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MINIREVIEW

Antifungal Agents: Chemotherapeutic Targets and Immunologic Strategies

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INTRODUCTION

During the past two decades the frequencies and types of life-threatening fungal infections have increased dramatically in immunocompromised patients (7, 220, 232, 282). Several factors have contributed to this rise: the expansion of severely ill and/or immunocompromised patient populations with human immunodeficiency virus (HIV) infection, with chemotherapy-induced neutropenia, and receiving organ transplant-associated immunosuppressive therapy; more invasive medical procedures, such as extensive surgery and the use of prosthetic devices and vascular catheters; treatment with broad-spectrum antibiotics or glucocorticosteroids; parenteral nutrition; and peritoneal dialysis or hemodialysis (25, 63, 66). The major opportunistic pathogen has been *Candida albicans* (17, 25, 142); however, the frequency of non-*C. albicans Candida* species is increasing (232, 287). Invasive pulmonary aspergillosis is a leading cause of attributable mortality in bone marrow transplant recipients (209). HIV-infected patients are particularly susceptible to mucosal candidiasis, cryptococcal meningitis, disseminated histoplasmosis, and coccidioidomycosis (5, 66, 294), while *Pneumocystis carinii* pneumonia is a leading cause of death in HIV-infected patients in North America and Europe (121). *P. carinii* was considered, until recently, a protozoal parasite on the basis of its resistance to classical antifungal agents. However, it has been reclassified as being most closely related to ascomycetous fungi on the basis of rRNA and β-tubulin homologies, the presence of the typical fungal cell wall polymers glucan and chitin, and separate dihydrofolate reductase and thymidylate synthase enzymes (in protozoa, both activities reside on a single protein) (74, 163).

Treatment of invasive mycoses is complicated by problems in diagnosis (285) and susceptibility testing (8, 79, 90, 230) of fungi. Opportunistic fungal infections are often treated empirically in profoundly neutropenic patients when there is fever of unknown origin refractory to broad-spectrum antibacterial agents (233, 266, 284). Treatment of deeply invasive fungal infections has consistently lagged behind bacterial chemotherapy (27, 178). Amphotericin B, still the "gold standard" for the treatment of most severe invasive fungal infections, was discovered in 1956 (102). One reason for the slow progress is that, like mammalian cells, fungi are eukaryotes, and thus, agents that inhibit protein, RNA, or DNA biosynthesis have greater potential for toxicity. A second reason is that, until recently, the incidence of life-threatening fungal infections was perceived as being too low to warrant aggressive research by the pharmaceutical industry. In the past decade, however, there has been a major expansion in the number of antifungal drugs available (99). Nevertheless, there are still major weaknesses in their spectra, potencies, safety, and pharmacokinetic properties. This minireview briefly discusses the antifungal agents currently in clinical use. It then considers the use of promising new biochemical targets in fungi as well as host-based, immunological approaches as evolving strategies for antifungal therapy.

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ANTIFUNGAL AGENTS IN CLINICAL USE

Four major classes of systemic antifungal compounds are currently in clinical use: the polyene antibiotics, the azole derivatives, the allylamines and thiocarbamates, and the fluoropyrimidines (Fig. 1 and Table 1). The first three are targeted against ergosterol, the major fungal sterol in the plasma membrane. They are thus ineffective against *P. carinii* (20, 21), which has cholesterol instead of ergosterol, possibly acquired from its mammalian host (134).

Polyenes. The polyene antibiotics, produced by *Streptomyces* species, are fungicidal and have the broadest spectrum of activity of any clinically useful antifungal compound (34, 92). They complex with ergosterol in the plasma membrane, causing membrane disruption, increased permeability, leakage of cytoplasmic contents, and cell death (32). Recent evidence suggests that they also cause oxidative damage, which may contribute to their fungicidal activity (34). The clinically useful polyenes, amphotericin B (Fig. 1), nystatin, and natamycin (pimaricin), have a higher affinity for ergosterol than its mammalian counterpart, cholesterol, and are thus less toxic to mammalian cells (290). The acute and chronic side effects of amphotericin B may be reduced in newer formulations, such as liposomes (1, 84, 167, 234), lipid complexes (181), and colloidal dispersions (107, 213). Derivatization of amphotericin B has also been attempted with the aim of reducing toxicity (168).

Microbiological resistance to polyenes is associated with altered membrane lipids, particularly sterols (138, 221, 295). Other mechanisms of resistance may involve altered phospholipids and increased catalase activity with decreased susceptibility to oxidative damage (262). Amphotericin B resistance, although still rare in *Candida* species other than *Candida lusitaniae* (68, 89, 218, 224), is common in emerging pathogens, such as *Trichosporon* and *Fusarium* species (6, 286).

Azoles. The azole derivatives, discovered in the late 1960s, are totally synthetic and are the most rapidly expanding group of antifungal compounds (86, 277) (Fig. 1 and Table 1). They are classified as imidazoles or triazoles on the basis of whether they have two or three nitrogens in the five-membered azole ring. Depending on the particular compound, azole antifungal

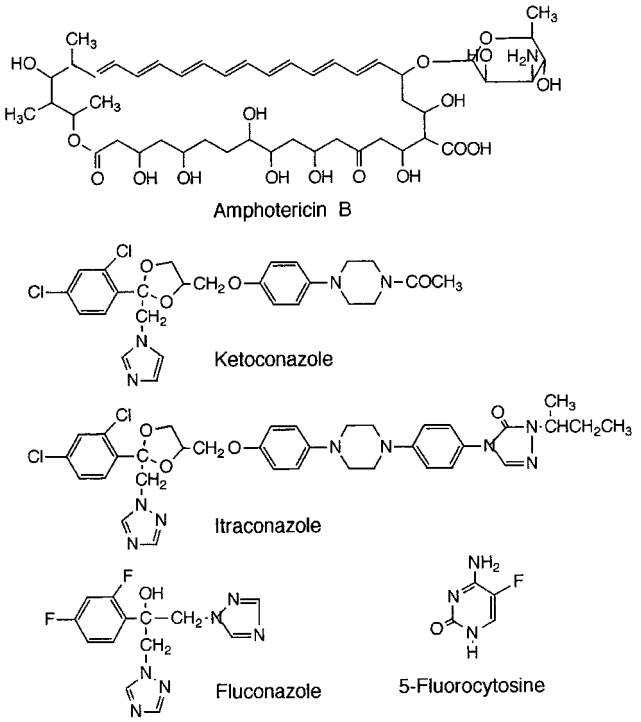

FIG. 1. Structures of some antifungal agents used systemically.

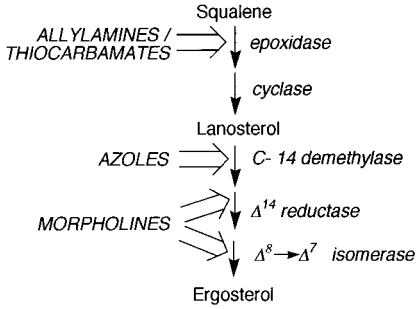

FIG. 2. Ergosterol synthesis pathway, showing sites of inhibition of different antifungal agents.

agent have fungistatic, broad-spectrum activity that includes most yeasts and filamentous fungi. They act primarily on ergosterol biosynthesis at the C-14 demethylation stage, a three-step, oxidative reaction catalyzed by the cytochrome P-450 enzyme 14α-sterol demethylase ($P\text{-}450_{DM}$) (Fig. 2). Azole antifungal agents form, through their azole ring, a stoichiometric complex with the heme iron of $P\text{-}450_{DM}$, which can be measured spectrophotometrically, by the red shift of the Soret band of the heme from 417 to 447 nm (113). The resulting ergosterol depletion and the accumulation of lanosterol and other 14-methylated sterols interfere with the "bulk" functions of ergosterol as a membrane component (212); they disrupt the structure of the plasma membrane, making it more vulnerable to further damage, and alter the activity of several membrane-bound enzymes, such as those associated with nutrient transport and chitin synthesis (19, 96, 277). Severe (>99%) ergosterol depletion may additionally interfere with the hormone-like ("sparking") functions of ergosterol, affecting cell growth and proliferation (194, 212).

The older, primarily topical, imidazoles also interact and damage the cell membrane directly at higher concentrations and are fungicidal and toxic (86, 97, 264). Azole antifungal agents are generally free of serious toxicity; however, rare cases of fatal hepatotoxicity have been reported, particularly with ketoconazole. Because of their ability to inhibit the cytochrome P-450-dependent enzymes involved in the biosynthesis of steroid hormones in mammalian cells, azole antifungal agents may produce endocrine-related side effects, such as depletion of testosterone and glucocorticoids, resulting in gynecomastia and adrenal insufficiency, respectively (18, 106). Nonazole inhibitors of $P\text{-}450_{DM}$ have been reported recently (10, 44), but apparently, they have not been further pursued.

Resistance to azoles, particularly fluconazole, is emerging in *C. albicans*, the most common cause of mucosal candidiasis in HIV-infected patients, after long-term suppressive therapy (114, 129, 182). Resistance to fluconazole in other *Candida* species (198, 231) and in *Crytococcus neoformans* (53) has also

TABLE 1. Mechanisms of action of some antifungal agents used clinically

Class and compound	Route of administration	Mechanism of action
Polyenes		Interact with ergosterol, thereby disrupting the cytoplasmic membrane
Amphotericin B	Systemic	
Nystatin	Topical	
Azoles		Interact with cytochrome P-450; inhibit C-14 demethylation of lanosterol, thereby causing ergosterol depletion and accumulation of aberrant sterols in the membrane
Miconazole	Topical	
Ketoconazole	Systemic	
Itraconazole	Systemic	
Fluconazole	Systemic	
Allylamines and thiocarbamates		Inhibit oxidosqualene cyclase, thereby causing ergosterol depletion and accumulation of squalene oxides in the membrane
Naftifine	Topical	
Terbinafine	Systemic	
Tolnaftate	Topical	
Morpholine, amorolfine	Topical	Inhibit sterol Δ^{14} reductase and Δ^7-Δ^8 isomerase; only the former is essential
Nucleoside analog, 5-FC	Systemic	Is deaminated to 5-FU, which (i) is converted to triphosphate and incorporated into RNA, thereby causing miscoding, and (ii) is converted to deoxynucleoside which inhibits thymidylate synthase and thereby DNA synthesis

been reported. Resistance is due to decreased membrane permeability, resulting from changes in membrane sterols (115), active efflux, altered or overproduced target enzyme (278, 279), and compensatory mutations in $\Delta^{5,6}$ desaturase (139, 291). Suppressor mutations in $\Delta^{5,6}$ desaturase also occur after sterol 14α-sterol demethylase gene disruption (137), suggesting that accumulation of 14-methyl-3,6-diol, rather than accumulation of 14-methylated sterols in general or ergosterol depletion, is the cause of growth inhibition. Transport-associated resistance affects different azoles differently; for example, fluconazole resistance may not affect itraconazole (279) or ketoconazole (129). The extent and degree of azole resistance is difficult to assess because of limitations in correlating susceptibility data for ergosterol synthesis inhibitors with clinical response results (197, 230).

Allylamines and thiocarbamates. There are two allylamine antifungal agents in clinical use, naftifine and terbinafine, and one thiocarbamate, tolnaftate (245) (Table 1). All three are reversible, noncompetitive inhibitors of squalene epoxidase (217, 245, 246), an enzyme which, together with (2,3)-oxidosqualene cyclase, is responsible for the cyclization of squalene to lanosterol (Fig. 2). The resulting ergosterol depletion and squalene accumulation affect membrane structure and function, such as nutrient uptake (95, 245). The benzylamine butenafine has a mechanism of action similar to that of allylamines and, in addition, causes direct membrane effects in ergosterol-depleted cells (127). Squalene epoxidase has also been considered an anticholesteremic target, and an allylamine inhibitor, NB-598, with specificity for the mammalian enzyme has been reported (116).

Pharmacokinetic properties confine the clinical efficacies of allylamines and thiocarbamates largely to dermatophytes, even though allylamines have broad-spectrum in vitro activity. Resistance has not been reported for human pathogens, although it has been described in the corn pathogen *Ustilago maydis* (206), where it involves decreased affinity of the target enzyme and decreased drug accumulation in the fungal cell.

Morpholines. The morpholines, discovered in the 1970s, are totally synthetic and, with the exception of amorolfine, which is used in the topical treatment of nail infections, are agricultural fungicides (15). They also act on the ergosterol pathway, inhibiting two reactions, Δ^{14} reductase and Δ^{7}-Δ^{8} isomerase (15, 223) (Fig. 2). Morpholines are analogs of the carbocationic high-energy intermediates involved in both reactions (15, 227). The result, at least in *Saccharomyces cerevisiae*, is the accumulation of 24-methylene ignosterol in the plasma membrane and, hence, altered membrane properties (170, 263). The antifungal activity of morpholines is probably due solely to sterol Δ^{14} reductase inhibition (136); Δ^{14} reductase is essential (171), while Δ^{7}-Δ^{8} isomerase is not (11). Interestingly, the morpholine fenpropimorph also inhibits cholesterol biosynthesis in mammalian cells, but it affects the demethylation of lanosterol rather than sterol reductases or isomerases (56). Resistance to amorolfine has not been reported for human pathogens. Toxicity considerations have precluded the use of morpholines systemically.

Flucytosine. The fluoropyrimidine flucytosine (5-FC) (Fig. 1 and Table 1) has a limited spectrum of activity and is mainly used in combination with amphotericin B in cryptococcal meningitis, as well as in cases of disseminated candidiasis (85). It is also used as a single agent for the treatment of chromoblastomycosis and mycoses in the urinary tract, where 5-FC achieves concentrations up to 100-fold higher than those in serum (82, 281). Recent work shows encouraging activity of 5-FC in combination with fluconazole against cryptococcal meningitis (157).

5-FC is taken up into fungal cells by a cytosine permease, deaminated to 5-fluorouracil (5-FU), converted to the nucleoside triphosphate, and incorporated into RNA, where it causes miscoding (67, 223, 252). In addition, 5-FU is converted to deoxynucleoside, which inhibits thymidylate synthase and thereby DNA biosynthesis. 5-FC is relatively nontoxic to mammalian cells because of the absence or very low level of activity of cytosine deaminase. 5-FU, on the other hand, is a potent and widely used anticancer agent.

Emergence of resistance to 5-FC is common when the compound is used alone and can result from mutations in any of the enzymes necessary for 5-FC action, particularly uracil phosphoribosyltransferase (81, 141). The lack of a parenteral formulation of 5-FC in the United States has been associated with toxicity, stemming from the conversion of oral 5-FC to 5-FU by intestinal bacteria. Toxicity is exacerbated by amphotericin B-induced renal insufficiency (281).

NEW TARGETS FOR ANTIFUNGAL AGENTS

New antibiotics originate from the random or target-based screening of microbial products and synthetic compounds and from rational drug design. Different strategies have been used in the screens to select against known antibiotics, such as polyenes (80), and to target specific fungal structures or functions with no mammalian counterpart. Rational drug design is limited to well-characterized targets and mechanistically understood reactions, in which structure optimization, including computer-aided modeling, is feasible.

Fungal cell wall. The fungal cell wall, a structure essential to fungi and lacking in mammalian cells, is an obvious target for antifungal agents (98, 104, 105). Its major macromolecular components are chitin, β-glucan, and mannoproteins (39, 41, 147). Chitin (chitosan in some fungi) and β-glucan fibrils form the scaffolding responsible for the strength and shape of the wall, while mannoproteins are interstitial components, responsible for the wall's porosity, antigenicity, and, in *C. albicans*, adhesion (39, 43).

(i) Chitin. Chitin is a linear homopolymer of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) residues. It is synthesized on the cytoplasmic surface of the plasma membrane, extruded perpendicularly to the cell surface as microfibrils, and crystallized outside the cell through extensive hydrogen bonding as α-chitin (the poly-GlcNAc chains run antiparallel). The polymerization of GlcNAc is catalyzed by chitin synthases, membrane-bound enzymes found in cell homogenates largely as zymogens (36, 94). There are three chitin synthases (Chs) in *S. cerevisiae* and *C. albicans* (13, 253, 265). In the former organism, the major in vitro chitin synthase (Chs1) is a nonessential, repair enzyme (42, 205, 260), while the other two are involved in septum formation (Chs2) and cell wall maturation and bud ring formation (Chs3) (253). Each Chs isoenzyme can be assayed individually in the absence of the other two, by making mutants, or by the inhibition of the other two, by changing the pH and the divalent cations present (51).

Chitin synthesis is inhibited competitively by polyoxins and nikkomycins, nucleoside-peptide antibiotics produced by streptomycetes. They act as analogs of the substrate UDP-GlcNAc, inhibiting chitin synthase with K_is of 0.1 to 1 μM (62, 125). The effect on the fungus is inhibition of septation and osmotic lysis (24). Different isozymes of chitin synthase may be inhibited to different degrees; in *S. cerevisiae*, Chs1 and Chs3 are more susceptible than Chs2 to nikkomycin derivatives (38, 93). The nucleoside-peptide inhibitors are taken up by a dipeptide permease, and thus, peptides in body fluids antagonize their transport. *C. albicans* and other medically important fungi are resistant to polyoxins owing to their poor transport across the cell

membrane (176, 297). Bypassing peptide transport is an obvious goal, and appropriate polyoxin derivatives have been synthesized (101). Another hopeful development is the observed synergy between chitin synthase inhibitors and glucan (110) or ergosterol (111) synthesis inhibitors.

An essential component in the maintenance of cell wall plasticity during fungal growth and proliferation is chitinase (37); disruption of its structural gene in *S. cerevisiae* results in cell clumping and failure of the cells to separate after cell division (153). A similar effect is produced by demethylallosamidine, a specific inhibitor of chitinase (247). There are three chitinase genes in *C. albicans* (*CHT1*, *CHT2*, *CHT3*), two of which (*CHT2*, *CHT3*) encode for proteins with a predicted size of 60 kDa and which are preferentially expressed in the yeast phase (177). Although allosamidine lacks antifungal activity, further work on chitinases may be necessary to determine whether they are potential antifungal targets.

(ii) Glucan. Glucans are glucose homopolymers, arranged as long (≥60 units [255]) coiling chains of β-(1,3)-linked residues with occasional side chains involving β-(1,6)-linkages (108). The enzyme catalyzing the polymerization, β-(1,3)-glucan synthase, has at least two functional components: a catalytic component, which acts on the UDP-glucose substrate, and a regulatory component, which binds GTP (40, 135, 183). The latter is a 20-kDa protein that is regulated by a GTPase-activating protein and that may link glucan synthesis to the cell cycle via a phosphorylation-dephosphorylation relay system. Accordingly, calcineurin, a protein phosphatase, has been shown to be involved in the regulation of glucan synthase activity (71). There are two glucan synthase systems in *S. cerevisiae*, and most likely in pathogenic fungi as well (98).

β-(1,3)-Glucan synthase is inhibited noncompetitively by papulacandins and echinocandins, natural products discovered in the 1970s (272). Papulacandins are fatty acid derivatives of the disaccharide β-(1,4)-galactosylglucose (14), while echinocandins are fatty acid derivatives of cyclic hexapeptides. The K_i for cilofungin, an echinocandin derivative, is 2.5 μM for the enzyme from *C. albicans* (87, 270) and 0.2 μM for the enzyme from *Aspergillus fumigatus* (22, 23). Its in vitro activity against *A. fumigatus* is best measured by its effects on morphology rather than growth inhibition in conventional broth microdilution assays (154). Papulacandins and echinocandins may be bound to different sites on glucan synthase, since some, although not all, yeast strains resistant to the echinocandins are still susceptible to papulacandins (72). Interestingly, echinocandin resistance may result in increased susceptibility to nikkomycin (77). Papulacandins are no longer being pursued as antifungal agents since their in vitro activity is limited to *Candida* species and, most importantly, does not translate to in vivo activity (98). Echinocandins, on the other hand, have fungicidal activity both in vitro and in animal models (48, 272, 283). It has been suggested that the limited spectrum of echinocandins and other β-(1,3)-glucan synthase inhibitors may be due in part to their interaction with the fungal membranes (150, 151). Echinocandins have been chemically modified to produce semisynthetic analogs with improved pharmacological properties. Cilofungin (61) reached phase II clinical trials, but it was abandoned because of toxicity associated with the formulation vehicle (the compound is extremely hydrophobic). Two water-soluble semisynthetic derivatives, one (LY-303,366) of echinocandin B (58) and the other (L-733,560) of pneumocandin A_0 (33, 251) (Fig. 3), have promising in vitro and in vivo activities against *Candida* species, *P. carinii*, and other fungi and are currently in clinical development. In vitro β-(1,3)-glucan synthase assays based on *C. albicans*, *A. fumigatus*, and *Neurospora crassa* have been developed for use in high-throughput screens (268).

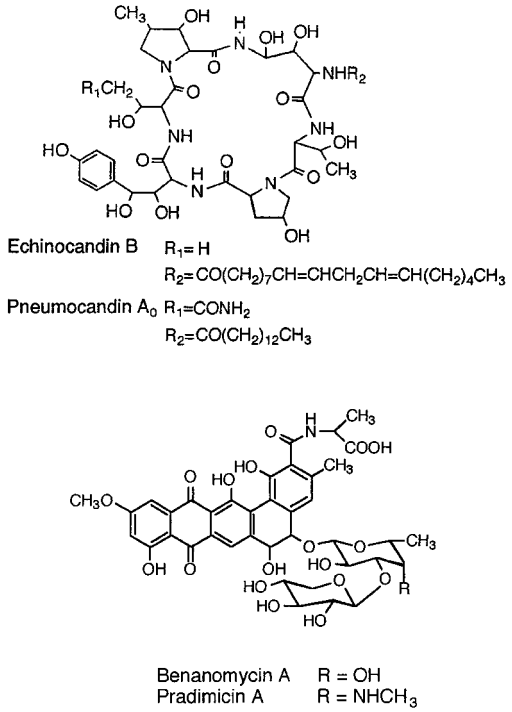

FIG. 3. Structures of some promising antifungal prototypes.

(iii) Mannoproteins. The interstitial cell wall components known as mannoproteins are involved in the mechanism of action of benanomicins and pradimicins, benzonaphthacene quinones conjugated with a D-amino acid and a disaccharide side chain (203, 269) (Fig. 3). Their mechanism of antifungal action involves initial calcium-dependent complexing of their free carboxyl group with the saccharide portion of cell surface mannoproteins (250, 273). They then act primarily on the membrane, causing leakage of intracellular potassium. Pradimicins are active in animal models of cryptococcosis, candidiasis, and aspergillosis, with potencies intermediate between that of ketoconazole and that of amphotericin B (204). Water-soluble derivatives are under development (202).

Plasma membrane. Like its mammalian counterpart, the fungal plasma membrane contains sterols and phospholipids as its major lipid components and functions as a permeability barrier, a conduit for the transport of small molecules and signals, and a matrix for proteins. A key factor for its functions is its fluidity, determined by its lipid composition. Anchored to or embedded into the membrane are proteins, whose co- or posttranslational modification may also yield therapeutic targets (see section on lipoproteins below).

(i) Ergosterol synthesis. Most rational drug design efforts have focused on fungal sterols, since they are structurally distinct from their mammalian counterparts and their biosynthesis has been studied extensively (179, 180, 200, 293). New targets actively pursued in the ergosterol biosynthesis pathway are oxidosqualene cyclase and Δ^{24} methyltransferase (131, 200). The latter has no mammalian counterpart (cholesterol is not methylated at C-24) and is thus a particularly attractive target. With both oxidosqualene cyclase and Δ^{24} methyltransferase, research has focused on designing high-energy intermediates or transition state analogs (12, 70, 271, 299). Inhibi-

tors of the postsqualene steps need not be selective unless they cause the accumulation of compounds toxic to mammalian cells, since mammalian cells can take up dietary cholesterol via the low-density lipoprotein pathway (35), while fungi have no uptake system for exogenous sterols.

Targets in the presqualene segment of the ergosterol pathway are less attractive, not so much because the reactions are the same in fungi and mammalian cells (235) (the targets for the clinically useful allylamines and azoles are also present in mammalian cells) but because inhibitors may affect the synthesis of other essential terpenoids, such as coenzyme Q, farnesyl pyrophosphate (involved in protein farnesylation), and dolichol, among others (103). Nevertheless, inhibitors of hydroxymethylglutaryl coenzyme A (CoA) and mevalonic acid synthesis are potential or commercial cholesterol-lowering agents, suggesting that enzymes at the branch points of the sterol pathway may have different affinities for substrates, sparing critical but quantitative minor pathways during depletion of key intermediates (200). Inhibitors of squalene synthase, the squalestatins and zaragosic acids, have also been reported (50, 60), although none has promising antifungal activity, probably because of membrane permeability constraints.

(ii) Phospholipid synthesis. Fungal phospholipids are synthesized by pathways that are basically similar to their mammalian counterparts (46, 276). The only known difference is in the biosynthesis of phosphatidylserine, which is synthesized from CDP-diacylglycerol in fungi but from phosphatidylethanolamine and serine in mammalian cells. As with other envelope components, most studies have centered on *S. cerevisiae* (46), although there are recent reports on the phospholipids of *C. albicans* and other fungal pathogens (145, 169). There are also assays for phospholipid synthesis which lend themselves to high-throughput screens (146).

(iii) Sphingolipid synthesis. Sphingolipids are essential membrane components of both mammalian cells and fungi and are localized primarily on the outer leaflet of the fungal cytoplasmic membrane (214). The first step in their biosynthesis is the condensation of a fatty acyl CoA, usually palmitoyl CoA, with serine in a reaction catalyzed by serine palmitoyltransferase. Sphingofungins inhibit this reaction at nanomolar concentrations (300) and are broad-spectrum antifungal agents (117, 280). Serine palmitoyltransferase is present in both mammalian and fungal cells and is thus unlikely to be a selective target. However, other enzymes of the sphingolipid pathway may be different, since fungal sphingolipids differ from their mammalian counterparts (160).

(iv) Proton ATPases. The plasma membrane H^+ ATPase is an integral membrane protein belonging to the P-type class of ion-translocating ATPases (185). It is an abundant, essential enzyme involved in the maintenance of electrochemical proton gradients and the regulation of intracellular pH. Plasma membrane H^+ ATPases are known in sufficient molecular detail to be targets for rational drug design, provided that there are exploitable differences between the fungal and mammalian enzymes. The vesicular H^+ ATPase (V-ATPase) is inhibited specifically by folimycin, an antifungal agent structurally related to bafilomycins (191). These compounds block acidification of intracellular organelles and thereby affect intracellular protein trafficking and translocation to the cell surface. As with the plasma membrane ATPase, the selectivity between fungal and mammalian enzymes is unclear at present.

(v) Efflux pumps. Proteins with pump function have been reported in *Candida* species (16, 29, 249) and may be responsible for the observed broad resistance of these organisms to azoles and perhaps to other antifungal agents. Although they are functionally similar to multidrug resistance proteins reported in bacteria, parasites, and mammalian cells, their encoding genes may be different from the multiple-drug resistance genes of the P-glycoprotein family (112). The lack of structural similarity to mammalian P glycoproteins may be exploited in designing specific inhibitors of the fungal efflux pumps. Recent studies have shown that deletion of a multidrug resistance gene in *C. albicans* results in a marked attenuation of virulence of the organism (28), suggesting the possibility of simultaneously potentiating antifungal activity and attenuating virulence.

DNA and topoisomerases. Topoisomerases I and II control the topological state of DNA so that it can undergo replication, transcription, repair, and chromosomal segregation (164, 207). Topoisomerase II is a universally essential enzyme (292). Mammalian topoisomerase II is the target of the widely used anticancer agents anthracyclines and epipodophylotoxins; its prokaryotic counterpart, DNA gyrase, is the target of the highly successful quinolones. Topoisomerase I, although not an essential enzyme, is also a lethal target by virtue of the fact that it forms a cleavage complex with DNA and inhibitors, such as camptothecin (derivatives are in clinical trials as anticancer agents), thereby derailing the oncoming replication fork (164). The success of topoisomerase inhibitors in antibacterial and anticancer chemotherapy has underscored the potential of fungal topoisomerases as drug targets (256). While it is not yet known whether fungal topoisomerase II has exploitable differences relative to its mammalian counterpart, recent studies suggest fungal topoisomerase I can be inhibited selectively (83).

Toxicity considerations would preclude DNA itself as an antifungal target, although it may be the target of the anti-pneumocystis drug pentamidine (132). Recent studies on related dicationic-substituted bisbenzimidazoles have shown that they bind to the minor groove of DNA, and some are selective inhibitors of topoisomerases I and II from *P. carinii* (75).

Protein synthesis. Both fungal and mammalian cells require two soluble protein factors, elongation factor 1 (EF-1α) and EF-2 for the polypeptide chain elongation reactions of protein synthesis (184). However, fungi require an additional factor, EF-3, which is absent from mammalian cells (133, 261). This 120- to 125-kDa protein (274) is present in most fungi, including *C. albicans* and *P. carinii* (55), and is essential for cell viability since disruption of its gene is lethal to the organism (55, 226). EF-3 has ATPase activity, is specifically required by the yeast 40S ribosomal subunit, and may be involved in the translocation of the growing peptide, although its exact function in the elongation cycle is unclear. A major drawback for rational drug design is the absence of known EF-3 inhibitors.

Fungi synthesize a small number of *N*-myristoylated proteins, the most prominent being 20-kDa ADP-ribosylation factors. Myristoylation involves the cotranslational transfer of myristate, a 14-carbon saturated fatty acid, from CoA to the amino-terminal glycine of proteins (128). The reaction is essential in *C. neoformans* (165) and other fungi (73) and is catalyzed by myristoyl CoA:protein *N* myristoyltransferase (NMT). An analog of myristic acid with an oxygen substituted for a methylene at position 4 inhibited NMT and had in vitro fungicidal activity (156). Since the peptide substrate specificities of fungal and mammalian NMTs are different (144, 166, 236), it may be possible to design specific inhibitors for the fungal enzyme.

Another target within the posttranslational modification of proteins is the enzyme involved in the transfer of glycophosphatidylinositol mannoproteins from their glycophosphatidylinositol membrane anchors to β-(1,3)-glucan (64). This reac-

tion has no mammalian counterpart and may thus be a particularly attractive target.

Intermediary metabolism. (i) Nucleic acids. The success of trimethoprim-sulfomethoxazole in treating *P. carinii* pneumonia has validated their sites of action in the folate pathway as drug targets for this organism (244), although not for other fungi. Phosphoribosylaminoimidazole carboxylase, an enzyme of the purine pathway, may be a target for *C. neoformans*, possibly because of the low levels of free adenine in cerebrospinal fluid (215). The *ADE2* gene encoding for this enzyme is different from its mammalian counterpart. However, it is not yet clear whether these differences translate into differences in protein structure that could permit the design of specific inhibitors.

(ii) Amino acids. The discovery of the amino acid analog cispentacin (152), an antifungal agent with excellent in vivo activity (201) and multiple cellular targets (45), raised the possibility of interfering with amino acid synthesis. Other amino acid analogs with antifungal activity are RI-331, which inhibits homoserine dehydrogenase (298), a particularly attractive target since it is absent from mammalian cells, and azoxybacillin, which inhibits the biosynthesis of sulfur-containing amino acids (9).

(iii) Polyamines. Ornithine decarboxylase, the rate-limiting enzyme in polyamine synthesis (267) and a favorite target in anticancer chemotherapy (175), may also be an antifungal target. Ornithine decarboxylase is inhibited by the substrate analogs α-hydrazino-γ-aminovaleric acid and α-difluoromethyl ornithine (eflornithine). The former is a competitive inhibitor, and the latter is an irreversible suicide inhibitor of the enzyme. Difluoromethyl ornithine has some antifungal activity (219). *P. carinii* ornithine decarboxylase is far less susceptible than the mammalian enzyme to difluoromethyl ornithine (248), suggesting differences in the active sites, although the available information is not yet sufficient for rational drug design.

Other cellular functions. (i) Microtubules. Microtubules are dynamic polymers of α- and β-tubulin dimers (52). They form a highly organized cellular skeleton in all eukaryotic cells, and their aggregation-disaggregation plays a key role in cell morphology and growth. Microtubule aggregation is inhibited by griseofulvin, the agricultural fungicide benomyl, and the anticancer drugs vincristine and vinblastine; disaggregation is inhibited by taxol (126). These agents interact with β-tubulin, a protein highly conserved in eukaryotes. Nevertheless, there appear to be differences between mammalian and fungal tubulins; for example, colchicine binds preferentially to mammalian tubulin (143).

(ii) Signal transduction and cell cycle. Yeasts, in particular *S. cerevisiae* and *Schizosaccharomyces pombe*, have been extensively used as models for intracellular signal transduction and the cell cycle of mammalian cells (109, 196, 216, 254, 257, 258). Kinases (57) and phosphatases (120) have been implicated in fungal cell morphogenesis and growth (78), including those of *C. albicans* (211). Mitogen-activated protein kinase-kinase (124) and mitogen-activated protein kinase homologs (159) exist in yeasts, although their exact functions, multiplicity, and suitability as antifungal targets are unknown. A yeast protein kinase C isozyme, PKC1, regulates cell wall synthesis (162); mutants defective in the PKC1 gene exhibit cell cycle-specific osmotic instability (161).

Virulence factors. Virulence factors in medically important fungi are best defined by molecular genetic approaches in which putative virulence genes are examined by deletion and transformation. Attenuating fungal mechanisms for tissue invasion is conceptually appealing, although the species specificity of virulence factors limits their potential as therapeutic targets. Nevertheless, several general principles can be outlined as a guide to developing virulence-targeted therapeutics.

With *C. albicans* as a model pathogen, infection proceeds through an initial adhesion phase; this is followed by invasion of epithelial or endothelial cells, depending on its portal of entry (140). *Candida* species are able to bind to fibronectin on epithelial and endothelial cell surfaces and to extracellular matrix proteins (fibrinogen, laminin, and collagen IV) in subepithelial and subendothelial tissues (26, 43, 59, 118, 148, 193). Blockade of adhesion molecules, especially with Arg-Gly-Asp-containing peptides, can reduce the burden of *C. albicans* in tissue (149). Studies of *Candida tropicalis* also demonstrate that such an attachment-blocking strategy may be effective in reducing adherence in vitro to epithelial cells (26). Another attachment-blocking strategy may be to modulate the expression of the fungal proteins involved in adhesion (119) or to block uptake of iron (187) in *C. albicans*. Blockade of adhesion with other cell surface components is another possible approach; for example, extracts of the *Candida* cell wall inhibit the adhesion of *C. albicans* to endothelial cells (76).

Germ tube formation and the penetration of cutaneous and nonkeratinizing squamous epithelial alimentary and vaginal mucosal surfaces by *C. albicans* is another potential virulence target. Subinhibitory concentrations of azole and polyene antifungal agents inhibit germ tube formation and modulate attachment to the epithelial surface (100). Proteinase production by *C. albicans* is an important factor in the penetration of keratinized epithelial cell surfaces (88, 228, 296). While inhibition of fungal proteinases is an appealing target, the role of proteinases in pathogenesis requires further clarification. Phospholipase may be another important virulence factor in disseminated candidiasis caused by *C. albicans* (122), and thus a potential target for drug development.

Additional potential virulence factors in other fungi with chemotherapeutic implications are the melanin biosynthetic pathways of *C. neoformans* and dematiaceous fungi, such as *Wangiella dermatitidis* (69, 289). *C. neoformans* elaborates melanin through the phenol oxidase pathway and *W. dermatitidis* elaborates melanin through the pentaketide pathway, both of which are amenable to inhibition by small molecules. Molecular genetic and functional studies have demonstrated the critical role of the capsule in *C. neoformans* virulence and have suggested that it may be an antifungal target (49, 155) (see also next section).

AUGMENTATION OF HOST IMMUNE RESPONSE

Effects of antifungal compounds. Currently used antifungal agents, although selective toward ergosterol, nevertheless also affect the function (polyenes) or biosynthesis (azoles, allylamines) of mammalian sterols, and thereby the host immune response (241). For example, amphotericin B increases the aggregation, adherence, and fungicidal action of polymorphonuclear leukocytes (30, 172, 208). Azoles inhibit chemotaxis of and superoxide production by polymorphonuclear leukocytes (130, 199), while allylamines have no effect on chemotaxis and only slightly increase fungicidal activity (275). It has been recently suggested that azoles, by inhibiting P-450$_{DM}$, may sensitize fungal cells to the oxidative metabolites produced by phagocytes (259).

Antifungal vaccines and antibodies. Active and passive immunization against pathogenic fungi is a promising strategy for prevention and treatment. *C. neoformans*, *Coccidioides immitis*, and *Histoplasma capsulatum* have the greatest potential as targets for vaccines in high-risk patients. Monoclonal antibodies to the capsular polysaccharide of *C. neoformans* can pro-

long survival and decrease the fungal burden in organs in experimental murine cryptococcosis (188, 189). These capsule-binding monoclonal antibodies also can enhance the effect of amphotericin B against *C. neoformans*, providing further support for considering combination therapy in humans (190). Active immunization with a tetanus toxoid conjugate of glucuronoxylomannan antigen elicits antibody formation (47, 65), which may be protective in animal models of disseminated cryptococcosis (192). This vaccine is in phase I clinical trials.

The striking increase in the incidence of coccidioidomycosis has given impetus to vaccine development against this potentially lethal pathogen. A vaccine consisting of formaldehyde-killed spherules of *C. immitis*, previously shown to provide protection against the development of lethal coccidioidomycosis in laboratory animals, was evaluated in more than 2,800 human volunteers (210). Although there was no significant difference in the frequency of coccidioidomycosis in that study, further work is being pursued to identify the purified immunogenic protective antigens (54, 91).

Histoplasmosis also lends itself to the development of vaccines. Recent studies indicate protection from disseminated histoplasmosis in murine models (3). While promising immunoprotective antigens have been identified, further work is necessary before clinical trials can be initiated.

Antibodies to *C. albicans* HSP90 may be protective against experimental disseminated candidiasis. Patients recovering from systemic candidiasis produce antibodies to HSP90, both to species-specific epitopes and, more commonly, to epitopes shared with human HSP90 (173, 174). One such anti-HSP90 antibody was protective in a mouse model of systemic candidiasis.

Recombinant human cytokines and immune reconstitution of effector cells. Augmentation of the host defense response, treatment of the underlying neoplastic disease, and resolution of the principal immune impairment are paramount to successful treatment of invasive mycoses in immunocompromised patients (222). The availability of recombinant cytokines offers hope for the prevention and treatment of invasive fungal infections (158).

In a large randomized, prospective, placebo-controlled trial in patients with chronic granulomatous disease, gamma interferon was found to significantly reduce the frequency of serious primary infections (123). Among patients with chronic granulomatous disease who were receiving gamma interferon three times per week, there was enhancement of damage to hyphae of *A. fumigatus* (229).

Administration of recombinant hematopoietic human cytokines, such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF), may decrease the duration of neutropenia (G-CSF, GM-CSF), increase the microbicidal functions of neutrophils, monocytes, and macrophages (G-CSF, GM-CSF, M-CSF), and possibly improve mucosal integrity (G-CSF, GM-CSF) following cytotoxic chemotherapy under experimental and clinical conditions (239, 288). Shortening the duration of neutropenia by use of recombinant human cytokines may permit more intensive cytotoxic chemotherapy. However, concerns have recently been expressed about possible damage to proliferating stem cells by repeated cytokine exposure, which could manifest as prolonged cytopenia after three or four cycles of chemotherapy (186). Nevertheless, decreasing the duration of granulocytopenia may decrease the frequency of invasive fungal infections. As the result of recombinant human cytokines and the administration of peripheral blood stem cells, autologous bone marrow transplantation with intensive cytotoxic chemotherapy is being conducted in some centers as an outpatient procedure.

When they are used alone, recombinant cytokines may be most effective in the prevention of fungal infections. When they are used for the treatment of established infections, they may be most effective in combination with an antifungal compound. Recent studies with GM-CSF suggest that this recombinant cytokine may be active as adjunctive therapy in the management of invasive fungal infections in cancer patients (31). A phase I clinical trial of recombinant human M-CSF in patients with invasive fungal infections demonstrated that M-CSF was well tolerated but did produce a transient dose-related thrombocytopenia (195). The study design did not permit evaluation of the potential antifungal properties of M-CSF versus optimal antifungal therapy alone, but such a trial is being planned.

The American Society for Clinical Oncology recently provided guidelines for patients and adults receiving G-CSF or GM-CSF. These cytokines should be used when the expected incidence of febrile neutropenia is $\geq 40\%$ in order to avoid infectious complications and to maintain dose intensity in subsequent treatment cycles. These cytokines were also recommended, in combination with autologous progenitor cell transplantation, after high-dose chemotherapy (4). We would further recommend the administration of recombinant G-CSF or GM-CSF to persistently neutropenic patients who have a proven invasive fungal infection and who are receiving an appropriate antifungal compound.

Recombinant hematopoietic cytokines also augment the functional activity of immunosuppressed nonneutropenic hosts against fungi. For example, G-CSF reverses the neutrophil dysfunction against *Aspergillus* hyphae in HIV-infected patients (238). G-CSF also reverses the corticosteroid-induced immunosuppression of neutrophils against *Aspergillus* hyphae (240), although the clinical significance of these observations is unclear.

While the gamma interferon T-helper 1 (T_H1) cytokine augments the host response to *Candida* species, the T_H2 type cytokines interleukin-4 (IL-4) and IL-10 may suppress the immunologic clearance of *Candida* species from tissues. Inhibition of their production or action may thus constitute possible immunologic strategies. As a case in point, administration of recombinant soluble IL-4 receptor to mice had a significant impact on the course of infection in experimental murine disseminated candidiasis, with a shift from the T_H2 response to the T_H1 response (225). Neutralizing antibodies to IL-4 and IL-10 had a similar effect in experimental murine disseminated candidiasis (242, 243).

Newer recombinant cytokines, such as IL-1, IL-3, IL-6, stem factor, and hematopoietic dipentapeptides, may result in improved recovery from marrow aplasia, lead to increased microbicidal function, and reduce the risk of invasive mycoses. Transfusion of elutriated monocytes or neutrophils from donors treated with G-CSF may be important therapeutic or preventive adjuncts which merit further study. Ultimately, these immunologic approaches will likely have their greatest impact in conjunction with antifungal therapy.

SUMMARY AND OUTLOOK

The recent surge in the use of antifungal agents, particularly azoles, is selecting resistant strains of susceptible species and is shifting the population of fungal pathogens toward species that are intrinsically resistant, such as the non-*C. albicans Candida* species *Candida krusei* (2, 237) and *Candida glabrata* (198). The conditions that have led to the emergence of fungal in-

fections in the past 10 years are likely to persist in the future. New approaches are urgently needed for improved diagnosis, including species identification, rapid and predictive susceptibility assays, and effective treatment.

Two classes of selective antifungal agents, the echinocandins and pneumocandins and the pradimicins and benanomycins, are currently in clinical development. Other targets have yet to produce clinical candidates because of limited knowledge concerning selectivity (topoisomerases, protein kinases), absence of lead compounds (EF-3), or permeability constraints (chitin synthase). Concerning permeability, which has been studied far less rigorously in fungi than in bacteria, a picture is emerging in which both efflux and influx contribute to intracellular drug accumulation and fungal susceptibility. Better biochemical understanding of targets is also necessary, especially in cases, such as chitin synthase, in which the structural homogeneity of inhibitors impedes progress in rational drug design.

In addition to "classical" targets, treatment of fungal infections may be directed to "soft" targets such as fungal adhesion, phase transition to the more invasive form, and virulence. It may also include host-directed immunomodulators, since the vast majority of systemic mycoses occur in immunocompromised patients. For the same reason, the importance of broad-spectrum, fungicidal agents of acceptable toxicity cannot be overemphasized. The continued broad use of amphotericin B, despite the advent of less toxic agents, underscores the critical need for potent, fungicidal drugs.

This minireview has aimed at providing a conceptual framework for rational, novel approaches to antifungal drug discovery on the basis of fungal physiology and the host immune response. As the population of immunocompromised patients expands and as the patterns of mycoses evolve, such approaches will undoubtedly assume increasing importance.

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