



# Fungal glucosylceramides: from structural components to biologically active targets of new antimicrobials

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The first work reporting synthesis of glucosylceramide (cerebrin, GlcCer) by yeasts was published in 1930. During approximately 70 years members of this class of glycosphingolipids (GSL) were considered merely structural components of plasma membrane in fungi. However, in the last decade GlcCer was reported to be involved with fungal growth, differentiation, virulence, immunogenicity, and lipid raft architecture in at least two human pathogens. Fungal GlcCer are structurally distinct from their mammalian counterparts and enriched at the cell wall, which makes this molecule an effective target for antifungal activity of specific ligands (peptides and antibodies to GlcCer). Therefore, GSL are promising targets for new drugs to combat fungal diseases. This review discusses the most recent information on biosynthesis and role of GlcCer in fungal pathogens.

**Keywords:** glucosylceramide, fungal pathogens, antifungal targets

## INTRODUCTION

The family of glycosphingolipids (GSL) combines a diversity of molecules consisting of at least one saccharide unit covalently linked to a ceramide (Schnaar et al., 2009). The biosynthesis of GSL is complex and involves a repertoire of enzymes that must be carefully expressed and distributed over endoplasmic reticulum (ER) and Golgi in all eukaryotic cells (Halter et al., 2007; Daniotti and Iglesias-Bartolome, 2011). Due to their structural properties and primary cellular location, GSL were initially described as architectural components of cell membranes, straightly connected with fluidity and stability (Feinstein et al., 1975; Tinker et al., 1976; Aaronson and Martin, 1983; Campanella, 1992). Recent findings, however, clearly demonstrate that their role goes beyond the former concepts, since these molecules are major components of specialized membrane domains called lipid rafts (Bagnat et al., 2000; Hakomori, 2003, 2008; Wachtler and Balasubramanian, 2006). In addition, GSL have been characterized as relevant structures in events of cell–cell interaction, cell signaling and protein sorting (Bagnat et al., 2000; Bagnat and Simons, 2002; Nimrichter et al., 2008; Lopez and Schnaar, 2009; Schnaar et al., 2009; Staubach and Hanisch, 2011).

In fungal organisms two types of GSL have been reported. Inositol phosphorylceramides (IPC) are used to make complex fungal GSL and have been extensively studied in the yeast model *Saccharomyces cerevisiae* (Sugimoto et al., 2004; Dickson et al., 2006; Dickson, 2008). They consist of a ceramide usually containing phytosphingosine as long chain base, associated with a very long fatty acid (C<sub>24:1</sub>–C<sub>26:1</sub>). A unit of inositol is covalently linked to the ceramide and the preformed molecule, which is not found in mammalian cells, is the substrate for mannosyltransferases for the synthesis of mannosylinositol phosphorylceramide (MIPC) and mannosyldiinositol phosphorylceramide [M(IP)<sub>2</sub>C]. The second

major class of GSL synthesized by fungal cells is the monohexosylceramide (CMH, glycosylceramide, cerebroside, or cerebrin). The sugar moiety covalently linked to a distinct ceramide is usually glucose (GlcCer) or, to a minor extent, galactose (GalCer; Boas et al., 1994; Takahashi et al., 1996; Levery et al., 2000, 2002; Rodrigues et al., 2000; Toledo et al., 2000; Pinto et al., 2002; Barreto-Bergter et al., 2004; da Silva et al., 2004; Nimrichter et al., 2004, 2005a). GlcCer and GalCer are not synthesized by *S. cerevisiae* but are part of the lipid arsenal of classical fungal pathogens, excepting *Candida glabrata* (Saito et al., 2006; Tavares et al., 2008).

A very conserved structure was described as the major GlcCer in fungal pathogens, the *N*-2'-hydroxy(hexa/octa)de(ca/ce)noyl-1-beta-D-glucopyranosyl-9-methyl-4,8-sphingadienine. GlcCer distribution is not limited to fungal membranes, since large amounts of this GSL have been also found at cell wall (Rodrigues et al., 2000; Nimrichter et al., 2005a; Rhome et al., 2011). Although GlcCer have been identified as fungal components decades ago, knowledge about their functions during fungal growth/dimorphism, lipid raft formation, and correlation with virulence has been recently reported. In this review we discuss the most recent findings that characterize the functions of GlcCer and its biosynthetic steps as potential targets for new antifungal drugs. The putative correlation between GlcCer, lipid rafts and secretion and the potential role of this GSL as virulence regulators are also reviewed.

## THE UNCOVERED PATHWAY FOR FUNGAL GlcCer SYNTHESIS

Sphingoid bases of fungal GlcCer are structurally distinct from mannosylated IPCs and from their counterparts in animal cells. They have a methyl group at C-9 and an extra unsaturation at C-8, forming the typical fungal base mentioned above (for review please see Barreto-Bergter et al., 2004). However, initial steps of ceramide biosynthesis are the same independent of the cell type studied.

Ceramide synthesis always begins with condensation of palmitoyl-CoA and serine, followed by sphinganine formation through the activity of a 3-ketosphinganine reductase (Barreto-Bergter et al., 2004; Li et al., 2006; Schnaar et al., 2009; Gault et al., 2010). The product, sphinganine, is then *N*-acylated to generate ceramide. Two distinct pools of ceramide operate as building blocks for major fungal GSL (CMH and IPCs). They differ according to the hydroxylation level of long chain base (dihydroxy or trihydroxy) and the extension of hydroxylated fatty acids (C<sub>16:1</sub>–C<sub>18:1</sub> or C<sub>24:1</sub>–C<sub>26:1</sub>) (Barreto-Bergter et al., 2004; Dickson et al., 2006; Takakuwa et al., 2008; Rittenour et al., 2011; Ternes et al., 2011). All enzymes involved in fungal ceramide synthesis operate specifically according to their substrate leading to precise production of GlcCer or MIPCs. So far, the only exceptions appear to be *Magnaporthe grisea* and *Neurospora crassa*, discussed later on (Lester et al., 1974; Maciel et al., 2002). It implies that preformed ceramide must be considerably modified before GlcCer or IPC synthesis. This section is focused on fungal GlcCer biosynthesis. Approaches utilized for structural characterization of these molecules are discussed in detail by Barreto-Bergter et al. (this issue).

Synthesis of IPC and GlcCer must include a branching point, since the enzymes required for this process make part of common and specific biosynthetic pathways. A very elegant study published by Ternes et al. (2011) contributed significantly to the understanding of GlcCer biosynthesis. Briefly, these authors demonstrated that in *Pichia pastoris*, similarly to *S. cerevisiae*, *Lag1* and *Lac1* appear to be the genes responsible for coding the enzymes that make trihydroxy ceramides carrying very long chain fatty acids (Ternes et al., 2011). On the other hand, *BarA* (biocontrol agent resistance) is related to synthesis of dihydroxy ceramides bearing hydroxylated C<sub>16</sub>–C<sub>18</sub> fatty acids in *P. pastoris*. *BarA* was originally identified by a screen assay performed to isolate mutants from *A. nidulans* resistant to the polyketide HSAF, a heat-stable antifungal factor isolated from the biocontrol agent *Lysobacter enzymogenes* (Zhang and Yuen, 1999). In *A. nidulans*, exposure to HSAF leads to a rapid loss of the formin SepA at growing hyphal tips and disturbs the polarized growth (Li et al., 2006). Phylogenetic analysis indicated that BarAp belongs to a distinct clade of ceramide synthases when compared to *Lag1p* and *Lac1p*. Accordingly, *A. nidulans* lacking BarAp do not synthesize GlcCer (Li et al., 2006). In *Kluyveromyces lactis*, overexpression of *Lac1* increased GlcCer synthesis while gene disruption blocked this pathway, leading to IPC accumulation (Takakuwa et al., 2008). Disruption of *Lag1* appears to be lethal in *K. lactis*. Orthologous expression of *Lac1* from *K. lactis* in *S. cerevisiae* resulted in IPC with C<sub>18:1</sub> fatty acids, which are presumably exclusive to GlcCer. Sequence analysis suggests that *Lac1* of *K. lactis* belongs to the same clade as *BarA* (Rittenour et al., 2011). These data indicated that when *BarA* is absent in fungi that express GlcCer, *Lac1* is the gene involved with GlcCer production.

The biosynthetic steps occurring right after ceramide hydroxylation at C-2 of fatty acid are  $\Delta$ 4-desaturation,  $\Delta$ 8-desaturation, C-9-methylation, and then glucose addition by ceramide glucosyl ceramide-synthetase (GCS; Ternes et al., 2002, 2011; Michaelson et al., 2009). Membrane-bound desaturases are part of a superfamily defined by the histidine boxes HX3–4H, HX2–3HH, and (H/Q)X2–3HH that usually require NAD(P)H and O<sub>2</sub> as cofactors

(Sperling et al., 1995; Zauner et al., 2008). Searching proteins with histidine box sequence motifs a family of  $\Delta$ 4-desaturase was discovered. *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, and *Candida albicans*  $\Delta$ 4-desaturases were expressed in *S. cerevisiae* and biochemically characterized (Ternes et al., 2002). Sphing-4-ene and 4-hydroxysphinganine were characterized as products in all situations, indicating a bifunctional activity ( $\Delta$ 4-desaturase and C14-hydroxylase). This enzymatic step appears to be essential, given that *P. pastoris* mutant lacking  $\Delta$ 4-desaturase is not able to make GlcCer (Ternes et al., 2011). Insertion of a double bond between C-8 and C-9 of long chain base, the step catalyzed by a  $\Delta$ 8-desaturase, does not appear to be a requirement for GlcCer. *P. pastoris* lacking the enzyme is still able to build regular amounts of GlcCer.

Transference of the C-9-methyl group to the sphingoid base is the last step of ceramide synthesis used to construct fungal GlcCer. The enzyme responsible for this step belongs to the superfamily of *S*-adenosylmethionine-(SAM)-dependent methyltransferases, appears to be membrane-bound and requires a  $\Delta$ 4,8-desaturated ceramide as substrate (Ternes et al., 2006). As observed for  $\Delta$ 8-desaturase, absence of C-9 methyltransferase in *P. pastoris* (coding the enzyme Ppmt1) does not impair GlcCer synthesis, resulting in the exclusive formation of methylated ceramide. In *C. albicans*, strains lacking GlcCer, or C-9 methyltransferase were also viable, although a deficient hyphal growth was observed when compared to the wild type strain (Oura and Kajiwarra, 2010). Two genes encoding putative C-9 methyltransferase were found in *Fusarium graminearum*, FgMT1, and FgMT2 (Ramamoorthy et al., 2009). Both genes complemented the lack of C-9 methyltransferase activity in *P. pastoris* mutant Ppmt1. FgMT1 disruption did not change the ceramide methylation and mutants grew as wild type cells. On the other hand, disruption of FgMT2 reduced the enzymatic product in approximately 35%, decreased growth ratio, altered shape, and size of conidia, reduced mycelial formation and virulence to *A. thaliana* leaves and wheat. Double-knockouts were not viable, suggesting that C-9 methyltransferase is essential to fungal growth in *F. graminearum*.

Glucose transfer to ceramide occurs at Golgi stacks in animal cells through a CGS (Halter et al., 2007). A fraction of newly synthesized GlcCer returns to ER and is transported to Golgi lumen to synthesize LacCer and other complex GSL. Using a second pathway, intact GlcCer reaches the cell surface through a non-vesicular pathway. In yeast, ceramide is also transported to Golgi through vesicular and non-vesicular routes (Funato and Riezman, 2001) where complex GSL will be synthesized. It is not clear whether *N*-2'-hydroxy(hexa/octa)de(ca/ce)noyl-1- $\beta$ -D-glucopyranosyl-9-methyl-4,8-sphingadienine follows a similar pathway.

The complexity of fungal GSL biosynthesis is supported by studies on the phytopathogens *F. graminearum* and *M. grisea*. *F. graminearum* is a causative agent of head blight, a plant disease that results in crop loss or grain unsuitability for animal consumption due to mycotoxin production (Xu and Nicholson, 2009). As observed for *A. nidulans*, *Bar1* mutants of *F. graminearum* do not synthesize GlcCer and are resistant to HSAF (Rittenour et al., 2011). GlcCer was also isolated from lipid extracts of the rice blast agent *M. grisea* (Maciel et al.,

2002). The typical fungal GlcCer structure was characterized in this model, consisting of *N*-2'-hydroxy(octa/hexa)decanoyl-1-*O*- $\beta$ -D-glucopyranosyl-9-methyl-4,8-sphingadienine. The *M. grisea* crude extract showed an extra orcinol positive TLC band migrating above GlcCer, indicative of a dihexosylceramide (CDH). The purified molecule was identified as lactosylceramide (LacCer). Unusually, the lipid moiety of LacCer consisted of a phytosphingosine covalently linked to a C<sub>24:2</sub> fatty acid. A tetrahexosylceramide has been also reported in *N. crassa* (Lester et al., 1974). By using gas and thin layer chromatography the identified components were Gal<sub>3</sub>Glc, sphinganine, and a C<sub>24:1</sub> fatty acid.

These results establish that GSL biosynthesis is conserved in fungal cells; however, it cannot be summarized by a simple pathway. It seems important to consider that different species must be investigated individually. Reconstituted strains on GSL pathways also should be carefully investigated, since proper enzyme location usually cannot be assured, which may result in unusual products.

### CORRELATION BETWEEN FUNGAL GlcCer, LIPID RAFTS, AND SECRETION MECHANISMS

To our knowledge, the mechanisms displayed by fungal organisms to traffic GlcCer intracellularly have never been investigated. Fungal GlcCer destiny includes plasma membrane and cell wall (Rodrigues et al., 2000; Nimrichter et al., 2005a), as well as the extracellular space (Rodrigues et al., 2007a, 2008a,b; Nosanchuk et al., 2008).

As mentioned previously, along with sterol and GPI-anchored proteins, GSL are components of lipid domains or lipid rafts (Hakomori, 2003, 2008). These lipid platforms are found in different cell types, including non-pathogenic and pathogenic fungi, where they participate in polarization and compartmentalization steps of the plasma membrane (Bagnat et al., 2000; Bagnat and Simons, 2002; Martin and Konopka, 2004; Siafakas et al., 2006; Wachtler and Balasubramanian, 2006). In the *C. albicans* model, the ability of fungal cells to switch from yeast to hyphae is considered a crucial step for host cell colonization (Mitchell, 1998). Such morphological changes are dependent on a polarized growth mediated by cytoskeleton (Whiteway and Bachewich, 2007). The first correlation between lipid rafts and polarization in *C. albicans* was suggested by Martin and Konopka (2004). They observed accumulation of sterols at the growing tip of hyphal cells and at sites of septum formation in mature hyphae. In addition, treatment with myriocin, an inhibitor of serine palmitoyltransferase, decreases filipin staining, and reduces Pma1p recruitment to lipid rafts. Pma1p is a plasma membrane proton pump that is considered a raft marker in *C. albicans* and *S. cerevisiae* (Bagnat et al., 2001; Insenser et al., 2006). Along with Pma1p, heat shock proteins (HSP70, HSP90) and molecules required for sterol metabolism, energy production, and polysaccharide synthesis were compartmentalized in lipid domains in *C. albicans* (Insenser et al., 2006). A connection between polarized growth, lipid rafts, and GlcCer was also observed in *F. graminearum* and *A. nidulans* lacking Bar1p and GlcCer as well. In *F. graminearum*, germ tube formation occurs regularly in the absence of Bar1p, but hyphal tip organization is considerably altered. Although sterols accumulate in hyphal tips of a wild type strain,  $\Delta$ *bar1* mutants lack this distribution. Similarly, no filipin staining was visualized at

hyphal tips of *A. nidulans* lacking Bar1p. These mutants do not display regular Spitzenkörper when stained with FM4-64, a fluorescent lipid marker. Polarization defects were confirmed by the altered tropomyosin distribution. Together, these data suggested a requirement of GlcCer and intact lipid rafts for hyphal growth and polarization of fungal cells.

Lipid rafts were also isolated from the opportunistic pathogen *C. neoformans*. GlcCer, phospholipase B1 (PLB1), and Cu/Zn superoxide dismutase (SOD1), molecules involved with fungal virulence, were characterized as raft components in this fungus (Siafakas et al., 2006). It was hypothesized that membranes of *C. neoformans* contain lipid domains that concentrate some virulence factors at fungal surface. Secretion of virulence factors could be associated to their aggregation to lipid rafts.

A few years ago, we discussed aspects of cell wall that, at that point, were considered unusual (Nimrichter et al., 2005b). We then raised the possibility that compact vesicles capable of crossing polar environments, such as the fungal cell wall, could be used by fungal organisms for the extracellular release of macromolecules. Lipid rafts would be likely necessary to increase vesicle stability. In fact, we recently demonstrated that *C. neoformans* produces extracellular vesicles containing GlcCer, sterol, and SOD1 (Rodrigues et al., 2007a, 2008a). Trans-cell wall traffic of GlcCer-containing vesicles could in fact explain the high amounts of GlcCer that are found at the cell wall of fungal pathogens (Rodrigues et al., 2000; Nimrichter et al., 2005a). Further studies revealed that extracellular vesicles are produced by different fungal organisms, possibly representing a conserved mechanism of trans-cell wall transport (Nosanchuk et al., 2008; Oliveira et al., 2010a,b). A number of proteins can reach the extracellular environment through these vesicles, including other lipid raft components, such as Pma1p, HSP70, and HSP90 (Albuquerque et al., 2008; Rodrigues et al., 2008a). The putative correlation between extracellular vesicle formation and fungal lipid rafts is then suggested.

Molecular exportation to the extracellular space through vesicles is not limited to proteins. The polysaccharide glucuronoxylomannan (GXM), a major capsule component of *C. neoformans*, follows a vesicle association pathway to leave the cells, in a process that apparently requires a close association with GlcCer (Rodrigues et al., 2007a; Oliveira et al., 2009). These data indicate that GlcCer regulates/participates on polysaccharide and protein sorting in fungal cells. Such processes involve compartmentalization and changes in membrane shape. This attribute could be correlated with another property of some complex GSL, the capacity to control membrane curvature and vesicle formation (Sonnino et al., 2007). Recently, a correlation between GlcCer and Golgi morphology was observed in plant cells (Melser et al., 2010). Treatment of *A. thaliana* with the inhibitor of GlcCer synthesis PDMP disturbed transport of soluble and membrane-associated secretory proteins to plant cell surface. This phenotype was accompanied by remarkable morphological changes and protein retention in the Golgi of treated cells. Reduction on Golgi cisternal thickness and stack width together with an increase in the number of vesicular structures surrounding this organelle was visualized after treatment with drugs that inhibit ceramide glycosylation.

As depicted above, pharmacological drugs that inhibit GSL synthesis are important tools to investigate the role of GSL and lipid

rafts physiological events in animal cells (Tiffit and Proia, 2000). Their activity against fungi is controversial, as discussed below.

Treatment of *Aspergillus* species with D-threo-1-phenyl-2-palmitoyl-3-pyrrolidino propanol (P4) and D-threo-3P,4P-ethylenedioxy-P4 (EDO-P4), inhibitors of ceramide glycosylation, was followed by a decrease on GlcCer expression and defects in spore germination, cell cycle, and hyphal growth (Leverly et al., 2002). Hillig et al. (2005) extended these findings by evaluating the activity of GSL inhibitors on *P. pastoris* expressing heterologous GCS. The activity of these enzymes was measured in cell-free membrane fractions in the presence of EDO-P4, a GCS inhibitor. Although the human enzyme was strongly inhibited by this drug, it showed no effect over fungal GCS (for instance, *C. albicans*). These results, however, must be carefully interpreted, given the lack of information in the literature on the properties of GCS activity from other fungal species. However, we could not exclude the possibility that other fungal species are sensitive to derivatives of these inhibitors.

### GlcCer AND FUNGAL VIRULENCE IN MURINE MODELS

As discussed above it is unquestionable that GlcCer is a key molecule during physiological events in fungal organisms. However, mutants lacking GCS are usually viable. These mutant strains have been used to evaluate the role of GlcCer during infection. In this section we will debate the capacity of fungal GlcCer to regulate virulence in animal models of mycoses.

The first report showing that GlcCer could influence the capacity of a fungal organism to cause disease was established by Rittershaus et al. (2006). *C. neoformans* lacking GlcCer synthesis was generated after GCS disruption and used in murine models of cryptococcosis. Although the capacity to produce key virulence factors of *C. neoformans* was intact in the mutant, this strain was avirulent when inhaled by CBA/J mice. Dissemination to the brain was not observed even after 90 days of infection. When injected intravenously using the same mouse background, yeasts of *C. neoformans* reached different tissues, including the brain, with a similar outcome observed for WT strain. Histopathological sections confirmed these data and also showed yeast cells confined to granulomas in the lung of mice after intranasal infection. These granulomas were necessary to control fungal growth since brain dissemination was observed after inhalation of GCS mutants by T- and NK-cell-immunodeficient mice, where granuloma formation is impaired (Kechichian et al., 2007). A refined mechanism of yeast cell cycle transition correlated with pH and CO<sub>2</sub> concentration was suggested. For establishment of cryptococcosis, GlcCer appears to be required for fungal growth in the blood and alveolar spaces of the lung, which, in contrast to the acidic intracellular environment in macrophages, have a neutral pH. Corroborating with these data T- and NK-cell-immunodeficient mice depleted of alveolar macrophages had decreased dissemination to the brain and prolonged survival. The hypothesis raised by Rittershaus et al. (2006) is also in agreement with experiments published by Saito et al. (2006). These authors reported a relationship between GlcCer synthesis and the capacity of fungal cells to grow under neutral or alkaline pH.

Results from a meticulous work developed by Noble et al. (2010) using the model *C. albicans* reinforce the role of GlcCer

as a virulence regulator. These authors disrupted 674 genes in *C. albicans* using auxotrophic markers with no effect on virulence. Mutants were then screened for three major phenotypes: (i) pathogenicity in mouse model of candidiasis, (ii) morphological switching (yeast to hypha conversion), and (iii) cell proliferation *in vitro*. The experiments *in vivo* disclosed 115 mutants with impaired pathogenicity. Among these only 15 mutants displayed impairment to differentiate and grow as hypha. In addition, in the group of 115 mutants with impaired pathogenicity, 89 exhibited growth rates that were close to the WT strain. Therefore, delay in fungal growth did not correlate with capacity to cause disease in mice. The authors then analyzed the mutants with impaired capacity to colonize mice but efficient in proliferating and in promoting morphological changes. Strikingly, four mutants were involved with synthesis of GlcCer.

Previous studies by Oura and Kajiwara (2010) have shown that mutants of *C. albicans* lacking C-9 methyltransferase, GCS, and  $\Delta 8$ -saturase have growth rates that are similar to those observed in WT cells. Delayed morphological changes were visualized when these mutants were cultivated in spider medium, but hypha formation was observed in all situations. Contrasting with *C. neoformans* mutants lacking GlcCer, mutants of *C. albicans* isolated by Noble and colleagues were able to grow under neutral and alkaline pH similar to WT. Thus, a distinct mechanism of virulence regulation is dependent on GlcCer in *C. albicans*. The authors identified GlcCer as the first molecule specifically required to govern virulence in *C. albicans* (Noble et al., 2010).

### FUNGAL GlcCer AS TARGETS TO NEW ANTIFUNGAL DRUGS

Together with other groups we have demonstrated the use of anti-GlcCer antibodies to prevent fungal differentiation and growth of fungal pathogens. For instance, these antibodies blocks germ tube formation in *C. albicans*, *Colletotrichum gloeosporioides*, and *Pseudallescheria boydii* (Pinto et al., 2002; da Silva et al., 2004). Furthermore, anti-GlcCer antibodies were able to protect mice in a lethal infection by *C. neoformans* (Rodrigues et al., 2007b). The mechanism of action for these antibodies is still unknown. The enrichment of GlcCer at budding sites in *C. neoformans*, suggested that the antibodies could interfere with the cell wall biosynthesis and organization as previously described for the lectin WGA (Cio-praga et al., 1999). Alternatively, these antibodies could associate to GlcCer on lipid rafts, impairing cleavage of GPI-anchored proteins, essential to cell wall assembly. Cross linking of components found on lipid rafts by antibodies or ligands could also initiate a cell signaling response as observed in mammalian cells (Landry and Xavier, 2006; Marta et al., 2008; Bingaman et al., 2010). In this context, the only information available was the prominent cellular damage, with organelle destruction, membrane retraction, and increased vacuolization observed in yeasts from *C. neoformans* treated with anti-GlcCer (Nimrichter et al., 2005b). Some TEM images suggested an apoptotic mechanism, but no biochemical evidences were characterized.

A set of experiments published by Thevissen et al. (2004) strongly suggested that targeting fungal GlcCer could in fact initiate a cell signaling response in fungal organisms (Thevissen et al., 2004; Aerts et al., 2007). These authors demonstrated that a peptide isolated from radish seeds and named RsAFP2 was able to

specifically bind to fungal GlcCer (Thevissen et al., 2004). The peptide displayed a potent microbicidal activity in micromolar concentrations against fungal species that express GlcCer. The exact region of GlcCer where the peptide binds is not known, but apparently C-9 methylation at ceramide is not required, since mutants where this enzyme was impaired remained susceptible to the peptide (Ramamoorthy et al., 2009). Binding of peptide was followed by ROS production and fungal death (Aerts et al., 2007). The peptide activity was impaired by anti-oxidative molecules, confirming that fungal death was caused by an oxidative activity. Further studies by our group demonstrated a prophylactic activity for RsAFP2 in a murine model of candidiasis (Tavares et al., 2008). The peptide was biologically active against distinct *Candida* species and its potency was proportional to the amount of GlcCer expressed by the *Candida* strain tested.

Although the use of current GCS inhibitors is still questionable, a new alternative to decrease fungal GlcCer content in pathogenic fungi was recently proposed by Rhome et al. (2011): the administration of cerezyme (Cz) to mice lethally infected with *C. neoformans*. Cz is a human recombinant enzyme that removes glucose from GlcCer. It has been used as an alternative treatment for patients with Gaucher's, disease where hydrolysis of GlcCer is impaired and the GSL accumulates in lysosomes (Charrow, 2009). This enzyme was proven to hydrolyze fungal GlcCer and reduce its content in *C. neoformans in vitro* and *in vivo*. Cz-induced GlcCer decrease was associated with reduction of fungal membrane stability. In a murine model of cryptococcosis Cz was also able to increase mice survival (Rhome et al., 2011).

## CONCLUSION AND PERSPECTIVES

For over half a century, GlcCer was classified as a structural membrane component of eukaryotic cells. In the last decade,

however, a number of studies have uncovered sophisticated functions for GlcCer in eukaryotes. In fungi, it is now clear that knowledge on functions related to virulence, growth, and morphological transitions can be connected to specific structural features and particular biosynthetic steps to validate GlcCer as a potential target to development of new antifungal drugs. A major challenge, however, is to identify these drugs. The literature suggests several inhibitors of fungal GlcCer synthesis as candidates. So far, none of them have appeared to be sufficiently specific to allow use in human patients. In parallel, GlcCer ligands are apparently efficient and selective antifungal agents where in fact, peptides and monoclonal antibodies were already administered in murine models with encouraging results. Other candidates, including the antifungal factor HSAF and the enzyme Cz (which is already approved for use in humans), are also promising GlcCer-binding candidates. We need now more details on how antibodies, peptides, and enzymes interfering with the structure of GlcCer alter the biology of fungal cells, as well as how they impact human/animal physiology. Considering the critical side effects usually observed during antifungal therapy, the low efficacy of currently approved antifungal drugs and the emerging resistance of fungal cells against antifungals, we believe GlcCer is a very promising target for the activity of new compounds with potential to be used in human and animal individuals with severe mycoses.

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