REVIEW

Microbial natural products as a source of antifungals

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The vast number and variety of chemotherapeutic agents isolated from microbial natural products and used to treat bacterial infections have greatly contributed to the improvement of human health during the past century. However, only a limited number of antifungal agents (polyenes and azoles, plus the recently introduced caspofungin acetate) are currently available for the treatment of life-threatening fungal infections. Furthermore, the prevalence of systemic fungal infections has increased significantly during the past decade. For this reason, the development of new antifungal agents, preferably with novel mechanisms of action, is an urgent medical need. A selection of antifungal agents in early stages of development, produced by micro-organisms, is summarized in this review. The compounds are classified according to their mechanisms of action, covering inhibitors of the synthesis of cell wall components (glucan, chitin and mannoproteins), of sphingolipid synthesis (serine palmitoyltransferase, ceramide synthase, inositol phosphoceramide synthase and fatty acid elongation) and of protein synthesis (sordarins). In addition, some considerations related to the chemotaxonomy of the producing organisms and some issues relevant to antifungal drug discovery are also discussed.

Keywords Secondary metabolites, antifungals, natural products, fungi

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INTRODUCTION

The exploration of micro-organisms as sources of therapeutically useful compounds has a much shorter and less well-known history than the use of plants and plant extracts in human medicine. Secondary metabolites are defined as naturally produced substances which do not play an explicit role in the internal economy of the organisms that produce them. These micro-organisms may have evolved the ability to produce such compounds because of the selection advantages conferred upon them as a result of the interactions of the compounds with specific receptors in other organisms [1,2]. Although almost 20 000 microbial metabolites and approximately 100 000 plant products have been described so far, secondary metabolites still appear to be an inexhaustible source of lead structures for new antimicrobials, antivirals, antitumour drugs, and agricultural and pharmacological agents. In addition, numerous secondary metabolites, such as benzylpenicillin, cephalosporins, erythromycin, strobilurin, etc. were lead structures that later became the basis for synthetic and semisynthetic derivatives with improved pharmacological properties.

Fungal infections range from superficial conditions of the skin (e.g. ringworm and athlete's foot) and nails (onychomycoses) to disseminated life-threatening diseases. Serious invasive fungal infections caused by *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., *Pneumocystis carinii* and *Histoplasma capsulatum*, represent an increasing threat to human health. The prevalence of these systemic fungal infections has increased significantly during the past decade.

Major factors responsible for this dramatic rise include greater use of broad-spectrum antibiotics, marked increases in the numbers of

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immunocompromised persons (AIDS, cancer and transplant patients), the use of central venous catheters, and an aging patient population [3,4].

Until the 1970s, fungal infections were considered largely treatable and the demand for new medicines to treat them was very small. Before this period, antifungal chemotherapy included only two kinds of compounds: potassium iodide, effective in the treatment of sporotrichosis; and two useful polyenes, nystatin and amphotericin B, which were introduced in the 1950s. Except for the development of flucytosine (1964), there was little progress until the development of the azole drugs in the early 1970s. Therefore, only a limited number of antifungal agents (polyenes and azoles plus the recently introduced Cancidas) are currently available for the treatment of life-threatening fungal infections. These antifungal agents show some limitations, such as the significant nephrotoxicity of amphotericin B [5] and emerging resistance to the azoles [6], despite several recent improvements, such as lipid formulations of polyenes with lower toxicity and new triazoles (voriconazole, rovuconazole and pasaconazole) with a wider spectrum of action, including activity against some azole-resistant isolates [7]. The development of new antifungal agents, preferably naturally occurring with novel mechanisms of action, is an urgent medical need.

This review presents a selection of antifungal agents produced by micro-organisms, classified according to their mode of action. Some considerations related to the chemotaxonomy of the producing organisms and some issues relevant for antifungal drug discovery are also discussed.

CELL WALL BIOSYNTHESIS INHIBITORS

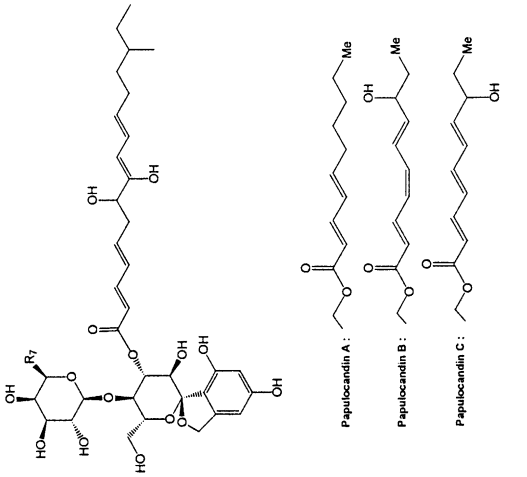
One of the targets for novel antifungals under active investigation is the fungal cell wall. Antifungal agents acting on this target are inherently selective. This characteristic and the fact that they are fungicidal for *Candida* make them particularly attractive for clinical development. Fungal cell wall composition varies among species, but it generally has three polymeric components: glucan, chitin and mannoproteins. The subcellular mechanisms of their synthesis and assembly have been used as potential targets to search for new antifungals and numerous natural products have been identified as inhibitors acting at these levels.

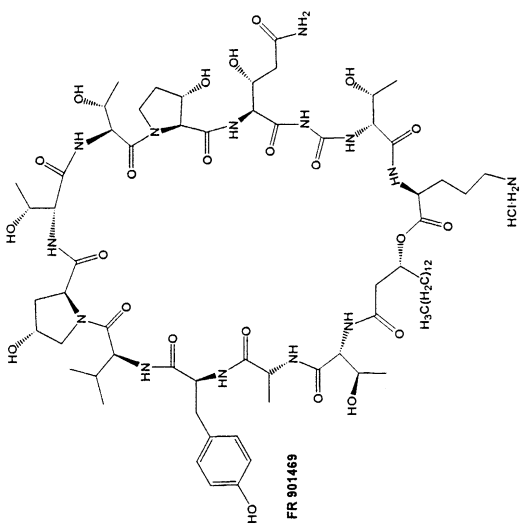
Glucan synthesis inhibitors

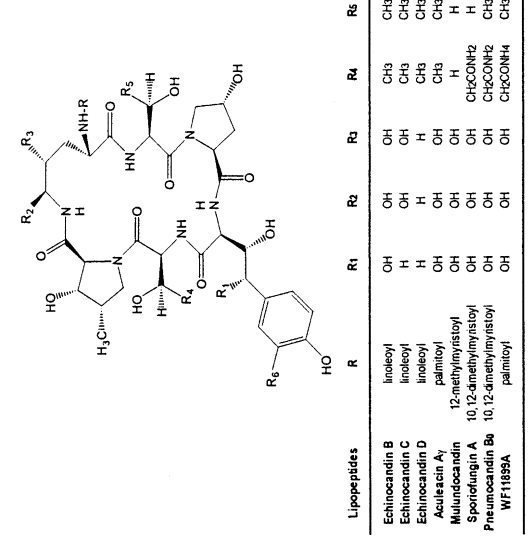
At present, glucan synthesis is the only component of the cell wall synthesis machinery that has successfully led to the development of a new drug on the market. Glucan is a polysaccharide constituted by glucose monomers linked by (1,3)-β or (1,6)-β bonds, and it is an essential component of the cell wall, guaranteeing many of its physical properties [8]. Inhibitors of glucan synthesis have been shown to possess antifungal activity in vitro as well as in vivo in many different animal models. The most classical examples of inhibitors of glucan synthesis are the echinocandins. These are cyclic hexapeptides *N*-acylated with an aliphatic chain of different length [9]. The various echinocandins differ in having different substituents in the hexapeptide ring or a distinct fatty acid chain (Figure 1). Since the first echinocandin was discovered in the early 1970s [10], many members of the family have been discovered in diverse fungi (Table 1).

The pneumocandins in particular have been successfully used to develop an antifungal drug that has been recently approved by the FDA. This semi-synthetic pneumocandin, caspofungin acetate, is an aza-substituted derivative of pneumocandin B_0 [11]. Pneumocandins are natural products derived from the fermentation of the fungus *Glarea lozoyensis* [12,13]. The introduction of additional amino groups in the peptide ring of pneumocandin B_0 increased the solubility of the molecule and the potency against fungal pathogens by two orders of magnitude [14]. The compound has been shown to be effective in animal models of disseminated candidiasis, aspergillosis, coccidiomycosis and pneumonia caused by *Pneumocystis carinii* [15–18]. The clinical trials have demonstrated good tolerance of the compound and its efficacy in the treatment of oropharyngeal and oesophageal candidiasis, as well as in invasive aspergillosis [19,20]. Cancidas has recently been approved by the FDA for use against invasive aspergillosis, refractory to, or intolerant of, other therapies.

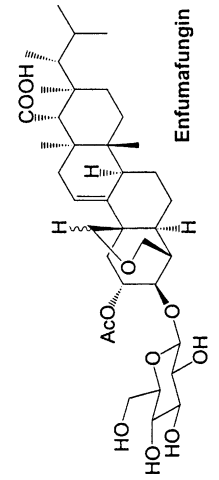
Other echinocandin-like antifungal agents in advanced phases of drug development include FK463 (micafungin), an echinocandin derivative with a sulphate ester moiety in the hexapeptide nucleus [21], and LY303366 (V-echinocandin, anidulafungin), which has a terphenyl head group and a C5 chain [22]. Other echinocandin derivatives have also been reported from Fujisawa [23],

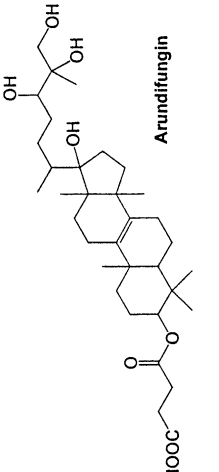






Lipopeptides	R	R_1	R_2	R_3	R_4	R_5	R_6
Echinocandin B	linoleoyl	OH	OH	OH	CH_3	CH_3	H
Echinocandin C	linoleoyl	H	OH	OH	CH_3	CH_3	H
Echinocandin D	linoleoyl	H	H	H	CH_3	CH_3	H
Aculeacin A_7	palmitoyl	OH	OH	OH	CH_3	CH_3	H
Mulundocandin	12-methylmyristoyl	OH	OH	OH	H	H	H
Sporiofungin A	10,12-dimethylmyristoyl	OH	OH	OH	CH_2CONH_2	H	H
Pneumocandin B_0	10,12-dimethylmyristoyl	OH	OH	OH	CH_2CONH_2	CH_3	H
WF11899A	palmitoyl	OH	OH	OH	CH_2CONH_4	CH_3	OSO_3H





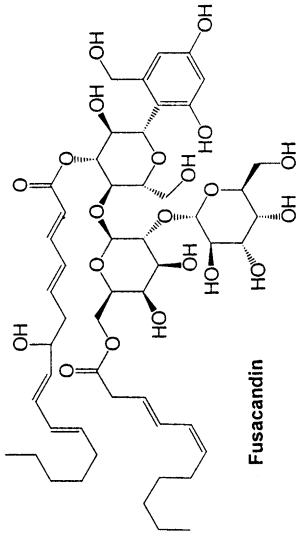


Figure 1 Chemical structures of some glucan synthesis inhibitors.

Table 1 Natural products inhibitors of glucan synthesis

Compound	Producing species	Reference
Lipopeptides		
Echinocandin B	*Aspergillus nidulans*	Nyfeler and Keller 1974 [10]
	A. rugulosus	
Aculeacin	*Aspergillus aculeatus*	Mizuno et al. 1977 [107]
Mulundocandin	*Aspergillus sydowii*	Roy et al. 1987 [25]
Sporiofungins	*Penicillium arenicola*	Tscherter and Dreyfuss 1982 [108]
	Cryptosporiopsis sp.	
Pneumocandins	*Glarea lozoyensis*	Schwartz et al. 1992 [12]
	Pezicula sp.	Bills et al. 1999 [13]
	Cryptosporiopsis sp.	Noble et al. 1991 [109]
Cryptocandin	*Cryptosporiopsis quercina*	Strobel et al. 1999 [110]
WF11899 and related sulfate-derivatives	*Coleophoma empetri*	Hori 1999 [111]
	Coleophoma crateriformis	
	Tolypocladium parasiticum	
	Chalara sp.	
FR901469	Unidentified fungus	Fujie et al. 2000 [27]
Arborcandins	Unidentified fungus	Ohyama et al. 2000 [26]
Clavariopsins	*Clavariopsis aquatica*	Kaida et al. 2001 [28]
Glycolipids		
Papulacandins	*Papularia sphaerosperma*	Traxler et al. 1977 [29]
Corynecandin	*Coryneum modonium*	Gunawardana et al. 1997 [112]
Mer-WF3010	*Phialophora cyclaminis*	Kaneto et al. 1993 [113]
Fusacandin	*Fusarium sambucinum*	Yeung et al. 1996 [31]
BU-4794F	*Gilmaniella* sp.	Aoki et al. 1993 [114]
L-687781	*Dictyochaeta simplex*	VanMiddlesworth et al. 1991 [115]
Acidic terpenoids		
Efumafungin	*Hormonema* sp.	Peláez et al. 2000 [34]
Arundifungin	*Arthrinium arundinis*	Cabello et al. 2001 [33]
	A. phaeospermum	
	Leotiales anamorphs	
	Coelomycete undetermined	
Ascoteroside	*Ascotricha amphitricha*	Onishi et al. 2000 [32]
	Mycoleptodiscus atromaculans	
Ergokonin A	*Trichoderma longibrachiatum*	Vicente et al. 2001 [35]
	T. koningii	
	T. viride	

Roche (aerothricins) [24] and Aventis (mulundocandins) [25].

The success of the lipopeptide class of glucan synthesis inhibitors has prompted interest from the industry in the search for other structural types with improved features over the echinocandins (especially for their lack of oral absorption). Besides echinocandins and the like, other cyclic peptides have been described as acting as glucan synthesis inhibitors. Arborcandins are recently described antifungal agents, containing a 10-amino-acid ring and two lipophilic tails [26]. Likewise, the compound named FR901469 is a macrocyclic lipopeptidolactone composed of 12 amino acids and a 3-hydroxypalmitoyl moiety [27]. Clavariopsins, cyclic depsipeptides lacking a long lipophilic radical, have also been suggested as acting as inhibitors of glucan synthesis [28]. However, to date only two other types of glucan synthesis inhibitors are known, besides cyclic lipopeptides, the papulacandins and related compounds, and the acidic triterpenes.

The papulacandins are glycolipids discovered in the late 1970s [29]. A series of related compounds has been discovered over the years, all of them produced by fungi (Table 1 and Figure 1). Despite medicinal chemistry efforts, neither papulacandins nor any of their relatives have been developed as drugs, basically due to their limited potency in animal models [30,31].

The most recently discovered compound class of glucan synthesis inhibitors are triterpenes

containing a polar (acidic) moiety [32]. This polar moiety can be a glycoside (in enfumafungin and ascosteroside), a succinate (in arundifungin) or a sulphate-derivative amino acid (in ergokonin A) (Figure 1). The conclusion that these compounds are acting as inhibitors of glucan synthesis is based on several lines of evidence: first, the spectrum of activity, being active against *Aspergillus* and *Candida* species, but inactive against *Cryptococcus*; second, they induced the same alterations in the micromorphology of *Aspergillus fumigatus* as other inhibitors of glucan synthesis [33–35]; and third, direct measurement of the effect of these compounds on synthesis of cell wall macromolecules indicates that glucan is the only polymer of which the synthesis is significantly altered upon treatment with these agents [32]. Although these compounds were inactive or only weakly active in the in vivo mouse model [33–35], they represent a new paradigm in the search for antifungal compounds with this mode of action, and they could be useful as a base for the development of improved drugs.

Clearly, a weak point in all the glucan synthesis inhibitors discovered or developed up to date is their lack of activity against *C. neoformans*. The reasons for this lack of activity are unclear. The hypothesis that echinocandins do not inhibit β-(1,6) glucan synthesis, which seems to be the main glucan in *C. neoformans* cell wall [30], has been recently contradicted experimentally [36]. Moreover, the FKS1 homologue gene, coding the catalytic subunit of β-(1,3)-glucan synthase, has been shown to be essential in *C. neoformans*. However, the enzyme could be relatively resistant to the action of echinocandins and the rest of the glucan synthesis inhibitors [36].

Chitin synthesis inhibitors

Chitin is an insoluble polysaccharide made of β-(1,4)-linked *N*-acetylglucosamine units. This biological polymer is one of the structural microfibrillar components of the fungal cell wall structure which maintains the morphological shape of the cells and plays an essential role in fungal morphogenesis [37]. In yeasts, chitin accounts for 1% of the cell wall and is distributed differently from glucan. Chitin also links covalently to the cellular glucan, thereby strengthening the wall. There are at least three different chitin synthases in *Saccharomyces cerevisiae*: chitin synthase I, which is involved in a repair function at the time of cytokinesis; chitin synthase II, an essential enzyme for primary septum formation between mother and daughter cells; and chitin synthase III, which synthesizes lateral chitin in the cell wall [38].

The classical inhibitors of chitin synthesis are nikkomycins and polyoxins [39]. Both belong to a family of peptide-nucleoside antimycotic agents that are substrate analogues of UDP-N-acetylglucosamine, the essential building block for chitin biosynthesis and were isolated from two different *Streptomyces* species: *S. tendae* (nikkomycin) and *S. cacaoi* var. *asoensis* (polyoxin). *Candida albicans* is resistant to polyoxins due to the difficulty that these agents present in being transported to the interior of the cell wall. Nikkomycins exhibit activity against dimorphic fungi but low activity against yeast and filamentous fungi. However, both demonstrated synergy with the azole compounds and with β-glucan inhibitors [40,41]. Nikkomycins and polyoxins are currently used exclusively as agricultural fungicides, due to their modest activity against human pathogens [42].

Recently, as a part of the continuing screening for new chitin synthase inhibitors, two novel antifungal compounds were found: phellinsin A and arthrichtin. Phellinsin A is a phenolic compound which selectively inhibited chitin synthase I and II of *S. cerevisiae*. In addition, this compound exhibited antifungal activity against human pathogens such as *Trichophyton mentagrophytes* and *A. fumigatus* and very weak activity against other human pathogens such as *C. neoformans* and *Coccidioides immitis*. However, it showed no activity against *C. albicans*, *C. lusitaniae*, *C. krusei*, *C. tropicalis* and *Fusarium oxysporum* [43]. Arthrichitin is a cyclic depsipeptide which was isolated from *Arthrinium phaeospermum* and (as LL156256g) from the marine fungus *Hypoxylon oceanicum* [44,45]. This compound showed activity against *Candida* spp., *Trychophyton* spp. and several phytopathogens. Although its in vitro potency is too low for its use in the clinic, it has been suggested that analogues with improved activity could be developed [44].

Mannoprotein synthesis inhibitors

Mannoproteins are the third main component of the fungal cell wall. They form the outer layer of the cell wall and contain as much as 50% carbohydrate. The majority of the cell wall mannoproteins are anchored by β-(1,6)- and β-(1,3)-glucan [46]

and play several roles in the function of fungal membranes. Therefore, they have been considered another potential target in fungal membranes for antimycotic agents. Until now, any mannoprotein associated with the cell wall has been considered as essential by examining for viability after gene disruption experiments. Inhibitors of the mannoproteins function are the pradimicin/benanomycin family, whose chemical structure possesses a benzo [a] naphthacenequinone skeleton [47,48]. The free carboxyl group of these compounds interacts with the saccharide portion of cell-surface mannoprotein, which is followed by disruption of the plasma membrane and leakage of intracellular potassium. These are antifungal (and antiviral) agents without significant acute toxicities, produced by *Actinomadura* (Actinomycetes) species. These antibiotics exhibited remarkable in vivo activity against systemic fungal infections caused by *C. albicans*, *A. fumigatus* and *C. neoformans* in mice. Pradimicin/benanomycin and analogues were studied in experimental animal models with good success rates; however, phase I clinical trials suggested drug-related toxicities and development was stopped [49].

SPHINGOLIPID SYNTHESIS INHIBITORS

Sphingolipids, although present in relatively small proportion in the fungal cytoplasmic membrane, are essential for cellular functions [50], and inhibition of sphingolipid synthesis results in growth inhibition and cell death [51,52]. Ceramide has been implicated as a component of an essential cell-signalling pathway in *Saccharomyces* [53]. Sphingoid bases also have a regulatory role in yeast where they have been shown to inhibit several key enzymes in phospholipid biosynthesis [54]. Sphingolipids are also involved in the synthesis of glycosylphosphatidylinositol anchors in *Saccharomyces*, and they appear to be the major repository for very long chain fatty acids (C24 and C26 species) in fungi [55].

Although many steps in the human and fungal sphingolipid biosynthetic pathway are similar, there are several enzymes uniquely found in fungi that make sphingolipid synthesis attractive for antifungal therapy (Figure 2). Three key enzymes in the sphingolipid synthesis pathway have been targeted to search for novel antifungals: serine palmitoyltransferase, ceramide synthase and inositol phosphoceramide (IPC) synthase; the latter lacks a mammalian counterpart and, as shown in Figure 2, inhibitors to all three have been discovered from natural sources. Sphingofungins [51,56], lipoxamycin [57] and viridiofungins [58] inhibit serine palmitoyltransferase. Fumonisin B1 [54,59] and australifungin [52] inhibit ceramide synthase, and aureobasidins [60], khafrefungin [61], and rustmicin [62] inhibit IPC synthase. Furthermore, minimoidin, an inhibitor of the fatty acid elongation pathway, was isolated from a coprophilic fungus [63]. The chemical structures of these compounds are presented in Figure 3, and the micro-organisms from which they were isolated are shown in Table 2.

Serine palmitoyltranferase inhibitors

Sphingofungins A to F constitute a family of novel chemical structures that resemble the long-chain base intermediates in the sphingolipid pathway. They were initially identified as broad spectrum antifungal agents, inhibiting the growth of various *Candida* species and showing an especially potent activity against *C. neoformans*, but they are inactive against filamentous fungi [56,64].

Lipoxamycin was discovered in 1970 as an antifungal compound of unknown mechanism of action [65]. Lipoxamycin and hydroxylipoxamycin, an analogue co-produced in the fermentation, have a long alkyl chain and an amino-containing polar head group, but otherwise do not resemble the sphingoid bases as closely as the sphingofungins do. Both compounds have antifungal activity against a panel of human pathogenic fungi, with better potency against some of the *C. neoformans* and *Candida* species. *A. fumigatus* was not inhibited in broth dilution assays, but other filamentous fungi were sensitive to the lipoxamycins in disk diffusion assays [57].

Viridiofungins A, B and C comprised a novel family of amino alkyl citrates that have potent, broad spectrum antifungal activity, inhibiting the growth of pathogenic fungi such as *C. neoformans*, *Candida* species and *A. fumigatus* [66].

It has been demonstrated by a variety of biological and biochemical means that sphingofungins, lipoxamycin, hydroxylipoxamycin and viridiofungins are specific inhibitors of serine palmitoyltransferase in fungi at nanomolar concentrations [51,56,58]. The potent in vitro inhibition of serine palmitoyltransferase and inhibition of whole cell

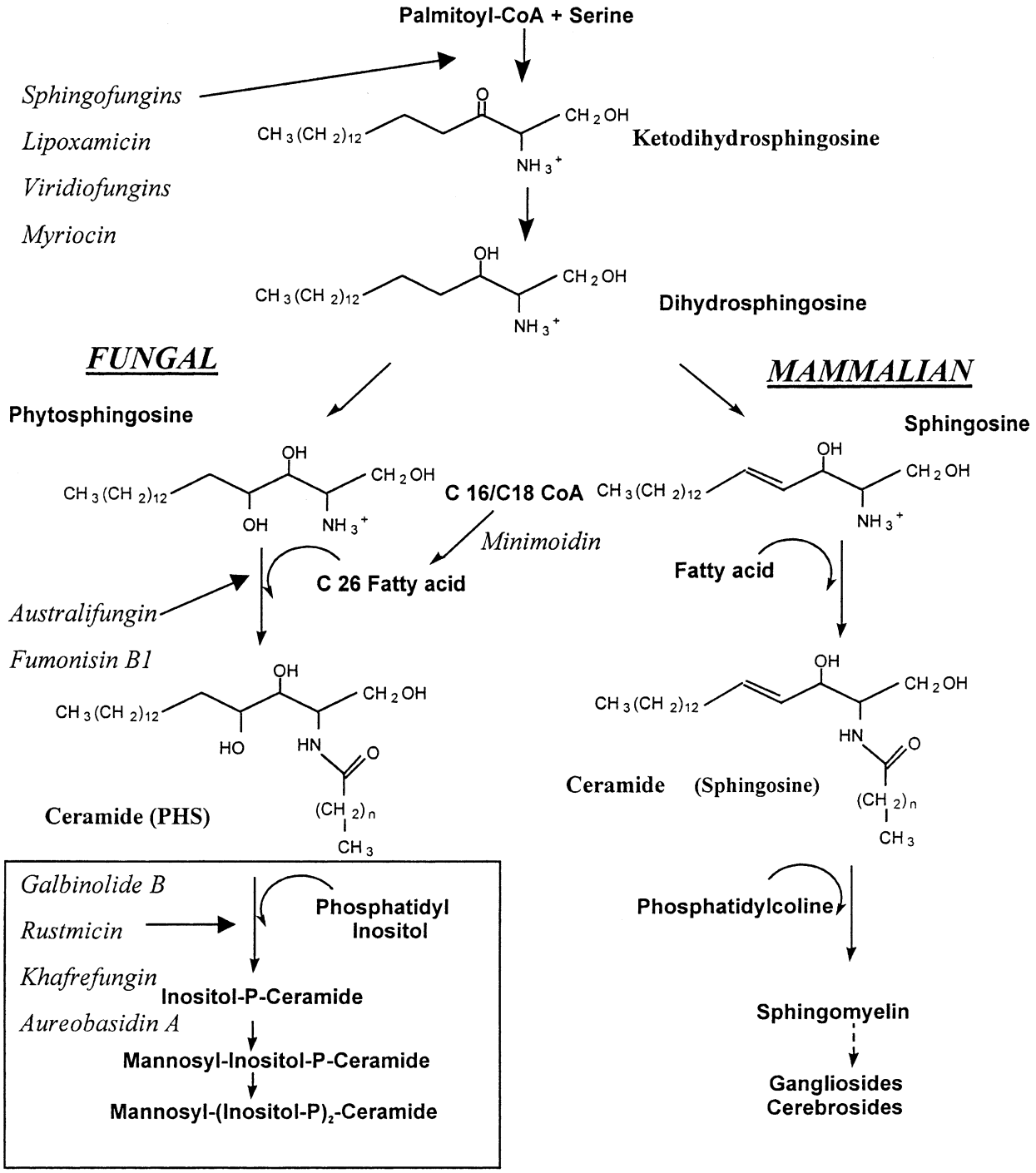


Figure 2 Sphingolipid biosynthesis pathway in fungal and mammalian cells. The different inhibitors of sphingolipid synthesis are indicated in the corresponding steps of the pathway they are blocking.

sphingolipid synthesis at minimum inhibitory concentrations (MICs) pointed to inhibition of serine palmitoyltransferase as the likely mechanism of their antifungal activity.

Viridiofungins are not as specific for serine palmitoyltransferase inhibition as the other inhibitors of the enzyme. They also inhibit squalene synthase and other enzymes that are sensitive to

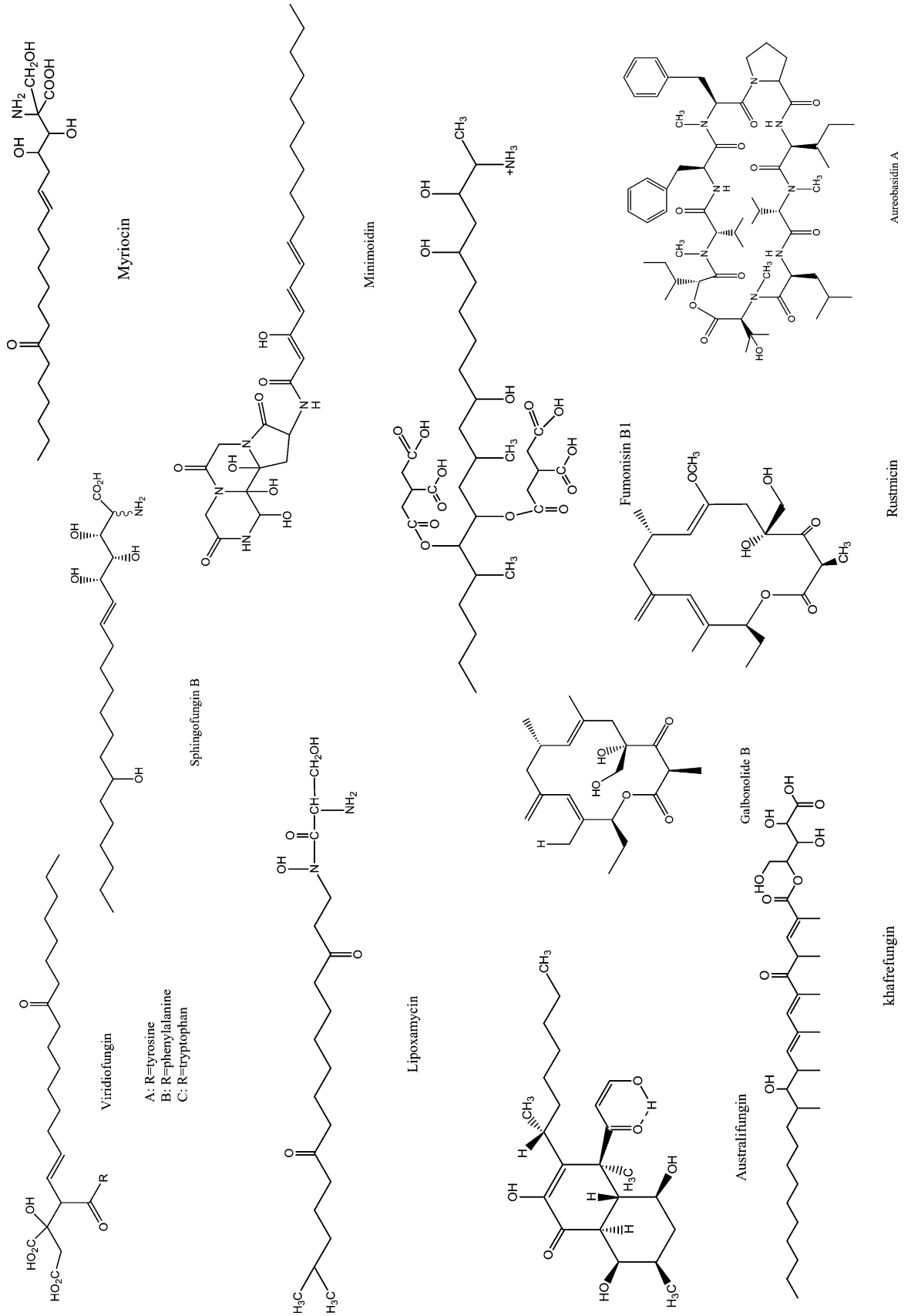


Figure 3 Chemical structures of the most relevant inhibitors of sphingolipid biosynthesis.

Table 2 Natural products inhibitors of shingolipid biosynthesis and protein synthesis

Compound	Producing species	Reference
Sphingolipid biosynthesis		
Sphingofungins	*Aspergillus fumigatus*	Zweerink et al. 1992 [51]
	Paecilomyces variotii	Horn et al. 1992 [56]
Lipoxamycin	*Streptomyces* sp.	Mandala et al. 1994 [57]
Viridiofungins	*Trichoderma viride*	Mandala et al. 1997 [58]
Myriocin	*Isaria sinclair*ii	Miyake et al. 1995 [67]
Fumonisin B1	*Fusarioum moniliforme*	Wang et al. 1991 [59]
Australifungin	*Sporormiella australis*	Mandala et al. 1995 [52]
Aureobasidin A	*Aureobasidium pullulans*	Nagiec et al. 1997 [60]
Khafrefungin	Unidentified sterile fungus	Mandala et al. 1997 [61]
Rustmicin	*Micromonospora chalcea*	Takatsu et al. 1985 [69]
	Streptomyces galbus	Fauth et al. 1986 [70]
	Micromonospora sp.	Mandala et al. 1998 [62]
Galbonolide B	*Micromonospora* sp.	Harris et al. 1998 [71]
Minimoidin	*Sporomiella minimoides*	Mandala et al. 2001 [63]
Protein synthesis		
Sordarin	*Sordaria araneosa*	Sigg et al. 1969 [79]
Zofimarin	*Zopfiella marina*	Ogita et al. 1987 [83]
BE31405	*Penicillium minioluteum*	Okada et al. 1998 [85]
SCH57404	Unidentified sterile fungus	Coval et al. 1995 [86]
Xylarin	*Xylaria* sp.	Schneider et al. 1995 [87]
Hypoxysordarin	*Hypoxylon croceum*	Daferner et al. 1999 [88]
GR135402	*Graphium putredinis*	Kinsman et al. 1998 [92]

dicarboxylic acids, although at higher concentrations than required for serine palmitoyltransferase inhibition [66].

Unfortunately, the serine palmitoyltransferase inhibitors described above also have potent activity against the mammalian enzyme [51,56,58], and other studies showed that lipoxamycin is highly toxic in mice when applied either subcutaneously or topically [65]. Toxicity may be mechanism based, since studies with a Chinese hamster ovary (CHO) cell mutant have shown that this enzyme is essential in mammalian cells. Another natural product isolated from the fungus *Isaria sinclairii*, the potent immunosuppressant ISP-1/myriocin, has also been reported to inhibit serine palmitoyltransferase at picomolar concentrations in an interleukin-2-dependent mouse cytotoxic T-cell line [67].

Ceramide synthase inhibitors

The fumonisins are mycotoxins initially characterized as tumour-promoting agents associated with severe toxicological effects in animals. They inhibit *de novo* sphingolipid biosynthesis blocking the reaction catalyzed by ceramide synthase in rat hepatocytes, and their toxicity and carcinogenicity have been attributed to inhibition of ceramide synthesis and the concomitant accumulation of sphingoid bases [59]. Although fumonisin B1 does inhibit fungal ceramide synthase in vitro [54] the fumonisins have very poor activity against whole cell fungal sphingolipid synthesis or growth; limited penetration could account for their poor antifungal activity.

Australifungin is a highly potent, broad-spectrum antifungal compound containing a unique combination of α-diketone and β-ketoaldehyde functional groups. It was the first non-sphingosine-based inhibitor described for the sphingolipid biosynthetic pathway. Australifungin inhibits ceramide synthase in vitro at nanomolar concentrations and the enzyme inhibition accounts for the arrest of sphingolipid synthesis. Australifunginol, an analogue isolated from the same fungus, also blocked the enzyme converting sphinganine to ceramide, but it was at least 50-fold less potent [52]. Australifungin had MICs of 1 mg/L or less against all the species tested, with particularly good activity against *Candida pseudotropicalis*, *C. tropicalis*, and *C. neoformans*. Much weaker activity was detected for australifunginol with MICs between 8 and 64 mg/L [52].

Although the activity of australifungin in mammalian systems has been largely uncharacterized,

it is known to inhibit ceramide synthesis in HepG2 cells [58], and therefore constitutes a major limitation to the therapeutic use of australifungin for the treatment of fungal infections.

IPC synthase inhibitors

Aureobasidins A to R are cyclic depsipeptides described as antifungal agents with high in vitro activity, particularly against *C. albicans*, aureobasidins A, B, C and E being the most potent [68]. Their mechanism of action remained unknown until it was shown that aureobasidin A inhibits the IPC synthase from *S. cerevisiae* with an IC_{50} of about 0.2 nM [60].

Khafrefungin is a novel 22-carbon linear polyketide acid esterified to an aldonic acid that shows a broad antifungal spectrum, with *C. albicans* being the most susceptible organism in vitro. The compound causes the accumulation of ceramide and inhibits the IPC synthase of *S. cerevisiae* and pathogenic fungi at picomolar to nanomolar concentrations [61].

Rustmicin (also named galbonolide A) is a macrolide antifungal agent with potent activity against wheat stem rust fungus *Puccinia graminis* [69], *Botrytis cinerea* and several other phytopathogens [70]. The mechanism of its fungal growth inhibition was not determined until 13 years later, when its extraordinarily potent antifungal activity against several human pathogens, especially *C. neoformans* was observed, and it was found that its antifungal activity was due to inhibition of sphingolipid synthesis at the IPC synthase level at picomolar to low nanomolar concentrations of the compound [62]. The rustmicin-related macrolide galbonolide B was also reported to inhibit IPC synthase, but with less potency [71].

A. fumigatus is one of the few human pathogens that is insensitive to any of the known IPC synthase inhibitors, although some inhibitors of earlier steps in the sphingolipid biosynthetic pathway do inhibit the growth of this organism [52,66]. The reason for this resistance is unknown. Sphingolipids are thought to be essential in *Aspergillus* [52,66] and *A. fumigatus* does synthesize alkali-stable inositol lipids, and their synthesis is inhibited by khafrefungin, but at much higher concentrations than those required for the other fungi. Lack of growth inhibition may be due to a resistant enzyme or poor uptake of the drug. Alternatively, the inositol-containing sphingolipids may not be essential in this organism, which also contains glycosylated sphingolipids.

Unlike inhibitors to earlier steps in sphingolipid synthesis, khafrefungin and rustmicin did not have any detectable effect on lipid synthesis in mammalian cells [61]. Rustmicin and aureobasidin A were non-toxic in animal studies [62,72], supporting the idea that inositol phosphoceramide synthase is a fungal selective target, and thus preferable in the search for novel antifungals blocking sphingolipid biosynthesis. Although khafrefungin was lytic to washed red blood cells at 12.5–25 mg/L, the toxicity may be due to the detergent-like properties of the compound.

Among the three structurally diverse IPC synthase inhibitors, rustmicin is unique in its remarkable activity against *C. neoformans*. However, although encouraging, the level of in vivo efficacy is far less than expected from the in vitro susceptibility of this organism to rustmicin. Poor in vivo efficacy in animals is probably due to chemical instability. Even at its optimal pH, rustmicin degrades relatively rapidly in aqueous media, converting to the inactive γ-lactone. Despite these limitations, concentrations of rustmicin that are required to completely inhibit phosphosphingolipid synthesis and accumulate ceramide in fungi are easily achieved [62].

Fatty acid elongation inhibitors

Minimoidin is a novel compound that indirectly inhibits sphingolipid synthesis by blocking the fatty acid elongation pathway, thus depriving the ceramide synthase of substrate [63]. In experiments of ^{14}C-acetate uptake, minimoidin blocked radioactivity uptake into C26 fatty acids, while the pattern of incorporation into other fatty acids was not modified. Moreover, minimoidin inhibited the incorporation of ^{14}C-malonyl-CoA into long-chain fatty acids.

PROTEIN SYNTHESIS INHIBITORS

Protein synthesis has always been considered one of the more attractive targets in the development of antimicrobial agents [73]. However, application of this idea to the field of antifungal therapy is not an easy task, due to the eukaryotic nature of fungi and therefore the great degree of similarity between the fungal and mammalian protein synthesis machineries. Two soluble elongation factors show

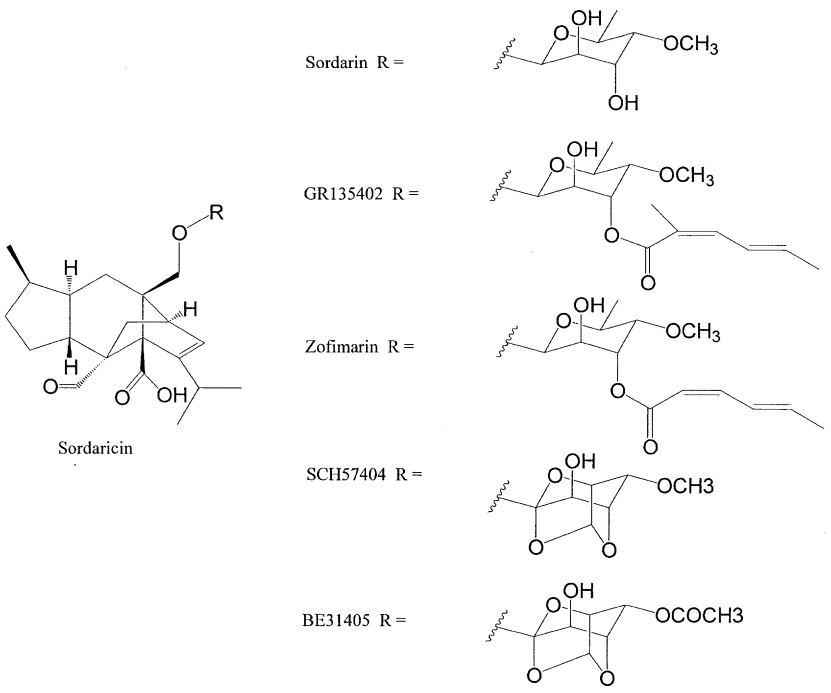


Figure 4 Chemical structures of some natural sordarins, selective inhibitors of fungal protein synthesis.

some fungal-specificity: EF3, a factor that is required by fungal ribosomes only [74,75], and EF2, which has been demonstrated to possess at least one functional distinction from its mammalian counterpart [76,77]; the fact that these fungal soluble factors are essential for protein synthesis makes them obvious targets for antifungal drug discovery [78].

The most important family of antifungal agents acting at the protein synthesis level are the sordarins. Sordarin was isolated by scientists at Sandoz from fermentations of the fungus *Sordaria araneosa* (Table 2). The compound was patented in 1969 under the name SL [79]. Its purification and degradation by acid hydrolysis to a diterpenic aglycon, sordaricin (Figure 4), and a novel sugar, 6-desoxy-4-*O*-methyl-D-altrose, were described in 1971 [80], and its full structure was reported shortly afterwards [81]. Recent publications from Merck and Glaxo Wellcome demonstrated that the sordarins are potent inhibitors of translation in fungi with an extremely high level of selectivity. They act via a specific interaction with EF2, by stabilizing the fungal EF2-ribosome complex, despite the high degree of amino acid sequence homology exhibited by EF2 from various eukaryotes. The fungal specificity of the sordarins makes EF2 an attractive antifungal target [77,82]. All compounds in this class inhibited in vitro translation in *C. albicans*, *C. tropicalis*, *C. kefyr* and *C. neoformans*, but to varying degrees. The lack of activity of the sordarins against *C. krusei*, *C. glabrata* and *C. parapsilosis*, in comparison with their extremely high levels of potency against *C. albicans*, suggests that these compounds have a highly specific binding site, which may also be the basis for the greater selectivity of these compounds in inhibiting the fungal, but not the mammalian, protein synthesis.

After the discovery of sordarin, several compounds structurally related, sharing the common aglycone of sordarin, sordaricin, were isolated from diverse fungal species. Those compounds and the micro organisms from which they were isolated are shown in Figure 4 and Table 2.

The compound called zofimarin was patented by Sankyo in 1987 [83]. Like sordarin, zofimarin is active against *C. albicans*, *S. cervisiae* and *C. neoformans*, but additional activity against *Aspergillus* was also described for this compound at higher concentrations. No further development appears to have been undertaken with zofimarin.

BE31405 was patented by Banyu in 1994 [84]. This compound contains a unique sugar with an unusual tricyclic structure. It presented broad spectrum and more potent antifungal activity against *C. albicans*, *C. glabrata* and *C. neoformans* than sordarin. The MIC against *C. albicans* (1.56 mg/L) was between those of amphotericin

B (0.39 mg/L) and miconazole (6.25 mg/L). This compound did not show toxicity against mammalian cells such as P388 mouse leukemia when tested at up to 50 mg/L [85].

SCH57404 was isolated from an unidentified fungus by Schering-Plough. It differs from BE31405 by the presence of a methoxy in place of the acetate at C-4 of the tricyclic sugar moiety. It has a narrow spectrum in vitro, with antifungal activity against *C. albicans* (MIC 16 mg/L), but ony poor activity against dermatophytes and *Aspergillus* [86]. In a concurrent study another group identified the same compound from a *Xylaria* sp. The isolated compound, named xylarin, exhibited potent activity against yeasts and filamentous fungi, and, as expected, was only weakly cytotoxic towards mammalian cells [87].

A new natural sordarin derivative, hypoxysordarin, has been isolated from cultures of *Hypoxylon croceum*. Like the parent compound sordarin, this new derivative presented potent antifungal activities against yeasts and several filamentous fungi [88].

During recent years, patents for sordarin derivatives have been filed by Glaxo Wellcome [89], Merck [90] and Banyu [91].

During the screening program carried out by Glaxo Wellcome to search for compounds inhibiting fungal protein synthesis, the most promising compound to emerge was a natural product that showed a close similarity to zofimarin, GR135402 [92]. The spectrum of activity included *C. albicans*, *C. tropicalis* and *C. neoformans*, but not some other *Candida* species or *Aspergillus* species. GR135402 showed a therapeutic effect in mice with systemic candidiasis following subcutaneous dosing at 100 mg/kg/dose [92]. Because this compound is a selective and potent inhibitor of *C. albicans* protein synthesis, a synthetic chemical program at Glaxo Wellcome was initiated to produce novel analogues. Several sordarin derivatives with a broad spectrum of activity and remarkable potencies in vivo were synthesized by modifications of the basic sordarin molecule sugar moiety (different types of fused rings at position C3′ to C4′) [93]. Four of these sordarin derivatives (GM222712, GM237354, GM193633 and GM211676) have demonstrated good in vitro and in vivo antifungal activity against most *Candida* species, including azole-resistant isolates, *C. neoformans* and *Pneumocystis carinii* [94,95]. Moreover, these derivatives demonstrated potent fungicidal activity against important dimorphic endemic fungal pathogens, such as *Histoplasma capsulatum* [96] and *Coccidioides immitis* [97], and significant in vitro activity against yeast-like fungi, such as *Blastoschyzomyces capitatus*, and dematiaceous fungi, such as *Cladosporium carrioni* [93]. In contrast, the new sordarin derivatives have limited activity against *A. fumigatus*, which constitutes a serious limitation [98].

Recent efforts directed toward the synthesis and development of new sordarin antifungal agents with improved activity against pathogenic fungi and better pharmacological properties have resulted in the discovery of a new series of derivatives, the azasordarins [99]. These are compounds characterized by the presence of a 6-methylmorpholin-2-yl group with different *N*-4′ substituents at position 8a of the sordaricin indacene ring system instead of the 4′ sugar moiety present in sordarin, and they have the additional advantage of an easier chemical synthesis. Azasordarins displayed significant activities against *Candida* species, including fluconazole-resistant strains, with the exception of *C. krusei*. In addition, some azasordarin derivatives were active against *C. parapsilosis*. Furthermore, these compounds were extremely potent against *P. carinii*, *Rhizopus arrhizus* and *B. capitatus*. However, *C. neoformans* was resistant to all these new antifungal agents. In general, the levels of cytotoxicity presented by the azasordarin derivatives were low. These findings suggest that the azasordarins possess an important antifungal therapeutic potential [100].

The potent broad-spectrum in vitro activity, and the fact that some of the sordarins have shown oral efficacy in animal models, are significant advantages of these compounds, sufficient to justify an ongoing effort into developing the full clinical potential of the sordarin class.

OTHER TARGETS

Other targets that have been used for antifungal drug discovery include electron transport and membrane integrity. Some inhibitors of these targets are produced by micro-organisms. The polyenes (amphotericin B and nystatin) are prototypic antifungal agents affecting the integrity of the fungal cell membrane. Their mechanism of action is due in part to their selective binding to ergosterol, the major fungal sterol, in the cell membrane of susceptible fungi. This induces changes in

membrane permeability, causing leakage of cytoplasmic contents and cell death [101]. Their toxicity is associated with the fact that they bind cholesterol as well, although with less affinity than ergosterol, producing disruptions in mammalian cell membranes as well.

With respect to mitochondrial electron transport inhibitors, a series of related antifungal agents with this mode of action has been described. These compounds, named as UK2A, UK3A, and the structurally related antimycin A, consist of a nine-membered dilactone ring, and have been isolated from different species of *Streptomyces* [102,103]. The antifungal activities of UK-2A and UK-3A compounds were relatively broad-spectrum, including *C. albicans* and *Aspergillus* spp., equipotent with antimycin A, while less toxic to host cells.

GENERAL CONSIDERATIONS ON ANTIFUNGAL DRUG DISCOVERY

The production of antifungal agents is not at all infrequent in microbes. The results from our own screening program over the years have shown that a remarkable percentage of the strains isolated may produce at least one antifungal agent. Thus, in a study in which different families and orders of basidiomycetes were compared in their ability to produce antimicrobial activities, 20% of the isolates examined were observed to produce some kind of antifungal activity [104]. Likewise, studies on fungal communities from specific environments showed percentages of 14–41% of antifungal activity in fungal endophytes from halophytic plants [105,106], depending on the panel of target organisms used. For actinomycetes the figures are similar: in a study performed with *Streptomyces* and other genera from diverse geographies, isolated under conditions of extreme pH or salinity, between 25 and 50%, depending on the taxonomic groups, produced antifungal activities (Basilio et al., unpublished results). The question, then, is: why, if antifungal agents are so common, is it so complicated to take an antifungal drug to the clinical stage? The reasons are several. First, many of those activities seen in any screening effort do not represent different chemical entities. The same antifungal metabolite can be produced by different organisms, in some cases by many species that are phylogenetically unrelated. The cases of compounds restricted to single strains/species or to few strains of a given biological species are rare in comparison. Enfumafungin, for instance, was found only in three isolates from a *Hormonema* endophytic species, growing on the same host species (*Juniperus communis*) in the same forest [34]. However, much more common is the case of arundifungin, which was found in several *Arthrinium* species from different locations, plus a psychotolerant *Leotiales* anamorph and other species, all of them phylogenetically and ecologically unrelated [33]. Thus, the number of different compounds is far fewer than the number of activities seen in any screening. A screen such as the one that led to the discovery of the new triterpene class of glucan synthesis inhibitors resulted in a very high number of hits, most of which were later shown to be echinocandins, papulacandins and related compounds (Cabello et al., unpublished results). In particular, the metabolic pathways leading to the synthesis of the echinocandin class of antifungals seem to be widely distributed across the fungal kingdom, as shown in Table 1, and the same is true for the papulacandins.

Besides the matter of the number of compounds, issues of potency, spectrum and mode of action are probably the most relevant. Most of the antifungal activities observed in any screening of microbial natural products are not potent enough or do not show the spectrum that can make them attractive enough for industrial groups to dedicate resources to their identification. Others are simply toxins that also affect mammalian cells, making them undesirable leads. Finally, many compounds with promising in vitro activity are inactive or only weakly active when tested in animal models. This may result from a variety of reasons related to the pharmacokinetic properties of the compounds, from poor absorption to inactivation by serum-binding, high clearance rates, etc., but in many cases these features are not easy to solve by medicinal chemistry programs. In summary, although the available data indicate that the number of compounds with antifungal activity present in nature is really huge, taking one of these compounds to clinical trials is by no means an easy task, and the level of resources required to make this effort are not to be underestimated.

CONCLUSIONS

Until very recently, life-threatening fungal infections observed in the clinic could only be treated

with drugs from the azole family or with amphotericin B. Although the new generations of azoles are much improved compared to previous compounds in this class, concern remains about the development of resistance by pathogenic strains. Their fungistatic character is another weakness of these compounds. Amphotericin B shows a better activity profile, but also an undesirable toxicity. Although the new liposomal formulations have reduced toxicity, the lack of absolute specificity of amphotericin B mode of action will always be a concern. Recently, a new antifungal agent with a different mode of action has been included in the existing therapeutic options: caspofungin, approved for invasive aspergillosis in patients refractory, to or intolerant of, other therapies. It shows good activity against *Aspergillus* and *Candida*, and very acceptable tolerability. Other glucan synthesis inhibitors are expected for the near future. In addition, the sordarin derivatives could constitute a new and promising group of antifungals to be developed in a more distant future. An important point yet to be established is to specify the applicability and utility of the antifungal associations with different mechanisms of action.

The compounds discussed in this review represent a subset of all the antifungal agents of natural origin that have been isolated, characterized and described in published literature to date. There is a huge number of other molecules with reported antifungal activity that have not been included here, because their mode of action is unknown. In addition, what has been described in the literature would be only a fraction of the potentially existing antifungal compounds from natural sources. Research in the field of antifungal antibiotics should continue in order to obtain more effective and selective drugs in the near future. Because only a minor percentage of the microorganisms living in the biosphere have been described and studied to date, there is an enormous and still unexplored reservoir of natural compounds of large structural diversity that could be used for the development of new antifungals. Also, the advent of fungal genomics is expected to increase the number of molecular targets useful for antifungal drug discovery. Thus, the stage is set for the development of an antifungal portfolio to rival the diversity of antibacterial drugs and increase the armamentarium of drugs active against systemic fungal infections.

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