New cell wall-affecting antifungal antibiotics

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Abstract

Fungi have emerged worldwide as increasingly frequent causes of healthcare-associated infections. Invasive fungal infections can be life-threatening. However, the number of antifungal agents available and their use in therapy is very limited. Recently, a new family of specific fungal cell wall synthesis inhibitors has emerged as an alternative antifungal therapy and is gaining increasing relevance yearly. The cell wall is a multilayer dynamic structure, essential to the integrity and shape of the fungal cell, whose function is to counteract the osmotic forces that could otherwise produce fungal cell lysis. The cell wall is absent in nonfungal cells, therefore representing a useful target in discovering selective drugs for the treatment of fungal infections without causing toxicity in the host. Although fungi exhibit a considerable diversity in their cell wall structure, all present β(1,3)-, β(1,6)- and α(1,3)-glucans, chitin, and mannoproteins as their major cell wall components. Three different cell wall synthesis inhibitors of the lipopeptide family of echinocandins, named caspofungin, micafungin and anidulafungin, are commercially-available and new classes of cell wall synthesis inhibitors are emerging. This review provides an overview of what is so far known about the different classes of cell wall-affecting antifungal agents and their mechanism of action, offering new alternatives with clinical potential.

1. Introduction

Invasive fungal infections are devastating. Despite the progressive development of new antifungal therapies, the mortality rates for invasive mycoses often reach the 50% (Lai et al., 2008, Park et al., 2009). Although fungal infections affect to individuals with intact immune systems, invasive aspergillosis and mucormycosis continue increasing in parallel with the growth of immunocompromised patients (Brown et al., 2012b). Improved diagnostics, new epidemiological analysis and new antifungals available have changed the field of medical mycology in the past few decades (Ostrosky-Zeichner et al., 2010).

The high phylogenetic similarity between fungi and humans makes that relatively few differential targets, can be used for antifungal drug development. Fungi originate a huge collection of extracellular enzymes and secondary metabolites to counteract and digest other fungi and microorganisms from their environment; thus many antimicrobial agents have been isolated from fungi themselves. The best example is penicillin, which was isolated from *Penicillium notatum* (now *Penicillium chrysogenum*). Similarly, the echinocandins, a class of antifungal compounds now in extensive clinical use, are semisynthetic derivatives of fungal-produced cyclic hexapeptides (Denning and Hope, 2010, Chain et al., 1993).

Presently, five classes of antifungal drugs are used for the treatment of fungal infections in humans: allylamines, azoles, echinocandins, polyenes and pyrimidine analogues. These drugs show some limitations, such as the nephrotoxicity of amphotericin B and the emerging resistance to the azoles (Cartledge et al., 1997), despite several improvements, such as new lipid formulations of polyenes with lower toxicity and new triazoles with a wider spectrum of action (Granier, 2000). The development of new antifungal agents, preferably naturally occurring with novel mechanisms of action, is an urgent medical need (Vicente et al., 2003). In the last years, new synthetic and semi-synthetic inhibitors of the cell wall synthesis have emerged (Hector and Bierer, 2011), being effective against the main infectious agents of life-threatening mycoses. In addition to the more traditional classes of cell wall synthesis inhibitors, this chapter focuses and reviews some of the new and more promising

classes of such compounds, and their mechanism of action, concentrating on the most promising candidates that are nearing or currently in clinical development.

2. Sites of action of the antifungal agents

The sites of action of all the antifungal agents used in clinic therapy are summarized in figure 1, showing the extensive range of antifungal targets that already exists. Nevertheless, the classes of agents that can be used against life-threatening mycoses are heavily focused, directly or indirectly, on the pathogen cell envelope (cell wall and plasma membrane), and particularly on the membrane complex β(1,3)-glucan synthase (GS), in charge of the biosynthesis of the cell wall β(1,3)-glucan; and on the membrane ergosterol and its biosynthesis (Chapter 8 of this book). Targets elsewhere in the cell would therefore be a welcome advance for systemically bioavailable antifungal agents. The introduction of the echinocandins (anidulafungin, caspofungin and micafungin are the only new drugs licensed within the last 10 years) and the third-generation triazoles (voriconazole and posaconazole), in particular, have enhanced therapeutic options for many fungal infections (Brown et al., 2012a, Ostrosky-Zeichner et al., 2010).

3. The fungal cell wall

The extracellular fungal wall is responsible for the cell shape, provides mechanical protection, and supports the internal osmotic pressure of fungal cells. In addition, its rigid structure is useful for penetration into and colonization of insoluble substrates. This structure is not simply a rigid exoskeleton, but has the flexibility required for allowing cell growth, cell division and the formation of numerous cell types during the fungal life cycle. The functional equivalent of the fungal cell wall in animal cells is denominated extracellular matrix, but its composition and osmotic and structural properties are totally different from those of the cell wall. Therefore, although both structures are functionally equivalent, their differences permit that the cell wall-affecting antifungals specifically inhibit the fungal cell wall synthesis without affecting the extracellular matrix of animal cells (Durán and Pérez, 2004, Free, 2013, Latge, 2007, Lesage and Bussey, 2006, Levin, 2011, Lu et al., 2012, White and Bednarek, 2003, Oh et al., 2012)

The cell wall is also critical for biofilm formation, a process that many fungi participate in, and which is an important ecological niche for a variety of fungi. For fungal pathogenicity, the cell wall is the surface of interaction between pathogen and host, being critical for its virulence and pathogenicity, providing both adhesive properties critical for invasion of host tissue and protection against the host defense mechanisms. Alterations of cell wall structure have a deep impact on the growth, morphology and integrity of the fungal cell, often leading to lysis by plasma membrane breaking and release of cytoplasm content. Given the essential role of the cell wall in fungal survival, this structure has long been considered an excellent target for antifungal agents (Free, 2013).

To build their walls, the fungal cells must synthesize the wall components in or export them across the plasma membrane, and assemble them outside the cell (Figure 2). The wall is composed basically of polysaccharides (70-90%) and glycoproteins (10-30%). Although composition varies among fungal species, most walls have a common structure (Latge, 2007). When observed by transmission electron microscopy, the cell walls show two electron dense external layers rich in glycoproteins and an internal layer more transparent to the electrons, which mainly contains fibrillar polysaccharides (Figure 2).

The main fungal wall fibrillar components are: glucose homopolymers, β(1,3)-glucan with different percentages of β(1,6) branches depending on the fungal species; chitin, a β(1,4)-N-acetylglucosamine polymer; and α(1,3)-glucan. Chitin accounts for only 1–2% of the yeasts wall (Cabib et al., 1988, Lesage and Bussey, 2006), whereas filamentous fungi, such as *Neurospora* or *Aspergillus*, contain 10–20% of chitin in their walls (Latge, 2007). In both yeasts and filamentous fungi, chitin forms microfibrils by interchain hydrogen bonding, which have enormous tensile strength and significantly contribute to the overall integrity of the cell wall (Cabib and Kang, 1987). Similarly, α(1,3)-glucan is essential for the adhesion strength of the cell wall components (Cortés et al., 2012).

The wall polysaccharides are formed at the plasma membrane by synthase enzymes and extruded to the periplasmic space (Figure 2), where they bind to each

other. The linkages among the different components, which results in a tightly linked network, are generated by transglycosylation (Cabib et al., 2007, Cabib et al., 2008) and are responsible for the mechanical strength of the cell wall (Cabib and Kang, 1987, Kapteyn et al., 1997, Kollar et al., 1995, Kollar et al., 1997).

4. Cell wall components

4.1. β(1,3)-glucan

β(1,3)-glucan is the main structural polysaccharide of the wall and represents 50-60% of the total cell wall polysaccharides. Most of glucan polymers are composed of glucose units linked with β(1,3) bonds (65-90%), although there are also some β(1,6), β(1,4), α(1,3) and α(1,4) glucans. The β(1,3)-glucan chains display a coiled spring-like structure that confers elasticity and tensile strength to the cell wall. Usually the β(1,3)-glucan is formed by a main backbone of β(1,3)-glucan and by β(1,6)-linked side chains (Figure 1, upper panel). In these linear chains of β(1,3)-glucan, new glucose units bind forming β(1,6) branches in variable proportion depending on the organism, from almost linear to highly branched. Then, the β(1,6)-branched β(1,3)-glucan can bind to other glucans, to chitin or to glycoproteins, providing a great mechanical resistance to the wall, which is essential to maintain the fungal cell integrity (Klis et al., 2002, Kollar et al., 1995, Kollar et al., 1997, Lesage and Bussey, 2006).

4.1.1. β(1,3)-glucan synthase (GS) complex

β(1,3)-glucan chains are synthesized by the enzyme complex β(1,3)-glucan synthase (GS), which is located in the plasma membrane. The echinocandins interfere with the production of β(1,3)-glucan and target the GS directly (Douglas, 2001). This family of enzymes use uridine-diphospho-glucose (UDP-Glc) as substrate and catalyze the reaction 2n UDP-Glc→ [Glc-β-1,3-Glc]n in the presence of GTP, resulting in the formation of linear glucan chains composed of approximately, 1,500 β(1,3)-bound glucose residues (Figure 1, upper panel) (Cabib and Kang, 1987). The GS complex is composed by at least two subunits, which were identified by detergent and high ionic strength extraction from the plasma membrane, followed by an *in vitro* assay for GS

activity. Under these conditions, the GS complex was separated into a membrane bound and a cytosolic fraction, containing the catalytic and regulatory GTP-binding subunits, respectively (Kang and Cabib, 1986):

4.1.1.1. Catalytic subunit

The genes coding for the putative GS catalytic subunit were initially identified in *S. cerevisiae* and named *FKS1* and *GSC2*/*FKS2* (Douglas et al., 1994b, Mazur et al., 1995). *FKS1* (for ***FK***506 **s**ensitive) was initially cloned by complementation of a mutant hypersensitive to the calcineurin inhibitor FK506 (Parent et al., 1993). The discovery that *ETG1* and *PBR1* (whose mutants are resistant to GS inhibitors), *CND1* (the *cnd1* mutant requires a functional calcineurin pathway), and *CWH53* (required for resistance to calcofluor white) are all identical to *FKS1*, established the link between Fks1 and the cell wall (Castro et al., 1995, Douglas et al., 1994a, El-Sherbeini and Clemas, 1995, Garrett-Engele et al., 1995, Ram et al., 1995). *FKS1* mutants show decreased GS activity, and Fks1 is enriched in membrane fractions enriched in GS activity, suggesting that Fks1 could be a GS catalytic subunit (Douglas et al., 1994a, Inoue et al., 1995).

Fks2/Gsc2 is 88% identical to Fks1 and presents similar topology and domain organization to that of Fks1. Fks1 and Fks2 are thought to be alternative subunits of the GS complex, with a double *fks1*Δ *fks2*Δ mutant being nonviable. This synthetic lethality and the finding that membrane extracts from cells expressing only *FKS2*, show a GS activity that can be depleted after treatment with anti-Fks2 antibodies, suggested that Fks2 is involved in β(1,3)-glucan synthesis (Inoue et al., 1999, Mazur et al., 1995).

FKS1 and *FKS2* show differential expression patterns. *FKS1* transcription is cell-cycle regulated, and linked to cell wall remodeling. *FKS2* transcription is calcineurin-dependent (Kurtz and Rex, 2001). *FKS1* is expressed during mitotic growth, in agreement with its proposed function as the major GS subunit. *fks1*Δ cells show reduced GS activity and altered cell wall composition, with decreased β(1,3)- and β(1,6)-glucan levels, and increased chitin and mannan levels (Dijkgraaf et al., 2002, Douglas et al., 1994a, Lesage and Bussey, 2006). In contrast, during vegetative growth the *fks2*Δ mutant does not display cell wall or cell growth defects. However, during

sexual differentiation the *fks2*Δ cells are impaired in spore wall assembly. The strength of the phenotypes observed in *fks1*Δ compared to those observed in *fks2*Δ cells, suggest that Fks1 is the main responsible for GS activity and β(1,3)-glucan synthesis during vegetative growth, while Fks2 would function under more stressful conditions (Lesage and Bussey, 2006). The *S. cerevisiae FKS* family has a third member, *FKS3*, whose product is required for sporulation (Deutschbauer et al., 2002, Ishihara et al., 2007).

The Fks protein family of GS is very well conserved in fungi and plants, and are large multispan membrane proteins with a hydrophilic central region (Johnson and Edlind, 2012). Orthologs of these genes have been described in the main fungal genera, encoding proteins with a high degree of identity ranging from 56% (*Cryptococcus neoformans*) to 83% (*Candida glabrata*) (Latge, 2007, Lesage and Bussey, 2006, Pérez and Ribas, 2004, Cortés et al., 2002).

The fission yeast *Schizosaccharomyces pombe* has provided an appealing model for studies addressing cell wall synthesis and morphogenesis. The cell wall of *S. pombe* has no detectable chitin (Durán and Pérez, 2004), but it contains three different essential β(1,3)-glucans as follows: a β(1,6)-branched β(1,3)-glucan, which is the major contributor to the cell wall structure; a minor linear β(1,3)-glucan, concentrated in the primary septum, with residual amounts in the cell wall; and a minor branched β(1,6)-glucan (Cortés et al., 2007, Humbel et al., 2001). *S. pombe* contains four genes, *bgs1*$^{+}$ to *bgs4*$^{+}$, which encode four different GS catalytic subunits of the Fks protein family, and three of them (Bgs1, 3 and 4) are essential during vegetative growth. Differently to what has been described for *S. cerevisiae* and some other fungi, where Fks1 and Fks2 have been shown to have redundant functions (see above), *S. pombe* GS catalytic subunits display differential essential non-overlapping functions in the assembly and the transport of different β(1,3)-glucans during polarized cell wall growth, septum synthesis and ascospore wall assembly. The different essential functions of Bgs proteins in cell wall synthesis and morphogenesis make them good targets for the search and study of new improved antifungal drugs that specifically inhibit β(1,3)-glucan synthesis. *bgs1*$^{+}$ was initially cloned by phenotype complementation of a mutant that displays hypersensitivity to Cyclosporin A as well as Papulacandin B, and the encoded protein Bgs1 is responsible for the synthesis of the linear β(1,3)-glucan and primary septum

(Cortés et al., 2002, Cortés et al., 2007, Ishiguro et al., 1997, Liu et al., 2002). *bgs2*$^{+}$ encodes a protein essential for spore wall maturation, and *bgs3*$^{+}$ was identified as a suppressor of a mutant that shows hypersensitivity to echinocandin. Bgs3 is essential, although its function remains unknown (Carnero et al., 2000, Liu et al., 2000, Martín et al., 2000). *bgs4*$^{+}$ encodes the only subunit that has been shown to form part of the GS enzyme. It is responsible for most of the cell wall β(1,3)-glucan synthesis and *in vitro* GS activity, and it is essential for the maintenance of cell integrity during cell growth and mainly septum synthesis. To date the only identified mutants of *S. pombe* that display reduced levels of β-glucan and GS activity, or resistance to specific GS inhibitors, are due to point mutations in the Bgs4 sequence (Castro et al., 1995, Cortés et al., 2005, Martins et al., 2011, Ribas et al., 1991b, Ribas et al., 1991a).

The presence of multiple *FKS*/*bgs* genes in some fungi might be the result of the ancient and selective retention of duplicated genes through increased fitness conferred by their specialized application of GS functions (Lesage and Bussey, 2006). This complexity is also found in plants, where the Fks/Bgs protein family of GS is also present. Plant GS synthesize a β(1,3)-glucan polymer denominated callose. Although β(1,3)-glucan is less abundant in plants than in fungi, the number of GS genes is much higher in plants than in fungi. Thus, *Arabidopsis thaliana* or *Oryza sativa* contain 12–18 callose synthase genes whereas the highest number of genes in fungi are the 4 *bgs* genes identified in *S. pombe*. Plant callose is involved in pollen development (primary and secondary cell wall of the pollen, germination pore and pollen tube) as well as wound repair after injury or disease. Similarly to *S. pombe* β(1,3)-glucans, all steps in which callose participates should involve different callose synthase genes, which would explain the existence of multiple callose synthase genes in plants. Despite plant and fungi belong to different kingdoms, plant and fungal GS display high sequence identity (Cortés et al., 2007, Latge, 2007, Verma, 2001, Verma and Hong, 2001).

The GS catalytic subunit is thought to extrude β(1,3)-glucan chains produced on the periplasmic face of the plasma membrane for incorporation into the wall. Although the GS enzyme has never been purified to homogeneity, the central hydrophilic domain of partially purified *Neurospora crassa* Fks protein was shown to crosslink to azido-UDP-glucose, supporting the conclusion that this protein is the catalytic subunit

(Schimoler-O'Rourke et al., 2003). Purification of the complete and active GS catalytic subunits will require new advances in our knowledge of enzymatically active integral membrane protein complexes (Lesage and Bussey, 2006, Levin, 2011).

4.1.1.2. The regulatory subunit Rho1

Besides the catalytic subunit, fungal GS require GTP-bound Rho1 GTPase for their activity. Essential Rho1 is the prototype of small G proteins, which in their active GTP-bound state bind and activate their effectors. After synthesis in the endoplasmic reticulum, Rho1 is geranylgeranylated, allowing its anchoring to the membrane through a C-terminal prenylated tail, which is required for Rho1-membrane association and activation of GS activity (Arellano et al., 1998, Díaz et al., 1993, Inoue et al., 1999). Different biochemical and genetic approaches have pointed out to Rho1 GTPase as a regulator of GS: (i) Rho1 colocalizes and copurifies with Fks1 and cofractionates with GS activity, (ii) thermosensitive *rho1* mutations cause thermolabile GS activity, (iii) a hyperactive *rho1* allele shows a GTP-independent GS (iv) overproduction of Rho1 is able to partly suppress the GS deficiency of a geranylgeranyltransferase type I mutant (Arellano et al., 1996, Drgonova et al., 1996, Mazur and Baginsky, 1996, Qadota et al., 1996, Kondoh et al., 1997). Geranylgeranylated Rho1p is transported to the plasma membrane, where is thought to be activated by its GDP/GTP exchange factors. This localized activation on the plasma membrane is required for proper cell wall β(1,3)-glucan synthesis (Abe et al., 2003, Díaz et al., 1993, Inoue et al., 1999, Perez and Rincon, 2010). Although the Fks family members are well established as Rho1 effectors, the interaction domains between the GS catalytic and regulatory subunits and the basis for Rho1 activation on the GS catalytic subunits remain unknown (Lesage and Bussey, 2006).

4.2. β(1,6)-Glucan

In addition to β(1,3)-glucan, most of fungal walls contain a second β-linked glucan, the β(1,6)-glucan. This polymer is shorter than β(1,3)-glucan, it does not form a fibrillar structure, and acts as a flexible glue by forming covalent cross-links to β(1,3)-glucan, chitin, and glycoproteins (Kollar et al., 1997). Comparative studies show the

variability of β(1,6)-glucan structures among fungi, with the most highly branched *S. pombe* diglucan, likely a variant of the β(1,6)-glucan found in *S. cerevisiae* and *C. albicans* (Lesage and Bussey, 2006). To date, most of the genes implicated in the synthesis of β(1,6)-glucan have been identified in *S. cerevisiae*. The site of β(1,6)-glucan synthesis has been controversial for many years. Defects in β(1,6)-glucan synthesis are observed in mutations of genes as *CWH41*, *ROT2, KRE5* and *CNE1*, encoding glucosidases I and II and UDP-glucose: glycoprotein glucosyltransferase-[UGGT]- and calnexin-related proteins, respectively. These proteins are involved in different steps of the secretory pathway (Page et al., 2003, Lesage and Bussey, 2006, Shahinian and Bussey, 2000), suggesting that biosynthesis of this polymer could start in the endoplasmic reticulum. In *S. pombe*, an immuno-electron microscopy analysis showed particles of β(1,6)-glucan associated to the Golgi apparatus (Humbel et al., 2001), suggesting that biosynthesis of this polymer progresses in the Golgi, and is completed at the cell surface. Indeed, *S. cerevisiae* glucosyl hydrolases (or transglucosylases) Kre6 and Skn1 are critical for β(1,6)-glucan synthesis and reside in the Golgi (Roemer and Bussey, 1991, Roemer et al., 1994). However, a late secretory pathway mutant displayed β(1,6)-glucan exclusively accumulated in the cell surface, indicating that a block of the secretion does not cause an accumulation of intracellular β(1,6)-glucan (Montijn et al., 1999), suggesting that β(1,6)-glucan, like β(1,3)-glucan, may be synthesized at the plasma membrane. An *in vitro* assay for β(1,6)-glucan synthesis using specific antibodies against β(1,6)-glucan has been developed (Vink et al., 2004). This assay requires membrane extracts, UDP-glucose and GTP and shows enhanced activity in cells overexpressing Rho1, suggesting that the β(1,6)-glucan could be synthesized at the sites of polarized cell growth where Rho1 is detected. Besides, the use of membrane extracts from defective β(1,6)-glucan mutants correlated the decreased *in vitro* β(1,6)-glucan synthase activity with the low levels of *in vivo* cell wall β(1,6)-glucan (Levin, 2011, Vink et al., 2004).

4.3. Chitin

Chitin is a β(1,4)-linked homopolymer of *N*-acetylglucosamine that forms microfibrils stabilized by hydrogen bonds. This polymer is present in the walls of all fungi studied to date with the exception of *S. pombe*. Chitin represents 1-2% of the yeast

cell wall whereas in filamentous fungi, it can reach up to 10-20% (Latge, 2007). Chitin is synthesized from N-acetylglucosamine units by the enzyme chitin synthase (CS) that deposits microfibrils of chitin outside of the plasma membrane. This family of enzymes use uridine-diphosphate-N-acetylglucosamine (UDP-GlcNAc) as substrate and catalyze the reaction 2n UDP-GlcNAc → [GlcNAc-β-1,4-GlcNAc]n. Chitin biosynthesis is best understood in *S. cerevisiae*, where it has been broadly studied. Three CS activities (CSI, CSII, and CSIII) have been identified in membrane extracts, and the corresponding catalytic subunits have been identified as Chs1, Chs2 and Chs3, respectively. The three Chs proteins are integral membrane proteins, each one responsible for the synthesis of a chitin (Cabib et al., 2001) at different times and places during cell growth. Chs1 acts as a repair enzyme during cell separation; Chs2 is responsible for synthesis of the primary septum chitin; and Chs3 synthesizes most of the cell wall chitin and is responsible for the increase in chitin synthesis observed when the cell wall is stress-affected (Roncero and Sanchez, 2010, Schmidt et al., 2002). The number of CS genes varies from 1 to 20 according to the fungal species. The large family of CS enzymes fall into seven classes according on the evolution of their amino acid sequences (Roncero, 2002). The multiplicity of enzymes suggests that they have redundant roles in chitin synthesis and makes it difficult to find functional significance to the different classes (Lenardon et al., 2010).

4.4. α(1,3)-glucan

Many fungi contain α(1,3)(1,4)-glucan in their cell wall. However, the corresponding *in vitro* α(1,3)-glucan synthase activity has not been detected yet. A putative catalytic subunit was first described in *S. pombe* (Cortés et al., 2012, Hochstenbach et al., 1998, Katayama et al., 1999). Ags1/Mok1 is a multidomain integral membrane protein with a predicted cytoplasmic synthase domain, multiple transmembrane domains and an extracellular transglycosylase domain. The cytoplasmic synthase domain would add glucose residues to the non-reducing end of an α(1,3)-glucan glucan chain. Interestingly, the large extracellular N-terminal region presents homology to transglucanases, which could function in cross-linking newly synthesized α(1,3)-glucan to other cell wall components (Grun et al., 2005, Vos et al., 2007). The α(1,3)-glucan synthesized by Ags1/Mok1 is vital for cell integrity during polar growth and mainly cell separation, and to maintain the adhesion between cell wall components,

primarily in the septum during cell separation (Cortés et al., 2012). *S. pombe* contains five genes coding for Ags/Mok proteins, and genomes of other fungi, including several pathogens in which the cell wall α-glucan accounts for around 35% of the total wall polysaccharides, present sequences of predicted proteins homologous to the Ags/Mok family (Edwards et al., 2011, García et al., 2006, Henry et al., 2011).

4.5. Glycoproteins

Glycoproteins represent 30-50% of the dry weight of the walls of *S. cerevisiae* or *Candida*, and around 15-20% of the dry weight of *S. pombe* and filamentous fungi walls. The glycoproteins present in the cell wall are expansively modified with both N- and O-linked carbohydrates, predominantly or exclusively formed by mannose residues known as mannan. In some cases, the mannan backbone presents single residues or side chains of different sugars, galactomannan, rhamnomannan, glucogalactomannan, rhamnogalactomannan, etc. (Bowman and Free, 2006, Leal et al., 2010). Most of the wall glycoproteins are attached through a glycosylphophatidyl inositol (GPI) remnant to β(1,3)-D-glucan or chitin, via a β(1,6)-glucan linker. Other wall glycoproteins are directly covalently attached to β(1,3)-D-glucan (Klis et al., 2006). The wall glycoproteins contribute to the preservation of the cellular shape, participating in adhesion processes, transmitting signals to cytoplasm, and remodeling the components of the wall (Latge, 2007).

5. Inhibitors of cell wall biosynthesis

As described above, the cell wall structure and rigidity depend on the layering and interlinking of β(1,3)-glucan, β(1,6)-glucan, chitin, α(1,3)-glucan and mannoproteins. Because the cell wall is absent in mammalian cells, its polysaccharides are attractive targets for the discovery and development of antifungal drugs. However, the types of polysaccharides, their extent and the linkages between them are quite variable across the fungal kingdom, and can change during the different growth phases. Therefore, the use of inhibitors of the synthesis of a specific wall component might result in different effects depending on the fungus or its stage of growth.

5.1. Inhibitors of the β(1,3)-glucan synthase (GS)

To date, the GS is the only component of the cell wall synthesis machinery that has successfully led to the development and commercialization of new drugs on the market. In 2001, the Merck inhibitor of the GS caspofungin (Cancidas®, Merck, Sharp and Dhome, MSD) was approved for clinical use. Since then, antifungal drugs that inhibit the GS have extensively been used in clinic. The collection of marketed echinocandins also includes micafungin (Mycamine®, Astellas Pharma) and anidulafungin (Eraxis® or Ecalta®, Pfizer), which were approved for the treatment of invasive candidiasis and aspergillosis in 2005 and 2006-7, respectively (Sable et al., 2008, Walsh et al., 2008, Chapman et al., 2008). Their structures are shown in figure 3. Additional members of this general class of GS inhibitors have emerged as preclinical candidates over the years, including papulacandins (Ciba-Geygy, Novartis), aerothricin analogs (Chugai Pharmaceutical and Basilea Pharmaceutica), cryptocandins (HMV Corporation), and other analogs (MSD, Astellas Pharma and Eli Lilly) (Hector and Bierer, 2011). However, this class of antifungals displays limitations as the lack of oral formulations, restricting them to parenteral formulations, and their semi-synthesis based on fermentation products. In addition, these compounds have a limited spectrum of activity showing no activity against, for example, *C. neoformans*. New β-glucan inhibitors with a broad spectrum activity against the main fungal pathogens, some of them with oral bioavailability, have started to emerge (Hector and Bierer, 2011).

5.1.1 Mode of action

The primary mode of action of this class of antifungals is the obstruction of the biosynthesis of the fungal cell wall by inhibiting the GS enzyme (Douglas et al., 1994b, Pérez et al., 1981, Yamaguchi et al., 1985), but little is known about their mechanisms of action. Inhibitors of β(1,3)-glucan synthesis also have secondary effects on other components of the cell, including a decrease in the ergosterol and lanosterol content and an increase in the chitin content of the cell wall (Pfaller et al., 1989). Echinocandins bind non-competitively to the catalytic subunit of GS. The inhibition of β(1,3)-glucan synthesis requires the uptake of echinocandins by sensitive cells. At low concentrations (<1 mg/ml), a high-affinity facilitated-diffusion transporter mediates the uptake of caspofungin, a semisynthetic pneumocandin B_0 derivative (see below) in *Candida albicans*. At higher concentrations, nonspecific uptake can also progress (Paderu et al.,

2004). The specificity of this class of antifungals for β(1,3)-glucan synthesis was demonstrated by the observation that echinocandins were ineffective in inhibiting chitin or mannan synthesis (Pérez et al., 1981). Substantial work has gone into the mechanistic understanding where echinocandins bind to the GS, but this question still remain obscure, largely because a membrane-associated protein is involved (Denning, 2003).

In yeasts, inhibitors of the GS increase the osmotic sensitivity of the cells and cause cell lysis; however resistant mutants with different behaviors against distinct antifungals compounds have been described, suggesting specific modes of action against the GS within this antifungal class (Martins et al., 2011). Only some *S. cerevisiae* and *S. pombe* mutants have been identified as resistant to the glycolipid papulacandin (Castro et al., 1995, Ribas et al., 1991b), in each case defining a single gene called *pbr1*, later found to be allelic to *FKS1* and $bgs4^{+}$, respectively. In *S. pombe*, wild type and the resistant mutant strains *pbr1-8* and *pbr1-6*, display differences both *in vivo* and *in vitro* between papulacandin, enfumafungin and echinocandins (Martins et al., 2011). In *S. pombe* wild type cells, papulacandin and enfumafungin produce generalized cell lysis, while echinocandins are different; the lysis of wild type cells is incomplete, and the surviving cells become rounded and maintain a residual cell growth. Besides, whereas *pbr1-8* and *pbr1-6* are highly resistant to papulacandin and enfumafungin, they exhibit opposite behaviors with regard to aculeacin; *pbr1-8* is highly resistant and *pbr1-6* is sensitive (Martins et al., 2011). No GS activation by GS inhibitors has been reported, except for papulacandin in *S. cerevisiae* wild type GS and for enfumafungin, pneumocandin and caspofungin in *S. pombe pbr1-8* GS (Kang et al., 1986, Martins et al., 2011). In the case of *S. cerevisiae*, the drug activation affects the wild type GS and is dependent on low substrate concentrations. It is possible that some mutations, such as that *S. pombe pbr1-8* GS, in the presence of an antifungal could mimic the proposed preferential binding of substrate to the active form of the enzyme. Caspofungin displays special properties as regards the *in vitro* GS activity, with an inhibitory concentration lower than that of other drugs, except papulacandin, and with two previously unreported inhibitory effects of high and low affinity (Figure 4), suggesting the presence of two GS interaction sites with caspofungin (Martins et al., 2011).

It is clear that the target to which these antifungals bind is the catalytic subunit of the GS, but their inhibitory effects on β(1,3)-glucan synthesis do not necessarily would involve the catalytic subunit itself, nor is it clear whether their binding site on GS catalytic subunit is external or internal to the plasma membrane (Odds et al., 2003). For example, it has recently been suggested that the acyl side chain of the echinocandins may interact with the plasma membrane (Chen et al., 2011).

5.1.2. Echinocandins

The commonly known as echinocandins (variously called lipopeptides, cyclic hexapeptides, pneumocandins, etc.) is a class of antifungals that specifically target the GS enzyme. Echinocandins (Figure 3) are large lipopeptide molecules, produced by fungi as secondary metabolites. The first echinocandin-type antimycotics, aculeacin A and echinocandin B, were isolated independently by random screenings in the 1970s. A modified form of echinocandin B, cilofungin, was developed to clinical use, but was abandoned when its formulation showed toxicity to patients in the trials. All the echinocandin derivatives in clinical use or development are amphiphilic cyclic hexapeptides with an *N*-linked acyl lipid side-chain (Denning, 2003, Kurtz and Rex, 2001, Odds et al., 2003). The three echinocandins approved for clinic use (caspofungin, micafungin and anidulafungin) exhibit linear pharmacokinetics, are highly protein bound (97%–99%) and are not dialyzable. The echinocandins present some advantages which make them useful when used as additional antifungal treatment. Among common fungal pathogens, only *C. neoformans* is excluded from the echinocandins spectrum; but they also lack activity against emerging pathogens, such as *Fusarium* spp. and *Scedosporium* spp. However, they are active against *Pneumocystis jiroveci* (Odds et al., 2003). Besides, echinocandins display an improved hepatic and renal safety profile compared with those of the azoles and polyenes, and decreased cytochrome-mediated drug interactions compared with those of the azoles (Walker et al., 2011). However, the main disadvantage of available echinocandins is that all of them have limited oral bioavailability, and therefore must be administered by intravenous infusion (Emri et al., 2013, Sable et al., 2008).

5.1.2.1 Caspofungin

In 2001 Caspofungin (Cancidas®) was the first cell wall antifungal, commercialized by MSD and approved for clinical use in both the United States and the European Union. Caspofungin is used as salvage therapy for invasive aspergillosis. Caspofungin is a semisynthetic echinocandin derived from pneumocandin B_0 via chemical modification of the hexapeptide scaffold (Figure 3). Pneumocandins are natural products derived from the fermentation of the fungus *Glarea lozoyensis*. The introduction of additional amino groups in the peptide ring of pneumocandin B_0 increased the solubility of the molecule and the potency against fungal pathogens by two orders of magnitude. The caspofungin group has lower rates of nephrotoxicity, infusion-related events, and drug-related adverse events (Denning, 2003, Emri et al., 2013, Sable et al., 2008, Vicente et al., 2003).

5.1.2.2 Micafungin

Micafungin (Mycamine®) was commercialized by Astellas Pharma and approved for clinical use in the United States and the European Union in 2005. It is a semisynthetic echinocandin produced from the echinocandin B FR901379 via substitution of the fatty acid side chain (Figure 3). Micafungin was approved for the treatment of esophageal candidiasis and for the prevention of *Candida* infections in patients undergoing hematopoietic stem cells transplantation. Micafungin is shown to be superior to fluconazole when given as prophylaxis in stem cell transplantation, and show less drug interactions than caspofungin (Denning, 2003, Odds et al., 2003, Sable et al., 2008).

5.1.2.3 Anidulafungin

Anidulafungin was commercialized by Pfizer and approved for clinical use in the United States in 2006 (Eraxis®) and in the European Union in 2007 (Ecalta®). It is a semisynthetic echinocandin produced from echinocandin B, via substitution of the fatty acid side chain (Figure 3). It is used against esophageal candidiasis, candidemia, and other *Candida* infections (intra-abdominal abscess and peritonitis). It is highly active *in vitro* against a wide range of *Candida* spp, including those that are resistant to azoles (*C. krusei*), amphotericin B (*C. lusitaniae*) or other echinocandins (*C. parapsilosis*), and

also against species of *Aspergillus* spp. (Denning, 2003, Emri et al., 2013, Kathiravan et al., 2012, Sable et al., 2008).

5.1.2.4 Echinocandin B derivatives

A series of cyclohexapeptide echinocandin semi-synthetic derivatives and formulations are the subject of several patents. In one of these patents it is claimed that the natural products echinocandin B, aculeacin, pneumocandin A_0, pneumocandin B_0, pneumocandin C_0 and cilofungin may also be used as synthetic starting points. Activity against several *Candida*, *Aspergillus* and *Cryptococcus* strains is mentioned for the described compounds, but biological data are not provided. Although not shown, it is stated that some of the echinocandin B derivatives present good activity *in vitro* against the GS of *C. albicans* and *A. fumigatus* (Hector and Bierer, 2011).

5.1.3 Enfumafungin and MK-3118

Enfumafungin is a hemiacetal triterpene glycoside (Figure 3) that was originally isolated by the fermentation of *Hormonema* sp. It exists as a mixture of two interconverting forms at the hemiacetal state, and it was determined that the natural product specifically inhibits the GS (Pelaez et al., 2000, Onishi et al., 2000, Martins et al., 2011). MK-3118 (Figure 3) is an orally active, semisynthetic derivative of enfumafungin with *in vitro* and *in vivo* activity against *Candida* spp and *Aspergillus* spp. MK-3118 and other derivatives of enfumafungin are potent inhibitors of fungal GS, yet these compounds are structurally distinct from the echinocandins. The sites of mutations in Fks GS that are associated with resistance to the echinocandins are in some cases distinctly different from those causing decreased susceptibility to the enfumafungin derivatives. MK-3118 displays *in vitro* an excellent activity against wild type *Candida* spp, and wild type and itraconazole-resistant strains of *Aspergillus* (Hector and Bierer, 2011, Pfaller et al., 2013b, Pfaller et al., 2013a, Walker et al., 2011). *In vivo*, the preclinical results demonstrate a comparable level of activity for MK-3118 against *Candida* spp. compared with caspofungin, while mouse efficacy results for aspergillosis suggest a somewhat inferior response compared with caspofungin. Importantly, the demonstration of oral bioavailability with MK-3118 suggests that formulations of this drug may be dosed either orally or parenterally, providing a much

needed flexibility to the class of GS inhibitors as antifungal agents (Hector and Bierer, 2011).

5.1.4 Papulacandins

The glycolipids papulacandins (Figure 3) are a series of naturally occurring antifungal agents containing a benzannulated spiroketal unit, which has been the signature of a wide series of bioactive natural products and has inspired ample synthetic activity. The papulacandins A–E were isolated from the fermentation broths of *Papularia sphaerosperma* (Traxler et al., 1977, van der Kaaden et al., 2012). They block the synthesis of β(1,3)-glucan by inhibition of GS (Baguley et al., 1979, Pérez et al., 1981, Varona et al., 1983). Interestingly, it has been observed a general decline in the inhibition of GS as the concentration of the substrate UDP-glucose is decreased. Surprisingly, at very low concentrations of UDP-glucose, papulacandin B even acted as a stimulator, suggesting that UDP-glucose might act as an allosteric ligand, shifting the enzyme from one conformation to another once its concentration increases. Within the range of substrate concentrations in which papulacandin B is inhibitory, the inhibition appears to be of the mixed type, although very close to non-competitive (Kang et al., 1986, Pérez et al., 1981). Papulacandins display a very high specific activity against several yeasts, but they are largely inactive against filamentous fungi, bacteria and protozoa (Traxler et al., 1977). Direct comparison (Figure 4 and not shown) between glycolipids (papulacandin B), echinocandins (pneumocandin, caspofungin and aculeacin) and acidic terpenoids (enfumafungin) has shown that papulacandin B inhibitory effect of GS activity is superior in several orders of magnitude to that of the other antifungals (Martins et al., 2011). Several new compounds structurally related to papulacandins have been isolated (van der Kaaden et al., 2012). Their structures diverge with respect to the two partially unsaturated acyl chains on the sugars. Drastic changes in these tails or the lack of one of the tails severely reduce their activity compared with the most active papulacandin B (van der Kaaden et al., 2012). Due to their limited potency in animal models, neither papulacandin B nor any of its derivatives have been developed as GS inhibitory drugs for clinical use (Vicente et al., 2003).

5.1.5. Pyridazinone derivatives

Since the echinocandins used in clinic are delivered only parenterally, there is a significant interest in identifying new and unrelated GS inhibitors. A search for antifungal bioactivities combined with mechanism-of-action studies identified a new class of piperazinyl-pyridazinones that target the GS. The generic form of pyridazinone compounds that inhibit the GS is shown in figure 3 (Hector and Bierer, 2011). These compounds exhibit *in vitro* activity comparable, and in some cases superior, to that of echinocandins (Walker et al., 2011, Hector and Bierer, 2011, Butts and Krysan, 2012). The pyridazinone compounds inhibit GS *in vitro*, with a strong correspondence between enzyme inhibition and *in vitro* antifungal activity. Moreover, the compounds cause cell lysis and release of cytoplasmic contents as other GS inhibitors. Importantly, this novel class of small-molecule GS inhibitors present oral efficacy in a murine model of disseminated *C. glabrata* infection. The oral availability of these pyridazinone derivatives distinguishes them from the echinocandins (Walker et al., 2011).

5.1.6 Other inhibitors of the GS

Besides echinocandins, other cyclic peptides have been described as inhibitors of β(1,3)-glucan synthesis (Vicente et al., 2003, Hector and Bierer, 2011). The echinocandin-related Cryptocandin has an inhibitory activity against *C. albicans* and *Trychophyton* spp (Strobel et al., 1999). Arborcandins are other antifungal agents described as GS inhibitors, containing a 10-amino-acid ring and two lipophilic tails (Ohyama et al., 2000). Similarly, Aerothricin3/FR901469 is a macrocyclic lipopeptidolactone composed of 12 amino acids and a 3-hydroxypalmitoyl moiety with GS inhibitory activity (Fujie et al., 2001, Kondoh et al., 2002). The piperazine propanol derivative GSI578 [(2,6-Difluoro-phenyl)-carbamic acid 3-(4-benzothiazol-2-yl-piperazine-1-yl)-propyl ester] is a synthetic antifungal drug described as GS inhibitor (Kondoh et al., 2005). Clavariopsins, cyclic depsipeptides lacking a long lipophilic radical, have also been described as inhibitors of glucan synthesis (Kaida et al., 2001). New chlorogenic, quinic and caffeic acid derivatives that were coupled with an H2N-orn-4-(octyloxy) aniline group have been described to display antifungal activities by partial inhibition of GS (Ma et al., 2010). Additionally, several new synthetized 4-aryl-4-*N*-arylamine-1-butene compounds display antifungal properties and specific inhibitory activity on the GS (Urbina et al., 2000).

5.1.7. Resistance to GS inhibitors: The importance of *FKS* hotspots

Nowadays, a significant problem of the public health is the rising prevalence of resistance to antimicrobial agents among important human pathogens, which is severely restricting the availability of treatments for common infections (Ben-Ami and Kontoyiannis, 2012). Although resistance to echinocandins and other GS inhibitors is still relatively uncommon, it is increasingly encountered; moreover, the clinical susceptibility breakpoints for echinocandins result in the inclusion of a greater proportion of clinical isolates in the resistant category (Pfaller et al., 2011a, Ben-Ami and Kontoyiannis, 2012).

Fungal resistance to GS inhibitors is clearly associated with mutations grouped in conserved short regions (hot spots) of the Fks proteins (Figure 5B), indicating that this resistance mechanism is well conserved in fungi (Perlin, 2007, Rocha et al., 2007, Walker et al., 2010). In addition, some fungi are naturally resistant to echinocandins, as they contain natural substitutions in the conserved Fks hot spot regions that are determinants of their resistance (Katiyar and Edlind, 2009, Perlin, 2007, Walker et al., 2010). *FKS* hotspot mutations which confer resistance to echinocandin are frequently associated with changes in cell wall thickness, attributed to increased cell wall chitin content due to upregulation of chitin synthesis as a result of activation of cell wall salvage pathways (Walker et al., 2008, Ben-Ami and Kontoyiannis, 2012). In these cases, the combination of echinocandins with chitin synthase inhibitors (see below) avoids the increase of cell wall chitin, preventing the growth of echinocandin-resistant strains that contain *FKS1* hot spot mutations (Walker et al., 2008, Munro, 2013).

Another resistance effect associated to just activation of compensatory mechanisms from the cell integrity and calcineurin pathways is the termed paradoxical growth effect, or Eagle effect, described for *Candida* spp. This paradoxical effect consists of an *in vivo* attenuation of growth inhibition at drug concentrations above the inhibitory concentration. The result is growth inhibition followed by a resumption of growth at higher antifungal concentrations and a new inhibitory effect when the drug concentration increases. In this case, the resistance is not due to Fks hot spot mutations and therefore, it is only observed on the *in vivo* cell growth but not on the *in vitro* GS activity (Fleischhacker et al., 2008, Wiederhold, 2007).

Clearly, a weak point in all the β(1,3)-glucan synthesis inhibitors discovered or developed up to date is their lack of activity against *C. neoformans*. This is something intriguing since the *FKS1* homologue gene of this fungus has been shown to be essential, leading to the proposal that its GS enzyme could be relatively resistant to the action of echinocandins and the rest of the β(1,3)-glucan synthesis inhibitors. However, the *in vitro* GS assays have demonstrated that the GS from *C. neoformans* is in fact very sensitive to caspofungin and cilofungin (Maligie and Selitrennikoff, 2005), indicating that *C. neoformans* is resistant to echinocandins through other mechanisms.

S. pombe is a good model for *in vivo* and *in vitro* studies of the resistance mechanisms to GS inhibitors (see above). It is interesting that exclusively some *S. cerevisiae* and *S. pombe* mutants display resistance or have been found resistant to papulacandin (see above and Figure 5A). Although *S. pombe* vegetative cells contain three essential Bgs subunits which all contain in their hot spots the conserved aminoacid sequences associated to natural antifungal sensitivity, the antifungal resistance is only associated with Bgs4 hot spot mutations, suggesting that Bgs1 and Bgs3 are natural intrinsic resistant subunits (Figure 5B). The analysis of mutants resistant to papulacandin expanded the resistance hot spot 1 to 13 aminoacids and defined a new resistance hot spot 1-2 (Martins et al., 2011). These new sites, which are important for resistance and interaction with antifungals, should help to understand the mechanism of action of antifungals, and the resistance mechanism to GS inhibitors of the Fks proteins.

5.2 New inhibitors of β(1,6)-glucan synthesis

C. neoformans is an encapsulated pathogenic yeast that is responsible for pulmonary infections and fatal meningoencephalitis in humans. Disseminated cryptococcosis is one of the main causes of death among immunocompromised patients, while cryptococcal pneumonia is one of most common manifestation of cryptococcosis in AIDS patients. As stated above, to date all the described or developed β(1,3)-glucan synthesis inhibitors are ineffective against *C. neoformans*. Since its GS is very sensitive to echinocandins (Maligie and Selitrennikoff, 2005), one hypothesis for the resistance of *C. neoformans* is that the pathogen capsule could inhibit the access of the drug to the

transmembrane GS. However, limitation of accessibility is an unlikely explanation since the minimal inhibitory concentrations of echinocandins for acapsular strains are similar to those for encapsulated strains (Feldmesser et al., 2000). Other theoretical explanation may be that echinocandins do not inhibit β(1,6)-glucan synthesis, which seems to be the main glucan in *C. neoformans* wall (Free, 2013, Feldmesser et al., 2000). Besides *C. neoformans*, in other fungal species (*Candida parapsilosis*, *Candida guilliermondii*, *Neurospora crassa*, *Fusarium graminearum*, *Fusarium solani*, *Fusarium verticilliodes* and *Magnaporthe grisea*) it has been found that their wild type Fks1 sequence contains natural substitutions in their hot spot region that are determinants of their resistance (Walker et al., 2010). Therefore, the search, design and development of novel drugs that specifically target the synthesis of β(1,6)-glucan might be essential to bypass the resistance of *C. neoformans* and other fungi, which are intrinsic resistant to GS inhibitors. Besides the importance of the β(1,6)-glucan as new target in those resistant species, this cell wall polymer is essential for virulence in *C. albicans* (Herrero et al., 2004, Umeyama et al., 2006), representing an additional target for antifungals.

Recently several works and a patent have described new bicyclic heteroaryl ring derivatives as inhibitors of β(1,6)-glucan synthesis (Figure 3 shows the generic bicyclic heteroaryl ring), which have been reported to have *in vitro* and *in vivo* activity against a range of *Candida* spp (Kitamura et al., 2009a, Kitamura et al., 2009b, Kitamura et al., 2010, Takeshita et al., 2010, Hector and Bierer, 2011). Unfortunately, like the β(1,3)-glucan synthesis inhibitors reported to date, the β(1,6)-glucan synthesis inhibitors have little or no *in vitro* activity against *C. neoformans*. As stated above, most of wall glycoproteins are bound to the cell wall through a glycosylphophatidyl inositol (GPI) residue via a branched β(1,6)-glucan linker (Figure 1). Recently, the novel compound E1210, orally active isoxazole-based inhibitor of glycosylphosphatidylinositol (GPI)-linked protein biosynthesis, has been shown to have a good activity against *C. neoformans*, as well as a wide range of medically relevant yeasts and molds (Pfaller et al., 2011c, Pfaller et al., 2011b, Miyazaki et al., 2011).

5.3 Chitin synthase (CS) inhibitors as a target for antifungal therapy

Chitin is one of the main wall polysaccharides, which is vital for the maintenance of cell wall structure and integrity. Therefore, inhibition of chitin synthesis has been proposed as an attractive target for antifungal treatments. Differently from GS, no CS inhibitor has ever been developed into drug for clinical use (Munro, 2013). Existing CS inhibitors such as peptidyl nucleoside antibiotics polyoxins (or nikkomycins) are more potent and specific against class I enzymes, and less effective against the other classes. Their structures imitate the structure of the Chs substrate and thus, they act as competitive inhibitors (Gaughran et al., 1994, Munro, 2013, Lenardon et al., 2010). The finding that *CHS1* is essential for the viability of *C. albicans* (Munro et al., 2001) pointed to the search of novel inhibitors of the class II CS. The compound RO-09-3143 inhibits specifically to Chs1, and it is only fungicide to *C. albicans* in the absence of Chs2 (Sudoh et al., 2000), suggesting the existence of compensatory mechanisms between the different CS enzymes. Supporting this observation, *C. albicans* yeast cells harboring *chs1*Δ deletion (absence of Chs1) were able to grow when treated with Ca^{2+} and the chitin-interfering fluorochrome Calcofluor white, due to an increase of the chitin content and the synthesis of a new remedial septum (Walker et al., 2008). In conditions of stress or when the integrity of the cell wall is affected, this structure is reinforced by increasing the synthesis of chitin, either in *S. cerevisiae* or in *C. albicans* (Lenardon et al., 2010). In addition, *C. albicans fks1* mutants display increased cell wall thickness, which is attributed to higher cell wall chitin content. Something similar is observed when fungi are grown in the presence of sub-lethal concentrations of echinocandins (Ben-Ami and Kontoyiannis, 2012).

Synthesis of chitin-rich cell walls results from activation of cell wall salvage pathways, including the high osmolarity mitogen activated protein kinase, the protein kinase C and the Ca^{2+}/calcineurin pathways. These compensatory mechanisms are though to reduce the sensitivity of *C. albicans* to echinocandins. Therefore, the combined treatment of echinocandins with CS inhibitors is more effective than individual drug treatments (Walker et al., 2008, Munro, 2013, Lenardon et al., 2010). In addition, the treatment with antagonists of the calcineurin pathway, which regulates chitin synthesis in *C. albicans* and *A. fumigatus*, as well as the response to echinocandin drugs, have shown a synergistic effect when combined with echinocandins and CS inhibitors (Fortwendel et al., 2009, Hill et al., 2013, Lamoth et al., 2012, Lenardon et al., 2010, Munro, 2013, Walker et al., 2008, Wiederhold et al., 2005).

6. Perspective

In the past decade there have been important advances in antifungal therapy. Several new antifungal agents have received authorization for clinical use in the United States and European Union. Sadly, with some exceptions against certain fungi, available antifungal compounds are fungistatic rather than fungicidal; and therefore, treatments for life-threatening mycoses require prolonged periods of time, conducing to the emergence of drugs resistance in fungal pathogens, which is compromising the efficiency of the available antifungal drugs. The combined treatment of inhibitors of the main cell wall components, together with azole antifungals and antagonists of pathways involved in mechanisms of antifungal resistance, is a powerful strategy to abrogate fungal resistance and combat invasive fungal infections. In addition to orally available azole compounds, the new formulations with oral bioavailability, the broad array of natural, semi-synthetic and synthetic compounds that have shown an specific activity against β(1,3)-glucan synthesis, together with the new inhibitors of β(1,6)-glucan synthesis, will supply more powerful increased activity and spectrum of activity against most of the pathogenic fungi. Unfortunately, with the exception of the three closely related echinocandins now on the market, no new antifungals have been develop to advance clinical testing. However, the diversity of antifungal classes with activity against β-glucans reviewed in the present chapter, plus the prospect of oral bioavailability, will open new strategies to develop novel, safe and more effective drugs as additional treatments and managements of life-threatening mycoses.

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FIGURE LEGENDS

Figure 1. Sites of action of antifungal agents. Illustration of the sites of action of currently available antifungals. **A.** Echinocandins inhibit the β(1,3)-glucan synthase (GS) and the formation of the β(1,3)-glucan of the fungal cell wall. It is shown a schematic representation of the synthesis and organization of the β-glucans. The synthesis of linear β(1,3)-glucan chains starts in the plasma membrane by the GS. Then cell wall transglycosidases form branched β(1,3)β(1,6)-glucan bound to chitin via β(1,4) linkages. Proteins are covalently attached to β(1,3)β(1,6)-glucans through the GPI remnant or through a glutamine residue following a transglutaminase reaction. **B.** Polyene antifungals bind to fungal ergosterol, disturbing the plasma membrane function. **C.** Azole agents target the biosynthesis of ergosterol at the endoplasmic reticulum, leading to an altered plasma membrane depleted of ergosterol. **D.** Griseofulvin presumably binds to tubulin, thus interfering with the assembly of microtubules and inhibiting the nuclear mitosis. **E.** 5-Fluorocytosine or Flucytosine interferes with DNA and RNA synthesis. **F.** Sordarins block the fungal translation thus interfering with protein synthesis.

Figure 2. **Structure and composition of fungal cell wall.** The upper panel shows a cartoon of the organization and composition of the main fungal cell wall layers with the cell wall synthases embedded in the plasma membrane. Transmission electron micrographs of a fission yeast cell and a detail of the cell wall are presented in the lower panel.

Figure 3. Chemical structures of the main inhibitors of cell wall β-glucan biosynthesis. Echinocandins (pneumocandin, caspofungin, micafungin and anidulafungin), papulacandins (papulacandin B), acidic terpenoids (enfumafungin and MK-3118) and pyridazinone derivatives inhibit the GS activity and thus the synthesis of β(1,3)-glucan. Derivatives of bicyclic heteroaryl ring inhibit the synthesis of β(1,6)-glucan. For simplicity the generic forms of pyridazinone and the bicyclic heteroaryl ring are shown. See the text for more details.

Figure 4. Differential inhibitory effect of pneumocandin B0, caspofungin and papulacandin B on the *in vitro* GS activity of *Schizosaccharomyces pombe* wild type

cell extracts. Caspofungin exhibits a higher inhibitory capacity than pneumocandin and lower than papulacandin B. Besides caspofungin displays a dual inhibitory effect, at low and high concentrations (1st and 3rd sections marked by dotted lines), separated by a plateau of a 100-fold drug increase with no increase in inhibition (2nd section marked by dotted lines). Adapted from Martins et al., 2011.

Figure 5. **Mapping of the Fks hot spots that confer fungal resistance to GS inhibitors. A.** Hydropathy profile of Bgs4. The two predicted transmembrane (TM) regions and the sites where Bgs4$^{pbr1\text{-}8}$-E700V and Bgs4$^{pbr1\text{-}6}$-W760S are located in the hot spot 1 in the first transmembrane region and the sites where the hot spot 2 is located in the second transmembrane region are shown. **B.** Sequence alignment of two conserved regions of 70 and 24 amino acids of Bgs1, Bgs2, Bgs3, and Bgs4 from *S. pombe*, Fks1 and Fks2 from *Saccharomyces cerevisiae* (Sc), Gsc1 (Fks1) from *Candida albicans* (Ca), and Fks1 and Fks2 from *Candida glabrata* (Cg). The amino acid mutations described to confer resistance to echinocandins in *S. cerevisiae*, *C. albicans*, and *C. glabrata*, defining two resistance hot spot 1 and hot spot 2 of 9 and 4 aminoacids respectively, are shown. The Bgs4$^{pbr1\text{-}8}$ mutation is located 4 amino acids N-terminal from hot spot 1, increasing the cluster to a 13-amino acid hot spot 1-1 of resistance to papulacandin, enfumafungin, and echinocandins. The Bgs4$^{pbr1\text{-}6}$ change is located 48 amino acids C-terminal from hot spot 1-1, defining a novel hot spot 1-2 of resistance to the three antifungal families. Adapted from Martins et al., 2011.

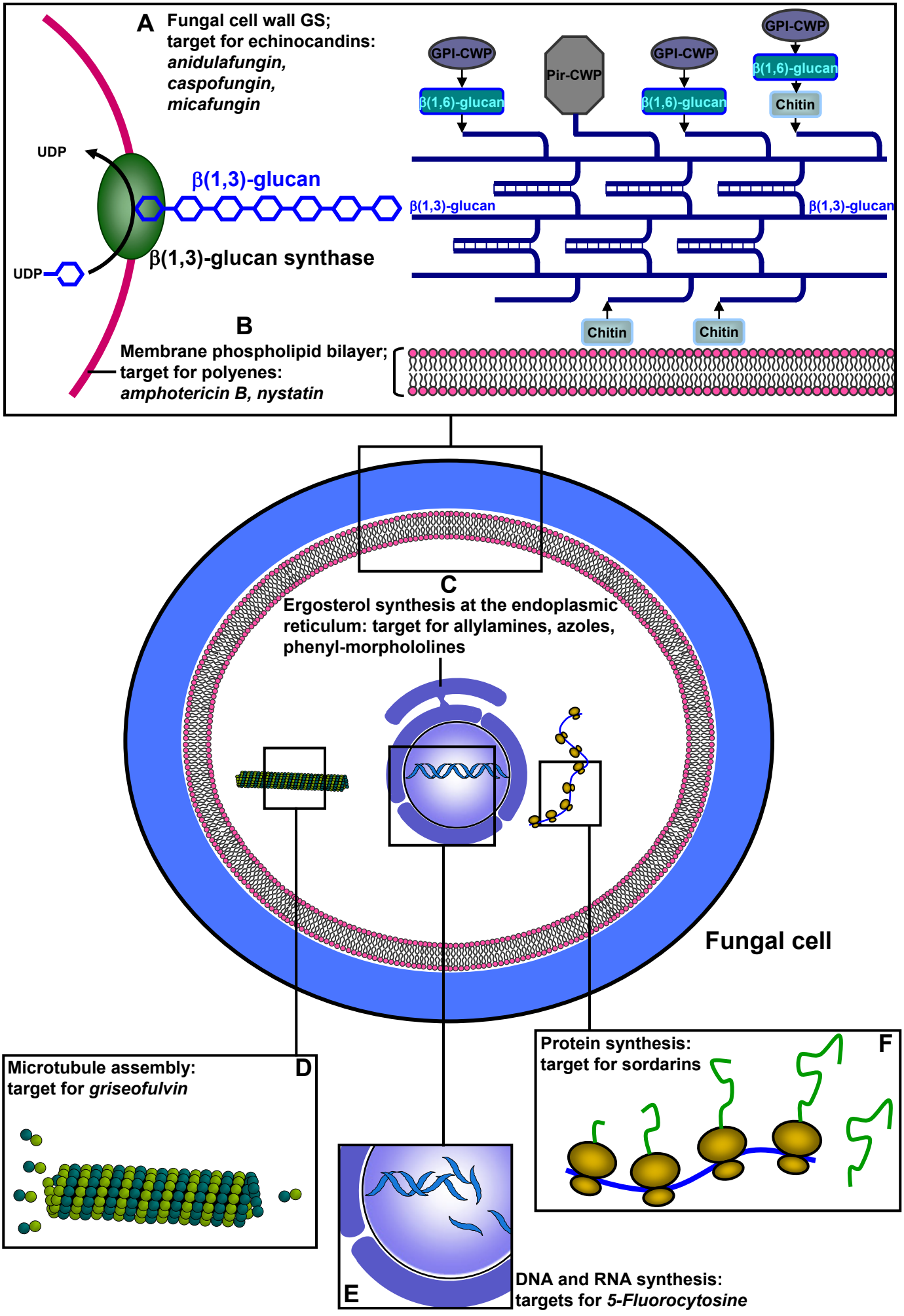
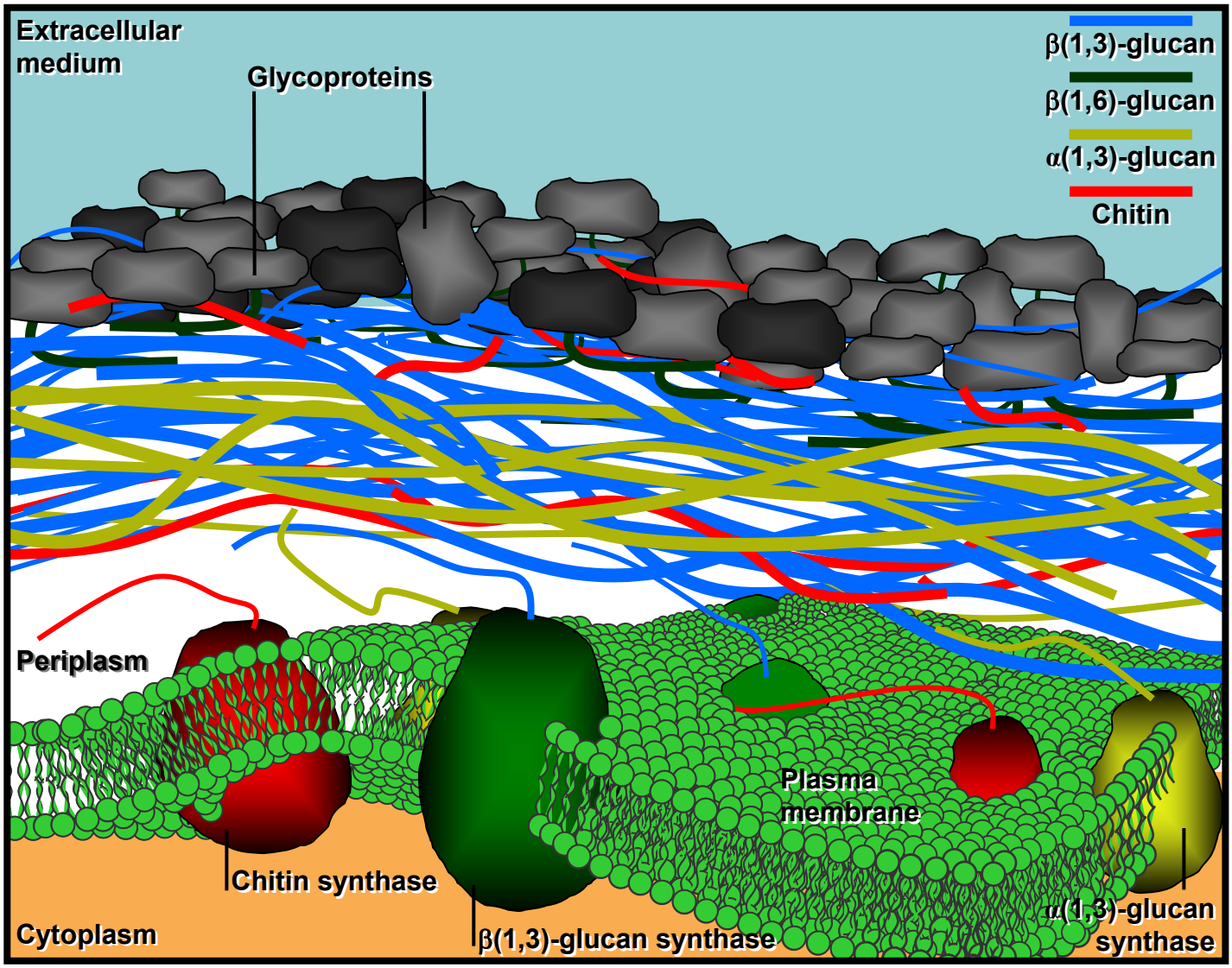

A
Fungal cell wall GS;
target for echinocandins:
anidulafungin,
caspofungin,
micafungin
UDP
β(1,3)-glucan
UDP
β(1,3)-glucan synthase
GPI-CWP
β(1,6)-glucan
Pir-CWP
GPI-CWP
β(1,6)-glucan
GPI-CWP
β(1,6)-glucan
Chitin
β(1,3)-glucan
β(1,3)-glucan
Chitin
Chitin
B
Membrane phospholipid bilayer;
target for polyenes:
amphotericin B, nystatin
C
Ergosterol synthesis at the endoplasmic
reticulum: target for allylamines, azoles,
phenyl-morpholines
Fungal cell
Microtubule assembly:
target for griseofulvin
D
E
DNA and RNA synthesis:
targets for 5-Fluorocytosine
Protein synthesis:
target for sordarins
F

Figure 1


Extracellular medium
Glycoproteins
β(1,3)-glucan
β(1,6)-glucan
α(1,3)-glucan
Chitin
Periplasm
Plasma membrane
Chitin synthase
Cytoplasm
β(1,3)-glucan synthase
α(1,3)-glucan synthase

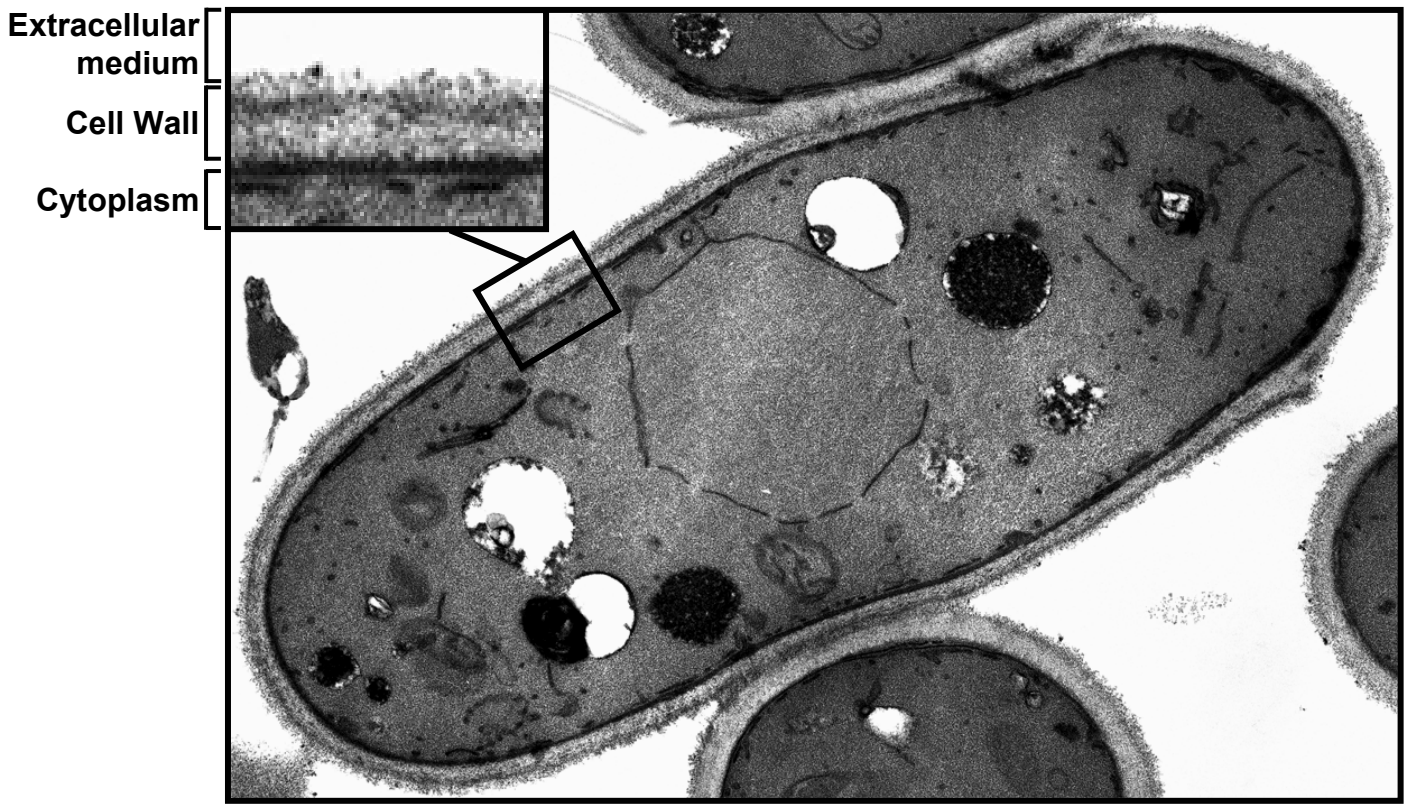
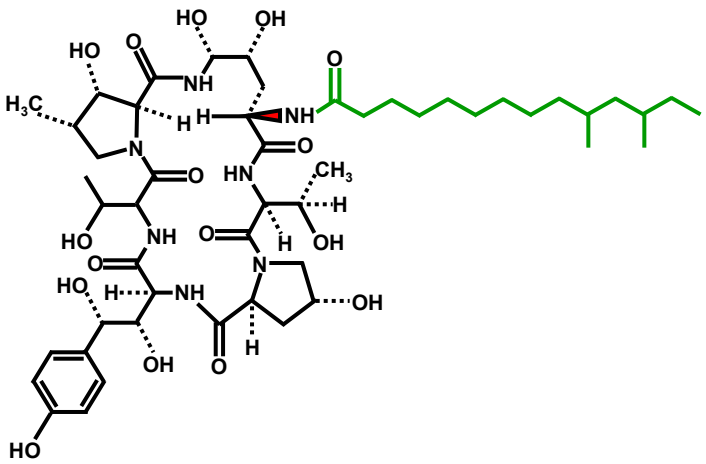
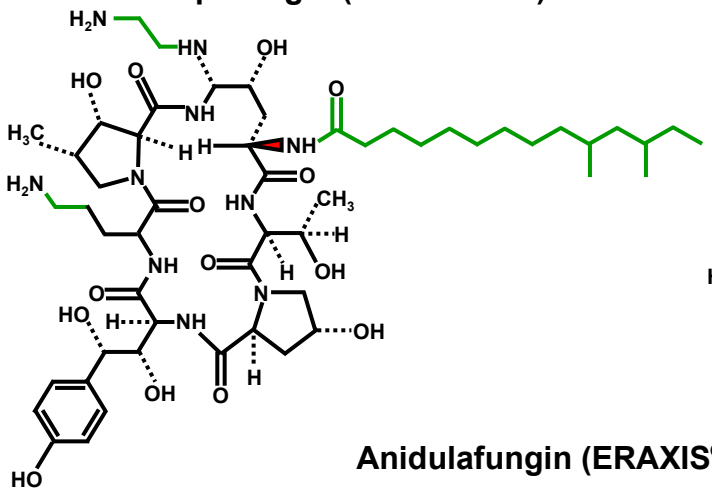

Extracellular medium
Cell Wall
Cytoplasm

Figure 2

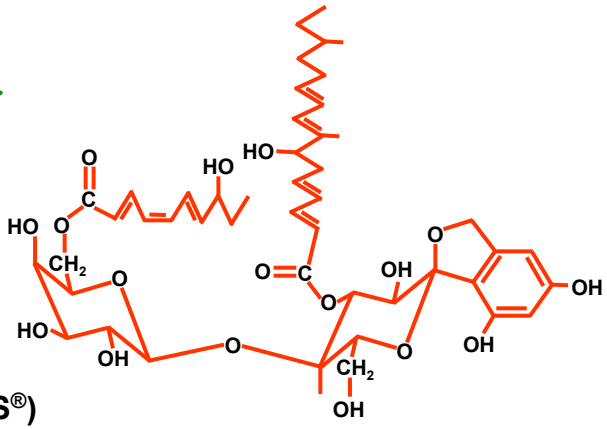
Pneumocandin



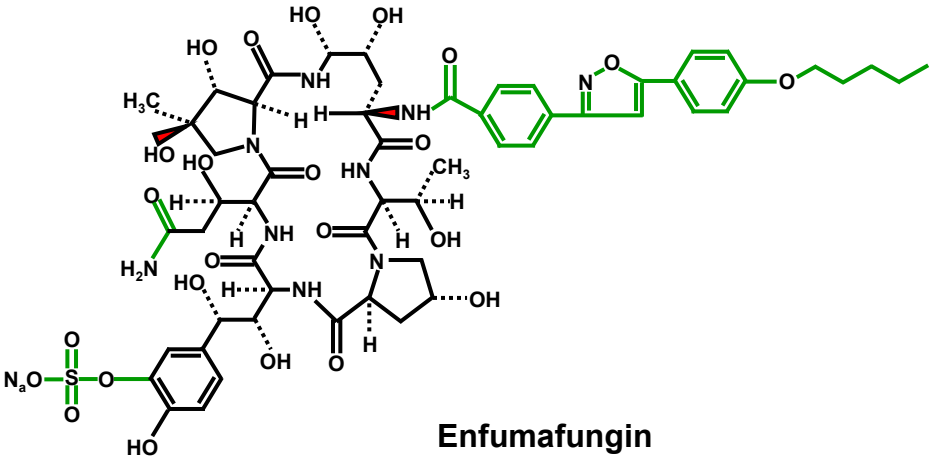
Caspofungin (CANCIDAS®)



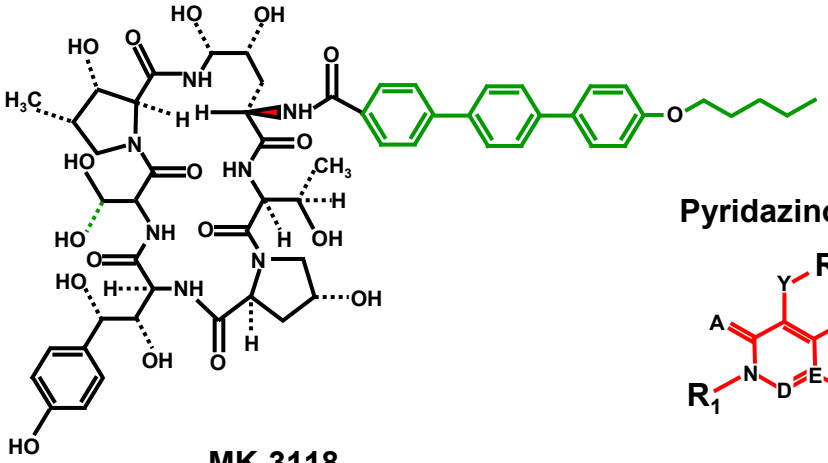
Papulacandin B



Micafungin (MYCAMINE®)

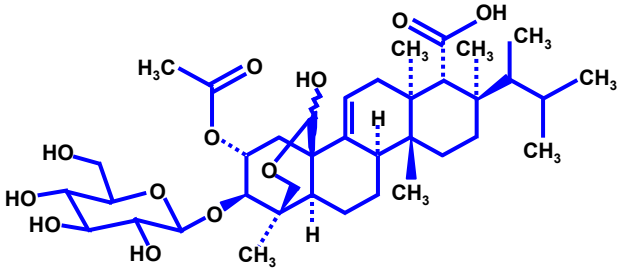


Anidulafungin (ERAXIS®)

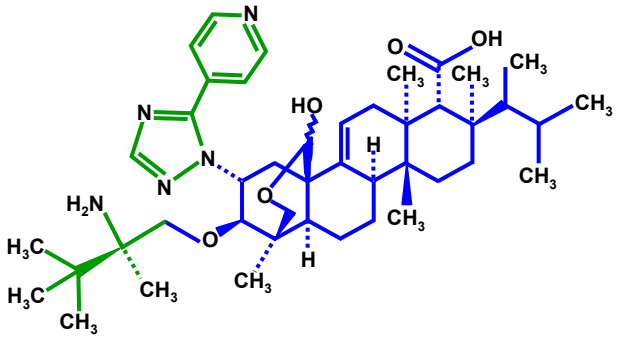


Pyridazinone

Enfumafungin



MK-3118



Bicyclic heteroaryl ring
(generic for β(1,6)-glucan inhibitors)

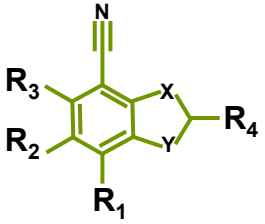


Figure 3

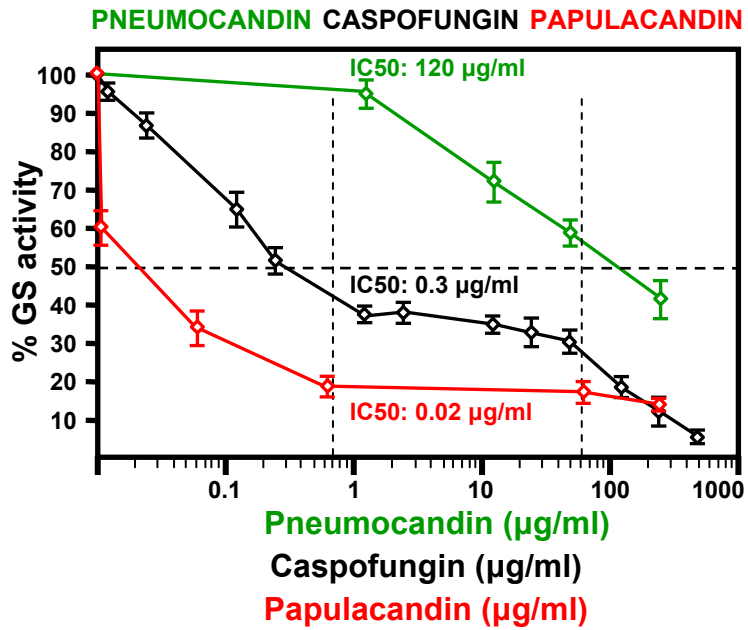

PNEUMOCANDIN CASPOFUNGIN PAPULACANDIN
IC50: 120 µg/ml
IC50: 0.3 µg/ml
IC50: 0.02 µg/ml
% GS activity
100
90
80
70
60
50
40
30
20
10
0.1
1
10
100
1000
Pneumocandin (µg/ml)
Caspofungin (µg/ml)
Papulacandin (µg/ml)

Figure 4

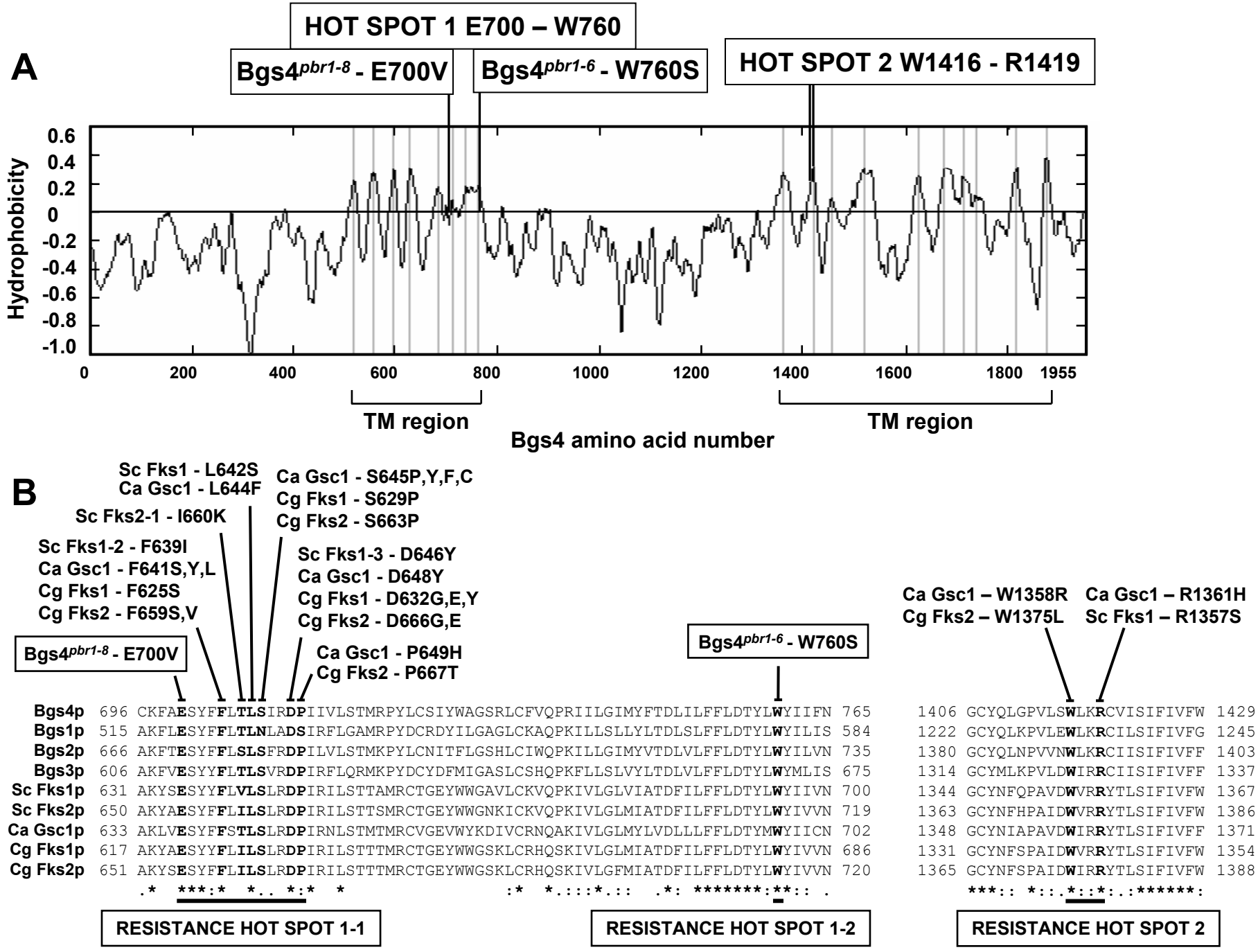

A
HOT SPOT 1 E700 – W760
Bgs4pbr1-8 - E700V
Bgs4pbr1-6 - W760S
HOT SPOT 2 W1416 - R1419
Hydrophobicity
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
0
200
400
600
800
1000
1200
1400
1600
1800
1955
TM region
TM region
Bgs4 amino acid number
B
Sc Fks1 - L642S
Ca Gsc1 - L644F
Ca Gsc1 - S645P,Y,F,C
Cg Fks1 - S629P
Cg Fks2 - S663P
Sc Fks2-1 - I660K
Sc Fks1-2 - F639I
Ca Gsc1 - F641S,Y,L
Cg Fks1 - F625S
Cg Fks2 - F659S,V
Sc Fks1-3 - D646Y
Ca Gsc1 - D648Y
Cg Fks1 - D632G,E,Y
Cg Fks2 - D666G,E
Ca Gsc1 - P649H
Cg Fks2 - P667T
Bgs4pbr1-8 - E700V
Bgs4pbr1-6 - W760S
Ca Gsc1 – W1358R
Cg Fks2 – W1375L
Ca Gsc1 – R1361H
Sc Fks1 – R1357S
Bgs4p 696 CKFAESYFFLTLSIRDPIIVLSTMRPYLCSIYWAGSRLCFVQPRIILGIMYFTDLILFFLDTYLWYIIFN 765
Bgs1p 515 AKFLESYFFLTLNLADSIRFLGAMRPYDCRDYILGAGLCKAQPKILLSLLYLTDLSLFFLDTYLWYILIS 584
Bgs2p 666 AKFTESYFFLSLSFRDPILVLSTMKPYLCNITFLGSHLCIWQPKILLGIMYVTDLVLFFLDTYLWYILVN 735
Bgs3p 606 AKFVESYYFLTLSVRDPIRFLQRMKPYDCYDFMIGASLCSHQPKFLLSLVYLTDLVLFFLDTYLWYMLIS 675
Sc Fks1p 631 AKYSESYYFLVLSLRDPIRILSTTAMRCTGEYWWGAVLCKVQPKIVLGLVIATDFILFFLDTYLWYIIVN 700
Sc Fks2p 650 AKYAESYFFLILSLRDPIRILSTTSMRCTGEYWWGNKICKVQPKIVLGLMIATDFILFFLDTYLWYIVVN 719
Ca Gsc1p 633 AKLVESYFFSTLSLRDPIRNLSTMTMRCVGEVWYKDIVCRNQAKIVLGLMYLVDLLLFFLDTYMWYIICN 702
Cg Fks1p 617 AKYAESYYFLILSLRDPIRILSTTTMRCTGEYWWGSKLCRHQSKIVLGLMIATDFILFFLDTYLWYIVVN 686
Cg Fks2p 651 AKYSESYFFLILSLRDPIRILSTTTMRCTGEYWWGSKLCRHQSKIVLGFMIATDFILFFLDTYLWYIVVN 720
1406 GCYQLGPVLSWLKRCVISIFIVFW 1429
1222 GCYQLKPVLEWLKRCILSIFIVFG 1245
1380 GCYQLNPVVNWLKRCIISIFIVFF 1403
1314 GCYMLKPVLDWIRRCIISIFIVFF 1337
1344 GCYNFQPAVDWVRRYTLSIFIVFW 1367
1363 GCYNFHPAIDWVRRYTLSIFIVFW 1386
1348 GCYNIAPAVDWIRRYTLSIFIVFF 1371
1331 GCYNFSPAIDWVRRYTLSIFIVFW 1354
1365 GCYNFSPAIDWIRRYTLSIFIVFW 1388
RESISTANCE HOT SPOT 1-1
RESISTANCE HOT SPOT 1-2
RESISTANCE HOT SPOT 2

Figure 5