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Plasma membrane lipids and their role in fungal virulence

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There has been considerable evidence in recent years suggesting that plasma membrane lipids are important regulators of fungal pathogenicity. Various glycolipids have been shown to impart virulent properties in several fungal species, while others have been shown to play a role in host defense. In addition to their role as virulence factors, lipids also contribute to other virulence mechanisms such as drug resistance, biofilm formation, and release of extracellular vesicles. In addition, lipids also affect the mechanical properties of the plasma membrane through the formation of packed microdomains composed mainly of sphingolipids and sterols. Changes in the composition of lipid microdomains have been shown to disrupt the localization of virulence factors and affect fungal pathogenicity. This review gathers evidence on the various roles of plasma membrane lipids in fungal virulence and how lipids might contribute to the different processes that occur during infection and treatment. Insight into the role of lipids in fungal virulence can lead to an improved understanding of the process of fungal pathogenesis and the development of new lipid-mediated therapeutic strategies.

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1. Introduction

Lipids are essential molecules that serve diverse structural and signaling functions in multiple organisms. In recent years, due to the emerging interest in lipids as therapeutic targets, numerous studies have begun to address their biological properties and immunological functions. Most

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importantly, certain glycosphingolipids, such as glucosylceramide (GlcCer), have been shown to be involved in the regulation of virulence in fungi affecting plants [1,2] and humans [3,4]. This regulation is important for pathogenicity of fungi and opens avenues for new, lipid-targeted, therapeutic approaches. For example, blocking the synthesis of fungal GlcCer can be achieved by pharmacological approaches without affecting the synthesis of mammalian GlcCer [5]. This is important because drugs that directly target fungal enzymes and are not toxic towards mammalian cells are not available. There is still much to know on the role of lipids in fungal virulence, highlighting the need for more mechanistic studies on how lipids regulate fungal pathogenicity.

The physical properties of membrane lipids in pathogenic fungi have received significant attention in recent years. Studies have shown that lipid microdomains consisting of glycosphingolipids and sterols might serve to concentrate virulence factors [6,7] and affect infectivity [8] and pathogenicity [9]. Thus, the physical properties of the plasma membrane appear to affect the outcome of the infection. In this review, an account of the recent studies exploring the role of lipids in fungal virulence will be provided and discussed. In the first part, lipids that have been characterized as fungal virulence factors will be introduced and their structure and function will be examined. In the second part, the role of lipids in regulating the physical properties of the fungal membrane and the role of membrane lipids in fungal pathogenicity will be discussed.

2. Lipids as fungal virulence factors

Until recently, limited data were available on the virulence factors for medically relevant fungi. A virulence factor is defined as any fungal component or mechanism that contributes to its pathogenicity in a susceptible host [10]. With the advent of new molecular biology techniques such as genetic manipulation, rapid progress has been made to reveal and disrupt the most important virulence factors in fungi. This has important consequences, as studying these factors could provide novel therapeutic avenues to treat fungal infections.

Fungi have many biological properties that are considered virulence factors, which facilitate their ability to colonize and proliferate in human hosts. Established virulence factors include: 1) the ability to grow at 37 °C and physiological pH [3,10] 2) secretion of many proteases, distinctive of *Aspergillus* species [11–13] 3) production of a thick polysaccharide capsule and the synthesis of melanin, observed in *Cryptococcus* species [14] 4) production of 1,3-glucan carbohydrate polymer, a virulence factor in *Paracoccidioides brasiliensis* and *Histoplasma capsulatum* [15–17] 5) having a spore size, which is comparable to alveolar space dimensions; this is particularly important for fungi that cause infection by inhalation 6) secretion of phospholipase enzymes in *Candida species*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* [18,19] and, 7) modulation of lipid metabolism, characteristic of *Cryptococcus* species and dimorphic fungi [20] [21].

Other less established virulence factors are proteins (e.g. glucosylceramide synthase 1 (Gcs1), sphingolipid C9 methyltransferase 1 (Smt1), sterylglucosidase 1 (Sgl1), superoxide dismutase (Sod)) and very few lipids. Importantly, recent characterization has revealed that fungal glycolipids have a unique structure, which is different from mammalian glycolipids and has ignited a considerable interest in these microbial molecules as therapeutic targets. The following section will focus on lipids as virulence factors, in particular we will discuss new insights on how glycolipids, such as sterylglucosides and glucosylceramide, regulate fungal pathogenicity.

2.1. Sterylglycoside

Sterylglycosides (SGs), are found in plants, algae, and fungi, but are rarely distributed in bacteria and mammalian cells [22,23]. While the literature is beginning to appreciate the functions of SGs in mediating immune cell proliferation and activation, the underlying mechanisms of proliferation as well as the functional significance of SGs are largely unknown and few studies have attempted to answer these questions. In the following sections, a review of the structure of fungal SGs, differences between fungal and mammalian SGs (glycosylated-cholesterol), specific functions of SGs as regulator of host immune response, and an overview of the role of SGs in fungal virulence, will be provided. The role of SGs as anti-fungal and anti-cancer compound will also be discussed.

2.1.1. Structure of sterylglycoside

SGs are sugar derivatives of a membrane-bound sterol. The sterol consists mainly of sitosterol, sigmasterol, and campesterol in plants [24], ergosterol in fungi [25], and cholesterol in animals [26]. SGs are characterized by a planar sterol backbone made up of four condensed aliphatic rings and a hydrocarbon side chain at C17 with the sugar moiety attached to the 3β-hydroxy group at carbon 3 of the sterol. A double bond is also present between C5 and C6 (Δ^5-sterol) and/or between C7 and C8 (Δ^7-sterol) in the second ring. SG structures can vary significantly from plants to animals depending on the sterol heterogeneity, the number and type of sugar moieties, and the presence of an acyl group attached to the sugar moiety. The most common sugar moiety is the pyranose form of D-glucose in β configuration [22,23]. The most abundant SG chemical structure in fungi is ergosterol-3β-glucoside, which has been characterized by mass spectrometry and two dimensional nuclear magnetic resonance analyses (NMR) by various research groups (Fig. 1) [27,28].

2.1.2. Function of sterylglycoside

Studies on the role of SGs have shown that these glycolipids are important regulators of the host immune response to fungal infections. For example, administration of plant SGs has been associated with better outcome in pre-clinical murine models of fungal infections. In particular, the administration of daucosterol (DS-plant β-sitosterol glucoside) and ginsenoside Rg1 (steroid glycoside) in mice prior to a challenge with *Candida albicans* has been reported to improve their survival [29, 30]. The protective function of the SGs was abrogated when mice were pre-treated with anti-$CD4^+$ antibody. This suggested that $CD4^+$ T cells may be required for the protective effect of SGs. Indeed, ELISA analysis on primed splenic $CD4^+$ T cells from DS or ginsenoside-treated mice revealed higher production of Th1 cytokines (INF-γ and IL-2) compared to Th2 (IL-4 and IL-10) suggesting that SGs might change Th1/Th2 balance towards a more dominant Th1 response.

Our studies in *C. neoformans* reported similar protective effects when the endogenous pool of fungal SGs was manipulated. The *C. neoformans* strain lacking the sterylglucosidase gene (Δ*sgl1*), accumulated SGs and was non-pathogenic in a murine model of cryptococcosis. Additionally, Δ*sgl1* acted as a vaccine since mice exposed to this strain survived a subsequent challenge with a lethal dose of *C. neoformans* wild-type serotype A H99 or *Cryptococcus gattii* R265. Interestingly, this effect was also observed in mice depleted of $CD4^+$ T-cells suggesting potential benefits in human immunodeficiency syndromes targeting the $CD4^+$ T-cells such as HIV/AIDS [21]. Although the molecular mechanism(s) of this protection is under investigation, these studies clearly suggest that the loss of Sgl1 and/or accumulation of SGs not only abolishes fungal virulence, but also protects the host from a subsequent infection. Thus, further studies focused on the underlying mechanisms of SG-induced immunity are needed. For instance, the administration of purified fungal SGs, or fungal membranes, could provide a potential prevention strategy in susceptible patients or/and a supplemental approach to boost the host immunity along with the administration of the standard antifungal therapy.

In addition to fungal infections, SGs appear to play a protective role in patients suffering from allergic diseases such as rhinitis and sinusitis in which the administration of SGs promoted beneficial effects and general recovery [31,32]. Also, in patients with pulmonary tuberculosis, the oral intake of certain SGs, along with the administration of regular treatment, was shown to increase the lymphocyte and eosinophil counts and

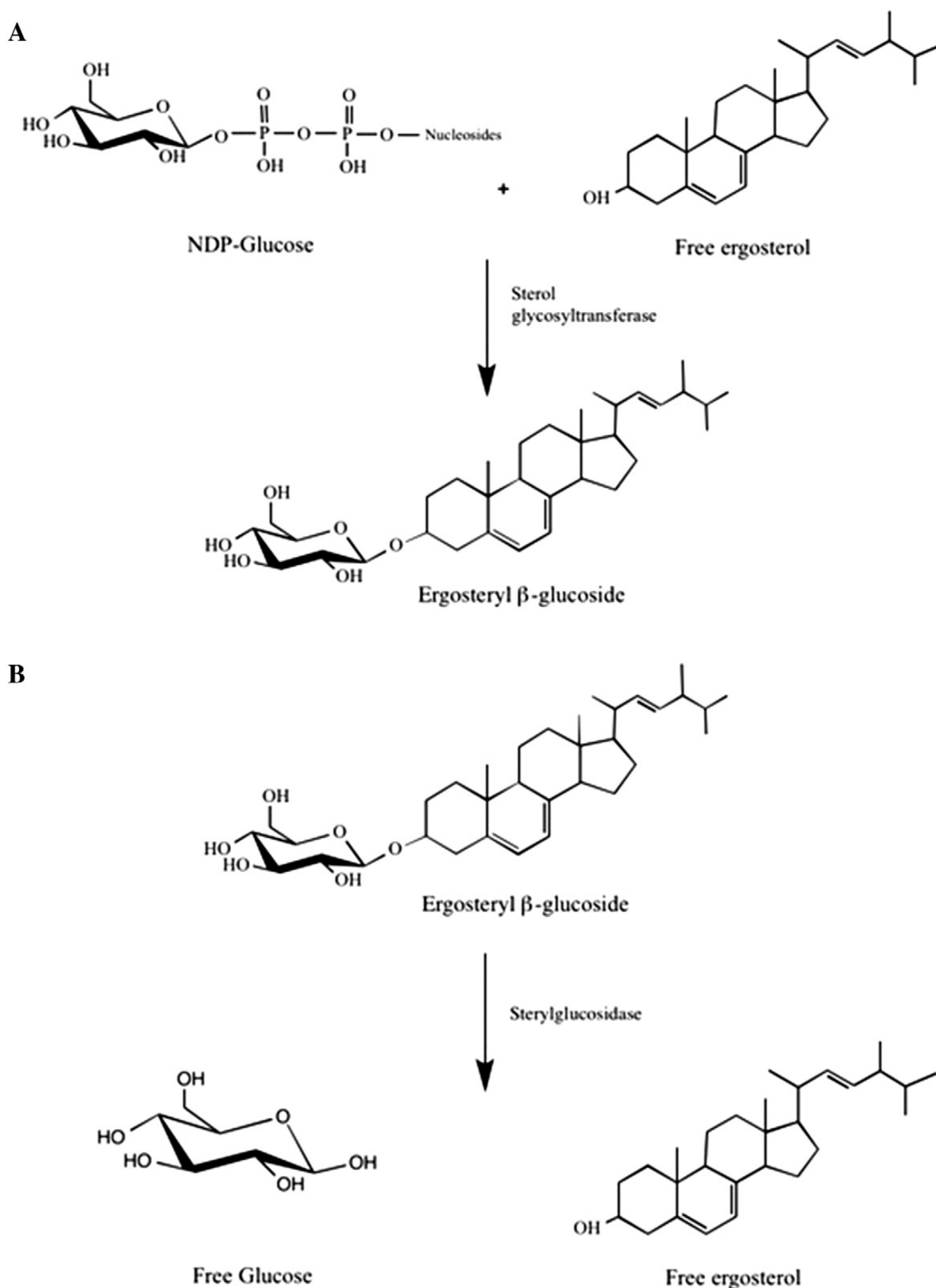

Fig. 1. Schematic representation of ergosteryl β-glucoside biosynthesis and breakdown. A) The first step in the sterylglucosides (SGs) biosynthesis is the condensation of NDP-glucose with free sterol by a sterol glycosyltransferase. B) The degradation of SGs is due to the sterylglucosidase enzyme. The absence of the sterylglucosidase results in a dramatic accumulation of SGs.

improve patient outcome [31]. Similar proliferative effects, due to SG administration, have been observed on mononuclear cells obtained from healthy volunteers [33].

Recent studies are exploring the tumor suppressive properties of SGs [32,34]. SGs isolated from *Citrus aurantium* and supplemented to colon cancer cells, resulted in the inhibition of cell proliferation and induction of cell cycle arrest [35]. Similar studies showed that administration of SGs, isolated from *Castanopsis indica* leaves, induces apoptosis in Ehrlich's ascites carcinoma cells. This effect was attributed to the induction of DNA damage and up-regulation of the DNA damage effectors such as p53 and p21 [36]. Whether this effect is specific to malignant neoplasms in comparison to normal tissues, or whether it is tumor-type specific, remains to be seen. Taken together, and albeit the scarce literature available on their biological functions, the potential use of

SGs as anti-fungal/anti-cancer therapeutic compounds is paving the way for a new exciting area to explore with great benefit to human health.

2.1.3. Sterylglucosides transferases and hydrolases as virulence factors

The first reaction in the SGs biosynthetic pathway is the conversion of free sterol into SGs by the sterol-sugar dependent transferase (Fig. 1A) [22]. This enzyme transfers fatty acid, sugar and phosphatidyl residues, to form, respectively, acyl sterylglucoside, steryldiglucoside or oligoglucoside and phosphatidyl steryl glucosides. In contrast, very limited literature is available on the steryl hydrolase, the enzyme that degrades SGs into sterol and glucose. Sterylglycosyltransferases and sterylglucosidases have been identified in fungi, yeast and plants [27, 37–42]. The first sterolglycosyltransferase enzymes were described in fungi in 1999 by Warnecke and colleagues [37]. The UDP-glucose:sterol glucosyltransferase genes from *Saccharomyces cerevisiae* (gene *UGT51*), *Pichia pastoris* (gene *UGT51B1*), *C. albicans* (gene *UGT51C1*) and the mold *Dictyostelium discoideum* (gene *UGT52*) were identified by amino acid sequence similarities with the plant sterol glucosyltransferases (Ugt80A1, Ugt80A2) [37–39]. The expression products of these genes were purified from *Escherichia coli* and tested *in vitro* for SGs synthase activity using various radiolabeled sugar donors and sterols.

Recently, the first sterylglucosidase in fungi, endoglycoceramidase-related protein 2 (*EGCrP2*), was identified and characterized [27]. This protein exhibited glucosylceramidase activity on short chain C_6-NBD-GlcCer [43]. The disruption of EGCrP2 gene in *C. neoformans* resulted in the accumulation of ergosteryl-β-glucoside (Fig. 1B), but not GlcCer [27]. The homolog of EGCrP2 in *S. cerevisiae*, Egh1 (ORF name, Yir007w), was also recently described. The disruption of the *EGH1* gene in *S. cerevisiae* (Δ*egh1*) also resulted in accumulation of sitosteryl β-glucoside, otherwise undetectable in the wild type strain. Egh1 showed catalytic activity towards SGs and short chain C_6-NBD-GlcCer [40]. In agreement with Watanabe et al. we observed that EGCrP2 showed catalytic activity towards short chain C_6-NBD-GlcCer but not towards endogenous long chain GlcCer [21]. In addition, we showed that EGCrP2 exhibits a clear activity towards endogenous SGs. Given that C_6-NBD-GlcCer is a synthetic lipid, and considering that *in vivo* the most abundant species are long chain GlcCer, we suggested to re-name the EGCrP2 to sterylglucosidase 1 (Sgl1) to underline its specific activity towards SGs [21].

The Sgl1 enzyme is an important virulence factor that controls the intracellular fungal levels of SGs [21,22,37,44–49] in order to prevent a host immune response against the pathogen. The study of tissue burden after administration of Δ*sgl1* in murine models revealed a continuous decrease of Δ*sgl1* cells in the lungs starting at the third day post-infection in comparison to wild type. Furthermore, no fungal cells were found in the brain [21]. Since Δ*sgl1* possessed similar growth profile and similar common virulence factors of *C. neoformans*, we hypothesized that, following inhalation of Δ*sgl1*, the host immune response is rapidly activated, leading to the elimination of Δ*sgl1* in the lungs and preventing its dissemination to other organs such as the brain. The tightly regulated control of the levels of SGs is, most probably, an evolutionary mechanism developed by fungi to escape the host immune system. This can be achieved either through a basal activation of steryl glucosidase or through a functional balance that could exist between the synthesis and the catabolism of SGs and, in particular between the enzymes steryl glucosyltransferase and glucosidase, to maintain a basal level of SGs in physiological conditions.

SGs could also have diverse functions as illustrated in the bacterium *Helicobacter pylori. H. pylori* is a Gram negative bacterium, that causes chronic gastritis and predisposes to gastric cancers. It acquires cholesterol from the plasma membrane of gastric epithelial cells, which leads to their disruption. However, acquisition of high levels of cholesterol activates a T-cell dependent immune response. To circumvent this response, the bacterium glucosylates cholesterol to form cholesterol–glucosides. This glycosylation allows the bacterium to be "invisible" to the host immune response [50]. While the fungi have engineered mechanisms to down-regulate the level of SGs to avoid boosting of the host immuno-response, in *H. pylori*, the glycosylation of cholesterol is used as a mechanism to escape the host immune response. Although the functions of SGs in fungi and in *H. pylori* are different, both microorganisms regulate and modulate the levels of SGs in a way that is beneficial for the pathogen.

2.2. Glucosylceramide

Glucosylceramide (GlcCer) is a glycosphingolipid that is found in plants, fungi, and animals, but is absent in bacteria and some eukaryotes such as *S. cerevisiae*. In the following sections, the structure of GlcCer and its role in many biological events such as alkali tolerance, polar growth, morphogenesis, cell membrane physiology, budding, and cell division is discussed. Particular attention has been given to the enzymes involved in GlcCer biosynthesis and the newly discovered *C. neoformans* glucosylceramidase (Fig. 2).

2.2.1. Structure of glucosylceramide

GlcCer is synthetized in the Golgi apparatus and reaches the plasma membrane where it is primarily localized. It has also been found in the cell wall, mostly at the budding sites of dividing cells [51,52], and in extracellular vesicles [53,54]. The molecular structure of GlcCer varies significantly among microorganisms, resulting in a unique chemical structure specific for each organism [54–58]. For example, in fungi GlcCer possesses a β-linked glucose and ceramide backbone. The latter is composed of a characteristic sphingoid base with double bonds at carbon 4 and carbon 8 in trans conformation and a methylation in position 9 linked to a fatty acid via an amide bond. Mammalian GlcCer possesses glucose attached to ceramide via a β-glycosidic bond. Ceramide is composed of a sphingosine backbone linked to a fatty acyl chain via an amide bond at carbon 2. The sphingosine backbone includes a hydroxyl group at carbon 1 and 3 as well as a double bond at carbon 4. One of the major features that distinguishes mammalian and plants from fungal GlcCer is the absence of the C9-methyl group on the sphingoid base (Fig. 2).

2.2.2. Functions, synthesis and degradation of glucosylceramide

The sphingolipid biosynthetic pathway starts with the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyl transferase to generate 3-ketodihydrosphingosine [59]. The latter is reduced to form dihydrosphingosine, which is acylated by ceramide synthases to produce α-hydroxy-ceramide. This structure is then desaturated by Δ4, Δ8-desaturase and methylated by C9-methyl transferases to form mature ceramide [55]. GlcCer synthesis requires the transfer of glucose from UDP-glucose to ceramide by glucosyl-ceramide synthase-1 (Fig. 2) [3]. Leipelt and colleagues were the first researchers to characterize and clone the glucosylceramide synthase in fungi, and in particular in *C. albicans*, *Caenorhabditis elegans*, and *P. pastoris*, paving the way for more genetic and pathobiological studies [60]. Therefore, to better define the biological functions of fungal sphingolipids, and their role in pathogenicity, many researchers have started to disrupt the enzymes involved in the synthesis of GlcCer. Disruption of GlcCer synthase in *C. neoformans* led to the production of a mutant strain Δ*gcs1*, which was unable to grow at neutral/alkaline pH and was avirulent in an immunocompetent murine model. These results demonstrate the critical role of GlcCer in neutral/alkaline tolerance and fungal virulence [3]. The depletion of GlcCer synthase in *Fusarium graminearum*, resulted in a significant change in the conidia morphology and exhibited a polarized growth defect. Contrary to *C. neoformans*, the mutant strain lacking GlcCer synthase in *F. graminearum* (Δ*Fggcs1*) did not always show an avirulent phenotype even though its ability to spread in the host was significantly reduced [1].

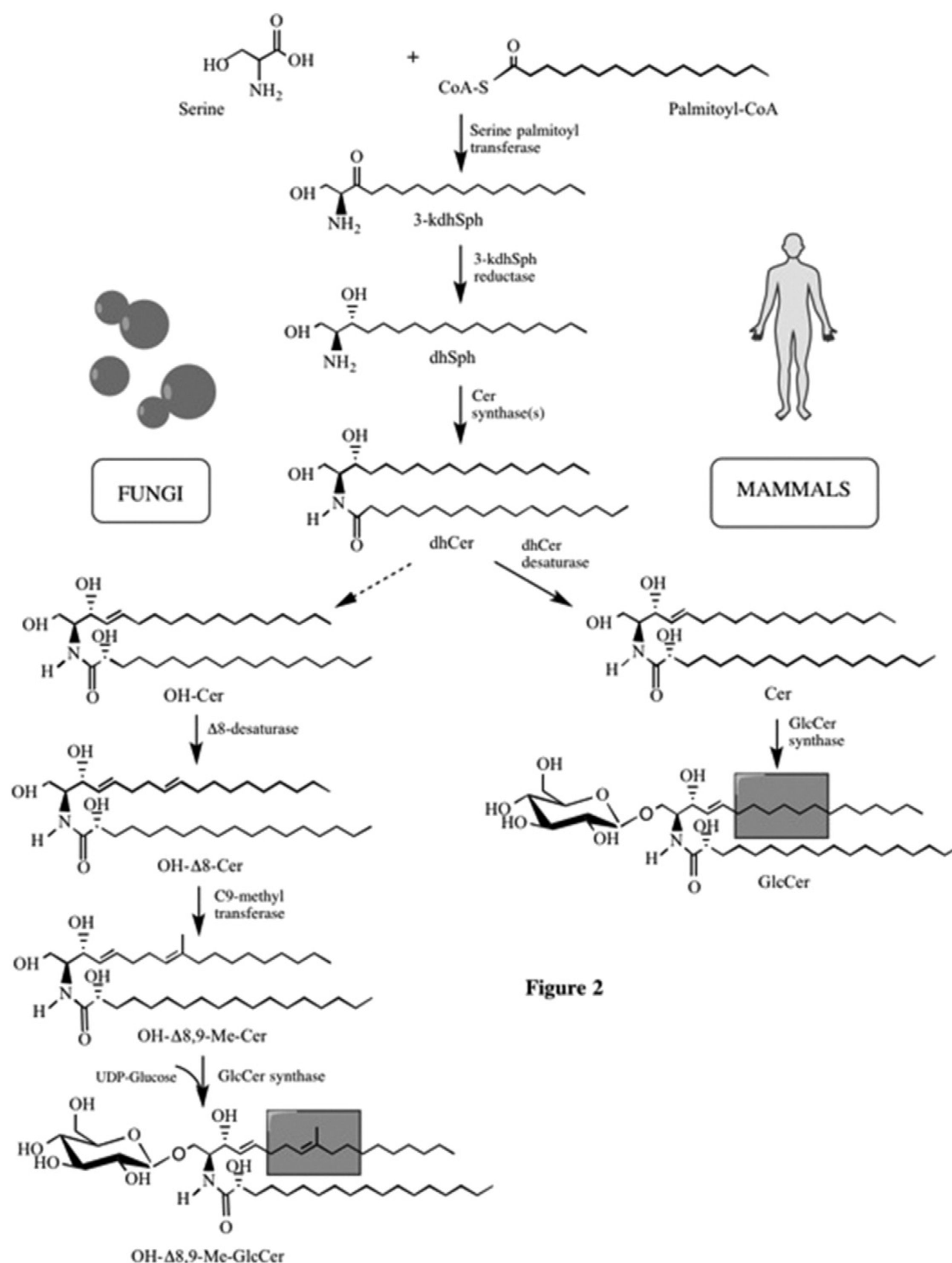

Fig. 2. Schematic representation of glucosylceramide biosynthetic pathway in yeast and mammals. The gray boxes indicate the differences between yeast and mammals glucosylceramide. 3-kdhSph: 3-ketodihydrosphingosine; dhSph: dihydrosphingosine; dhCer: dihydroceramide; OH-Cer: α-hydroxyceramide; OH-Δ8-Cer: α-hydroxy-Δ8-ceramide; OH-Δ8,9-Me-Cer: α-hydroxy-Δ8,9-ceramide; OH-Δ8,9-Me-GlcCer: α-hydroxy-Δ8,9-methyl-glucosylceramide; Cer: ceramide; GlcCer: glucosylceramide.

It has been reported that the structure of GlcCer is important in fungal morphogenesis and virulence [3,9,61,62]. Disruption of Δ8-desaturate and C9-methylated glucosylceramide in *C. albicans* showed a decrease in hyphal elongation compared to the wild-type strain [61, 62]. The effect of these mutations in *C. albicans* has not been elucidated. Few years later, the disruption of C9- methyltransferase gene (*SMT1*), in *C. neoformans* was studied by Singh et al. [9]. The Δ*smt1* mutant strain exhibited decreased virulence in a murine model of cryptococcosis and an increased susceptibility to sodium dodecyl sulfate (SDS), suggesting a defect in cell membrane structures confirmed by fluorescence spectroscopy and atomic force microscopy studies [9].

Although, the sphingolipid biosynthetic pathway has been well characterized over the past two decades, very little was known about GlcCer catabolism until recently. The first fungal glucosylceramidase, called endoglycoceramidase-related protein 1 (EGCrP1), was identified and characterized in *C. neoformans* by Ishibashi and colleagues [43]. The

disruption of *EGCrP1* gene led to the accumulation of mature and immature GlcCer in the Δ*egcrp1* mutated strain, suggesting that *EGCrP1* is important in the quality control of GlcCer and in particular in the elimination of the incorrectly synthetized GlcCer. Interestingly, it was reported that *EGCrP1* is a specific neutral glucosylceramidase, suggesting the presence of other glucosylceramidase enzymes working at different pH conditions.

Recently, a lot of progress has been achieved in fungal lipid research such as: 1) the characterization of new enzymes involved in the synthesis and catabolism of glycolipids that may be a promising target for the design of specific inhibitors, 2) the development of new anti-fungal drugs that have the potential to be more patient-friendly compared to the current ones, which often have intolerable and dose-limiting side effects [5], and 3) immunotherapy using specific antibodies against lipid virulence factors such as GlcCer, to inhibit fungal growth [63]. Even though many of these studies are preliminary, and have been done only *in vitro* or in murine models, the encouraging recent findings provide enough impetus to proceed with the study of these lipids as therapeutic targets.

3. Regulation of fungal virulence by plasma membrane lipids

Plasma membrane lipids can regulate various aspects of fungal virulence through lipid-lipid and lipid-protein interactions. In addition, lipid microdomains composed of ordered sphingolipid–ergosterol molecules have been suggested to play an important role in virulence by concentrating virulence factors or affecting cell membrane physical properties. In the following sections, a review of the literature on the role of plasma membrane lipids in fungal drug resistance, biofilm formation, and delivery of virulence factors is discussed and an overview of lipid microdomains in fungi and their role in virulence is provided.

3.1. Plasma membrane lipids and their role in drug resistance

Two of the main classes of antifungal drugs, azoles (such as fluconazole) and polyenes (such as amphotericin B), target the synthesis of ergosterol or bind to sterols [64]. As such, the connections between lipid biosynthesis and drug resistance have been investigated by many researchers. Drug resistance is generally not reported for polyenes; however, this class of drugs shows significant nephrotoxicity, which limits their therapeutic index [65]. It has been reported that mutations in the *ERG3* gene that reduce the amount of ergosterol causes drug resistance in various fungal strains by stripping the drug of its intended target [66–68]. It has also been reported that ergosterol is replaced by methylated sterols (mainly lanosterol) in a *C. albicans* strain that is resistant to both triazoles and polyenes [69]. However, polyene resistance without alterations in ergosterol biosynthesis has also been reported [70] suggesting that alteration in lipid biosynthesis is likely to be only one of the mechanisms of polyene resistance.

Lipids also play a role in resistivity to triazoles. In this case, while overexpression or mutations in the drug target, Erg11p, can cause resistance [71,72], drug efflux pumps that reduce the intracellular levels of drugs can also be involved [73,74]. Interestingly, the function of these pumps has been shown to depend on plasma membrane lipids. For example, when the CDR1 (*Candida* drug resistance) gene was expressed in *S. cerevisiae* mutants, which accumulated various intermediates of ergosterol biosynthesis and showed increased membrane fluidity; an increased susceptibility to fluconazole compared to the wild-type was observed [75]. This phenomenon suggests that either membrane fluidity or sterol composition affects the function of the CDR1p. In *C. albicans* strains, acquired resistivity to fluconazole was accompanied by an increase in the expression of the drug transporter genes *CDR1* and *CDR2* as well as the target enzyme *ERG11* and a decrease in ergosterol biosynthesis and membrane fluidity (likely as a result of reduction in ergosterol) [76]. In addition, defects in various genes encoding ergosterol or sphingolipid biosynthesis resulted in the defective transfer of Cdr1p to the plasma membrane suggesting that sphingolipids and sterols, and possibly lipid microdomains, play a role in the transfer of drug efflux pumps to the plasma membrane [77]. Despite this evidence, the role of sterol concentration and lipid microdomains in acquired drug resistance is not well understood. For example, in another study, triazole resistant strains of *C. albicans* have been shown to contain more ergosterol, but less mannosylinositolphosphorylceramides (MIPC) [78], which is another constituent of lipid microdomains [79]. While the overall effect of these changes on membrane fluidity are not exactly known, the changes in ergosterol concentration do not agree with the previous reports on the relationship between reduction of ergosterol and triazole resistivity and require further studies.

3.2. Plasma membrane lipids and their role in biofilm formation

Fungal biofilms are surface-associated communities of fungal cells that are protected by an extracellular matrix. Biofilms can form on medical implants and are associated with a high resistance to antifungal drugs and are a cause of persistent infections [80], which can lead to significant morbidity and mortality [81,82]. Molecular studies have shown that plasma membrane lipids play an important role in biofilm formation and drug resistance. Mukherjee et al. [83] demonstrated that as *C. albicans* biofilms mature, their ergosterol level reduces while their resistivity to triazole increases. The authors suggested that increased drug resistivity might be due to alterations in membrane permeability, which can affect the entry of antifungal agents into the cell, although the possibility of an indirect effect such as changes mediated by a cell wall protein was not ruled out. Ergosterol appears to be essential for the formation of biofilms; however, as shown by Mukherjee et al. [83] ergosterol levels dropped to almost half of their initial concentration after 48 h of biofilm maturation. The importance of ergosterol for biofilm formation was shown in the study of Liu et al. [84], where addition of simvastatin (a compound with inhibitory effect on ergosterol biosynthesis [85]) was reported to inhibit the formation of *C. albicans* biofilms. Interestingly, the effect of low simvastatin concentrations could be reversed by addition of exogenous ergosterol suggesting the importance of ergosterol in biofilm formation. Ergosterol-sphingolipid microdomains in the plasma membranes have also been shown to be important for biofilm formation. Staining of membrane sterol using the fluorescent dye filipin, showed significantly increased staining in *C. albicans* biofilms compared to planktonic cells [86]. Given that ergosterol levels in biofilms are shown to be equal or less than planktonic cells [83], these results suggest an increased incorporation of sterols in the membrane during biofilm formation. In addition, drugs such as myriocin and aureobasidin A that inhibit the biosynthetic pathway of sphingolipids led to a marked reduction in biofilm formation suggesting the importance of lipid microdomains in biofilm formation [86].

3.3. Extracellular vesicles

In contrast to drug resistance and biofilm formation, extracellular vesicles are a less well-known facet of fungal virulence. Extracellular vesicles were first isolated from *C. neoformans* and shown to have a lipid bilayer structure mainly composed of GlcCer and sterols [87]. Extracellular vesicles were shown to carry capsule components, enzymes such as urease and laccase, and a number of proteins related to virulence and as such were considered to be “virulence bags” used for delivery of virulence molecules [88]. These vesicles were also found to carry RNA, similar to mammalian exosomes [89]. Extracellular vesicles show immunomodulatory properties and due to their small size (vesicle diameters of 100–300 nm have been reported [90]) are more likely to reach various cells and elicit an immune response. Olivera and colleagues [91] reported that these vesicles are incorporated by mammalian macrophages and stimulate their antimicrobial activity such as tumor necrosis factor alpha, interleukin-10, and nitric acid production [91]. In addition, these vesicles were shown to fuse with human brain

microvascular endothelial cells (HBMECs), and increase the transversal of *Cryptococcus* cells across the blood–brain barrier both *in vitro* (in HBMECs) and *in vivo* [92].

In addition to *C. neoformans*, extracellular vesicles have been isolated from other pathogenic fungi such as *H. capsulatum*, *C. albicans*, *Candida parapsilosis* [93] and *P. brasiliensis* [94,95] as well as the non-pathogenic fungus *S. cerevisiae* [90,91,93] suggesting that the release of these vesicles might be a universal phenomenon in fungi. The exact mechanism of secretion of extracellular vesicles in fungi is not well known [96,97]. However, the importance of the post-Golgi secretory pathways has been demonstrated [98] and the fusion of vesicles to the plasma membrane and subsequent budding from the membrane has been proposed as a potential mechanism [97,99,100]. Regardless of the mechanism, extracellular vesicles demonstrate a new role for lipids in fungal pathogenesis, which can be a focus of future studies.

4. Membrane microdomains and virulence

A different mechanism by which lipids in the plasma membrane of pathogenic fungi have been suggested to affect fungal virulence is by partitioning membrane proteins into certain 'microdomains' in the plasma membrane. The concept of lipid microdomains or lipid rafts is still very controversial; however, it is now widely accepted that ordered domains made of sphingolipids and sterols exist in the cell membrane and might play a role in protein localization. Most of the information in this area is focused on mammalian cells and very few studies have focused on lipid microdomains in pathogenic fungi. The following sections focus on the molecular origins of lipid microdomains in fungi, their role in virulence, and finally the role of mammalian lipid rafts in the immune response against fungal infections. A thorough account of lipid rafts in pathogenic bacteria, methods to study lipid rafts in microorganisms, and connections between lipid rafts in fungi and bacteria is given elsewhere [101].

Much of the information on lipid microdomains is obtained by studies on model membranes. Early studies have shown that cholesterol has a 'condensing effect' and promotes phase segregation in lipid monolayers and bilayers [102,103]. Areas with high lipid order and low lateral diffusion are known as the liquid-ordered (Lo) phase whereas areas with low lipid order and high lateral diffusion are known as liquid-disordered (Ld) phase [104]. The condensing effect of cholesterol on lipids is proposed to be caused by the umbrella effect, through which the hydrophilic headgroup of sphingolipids or phospholipids is extended to form an 'umbrella' and protect cholesterol from the aqueous phase [105]. The hydrophobic interactions between cholesterol and the acyl chains of other lipids will then result in a condensation effect and phase segregation [105]. Lipids with long and saturated acyl chains promote phase segregation in combination with cholesterol, while lipids with short chain fatty acids or cis-double bond tend to disrupt Lo phases [106]. In mammalian cells, lipid microdomains are composed of cholesterol and saturated sphingolipids such as sphingomyelin [107]. Fungal cells do not make cholesterol and their main raft-forming sterol is ergosterol. Also, in fungal cells, saturated sphingolipids have a sugar or inositol headgroup and lipids such as GlcCer, IPC, MIPC, and MIP2C are suggested to partition into ordered domains [79].

Studies of lipid rafts in microorganisms are hindered by the scarcity of methods for *in vivo* characterization. Detergent insolubility has been a major technique in studies of lipid rafts in fungal (and mammalian) cells. Lipids in the Lo phase do not dissolve in low concentrations of cold, non-ionic detergents such as Triton X-100 and can be floated using density gradient centrifugation, leading to detergent resistant membranes (DRMs) [108]. DRMs isolated from fungal cells are highly concentrated in glycosphingolipids and ergosterol and have been also named detergent insoluble glycolipid-enriched complex (DIG) [109]. DRMs are not the same as lipid rafts and it is possible that incubation at low temperature and the use of detergent can cause experimental artifacts [110]. However, DRMs have been used as a surrogate for rafts and have increased the current understanding of lipid rafts in pathogenic fungi.

The non-pathogenic yeast *S. cerevisiae* has provided a useful model strain for researchers to study the existence and function of lipid rafts in yeast. The methods and knowledge base developed by studying *S. cerevisiae* has been used for studies in pathogenic fungi. In a pioneering study, Bagnat et al. [109] showed that Plasma membrane ATPase 1 (Pma1p) and Glycosylphosphatidylinositol-anchored surface protein 1 (Gas1p), two of the markers of lipid rafts in mammalian cells are concentrated in DRMs isolated from *S. cerevisiae* suggesting that membrane microdomains similar to those in mammalian cells exist in fungi. These proteins were misplaced from the DRMs in ergosterol auxotrophs and sphingolipid mutants [109] and disruption of lipid rafts resulted in the delivery of Pma1 to the vacuole instead of the plasma membrane suggesting that lipid rafts are important for cell surface delivery of proteins in yeast [111]. The absence of sphingolipids and ergosterol, the main components of lipid microdomains, has also been shown to lead to disruption in the transport of GPI anchored proteins and high affinity tryptophan permease-tat2p in *S. cerevisiae* [112,113]. Furthermore, it has been shown that pheromone-treated *S. cerevisiae* cells undergo plasma membrane reorganization, which leads to the clustering of lipid rafts at mating projections and the partitioning of mating proteins into these clusters [114,115]. Further evidence of lipid phase segregation in yeast was provided by microscopy studies on vesicles synthesized out of total lipid extracts of *S. cerevisiae*, which revealed the presence of micron-sized, ordered domains [116]. It was also shown that IPCs purified from *S. cerevisiae* show an increased degree of order compared to mammalian sphingomyelin, which supports the notion that they are capable of forming ordered domains [116].

Similar to studies in *S. cerevisiae*, many studies in pathogenic fungi have relied on DRM isolation and identification of proteins in DRMs. Studies in *C. neoformans* revealed that DRMs were concentrated in ergosterol, GlcCer, phosphatidylethanolamine, and phosphatidylcholine. Two virulence factors, superoxide dismutase and phospholipase B1, were localized in DRM fractions and were displaced upon ergosterol extraction [117]. Our studies revealed that another virulence factor of *C. neoformans*, Pma1 (required for survival in the acidic environment in the phagolysosome), is also localized in DRM fractions in agreement with the studies on *S. cerevisiae* [118]. Thus, lipid microdomains could contribute to virulence by concentrating a number of virulence factors.

The microstructure of plasma membrane could also have other roles in virulence. Studies from our group have shown that genetic modifications that result in loss of a methyl group from GlcCer lead to altered membrane structure, such as reduction in membrane order and the height difference between Lo and Ld phases, and inhibit the ability of the fungus to grow at neutral pH [119]. The ability of *C. neoformans* to grow at neutral pH is crucial for virulence and *C. neoformans* mutant strains, which cannot grow at neutral pH are unable to establish virulence in the mouse model [120]. It has been shown that ergosterol extraction inhibits the ability of the pathogenic yeast *H. capsulatum* to infect alveolar macrophages *in vitro* [79]. This effect was proposed to be due to the displacement of adhesion proteins, such as laminin-binding protein, involved in fungal-host interactions.

A number of studies have demonstrated the role of sterol-rich domains in cell division in pathogenic fungi. The evidence for these studies has been gathered by cell staining with filipin, a fluorescent polyene that binds to sterols [121]. Filipin staining has revealed the accumulation of sterols at bud tips and hyphal tips in *C. neoformans* [122], *C. albicans* [123,124], and *Aspergillus nidulans* [125–127]. Treatment with drugs that inhibit sterol or sphingolipid biosynthesis revealed a connection between these lipids and sterol-rich domains and suggested a role for lipid rafts in hyphal growth [124]. However, sterol-rich domains appear to create a platform for protein concentration, an ability that is often attributed to lipid rafts. Two proteins involved in polarized growth in *A. nidulans*, Tea1 and TeaR, were shown to be concentrated in sterol-rich domains and were displaced from hyphal tips after

treatment with high concentrations of filipin suggesting the importance of sterol-rich domains to hyphal growth [127]. It should be mentioned that sterol-rich domains do not directly correlate with lipid rafts, as it is not clear whether these areas show increased sphingolipid concentration or increased membrane order.

While not the focus of this review, it is important to note that lipid rafts in the mammalian cells infected with fungi appear to play an important role in host-pathogen interactions during the course of infection. Mammalian lipid rafts, which consist of cholesterol and sphingolipids, concentrate many of the receptors involved in the recognition and phagocytosis of fungal pathogens. Dectin-1 a C-type lectin receptor, which recognizes the glucans of the fungal cell wall [128] gets concentrated in lipid rafts upon stimulation with glucans [129]. In fact, disruption of lipid rafts by means of cholesterol extraction leads to a reduction in glucan uptake [129,130]. Furthermore, various fungal pathogens such as *C. neoformans* and *P. brasiliensis* bind to monosialotetrahexosylganglioside (GM1) [131, 132], a component of mammalian lipid rafts [133], or associate with GM1 localized areas during infection [134]. Disruption of lipid rafts via cholesterol extraction reduces the uptake of both pathogens, potentially by dispersion of GM1 and/or other receptors or binding molecules [131, 135]. Several other examples of pathogen-host lipid raft interactions exist in the literature (see for example [92,134]), suggesting that this could be an area of future focus for better understanding of the process of pathogenesis and host-pathogen interactions.

5. Conclusion and future directions

Over the years, studies in eukaryotic and prokaryotic cells have clearly demonstrated the role of plasma membrane integrity in cell survival. However, the roles of the plasma membrane lipids in pathogenicity remain a highly understudied topic. In recent years, researchers have started to address the role of lipids, lipid-lipid, and lipid-protein interactions in fungal pathogenicity and certain lipids have been identified as regulators of fungal virulence [120]. These findings have led to growing interest in targeting lipid virulence factors, and in particular glycolipids, for the treatment of fungal infections. Two strategies have been proposed; the first is through modulation of endogenous lipid levels by directly targeting enzymes responsible for their metabolism, such as Gcs1. This approach requires the identification of specific inhibitors/activators for these enzymes, and their testing in pre-clinical animal models as well as in clinical trials to evaluate their safety and efficacy. The second strategy is preventive and utilizes the immunomodulatory properties of lipids. It is illustrated by the protective effects of the non-pathogenic Δ*sgl1 C. neoformans* strain on murine models of cryptococcosis. This approach has significant potential as a vaccination strategy, but requires better vaccine formulations to be developed due to the potential hazards associated with live vaccines.

There is still a scarcity of studies on the role of lipid microdomains in fungal pathogenicity. While studies are starting to provide valuable information on the role of lipid microdomains in concentrating virulence factors, existing studies have been overly reliant on DRM isolation, filipin staining, or the use of antifungal drugs; all of which are aggressive methods and can introduce artifacts. Thus, the majority of studies have turned to the non-pathogenic yeast *S. cerevisiae*, which has led to a disconnect between the current understandings and their applicability in pathogenic fungi. As such, further studies on the role of microdomains in pathogenic fungi is needed to better characterize the lipid compositions, and physical and structural attributes of lipid microdomains, the importance of microdomains in the virulence process, and the potential changes in the composition of microdomains during the process of infection. Furthermore, while the presence of virulence factors in lipid domains has been demonstrated, little has been done to target them and use their therapeutic potential.

The role of membrane lipids in morphological changes in fungi is another area of future interest. Current studies have revealed significant changes in membrane properties (e.g. changes in local sterol composition) during changes in morphology. However, the role of the local lipid composition during morphological changes is not known. Also, it is not clear whether certain lipids promote or inhibit changes in morphology and if the composition of such lipids changes during the process of growth or infection. Finally, and importantly, it is not known whether lipids can be targeted to avoid changes such as hyphal formation or extracellular vesicle generation in fungi. Despite the valuable contributions made by many research groups, our understanding of lipids in fungal pathogenesis is still primitive and answering these questions, and many others, are likely to lead to increased understanding of fungal pathogenesis and novel therapeutics.

Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this review.

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