 33258-derived benzimidazole (Fig. 18.7(21)) led to a nearly five-fold increase in the neomycin affinity toward TAR RNA in a competitive binding assay with the *tat* protein (Ranjan et al. 2013). Further enhancement toward TAR RNA binding was achieved by synthesis of a multivalent, in principle, triple-recognition agent (Fig. 18.7(22)) that showed nanomolar affinity (Kumar et al. 2016). Triazole-linked dimeric neomycin conjugates (Fig. 18.7(23)) have also shown remarkable binding toward TAR RNA with affinity constants $K_a \sim 10^7$–10^8 M^{-1} and inhibit the release of RT at low concentrations (Kumar et al. 2012). Clearly, these promising results require further follow-up in terms of toxicity studies for them to be developed as specific *tat*-TAR interaction inhibitors.

18.4.1 Protease Inhibitors

HIV protease is one of the most important enzymes for the HIV life cycle and is responsible for production of all necessary viral proteins. Subsequent to HIV integrase function, the proviral DNA undergoes transcription and translation to give long chains

Fig. 18.8 Chemical structures of recently developed protease inhibitors

of proteins that contain Gag and Gag-Pol polyproteins. Protease cleaves the long polypeptides at nine processing sites to give mature active proteins eventually leading to final digested products as new protease, RT (p51), RNAse H (p15), and integrase. Not surprisingly, protease has been a central target of antiretroviral therapy.

The majority of FDA-approved anti-HIV drugs belong to the class of protease inhibitors and have been an essential part of combination therapies. Despite the success of this class of retrovirus inhibitors, a number of efforts have been going on toward combating resistance issues and increasing their efficacy both in monotherapy and combination therapies. These have included (Fig. 18.8) cyclic sulfonamide-based inhibitors (Fig. 18.8(24)) with picomolar affinities (Ganguly et al. 2014) that resulted from structural study-based design. Recently, a series of morpholine-based aspartate-binding group compounds were developed which identified MK-8718 (Fig. 18.8(25)) as an orally bioavailable agent with a good overall effect including a favorable pharmacokinetic profile (Bungard et al. 2016). A series of new HIV protease inhibitors with hydroxyethylamine core (Fig. 18.8(26)), which resemble the FDA-approved drug Darunavir, have been reported to have potent activity similar to Darunavir (Gao et al. 2011). Similar compounds, based on Darunavir, containing phenyloxazolidinone (Fig. 18.8(27)) showed picomolar binding affinities and low nanomolar antiviral activity against patient-derived HIV-1 virus (Ali et al. 2010). Tertiary alcohol containing transition state mimics bearing P2 and P1 substituents have shown excellent inhibitor properties (Ohrngren et al. 2011). A new class of C2 symmetric protease inhibitors (Fig. 18.8(28)) has been reported with low nanomolar

affinities (Wannberg et al. 2006). Novel isosorbide-derived P2 ligands (Fig. 18.8(29)) sharing the Darunavir cores have also shown picomolar affinities, thus opening several avenues to take drug-resistance challenges (Qiu et al. 2014).

18.4.2 Other New Targets

In addition to the above discussed inhibitors, a few other processes are being targeted to develop a new class of inhibitors. Prime of them are maturation and capsid assembly inhibitors. Maturation inhibitors, albeit inhibiting the functions of protease, are different from protease inhibitors in that they do not directly bind to the protease enzyme to block its catalytic activity. Rather they bind to a segment of Gag polyprotein disrupting the protease-mediated conversion of p25Gag (CA/SP1) to mature p24 protein (Liu et al. 2016). Proteolytic cleavage of Gag polyprotein releases HIV-1 capsid protein, which reassembles into a cone-shaped structure containing viral RNA and other necessary proteins (e.g. RT, integrase, protease) (Tremblay et al. 2012). Proper assembly of capsid is mandatory for the infectivity of the new virus. Therefore, capsid assembly inhibitors are also being explored as a new type of anti-HIV drug. The interaction of *rev* response element (RRE) with the *rev* protein is essential for viral replication (Luedtke and Tor 2003). A line of *rev*-RRE interaction inhibitors have been designed and are being explored as an alternative drug target. Immunotherapy and gene therapy-based efforts have also been tried for HIV treatment.

18.5 Future Perspectives

Development of combination therapies such as the HIV-AIDS-related HAART have helped us explore new cumulative and complementary approaches to a desired action. Advancements in the computational methods have significantly curtailed lengthy screening efforts for the lead identification and have provided alternatives for structural understanding of a drug-receptor binding. Despite this progress, the growing emergence of drug resistance and fast viral mutation has always reminded us to be vigilant to meet the challenges of the future. While lead optimization has been relatively faster in recent years, the long path of clinical trials has always been a limiting factor. A drug making it to the market has to go through all stringent checks for its adverse actions, which requires a longer time frame for data compilation and the willingness of several patient groups for a new drug trial. Clearly these issues are big roadblocks in a fast drug development and continue to be a problem whose efficient solutions are not in place yet.

However, advancements in structural biology, most notably by the development of high-resolution crystallographic and Nuclear Magnetic Resonance (NMR) techniques, have helped us understand several biologically relevant targets with fairly decent details of their active site. Understanding of the active site and chemically important functional groups in the vicinity of the active binding site has greatly aided in structure-guided drug-design efforts. Moreover, the biochemical functional

mapping has allowed us to think of novel targets at fusion, maturation, and capsid assembly stage. These new targets need to be followed with dedicated discovery programs to bring new drugs to the market.

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