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Antifungal Agents: In Vitro Susceptibility Testing, Pharmacodynamics, and Prospects for Combination Therapy

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Abstract As invasive fungal infections in immunocompromised patients become increasingly important, the field of antifungal chemotherapy continues to evolve rapidly. New agents have entered the clinical arena, providing physicians with a variety of choices for treatment of most infections. Standardized methods for testing the in vitro susceptibility of fungi have become available, and concentration-effect relationships are increasingly explored. Finally, the availability of an entirely new class of antifungal agents is opening new opportunities for combination therapy of infections that are notoriously difficult to treat and carry a dismal prognosis. However, the ongoing progress in these key areas has also made antifungal chemotherapy considerably more complex and susceptible to misconceptions. Continuing efforts in the laboratory and well designed collaborative clinical trials are needed more than ever to turn opportunities into lasting benefit for patients at risk for or suffering from life-threatening invasive mycoses.

Introduction

Over the past two decades, fungi have emerged as important causes of infectious morbidity and mortality in immunocompromised patients. The most significant risk factors include profound and prolonged granulocytopenia, immunosuppression with corticosteroids, acquired deficiencies in the number and/or function of T-helper cells, and severe illness requiring multiple invasive medical procedures, such as the use of intravascular devices and extensive abdominal surgery. While *Aspergillus fumigatus* and *Candida albicans*traditionally account for the majority of invasive opportunistic infections, more recent epidemiological trends indicate a shift toward infections by non-*fumigatus Aspergillus* spp., non-*albicans Candida* spp., and previously uncommon fungi that often display resistance to current antifungal agents in vitro and in vivo [1]. Human immunodeficiency virus (HIV)-infected patients with advanced immune dysfunction are particularly susceptible to cryptococcal meningitis, disseminated histoplasmosis, coccidioidomycosis, and penicillosis, and recent outbreaks highlight that endemic fungi can become a significant public health concern beyond their baseline prevalence [2, 3].

For many years, the treatment of invasive fungal infections was limited to amphotericin B deoxycholate with or without the addition of 5-fluorocytosine. It was not until the late 1980s that the first durably useful alternatives emerged through the advent of fluconazole and itraconazole. Prompted by the exponential increase of severely immunocompromised patients at risk for invasive fungal infections, however, the past 10 years have witnessed a major expansion in antifungal drug research, as reflected by the introduction of less toxic lipid formulations of amphotericin B as well as the ongoing development of novel echinocandin lipopeptides and improved antifungal triazoles [4] (Fig. 1). Improved blood culture, antigen, and nucleic acid detection techniques [5, 6, 7], the advent of high-resolution two-dimensional imaging [8, 9], and an increased awareness among physicians of the fungal pandemic have all had considerable impact on improving the early clinical diagnosis of invasive fungal infections, and major advances have been achieved in harmonizing disease definitions, in defining paradigm for antifungal interventions, and in designing and implementing clinical trials [10, 11, 12]. Last, but not least, standardized methods for testing the in vitro susceptibility of fungi have become available and are continuously refined [13], and concentration-effect relationships in vitro and in vivo are increasingly explored [14, 15]. Nevertheless, despite these advances, invasive fungal infections remain difficult

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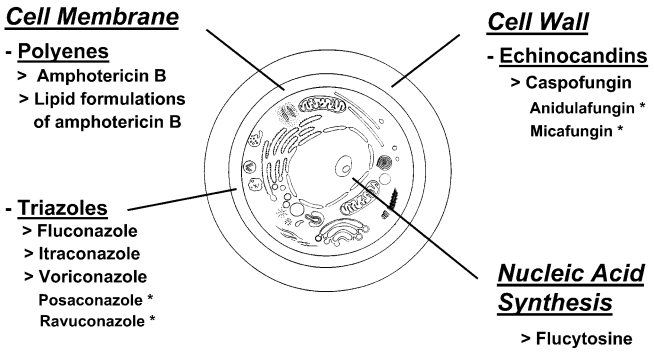

Fig. 1 Schematic diagram of current antifungal agents and their targets in the fungal cell. *, investigational agents

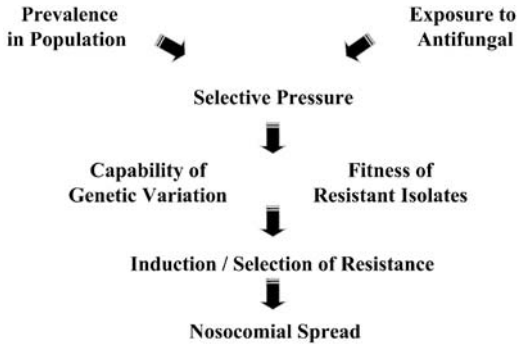

Fig. 2 Evolution of antimicrobial resistance. Selective pressure arises upon prolonged exposure to a given habitat in which the microorganism in question is prevalent. Induction of resistance is dependent on the genetic versatility of the microorganism, and evolutionary success of resistant clones relies on their biological fitness and opportunities for nosocomial transmission

to diagnose and to manage, and there is a continuing need for improved antifungal therapy.

Current Role of Antifungal Susceptibility Testing

Establishing reproducible in vitro methods to assess antimicrobial susceptibility is an important tool for the identification of microbiologically resistant organisms and for optimal selection of antimicrobial therapy. The experience with antibacterial chemotherapy indicates superior outcomes for therapies that are guided by the results of in vitro susceptibility testing as opposed to a merely species-based therapy [16]. Standardized methods for testing the in vitro susceptibility of yeasts [17, 18] and filamentous fungi [19] to current antifungal agents have become available. Tentative breakpoints have been established for fluconazole, itraconazole, and 5-fluorocytosine against-*Candida* spp. [13, 17]; for azole antifungal agents against *Candida* spp., these breakpoints appear to have predictive utility similar to that observed with in vitro susceptibility testing of antibacterial agents [20]. However, for other organism-drug combinations, correlation of in vitro susceptibility with antifungal activity in vivo remains difficult to establish [20, 21]. This is partly related to ongoing methodological problems associated with the selection of optimal assay conditions and endpoints, particularly for the polyenes and the echinocandins, but also is due to the prominent role of host- and disease-related factors in the outcome of most invasive opportunistic fungal infections.

Unlike pathogenic bacteria, in which resistance may emerge rapidly and spread, fungi do not become rapidly resistant because of their eukaryotic nature, their longer replication time, and their lack of genetic mechanisms for the exchange of resistance and of drug-degrading substances. Currently, the emergence of resistance is essentially limited to the following two scenarios [22]: (i) primary emergence of a naturally resistant species, such as*Trichosporon asahii* or *Pseudallescheria boydii*, that is resistant to the fungicidal activity of amphotericin B; and (ii) the selection of a resistant species during antifungal therapy, as exemplified by breakthrough infections with *Candida krusei* or *Candida glabrata* during systemic prophylaxis with triazoles [4]. Stepwise, cumulative molecular events that lead to progressively decreased susceptibility and stable resistance during exposure to current azoles are rarely encountered in patients but have been reported following longstanding exposure to azoles in conjunction with HIV-associated oropharyngeal candidiasis [23, 24] and, less well characterized, chronic granulomatous disease [25].

As the use of antifungal azoles in medicine, agriculture [26], and animal health [27] becomes more widespread, the selection and nosocomial spread of azole-resistant-*Candida* spp. appears inevitable (Fig. 2). To meet this challenge, a thorough understanding of the molecular mechanisms of antifungal drug resistance is required. During the past few years, increased target expression, alterations at the target binding site, and the presence of inducible efflux pumps have been identified as mechanisms of azole resistance and may offer targets for intervention [28, 29]. Comparatively little is known about resistance mechanisms against polyenes and echinocandins; changes in the composition of the fungal cell wall and in the sterol chemistry of the cell membrane have been described for fungi exposed to amphotericin B [24, 30], and mutations of the FKS1 gene have been observed in fungi exposed to echinocandins [31].

In clinical practice, the microbiological diagnosis should be attempted as feasible in all cases of suspected invasive fungal infection, with the organism identified to the species level. Because of the lack of its predictive value in other settings, in vitro susceptibility testing is currently limited to *Candida* spp. versus fluconazole and flucytosine, respectively. However, additional in vitro testing of other organism/drug combinations may be indicated in refractory infections and within surveillance programs [20, 21] (Fig. 3).

Pharmacodynamics of Antifungal Compounds

In a broad sense, the term pharmacodynamics encompasses the description of concentration-over-time relationships of antifungal drugs and drug combinations in vitro

- **Appropriate microbiological diagnostic procedures**
- **Identification of the isolate to the genus if not species level**
- **Susceptibility testing *in vitro***
  - **Recommended testing:**
    - **Fluconazole and flucytosine for *Candida* spp. from sterile sites**
  - **Adjunctive testing:**
    - **Failure of initial therapy for invasive disease**
      - **Amphotericin B for *Candida* isolates**
      - **Amphotericin B, fluconazole, flucytosine for *C. neoformans***
      - **Fluconazole for *H. capsulatum***
    - **For isolates with high rate of intrinsic resistance, use alternative agents**
    - **For all other organism-drug combinations, select therapy based on guidelines or survey data**

Fig. 3 Proposed approach to identification and antifungal susceptibility testing of fungal organisms for selection of antifungal therapy (modified from Rex and Pfaller [20])

- **Determination of MICs and MFCs**
- **Kinetics of activity**
  - **time-kill assays**
- **Persistent effects**
  - **postantifungal effect**
  - **subinhibitory effects**
- ***in vitro* PK/PD models**
- **microscopy, vitality staining, metabolic assays**

Fig. 4 Overview of currently available methods to assess concentration-time-effect relationships of antifungal agents in vitro

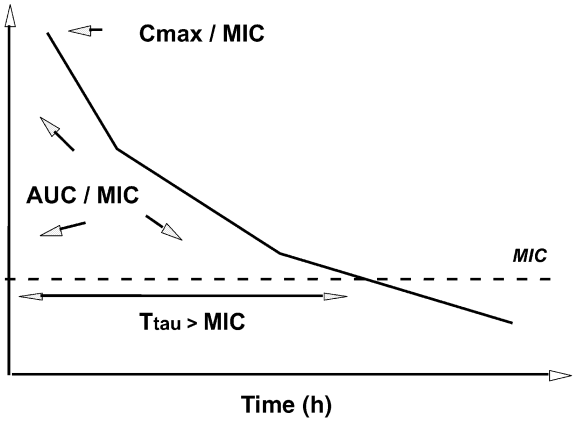

Fig. 5 Commonly used pharmacodynamic parameters to assess pharmacokinetic-pharmacodynamic relationships of antifungal agents in vivo. Correlation of these parameters with endpoints of outcome through mathematical equations in experimental models of invasive fungal infections allows for determination of 50% or 90% effective parameter values. In addition, fractionating the identical daily dose in 1–4 divided doses and of comparison of pharmacodynamic parameters and therapeutic effect among the different cohorts may enable identification of the parameter most predictive of therapeutic success. Pharmacodynamic modelling and dose-fractionating studies can be used as guidance for setting susceptibility breakpoints and for selection of the optimal dose and dosage schedule in patients. C_{max}/MIC, ratio of peak plasma level and MIC; AUC/MIC, ratio of the area under the concentration-vs.-time curve and the MIC; T_{tau}>MIC, time during the dosing interval that plasma concentrations exceed the MIC for the investigated isolate(s)

and in vivo. Common, nonstandardized tools to study the pharmacodynamics of antifungal drugs in vitro are listed in Fig. 4. While these methods provide important information on the mode of action of antifungal drugs, they have major technical and biological limitations. Changing assay conditions such as inoculum size, medium, or pH may result in conflicting observations and, therefore, uncertainty as to their therapeutic relevance. Biological factors, such as different growth characteristics of the organism in vivo and the absence of host defense factors, plasma pharmacokinetics and tissue distribution, and protein binding and carrier effects, are further impediments to an immediate translation to the therapeutic setting. Therefore, results and observations from pharmacodynamic in vitro studies should always be interpreted with caution and further investigated in appropriately designed animal models.

One of the principal aims of antimicrobial drug therapy is the characterization of the relationships between dose, dosage interval, drug concentrations in the body, in vitro susceptibility of the microorganism, and drug effects. Understanding these pharmacokinetic-pharmacodynamic relationships provides important knowledge of a drug's mode of action and can be instrumental in setting susceptibility breakpoints and in guiding optimal dosing regimens [32]. Due in large part to the importance of host- and disease-related factors for patient outcome and the lack of reliable surrogate markers in invasive mycoses, the evaluation of pharmacokinetic and pharmacodynamic relationships relies on well-controlled infection models that provide true endpoints. Such models, by virtue of pharmacodynamic and dose-fractionating studies, allow for the exploration of the relationships of pharmacodynamic parameters such as C_{max}/MIC, AUC/MIC, and the length of time that plasma concentrations stay above the MIC (T_{tau}>MIC) with antifungal efficacy (Fig. 5). Contemporary approaches to treating most invasive mycoses are based on doses and dosage schedules that have been empirically derived over time. Experimental exploration of pharmacokinetic-pharmacodynamic relationships has begun only recently [14, 15], and incorporation of pharmacodynamic endpoints into clinical studies remains an important goal.

Although the target sites of current antifungal drug classes are quite limited, the existence of these targets and the pharmacodynamic consequences of the drug-target interaction are quite diverse due to the enormous variety of fungal organisms that are biologically very different. In the following paragraphs, we will briefly review mechanism of action, antifungal spectrum, concentration-effect relationships in vitro, and pharmacokinetic/pharmacodynamic relationships of the existing classes of antifungal compounds, along with resistance of fungi to these compounds, with a focus on clinical implications and open questions.

I. Antifungal Polyenes

Class and Mechanism of Action

The antifungal polyenes consist of a family of some 200 natural macrolide antibiotics, of which only amphotericin B and nystatin have been developed for systemic therapy. There are currently four licensed amphotericin B formulations: amphotericin B deoxycholate, amphotericin B colloidal dispersion, amphotericin B lipid complex, and a small unilamellar liposomal amphotericin B. The lipid carriers of these formulations have distinct physicochemical and pharmacokinetic characteristics. However, it is largely unknown whether the marked differences in pharmacokinetics also have a clinically relevant impact on the pharmacodynamics of these agents [33, 34, 35]. A multilamellar liposomal formulation of nystatin entered clinical trials in the 1990s [4, 36] but has not been further developed.

The principal mechanism of action of the polyenes is specific binding to ergosterol in the fungal cell membrane. This binding results in the disorganization of the membrane, possibly by formation of specific pores composed of small aggregates of drug and ergosterol. These defects cause depolarization of the membrane, an increase in membrane permeability to protons and monovalent cations, and eventually, cell death. The polyenes also bind to other sterols such as cholesterol, although with less avidity; nevertheless, this accounts for much of their toxicity. A contributory mechanism of action of amphotericin B may be the generation of oxidative metabolites, possibly due to auto-oxidation of the compound with formation of free radicals or an increase in membrane permeability [4, 37].

Spectrum of Antifungal Activity, and Resistance of Fungal Pathogens

Amphotericin B has broad-spectrum antifungal activity that includes most opportunistic and endemic fungi. Notable exceptions are *Candida lusitaniae*,*Aspergillus terreus*, and some of the emerging pathogens such as *Trichosporon asahii*, *Fusarium* spp.,*Pseudallescheria boydii*, *Scedosporium prolificans*, and *Paecilomyces lilacinus* [1, 4]. Given the still-evolving methodology for resistance testing of amphotericin B in vitro [20], it is unclear whether primary resistance among fungal pathogens is truly uncommon or just difficult to detect. Primary microbiological resistance to amphotericin B appears to be due predominantly to quantitative or qualitative alterations in membrane-associated ergosterol. The description of secondary resistance is restricted to anecdotal cases of patients who received nonresorbable polyenes as antifungal prophylaxis [21, 30].

Pharmacokinetics

After intravenous administration of the conventional formulation in deoxycholate, the compound dissociates from its lipid carrier, becomes highly (>95%) protein bound, and distributes preferentially into organs of the mononuclear phagocytic system. The drug follows a biphasic pattern of elimination from plasma with an initial (beta-) elimination half-life of 24–48 hours, followed by a long terminal (gamma-) half-life of several days. The drug is slowly eliminated into urine and bile, with 62% of a dose recovered in unchanged form in urine and feces at 1 week. No metabolites have been identified thus far, and recent mass balance studies suggest that metabolism plays at most a minor role in amphotericin B elimination [4, 38, 39].

In comparison to amphotericin B deoxycholate, the so-called amphotericin B lipid formulations (amphotericin B colloidal dispersion, amphotericin B lipid complex, and the small unilamellar liposomal amphotericin B) have reduced nephrotoxicity yet they preserve the antifungal activity of the parent. Whereas amphotericin B colloidal dispersion (Amphotec) is not fundamentally different from conventional amphotericin B with regard to plasma pharmacokinetics, amphotericin B lipid complex (Abelcet) is more rapidly taken up by the mononuclear phagocytic system, and the small unilamellar liposomal formulation (AmBisome) achieves comparatively higher peak plasma levels and a prolonged and stable circulation in plasma [33, 34, 39, 40]. Independent of its formulation and on the basis of its prolonged half-life in plasma, amphotericin B is usually administered once daily. Due to their reduced nephrotoxicity, the lipid formulations allow for the delivery of higher doses than with conventional amphotericin B. However, the majority of animal models have also demonstrated that higher doses are usually required to achieve antifungal efficacy equivalent to that of conventional amphotericin B [34].

In Vitro Pharmacodynamics

Investigations on the impact of drug concentrations on the rate and extent of organism killing in vitro by time-kill studies consistently revealed concentration-dependent fungicidal activity of amphotericin B against *Candida albicans* and *Cryptococcus neoformans* [41, 42]. As drug concentrations are increased, both the rate and extent of antifungal activity is enhanced. However, the in vitro fungicidal properties of amphotericin B are organism-dependent. While amphotericin is highly fungicidal against *Candida albicans*, the drug is not fungicidal against various emerging fungal pathogens at safely achievable concentrations. An example is *Trichosporon asahii*, an uncommon but life-threatening cause of disseminated infection in granulocytopenic patients [43]. In vitro pharmacodynamic studies have demonstrated that amphotericin B inhibits but does not kill *Trichosporon*, and these findings correlated with lack of fungicidal

activity in experimental disseminated trichosporonosis and clinical resistance to maximum tolerated doses [44, 45]. Consistent with its mechanism of action, amphotericin B also exerts prolonged postantifungal effects (PAFEs) in vitro, ranging from 0.5 to 10.6 hours and from 2.8 to 10.4 hours for *Candida albicans* and*Cryptococcus neoformans*, respectively [46]. More recent data with the same fungal species demonstrate PAFEs of greater than 12 hours with amphotericin B concentrations above the MIC and shorter PAFEs of 0–2 hours for concentrations below the MIC [47].

In Vivo Pharmacodynamics

Experimental pharmacodynamic in vivo studies support the concentration-dependent killing effects observed in vitro. In a dose-fractionating study in neutropenic mice with disseminated candidiasis, animals received total doses ranging from 0.8 to 20 mg/kg over 72 hours divided into 1, 3, or 6 fractions. The peak/MIC ratio was the parameter that provided the best relationship with the residual fungal burden in kidney tissue (r^2=0.93), followed by time above the MIC (T_{tau}>MIC; r^2=0.82) and the AUC/MIC ratio (r^2=0.61). This study also demonstrated prolonged in vivo PAFEs ranging from 23 to 30 hours [48]. In a *Candida albicans* neutropenic murine lung infection model investigating the effects of escalating doses of liposomal amphotericin B in single or divided daily doses, single daily high doses (20–30 mg/kg/day) had a greater effect on fungal burden than lower- or divided-dose regimens [49]. Similarly, in a rabbit model of central nervous system candidiasis examining all four licensed amphotericin B formulations, a strong inverse correlation was observed between fungal burden in brain tissue and plasma concentrations of total amphotericin B, with higher concentrations demonstrating a more pronounced effect [50].

The distinct pharmacokinetic and pharmacodynamic properties of the lipid formulations have been used to investigate dose escalation of amphotericin B in patients with invasive fungal infections. In a formal maximum tolerated dose study, escalating doses of liposomal amphotericin B were investigated in 21 patients with proven or probable aspergillosis, zygomycosis, or fusariosis [51]. Doses as high as 15 mg/kg were well tolerated, and 68% of patients achieved a successful outcome by intent-to-treat analysis. Similarly, the accumulation of large concentrations of amphotericin B in the mononuclear phagocytic system achieved by amphotericin B lipid complex [52] was harnessed to explore a strategy for treatment of hepatosplenic candidiasis. Loading of tissues with amphotericin B lipid complex in the amount of 100 mg/kg over 6 weeks resulted in a continued resolution of hepatic and splenic lesions for 6 months after discontinuation of therapy [53].

Table 1 In vitro pharmacodynamic characteristics of antifungal drug classes against *Candida*and *Aspergillus* spp. (modified from Groll et al. [14] and Andes [15])

Antifungal class	*Candida*	*Aspergillus*
Polyenes	fungicidal	fungicidal
Flucytosine	fungistatic	n/a
Triazoles	fungistatic	fungicidal
Echinocandins	fungicidal	fungistatic

n/a, not applicable due to lack of activity of flucytosine against *Aspergillus*spp.

Table 2 In vivo pharmacodynamic characteristics of antifungal drug classes in models of invasive*Candida* and *Aspergillus*infections (modified from Groll et al. [14] and Andes [15])

Antifungal class	*Candida*	*Aspergillus*
Polyenes	C_{max}/MIC	unknown
Flucytosine	T_{tau}>MIC	n/a
Triazoles	AUC/MIC	unknown
Echinocandins	C_{max}/MIC AUC/MIC	unknown

n/a, not applicable due to lack of activity of flucytosine against *Aspergillus*spp.

Clinical Implications

The collective evidence from these studies implies an important consideration for the use of amphotericin B in clinical practice. The concentration-dependent fungicidal effects, the prolonged PAFEs, and the dose- and concentration-dependent antifungal efficacy in experimental models of invasive fungal infections (Tables 1and 2) all suggest that large, daily doses will be most effective and that achievement of optimal peak concentrations is important. As a consequence, the dose of amphotericin B should not be reduced injudiciously, and infusion for longer durations than approved by the regulatory authorities should be avoided. Finally, dose escalation appears to be a valid strategy for treatment of clinically refractory infections by amphotericin B-susceptible organisms that should be further pursued.

II. Flucytosine

Mechanism of Action

Flucytosine (5-fluorocytosine) is a low-molecular-weight, synthetic, fluorinated, pyrimidine analogue. Following uptake by the fungus-specific enzyme cytosine permease, it is converted to 5-fluorouracil, a potent anticancer agent that causes RNA miscoding and inhibits DNA synthesis [54].

Spectrum of Antifungal Activity, and Resistance of Fungal Pathogens

Flucytosine has broad-spectrum antifungal activity against *Candida* spp., *Cryptococcus neoformans*,*Saccharomyces cerevisiae*, and certain dematiaceous moulds [55, 56]. Resistance to 5-fluorocytosine in susceptible species may involve either mutations in enzymes necessary for cellular uptake, transport, or metabolism, or competitive upregulation of pyrimidine synthesis [57]. Primary resistance in invasive isolates of *Candida albicans* and *Cryptococcus neoformans* is currently reported in 0–8% [29] and <2% [58], respectively, and is due mostly to defects in cytosine deaminase. Secondary resistance is caused primarily by defects in the downstream intracellular metabolism and has been observed in up to 40% of patients receiving flucytosine monotherapy [21]. Because of the potential for development of secondary resistance, flucytosine is rarely given alone but in combination with amphotericin B or, more recently, fluconazole. Since marked increases in the frequency of resistance in vitro have been observed below 25 μg/ml, it has been recommended that serum levels in patients be maintained above that concentration [57].

Pharmacokinetics

Flucytosine is readily absorbed from the gastrointestinal tract, has negligible protein binding, and distributes evenly into tissues and body fluids. It undergoes only minor hepatic metabolism and is eliminated predominantly in active form into the urine. On the basis of an elimination half-life of 3–6 hours in patients with normal renal function, flucytosine is usually administered in 3–4 equally divided doses [4]. Close monitoring of plasma levels and dosage adjustment is recommended, particularly with impaired renal function. Peak plasma levels between 40 and 60 mg/l correlate with antifungal efficacy and are rarely associated with hematopoietic toxicity [59]. The drug is available in oral and parenteral form, but only the oral formulation is approved in the USA.

In Vitro Pharmacodynamics

Time-kill assays performed against*Candida* spp. and *Cryptococcus neoformans* suggest a predominantly concentration-independent fungistatic (≤99% reduction in cfu) activity of flucytosine at clinically achievable plasma concentrations [60, 61], i.e. the rate and extent of activity is generally not increased when concentrations of flucytosine exceed the MIC for the isolates. Prolonged PAFEs against*Candida* spp. and *Cryptococcus neoformans* have been consistently noted. Growth suppression was dependent on concentration and duration of exposure and ranged from 0.8 hours to up to 10 hours [61, 62].

In Vivo Pharmacodynamics

The pharmacokinetic and pharmacodynamic relationships of flucytosine have been investigated using dose fractionating in a neutropenic kidney target mouse model of disseminated candidiasis. Increasing doses produced minimal concentration-dependent killing; nevertheless, flucytosine produced a dose-dependent suppression of growth after plasma concentrations had fallen below the MIC. The fungistatic dose increased with lengthening dosing interval. Nonlinear regression analysis revealed that both the time above the MIC and the AUC/MIC ratio were important in predicting efficacy, whereas the peak level/MIC ratio was the least important parameter. Maximum efficacy was observed when levels exceeded the MIC for only 20–25% of the 24-hour dosing interval [63].

Clinical Implications

The published in vitro and in vivo data suggest that flucytosine exhibits concentration-independent or time-dependent activity (Tables 1 and 2). Since current dosing regimens achieve plasma levels that stay severalfold above the MIC90 of *Candida albicans* isolates for periods exceeding the usual dosing interval of 6 hours, the use of lower doses or less frequent dosing may be worthwhile to explore [15]. New dosing regimens may yield identical antifungal efficacy while further reducing potential toxicities that are believed to be concentration dependent [59].

III. Antifungal Triazoles

Mechanism of Action

The antifungal triazoles (fluconazole, itraconazole, voriconazole, and investigational agents posaconazole and ravuconazole) are synthetic compounds that have one or more azole rings with three nitrogen atoms in the five-member ring. They act predominantly by inhibition of the CYP450-dependent conversion of lanosterol to ergosterol. This leads to an accumulation of toxic 14-a-methylsterols and a depletion of membrane-associated ergosterol, which alters cell membrane properties and function and results in inhibition of cell growth and replication [4].

Spectrum of Antifungal Activity, and Resistance of Fungal Pathogens

The triazoles are active against dermatophytes, *Candida albicans*, non-*albicans Candida* spp.,*Cryptococcus neoformans*, and the dimorphic (endemic) fungi [64, 65, 66]. Among non-*albicans Candida* spp., they are less active against*Candida glabrata* and, with the exception of voriconazole and the investigational triazoles, inactive against *Candida krusei* [20]. Clinically useful activity

against *Aspergillus* spp. and dematiaceous moulds is restricted to itraconazole and voriconazole [67]. Voriconazole also has activity against *Fusarium* spp. [66]. The mechanisms of resistance to antifungal azoles include differences or alterations in the composition of membrane-associated sterols, alterations in the biosynthetic pathway of ergosterol, genetic changes in the target enzyme (mutation, overexpression, gene amplification), and enhanced efflux by ABC transporters and major facilitators [24, 29]. Secondary resistance has been observed following prolonged exposure to azoles in the settings of chronic recurrent oropharyngeal candidiasis, allogeneic hematopoietic stem-cell transplantation, and chronic granulomatous disease and is thought to be primarily due to the selection of resistant clones. Stepwise induction of stable secondary resistance, however, has been observed in HIV-infected patients with chronic recurrent oropharyngeal candidiasis [23, 24], highlighting the potential epidemiological threats by injudicious azole prophylaxis.

Pharmacokinetics

The antifungal triazoles are generally well tolerated, but they have significant potential for drug-drug interactions through their interference with cytochrome P450-dependent oxidative metabolism.

The three currently approved compounds have quite different pharmacokinetic properties. While fluconazole has nearly complete oral bioavailability, circulates in plasma mostly as free drug, undergoes only negligible hepatic metabolism, and is excreted predominantly through the kidneys in unchanged form, itraconazole, in contrast, is not as well absorbed by the gastrointestinal tract, displays high protein binding extensive hepatic metabolism, and is excreted in inactive form via the liver and kidneys [4]. Voriconazole has good oral bioavailability but exhibits nonlinear pharmacokinetics. Protein binding is low, and tissue and CSF levels exceed those of trough plasma levels severalfold. Elimination occurs primarily by oxidative hepatic metabolism, and only small amounts of voriconazole are excreted in unchanged form into the urine. Unlike fluconazole and itraconazole, where CYP3A4 is the predominant isoenzyme, oxidative metabolism of voriconazole occurs primarily by CYP2C19, although CYP2C9 and CYP3A4 also contribute. Of note, there is wide between-subject variability in disposition that is related to genetic CYP2C19 polymorphism [66, 68].

It is unclear whether the differences in the disposition of the current triazoles may result in different efficacy at different sites. Also unclear is the clinical impact of genetic polymorphisms in the hepatic metabolization of voriconazole. All agents are available as oral and parenteral formulations. On the basis of their extended half-lives, fluconazole and intravenous itraconazole are approved for once-daily dosing; the oral formulations of itraconazole as well as voriconazole are administered on a twice-daily schedule.

Fluconazole

In Vitro Pharmacodynamics On the basis of in vitro observations, fluconazole is generally considered to be a fungistatic agent [14, 15]. Time-kill studies for susceptible *Candida albicans*, *Candida tropicalis*, and *Candida glabrata*conducted over an incubation period of 24 hours at multiples of the MIC showed fungistatic activity (<99.9% decrease in the log cfu/ml compared with starting inoculum), with minimal concentration-related growth effects observed between concentrations equal to 0.5 times and 2 times the MIC, but not beyond [41]. Against three susceptible clinical isolates of *Cryptococcus neoformans*, similar pharmacodynamics were observed at concentrations ≥0.5 times the MIC, with the rate and extent of antifungal activity being concentration independent [42]. However, time-kill studies with *Candida albicans* in which extended incubation periods and nonproliferating growth conditions were used demonstrated direct fungicidal activity of fluconazole against nonproliferating *Candida albicans* [69]. While the underlying mechanism is unclear, these findings raise the possibility that fluconazole might eliminate *Candida* spp. over extended periods without help from host defenses. This would be consistent with the efficacy of fluconazole in the treatment of disseminated candidiasis in persistently neutropenic rabbits [70] and in neutropenic patients [71, 72].

At various concentrations and exposure times and in different serum-free growth media, fluconazole displayed no measurable PAFE against *Candida albicans* or *Cryptococcus neoformans* [47, 73]. However, when the assays were performed in the presence of fresh serum, concentration-dependent PAFEs against *Candida albicans* in the range of 1.1–3.6 hours were noted; pretreatment also increased the vulnerability of*Candida albicans* to killing by polymorphonuclear leukocytes [73].

In Vivo Pharmacodynamics Consistent with the results of time-kill assays, fluconazole demonstrated fungistatic (<99.9% reduction in cfu/ml) activity against *Candida albicans* in a dynamic 48 hour one-compartment in vitro bloodstream infection model; doubling of the fluconazole dose did not increase rate or extent of activity against either of the two isolates [74]. The pharmacodynamics of fluconazole in vivo have been investigated in murine models of disseminated candidiasis that used the fungal burden in kidney tissue as endpoint for antifungal efficacy. In a non-neutropenic mouse model, dose fractionating revealed similar reductions in fungal densities between groups that received the same total dose of fluconazole in one, two, or four equally divided doses. Calculation of pharmacodynamic parameters suggested that the pharmacodynamic parameter of fluconazole that best predicted outcome was the AUC/MIC ratio [75]. These findings were corroborated in neutropenic mice using three*Candida albicans* strains for which MICs of fluconazole spanned the range of susceptible and susceptible dose-dependent MICs (0.5, 16, and 32 μg/ml). The magnitude of the AUC/

MIC ratio required to reach 50% of the effective dose (ED50) was similar for all three organisms and ranged from 12 to 25 [76].

Clinical Implications Investigations of pharmacokinetic and pharmacodynamic relationships in patients have not been presented to date. The dose-independent pharmacokinetics (Tables 1 and 2) as well as the available experimental and clinical data are in support of once-daily dosing regimens. Current susceptibility breakpoints and dosing recommendations for fluconazole against *Candida* spp. have been derived from MIC and outcome information of a limited set of patients with mostly superficial *Candida* infections [13]. The principal feasibility of this approach is supported by several animal studies that have demonstrated a correlation between MIC and antifungal efficacy [14] and supporting evidence from clinical studies [20]. However, given the still-evolving state of antifungal susceptibility testing methodologies and the limited clinical database with which it is correlated, treatment of serious invasive mycoses caused by organisms for which MICs are in the susceptible dose-dependent range by dose escalation warrants controlled investigation before its injudicious incorporation into clinical practice.

Itraconazole

In Vitro Pharmacodynamics Itraconazole exerts species- and strain-dependent fungicidal activity against filamentous fungi [77, 78, 79] and, with the exception of some strains of *Cryptococcus neoformans* [80], is generally fungistatic in vitro against yeast-like fungi. The biological background for this differential mode of action is not clear.

Time-kill experiments in conventional media have demonstrated concentration-independent, fungistatic activity of itraconazole against *Candida* spp. and *Cryptococcus neoformans*, with maximum effectiveness observed at two times the MIC and four to eight times the MIC, respectively [72, 81]; performance of time-kill assays in the presence of serum (80%) did not alter the dynamics observed [82]. Against *Aspergillus* spp., itraconazole displayed time- and concentration-dependent fungicidal activity with >87–>97% killing within 24 hours of drug exposure [72]. Studies on persistent effects or using in vitro pharmacodynamic models have not been published to date.

In Vivo Pharmacodynamics The principal possibility of a correlation between in vitro susceptibility data and outcome has been demonstrated for itraconazole in a murine model of disseminated aspergillosis [83]. In a model of invasive pulmonary aspergillosis in methylprednisolone/cyclosporine-immunosuppressed rabbits receiving oral itraconazole, there was a significant pharmacodynamic relationship (r=0.87, P<0.001) between itraconazole concentrations in plasma and antifungal efficacy as a function of the burden of *Aspergillus fumigatus* in lung tissue [84]. In persistently neutropenic rabbits with invasive pulmonary aspergillosis receiving intravenous itraconazole, best correlations were found between AUC, C_{max}, and C_{min}, suggesting concentration- and time-dependent pharmacodynamic relationships [85]. These studies provide the experimental support for the concept of a critical threshold concentration as a surrogate marker for prediction of the drug's concentration and efficacy in the respiratory tract.

Clinical Implications The main rationale for monitoring plasma levels in patients has been the highly variable bioavailability of oral itraconazole. Initially, the target plasma level for oral itraconazole was set at 0.25 μg/ml (high-performance liquid chromatography) at trough on the basis of MIC90s in vitro; later, data on concentrations in patients who received oral itraconazole for prophylaxis suggested the maintenance of plasma levels between 0.25 and 0.5 μg/ml [86, 87]. More recently, the predictive value of threshold concentrations has been analyzed in a cohort of 190 patients with acute leukemia who received antifungal prophylaxis with oral itraconazole. A total of 27 patients developed a proven or probable invasive mould (n=25) or yeast (n=2) infection. The median itraconazole trough concentration after the first week of prophylaxis was significantly lower in patients who developed invasive fungal infections (0.46 vs. 0.82 μg/ml, P=0.008). Multivariate logistic regression analysis demonstrated a significant (P=0.028) statistical association of trough concentrations <0.5 μg/ml with the occurrence of invasive fungal infections [88]. This study strongly suggests that minimum trough levels of ≥0.5 μg/ml (high-performance liquid chromatography) should be achieved and maintained when oral itraconazole is given to neutropenic patients for prophylaxis.

In the therapeutic setting, pharmacodynamic relationships of oral cyclodextrin itraconazole have been investigated in a cohort of 27 HIV-infected children with oropharyngeal candidiasis using a standardized scoring of mucosal involvement as endpoint of efficacy and pharmacodynamic modeling [89]. Pharmacokinetic/pharmacodynamic relationships fitted into inhibitory maximum effect pharmacodynamic models. Best fits were observed for AUC, AUC/MIC, C_{max}, and C_{max}/MIC (r=0.483–0.595,P<0.01), and estimated EC50 values for peak and trough concentrations were 0.40 and 0.16 μg/ml, respectively.

Although several details remain to be investigated, the data presented in this section suggest that, similar to fluconazole, itraconazole exhibits concentration- and time- or exposure-dependent pharmacodynamics (Tables 1 and 2) and that it may be most effective when maintained at certain concentrations at the target site. Minimum trough levels of 0.5 μg/ml (high-performance liquid chromatograph) appear to be a valid surrogate for these concentrations and should be exceeded when itraconazole is given for prophylaxis or treatment of invasive fungal infections. Nevertheless, in the therapeutic situation, the optimum exposure is not well defined since no correlations have

been established between pharmacokinetic parameters and measures of efficacy.

Voriconazole

In Vitro Pharmacodynamics Similar to itraconazole, voriconazole exerts species- and strain-dependent fungicidal activity against opportunistic filamentous fungi, but, on the basis of currently accepted in vitro testing methods, is generally believed to be fungistatic against yeast-like fungi [72, 78, 90]. It is unclear at present whether voriconazole differs from itraconazole, posaconazole, or ravuconazole with respect to its ability to express fungicidal activity.

In vitro, against two strains each of *Candida albicans*, *Candida glabrata*, and *Cryptococcus neoformans* and one strain of *Candida tropicalis* exposed to concentrations of 0.06–16 times the MIC in RMPI, voriconazole exhibited non-concentration-dependent in vitro pharmacodynamics. Maximum fungistatic activity was noted at drug concentrations of approximately three times the MIC, and neither the EC50 nor the EC90 changed over time [91]. Similar observations of non-concentration-dependent fungistatic activity against *Candida albicans* have been made by other investigators [72]. In time-kill assays against *Aspergillus fumigatus*, at concentrations exceeding the MIC for the study isolate by 2.5- to 20-fold, voriconazole displayed time-dependent fungicidal activity [72]. Of note, against *Candida albicans*, a concentration-dependent PAFE of 0.2–4.1 hours has been observed for voriconazole at concentrations at or four times above MIC in the presence of serum but not in serum-free medium. Similarly, pretreated organisms were more susceptible to subsequent exposure to subinhibitory concentrations than untreated organisms and to killing by polymorphonuclear leukocytes in the presence of serum [92]. It is conceivable that the combination of these effects adds to the in vitro activity of voriconazole and enhances its efficacy against *Candida* in vivo.

In Vivo Pharmacodynamics and Clinical Implications The in vivo pharmacodynamics of voriconazole have been investigated in a murine kidney target model of disseminated candidiasis. Similar to other antifungal triazoles, the AUC/MIC ratio was the pharmacodynamic parameter that correlated best with efficacy. Using 10 *Candida albicans* isolates of different voriconazole susceptibilities, the free drug AUC/MIC ratios were similar for all of the organisms studied (mean±SD, 24±17; P=0.45) and similar to those observed for fluconazole and ravuconazole in the same model [93]. Current dosage recommendations for patients are based on interspecies scaling and exposure-dependent pharmacodynamics such as class effect, dose escalation in non-life-threatening fungal infections, and documented efficacy in the treatment of invasive fungal infections. Since voriconazole has nonlinear pharmacokinetics at therapeutic dosages and exhibits considerable interindividual variability in hepatic metabolization, correlating its pharmacokinetics with antifungal efficacy in patients will be a formidable challenge.

Investigational Triazoles

Investigational antifungal triazoles include posaconazole (SCH 56592; Schering-Plough, Kenilworth, NJ, USA) and ravuconazole (Bristol-Myers Squibb, Wallingford, CT, USA). While ravuconazole is structurally related to fluconazole, the structure of posaconazole is very similar to that of itraconazole. Similar to voriconazole, these new agents have enhanced target activity and specificity. They are active against a wide spectrum of clinically important fungi, including*Candida* spp., *Trichosporon asahii*, *Cryptococcus neoformans*, *Aspergillus*spp., *Fusarium* spp., and other hyaline moulds, and against dematiaceous as well as dimorphic moulds [66, 94, 95].

Posaconazole and ravuconazole are highly (>95%) protein bound, exhibit linear pharmacokinetics over the anticipated dosage range, undergo hepatic metabolism, have the potential for significant drug-drug interactions through handling by the CYP450 enzyme system, and are eliminated predominantly via the feces. A hallmark of ravuconazole is its prolonged elimination half-life [96, 97, 98, 99]. Whether the described differences in metabolism and elimination are of clinical significance remains to be determined.

Although posaconazole and ravuconazole follow the general pattern of extended-spectrum triazoles with respect to potential for fungicidal activity against opportunistic moulds and for fungistatic activity against yeasts [79, 100, 101], detailed in vitro pharmacokinetic studies have not been published. The relationships between concentration, time, and antifungal efficacy in vivo have been studied for posaconazole and ravuconazole in animal models of pulmonary aspergillosis and disseminated candidiasis, respectively. The results of these studies are in support of exposure-dependent antifungal efficacy in vivo [102, 103].

IV. Echinocandin Lipopeptides

Class and Mechanism of Action

The echinocandins are a novel class of antifungal lipopeptides. They inhibit the synthesis of 1,3-beta-D-glucan, a polysaccharide in the cell wall of many pathogenic fungi. Together with chitin, the rope-like glucan fibrils are responsible for the cell wall's strength and shape. They contribute to maintaining the osmotic integrity of the fungal cell and play an important role in cell division and cell growth [104, 105]. While caspofungin (MK-0991; Merck, Rahway, NJ, USA) has been approved in the USA and most of Europe, micafungin (FK463; Fujisawa, Deerfield, IL, USA), and anidulafungin (VER-002, formerly LY303366; Vicuron Pharmaceuticals,

Fremont, CA, USA) are in advanced stages of clinical development.

Antifungal Spectrum and Pharmacokinetics

The three current echinocandin agents appear to possess similar pharmacological properties. All three compounds have potent, broad-spectrum fungicidal in vitro activity against*Candida* spp. and potent fungistatic activity against *Aspergillus* spp.; their antifungal efficacy against these organisms has been demonstrated in various animal models and in patients. The echinocandins are not metabolized through the CYP450 enzyme system and are generally well tolerated due to the lack of mechanism-based toxicity. Although only available in parenteral formulations, the echinocandins possess favorable pharmacokinetic properties and are dosed once daily [106, 107].

Pharmacodynamics In Vitro

The current echinocandins possess a species-dependent mode of antifungal activity. Whereas potent inhibition of glucan synthesis has been demonstrated in membrane preparations of*Candida* and *Aspergillus* spp., whole cell in vitro assays reveal fungicidal activity against most *Candida* spp. but not against*Aspergillus* spp. [108, 109]. Broth-based assays show a dose-dependent formation of microcolonies [110, 111] with progressively truncated, swollen hyphal elements that appear to be cell-wall deficient but regain their cell walls following withdrawal of the drug [110, 111, 112]. Vitality fluorescence staining of *Aspergillus fumigatus* exposed to caspofungin revealed a differential killing effect on apical and subapical branching cells, with little reduction in vital staining of subapical cells, suggesting that only cells at the active centers for new cell wall synthesis within *Aspergillus fumigatus* hyphae are killed [113]. These differences may have implications for antifungal therapy of the neutropenic host. In infection models in persistently neutropenic rabbits, echinocandins achieved a dose-dependent elimination of *Candida* spp. in experimental disseminated candidiasis [114, 115]; however, they had no effect on the residual fungal burden in experimental pulmonary aspergillosis and were less effective than amphotericin B in attenuating tissue injury and improving survival [115, 116, 117].

In vitro time-kill assays have demonstrated concentration-dependent fungicidal activity (≥99.9% reduction in cfu) against *Candida* spp. for caspofungin [118], micafungin [115], and anidulafungin [114, 119, 120]. In addition, a concentration-dependent PAFE exceeding 12 hours has been observed for both caspofungin and anidulafungin against *Candida* spp. [47]. In a one-compartment in vitro pharmacodynamic model of anidulafungin against fluconazole-sensitive and fluconazole-resistant *Candida albicans*, a positive correlation of C_{max} and AUC with optimal killing and minimal fungal regrowth has been demonstrated [121].

Pharmacodynamics In Vivo

In vivo, in persistently neutropenic rabbit models of subacute disseminated candidiasis and invasive pulmonary aspergillosis, anidulafungin showed highly predictable concentration-effect relationships in experimental disseminated candidiasis; however, no concentration-effect relationships were observed in experimental pulmonary aspergillosis using the residual fungal burden and survival as endpoints of antifungal efficacy, despite full exploration of the dosage range [122]. Similar concentration-dependent activity against *Candida albicans* was found in a thigh infection model: the ratio between tissue concentrations and MIC was found to be highly predictive of the therapeutic efficacy of micafungin[123]. In murine kidney target models of disseminated candidiasis, the AUC/MIC ratio was most strongly correlated with efficacy of caspofungin [124], whereas the peak/MIC ratio was most predictive of the efficacy of HMR 3270, a novel investigational glucan synthesis inhibitor [125].

Clinical Implications

Concentration-dependent pharmacodynamics in vitro and in vivo (Tables 1 and 2) and the existence of prolonged PAFEs support the current once-daily dosing regimen and, at the same time, provide a rationale for exploration of dose escalation for treatment of complicated invasive *Candida* infections. The pharmacodynamics of echinocandins against*Aspergillus* spp. are more complex and difficult to interpret, and perhaps suggest concentration-independent dynamics beyond a certain threshold dosage.

Pharmacodynamics of Antifungal Combinations

The availability of drugs with different molecular targets has opened new avenues for exploring combination therapies of two or even three drugs. The obvious aims of combination therapies are to broaden the antifungal spectrum, to decrease the selection of resistant organisms, and to improve overall antifungal efficacy without compromising patient safety. However, combination therapy is not to be perceived as the universal approach for all proven or suspected fungal infections but will be reserved for selected subgroups. These include patients with invasive infections due to opportunistic filamentous fungi and poor prognosis, those with acute and fulminant or refractory infections, and those with infections at anatomically privileged sites such as the brain [126, 127].

The paradigm for this approach is the combination of amphotericin B deoxycholate and flucytosine, which has documented synergistic activity against *Cryptococcus neoformans* in vitro and in animal models [128], activity

that also translated into superior outcome in patients with cryptococcal meningoencephalitis [129, 130]. Beyond cryptococcal meningitis, clinical experience with combination therapies is mostly anecdotal. However, there is an understandable trend to utilize in desperately ill patients whatever combination appears to have a theoretical advantage. Nevertheless, irrespective of the pressing clinical need, systematic preclinical investigation of antifungal combination therapies is warranted, followed by appropriately designed randomized clinical trials. This may be illustrated by a drug- and fungus-specific antagonism between amphotericin B deoxycholate and antifungal azoles in vitro and in animal models that has been consistently noted with lipophilic azoles against *Candida* spp. and, in particular, against *Aspergillus* spp. [131, 132, 133]. A recently completed placebo-controlled study comparing fluconazole 800 mg/day plus placebo versus fluconazole 800 mg/day plus amphotericin B (0.7 mg/kg/day) for treatment of non-neutropenic candidemia, however, revealed no evidence of antagonism but more rapid clearance of the bloodstream and a trend toward improved antifungal efficacy of the combination [134]. While the combination of amphotericin B and itraconazole continues to be controversial [126, 127], the body of current preclinical studies indicate no antagonism of combinations of echinocandins with azoles, amphotericin B, or the chitin-synthase inhibitor nikkomycin Z against common opportunistic fungal pathogens [135, 136]. In fact, synergistic efficacy has been observed for the combination of echinocandins and novel triazoles against experimental invasive aspergillosis [137, 138], and first preliminary reports are emerging that point to the potential clinical usefulness of combination therapies in the treatment of invasive aspergillosis [139, 140].

Over the next decade, combination therapy will probably become the standard of care for fungal infections that are notoriously difficult to treat. However, the critical question of how these combinations can be harnessed to improve antifungal therapy can only be evaluated in sufficiently powered, randomized clinical trials that are founded on discriminative animal infection models that incorporate pharmacokinetic and pharmacodynamic endpoints [141].

Future Directions

Invasive fungal infections will likely remain a frequent and important complication in the ever-expanding population of immunocompromised patients. However, not unlike the current situation with antibacterial agents, the road to identification of novel targets and the development of entirely new antifungal therapeutics is difficult and paved with failed projects. One reason for the slow progress in this area is that, owing to their eukaryotic nature, fungal cells have a restricted set of specific molecular targets that do not overlap with their mammalian counterparts and carry the potential of mechanism-based toxicity. In the long range, whole-genome sequencing, bioinformatics, and advances in proteomics and stereochemistry hold great promise for accelerated identification and development of selective antifungal compounds [142]. Nevertheless, for the next foreseeable future, our antifungal armamentarium is likely to remain limited. For this reason, continuing efforts are needed to further capitalize on the current experimental and clinical foundations of antifungal drug research and therapy. The now considerably expanded antifungal drug arsenal, predictive resistance testing in vitro, integration of pharmacokinetic and pharmacodynamic principles, and multiple-drug regimens offer hope for further substantial progress in prevention and treatment of invasive fungal infections.

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