WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 11, Issue 2, 478-494. Review Article ISSN 2277– 7105

ANTIFUNGAL AGENTS: MECHANISMS OF ACTION

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Article Received on
25 Nov. 2021,

Revised on 15 Dec. 2021,
Accepted on 05 January 2022

DOI: 10.20959/wjpr20222-22850

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ABSTRACT

Clinical needs for novel antifungal agents have altered steadily with the rise and fall of AIDS-related mycoses, and the change in spectrum of fatal disseminated fungal infections that has accompanied changes in therapeutic immunosuppressive therapies. The search for new molecular targets for antifungals has generated considerable research using modern genomic approaches, so far without generating new agents for clinical use. Meanwhile, six new antifungal agents have just reached, or are approaching, the clinic. Three are new triazoles, with extremely broad antifungal spectra, and three are echinocandins, which inhibit synthesis of fungal cell wall polysaccharides – a new mode of action. In addition, the sordarins represent a novel class of agents that inhibit fungal protein synthesis. This review describes the targets and mechanisms of action of all classes of antifungal agents in clinical use or with clinical potential.

KEYWORD: Antifungal agents, Immunosuppressive therapies, Sordarins, Clinical potential.

INTRODUCTION

The massive emergence of fungal diseases associated with AIDS in the 1980s and the rising frequency of fatal mycoses associated with increasing use of immunosuppressive medical therapies since the 1970s stimulated research directed towards the discovery of novel anti-fungal agents. The results can be seen in six new agents, representing two antifungal classes, which were recently licensed for human use or are undergoing phase 3 clinical trials. Other novel antifungal classes have been reported, but these have so far been unsuccessful in the transition from discovery to therapeutic use. The objective of this Review is to summarise

current knowledge of the targets and modes of action of both new and old antifungal agents, and our understanding of resistance to them.

New targets, new Agents and Clinical realities

The biomedical literature abounds with reports of macro- molecules essential for fungal survival, growth, virulence or cellular morphogenesis that have been proposed as potential targets for novel antifungal agents. The arrival of whole-genome sequence data for pathogenic fungi, such as Candida albicans,[1] Aspergillus fumigatus[2] and Cryptococcus neoformans,[3] as well as for non-pathogens, such as Saccharomyces cerevisiae,[4] has paved the way for discovery of genes encoding candidate antifungal targets on a previously unprecedented scale[5] However, the antifungal discovery process based on screening compounds against molecular targets, which dates back before the genomics era, has so far not resulted in a single new agent emerging into the clinic or even the development pipeline. (The same can be said for most areas of drug discovery research.[6]) It takes time to generate practically useful results from fundamental biomedical research, and it is probably still too early to expect new antifungal agents to be discovered this way. However, the shift from random screens for antifungal effects towards screens against specific molecular targets has led to a re-appreciation of formerly abandoned classes of antifungals, such as the sordarins.[7]

Antifungal targets

The targets of all antifungal agents used in the clinic (and of some agents that entered or approached clinical development but have not been marketed) are summarised in Figure 1. This figure shows that, contrary to occasional pessimistic comments, a considerable diversity of anti- fungal targets already exists. Nevertheless, in terms of numbers of classes of agents that can be used to treat life- threatening mycoses, the targets are heavily focused, directly or indirectly, on the cell envelope (wall and plasma membrane), and particularly on the fungal membrane sterol, ergosterol, and its biosynthesis. Targets elsewhere in the cell would therefore be a welcome innovation for systemically bioavailable antifungal agents.

There are six new antifungal agents that are currently generating excitement as they pass through the final developmental stages of clinical trials. Three of them (posaconazole, ravuconazole and voriconazole) are triazole compounds, a subset of the azoles, which are the most successful antifungal class in the clinic since the

late 1960s. The other three (anidulafungin, caspofungin and micafungin) are echinocandins, and are successors to cilofungin, which was abandoned in the 1980s. The echinocandins inhibit synthesis of fungal b-1,3 glucan, and this represents the first novel target in 20 years of antifungal drug discovery in terms of clinically useful drugs.

Changes in clinical needs

The pace of antifungal discovery and development is exceedingly slow; the six new agents started on their paths to the clinic in the 1980s. Since that time, the clinical needs for antifungal agents have changed considerably.

Problems with oropharyngeal *Candida* infections in AIDS patients were seen as a major therapeutic area in the 1980s, adding to an often-documented rise in fatal *Candida* infections in iatrogenically immunosuppressed patients and those undergoing intensive care.[8] However, the advent of highly active antiretroviral therapy (HAART) has enormously diminished the incidence of AIDS-associated fungal diseases, and judicious prophylactic and therapeutic use of existing antifungal agents, particularly amphotericin B and fluconazole, has considerably affected the incidence of disseminated *Candida* infections. Hence, the most recent epidemiological surveys suggest a decreasing, rather than an increasing, trend. [9]

Meanwhile, invasive aspergillosis, most commonly due to *Aspergillus fumigatus*, has risen in incidence through the 1990s to the point that many infectious disease physicians now regard aspergillosis, not *Candida* infection, as their major fungal problem. The trend away from *Candida* towards *Aspergillus* infections is perhaps illustrated most clearly in a huge survey of autopsy data involving almost 8000 Japanese patients between 1969 and 1994.[10] This study showed that *Aspergillus* spp. was involved in an average of 23% of deaths due to fungi in the 1970s, 27% in the 1980s, and 36% in the 1990s. In addition, cases of disseminated infection due to *Fusarium* and *Scedosporium* species have occurred on a scale sufficient to cause concern, as neither genus is particularly susceptible to existing agents. Caspofungin and voriconazole, the first two of the ‘new six’ antifungal agents to gain FDA approval, both began their development with clinical trials against AIDS-associated oral *Candida* infections, but were approved on the basis of trials

proving efficacy in aspergillosis, thus reflecting the change in clinical needs that has occurred since the 1980s. One compelling conclusion to be drawn from this recent history is that any novel antifungal agent needs to have as broad a spectrum of susceptible fungal species as possible, whatever its mechanism of action. Genomics-based target searches must therefore emphasise genes that are widely represented in the fungal kingdom, but are absent or demonstrably of a different structure in mammalian cells. If a target is shared between host and pathogen, additional constraints are placed on the selectivity and toxicity of potential antifungal agents. This greatly reduces the apparently large number of potentially useful targets that is found from genomics searches based only on *C. albicans and S. cerevisiae.*

Mechanisms of antifungal action

Griseofulvin

The earliest inhibitory agent specific to fungal species was griseofulvin (Fig. 2). The precise mechanism of action of this compound is still unknown,[11] but the favoured explanation is that it interferes with microtubule assembly. The selective toxicity of griseofulvin for fungi is only moderate (liver toxicity is recognised as an occasional hazard) and its spectrum of action is restricted mainly to the dermatophyte fungi – causes of ringworm and athlete's foot. However, other types of compound are known to interfere with microtubule assembly and function in pathogenic fungi, such as *C. neoformans.*[12] Effects on microtubules explained the antifungal activity of early compounds, such as benzimidazole. However, unlike the situation in fungicide research against plant pathogens, relatively little research attention seems to have been paid in recent years to microtubules as possible antifungal targets for clinical use.

Flucytosine

Flucytosine (5-fluorocytosine; Fig. 2) works as an anti- fungal agent through conversion to 5-fluorouracil within target cells. Fluorouracil becomes incorporated into RNA, causing premature chain termination, and it inhibits DNA synthesis through effects on thymidylate synthase. For this mechanism of action, the target cells must possess cytosine permease to internalise the flucytosine molecule, cytosine deaminase to convert it to 5-fluorouracil, and uracil phosphoribosyl transferase to convert 5-fluorouracil into a substrate for nucleic

acid synthesis. Most filamentous fungi lack these enzymes and hence the useful spectrum of flucytosine is restricted to pathogenic yeasts (*Candida* species and *C. neoformans*). Flucytosine is used as adjunctive, rather than primary therapy, in the clinic, because primary and secondary resistance (resulting from defects in the permease, deaminase and/or phosphoribosyl transferase enzymes) were thought to occur at a high frequency. However, recent large-scale susceptibility testing of *Candida* species by current reference methodology found surprisingly few primary resistant isolates,[13] in contrast to the results of non standardised tests done in the 1970s. Therefore, flucytosine might deserve more use in the clinic than it has previously enjoyed.

Polyene antifungal agents

Amphotericin B (Fig. 2) has for many years been the only antifungal polyene that can be administered systemically to treat a visceral infection. Its mode of action is atypical for an antimicrobial molecule: instead of inhibiting an enzyme, it binds to ergosterol, the principal sterol in fungal membranes, thereby perturbing membrane function to the point of causing leakage of cellular contents. The precise way in which this fungicidal effect occurs still remains unclear. The ergosterol molecule of fungi has a cylindrical three-dimensional structure, unlike cholesterol, the major sterol in mammalian membranes, which has a sigmoid shape (Fig. 3). This conformational difference is probably sufficient to explain the greater binding affinity of amphotericin B for ergosterol over cholesterol. This difference and the higher ratio of ergosterol: phospholipids in fungi is the basis for the antifungal selectivity of amphotericin B.[14] However, this selectivity is low and suggests that amphotericin B will have potential toxicity for mammalian cells. Binding to a sterol leaves the amphoteric amphotericin B molecule with its hydrophilic edge unbalanced relative to the larger hydrophobic portion of the complex. Almost certainly, such a complex when formed in a membrane, will migrate to align the hydrophilic faces within aggregations of complexes, thus creating areas of local tension within the membrane. The mechanism of action correctly predicts that amphotericin B will act on a broad spectrum of fungal species. Indeed, it is undoubtedly this breadth of action of this molecule that has retained its clinical popularity despite considerable toxicity problems.

As predicted on the basis of its mechanism of action, amphotericin B is toxic to mammalian cells, particularly causing nephrotoxicity. This was observed from the earliest days of clinical use of the drug. To overcome amphotericin B toxicity, a variety of reformulated versions of the agent have been introduced. They probably reduce nephrotoxicity by slowing the rate at which amphotericin B is delivered to the kidneys. The most successful, clinically proven versions of novel formulations are based on lipid combinations with amphotericin B, encapsulated in liposomes or in ribbon-like and disc-like lipid complexes.[15] Other formulations are under investigation, including an amphotericin B– cochleate preparation[16] and an arabinogalactan complex.[17] The antifungal polyene nystatin (Fig. 2) is also being developed in a liposomal formulation for systemic use.[18]

Antifungal azoles

Imidazoles and triazoles (‘azoles’) are the largest class of antifungal agents in clinical use (Fig. 2). Their main effect is to inhibit 14*a*-demethylation of lanosterol in the ergosterol biosynthetic pathway,[19] but in some fungal species, they can also inhibit the subsequent *D*22-desaturase step.[20] With ergosterol depleted and replaced with unusual sterols, the normal permeability and fluidity of the fungal membrane is altered, with secondary consequences for membrane-bound enzymes, such as those involved in cell wall synthesis.[21] The principal molecular target of azole antifungals is a cytochrome P450– Erg11p or Cyp51p, according to different gene-based nomenclatures, which catalyses the oxidative removal of the 14*a*-methyl group of lanosterol and/or eburicol in fungi by a typical P450 mono-oxygenase activity. This protein contains an iron protoporphyrin moiety located at the active site, and the antifungal azoles bind to the iron atom via a nitrogen atom in the imidazole or triazole ring (Fig. 4). The remainder of the azole molecule binds to the apoprotein in a manner dependent on the individual azole’s structure. The exact conformation of the active site differs between fungal species and amongst the many mammalian P450 mono-oxygenases.[19] The precise nature of the interaction between each azole molecule and each kind of P450 therefore determines the extent of the azole’s inhibitory effect in different fungal species. So far, the only crystal structure of a Cyp51p molecule to have been published is for the one from *Mycobacterium tuberculosis*, and studies with this enzyme should be consulted for details of the precise interactions between sterols, azoles and the active site protoporphyrin moiety.[22,23]

Azole molecules have been refined during the past 30 years (Fig. 2). A triazole nucleus has replaced imidazole in the active pharmacophore, to enhance the of specificity binding to fungal P450. For molecules derived from the fluconazole prototype, the inclusion of an *a*-O-methyl group confers activity against Aspergillus species and many other filamentous fungi. For molecules derived from the ketoconazole prototype, extension of the side chain enhances binding of the azole to the P450 apoprotein.

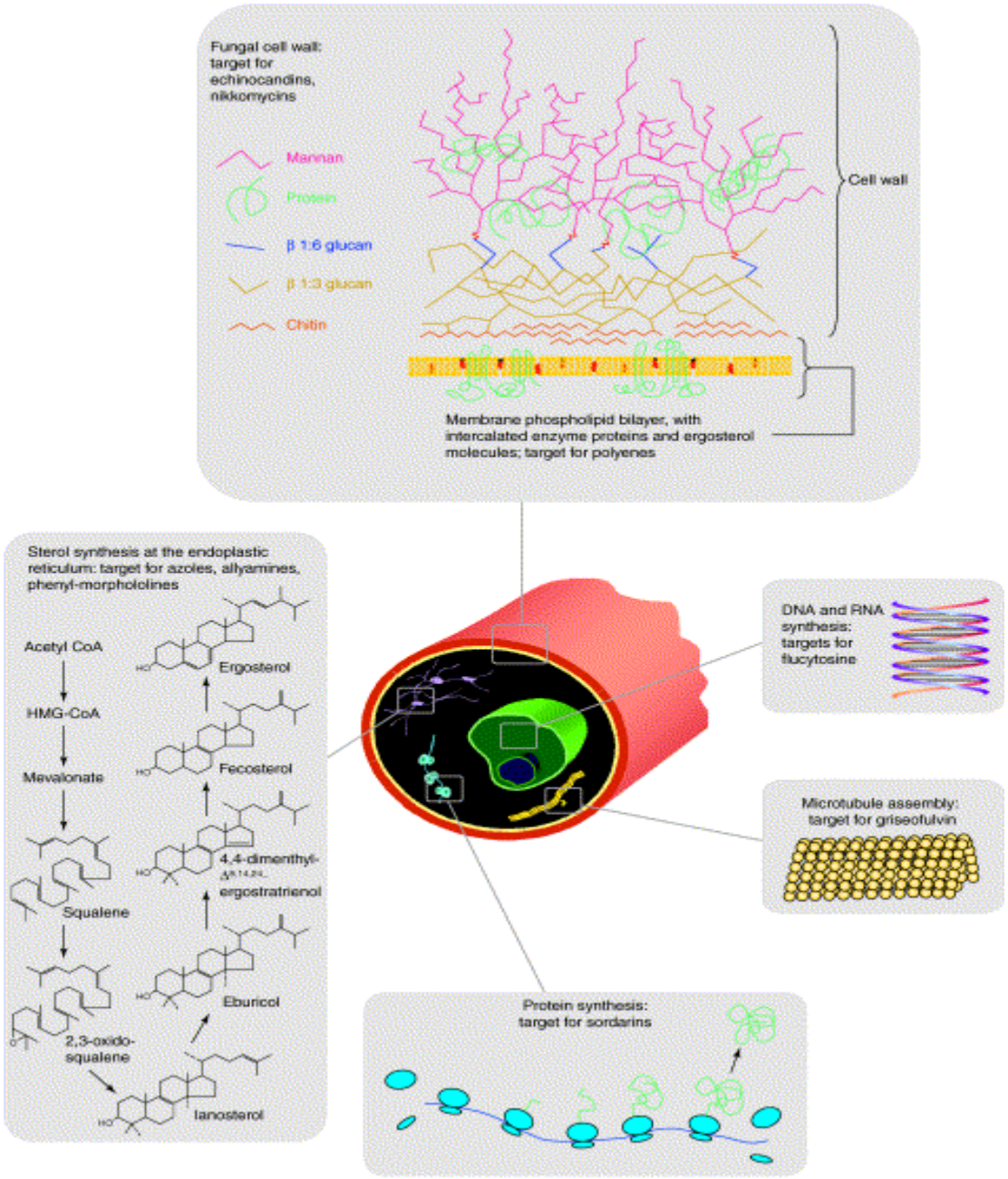


Fig. 1: Generalised cartoon showing target areas for antifungal agents. The cross-section through a fungal hypha shows the intracellular sites of action of antifungal agents. The callouts show details for each site. The cell envelope structure

illustrated is based on data for *Candida albicans*. Other fungal species differ in the details of their cell wall composition. The steps illustrated for ergosterol synthesis are the major steps found in all fungi; species can differ in having additional steps that bypass or parallel those shown.

Resistance to azoles can occur by mutations that modify the target molecule or by overexpression of membrane efflux pumps that export the antifungals from the cell. Combinations of both mechanisms have been detected in some C. albicans isolates, although in clinical terms, the consequences of azole resistance have so far been minimal, except in the case of AIDS-associated oropharyngeal infections.[24]

New triazoles

Of the three newest triazole antifungals, voriconazole and ravuconazole are structurally related to fluconazole, whereas posaconazole bears a close resemblance to itraconazole, but with the dioxolane ring altered to a tetrahydrofuran (Fig. 2). The structural differences might seem small, but they dictate antifungal potency and spectrum, bioavailability and drug interaction and toxic potential – very important considerations for compounds that bind to haem groups in P450s. All of these properties (not to mention the chemical stability of a compound, its ease of manufacture and many other parameters) are vital factors in the success of an antifungal drug. Hence, the successful design of an optimum inhibitor by three-dimensional (3-D) imaging of target molecules alone is not possible.

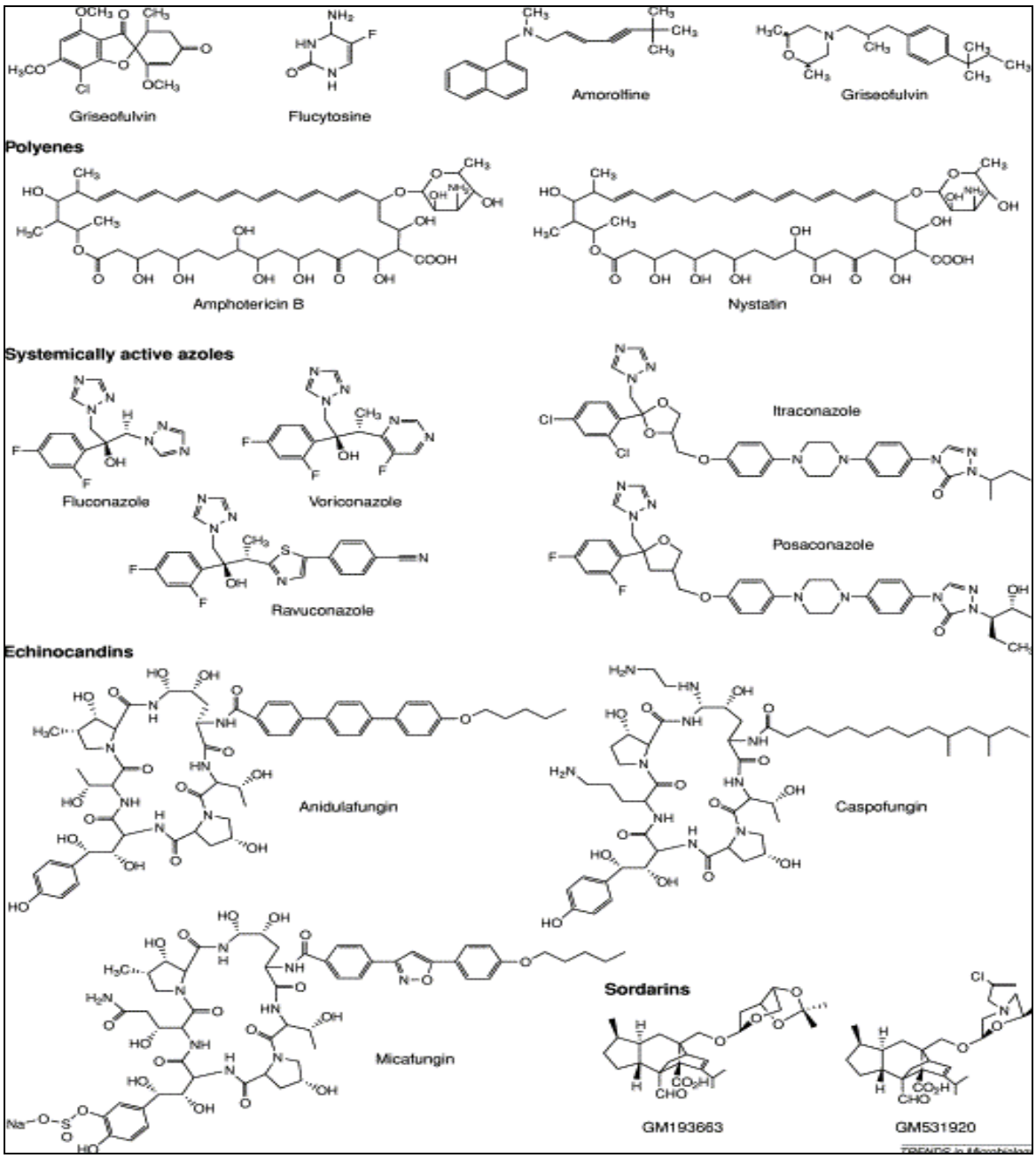
Voriconazole, which is the first approved and so far the most fully characterised of the three new triazoles, enjoys a very broad spectrum of target fungal species and, like itraconazole, is even fungicidal against some isolates of filamentous species.[25] Furthermore, it shows activity against the hard-to-treat *Fusarium* and *Scedosporium* infections. However, there is a long list of other drugs with which voriconazole interacts (similar to the list for itraconazole), including some immune suppressants, benzodiazepines, prednisolone, digoxin and other drugs in common use, which can cause problems for physicians treating seriously ill patients who receive multiple medications.[26]

Posaconazole also acts against a broad spectrum of susceptible fungi, and shows

interestingly promising efficacy against coccidioidomycosis in preclinical studies.[27] Ravuconazole, with an identical pharmacophore but a longer side-chain than voriconazole, stands out for its unusually long plasma half-life in humans (Olsen, S.J. *etal.*, Abstracts of the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 2000, page 22).

Other sterol synthesis inhibitors: Allylamines and Morpholines

The ergosterol biosynthetic pathway is the target for two other classes of antifungal agent. The allylamines, notably terbinafine (Fig. 2), inhibit squalene



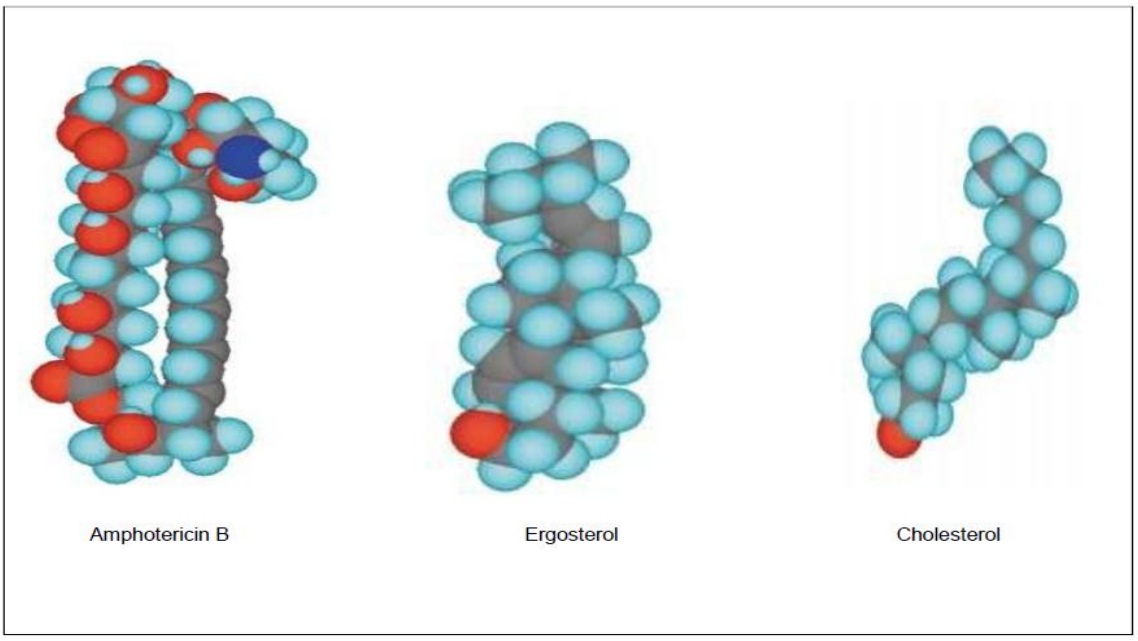


Fig. 3: The polyene antifungal agent, amphotericin B, ergosterol and cholesterol, visualised in three dimensions. Ergosterol, the sterol found in fungal cell membranes, retains a cylindrical shape in all rotations and binds better to the hydrophobic (right-hand) side of the amphotericin B molecule than does cholesterol, with its sigmoid structure. Cholesterol is the membrane sterol found in mammalian cells; the differential binding affinity of amphotericin B for the two sterols is the basis of its selective antifungal action.

Epoxidase, an early step in the pathway, with fungicidal consequences in susceptible species. These include many filamentous fungi but few pathogenic yeasts. Although terbinafine is not approved for treatment of visceral mycoses, there is interest in the possibility of combining terbinafine with other ergosterol synthesis inhibitors to achieve synergistic inhibitory effects. The phenylmorpholine class, of which amorolfine (Fig. 2) is the sole representative in human medicine, affects two targets late in the ergosterol pathway: Erg24p, the D^{14} reductase enzyme, and Erg2p, the D^{8}-D^{7} isomerase enzyme. Amorolfine can be used only for topical treatment of superficial mycoses, and neither of its targets has attracted recent research interest.

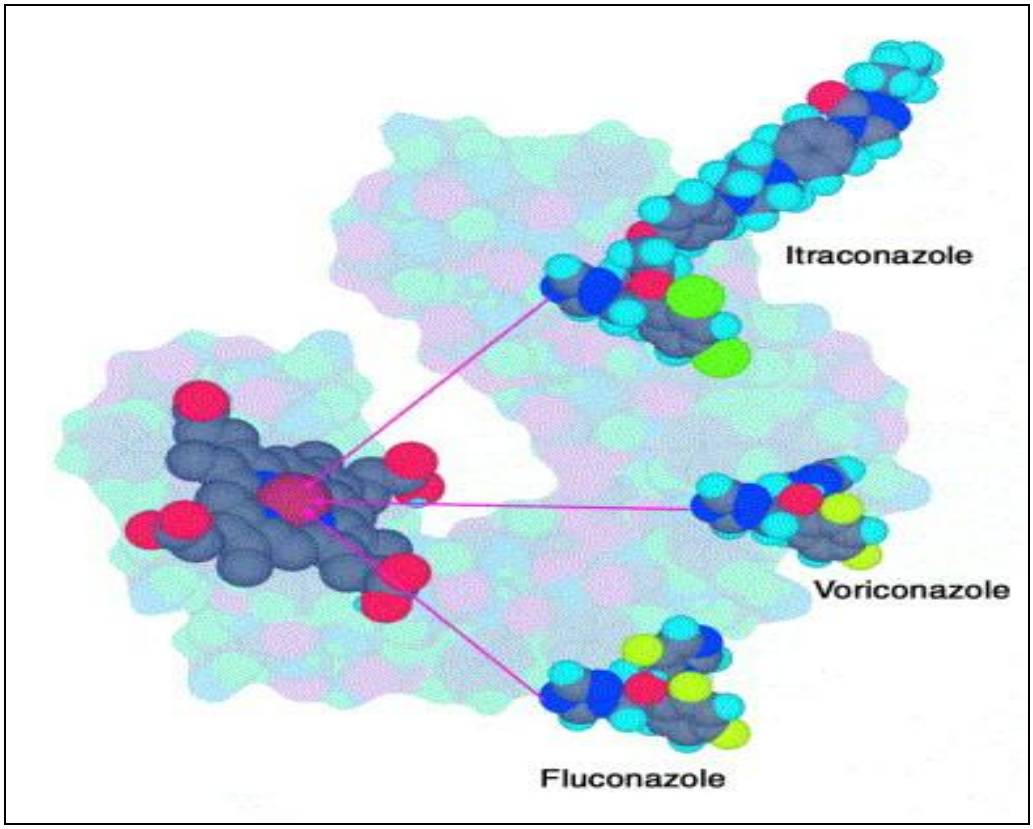


Fig. 4: Cartoon giving an approximate impression of the protoporphyrin moiety located at the active site of Erg11p (Cyp51p), the cytochrome P450 enzyme target for imidazole and triazole antifungals. Three triazole antifungals, itraconazole (top), fluconazole (centre) and voriconazole (bottom) are shown in comparable orientations. Arrows link the azole nitrogen atom to the iron atom where the azoles bind to block the active site of the enzyme. The different side chains attached to the common azole pharmacophore in the three examples shown will obviously bind differently to the surrounding regions of the whole P450 protein. See Refs[22] and[23] for precise molecular details on binding interactions between azoles and Cyp51p.

Echinocandins

The echinocandins are fungal secondary metabolites comprising a cyclic hexapeptide core with a lipid side chain responsible for antifungal activity (Fig. 2) Antifungal activity in the prototypes, echinocandin B and aculeacin A, was discovered by random screening in the 1970s. A modified form of echinocandin B, cilofungin, was developed to the point of phase 2 trials, but was abandoned when its formulation showed toxicity to patients. In the late 1990s, three echinocandin-class compounds, anidula- fungin, caspofungin and micafungin (Figs 2,5), all

entered clinical development.[28] The three-dimensional configur- ation of all three molecules is similar.

A central, common core bears a long, 'gun-barrel'-like side chain known to be a determinant of the spectrum of susceptible species (Fig. 5), and a hydroxylated side chain that appears opposite the 'gun barrel' in flat structural representations (Fig. 2) but is adjacent in energy-minimised 3-D structures (Fig. 5).

The target for the echinocandins is the complex of proteins responsible for synthesis of cell wall β -1,3 glucan polysaccharides (Fig. 5). In S. cerevisiae, where the enzyme complex has been best studied, two proteins (Fks1p and Fks2p) are regulated by a GTP-binding peptide, Rho1p, and by elements of the calcineurin pathway.[29] Homologues for all three gene products have been found in C. albicans, but it seems that the Fks2p homologue is not expressed in growing cells.[28,29] Mechanistic details of glucan synthesis and its inhibition by echinocandins still remain obscure, largely because a membrane-associated protein complex is involved. There is no doubt that the component to which echinocandins bind is Fks1p, but their non-competitive inhibitory effects on glucan synthesis do not necessarily imply that Fks1p itself is the catalytic subunit, nor is it clear whether the echinocandin-binding site on Fks1p is external or internal to the cell membrane. Caspofungin was approved by the US Federal Drug Agency in 2001 for therapy of aspergillosis unresponsive to other agents, and approval is likely to be obtained for treatment of disseminated Candida infections in 2003. Among common fungal pathogens, only Cryptococcus neoformans is excluded from the echinocandin spectrum; they also lack activity against emerging pathogens, such as *Fusarium* spp. and *Scedosporium* spp.

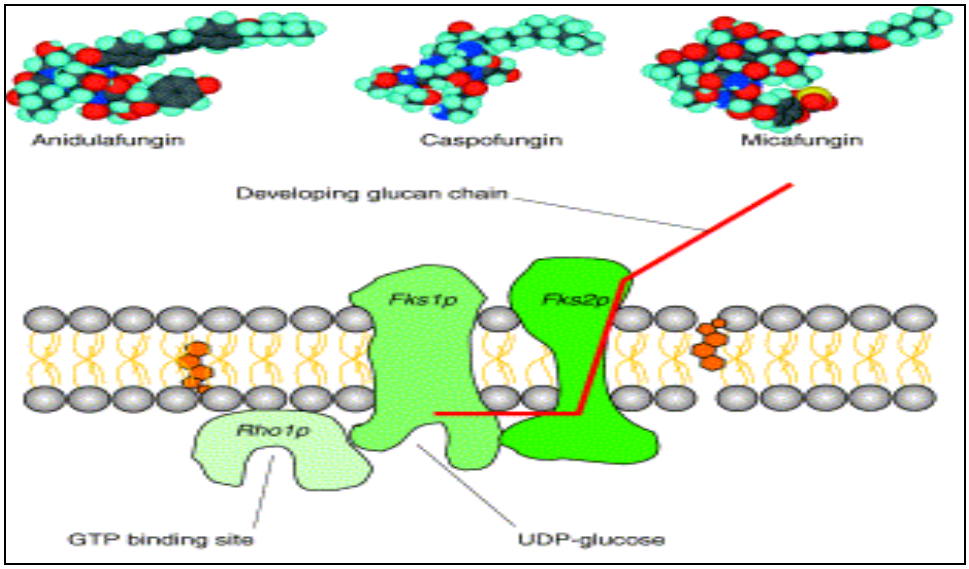


Fig. 5: Cartoon depicting the transmembrane complex of two proteins, Fks1p and Fks2p, involved in synthesis of b-1:3 glucan in the cell walls of Saccharomyces cerevisiae. Activity of the complex is regulated by GTP- binding peptide, Rholp. Fks2P is the target of the echinocandins, with the structures of the three agents now entering clinical use illustrated above. Evidence for Candida albicans suggests that the Fks2p homologue in this species might not be expressed in growing cells. Much remains unknown about the glucan synthase protein complex and the manner of interaction of echinocandins with the complex.

However, they are active against Pneumocystis jiroveci.[30] All of the three new echinocandins can only be administered by intravenous injection, which also places constraints on their use. Nevertheless, the echinocandin class rep- resents a particularly interesting advance in antifungal therapeutics because their target is a new one. Already, there are clear signs of interest from clinicians to explore combination therapy with echinocandins and azoles. The rationale is similar to that used to treat tuberculosis and HIV infection, in which effects on two or more disparate targets are a better guarantee of clinical efficacy than effects on a single target. So far, resistance to caspofungin has only appeared by mutation of the FKS1 gene in C. albicans. The drug is not exported by known fungal efflux pumps.

Sordarins

The sordarin antifungal class, although not developed for clinical use, merits mention among the new mechanisms of action. Sordarins (two examples of structures are shown in Fig. 2) inhibit protein synthesis by blocking the function of

fungal translation Elongation Factor 2 (EF2). The class was discovered by routine screening but was abandoned in the early 1970s.[7] Interest in sordarins was re-awakened as a result of a prospective screen for inhibitors of *C. albicans* protein synthesis *in vitro*, which pinpointed the nature of the sordarin antifungal effect. When refined experimentation revealed EF2 as the specific target of the sordarins,[31] the result engendered surprise because C. albicans EF2 displays more than 85% amino acid sequence identity to the human equivalent, and EF2 would never have emerged as a potential target from genomics-based screening. Different sordarin derivatives have different spectra of susceptible species, for reasons that are not yet clear. This might reflect problems of penetration of these agents into target fungi. Never- theless, their high specificity for the fungal target and the relative ease with which new sordarin variants can be synthesised holds promise for positive future developments with this series.

Antifungal agents and the future

This review has summarised all known types of clinically used antifungal agents, their molecular targets and modes of action. Over the 50 or more years in which specific antifungal agents have been discovered, the clinical needs for the agents have altered considerably and continuously. Superficial mycoses remain relatively easy to treat, with a large range of antimycotic products now available over the counter for the purpose. The spectrum of disseminated mycoses in highly immunocompromised individuals has undergone many changes, with the advent and subsequent therapeutic suppression of HIV-associated problems, and with changes in the nature of management of all types of serious illness. It is obvious that, at present, the prime requirement is for agents with a broad spectrum of susceptible species. The six newest agents in development go a long way to this requirement, particularly the three new triazoles. A consequence of the huge investment in genome-centred approaches is that antifungal research is currently rich in validated targets for new agents, but it is likely to be many years before clinically useful inhibitors of these targets are discovered and developed. Well-understood, fungal-specific targets, such as chitin synthesis, have so far eluded chemical exploitation, emphasising the gulf between target discovery and drug development. As with all antimicrobial agents, the spectre of emergence of resistance is a real one, and appropriate vigilance in the arms race between fungi and humans means that new targets and new inhibitors will continue to be required for effective antifungal therapy in the future.

ACKNOWLEDGEMENTS

We thank the British Society for Antimicrobial Chemotherapy, the Wellcome Trust and the Biotechnology and Biological Sciences Research Council for supporting related aspects of our research.

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