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## Review

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## Anti-infectives

# New potential targets for antifungal development

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With the increasing number of immunocompromised hosts and advances in medical technology there has been a concomitant rise in the number of cases of invasive fungal infections. Novel approaches for the discovery of new antifungal targets and their inhibitors will be needed. In this review we discuss how such approaches are being developed through the identification of novel biochemical and molecular targets to meet the challenges imposed by the scientific research in medical mycology.

**Keywords:** *antifungal agent, drug target, fungal infection, gene disruption, signal transduction, virulence*

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## 1. Introduction

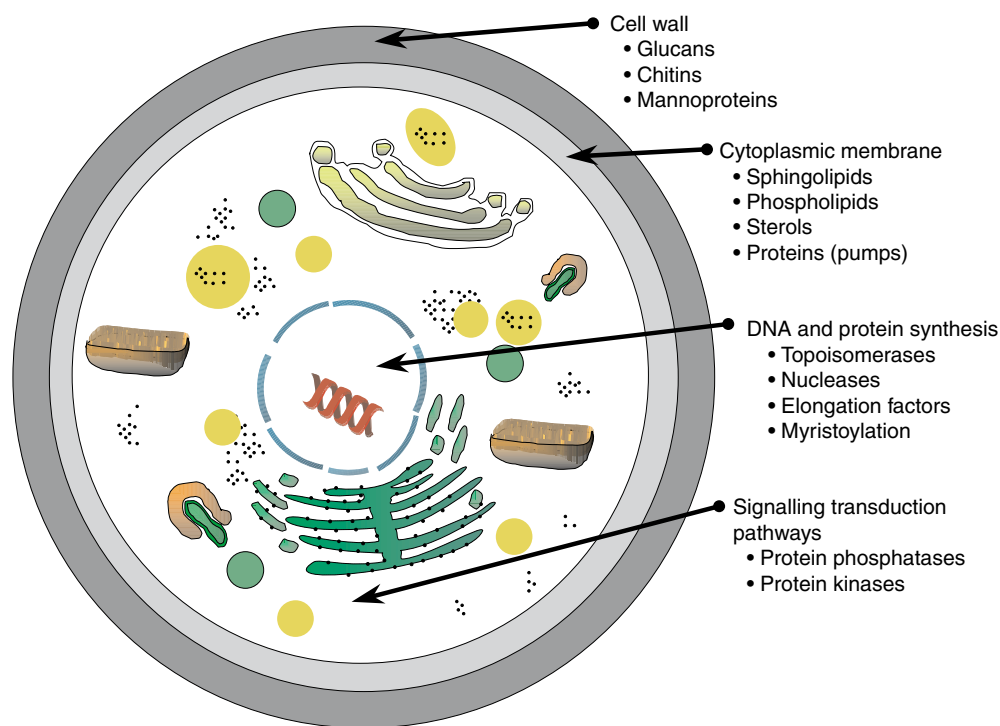
There has been an increase in the numbers of immunocompromised hosts due to advances in medical technology and a pan-epidemic with HIV infections. With the rise in at-risk patients, the number of invasive fungal infections has dramatically increased in both developed and undeveloped countries. These diseases range from life-threatening invasive mycoses to irritating, but relatively benign, mucocutaneous fungal infections. Despite the large number of patients, clinicians are limited to only four classes of antifungal drugs for treatment:

- polyenes (amphotericin B)
- flucytosine
- azoles (fluconazole and itraconazole)
- squalene epoxidase inhibitors (terbinafine)

Two therapeutic factors concerning antifungal agents have been clearly recognised. First, the financial market for these drugs remains large because of an expanding population at risk. Second, present antifungal agents tend

## 2 New potential targets for antifungal development

**Figure 1:** Schematic view of emerging targets for antifungal drug development.



to be fungistatic and/or toxic. Thus, relapses, failures or simply long courses of these antifungal drugs remain the standard clinical outcome. Of course, improvement in control of underlying diseases and better management of immune modulators will have a significant impact on patient outcome with these mycoses in the future. However, it is also clear that new and more potent antifungal agents would be extremely helpful, either alone or in combination with other antifungal agents, in the successful prevention or treatment of these life-threatening infections.

With this obvious need, there has been significant progress in developing and implementing strategies for development of new antifungal agents. Several reviews have elegantly discussed emerging antifungal targets [1-5] or the use of molecular pathogenesis for target identification [6]. There have also been reviews that identify drugs against other eukaryotic targets, such as cancer cells [7], or examine biological peptides [8]. In examining these reviews, there have been at least three prominent methods or strategies for the identification of new antifungal agents. The first is the classical screening of many classes of synthetic or natural products against a variety of fungi by classical *in vitro* susceptibility testing. This strategy can be successful because it directly targets growth of

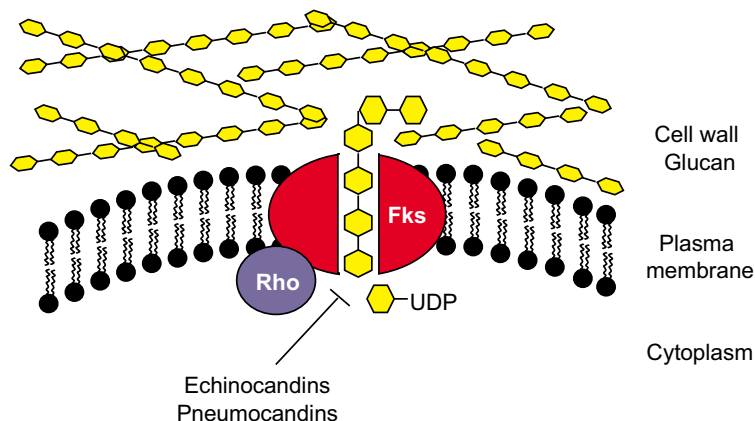
the whole organism. However, its drawbacks include its lack of focus and the identification of compounds too toxic to the host, difficulties in recreating the chemistry, and no appreciation for mechanism(s) of action or identification of target(s).

A second strategy for antifungal drug development has taken advantage of the molecular biological revolution. It is now possible to identify molecular targets that are essential for fungal virulence. The identification of unique genes and their signalling network, important to the pathobiology of fungi, could translate into their use as molecular targets for drug development. There are now a series of molecular techniques to identify gene expression(s) under certain conditions and the field of molecular pathogenesis has matured to the point that validation for these potential 'virulence' targets can be achieved. Functional genomics continues to rapidly progress so that pathogenic fungal genomes are being sequenced and microarray technology for gene expression will be made available for fungal pathogens. The power and breadth of virulence gene targeting will be realised.

Furthermore, functional genomics is now being analysed in model fungi such as *Saccharomyces*

**Figure 2:** Working model of glucan synthase.

Fks: Glucan synthase complex; Rho: GTP-binding regulatory subunit; UDP: Uridine diphosphate.



*cerevisiae* in which screens for compounds against the known essential genes of this fungus can now be made. It is also possible to use known mutant screens to identify compound target(s) with these yeasts. With the human genome sequence being completed, selectivity of target between these two eukaryotic cells will be rapidly approached and compared. The power of this approach is apparent. Soon functional genomics will identify the limits of antifungal target development and is likely to be exploited to develop immediate ideas regarding mechanism(s) of action. However, the strategy can be diffuse in its approach, allowing the identification of a series of targets with relative differences in potency and it has the potential to be fungal-species specific.

The third strategy is to focus on a known area of cell biology within pathogenic fungi and use all the molecular and biochemical tools and understanding to dissect it, and thereby attempt to find sensitive fungicidal targets with a potential for selectivity over mammalian cells. In this review, we have attempted to examine in-depth several of these focused strategies on antifungal development (**Figure 1**). It is hoped that the reader will appreciate how molecular biology, biochemistry, cell biology and pathophysiology can now be creatively merged to identify and validate new antifungal targets.

## 2. The fungal cell wall

The fungal cell wall acts as the interface between the fungus and its environment. It has several roles, which include providing the fungus with its shape and

supporting it against osmotic forces. Furthermore, it acts as a filter, controlling the secretion and uptake of molecules into the cell. Some enzymes are also retained by the cell wall and this allows for the enzymatic conversion of nutrients into metabolisable forms, prior to their entry into the protoplast [9]. As such, interference with its structure or synthesis will eventually lead to fungal cell lysis and death. This section examines the major structural components and enzymes contributing to the architecture of the fungal cell wall and reviews ways in which they can be targeted for drug design. This structure is not only important to viability of the fungal cell; it is also unique to fungi and not present in mammalian cells. These features make it an ideal antifungal target.

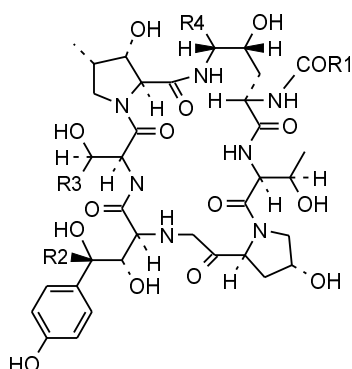
### 2.1 (1,3)- $\beta$ -D-Glucan synthase

The  $\beta$ -glucans are an abundant class of polysaccharides that are involved in structural, functional and certain morphological roles at the cell surface of fungi [10]. The membrane-bound enzyme (1,3)- $\beta$ -D-glucan synthase (GS) catalyses the synthesis of (1,3)- $\beta$ -glucan, an essential glucose polymer found in fungi. In fact, (1,3)- $\beta$ -glucan is the most prominent carbohydrate component of the cell wall. It forms a fibril composed of three helically entwined linear polysaccharides, which provide rigidity and integrity to the cell wall structure. Since the (1,3)- $\beta$ -glucan structure is not found in mammalian cells, the GS enzyme has become a target for research into antifungal agent development.

Isolation of fungal mutants is one approach to identify those genes that encode biosynthetic enzymes and

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**Figure 3:** The candin basic structure.



regulatory proteins needed for cell wall biosynthesis. This approach has been successful in the case of genes encoding enzymes responsible for the biosynthesis of cell wall polysaccharides, such as (1,3)- $\beta$ -glucan, mannan [11] and chitin [12]. In fact, mutant analysis has yielded data about the mechanism of action of the echinocandins, a new class of antifungals which target enzyme(s) for the cell wall. Resistant echinocandin mutants of *S. cerevisiae* were generated and their analysis led to the cloning of the echinocandin target gene *ETG1*, also known as *FKS1* [13], which encodes GS.

It has now been determined that GS is a multi-component enzyme consisting of at least two subunits which are inactive by themselves, but which are activated upon reconstitution [14]. The GS complex has now been described in *S. cerevisiae*, *Candida albicans*, *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Neurospora crassa* and *Cryptococcus neoformans* [15-22]. The current proposed model for GS is shown in **Figure 2**.

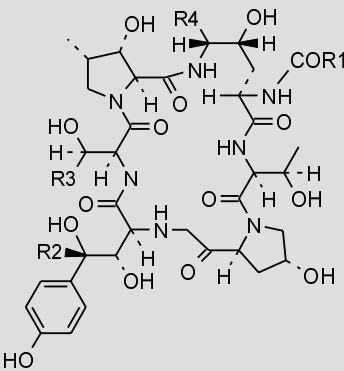

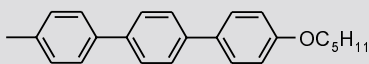
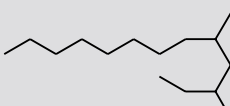
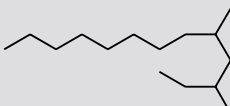
The GS complex includes a large, integral membrane subunit that polymerises the substrate (UDP-glucose) into fibrils of (1,3)- $\beta$ -glucan and extrudes the polymer through the plasma membrane. In *S. cerevisiae*, the GS complex is formed by two catalytic subunits, Fks1 and Fks2, encoded by *FKS1* and *FKS2* genes, respectively [13,15,23]. *FKS1* expression is more abundant during vegetative growth and is cell cycle-regulated. The *FKS2* gene is 88% homologous to *FKS1*. Its gene product is regulated by calcineurin and has been found to be important for sporulation [18]. Disruption of the *FKS1* gene alone in *S. cerevisiae* is not sufficient to produce a lethal mutant, but the disruption of *FKS1* and *FKS2* together is lethal to the yeast cell [13].

The second component of the complex is a small, GTP-binding regulatory subunit with GTPase activity. It is more loosely associated with the membrane and is required for activation of the large catalytic subunit. The GTP-binding subunit is encoded by *Rho1*, a Ras-like GTP-binding protein, that regulates polymer synthesis. Research has shown that *RHO1* overexpression will increase GS activity, although it is still dependent on GTP, and that overproduction causes aberrant morphology and a defective yeast cell wall [19].

Glucan synthesis can be inhibited by a variety of natural compounds, including lipopeptides and papulacandins, which act as non-competitive inhibitors. Other natural products include mulundocandins, sporiofungins and aculeacin [24]. Lipopeptide echinocandins and pneumocandins are structurally related and form the basis of many antifungal derivatives (**Figure 3** and **Table 1**). The novel mode of action and fungicidal activity of these compounds has led to the production and development of several analogues, namely LY303366 (based on Echinocandin B), L-733,560 or caspofungin, MK0991/caspofungin acetate (based on Pneumocandin B<sub>0</sub>) and FK463 [25-28]. These compounds have improved potency and water solubility, and have broadened the antifungal spectrum compared to parent compounds [29-35]. The fungicidal activity of LY303366 has recently been reported [35-37]. Several candins are now being studied in treatment of human candidiasis and aspergillosis.

The effectiveness of the candins is due to their extremely broad spectrum of activity and their great potency *in vitro* and in animal models [38-41]. However, present candins appear much less potent against *C. neoformans*, despite this yeast containing both (1,3)- $\beta$ -glucan in the cell wall and the *FKS1* homologue, which appears essential for growth [22]. Nevertheless, a GS inhibitor (MK-0991/caspofungin acetate) can enhance the *in vitro* activity of amphotericin B and fluconazole against *C. neoformans* [31]. In contrast, they are extremely potent against *Pneumocystis carinii* since the wall of the 'cyst' form of this fungus contains GS [33,42]. Currently, the GS inhibitors in clinical development are administered parenterally and, despite considerable efforts to modify the lipopeptides chemically or to formulate them to increase their oral bioavailability, the oral absorption of the present candins is low. New studies have looked to improve this feature and recently four acidic terpenoid natural products

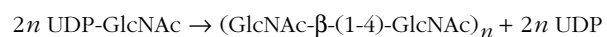
**Table 1:** Chemical structures and functions of echinocandin B, pneumocandin B<sub>0</sub> and their semi-synthetic derivatives.

|   |   | R1 | R2                | R3   | R4  | Activity | Ref. |
|---|---|----|-------------------|--|---|----------|------|
|  |   |    |                   |  |   |          |      |
| <b>Echinocandins: Inhibits fungal 1,3-β-glucan synthase</b>                       |   |    |                   |  |   |          |      |
| Echinocandin B  |    | OH | CH <sub>3</sub>   | OH   | Active against <i>Candida</i> spp., <i>Aspergillus</i> spp. and <i>P. carinii</i> | [25]     |      |
| LY303366 derivative   |    | OH | CH <sub>3</sub>   | OH   | Active against <i>Candida</i> spp., <i>Aspergillus</i> spp. and <i>P. carinii</i> | [26]     |      |
| <b>Pneumocandins: Inhibits fungal 1,3-β-glucan synthase</b>                       |   |    |                   |  |   |          |      |
| Pneumocandin B <sub>0</sub>   |  | OH | OCNH <sub>2</sub> | OH   | Active against <i>Candida</i> spp., <i>Aspergillus</i> spp. and <i>P. carinii</i> | [27]     |      |
| L-733,560 derivative  |  | OH | NH <sub>2</sub>   | OH <sub>2</sub> CH <sub>2</sub> CNH <sub>2</sub> | Active against <i>Candida</i> spp., <i>Aspergillus</i> spp. and <i>P. carinii</i> | [28]     |      |

(enfumafungin, ascosteroside, ergokonin A and arundifungin) have been discovered using a series of newly developed methods. Sensitivity of *Candida* and *Aspergillus* species for these compounds were equal to that seen for the other echinocandins and, similarly, *C. neoformans* was not inhibited or killed [43]. What is most encouraging about these new compounds is that other terpenoid glycosides are currently being administered orally and have been therapeutically effective for certain conditions. Further work needs to be carried out to determine the oral absorption properties of these particular antifungal compounds to allow assessment of their potential as oral GS fungal inhibitors for antifungal treatment.

## 2.2 Chitin synthase

Chitin is a major structural component of the cell walls of many fungi. It is a (1-4)-β-linked homopolymer of *N*-acetyl-D-glucosamine, and is produced by chitin synthase from the nucleotide UDP-GlcNAc and follows the reaction [44]:

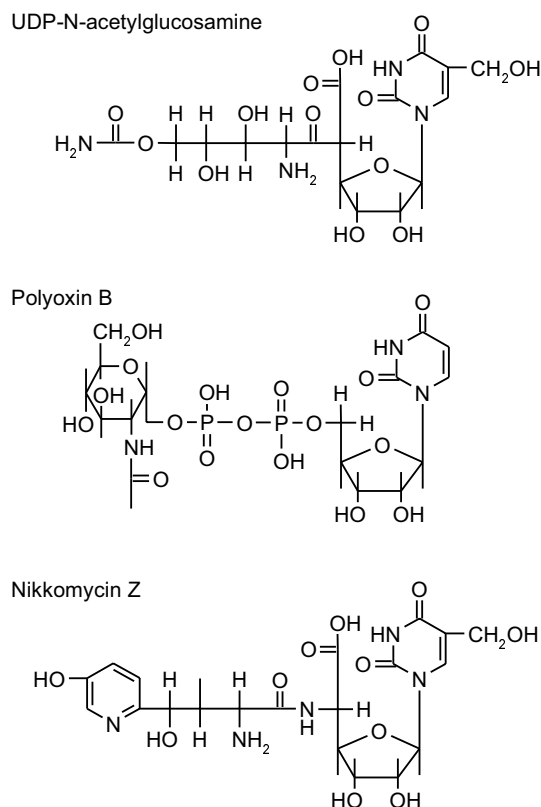


This section focuses primarily on the synthesis of chitin in *S. cerevisiae* since it is better understood in this yeast than in any other fungus.

In *S. cerevisiae*, the cell wall is relatively poor in chitin, but the primary septum that separates the mother and daughter cells, as well as the resulting bud scars, are

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**Figure 4:** Chemical structures of UDP-*N*-acetylglucosamine, polyoxin B and nikkomycin Z.  
UDP: Uridine diphosphate.



mostly composed of chitin [45,46]. It is also found in the cell wall and seems to play an important role in cell wall integrity. Chitin is also found associated with the cell membrane where it acts as a covalent anchor for other cell wall structural components, such as glucan-mannan complexes in maintaining cell integrity [47]. Since chitin is not present in mammalian cells, it has the potential to be a highly selective target for therapeutic use.

Chitin synthesis is cell cycle regulated; the amount and distribution of chitin in the cell wall changes as the cell proceeds from vegetative growth to diploid formation and then sporulation [48]. Gene disruption experiments have revealed that chitin biosynthesis is a complex process involving multiple catalytic and regulated proteins. *CHS1* [49] is the structural gene for chitin synthase 1 (Chs1) and it is thought to be involved in repair, synthesising chitin in response to an acidification event following separation of mother and daughter cells [50]. The *CHS2* gene [12] encodes Chs2, which is localised to the mother-bud septum and is responsible for primary septum formation

between mother and daughter cells [51,52]. The *CHS3* gene [53,54] encodes the catalytic subunit of the major Chs3 and it synthesises more than 90% of the chitin in the cell wall [51,54]. The role of *CHS4* is not yet clear, although Chs4 seems to be required for Chs3 activity both *in vivo* and *in vitro* [54,55]. It has been suggested that it acts as a post-translational regulator of the Chs3 complex [48]. The *CHS5* and *CHS6* genes also encode regulatory proteins involved with Chs3 activity [56-58], possibly acting as carrier proteins moving the Chs3 complex towards the cytoplasmic membrane [58,59]. Recently, the *CHS7* gene [60] has been isolated and its product is thought to be responsible for the export of Chs3 from the endoplasmic reticulum.

The *CHS1* gene has been cloned from *S. cerevisiae* [49], *C. albicans* [61], *C. neoformans* [62] and *Paracoccidioides brasiliensis* [63]. In *S. cerevisiae*, it has been shown that mutations affecting chitin synthesis, such as  $\Delta chs1$ , produce osmotic sensitivity [54,64], abnormal morphology and aggregation, as well as growth arrest of elongated buds [51,52,65]. Interestingly, in *S. pombe* deletion or overexpression of the *CHS1* gene does not lead to any apparent defects in vegetative growth [66]. The complexity of chitin synthases is revealed in the *C. albicans* *CHS3* studies. When *CHS3* was disrupted, the  $\Delta chs3$  mutant was attenuated in virulence in mice by survival. However, tissue census was the same as wild type and this makes it less attractive as a drug target [67].

There are two structurally related groups of secondary fermentation metabolites that act as specific inhibitors of chitin synthase. These are the polyoxins and the nikkomycins; structurally they bear strong similarities to UDP-*N*-acetylglucosamine, the precursor substrate for chitin (**Figure 4**). Both groups act as competitive inhibitors of fungal chitin synthase by inhibition of protein synthesis, which is similar to that seen with other aminoacyl nucleoside antibiotics (reviewed in Fox *et al.* [68]). Presently, 13 naturally-occurring polyoxins are identified (polyoxins A through to M) and 14 naturally-derived nikkomycins (nikkomycins Bx, Bz, Cx, Cz, D, E, I, J, M, N, X, Z, pseudo-J and pseudo-Z) [69-78]. These antifungal compounds appear to have potent inhibitory activity against isolated chitin synthases, but only limited *in vitro* antifungal activity against whole organisms [79,80]. The chitin synthase enzymes are located within the plasma membrane of the cell, so that potential inhibitors probably need to be transported into the cell for activity. As such, factors that hinder their transport or promote their efflux from the cell will influence the

antifungal activity of these potential antifungals. Studies have shown that isolated chitin synthase enzymes from both resistant and sensitive cells are equally inhibited by polyoxin B, suggesting that the mechanism of resistance is due to a transport defect of the inhibitor into the cell or efflux pumps, rather than an inherent resistance of the enzyme to the inhibitor.

Treatment with this class of antifungal agents has produced cells described as swollen, protoplast-like structures, similar to the effect seen in bacteria with  $\beta$ -lactam antibiotics [81]. Polyoxin D inhibits septum formation and produces morphological chaining and swelling of cells in both *C. albicans* and *C. neoformans* [82]. Becker *et al.* also demonstrated that cells treated with polyoxin D had far less chitin in their cell walls than control cells and that no chitin was seen at the site of septum formation, an area that is normally rich in chitin [82]. *S. cerevisiae* cells treated with such antifungals also exhibit a chaining effect, suggesting that chitin may be essential for cells to separate properly. *Candida* cells treated with polyoxin D under conditions of germination showed an inability to form germ tubes [83], while prolonged incubation of cells led to the disappearance of chitin from the inner cell walls, as well as a lack of septum formation [84].

Fungicidal activities with the nikkomycins have been reported and the dimorphic fungi *Coccidioides immitis* and *Blastomyces dermatitidis* were found to be highly susceptible, while *C. albicans* and *C. neoformans* were more resistant [85,86]. Furthermore, *Aspergillus fumigatus*, *Rhizopus arrhizus* and *Candida tropicalis* were all found to be resistant to nikkomycins X and Z. As these species have considerable amounts of chitin, the theory that fungal cells are more resistant if they have less chitin in their walls is not supported, and so the issue seems to be more complex. It is possible to produce additive effects when nikkomycin Z is added to a glucan synthase inhibitor *in vitro* against a series of filamentous fungi, such as *Aspergillus* sp. [86]. These studies support further investigation into the value of blocking two enzymes in cell wall synthesis to increase potency of the antifungal effect and broaden antifungal spectrum.

The major problem with chitin synthase inhibitors is that they must be able to permeate the fungal cell wall. Animal studies involving chitin synthase-targeted drug treatments are limited and somewhat conflicting [87,88]. However, in 1997, nikkomycin Z was

evaluated orally against pulmonary blastomycosis in mice, producing results equivalent to those seen with parenteral amphotericin B [89].

New formulations and combination therapies have been reviewed by Zhang and Miller [90]. Recently, nikkomycins have been investigated in combination with other drugs that might enable them to enter the cell more easily. Nikkomycin Z was evaluated in combination with fluconazole in the murine model of histoplasmosis. The drug combination was shown to be well-tolerated and more effective than either drug alone [91]. This combination was then investigated with several different fungi and additive interactions were observed against *C. albicans*, *C. neoformans* and *C. immitis* [92]. These results of drug combinations are encouraging for further development of this class of inhibitors.

### 2.3 Mannoproteins

Since mannose constitutes such a major portion of the cell wall of many fungi, as well as the glycoproteins that form the protective capsule in *C. neoformans*, it could be hypothesised that the biosynthetic pathway of this polysaccharide might be critically important to its survival in the host.

The GDP-mannose biosynthesis pathway involves three key enzymes: phosphomannose isomerase (Pmi), phosphomannomutase and mannose pyrophosphorylase. Pmi catalyses the interconversion of mannose-6-phosphate and fructose-6-phosphate sugars, linking mannose-6-phosphate into mannose metabolism with the generation of GDP-mannose, and subsequently dolichol-P-mannose and fructose-6-phosphate into glucose metabolism *via* glycolysis and the TCA cycle (**Figure 5**). Both GDP-mannose and dolichol-P-mannose structures are mannose donors essential for mannoprotein biosynthesis [116,117]. GDP-mannose is also necessary for the mannosylation of various structures including exopolysaccharides, lipopolysaccharides and glycoproteins [118], as well as being a precursor for other nucleotide sugars involved in the synthesis of these vital structures in cell membrane, wall and capsule.

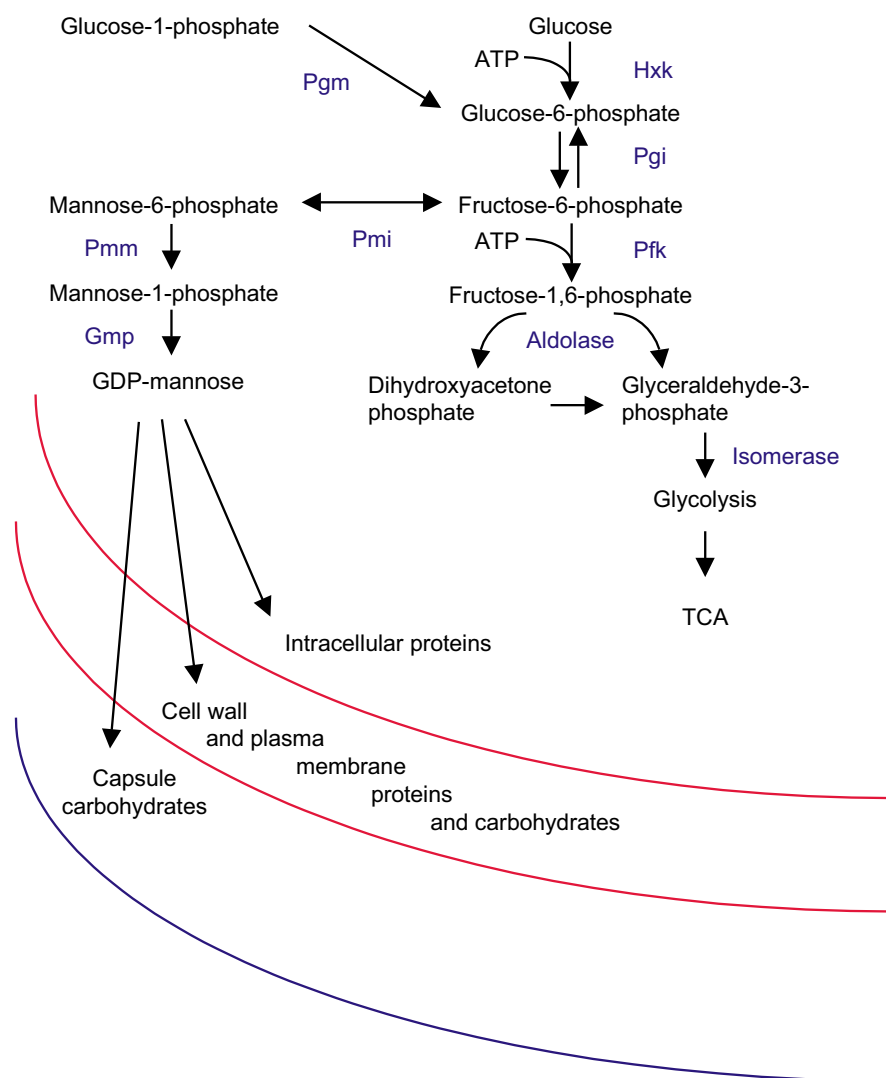
In *C. neoformans*, the major virulence factor is the polysaccharide capsule, which is composed primarily of glucans and mannose-containing polysaccharides. Capsule size varies depending upon environmental conditions [119-121] and has been implicated in mechanisms to avoid or weaken host defences [122],

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**Figure 5:** Mannose metabolism pathway in fungi.

The pathway shows the inter-relationship between the mannose and glucose metabolic pathway and the likely uses of GDP-mannose in the cell.

Gmp: GDP-mannose pyrophosphorylase; Hxk: Hexokinase; Pfk: Phosphofructose kinase; Pgi: Phosphoglucoisomerase; Pgm: Phosphoglucomutase; Pmi: Phosphomannose isomerase; Pmm: Phosphomannomutase; TCA: Tricarboxylic acid cycle.



such that acapsular cells are unable to cause disease *in vivo* [123-125].

Pmi is the essential enzymatic link between glucose and mannose metabolic pathways. Cells preferentially use glucose as a carbon source. However, if the cell requires mannose, either for structural or integral components of the cell, and no external sources of mannose are available, it can convert glucose into mannose using the Pmi enzymatic link. If the enzyme is not functional and no external mannose is available, the cell will begin to display defects in cellular morphology [116,118,126,127].

At the restrictive temperature, *S. cerevisiae*  $\Delta pmi$  mutant cells demonstrate abnormal cell clumping, inability to secrete extracellular glycoproteins and produce cell walls deficient in D-mannose [118]. In *A. nidulans*, hyphal tips show abnormal ballooning and the fungal cells eventually cease to grow [126]. Interestingly, in our recent studies we showed that the *C. neoformans*  $\Delta pmi$  mutant strain exhibits abnormal morphology, including capsule and clumping of cells. Furthermore, we demonstrated that the *C. neoformans*  $\Delta pmi$  mutant strain is not virulent *in vivo*, showing for the first time a linkage between this

pathway and the ability to produce infection, and validating its potential as an antifungal target [127].

Genes encoding for Pmi have been cloned from *S. cerevisiae*, *C. albicans*, *A. nidulans*, *C. neoformans* and *Pseudomonas aeruginosa* [116,126-129], and identified functionally as constituents of the mannose utilisation pathway. The *PMI* gene has also been isolated from humans [130] and a deficiency in the gene results in carbohydrate-deficient glycoprotein syndrome (CDGS) [131], the effects of which are well-documented. In order to utilise the enzyme as a drug target for fungal disease in humans, the targeted site for inhibitors would probably need to be carefully modulated to achieve specificity of fungi over mammalian cells. Recently, a Pmi inhibitor of *C. albicans* was isolated, but it was found to have only a minimal effect on whole cells [132]. Thus, new inhibitors of this enzyme need to be discovered and examined for their antifungal activities.

Once mannose has been synthesised, dolichol phosphate mannose synthase transfers mannose from GDP-mannose to dolichol phosphate, forming Dol-P-mannose, a key intermediate in protein glycosylation. The enzyme Dol-P-mannose synthase is involved *in vivo* in three glycosylation pathways: *N*-glycosylation, *O*-mannosylation and glycosyl phosphatidylinositol (GPI) membrane anchoring [133-135]. This enzyme has been studied in *S. cerevisiae* [136-138], *C. albicans* [139] and recently in *C. neoformans* [140]. The glycosylation of proteins occurs in the rough endoplasmic reticulum, after which they are transported to the cell wall. All these steps might become antifungal drug targets.

Finally, mannoproteins are formed by *O*-linkages joining mannose and small oligosaccharides to the hydroxyl groups of the amino acids serine or threonine. A second type of linkage connects high molecular weight and highly branched mannoproteins to the protein moiety *via* an *N*-glycosidic bond between two molecules of *N*-acetylglucosamine and asparagine [11,141-143]. Mannosyltransferases cover a multitude of mannose transfers within the cell. Some describe the binding of mannose to an  $\alpha$ -1,3-dimannoside acceptor, allowing the formation of a second  $\alpha$ -1,3 linkage [144]. Others are concerned with the linkage of mannose in  $\alpha$ -1,2 linkages to the same dimannoside acceptor [144,145]. Some specifically describe the transfer of mannose from GDP-mannose to seryl or threonyl residues in

proteins [146,147], a part of the *O*-glycosylation process in mannoprotein biosynthesis.

The *MNV* family of mannoprotein genes are involved in the synthesis of both *N*-linked and *O*-linked glycosylation and have been isolated from *C. albicans* and *S. cerevisiae* [148-152]. In *S. cerevisiae*, several genes have been identified as part of the *MNV* pathway, and construction of mutants has been important in the elucidation of their roles in the cell. In the mutants  $\Delta mnn2$  and  $\Delta mnn5$ , the addition of the first and second mannose is defective [152]. The gene *MNV9* encodes for a membrane-associated protein with unknown function, although it seems to be essential for the addition of the long 1,6-mannose backbone of mannan [149], such that one  $\alpha$ -1,6-mannose can be attached to the core polysaccharide, but further addition is blocked. This results in the formation of some very unusual structures [151] indicating the importance of *MNV9* in the glycosylation process. In general, these  $\Delta mnn$  mutants exhibit phenotypes characteristic of defects in cell wall biosynthesis and/or assembly, including poor cell wall growth, cell clumping and osmotic sensitivity. Therefore, inhibitors to these proteins may have profound impact on fungal viability.

### 3. The fungal cytoplasmic membrane

In contrast to the bacteria, the fungal plasma membrane is similar to its mammalian counterpart. It contains phospholipids, sphingolipids, sterols and proteins. The key factors for the plasma membrane to function are its fluidity, its rigidity and its transport mechanisms, determined by lipid composition, sterol composition and protein composition, respectively.

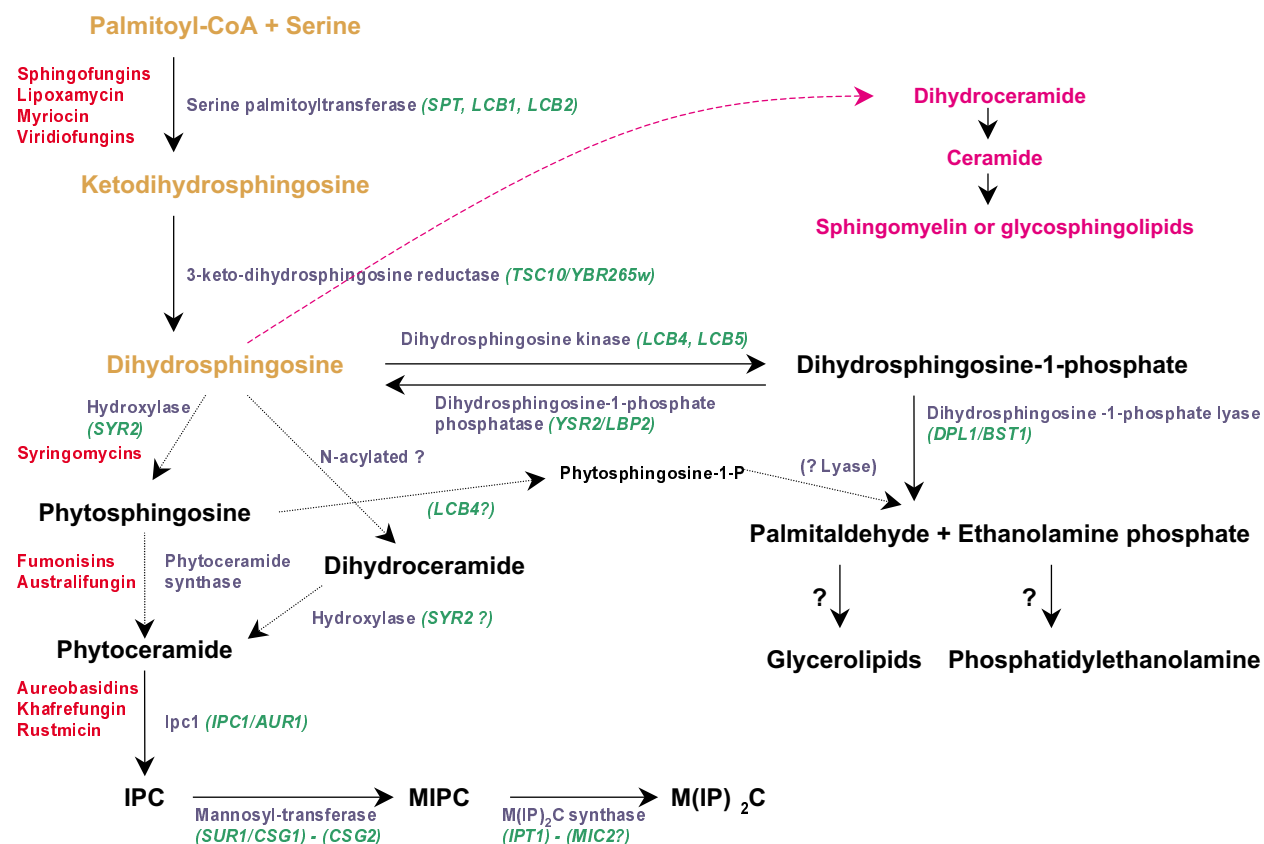
#### 3.1 Sphingolipids

Sphingolipids are essential components of all eukaryotic plasma membranes and modulation of them exerts a deep impact on cell viability [153,154]. Although the presence and role of sphingolipids are common to these two organisms, differences throughout their biosynthetic pathways have been described between mammalian cells and yeasts (**Figure 6**). Potentially, these differences may represent a new suitable target for the development of antifungal agents.

Sphingolipid synthesis and metabolism appear to be conserved among non-pathogenic and pathogenic fungi [155-158]. Enzymes in the sphingolipid pathway

**Figure 6:** Presumptive sphingolipids pathway.

Conserved pathway (orange), *Saccharomyces cerevisiae* (black), human (magenta), specific enzymes (blue), corresponding genes (green) and target sites of antifungal agents (red).



are targets of several natural compounds produced by many microorganisms [154,159,160].

In both yeast and mammalian cells, the sphingolipid biosynthetic pathway starts with the condensation of serine and palmitoyl-CoA to form ketodihydrosphingosine. The enzyme responsible for this reaction is serine palmitoyltransferase (Spt) (Figure 6). This enzyme contains at least two subunits, encoded by *LCB1/TSC2* and *LCB2/TSC1* genes [161,162]. Recently, a third subunit, encoded by the *TSC3* gene, has been isolated from *S. cerevisiae*. Tsc3 is a novel 80 amino acid protein that is required for optimal Spt activity in *S. cerevisiae* [163]. Although the human homologue has not yet been identified, the high degree of sequence homology between the yeast and the mammalian *LCB1* and *LCB2* genes suggests that the

Tsc3-like protein may be required for optimal activity in mammalian cells as well. Several natural products which inhibit the Spt enzyme and possess *in vitro* antifungal activity have been discovered, including sphingofungins [164,165], myriocin [166], lipoxamycin [167] and viridifungins [168,169]. However, the selectivity of these compounds to the fungal Spt enzyme and their toxicity to mammalian cells need to be further elucidated. For example, myriocin is a remarkably potent immunosuppressant [166] and the potential of this compound or its derivatives as antifungal agents remains hard to gauge.

Ketodihydrosphingosine is successively reduced to dihydrosphingosine by the 3-ketodihydrosphingosine reductase, encoded by the *TSC10* gene in *S. cerevisiae* [170]. No inhibitors are available against this enzyme.

Dihydrosphingosine is then hydroxylated to phytosphingosine. In fungi, this enzyme is encoded by the *SYR2* gene. This gene was originally cloned because it was required for the cytotoxicity exerted by syringomycin E, a cyclic lipodepsipeptide [171]. The Syr2 protein sequence has significant similarities with enzymes involved in the ergosterol biosynthetic pathway, such as the C-5 sterol desaturase (Erg3) and C-4 sterol demethylase (Erg25) [172]. Despite sequence homology between Syr2 and the enzymes involved in sterol metabolism, Syr2 has not been determined to have a role in sterol metabolism. However, recent studies have shown that sterols do influence the interaction of syringomycin E with Syr2 in the plasma membrane [173]. Interestingly, *in vivo* studies have shown that syringomycin E could be a promising antifungal compound, since evidence of the existence of phyto-sphingolipids in mammalian cells is controversial. In a murine model of vaginal candidiasis, syringomycin E resolved infection more rapidly compared to clotrimazole [174]. Note that the *SYR2* gene might also be involved in transforming dihydroceramide into phytoceramide (**Figure 6**). Whether fungal cells produce phytoceramide from phytosphingosine or dihydroceramide is not clear. Yeast phytoceramide synthase has not yet been cloned. However, the accumulation of phytosphingosine that occurs upon inhibition of phytoceramide synthase suggests that the hydroxylation reaction probably occurs before the acylation [175,176]. Two classes of natural product inhibitors of ceramide synthase have been described: fumonisins [177-179] and australifungin [175,180]. Fumonisin B1 has poor activity while australifungin has potent and broad spectrum antifungal activity. However, their activities are not selective against fungal cells and whether their potential derivatives would not have similar toxic effects on mammalian cells need further investigation.

In fungi, phosphorylinositol is transferred from phosphatidylinositol to phytoceramide to form inositol-phosphoryl-ceramide (IPC). This reaction represents the first of the fungus-specific steps in sphingolipid biosynthesis. It is catalysed by Ipc1 (also called IPC synthase), encoded by the *IPC1* gene [154,181]. Whereas mammalian cells synthesise sphingomyelin and cerebroside from ceramide, yeast use the same substrate to produce IPC and its mannosylated and phosphorylated forms (MIPC and M(IP)<sub>2</sub>C). Concomitant with IPC formation, Ipc1 produces diacylglycerol (DAG). Therefore, this enzyme regulates the levels of two lipid bioactive

molecules: ceramide, which is implicated in yeast stress responses; and DAG, a well-established mitogen [153]. There are several natural compounds that inhibit this reaction, including aureobasidin A [181], khafrefungin [180] and rustmicin [182]. Rustmicin is the most potent Ipc1 inhibitor, with IC<sub>50</sub> of 30 ng/ml, 25 ng/ml and 0.2 ng/ml against the enzyme from *S. cerevisiae*, *C. albicans* and *C. neoformans*, respectively. Also, rustmicin has the most potent *in vitro* antifungal activity, with minimum inhibitory concentrations (MIC) of 500 ng/ml, 15 - 400 ng/ml and 2 ng/ml against *S. cerevisiae*, *Candida* sp. and *C. neoformans*, respectively [182].

*Aspergillus* species are intrinsically resistant to Ipc1 inhibitors [182-184]. Recently, Georgopapadakou showed that the resistance of *A. fumigatus* to aureobasidin A is the result of increased efflux [157]. If the Ipc1 inhibitor is given together with verapamil, a known mammalian multi-drug resistance modulator [185], the MIC of aureobasidin A is decreased by ~15-fold. Although this synergistic effect is seen only in *A. fumigatus*, this study greatly expands the potential antifungal spectrum of Ipc1 inhibitors against one of the major deep-seated fungal infections in humans.

*C. albicans* and *S. cerevisiae* are sensitive to Ipc1 inhibitors, but less so than *C. neoformans* [182]. Mandala *et al.* have suggested that the plasma membrane H<sup>+</sup>ATPases (P-ATPase) of these yeasts could rapidly acidify the media of growing cultures, degrading rustmicin [182,186]. The P-ATPase controls both efflux and influx of cations (H<sup>+</sup>, Ca<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup>) and is encoded by the *PMA1* gene, which has been successfully isolated and characterised only in *C. albicans* and *S. cerevisiae* (see also below) [187-190]. However, studies regarding the activity of rustmicin in mutant yeast strains lacking P-ATPase activity are not available. Several multidrug efflux pumps have been identified in *S. cerevisiae*, *C. albicans* and *C. neoformans* [191-205]. In *S. cerevisiae*, disruption of pleiotropic drug resistance gene 5 (*PDR5*) increases rustmicin activity. Studies with other *PDR* genes are not available. However, studies in an animal model for both aureobasidin A and rustmicin are available against *C. albicans* and *C. neoformans* infections [182,183], and support the promising potential for these Ipc1 inhibitors and eventually their derivatives.

IPC is then mannosylated to yield mannose-inositol-phosphoceramide (MIPC) [206]. Deletion of either of the two non-essential genes, *SUR1* (also called *CSG1*)

## 12 New potential targets for antifungal development

[207] and *CSG2* [208] prevent synthesis of MIPC and thus accumulation of IPC. The *Sur1/Csg2* gene product is a mannosyltransferase and probably catalyses mannosylation of IPC [209,210]. The *Csg2* protein has 9 - 10 potential membrane-spanning domains and an elongation factor- $\text{Ca}^{2+}$ -binding domain, although it is unclear whether *Csg2* is necessary for mannosylation of IPC [211,212].

The final step in sphingolipid synthesis is the transfer of inositol phosphate from phosphatidylinositol to MIPC to yield  $\text{M(IP)}_2\text{C}$  (**Figure 6**). In *S. cerevisiae*, this reaction is catalysed by inositol phosphotransferase 1, encoded by the *IPT1* gene [213].  $\text{M(IP)}_2\text{C}$  is the most abundant and complex sphingolipid in *S. cerevisiae*, accounting for about 75%, the remaining 25% being divided between IPC and MIPC. In *S. cerevisiae*, *IPT1* is not essential for viability. The  $\Delta ipt1$  deletion mutant grows normally with increased level of MIPC [213,214]. Interestingly, *S. cerevisiae*  $\Delta mic2$ , another mutant also unable to make  $\text{M(IP)}_2\text{C}$ , showed decreased sensitivity to the antifungal drug nystatin, indicating that  $\text{M(IP)}_2\text{C}$ , in addition to ergosterol, is necessary for nystatin action [214,215]. These studies suggest that further investigations are warranted to elucidate the interaction among sterols and sphingolipids.

Other genes have been identified in *S. cerevisiae* encoding important enzymes in the degradation of sphingolipids, including long chain base kinases, encoded by *LCB4* and *LCB5* genes, yeast sphingosine resistance gene, encoded by the *YSR2* gene (also called *LBP2* gene) and the dihydrosphingosine-1-phosphate lyase, encoded by the *DPL1* gene (also called *BST1* [bestowed of sphingosine tolerance]) (**Figure 6**). All of these enzymes have been biochemically characterised in *S. cerevisiae* [216-222]. Recently, the murine and human sphingosine kinases have been identified [223-225]. Domain structure and alignment analysis showed similarity with yeast *LCB4* and *LCB5* genes [225]. Cloning and characterisation of human sphingosine kinase and analysis of its expression in yeast lacking *Lcb4* and *Lcb5* activities should help to clarify the role of the yeast kinases as antifungal targets. Also, the *DPL1* gene has been recently isolated from mammalian cells [226]. Expression of the mouse *DPL1* in yeast strain  $\Delta dpl1$  restored the wild type phenotype, indicating that the mouse gene can functionally complement the yeast defect [226]. Finally, the isolation of the human *YSR2* gene and its characterisation will help to understand

whether the yeast homologue has a future as a candidate for antifungal development.

In summary, the current focus should be on *Ipc1* inhibitors, since this essential step in yeast sphingolipid synthesis is not found in humans. Moreover, studies in *S. cerevisiae* showed that sphingolipids are necessary for growth at 37°C, low pH and under stress conditions [155,214,217,221,227-229]. There are indications that sphingolipids can regulate calcium-mediated signalling pathways [170,207,208,230-237]. Further studies are necessary to investigate this interaction and, eventually, the possibility of targeting these pathways with new antifungal compounds.

### 3.2 Phospholipids

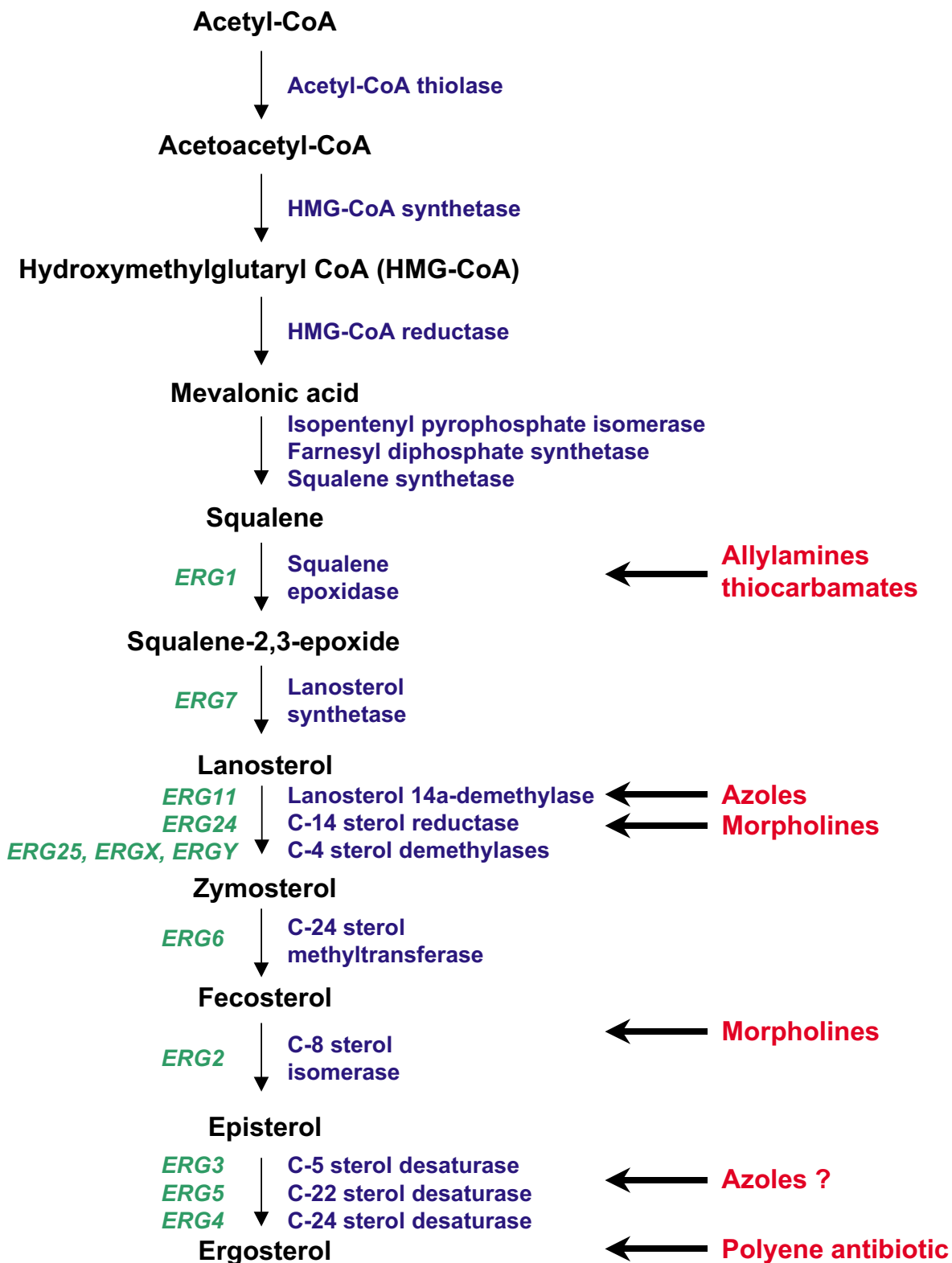
The fungal phospholipids pathway is structurally similar to the mammalian counterpart [238-241]. The only known difference is the synthesis of phosphatidylserine, which is synthesised from CDP-diacylglycerol in fungi, but from phosphatidylethanolamine and serine in mammalian cells [242]. Most studies have been performed on *S. cerevisiae* [238], although there are some recent studies on the phospholipids of *C. albicans* [243-245]. To our knowledge, no specific target or compound to inhibit fungal phospholipid biosynthesis has yet been reported.

### 3.3 Ergosterol synthesis

The association of sterols with the cell membrane serves to stabilise its structure and makes it less flexible. Why membrane rigidity is necessary in eukaryotes is not known. One possibility, however, is that the eukaryotic cell, which is much larger than the prokaryotic cell, must endure greater physical stresses on the membrane, thus necessitating a more rigid membrane structure in order to keep the cell stable and functional.

The ergosterol biosynthetic pathway and its target sites for antifungal agents are shown in **Figure 7**. Azole antifungal agents prevent the synthesis of ergosterol by inhibition of the cytochrome P450-dependent enzyme, lanosterol demethylase (also referred to as  $14\alpha$ -sterol demethylase or  $\text{P450}_{\text{DM}}$ ) [2,160,204,246]. This enzyme is also found in mammalian cells where it plays an important role in cholesterol synthesis [247]. However, azoles possess a much greater affinity for the fungal enzyme than its mammalian counterpart [248], and as such are

**Figure 7:** Ergosterol synthesis pathway (black), specific enzymes (blue), corresponding genes (green) and target sites of antifungal agents (red).



currently the most widely used and studied class of antifungal agents.

For detailed review of this 'well-established' target and past and future azole antifungal drugs, including those under development, we suggest the following recent reviews [2,160,204,246,249].

### 3.4 Plasma membrane ATPase

The plasma membrane ATPase (P-ATPase) is encoded by the *PMA1* gene and controls both efflux and influx of cations ( $H^+$ ,  $Ca^{2+}$ ,  $Na^+$  and  $K^+$ ) across the plasma membrane. The fungal Pma1 enzyme differs considerably from the homologous mammalian and plant enzymes, especially in transmembrane segments 1, 2, 3 and 4 [250,251]. Moreover, site-directed mutagenesis of these regions frequently results in lethal mutations in *S. cerevisiae*, demonstrating the importance of these fungal-specific domains to function [252-254]. Taken together, these observations suggest that the P-ATPase pumps can be considered potential targets for the development of new antifungal agents.

Bafilomycin A<sub>1</sub> is a macrolide antibiotic that inhibits the vacuolar  $H^+$ -ATPase (V-ATPase) in several organisms [255]. Although this class of antibiotic is considered a highly specific inhibitor of V-ATPase (nanomolar concentration) [255,256], recent studies have shown that the P-ATPase and the ATP-binding cassette (ABC) transporters are also sensitive to bafilomycin A<sub>1</sub> (micromolar concentration) [190,257-260]. In addition, our recent studies demonstrated that the calcineurin inhibitor, FK506, and the non-immunosuppressive L-685,818 exert strong synergistic effect with the antifungal action of bafilomycin A<sub>1</sub> in *C. neoformans* (see also below) [261]. Studies in animal models of cryptococcal meningitis will be helpful to determine if this drug combination is potential.

### 3.5 Efflux pumps

The resistance of pathogenic fungi to the available drugs is an increasing problem in antifungal therapy and the extent of this potential problem was observed in fluconazole-resistance of *C. albicans* during mucocutaneous candidiasis in HIV-infected individuals. An excellent review by White *et al.*, summarises the current knowledge of the clinical and cellular factors and the molecular mechanisms that contribute to the development of antifungal resistance [204]. Here, we emphasise the possibility of efflux pumps as new drug targets.

Any compound that inhibits the activity of these pumps would increase the intracellular concentration of other drugs when used in combination therapy. For example, recent studies showed that aureobasidin A (an Ipc1 synthase inhibitor, see above) also inhibits an ABC transporter (Cdr2) in cancer cell lines, resulting in an increased intracellular concentration of vincristine (a common anticancer drug) [262]. If confirmed in yeast studies, this characteristic could make the Ipc1 inhibitors even more attractive. Another example is given by the calcineurin inhibitor FK506, which is known to have activity on multi-drug resistance pumps in both humans and yeasts [263-267]. When FK506 is used in combination with fluconazole, the resulting effect is synergistic and makes the azole fungicidal against *C. neoformans* and *C. albicans* [261]. Since the synergistic effect is still found when either calcineurin or FKBP12 genes are disrupted, these studies are in accord with a model in which FK506 enhances fluconazole activity, probably by inhibiting one or more multi-drug resistance pumps that normally extrude fluconazole from the cell and thus potentiate its activity.

### 3.6 Antifungal peptides

Anthony De Lucca and Thomas Walsh have published an outstanding review on antifungal peptides, including their *in vitro* and *in vivo* activities, mechanisms of action and structure-activity relationships [8]. These molecules appear to act mainly on plasma membrane synthesis. A different class of peptides, lipopeptides, affect mainly cell wall synthesis (see above). For other recent reviews on lipopeptides, please see references [24,268]. It appears that these peptides may help both dissect important targets in the plasma membrane and themselves become antifungal agents.

## 4. DNA and protein synthesis

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### 4.1 Topoisomerases

Topoisomerases control the topological state of DNA by introducing transient DNA breaks (single-strand DNA for Type I and double-strand DNA for Type II) that allow for the manipulation of DNA strands relative to one another [269]. Topoisomerases stabilise the nicked DNA strands by forming a covalent phosphate-tyrosine linkage with either the 3'- or 5'-end of the DNA. Topoisomerase-specific inhibitors stabilise this covalent protein-DNA linkage,

effectively slowing the religation phase of catalysis and ultimately leading to DNA damage and cell death [270,271]. Recently, Redinbo *et al.* solved the crystal structures of human topoisomerase I (Top1) in covalent and non-covalent complexes with DNA [272,273]. In addition, two models for the interaction between the anticancer drug camptothecin and the human Top1-DNA complex have been proposed [273,274]. The sole cellular target of the camptothecins is human Top1 [275]. Two derivatives of camptothecin, topotecan and irinotecan, are currently in clinical use against ovarian, lung and colorectal cancers; additional camptothecin analogues are also in clinical trials against a variety of human cancers. The topoisomerase II (Top2) inhibitor etoposide is also in clinical use as an antineoplastic agent. An excellent review by James Wang covers both Top1 and Top2 inhibitors as antitumour agents [276].

Recent studies in the pathogenic fungus *C. neoformans* has revealed that *TOP1* is essential for viability [277]. In addition, studies in *C. albicans* also indicate that *TOP1* is essential [278]. Since *TOP1* appears critical for viability in fungal pathogens, it has the potential to be a potent antifungal drug target. Fungal Top1 enzymes contain an amino acid insertion, located in the linker domain region, not found in the mammalian enzyme. The linker region of Top1 (residues 636 - 709 of the human enzyme) joins the conserved core and C-terminal domains, which contain the enzyme's active site [272,273,279]. In human Top1, the linker domain is 77 amino acids long, whereas in both *C. neoformans* and *C. albicans* the Top1 linker domain is of 155 amino acids. The linker domain region of human Top1 is known to affect the cleavage/religation equilibrium of the enzyme and to be essential for camptothecin efficacy [280,281]. Thus, it is possible that selective antifungal compounds may be targeted to this fungal-specific region of the Top1 linker domain. Studies designed to elucidate the function of these fungal inserts are currently in progress.

The region around the active site residues of the human and fungal topoisomerases I exhibit a high degree of sequence similarity; only single-site conservative amino acid changes are observed adjacent to the catalytic tyrosine, two arginines, lysine and histidine that make up the active site [273,277]. However, selective inhibition of fungal Top1 catalytic activity over the human enzyme has already been observed. *C. albicans* Top1 can be specifically targeted by the aminocatechol A-3253, while this

compound is less effective against human Top1 [282]. In contrast, camptothecin has more activity against human rather than fungal Top1 [283]. These studies suggest that distinct structural differences do exist adjacent to the active site between human and fungal Top1 and that these structural differences may be exploited in the development of selective antifungal agents.

Recently, we tested the *in vitro* activity of a series of camptothecin and nitidine derivatives against fungal and cancer cells. Our findings reveal that several of these compounds exhibit good fungicidal activity and that some can act synergistically with existing antifungal compounds, such as amphotericin B [284]. If the selectivity of the camptothecins and nitidines for human Top1 relative to the fungal enzymes could be overcome by the design of novel derivatives, these particular compounds may hold great promise as antifungal drugs.

Topoisomerase II has not been studied as an antifungal drug target to the same degree as Top1. Its homologue in bacterial cells, however, is a well-known target of the antibiotic quinolones in the treatment of bacterial infections. In addition, the fact that *TOP2* is essential for viability in fungi makes it an attractive and potentially important target.

## 4.2 Nucleases

The dicationic aromatic compounds (DACs) are pentamidine derivatives that have been shown to possess excellent *in vitro* and *in vivo* activity against a number of pathogenic microorganisms, including *Giardia lamblia*, *Toxoplasma gondii*, *Plasmodium falciparum*, *Leishmania mexicana amazoniensis*, *Trypanosoma brucei* and *P. carinii* [285-291]. Recently, we reported that these compounds have *in vitro* antifungal activity against *C. neoformans*, *C. albicans* and *Candida* spp. other than *albicans* [292,293]. In particular, several of these agents exhibited excellent *in vitro* fungicidal activity against *Candida krusei*, *Candida glabrata*, *Candida lusitanae* and a *C. albicans* mutant strain containing a fluconazole-resistant mechanism [294]. Since these compounds have been administered safely to animals, they have the potential of being developed into potent antifungal agents for general use in humans.

The detailed mechanism of action of the DACs has yet to be established completely. It has been reported that DNA binding by these compounds is necessary, but

not sufficient, for antimicrobial activity [295]. It has been hypothesised further that the primary target of these compounds is an endo- or exonuclease in the pathogenic fungus *P. carinii* [296]. It has also been suggested, however, that the primary target of the dicationic compound, pentamidine, is the mitochondrion in *S. cerevisiae* [297]; since the DACs are derivatives of pentamidine, it is thought that they may act in the similar fashion. Studies to determine the mechanisms of action of these compounds are currently in progress, using *S. cerevisiae* as a genetic model.

### 4.3 Protein synthesis

Several well-characterised compounds are known to inhibit the RNA polymerases and elongation factors required for transcription and protein synthesis. Clancy *et al.* [298] investigated the *in vitro* antifungal activity of amphotericin B both alone and in combination with rifabutin, an inhibitor of bacterial RNA polymerase, against 26 clinical isolates of *Aspergillus* and 25 clinical isolates of *Fusarium*. Synergy or additivistic activity between these drugs was demonstrated against all isolates tested, suggesting that inhibition of the fungal RNA synthesis could be a potential target [298]. Other investigators have focused on two soluble proteins, elongation factor 1 (EF-1) and 2 (EF-2). Some sordarin derivatives, such as GM 222712 and GM 237354, showed excellent *in vitro* activities against a wide range of pathogenic fungi, including *Candida* spp., *C. neoformans* and *P. carinii*, as well as some filamentous fungi and emerging invasive fungal pathogens [299-302]. Recently, Dominguez *et al.* showed that sordarin blocks the elongation cycle at the initial steps of translocation, prior to GTP hydrolysis, preventing the formation of peptidyl-(3)H]puromycin on polysomes from *C. albicans* [303]. Sordarins are known to be a family of drugs of distinct medical interest. The evaluation of the degree to which these compounds are selective to fungi will determine whether this class of compounds has the potential of becoming novel antifungal agents.

Elongation factor 3 (EF-3) is a unique and essential requirement of the fungal translational machinery. Non-fungal organisms do not have and do not require a soluble form of the EF-3 for translation [304]; therefore, it is an ideal antifungal target [305]. To our

knowledge, no inhibitors of EF-3 have been identified.

### 4.4 The *N*-myristoyl transferase

The *N*-myristoyl transferase (Nmt), encoded by the *NMT* gene, catalyses the co-translational addition of myristic acid to the amino-terminal glycine residue of a number of important proteins with a wide variety of functions. This co-translational modification has only been observed in eukaryotes and it is required for the full biological activities of several *N*-myristoylated proteins. *NMT* genes have been identified in numerous organisms, including *S. cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, *C. albicans*, *C. neoformans* and human [306-312]. Genetic studies have revealed that *NMT* is essential for viability in *S. cerevisiae* and pathogenic fungi [307,309,313]. The crystal structures of Nmt from both *S. cerevisiae* and *C. albicans* have recently been determined [314,315]. The functional properties of the acyl-CoA binding site of human and *C. albicans* Nmt are very similar; however, there are distinct differences in their peptide binding sites [316]. Such differences have been exploited in the development of fungal-selective Nmt inhibitors with proven fungicidal activity [313,316]. For example, a novel imidazole-substituted dipeptide amide and new biologically active non-peptidic inhibitors have been recently designed and synthesised that selectively target the fungal Nmt enzyme [317,318]. If the *in vivo* effectiveness of these novel compounds can be established, the enthusiasm for the development of Nmt inhibitors as clinically useful antifungal agents will be significantly reinforced.

## 5. Signal transduction pathways

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The signal transduction cascades in fungi have become very attractive since their components are now emerging as targets for new natural antifungals. A recent review by Cardenas *et al.* focused on the mechanism of action of five natural products, cyclosporin A (CsA), FK506, rapamycin, wortmannin and geldanamycin on signalling [319]. Here, we focus on the calcineurin-mediated signal transduction, which is a direct target of these products.

### 5.1 Calcineurin

Calcineurin is a serine/threonine-specific  $\text{Ca}^{2+}$ -calmodulin-activated protein phosphatase that is conserved from yeast to human [320]. Calcineurin is

the target of CsA and FK506 in T-cells, *S. cerevisiae*, *C. albicans*, *C. neoformans* and *A. fumigatus* [321-324]. Because of their immunosuppressive effect, CsA and FK506 exacerbate cryptococcal meningitis in both murine and rabbit models [325,326]. However, a number of non-immunosuppressive FK506 and CsA analogues have been described, including L-685,818 (18-OH, 21-ethyl-FK506), which retains antifungal activity *in vitro* via inhibition of calcineurin [323]. Recently, we identified a series of CsA analogues, including 211-810 [ $\gamma$ -OH) CH<sub>3</sub>Leu<sup>4</sup>-CsA] and 209-825 [ $\alpha$ -SCH<sub>3</sub>)<sup>3</sup>-Val<sup>2</sup>-DH-CsA], that have dramatically decreased immunosuppressive activity but retain potent antifungal activity against *C. neoformans* [327]. Moreover, we showed that the compounds are toxic to *C. neoformans* via cyclophilin A-dependent inhibition of calcineurin. Most interestingly, the affinity of cyclophilin A-211-810 analogue complex for calcineurin is increased relative to that of cyclophilin A-CsA. These findings suggest that the  $\gamma$ -hydroxy substitution on the leucine 4 residue prevents binding to mammalian calcineurin but increases binding to the fungal calcineurin homologue [327]. If these non-immunosuppressive CsA analogues have antifungal activity, they will need to be tested in animal models for antifungal efficacy.

FK506 binds to a family of intracellular binding proteins, the FKBP immunophilins [328,329]. In particular, FK506 binds to FKBP12 and the FK506-FKBP12 complex binds to and inhibits calcineurin [330,331]. The non-immunosuppressive L-685,818 has the same mechanism of action [261,323]. Interestingly, we found that FK506 and L-685,818 have potent synergistic activity against *C. neoformans* when combined with bafilomycin A<sub>1</sub>, fluconazole and the pneumocandin MK0991/caspofungin acetate [261]. Bafilomycin A<sub>1</sub> inhibits the V-ATPase in several organisms and also P-ATPase and ABC transporters (see also above). Both calcineurin and V-ATPase regulate cation influx and efflux in fungi. We demonstrated that FK506 plus bafilomycin A<sub>1</sub> is a very potent combination *in vitro*. Our findings revealed that the FK506 synergistic action with bafilomycin A<sub>1</sub> requires FKBP12, since two FKBP12 mutant strains were completely resistant to its synergistic activity. In testing two calcineurin mutant strains, we found that calcineurin is not required for the activity of FK506 plus bafilomycin A<sub>1</sub>. Since both calcineurin mutant strains were sensitive to the synergistic activity of bafilomycin A<sub>1</sub>, several new

models must be invoked, including a novel target of the FK506-FKBP12 complex [261].

FK506 and fluconazole provide synergistic activity against *C. neoformans* and we have demonstrated that the synergistic action of FK506 and fluconazole is independent of both FKBP12 and calcineurin [261]. Presumably, FK506 increases the intracellular concentration of fluconazole by inhibiting multi-drug resistance pumps, as showed in *C. albicans* [332]. This drug combination might influence the appearance of azole-resistant *C. neoformans* and *C. albicans* strains in patients.

The pneumocandin MK0991/caspofungin acetate inhibits the fungal cell wall enzyme GS, which is encoded by *FKS1*, a single essential gene in *C. neoformans* [22]. We found that synergistic activity of FK506 plus MK0991/caspofungin acetate is dependent on both FKBP12 and calcineurin, because no synergistic activity was observed in the mutant strains lacking FKBP12 or calcineurin. Moreover, the *in vitro* activity of MK0991/caspofungin acetate was increased in the mutant strain lacking calcineurin compared to the wild type, suggesting a functional link between calcineurin and GS regulation in *C. neoformans* [261]. The value of drug combinations with existing antifungal agents and new potential compounds with a link in mechanism(s) should be further investigated.

Although this is one signal pathway, the further understanding of other signalling pathways involved in cell growth, differentiation and synthesis of both plasma membrane and cell wall in *S. cerevisiae* and in pathogenic fungi should identify other protein phosphatases and kinases involved in these cascades, which have the potential to become antifungal targets.

## 6. Virulence factors

### 6.1 Melanin

Melanin is produced by the enzyme laccase and has been thought to be a major virulence factor in the pathogenic fungus *C. neoformans*. The laccase gene (*LAC1*) was cloned from *C. neoformans* by Williamson in 1994 [333] and he has subsequently revealed a strong link between melanin production and pathogenesis in *C. neoformans*. Williamson has also written an excellent review detailing our knowledge on the subject [334]. To date, studies on melanin production as a virulence factor has been most fully developed in *C. neoformans* [335-340].

However, melanin production has also been discovered to be active in other pathogenic fungi, including the dematiaceous fungi, which produce infections classified as phaeohyphomycoses. There has been some suggestions from studies of albino mutants of these fungi that melanin production is a virulence factor [102,341,342], although the link between melanisation and pathogenesis is not complete.

The focus on *C. neoformans* and its melanin production has two potential benefits. First, it may help us to better understand the function of melanin not only *in vitro* but also in yeast cells within the host. Second, with further understanding of the biochemistry and molecular biology of melanin, it could become a unique target for antifungal drugs against *C. neoformans* and other dematiaceous fungi.

### 6.2 Mannitol

Other than mannose, another possible metabolic target associated with virulence in *C. neoformans* is the mannitol pathway. Chaturvedi *et al.* isolated one mutant with decreased mannitol production and found it to be more susceptible to polymorphonuclear leukocyte killing [343]. It was also demonstrated that the abilities of *C. neoformans* to produce and accumulate mannitol may influence its tolerance to heat and osmotic stresses, as well as its pathogenicity in mice [343,344]. Perfect *et al.* isolated the first *C. neoformans* gene (*MTL1*) related to mannitol synthesis, which was later found to regulate the *S. cerevisiae* mannitol dehydrogenase gene [345]. These studies promise to define the importance of the mannitol pathway in virulence, but will further require the isolation of specific genes and the studies of their respective mutants. Further genetic and biochemical investigations are needed to understand and validate the role of the mannitol pathway in fungal virulence.

### 6.3 Phospholipases

Phospholipases are a group of enzymes that hydrolyse specific ester linkages in glycerophospholipids. Invasion of host cells by microbes involves penetration and damage of the outer cell envelope. This likely happens by enzymatic or physical means and, thus, phospholipases are thought to be involved in the cell disruption process that occurs during infection. The enzymes could then promote the pathogen's penetration into the host cell [346].

Extracellular phospholipases have been found to be implicated with pathogenicity in fungi including *C. albicans*, *C. glabrata*, *Penicillium notatum*, *A. fumigatus* and *C. neoformans*. In *C. albicans*, Ghannoum *et al.* showed that a disruption in the phospholipase B gene (*PLB*) had no effect on adherence, but that the ability of the null mutant to penetrate the host cells was reduced and that the mutant showed attenuated virulence in a murine model [347]. In a *C. neoformans plb1* mutant, a similar attenuation in virulence was seen although the reconstitution of the strain did not return the virulence to that seen in wild type [348]. The potential of these enzymes to be targeted for drug design is undecided, however, since the construction of null mutants showed only attenuated virulence in animal models. Mammalian homologues have also been identified, indicating that careful drug selectivity would be required for therapeutic use.

### 6.4 Glucuronoxylomannan

The *C. neoformans* capsule is largely comprised of a major polysaccharide, glucuronoxylomannan (GXM). Both *in vitro* and *in vivo*, GXM, along with other capsular material, is shed from the cell and provokes an antibody response. Studies determining the efficacy of this response for the production of a vaccine have been initiated. A conjugate vaccine comprising of GXM covalently coupled to the tetanus toxoid (GXM-TT) has been constructed and evaluated in a cryptococcal mice model. The vaccine produced high levels of antibodies by both active and passive immunisation and was found to confer 70 - 80% protection after a challenge of  $10^3$  cryptococcal cells [349]. Further studies have indicated that the presence of the vaccine-induced antibodies is essential within the first 4 - 6 weeks of infection, for the vaccine to be useful. Currently, GXM-TT is being studied in human trials for safety and immunogenicity in both healthy and HIV-infected patients.

As well as using GXM as a vaccine to elicit protective antibodies, the antibodies themselves are being studied for therapeutic use [350]. To date, only monoclonal antibodies (mAbs) that recognise three epitopes of GXM (IgG1 mAbs 2H1 and E1, and IgM mAb 2E9) consistently provide protection against *C. neoformans* [351,352]. Studies have shown that, in the murine model, administration of the mAb 2H1 prior to infection can prolong survival and reduce fungal burden upon administration, although it seldom clears the infection [353]. Studies are underway to

further the potential use of mAbs to treat or prevent human *C. neoformans* infection.

## 7. Future considerations

In this review, we have taken a focused approach to examine the progress and potential for the development of antifungal targets and their inhibitors. Unavoidably, our discussion has been biased by our own work in the field and it should not be assumed that we have identified all of the most important targets or strategies. Instead, we hope to have covered some potential targets and to have demonstrated the depth of their database. It is clear from this review that as new targets are validated, the power of fungal biochemistry, molecular biology and robust animal models will make it possible to take these targets to the chemist for compound (inhibitor) identification.

With the age of functional genomics and sequenced genomes upon us, the identification of targets and their validation for specificity and selectivity will become commonplace. The molecular revolution has enabled great advances and has opened our understanding of fungal pathogenesis. In the next decade, this work will continue. A future challenge may not be so much the identification of important antifungal targets, but the ability to make or find inhibitors to a specific targets. Advances in biased combinatorial chemistry libraries and improved molecular modelling will be crucial to exploit the great promise of our present studies into antifungal targets. Drug discovery has never been more sophisticated, but both targets and compounds will still require careful strategies to bring them together for the realisation of new and improved antifungal agents for human mycoses.

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