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Fungal sphingolipids: role in the regulation of virulence and potential as targets for future antifungal therapies

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Abstract

Introduction: The antifungal therapy currently available includes three major classes of drugs: polyenes, azoles and echinocandins. However, the clinical use of these compounds faces several challenges: while polyenes are toxic to the host, antifungal resistance to azoles and echinocandins has been reported.

Areas covered: Fungal sphingolipids (SL) play a pivotal role in growth, morphogenesis and virulence. In addition, fungi possess unique enzymes involved in SL synthesis, leading to the production of lipids which are absent or differ structurally from the mammalian counterparts. In this review, we address the enzymatic reactions involved in the SL synthesis and their relevance to the fungal pathogenesis, highlighting their potential as targets for novel drugs and the inhibitors described so far.

Expert opinion: The pharmacological inhibition of fungal serine palmitoyltransferase depends on the development of specific drugs, as myriocin also targets the mammalian enzyme. Inhibitors of ceramide synthase might constitute potent antifungals, by depleting the pool of complex SL and leading to the accumulation of the toxic intermediates. Acylhydrazones and aureobasidin A, which inhibit GlcCer and IPC synthesis, are not toxic to the host and effectively treat invasive mycoses, emerging as promising new classes of antifungal drugs.

Keywords

fungi; sphingolipids; antifungal therapy; glucosylceramide; inositol phosphorylceramide; *Cryptococcus*; *Candida*; *Aspergillus*

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Declaration of interest

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1. Introduction

Fungal species affect billions of people worldwide, causing superficial infections of the skin or nails or invasive disease [1]. Despite the lack of epidemiological data, it is estimated that at least one and a half million individuals die every year due to invasive fungal infections (IFIs). This high number of deaths is comparable to those caused by tuberculosis [2], one of the top 10 global killers in 2016, according to the World Health Organization.

Cryptococcus, *Candida*, *Aspergillus* and *Pneumocystis* correspond to the most frequently isolated fungi from the clinical practice, accounting for more than 90% of the cases of invasive mycoses [2]. These fungal species are usually found in the environment or as commensal organisms, being cleared out by the immune system of healthy individuals. Immunosuppressive conditions, such as HIV/AIDS, chemotherapy, corticosteroid therapy and hematological malignancies, substantially increase the susceptibility to IFIs.

The main classes of antifungals currently used, and their simplified mechanism of action, are illustrated in Figure 1. The antifungal arsenal explores molecules or synthesis pathways unique to fungi, therefore limiting any potential damage to mammalian cells in host tissues. Ergosterol is the most abundant sterol in fungal membranes, playing a role in their fluidity and permeability [3]. Additionally, ergosterol differs in structure from its mammalian counterpart, cholesterol, making this lipid a suitable target for antifungal drugs. Indeed, two distinct classes of antifungal drugs disrupt the ergosterol pathway through binding to ergosterol (polyenes) or disrupting its synthesis (azoles).

The polyenes are natural products of *Streptomyces nodosus*, a soil actinomycete [4], and amphotericin B, its main prototype, has been used for systemic fungal infections since 1959. Despite its clinical use for several decades, amphotericin B is still one of the most potent antifungals, exhibiting a broad spectrum of activity, including against most isolates of *Cryptococcus* spp, *Candida* spp and *Aspergillus* spp. For a long time, it was thought that amphotericin B exerts its fungicidal activity through the formation of pores. However, Gray showed that the antifungal activity of this polyene relies on simply binding to ergosterol, subsequently disrupting membrane function [5]. The membrane permeabilization caused by channel formation constitutes a secondary mechanism which increases amphotericin B potency [6].

Azoles target ergosterol synthesis by inhibiting lanosterol 14α-demethylase, which converts lanesterol to ergosterol [7,8]. This enzyme is involved in a rate-limiting step of the late pathway in ergosterol synthesis [9,10]. The resulting inhibition of 14α-demethylase activates the alternate pathway, leading to the accumulation of the metabolite dienol, which exerts a fungistatic effect [11,12]. The first generation of azoles was introduced in 1969, being mainly used in the following decades as a miconazole parenteral formulation or as ketoconazole oral tablets which showed high gastrointestinal toxicity. The development of second (fluconazole, itraconazole) and third (voriconazole, posaconazole) generation azoles improved spectrum of activity, safety and oral availability [13].

The newest class of antifungals comprise echinocandins, particularly caspofungin, micafungin and anidulafungin, developed in the early 2000s. These drugs target the cell

wall, a branched matrix of polysaccharides, present in fungi but not in higher eukaryotes such as mammals [14]. Specifically, echinocandins inhibit the β−1,3-glucan synthase, which leads to cell death in *Candida* species and a modest, fungistatic effect in *Aspergillus* [15,16].

The widespread use of antifungal agents promoted the emergence of drug resistance, especially to azoles and echinocandins [17]. Fungal cells can circumvent the inhibitory activity of azoles by upregulating multidrug transporters and therefore increasing the drug efflux [18–21]. A second mechanism of acquired resistance is target modification or overexpression, which limits the deleterious effect of the drug. In *Candida*, amino acid substitutions in Erg11p have been reported in clinical isolates resistant to fluconazole [22,23]; this resistance phenotype might be explained by a lower affinity of the mutated proteins to azoles [24]. Additionally, an increased number of copies of chromosome 5 and gain-of-function mutations in the transcriptional regulator Upc2 lead to *ERG11* overexpression, reducing fungal susceptibility to azoles [25–27]. *A. fumigatus* resistance to azoles has also been associated with overexpression of the genes encoding 14α-demethylase (*cyp51A* and *cyp51B*) or mutations in its sequence [28]. Similarly, mutations in the catalytic subunit of the glucan synthases, called Fks, confer echinocandin resistance in yeasts and molds [29,30]. The development of resistance, along with the therapeutic limitations of the current drugs (e.g. polyenes' high toxicity, lack of echinocandins' oral formulations), emphasize the urgent need for new classes of antifungals.

Sphingolipids (SL) are composed of a sphingoid base or long chain base (LCB) amide-linked to a fatty acid, forming the ceramide backbone, which is attached to a polar group via C1-OH (Figure 2A) [31]. SL synthesis starts in the endoplasmic reticulum and, once produced, ceramides are transported to the Golgi, where the synthesis of complex SL occur [32]. In the fungal membrane, SL form microdomains along with sterols named lipid rafts, being crucial to growth, cell polarity establishment, hyphal formation and ultimately, virulence ([33–36], reviewed in [37]). SL are also found in the cell wall or secreted to the extracellular environment in vesicles, which stimulate cytokine production by macrophages [38–41]. More than structural components of membranes, SL act as signaling molecules, regulating cellular processes such as apoptosis and senescence [42]. Fungal SL are not produced by higher eukaryotes or differ structurally from their mammalian counterparts, highlighting the potential use of SL as novel targets for selective antifungal drugs.

The sphingolipid synthesis can be subdivided in three major branches, discussed in the distinct subsections of this review. The shared sphingolipid pathway (first subsection) includes the production of the precursor dihydrosphingosine (DHS), which is further acylated by ceramide synthases to generate dihydroceramide and phytoceramide (Figure 2B, blue arrows). The two pools of ceramides are used as building blocks for the synthesis of neutral (Figure 2B, green arrows) or acidic glycosphingolipids (Figure 2B, orange arrows), addressed in the second and third subsections of this review, respectively.

2. The shared sphingolipid synthesis

The first step of the sphingolipid synthesis consists of the condensation of serine and palmitoyl-CoA, catalyzed by serine palmitoyltransferase (SPT), generating 3-

ketodihydrosphingosine. SPT enzyme is a heterodimer encoded by the essential *LCB1* and *LCB2* genes, conserved from yeast to mammals [43–45]. The *Aspergillus* homologue of Lcb1, *lcbA*, is essential for fungal viability, and the conditional repression of *lcbA* severely impaired polarized growth and germling formation [46]. As the establishment of cell polarity is a crucial hallmark of fungal pathogenesis, these results suggest that pharmacological targeting of SPT might compromise virulence. Myriocin, a metabolite isolated from the insect fungus *Isaria sinclairii*, irreversibly inhibits SPT [47,48]. In *Aspergillus*, myriocin prevented spore germination and induced abnormal branching in hyphae, suggesting that the establishment of the cell polarity axis depends on the SL production [46]. Further applications of myriocin in antifungal therapy were limited as this compound is not specific to fungal SPT, showing toxicity towards mammalian and insect cells. In fact, myriocin severely impaired *de novo* SL synthesis and growth in Chinese hamster ovary (CHO) cells [49]. Additionally, the administration of myriocin to mice reduced the SPT activity in liver cells in a dose-dependent manner [50]. Myriocin also exerts an immunosuppressant effect in the host. Larvae of *Galleria mellonella* pre-treated with myriocin and infected with *C. albicans* showed a survival rate significantly lower than those infected with fungi only [51]. Therefore, the use of myriocin derivatives as novel antifungals depends on the synthesis of selective compounds, which do not show host toxicity. The drug repurposing might represent a promising alternative to circumvent the myriocin toxicity in SPT inhibition. Cycloserine, used for the treatment of tuberculosis and neurological disorders, did not show antifungal activity alone but improved the fluconazole activity [52]. Interestingly, heterozygous strains of *S. cerevisiae* lacking one copy of *LCB1* or *LCB2* were hypersensitive to cycloserine, suggesting that the compound targets the fungal SPT [52].

Once produced, 3-ketodihydrosphingosine (3-ketosphinganine, 3KS) is converted to dihydrosphingosine (DHS or sphinganine) through 3-keto dihydrosphingosine reductase (Ksr) activity. Ksr1 and KsrA proteins, from *C. albicans* and *A. fumigatus* respectively, were shown to reduce 3KS to DHS *in vitro* [53]. Also, deletion of *KSR1* impaired yeast-to-hypha transition, essential for *Candida* pathogenesis [53,54]. DHS structure is shared by fungi and higher eukaryotes [55]. In fact, FVT1, the mammalian homologue of KSR, can replace yeast KSR in DHS synthesis [56], suggesting a conserved role of the enzyme across species. Although FVT1 and fungal KSR share some sequence homology, these proteins are predicted to differ structurally [57]. Therefore, molecules targeting unique regions of fungal KSR might act as potent inhibitors of fungal differentiation.

The production of DHS represents a branching point in the sphingolipid synthesis [58]. DHS can be acylated by ceramide synthase, forming dihydroceramide, the precursor of the neutral glycosphingolipids (GSLs) as glucosylceramide (GlcCer). Alternatively, DHS can be hydroxylated, generating phytosphingosine (PHS), which will be used in the synthesis of acidic glycosphingolipids as inositolphosphoryl ceramides (IPCs) and mannosyl inositolphosphoryl ceramides (MIPCs). Distinct ceramide synthases use DHS or PHS as substrates; therefore, GlcCer and IPCs differ not only in their polar group (glucose or glycosylphosphoinositol), but also in the ceramide backbone structure (please compare GlcCer and IPC structures in Figure 2A) [55].

DHS conversion to dihydroceramide is catalyzed by Bar1p/BarA and its orthologs Lac1 in *C. albicans* and Cer1 in *C. neoformans*. The *barA* gene was first identified in a screen of *Aspergillus* mutants resistant to the antifungal compound HSAF [59]. Bar-like ceramide synthases show a preference for dihydroxi sphingoid bases and C16–18 fatty acids [60]. In addition, the disruption of *barA* or its homologs abolishes GlcCer synthesis, suggesting that dihydroxi ceramides bearing C16–18 fatty acids are the building blocks of GlcCer production [60–63]. Curiously, the role of this class of ceramide synthases diverge in fungal species. For instance, the *C. albicans lac1*Δ/*lac1*Δ mutant showed hyphal growth and lipid raft organization in hyphal tips similar to the wild type control [61]. In contrast, the disruption of *Aspergillus barA* impaired growth and raft formation [59]. *Cryptococcus* Cer1 was crucial for *in vitro* growth under acidic and neutral/alkaline conditions, mimicking the environments found in the alveolar macrophages and alveolar space respectively [63]. In agreement with these observations, lack of Cer1 rendered *C. neoformans* cells avirulent [63] . These results suggest that inhibitors of dihydroceramide synthesis might be more efficacious against *Aspergillus* and *Cryptococcus* infections rather than invasive candidiasis.

DHS can also be hydroxylated to phytosphingosine (PHS) by sphingolipid C4 hydroxylase prior to production of phytoceramide [58]. In *Aspergillus*, the gene encoding the C4 hydroxylase, *basA*, is essential for fungal viability [64]. This suggests that inhibitors of the C4 hydroxylase might compromise fungal virulence and pathogenesis.

The acylation of PHS with very long-chain fatty acids (VLCFA, C24 or C26) and consequent formation of phytoceramide is catalyzed by a clade of ceramide synthases that differ from those involved in dihydroceramide production [58,60]. LagA is an essential ceramide synthase in *Aspergillus*, producing the bulk of sphingolipids required for hyphal growth [59]. Similarly, the disruption of *Candida LAG1* gene also caused severe defects in polarized growth [61].

Besides the bulk of ceramides produced by *de novo* pathway as described above, this molecule can also be generated through the salvage pathway. This metabolic route occurs in the acidic lysosomes, where complex SL are broken down to sphingosine [65]. Finally, the sphingosine is re-acylated by ceramide synthases, producing ceramide [65].

The inhibition of ceramide synthesis can also lead to increased levels of DHS and PHS. These sphingoid bases inhibit *Aspergillus* growth and induce death by apoptosis [66]. Therefore, drugs targeting the ceramide synthases might be potent antifungals, showing a dual mechanism of action: depletion of the sphingolipids content and accumulation of toxic sphingoid bases. So far, two inhibitors of fungal ceramide synthase have been reported. Fumonisin B1 is a mycotoxin produced by *Fusarium* that competes with the sphingoid base as an enzyme substrate and also affects the binding of the fatty acid chain [67,68]. Although fumonisin B1 inhibits the ceramide synthases, this compound apparently showed poor antifungal activity [68]. In contrast, australifungin, a compound isolated from *Sporormiella australis*, inhibits the ceramide production and show a broad-spectrum activity against *Candida*, *Aspergillus* and *Cryptococcus* [69]. The use of these mycotoxins in the antifungal therapy has been limited due to their high reactivity and lack of specificity towards the fungal ceramide synthase, also inhibiting the mammalian enzyme [68,69]. In fact, fumonisin

B1 is toxic to animals and causes hepato and nephrotoxicity in rodents [70,71]. A fluorescent assay to detect ceramide synthase activity has been reported [72], enabling the screen of chemical libraries for more selective compounds, active against the fungal but not the mammalian enzyme.

3. The GlcCer synthesis

Dihydroceramide is the main substrate used by the sphingolipid Δ4-desaturase enzyme, which catalyzes a C4-reduction in the sphinganine backbone, producing ceramide [73,74]. This reaction occurs in both fungi and mammals [74], and the relevance of *(E)*-sphing-4-enine for fungal growth and virulence remains to be elucidated.

The unsaturation between C8–C9 and methylation in C9 of the LCB are catalyzed by sphingolipid Δ8-desaturase (Sld) and sphingolipid C9-methyltransferase (Smt) , respectively, generating Δ8–9,Me-Cer. These structural modifications are not observed in mammalian cells, which produce Cer. In *Aspergillus*, the deletion of sphingolipid Δ8-desaturase encoding gene compromised growth and the formation of sterol-rich rafts in the hyphal tip [75]. *Candida* cells lacking sphingolipid Δ8-desaturase also exhibited impaired hyphal growth, along with a striking reduction in fungal virulence [76,77]. Curiously, the C9-methylation of LCB had a modest relevance for *Candida* infection, but was crucial for *Cryptococcus* pathogenesis [77,78]. In fact, *C. neoformans* cells deficient in *SMT1* gene were not able to replicate in the murine lung, being restricted in granulomas [78]. These observations suggest that potential inhibitors of the sphingolipid C9-methyltransferase might be more effective against *Cryptococcus* than *Candida* infections. The pharmacological inhibition of the sphingolipid Δ8-desaturase remains as a promising strategy for novel antifungals, especially for the treatment of invasive candidiasis. The role of the sphingolipid Δ8-desaturase in *Cryptococcus* and *Aspergillus* virulence needs to be further investigated, so the use of desaturase inhibitors as panfungal drugs can be considered.

The last step of the GlcCer synthesis is the transfer of a glucose residue from UDP-glucose to the ceramide backbone [79]. This reaction occurs in the Golgi through the activity of glucosylceramide synthase (GCS) [80], which plays a key role in fungal virulence. Indeed, *C. neoformans* cells lacking *GCS1* are avirulent in a murine model of cryptococcosis [81]. As the deletion of *GCS1* also impaired *Cryptococcus* ability to grow at neutral/alkaline pH, it was suggested that GlcCer production is crucial for the colonization of alveolar spaces and the host extracellular environment [81]. The GlcCer synthesis is also important for the growth and virulence of *C. albicans* and *A. nidulans* [75,77,82]. Due to the relevance of GlcCer to fungal pathogenesis, GlcCer synthase inhibitors and targeting of GlcCer itself have already been reported. Levery *et al.* (2002) showed that analogs of the D-*threo*-PDMP (D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), known inhibitors of the mammalian GlcCer synthase, also prevented germination and hyphal growth in *Aspergillus* [83]. The development of new antifungals from D-*threo*-PDMP relies on the synthesis of fungal-specific compounds which ideally lack toxicity to the host.

In contrast to the PDMP derivatives, the acylhydrazones BHBM and D0 inhibited the GlcCer synthesis in fungi but not in mammalian cells [84]. BHBM and D0 also showed a

broad spectrum of antifungal activity, efficiently improving the survival of mice infected with *C. neoformans* and *C. albicans* [84]. Derivatives of these acylhydrazones retained the fungicidal activity *in vitro* and *in vivo*, while showing suitable pharmacokinetic properties and even lower toxicity towards mammalian cells [85,86]. Therefore, these compounds constitute promising candidates for antifungal therapy.

The immunological targeting of GlcCer might represent an efficacious strategy to prevent mortality caused by fungal infections. In fact, the administration of an anti-GlcCer antibody prior to a lethal *Cryptococcus* infection improved mice survival by 60% [87]. Vaccination of mice with the GlcCer extracted from the non-pathogenic *Candida utilis* also protected partially against cryptococcosis, preventing fungal dissemination to the brain [88]. These results highlight the potential of GlcCer synthesis pathway for: i) the development of novel inhibitors that treat invasive infections and ii) the isolation of lipids that differ structurally from the mammalian counterparts and can be used as antigens in fungal vaccines, preventing mortality, especially in the individuals more susceptible to the invasive mycoses.

4. IPC synthesis

IPCs are produced by the transfer of a phosphoinositol from phosphatidylinositol to the C1-hydroxyl group of phytoceramide [89]. This reaction is catalyzed by the product of the *IPC1* (or *AUR1*) gene, which is essential in fungal species [90,91]. The conditional repression of *IPC1* in *C. neoformans* reduced growth under acidic conditions, such as the macrophage intracellular environment, impairing the virulence in rabbits [92]. Similarly, the genetic inactivation of *Aspergillus aurA* was accompanied by growth defects in spores and germlings, which were unable to establish a normal polarity axis [46]. Since the IPC synthase plays a pivotal role in fungal virulence and is not found in mammalian cells, this enzyme constitutes a potential target for drug development. In fact, the IPC synthase encoding gene was first identified in *S. cerevisiae* mutants resistant to the compound aureobasidin A [90]. Aureobasidin A, a cyclic peptide isolated from *Aureobasidium pullulans*, inhibits the IPC synthase at nanomolar concentrations and shows antifungal activity against *Candida*, *Cryptococcus* and *Aspergillus* [93–95]. In addition, aureobasidin A shows low toxicity in mice and prolonged survival in a murine model of candidiasis [93]. The efficacy of aureobasidin A in treating infections caused by *Cryptococcus* and filamentous/dimorphic fungi remains to be determined. Other potent drugs that inhibit IPC synthase at nanomolar levels include khafrefungin and galbonolide A (rustmicin) [96,97]. Khafrefungin showed fungicidal activity against *C. albicans* and *C. neoformans*, promoting the accumulation of ceramides [98]. This suggests that Ipc1 relevance is related to the regulation of phytoceramide levels, which control growth arrest and stress response [99,100], rather than to the IPC production itself. Indeed, the conversion of phytoceramide to IPC enable *C. albicans* to tolerate fluconazole concentrations above the MIC (minimal inhibitory concentration) [101]. Inhibition of IPC synthase and consequently accumulation of phytoceramides abolish the tolerance phenotype, rendering fluconazole a fungicidal effect [101]. Therefore, the tight regulation of phytoceramide levels seems to play a crucial role in fungal survival and growth, including the slow growth of azole-tolerant subpopulations. Galbonolide A was mainly active against *C. neoformans in vitro* and reduced the brain fungal burden in a murine model of cryptococcosis [102]. However, this drug showed poor

stability in different pHs, quickly degrading into inactive derivatives [102]. SAR (Structure Activity Relationship) studies of galbonolide A derivatives might enable the development of stable compounds showing broad-spectrum activity.

To generate MIPC, the IPC is further mannosylated by MIPC synthase, which is encoded by the *MIT1* and *mitA* genes in *Candida* and *Aspergillus*, respectively [103,104]. The deletion of *C. albicans MIT1* did not impair growth or morphogenesis, but attenuated virulence in at least 50% [103]. In contrast, *A. fumigatus* cells lacking MitA were as virulent as the wild-type control [104]. So, inhibitors of the MIPC synthase might reduce the lethality of invasive candidiasis, albeit showing limited spectrum of activity.

The $M(IP)_2C$ synthase adds a second inositol phosphate to the MIPC, forming mannosyl diinositol diphosphoceramide, $M(IP)_2C$. In *C. albicans*, the disruption of the *IPT1* gene, encoding for the $M(IP)_2C$ synthase, led to the accumulation of MIPC and increased sensitivity to fluconazole and itraconazole [105]. The length of the fatty acid chain composing MIPC and $M(IP)_2C$ also affects azole susceptibility. Recently, it was shown that inactivation of *FEN1* or *FEN12* genes promote fluconazole resistance in *C. albicans* [106]. *FEN1* and *FEN12* encode fatty acid elongases, involved in the synthesis of VLCFAs which are transferred to phytosphingosine to form phytoceramide [106]. Indeed, the deletion of *C. albicans FEN1* or *FEN12* reduced the content of IPCs and MIPCs with 26-carbon fatty acids [106]. In the presence of fluconazole, Δ*fen1* and Δ*fen12* mutants produced higher levels of MIPC and DMCDD [14,24-dimethylcholesta-8,24(28)-dien-3β,6α-diol] than the wild-type strain [106]. DMCDD is the toxic sterol accumulated by Erg11 inhibition, which causes membrane permeabilization [107]. Together, these results suggest that the production of complex SL containing very long fatty acids mediates fungal resistance to fluconazole, possibly by reducing DMCDD insertion into the membrane and attenuating the azoles' effect.

5. The biological level of sphingolipids within fungal cells

GlcCer is the most abundant sphingolipid in *C. neoformans*, corresponding to 16 – 45 % of total SL content [108]. In contrast, IPCs correspond to 2.4 – 4.4 % of SL production [108]. Similarly, sphingolipidomic analysis suggest that GlcCer is also more abundant than IPCs in *A. fumigatus* [109]. To the best of our knowledge, the relative levels of GlcCer are not determined in Candida yet. Interestingly, in Candida, the IPC levels were associated with several processes, such as biofilm formation and susceptibly to azoles [110,111]. Variations in the SL level are also observed across fungal species: while IPC levels are higher than MIPC and $M(IP)_2C$ in Candida [111], $M(IP)_2C$ corresponds to the most abundant SL in S. cerevisiae [112]. Finally, LCB species (DHS, sphingosine and PHS) were found in C. neoformans in very low levels, accounting for 1.2 – 2.6 % of total SLs [108]. Together, these results suggest that SL content is highly regulated, draining the toxic LCBs into complex sphingolipids. Therefore, drugs altering the SL homeostasis might act as potent antifungals.

5. Expert opinion

The development of new drugs targeting sphingolipid synthesis might represent a therapeutic alternative to the antifungals that are currently available, overcoming some of the limitations faced when using azoles, polyenes and echinocandins. The polyene main prototype, amphotericin B, binds to ergosterol [5], utilizing the structural differences found between the fungal and mammalian sterol. Still, amphotericin B shows toxicity towards host tissues, possibly due to unspecific binding to cholesterol [13]. Fungi produce two main classes of sphingolipids, neutral (GlcCer/GalCer) and acidic (IPC, MIPC, $M(IP)_2C$). Like with ergosterol and cholesterol, fungal and mammalian GlcCer also show distinct architectures [79], and one might expect that inhibitors of the fungal GlcCer show some level of toxicity in higher eukaryotes. However, this does not seem to always be true as acylhydrazones were not toxic to mice [84]. Off-target effects caused by inhibiting the synthesis of IPC are also rare, as mammals lack the production of these sphingolipids.

Besides drug selectivity, another challenge in the development of new antifungals is to find molecules that show fungicidal rather than a fungistatic effect. Compounds that impair fungal growth, not leading to cell death, require the host immune system to clear the infection [113]. Many of the reactions involved in the SL synthesis are crucial for the fungal viability, suggesting that inhibitors might have fungicidal activity by inducing a lethal phenotype. Indeed, the inhibition of the IPC synthase by aureobasidin A killed *C. albicans* cells [93], likely by phytoceramide accumulation. Interestingly, drugs that target non-essential enzymes such as acylhydrazones can also exhibit fungicidal activity [86]. This might be explained by the fact that GlcCer regulates cell cycle progression in *C. neoformans* [81]. Therefore, the depletion of certain SL which regulate growth arrest, rather than the accumulation of toxic intermediates, might also cause a fungicidal phenotype.

Several questions remain to be elucidated by future studies. Current antifungals exhibit an underlying effect as immune modulators [114]. As GlcCer administration provoked a protective response for *Cryptococcus* infection in mice [88], it'd be valuable to determine if compounds targeting SL synthesis also induce a direct stimulation of immune cells, facilitate phagocyte activity or "unmask" potential antigens. In addition, GlcCer production has been reported in several species, including dimorphic and filamentous fungi (for review, [115]). If the GlcCer vaccination prevents disseminated infections caused by distinct species, it might constitute a valuable tool for prophylaxis.

Further advances in the use of SL inhibitors for antifungal therapy depend on some advances in this field of research. The structural determination of proteins involved in the SL synthesis pathway would bring new insights about their activity and regulation, enabling the design of novel inhibitors and high-throughput screenings. In addition, SAR studies of inhibitors that already have been reported would elucidate compounds' groups that drive antifungal activity and toxicity towards mammalian cells. This would allow the development of potent myriocin, fumonisin B1 and aureobasidin A derivatives that do not show unspecific or off-target effects in the host (reviewed by [116]).

SL synthesis inhibitors that emerge as promising candidates for clinical trials include the acylhydrazones and aureobasidin A derivatives. In fact, aureobasidin A derivatives that show enhanced activity against yeasts and filamentous fungi have been developed [13]. Importantly, aureobasidin A constitutes a promising candidate for combination therapy with fluconazole, as improved its antifungal activity [101]. SAR studies also enabled the synthesis of second and third acylhydrazone derivatives, with increased potency and selectivity towards fungal cells [86]. Therefore, acylhydrazones and aureobasidin A derivatives arise as new, promising drugs to be used in antifungal therapy.

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Article highlights

- Fungal sphingolipids play a crucial role in growth and virulence.
- The unique structure of fungal sphingolipids makes them promising targets for drug development.
- Novel inhibitors of ceramide synthases might act as potent drugs, by depleting the pool of complex sphingolipids and leading to the accumulation of toxic intermediates.
- Promising compounds for clinical trials include acylhydrazones and aureobasidin A, which inhibit the production of glucosylceramide and IPC, efficiently treating invasive fungal infections.

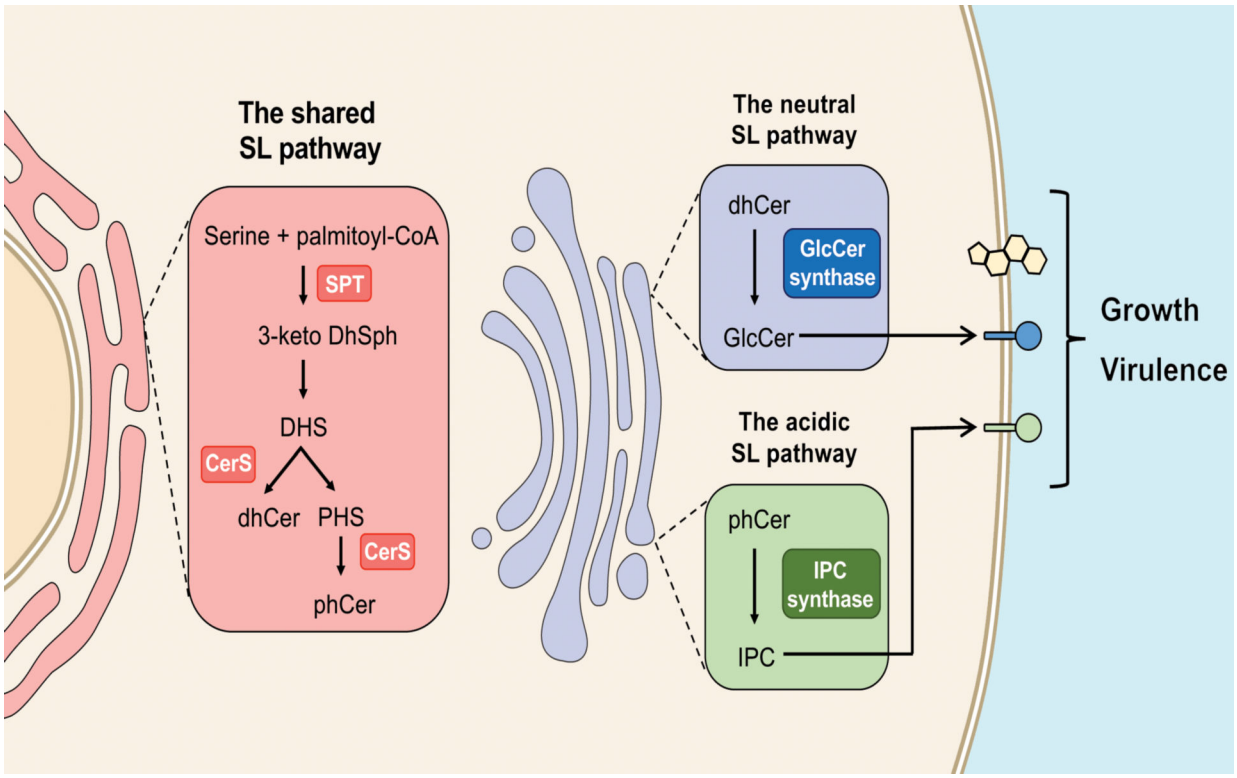

Figure 1.

Schematic representation of the mechanism of action of azoles, polyenes and echinocandins. Both the polyenes and azoles disrupt the major fungal sterol, ergosterol. Whilst the polyene amphotericin B binds to ergosterol and disrupts membrane function, the azoles inhibit 14α-demethylase enzyme, impairing ergosterol synthesis. β−1,3-glucan synthase, a key enzyme for cell wall integrity, is inhibited by the echinocandins.

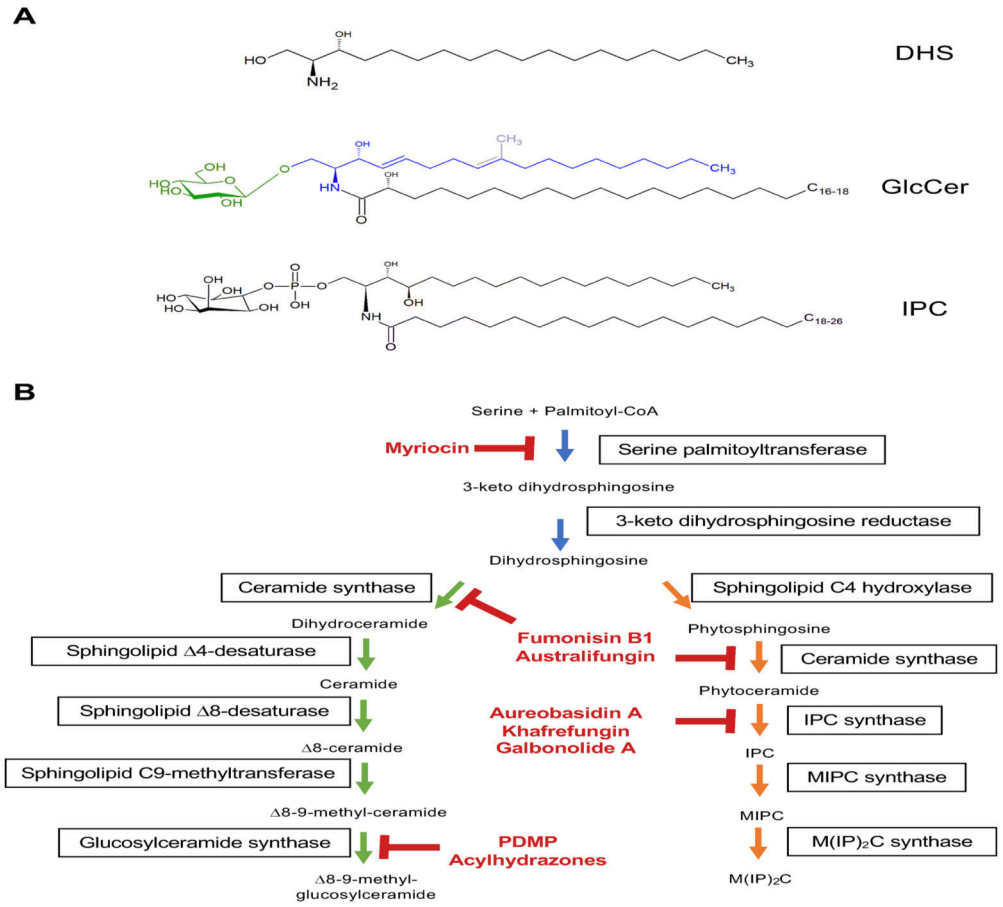

Figure 2.

(A) Structure of dihydrosphingosine, glucosylceramide (GlcCer) and inositol phosphorylceramide (IPC). The sugar and the long-chain base in GlcCer are highlighted in green and blue, respectively. The C8-unsaturation and C9-methyl group, present in the fungal but not in the mammalian GlcCer, are shown in gray. (B) The sphingolipid synthesis pathway in fungi. Inhibitors of SL production are highlighted in red and include: myriocin (targets Spt); fumonisin B1 and australifungin (targets ceramide synthases); aureobasidin A, khafrefungin and galbonolide A (targets the Ipc synthase); PDMP analogs and acylhydrazones (targets GlcCer synthase).

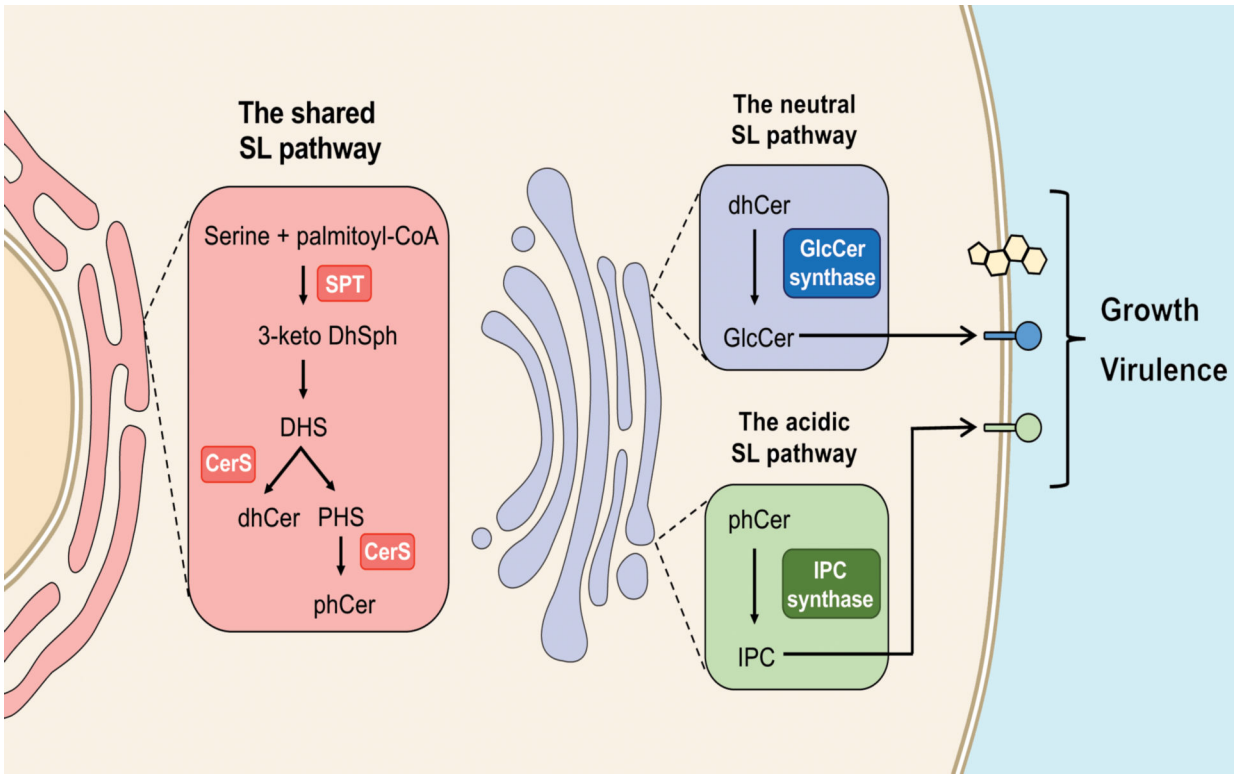

Figure 3.

Sphingolipid synthesis starts in the endoplasmic reticulum, with the reaction catalyzed by Spt. Once produced by distinct ceramide synthases (CerS), dihydroceramide (dhCer) and phytoceramide (phCer) are transported to the Golgi for the synthesis of complex sphingolipids (GlcCer and IPC). The conversion of toxic phCer to IPC and the production of GlcCer regulate fungal growth and virulence.