Sphingolipids as targets for treatment of fungal infections

Invasive fungal infections have significantly increased in the last few decades. Three classes of drugs are commonly used to treat these infections: polyenes, azoles and echinocandins. Unfortunately each of these drugs has drawbacks; polyenes are toxic, resistance against azoles is emerging and echinocandins have narrow spectrum of activity. Thus, the development of new antifungals is urgently needed. In this context, fungal sphingolipids have emerged as a potential target for new antifungals, because their biosynthesis in fungi is structurally different than in mammals. Besides, some fungal sphingolipids play an important role in the regulation of virulence in a variety of fungi. This review aims to highlight the diverse strategies that could be used to block the synthesis or/and function of fungal sphingolipids.

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During the last few decades, fungi have been considered an increasing concern due to the rise in cases of infection. Between 1979 and 2000, fungal infections increased by 207% in USA, reaching more than 16,000 cases of sepsis in 2000 [1]. The number of deaths due to fungal infections rose from 800 in the beginning of the 1980s to more than 2000 at the end of the 1990s and caused twice the amount of deaths as tuberculosis in 1997 [2]. Nowadays, it is estimated that one and a half million people die every year due to invasive fungal infections, although epidemiological data are lacking because these infections are often misdiagnosed and unreported [3]. The emergence of diseases caused by fungi is mainly related to the increased prevalence of immunocompromising infections, such as HIV/AIDS, and use of immunosuppressive interventions, such as organ transplantation and chemotherapeutic treatments [4,5].

Because of the increase in life-threatening fungal infections, the need for effective treatments for fungal disease is clear but, unfortunately,

the availability of antifungal drugs is limited. The first antifungal drug, amphotericin B, was discovered in the fifties and, since then, only few classes of antifungal drugs have been developed, such as fluoropyrimidine analogs, polyenes, azoles and echinocandins, directly targeting different fungal structures [6]. The most recently discovered class, echinocandins, was approved in 2000s and since then, no new class of drug has been developed [7]. Moreover, the currently used antifungal agents display a low efficacy in killing fungal cells and are highly toxic, due to the similarities between fungi and mammals at the biochemical and biological level [8]. For these reasons, discovery of new targets and drugs for treatment of fungal diseases is an urgent necessity, but it is also a challenge for scientists in the area of mycology.

In this context, sphingolipids have emerged as a potential new target for the development of new antifungal agents. Although synthesis of sphingolipids is highly conserved between mammalian and fungal cells, some structural

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differences and roles in pathogenesis allow for these molecules to be considered a good target for new drugs [9]. Sphingolipids are abundant components of membranes in eukaryotic cells, playing a variety of roles in fungal cells specifically, such as heat stress response, signal transduction, endocytosis and apoptosis [9–12]. Particularly, some sphingolipids play an important role in fungal pathogenesis, such as inositolphosphoryl ceramide (IPC) and glucosylceramide (GlcCer). A variety of studies have recently shown that affecting enzymes involved in these sphingolipid biosynthetic pathways using inhibitors or gene deletion can attenuate the virulence of fungal pathogens such as, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus* spp. [13–15]. Several natural inhibitors of fungal GlcCer synthesis have been described to exhibit a potent antifungal activity [16–18]. In addition, monoclonal antibodies against GlcCer are able to inhibit the growth of different fungi, including *C. neoformans*, *Colletotrichum gloeosporioides*, *Fonsecaea pedrosoi*, *Pseudallescheria boydii* and *Scedosporium apiospermum* [19–23]. Recently, synthetic compounds that target the synthesis of GlcCer was found to be effective against several fungi, including *C. neoformans*, *C. albicans*, *Aspergillus fumigatus* and *Histoplasma capsulatum* [24].

All these data suggest that sphingolipids are good targets for the development of new drugs and, therefore, should be studied further. This review aims to discuss these works and highlight the potential of these molecules as a target for new classes of antifungal agents.

Current classes of antifungals

Despite the increasing need for efficient antifungal treatment due to the emergence of life-threatening fungal infections, therapeutic options are still limited. There are only few classes of antifungal drugs used in clinical practices, such as polyenes, azoles, fluoropyrimidine analogs and echinocandins, which target three different metabolic pathways as shown in Figure 1 [25]. Fluoropyrimidines, such as 5-fluorocytosine and 5-fluorouracil, are analogs of the DNA nucleotide cytosine (Figure 2D–E). It was first synthesized in 1957 as an antitumor agent and later discovered to also possess antifungal activity. The use of this class of drugs is diminishing due to the occurrence of fungal resistance and to bone marrow toxicity and hepatotoxicity. The combination of flucytosine with amphotericin B for 2 weeks is the induction step of the gold standard treatment for AIDS-related cryptococcal meningitis [6,26]. However, flucytosine is not available and unregistered in most of Africa and Asia, where the burden of cryptococcal meningitis is greatest (reviewed in [27]).

The first fungal target studied for treatment of fungal infections is ergosterol, the primary sterol in the cell membranes of fungi that modulates cell membrane

thickness, membrane fluidity and permeability. Mutations in the late steps of its biosynthesis in different fungi showed defective plasma membrane and vacuolar fusion, cellular signaling, endocytosis and enzyme functions [28–31]. Because of its difference comparing to cholesterol, the mammalian sterol, ergosterol emerged in the past as a potential target for antifungal drugs, leading to the development of antifungal agents such as azoles and polyenes that affect its synthesis or function [6,32].

The oldest class of antifungal agents are the polyenes, from which amphotericin B, nystatin and natamycin are the most commonly used in clinical practice (Figure 2A–C). These drugs are produced by the bacterial genus *Streptomyces*, and its structure is composed of cyclic amphiphilic molecules, known as macrolides [33]. Polyenes directly target ergosterol due to their amphiphilic properties, forming pores and destabilizing cell membranes [33] and can be used in clinical practice due to its ability to preferably bind ergosterol rather than cholesterol. Nevertheless, they possess some low affinity to cholesterol, which causes high toxicity and side effects in patients [33]. Despite this, polyenes, especially amphotericin B, have been considered the gold standard for about 40 years and are still one of the main drugs used for invasive antifungal infections. Recently, it has been suggested that the primary mechanism of action of amphotericin B is to directly bind to ergosterol and affect several cellular events such as vacuole fusion, endocytosis, membrane compartmentalization and the function of membrane proteins [34]. In this case, membrane permeabilization and ion channel formation seems to be secondary mechanisms that increase drug potency [34]. On the other hand, there are some evidences indicating that pore formation is not enough for killing cells. Oxidative damage induced by amphotericin B treatment has been reported, but its role in antifungal effect is still unknown [35]. In *C. neoformans*, it has been demonstrated that amphotericin B treatment leads to a strong intracellular oxidative burst and protein carbonylation, indicating that intracellular damage plays an important role in killing fungal cell along with its effect on the plasma membrane [36].

Azoles inhibit ergosterol synthesis by targeting the enzyme lanosterol 14 α -demethylase and thus cause accumulation of toxic intermediates [37]. The first group of azoles developed was the imidazoles between 1960s and 1980s, such as econazole, miconazole and ketoconazole (Figure 2F–H) [38]. However, these drugs displayed a variety of limitations, including high toxicity, low efficacy and poor absorbance as well as severe side effects such as liver and gastrointestinal complications [6,39]. Therefore, in 1990s another group of azoles, called triazoles, was developed, such as fluconazole and itraconazole (Figure 2I & J), which display a higher solubility in mam-

Figure 1. Cellular targets of the current antifungal drugs.

ER: Endoplasmic reticulum; M: Mitochondria; V: Vacuole.

malian body fluids and absorbance by host tissues, allowing them to be widely used for the treatment systemic fungal infections [40]. Despite this success, resistance cases have been increasingly reported, thus a new generation of triazoles, such as voriconazole, posaconazole and ravuconazole (Figure 2K–M), has been developed to overcome fluconazole and itraconazole resistance, although the emergence of resistance has been recently observed also for these new drugs [41–45].

The most recently discovered antifungal class is echinocandins, which were introduced more than 15 years ago (Figure 2Q–S). The first licensed echinocandin product is caspofungin acetate (Cancidas; Merck & Co.), followed by subsequent members of the class such as micafungin (Mycamine; Fujisawa Ltd.) and anidulafungin (Versicor Inc.) [46]. The echinocandins have a unique mechanism of action, which consist of blocking the synthesis of β -1,3-D-glucan polymers, key components of the cell wall in pathogenic fungi and thus inhibiting the function of the genes *fks1*, *fks2* and *fks3* that encode β -1,3-D-glucan synthase. This complex structure is a

useful target for antifungal drugs, since mammalian cells do not present it and do not share many of the polysaccharides found in fungal cell wall [47]. Moreover, this fungal structure plays essential roles in fungi, such as protective barrier when in hostile environments and osmolarity control as well as present toxic components important for virulence [48–50]. In this context, 1,3- and 1,6-D-glucans compose the central core of fungal cell wall when bound to chitin [48,49]. Echinocandins however display a narrow spectrum of activity against fungi, such as *Candida* and *Aspergillus* species, whose cell wall is rich in β -glucans. However, zygomycetes usually lack this molecule and, consequently, are resistant to these drugs [51]. In addition, although echinocandins can enhance the activity of amphotericin B and fluconazole against *C. neoformans in vitro* [52] they are ineffective against disseminated cryptococcosis [53] and in some cases they could be detrimental [54]. Besides this limited spectrum, echinocandins present other limitations. The current formulation of the drug must be administered intravenously making it more difficult for use in

Amphotericin B

Nystatin

Natamycin

5-fluorocytosine

5-fluorouracil

Econazole

Ketoconazole

Miconazole

Fluconazole

Itraconazole

Voriconazole

Ravuconazole

Figure 2. Structures of the main classes of antifungal drugs (cont. from facing page). Polyenes: (A) amphotericin B, (B) nystatin, (C) natamycin; fluoropyrimidines: (D) 5-fluorocytosine, (E) 5-fluorouracil; imidazoles: (F) econazole, (G) ketoconazole, (H) miconazole; triazoles: (I) fluconazole, (J) itraconazole; next generation azoles: (K) voriconazole, (L) ravuconazole, (M) posaconazole; echinocandins: (Q) caspofungin, (R) micafungin, (S) anidulafungin.

the clinical setting [51]. Additionally, the emergence of echinocandin resistance has been reported, especially for *Candida* species, and it is associated with alterations in the FKS amino acid sequence. It has been shown that substitution in the first and fifth amino acid (phenylalanine and serine, respectively) confers higher resistance pattern in *C. albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis* [55,56].

The close relation between fungal and mammalian cells is a challenge for the development of new antifungal drugs. This is one of the main reasons why the development of antifungal drugs is considerably slower comparing to other areas. All antifungals mentioned above were developed in the last 65 years, and it is notable that many more antibiotics were approved during the same time [7].

Another molecule and its biological pathway, sphingolipids are emerging as a new target option. The next sections will present the reasons why sphingolipids are now considered a potential target for new drug develop-

ment, as well as some works suggesting that the inhibition of these molecules will be able to decrease fungal growth and virulence. Drugs that are available to inhibit the synthesis of sphingolipids (Figure 3) will also be described.

Role of fungal sphingolipids in virulence

Sphingolipids also play an important role in fungal virulence. IPCs have been associated to fungal pathogenesis since the downregulation of inositol-phosphoryl ceramide synthase 1 (Ipc1) in *C. neoformans* led to a decreased growth in acidic conditions, such as *in vitro* and intracellular growth in J774.16 macrophages, impaired pathogenesis in a rabbit model and also affected other virulence factors such as melanin formation [57]. However, IPCs are well studied in nonvirulent fungi, resulting in few works about the role of IPCs in fungal pathogenesis. In *S. cerevisiae* model, it is known that M(IP)₂C affects plant defensin susceptibility and oxidative stress resistance, as shown by Aerts *et al.* using

Figure 3. Structures of sphingolipid biosynthesis inhibitors. (A) Myriocin (2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoicos-6-enoic acid). (B) THL. (C) Australifungin. (D) Scyphostatin. (E) BHBM. (F) D0. (G) D-threo-PDMP. (H) Fumonisin B1. (I) Aureobasidin A.

an *S. cerevisiae* strain that lacks $M(IP)_2C$ in its membranes and becomes more resistant to H_2O_2 [58]. In addition, the *S. cerevisiae mic* mutant, which is deficient in conversion of mannosylinositol phosphorylceramide to $M(IP)_2C$, exhibits tolerance to nystatin suggesting that this class of lipid may compose membrane domains in combination with ergosterol and, thus, could also be a target for polyenes in the yeast model [59].

The role of GlcCer in virulence was first studied in *C. neoformans*, which have significantly contributed to the understanding of GlcCer importance in fungal pathogenesis. *C. neoformans* strain lacking GlcCer synthase gene ($\Delta gcs1$) is not virulent in the animal model [15]. Interestingly, other virulence factor, such as capsule and melanin, are not affected in $\Delta gcs1$ mutant. Interestingly,

deletion of *gcs1* in *C. neoformans* results in a dramatic growth defect at neutral/alkaline pH, 5% CO_2 and 37°C [15], suggesting that GlcCer may be important in fungi to promote alkaline tolerance [60]. When exposed to a neutral/alkaline environment, *C. neoformans* cells do concentrate GlcCer at the plasma membrane/cell wall/bud necks without upregulating its synthesis [61]. This phenomenon was observed not only during *in vitro* growth, but also in cells recovered *ex vivo* from murine infection model [61]. This redistribution of GlcCer at fungal membranes under these physiological environments (neutral/alkaline pH) may be important for proper function of specific transmembrane transporters [62], which may be particularly important for promoting fungal cell replication at neutral and alkaline pH.

These phenomena were also observed in a *C. neoformans* mutant lacking the sphingolipid methyltransferase 1 ($\Delta smt1$), which produces GlcCer but it is not methylated [62,63]. This suggests that a specific fungal structure of GlcCer involved in the regulation of specific fungal lipid domains is responsible for the regulation of these phenotypes. This is supported by studies in other fungal systems, such as *Fusarium graminearum*, where methylation of GlcCer is an essential step for the appropriate function of this molecule at the membranes/cell wall [64].

In dimorphic fungi, such as *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*, the production of GlcCer is mainly related to the yeast-pathogenic form, leading to the presence of antibodies to GlcCer in patient sera and suggesting its importance for fungal virulence [63,65,66]. In the plant pathogen *Penicillium digitatum*, knocking out the GlcCer synthase gene impairs its virulence in a citrus fruit infection model [67], suggesting that GlcCer plays an important role in virulence not only of mammal pathogens but also of plant pathogens.

In *C. albicans*, disruption of three different genes encoding enzymes from GlcCer biosynthetic pathway, such as *smt1*, *sld8* and *gcs1* genes, lead to a decreased cel-

lular elongation in hyphal-inducing solid media. Moreover, when *gcs1* is knocked out, this mutant presents defects in cell membrane structures as well as higher susceptibility to SDS and fluconazole treatment [68,69] and *Gcs1* also regulates virulence in *C. albicans* [70]. *Fusarium graminearum* mutant strain $\Delta Fgmt2$, which lacks C-9 methyltransferase and produce about 65–75% non-methylated GlcCer, also present mycelial growth defect as well as size and shape changes in conidia [64]. These studies clearly show that sphingolipids play an essential role in controlling fungal virulence.

Targeting the function of sphingolipids directly

As fungal and mammalian sphingolipids are structurally different (Figure 4), a variety of natural products have been shown to target GlcCer directly and affect fungal growth. Defensins from plants and insects, such as RsAFP2 (*Raphanus sativus* Antifungal Protein 2) and heliomicin, are active against *C. albicans* and *P. pastori*, and strains lacking GlcCer synthase gene are resistant, suggesting that GlcCer could be the target for these defensins. Moreover, RsAFP2 and heliomicin are not able to interact with human GlcCer, indicating a poten-

Figure 4. Main sphingolipid structures found in mammalian and fungal cells. Mammalian sphingolipids are shown in (A) and fungal in (B). Major sphingolipid structures in mammals: GlcCer (d18:1/16:0), GalCer (d18:1/16:0) and SM (d18:1/16:0). Major sphingolipid structures in fungi: GlcCer (d19:1/18:0h), GalCer (d19:1/18:0h) and IPC (t18:0/26:0(2OH)). GlcCer: Galactosylceramide; GlcCer: Glucosylceramide; IPC: Inositolphosphorylceramide; SM: Sphingomyelin.

Figure 5. Outlines of sphingolipid metabolic pathways (see facing page). Metabolic pathways for (A) mammals and (B) fungi are shown. Glucocerebrosidases can metabolize complex glycolipids, represent by dotted arrow. Sphingolipid biosynthetic pathway inhibitors are shown in bold italic. These are: Myriocin (SPT inhibitor); Fumonisin B1 (CERS inhibitor); THI (2-acetyl-5-tetrahydroxybutyl imidazole, SPL inhibitor); PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, GCS inhibitor); Aureobasidin A (SM synthesis and IPC synthase inhibitor), Scyphostatin (inhibitor of neutral sphingomyelinase), Australifungin (fungal CERS inhibitor), BHBM(N'-(3-bromo-2-hydroxybenzylidene)-2-methylbenzohydrazide, GlcCer biosynthesis inhibitor) and D0 (3-bromo-N'-(3-bromo-4-hydroxybenzylidene) benzohydrazide, GlcCer biosynthesis inhibitor). ASA: Arylsulfatase A; CDase: Ceramidase; CERS: Ceramide synthase; CERK: Ceramide kinase; CGT: Ceramide galactosyltransferase; CST: Cerebroside sulfotransferase; DES: Desaturase; GCS: Glucosylceramide synthase; HYD: Sphingolipid hydroxylases; IPCS: Inositol phosphorylceramide synthase; IPT: Inositol phosphoryl transferase; ISC: Inositol sphingolipid phospholipase C; KSR: 3-ketosphinganine reductase; LCS: Lactosylceramide synthase; MIPCS: Mannosylinositol phosphorylceramide synthase; SK: Sphingosine kinase; SPL: Sphingosine-1-phosphate lyase; SMase: Sphingomyelinase; SMS: Sphingomyelin synthase; SMT: Sphingolipid methyltransferase; SPP: Sphingosine-1-phosphate phosphatase; SPT: Serine palmitoyl transferase.

tial selectivity pattern for them [71]. RsAFP2 also acts in other *Candida* species and their susceptibility is related to their ability to synthesize GlcCer. *Candida glabrata*, for instance, does not produce GlcCer and is resistant to RsAFP2 [17]. A deeper study regarding the effects of RsAFP2 in *C. albicans* cells showed that this molecule interacts with the fungal cell wall, where GlcCer is mainly localized, to cause an aberrant budding profile and alter the cell shape. In addition, the binding of RsAFP2 with fungal GlcCer impairs the yeast-hyphae transition, which is a crucial step for *Candida* pathogenesis, and leads to the accumulation of ceramides, which is known to cause apoptosis in fungal cells [18].

During the last few years, another useful tool has been used to help the understanding of GlcCer role in fungal growth and pathogenesis: the use of monoclonal antibodies produced against fungal GlcCer. One of the first studies using antibodies against GlcCer, purified them from human sera of patients with cryptococcosis, showing that GlcCer is immunogenic during the infection. In addition, these antibodies helped to localize GlcCer on the surface of *C. neoformans* cells, and were also shown to inhibit bud's growth by inhibiting cytokinesis, which is one mechanism by which GlcCer regulate fungal growth [21,72]. Additionally, *in vivo* work has shown that these antibodies are able to protect mice from *C. neoformans* infections [72]. During the following years, a variety of studies have been done using monoclonal antibodies against GlcCer in other fungal systems. For instance, *Fonsecaea pedrosoi* and *Colletotrichum gloeosporioides* treated with antibodies against GlcCer showed reduced growth and conidia germination. Interestingly, treatment with these antibodies enhanced macrophage activity, suggesting a possible role on stimulating host cells against fungi [19,20]. This is supported by studies in *Pseudallescheria/Scedosporium* complex, where it was also shown that antibodies against GlcCer are able to inhibit conidia germination and to enhance phagocytosis by macrophages, as well as being able to present a synergic effect with itraconazole [22]. All these data

contributed significantly to understand the roles of GlcCer in fungi and helped to raise it as a specific lipid target to better control fungal infections.

Biosynthesis of sphingolipids in fungi

Sphingolipids are present in eukaryotic cells, such as mammals and fungi. Although the most frequent sphingolipid in mammalian cells is sphingomyelin, there are two main groups described in fungal cells, IPCs and hexosylceramides, such as GlcCer and GalCer [73]. The first steps of sphingolipids biosynthesis are conserved among mammalian, fungi and plants, starting by the condensation of serine to palmitoyl-CoA, forming 3-keto-dihydrosphingosine and then dihydrosphingosine through a reduction reaction (Figure 5). From sphingosine, fatty acids are transferred to the structure to form ceramide or phytoceramide, the latter being known to present very long chain fatty acids (C24 or C26) and to be exclusive to fungi [74]. From these two structures, IPCs and GlcCer are formed (Figure 5). Several works have elucidated the entire fungal sphingolipid pathway [74–79]. Certain fungi, particularly molds, can also make GalCer in addition to GlcCer [80] but studies addressing the enzyme(s) involved in its synthesis or its role in fungal biology and pathobiology are missing.

IPCs are formed by the transferring of an inositol phosphate unit to phytoceramides, which is composed by phytosphingosine as sphingoid base and by a series of fatty acids (saturated, unsaturated, hydroxylated and nonhydroxylated). IPC can be converted to mannosylinositol phosphorylceramide and eventually to mannosyldiinositol phosphorylceramide [M(IP)₂C] (Figure 5B) [75]. IPCs are the major glycoinositolphospholipid in fungi and, interestingly, it can also be found in some protozoan species, such as *Trypanosoma cruzi* [81,82]. Since IPCs are not found in mammalian cells, which produce sphingomyelin instead of IPCs, it is considered a potential target for antifungal drug development.

GlcCer has been well studied during the last decades in a variety of fungi. It is composed of a

Table 1. Potential drugs targeting sphingolipids.

Drugs	Pros	Cons
Targeting GlcCer directly		
Defensins (RsAFP2)	Active against yeast and mold; no interaction with human GlcCer	Not active against fungi without GlcCer (<i>Candida glabrata</i>)
Antibodies against GlcCer	Active against yeast and mold; synergism with antifungals	No wide-spectrum effect
Targeting sphingolipid biosynthesis		
SPT inhibitors (myriocin, sphingofungin)	Active against yeast and mold in planktonic and biofilm forms	Toxic to mammalian
Ceramide synthase inhibitors (fumonisins, australifungin)	Active against yeast and mold; differences in ceramide synthase from fungi and human	Toxic to mammalian
S1P and S1P lyase	Highly toxic to fungi; enhance host immune response	No drug known so far as an antifungal treatment
IPC inhibitors (aureobasidin, khafrefungin, galbonolide)	Active against different fungi; mammalian does not present IPC	Less or no activity against mold
GlcCer synthase inhibitors (D-threo-PDMP)	Partially active against some fungi	Toxic to mammalian (active against mammalian GlcCer synthase)
GlcCer inhibitors (BHBM, D0)	Highly active against yeasts and molds	More studies needed

GlcCer: Glucosylceramide; IPC: Inositolphosphoryl ceramide; SPT: Serine palmitoyltransferase.

sphingoid base backbone linked to a fatty acid with a chain length ranging from 16 to 26 carbons via an amide bond, and to glucose via a β -glycosidic bond (Figure 4B) [63]. The biosynthetic pathway from ceramide to the final GlcCer molecule includes ceramide C-8 desaturations (SLD8) and C-9 methylation (SMT1), and the transferring of the glucose moiety from UDP-glucose to ceramide by the GlcCer synthase (GCS1) [77]. Both the $\Delta 8$ desaturation (SLD8) and C-9 methylation (SMT1) are exclusively found in fungi (Figure 5). GlcCer plays crucial roles in fungal cells, being one of the main components, along with ergosterol, of lipid domains in plasma membrane, which play important roles in polarized growth, an essential feature for germination of filamentous fungi and for dimorphic fungi to switch from yeast to hyphal form [83]. In *Fusarium graminearum* and *Aspergillus nidulans* lacking GlcCer, for example, hyphal tip organization and sterol accumulation are affected [14,84]. These lipid domains are responsible for compartmentalization of a variety of molecules required for energy production and polysaccharide and sterol metabolism [85], as well as some virulence factors such as phospholipases and superoxide dismutase [75,86]. As a component of lipid domains, GlcCer is present not only in plasma membranes but also in vesicles and the cell wall [72,87–89], suggesting that this molecule is crucial not only for

fungal growth but also for secretion processes and cell protection.

Targeting the biosynthesis of fungal sphingolipids

Elucidation of the sphingolipid biosynthesis has been extremely important to understand the role of sphingolipids in fungal cells [63,75] and has also contributed to the discovery of inhibitors of the sphingolipid biosynthetic pathway. Along the biosynthetic pathway of sphingolipids, many inhibitors are known and some of them are related to antifungal activity (Figure 3) [90]. Some inhibitors are described according to their targets and their pros and cons are summarized in Table 1.

Inhibitors of the first step of sphingolipid biosynthesis act on the enzyme serine palmitoyltransferase (SPT) responsible for the sphingosine formation. SPT inhibitors such as sphingofungin, and myriocin are the most studied in fungi (Figure 3A). Although this step is conserved between mammalian and fungal sphingolipids biosynthesis, the use of myriocin has significantly contributed to understand the importance of sphingolipids in fungi [91]. Myriocin treatment against *Candida* and *Aspergillus* species impairs biofilm formation through inhibition of sphingolipid formation, leading to changes in lipid rafts distribution in fungal membranes and cell morphology [92,93]. Nevertheless, since

SPT is also found in host cells, myriocin is clearly not a good option, as seen with *Galleria mellonella* model infected with *C. albicans*, in which pretreatment with myriocin enhances the mortality of host [94].

Another class of inhibitors of sphingolipid metabolizing enzymes are the inhibitors of ceramide synthases, such as the family of fumonisins, especially fumonisin B1 (Figure 3H), comprises mycotoxins produced by *Fusarium* species presented in corn and other grains [95]. Fumonisin inhibit ceramide synthase in mammalian cells and also in fungi, although studies in fungi are limited [90]. Australifungin (Figure 3C) is another inhibitor of ceramide synthase with potent broad spectrum antifungal activity observed against *Candida* and *Aspergillus* species, as well as against *C. neoformans* [96]. Due to their unspecific effect, current ceramide synthase inhibitors would most likely be not effective against fungal infections. However, fungal ceramide synthases may be significantly different than human homologs and because a rapid ceramide synthase activity assay has been developed [97] one could envision a high-throughput screening of compounds that would inhibit the fungal but not the mammalian ceramide synthases.

Sphingosine-1-phosphate (S1P) and sphingosine kinases are also important target of the sphingolipid pathway in mammalian cells. S1P is a bioactive signaling molecule that modulates immune cell development, differentiation, activation and migration. S1P function can be suppressed by FTY720 (fingolimod), which is currently used for the treatment of multiple sclerosis (reviewed in [98,99]). By acting as a S1P receptor agonist, it prevents the endogenous S1P to bind to its receptor(s). As a consequence, it blocks several biological effects of S1P. To our knowledge, a specific fungal S1P or sphingosine kinase inhibitor does not exist and targeting the mammalian S1P or sphingosine kinase during fungal infection would be detrimental because they play an important role in macrophage activation and phagocytosis as well as in granuloma formation [100–102].

An interesting target however could be the enzyme sphingosine-1-phosphate lyase. Mammalian inhibitors for this enzyme exist (Figure 3B) [103–105]. If these compounds would also target the fungal in addition to the mammalian lyase, one could get two pigeons with one stone. The inhibition of the mammalian lyase would enhance S1P and thus, improving the host immune response; the inhibition of the fungal lyase would enhance fungal S1P, which is highly toxic to fungi. Previous studies suggest that lung S1P is important for phagocytes recruitment and granuloma formation against *C. neoformans* [100].

In the context of IPCs, aureobasidin, khafrefungin and galbonolide are known as IPC inhibitors [106–109].

Aureobasidin A (AbA) (Figure 3I) is a natural compound that inhibits Ipc1 particularly in yeasts, such as *C. albicans*, *S. cerevisiae* and *C. neoformans* [110]. Discovered in 1995 [109], novel chemistry for the modification of AbA to broaden its spectrum of activity is very promising [111]. In addition to decrease the production of IPCs, AbA treatment results in an accumulation of ceramide to a toxic level [112]. AbA is known to inhibit *Candida* planktonic cells by impairing germ tube elongation and also affects *in vitro* biofilm formation [113]. In plant pathogenic fungi, AbA also presents a fungicidal effect, as shown with *Botrytis cinerea* in which AbA affects some virulence factors, such as cellulase and pectinase activity, and it is also fungicidal in a tomato infection model [114]. Other IPC synthase inhibitors include khafrefungin, rustmicin and galbonolide A, both presenting antifungal activity against *Candida* spp., *C. neoformans* and to a lesser extent against *Aspergillus* spp. [115]. Ipc1 inhibitors are highly desirable because of their specificity (Ipc1 is not present in mammalian cells) and because Ipc1 is essential in fungi. Thus, the R&D of these inhibitors is highly warranted.

In the context of GlcCer, little is known about inhibitors of this branch of sphingolipid synthesis. A mammalian GlcCer synthase inhibitor, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-threo-PDMP) (Figure 3G), is able to decrease GlcCer synthesis in few fungi, affecting fungal growth, but more studies are needed on this subject [14]. In *Aspergillus fumigatus* and *Aspergillus nidulans*, it was shown that D-threo-PDMP and other analogs (Figure 3G) are able to affect fungal germination and reduce growth in a colony-forming assay [14]. However, whether this effect is due directly to the inhibition of fungal GCS1 or to an off-target effect is not known. In *C. neoformans*, for instance, these inhibitors have no significant effect against *C. neoformans* GCS1 [61].

Although there is an evident need for the development of new antifungal drugs, pharmaceutical companies have discontinued the research and development of antifungals targeting the synthesis of fungal sphingolipids, mainly because many biosynthetic steps between mammals and fungi are conserved. However, AureoGen, a small pharmaceutical company involved in the R&D of aureobasidin A, has recently reached an agreement with Merck for the further development of these Ipc1 inhibitors using the new chemistry platform that AureoGen developed. This is a step forward for the potential commercialization of the first fungal Ipc1 inhibitor.

A recent drug screening targeting the synthesis of fungal and not mammalian GlcCer led to

the discovery of two synthetic compounds, N'-(3-bromo-2-hydroxybenzylidene)-2-methylbenzohydrazide (BHBM) and 3-bromo-N'-(3-bromo-4-hydroxybenzylidene) benzohydrazide (D0) (Figure 3E & F) [24]. These compounds exhibit *in vitro* and *in vivo* antifungal activity against *C. neoformans* and other important fungi, such as *Candida* species, *Aspergillus fumigatus* and *Histoplasma capsulatum*. In addition to be efficacious against a variety of pathogenic fungi, these compounds were well tolerated by animals and their effect was additive to current antifungals [24]. This is also a step forward the R&D of the first inhibitor(s) of fungal GlcCer synthesis.

Conclusion

As the current antifungal therapies available today present many limitations and the need of new alternatives for fungal infection treatments is undeniable, the interest of using sphingolipids as a target for new drugs has finally increased. The fungal sphingolipid pathway represents a unique platform to exploit for the R&D of new antifungal compounds, particularly against IPCs and GlcCer synthesis. Both natural and synthetic fungal inhibitors have been identified and the R&D of these inhibitors has been significantly intensified during the last decade. We hope that pharmaceutical companies will be able to further develop such compound(s) and bring into the clinic a much needed drug(s) with a new mechanism of action different to those currently in use.

Future perspective

Fungal infections are now considered an increasing concern in immunocompromised and immunocom-

petent subjects. Current antifungal treatment is limited considering its efficacy and drug options. In this context, studies in new fungal targets and new antifungal agents are highly warranted. A variety of compounds have been reported to act against the fungal sphingolipid pathway. Such compounds include natural and synthetic molecules as well as antibodies directed against fungal sphingolipids. These studies bring new perspectives for the treatment of fungal infection and open a new field for the R&D of these compounds.

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Executive summary

Current classes of antifungals

- There are only few classes of antifungal drugs used in clinical practice.
- All drugs available present some limitations.

Role of fungal sphingolipids in virulence

- Inositolphosphoryl ceramide (IPCs) are important for fungal pathogenicity.
- Glucosylceramide (GlcCer) plays important roles for fungal growth and virulence in yeast and molds.

Targeting the function of sphingolipids directly

- Natural products, such as plant defensins, target the fungal sphingolipid GlcCer.
- Monoclonal antibodies against fungal GlcCer are able to inhibit fungal growth.

Biosynthesis of sphingolipids in fungi

- Some biosynthetic steps are conserved between mammalian and fungal cells, but others are fungal-specific.
- Mammalian cells produce sphingomyelin while fungal cells produce IPCs.
- Sphingolipids are structurally different between mammalian and fungal cells.

Targeting the biosynthesis of fungal sphingolipid

- Inhibitors of different steps of biosynthetic pathway are known.
- Some inhibitors, such as serine palmitoyltransferase inhibitors, also block mammalian sphingolipid synthesis.
- The inhibition of IPCs and fungal GlcCer is highly promising.

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