

# Combinations of antifungal agents in therapy—what value are they?

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Concurrent or sequential antifungal treatment for invasive mycoses has been typically considered as an option to improve results of monotherapy. However, data on the efficacy of combination therapy are sparse and consist largely of results from studies *in vitro* and experimental animal models. These studies have yielded controversial results depending on the criteria used to evaluate the antifungal interaction. Several combinations that showed synergy *in vitro* failed to do so in animal models. Overall, apart from cryptococcal infections, combined antifungal therapy is not significantly better than monotherapy in terms of clinical efficacy. It is questionable whether combination therapy should be used in most cases as there is a lack of evidence from well-designed clinical trials. However, combination therapy could be an alternative to monotherapy for patients with invasive infections that are difficult to treat, such as those due to multi-resistant species and for those who fail to respond to standard treatment.

Keywords: concurrent antifungal treatments, interactions *in vitro* of antifungal agents, clinical efficacy of antifungal combinations

## Introduction

Systemic fungal infections are a major cause of morbidity and mortality in debilitated patients. The antifungal therapies that are currently available exhibit limited effectiveness and a complete response depends mainly on correction of the underlying disease. The increase in available antifungal compounds has prompted the search for better therapeutic strategies, such as using the newer antifungal agents in combination.<sup>1–4</sup> For instance, the echinocandins inhibit the synthesis of  $\beta$  1–6 D-glucan, an essential component of the cell wall, thereby providing an additional target for combined agents to act synergistically.

Antifungal compounds used in combination might promote the effectiveness of each drug, with efficacy being achieved using a lower dose of each drug. Pharmacological benefits would accrue, with one drug clearing infection from one body system while the other clears it from a different site. In addition, combination therapy could be utilized in an attempt to prevent or delay the emergence *in vivo* of resistant populations of the pathogenic fungus.<sup>5,6</sup>

Concurrent and sequential antifungal treatment has been typically considered for invasive mycoses to improve the results of monotherapy.<sup>7–9</sup> However, the data on efficacy are sparse and consist largely of the results of *in vitro* studies and experimental animal models. There are no data from clinical trials regarding

the safety and efficacy of combination therapy. Nonetheless, many practitioners are giving combinations of drugs on the basis that they may do some good, but unexpected adverse effects can reverse any putative benefits of combination therapy by worsening the clinical outcome. In fact, large and expensive clinical trials are required to show significant differences between adverse events and the efficacy of a given combination compared with those of the monotherapy, but these are unlikely to take place in the current climate of budgetary restraints.<sup>10–12</sup>

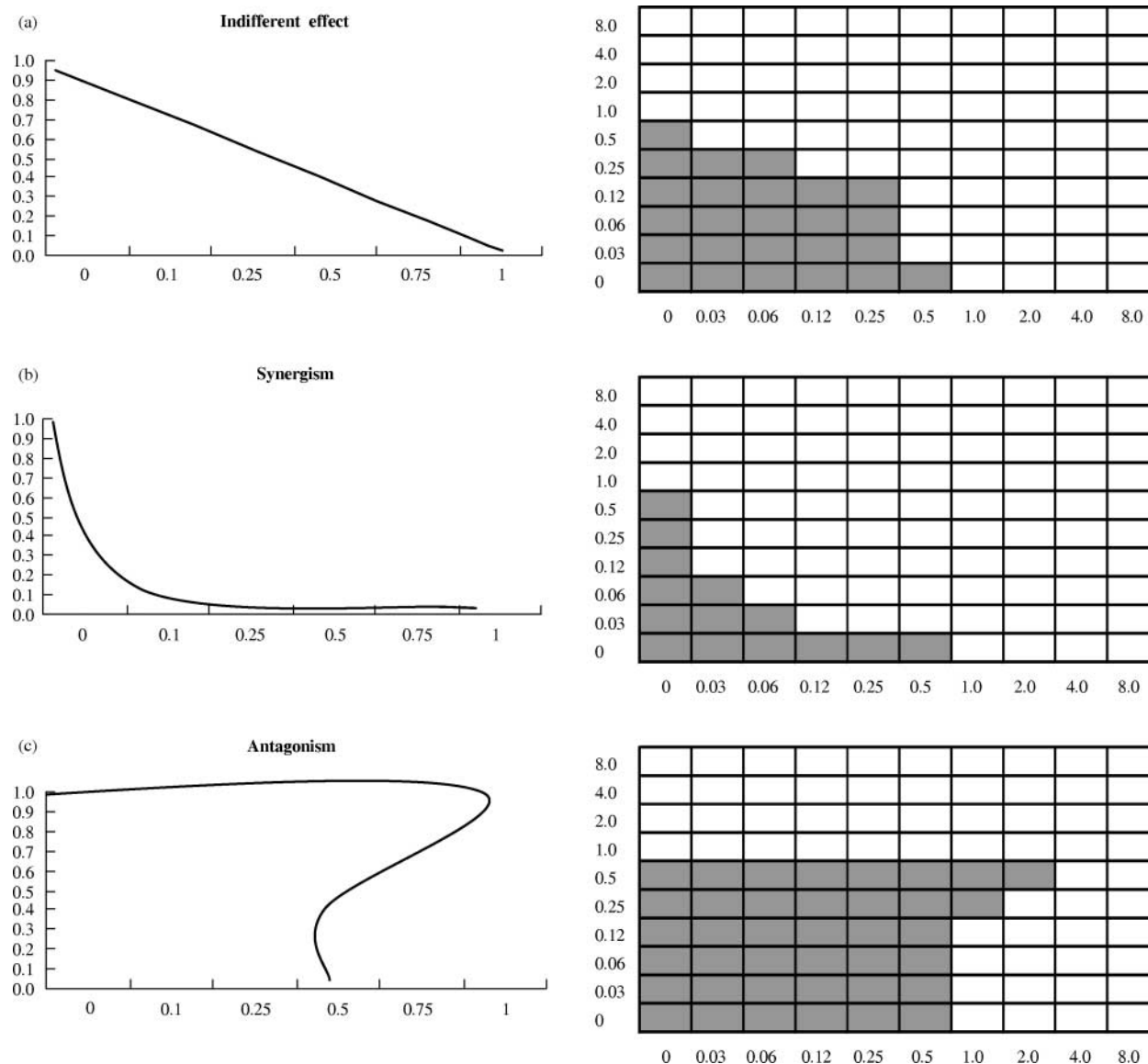
Animal models and susceptibility testing can help to predict efficacy of antifungal compounds in humans, and their results—although obtained using simplified methodologies—can be used to establish the dosing regimens for combination therapy, or to demonstrate synergy, thereby optimizing the design of feasible, reliable and powerful clinical trials.<sup>13</sup>

This review is a summary of antifungal susceptibility testing results, animal studies and clinical reports on combination antifungal agents used to treat systemic mycoses. The review is divided into three sections, in which combinations of various antifungal agents are discussed.

## Combination studies *in vitro*

Susceptibility testing of combinations of antifungal agents has yielded conflicting results due mainly to the different

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**Figure 1.** Chequerboard technique. The term chequerboard refers to the pattern, tubes or microtitration trays, formed by multiple dilutions of the two antifungal agents being tested, in concentrations equal to, above and below their MICs. Here are displayed results of testing combinations of two drugs diluted in two-fold increments in mg/L. Shading is visible growth. Also shown are isobolograms plotted on an arithmetic scale.<sup>14</sup>

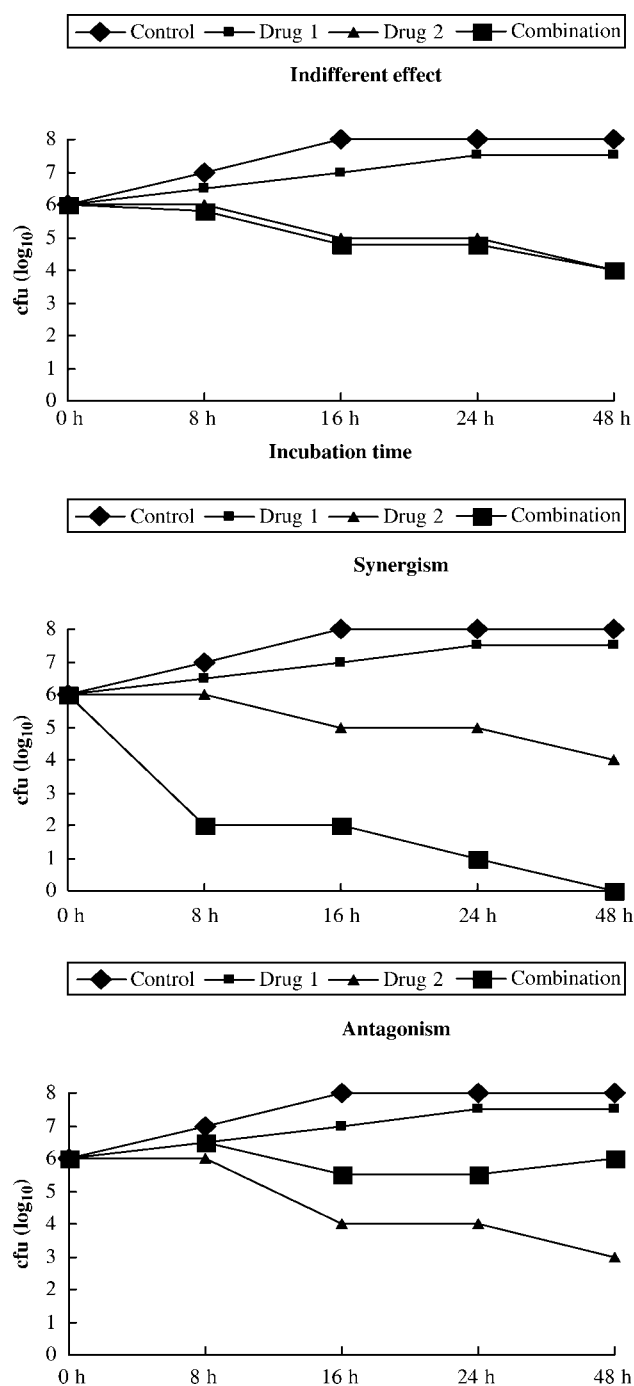
methodologies used, such as agar dilution, agar diffusion and broth dilution. The chequerboard method and the killing curves technique are most frequently used to assess antimicrobial combinations *in vitro*. The term chequerboard refers to the pattern, tubes or microtitration trays, formed by testing two antifungal agents, in concentrations several dilutions above and below the MICs for the fungi being tested. The method has been used almost exclusively for determining the inhibitory concentration (Figure 1).<sup>14</sup>

In contrast, the killing curve or time–kill curve technique measures the microbicidal activity of the combination being tested and provides a dynamic illustration of the interaction over time (Figure 2). This technique has been used for testing fungicidal agents such as amphotericin B, but the repetitive counting of colony-forming units that the technique entails is labour intensive, tedious and seriously limits the number of antifungal concentrations and combinations that can be tested at any one

time.<sup>14,15</sup> In addition, there is also controversy about ensuring that residual drug is removed. There is also no consensus about how to deal with sampling error, how to estimate survivors and how to define the minimum lethal concentration, for instance >99% kill or a reduction of at least two  $\log_{10}$ .

#### *In vitro* techniques

Chequerboard dilutions can be readily performed in clinical laboratories using microdilution or macrodilution systems, are easier to standardize and thus are more commonly reported. Although the dilutions used in the chequerboard are exponential, typically two-fold dilutions, the results are interpreted by the pattern they form on an isobologram, which displays fractional inhibitory concentration indices (FICI) on an arithmetic scale. A single FICI is the most common way in medical mycology to report the results of studies with chequerboard dilutions,



**Figure 2.** Time-kill curves technique, which measures the microbicidal activity on the combination being tested and provides a dynamic picture of interaction over time. Also displayed are cfu of viable organisms in log<sub>10</sub> by incubation time.

and is the lowest concentration of each drug that inhibits growth. It is calculated by the following formula:  $(A)/(MIC_A) + (-B)/(MIC_B) = FIC_A + FIC_B = FIC$  index, being (A) the concentration of drug A in a tube that is the lowest inhibitory concentration in its row, (MIC<sub>A</sub>) the MIC to drug A alone, and FIC<sub>A</sub> the fractional inhibitory concentration of drug A. (B), (MIC<sub>B</sub>) and FIC<sub>B</sub> are defined in the same fashion for drug A.<sup>14</sup>

The technical ease of the checkerboard technique is offset by some drawbacks. To begin with, controversial results can be obtained depending on the criteria used to evaluate the antifungal interaction, such as MIC endpoint definition, assay medium, reading method and analysis of results. A second flaw to consider is that the FICI calculation assumes incorrectly that all antimicrobial compounds have linear dose-response curves, providing a static, all-or-none view of antimicrobial interaction, creating artificial FICs.<sup>16,17</sup>

Alternative methods for assessing drug interaction have been developed recently in order to overcome the limitations of studies on combination antimicrobial agents *in vitro*. They rely on the response surface approaches generated by the three-dimensional nature of antimicrobial interactions, in contrast to the one-dimensional FICI. The drug effect is measured by the proportion of growth with respect to a drug-free control and is related to any drug combination, generating a surface when this relationship is plotted three dimensionally. Response surface models incorporate interaction parameters, as well as the uncertainty of the estimates, by taking into account the variation of the data. These approaches are not easy to understand and the mathematics necessary to calculate and interpret the results are complex. However, they constitute an alternative to isobolograms and the FIC index for determining drug interactions.<sup>16,18</sup>

### Definitions

There is general agreement on definitions of synergy and antagonism. Synergy is a positive interaction created when two agents combined exert an inhibitory effect that is greater than the sum of their individual effects. Antagonism, on the other hand, is a negative interaction observed when the combined effect of the drugs is markedly less than when the drugs are tested separately.<sup>14,19</sup> However, there is still confusion about the definition for 'no interactions' or 'zero interactions' and several terms are still widely used, such as 'additivity', 'sub-additivity', 'indifference', 'independence' and 'autonomy'. The absence of an interaction could be defined by the lack of any significant interaction between the antimicrobial agents being tested, as suggested by Greco *et al.*<sup>20</sup> Therefore, a combination is deemed synergistic or antagonistic when its effect is significantly greater or less, respectively, than that expected when there is no interaction.

However, many of the published criteria to determine interactions between antifungal agents are too lenient, and thus the clinical relevance of synergy or antagonism remains undefined.<sup>2,17,18,21</sup> In addition, most reports on antimicrobial combinations divide the no interaction into two categories, additivity and autonomy or indifference, making the interpretation of interactions more complicated. Additivity is observed when the result of a combination is the sum of the separate effects of the drugs being tested, whereas indifference suggests that the combined effect is simply the effect of the most active drug alone.

Nowadays, most experts agree that there is no real difference between additivity and indifference, and assert that FICI values slightly above or below the theoretical cut-off value of 1.0 really indicate no interaction between agents.<sup>19</sup> Synergy is then defined by an FICI or fractional microbicidal concentration index (FMCI)  $\leq 0.5$ , antagonism by a FICI or FMCI  $> 4$ , and no interaction by a FICI or FMCI  $> 0.5$ , but  $\leq 4$ .

## Review

**Table 1.** Interactions *in vitro* of combination antifungal agents classified per fungal species. The table displays the type of interaction in order of frequency according to literature reports; interactions described most frequently are marked in bold type

Combination antifungal agents	Interactions <i>in vitro</i> by fungal species		
	<i>Candida</i> spp.	<i>C. neoformans</i>	<i>Aspergillus</i> spp.
AMB + FC	<b>no interaction</b> <sup>d</sup> synergy	<b>synergy</b> no interaction	<b>no interaction</b> synergy antagonism
AMB + azole agents	<b>no interaction</b> antagonism synergy	<b>no interaction</b> antagonism synergy	<b>no interaction</b> antagonism synergy
Azole agents + FC	<b>no interaction</b> antagonism synergy	<b>synergy</b> no interaction	<b>no interaction</b>
AMB + TBF	<b>no interaction</b>	ND	<b>antagonism</b> no interaction
Azoles + TBF	<b>synergy</b> no interaction	ND	<b>synergy</b> no interaction
AMB + echinocandins	<b>no interaction</b> synergy	<b>no interaction</b> synergy	<b>no interaction</b> synergy
Azole agents + echinocandins	<b>no interaction</b> synergy	<b>no interaction</b> synergy	<b>no interaction</b> synergy

AMB, amphotericin B; FC, flucytosine; TBF, terbinafine; ND, no data.

<sup>d</sup>No-interaction includes both indifference and additivity.

### Drug combinations

**Amphotericin B plus flucytosine.** This combination has been the most frequently tested *in vitro* and generally showed no interaction or synergy, with little evidence of antagonism (Table 1).

Studies for *Candida* spp. date from the 1970s<sup>22–25</sup> and results obtained using the checkerboard technique showed that this combination exhibited no interaction or synergy against most of the *Candida* isolates tested.<sup>24,26,27</sup> Data from studies using the killing curve technique indicated that the combination of amphotericin B and flucytosine was indifferent against *Candida albicans*,<sup>28,29</sup> and synergistic against clinical isolates of *Candida lusitanae*.<sup>30</sup>

Concurrent therapy with amphotericin B and flucytosine is considered the standard treatment for cryptococcal meningitis and thus there are several studies of their interaction *in vitro* against *Cryptococcus neoformans*. Studies using both checkerboard and time–kill techniques showed overt synergy, but occasionally no interaction was observed. Antagonism has not been reported.<sup>23,24,26–28,31,32</sup> One *in vitro* study, based on the checkerboard method but using the response surface, showed variable interactions that were dependent on the strain tested.<sup>18</sup>

The combined effect, *in vitro*, of amphotericin B and flucytosine against other fungal species varied from synergy to evident antagonism. The finding of no interaction was the predominant effect against *Aspergillus* spp., but synergy was observed against a quarter of the strains tested.<sup>17,27,33</sup> Antagonism has also been described for six of 26 (23%) *Aspergillus* strains tested.<sup>34</sup> Finally, no interactions were reported for this combination against 35 strains of zygomycetes, although synergy was observed against *Exophiala spinifera*.<sup>35,36</sup>

**Amphotericin B plus azole agents.** There is a theoretical concern that amphotericin B and azole agents will lead to antagonism

because there will be less ergosterol in the cell membrane available for the polyene to bind to as a result of the azole inhibiting the lanosterol 14 $\alpha$ -demethylase in ergosterol synthesis.<sup>37</sup> Amphotericin B can also interfere with the influx of azole agents by damaging the membrane structure.<sup>17,38,39</sup> However, interaction studies *in vitro* of amphotericin B and azole agents have yielded divergent results (Table 1).

It has been hypothesized that antagonism depends on lipophilicity of azole agents.<sup>38</sup> Pre-incubation with lipophilic azoles such as miconazole, ketoconazole and itraconazole decreases the fungicidal effects of amphotericin B, whereas fluconazole, a hydrophilic compound, does not. These conflicting results could be explained largely by the technique and the criteria used for evaluating the interaction.

Amphotericin–azole combinations have been studied using different procedures. Using the checkerboard technique, no interactions were observed against *Candida* spp., *Aspergillus* spp. and *C. neoformans*.<sup>26,27,33,34,40</sup> Notably, synergy was described in some reports.<sup>27,41,42</sup> The finding that antagonism was rarely observed for combinations of amphotericin B with either fluconazole or itraconazole argues against the theoretical concept of antagonism between polyenes and azole agents.<sup>34</sup> However, negative interactions have been identified for *Candida*, *Aspergillus* and *Cryptococcus* species when the time–kill technique, Etest and agar diffusion methods were used and also when response surface modelling was employed to define the interaction.<sup>37,42–46</sup> **Antagonism was observed for combinations of amphotericin B with ketoconazole, miconazole, fluconazole, itraconazole and newer antifungal agents such as posaconazole.**<sup>41</sup>

Another point to consider is the efficacy *in vitro* of sequential combinations of amphotericin B plus azole agents. Sequential therapy with an azole agent followed by amphotericin B has been the focus of several experiments *in vitro*. Most of them have shown that the pre-incubation of fungal isolates with azole

agents before exposure to amphotericin B decreased their susceptibilities to the polyene. Pre-incubation of *C. albicans* with fluconazole or ketoconazole and *Aspergillus fumigatus* with ketoconazole, fluconazole or itraconazole generally showed antagonism.<sup>37,47,48</sup> One study *in vitro* described reversible antagonism against *A. fumigatus* when the Etest method was used for testing a combination of itraconazole and amphotericin B, the latter given sequentially.<sup>45</sup> However, pre-treatment with amphotericin B followed by azole agents resulted in positive interactions against fungal species.<sup>41,48</sup> For *Aspergillus* species, pre-incubation with the polyene followed by miconazole or fluconazole showed significantly greater synergy than when the drugs were tested simultaneously.<sup>47</sup>

Finally, the combined effect *in vitro* of amphotericin B and azole agents against other fungal species has been also tested. One study using the checkerboard technique showed no interaction between amphotericin B and fluconazole or itraconazole for 10 isolates of *Histoplasma capsulatum*. This finding was confirmed *in vivo*.<sup>49</sup> Another report described no interactions or synergy *in vitro* between amphotericin B and miconazole, fluconazole or itraconazole against clinical isolates of *Pseudallescheria boydii*. Antagonism was not observed.<sup>50</sup>

**Azole agents plus flucytosine.** Effects *in vitro* of an azole agent and flucytosine combinations have been less frequently investigated. Combinations of flucytosine with both older and newer azole agents (voriconazole and posaconazole) have exhibited synergy against *C. neoformans*.<sup>26,32,51–53</sup> This has led to a combination of an azole and flucytosine as an alternative for treating cases of cryptococcosis that fail to respond to conventional therapy. By contrast, interactions have not been identified *in vitro* for most *Candida* and *Aspergillus* species,<sup>26,27,30</sup> between flucytosine and miconazole, ketoconazole, fluconazole or itraconazole. However, there have been reports of antagonism between these antifungal compounds for some isolates of *Candida glabrata* and *C. lusitaniae*.<sup>54–56</sup> One study used surface response modelling and showed that the effect of flucytosine plus fluconazole depended on the *Candida* strain tested. In general, the combination was antagonistic, but synergy was found for some *Candida* isolates.<sup>18</sup>

**Combinations with terbinafine.** Terbinafine inhibits ergosterol biosynthesis at the level of squalene epoxidation. From the mechanistic point of view, combinations of azoles and terbinafine should exhibit synergy since they are acting at different points of the same pathway.<sup>16</sup> This has been corroborated in several studies *in vitro*. Combinations of terbinafine with fluconazole, itraconazole, voriconazole or posaconazole have shown synergy *in vitro* against species of *Candida*, *Aspergillus*, *Mucorales* and even against fluconazole-resistant *Candida* isolates and itraconazole-resistant *Aspergillus* strains.<sup>35,57–63</sup> One *in vitro* study using response surface modelling showed that itraconazole and terbinafine was the most potent combination against *Aspergillus* spp.<sup>43</sup> Others reported that combinations of terbinafine with miconazole, voriconazole or itraconazole showed synergy *in vitro* against the multidrug-resistant species *Scedosporium prolificans*.<sup>16,64</sup>

The interaction of terbinafine and amphotericin B or flucytosine has also been assessed. Checkerboard and time–kill curve studies have indicated that these combinations exhibit no interaction or are antagonistic against *Aspergillus* and other fungi.<sup>43,62</sup>

One study showed that amphotericin B plus terbinafine was synergistic against 20% of strains of zygomycetes tested,<sup>35</sup> whereas others reported no interactions against the majority of isolates tested.<sup>63</sup>

**Combinations with echinocandins.** Echinocandins are a new class of antifungal compound that interfere with cell wall biosynthesis by inhibiting 1,3- $\beta$ -D-glucan synthase.<sup>65</sup> Caspofungin is the first compound of this new drug class that has been approved for treating invasive aspergillosis in patients who are refractory to, or intolerant of, other therapies and candidaemia due to azole-resistant strains. Two other echinocandins, micafungin and anidulafungin, are also in development.<sup>3</sup>

The inhibition of cell wall synthesis can enhance the penetration of a second antifungal agent.<sup>3</sup> Several combinations of various antifungal compounds with echinocandins have been studied.<sup>66</sup> The combination of amphotericin B and caspofungin has been tested against 200 strains of *Candida* spp., *Aspergillus* spp. and isolates of *C. neoformans* against which the echinocandins are inactive *in vitro*. Synergy was described for some strains and antagonism was not found. Strains of *Aspergillus* spp. were exposed to the same combination and synergy was found for some isolates, with FICI in the range 0.39–0.66.<sup>67</sup>

The interaction between caspofungin and azole agents has been evaluated by several authors. Synergy was described for combinations of caspofungin and itraconazole and posaconazole against 20 clinical isolates of *A. fumigatus*, but combinations of the echinocandin with voriconazole and ravuconazole showed no interactions. The authors reported that the interaction was strain-dependent and hence was not predictable.<sup>68</sup> One study using a time–kill curve method found no interaction between fluconazole and caspofungin against clinical isolates of *Candida* spp. and *C. neoformans*,<sup>69</sup> whereas others reported synergy.<sup>66</sup> This combination displayed a measurable and prolonged post-antifungal effect against isolates of *C. albicans* and *C. neoformans*.<sup>70</sup> A recent study has reported voriconazole and caspofungin to be synergistic against itraconazole-resistant strains of *A. fumigatus*.<sup>71</sup>

There are limited data on combinations with the other echinocandins, but the results are similar to those described for caspofungin. Recent susceptibility data obtained by the checkerboard method found either no interaction between amphotericin B and micafungin or synergy against *Aspergillus* spp. mainly among *A. fumigatus* strains. The same study found synergy for micafungin combined with either itraconazole or flucytosine for some isolates.<sup>17</sup> There was no interaction found for the combination of anidulafungin and fluconazole *in vitro* against *Candida* spp. or *C. neoformans*.<sup>69</sup>

**Combinations of antifungal and antibacterial agents.** There are a large number of *in vitro* studies exploring the interactions between antifungal compounds and other classes of antimicrobial agents, but only those studies that have shown evident synergy between drugs will be considered here.

Combinations *in vitro* of antifungal and antibacterial compounds have been widely investigated. Rifampicin or rifabutin, a semisynthetic derivative closely related to rifampicin, form a stable complex with DNA-dependent RNA polymerase preventing DNA transcription.<sup>72</sup> Rifampicin exhibits no antifungal activity on its own, but amphotericin B appears to facilitate the drug's entry into the fungal cell, allowing it to inhibit DNA



## Review

transcription. Indeed, synergy has been found for amphotericin B plus rifampicin or its analogues against isolates of *Candida* spp., *Aspergillus* spp., *Fusarium* spp., *Mucorales* and *C. neoformans* and antagonism was not seen.<sup>31,33,35,72,73</sup> Rifampicin also enhances the effects *in vitro* of azole agents, but co-administration of these compounds is inappropriate in humans because the antibacterial agent is a potent inducer of P-450 enzymes that accelerate the metabolism of the azoles and result in lower concentrations of these agents.<sup>17</sup>

Several studies have also shown synergy between antifungal agents and the fluoroquinolones such as ciprofloxacin, levofloxacin and ofloxacin, and the macrolides against some fungal species.<sup>30,74–78</sup> A chitin synthase inhibitor, nikkomycin Z, is synergistic when combined with azole agents and echinocandins for *Aspergillus* spp. and other mould species that are difficult to treat.<sup>79–81</sup>

**Antifungal agents and non-antimicrobial agents.** Calcineurin inhibitors, particularly cyclosporin and tacrolimus, enhanced dramatically the activity *in vitro* of both fluconazole and caspofungin against *Candida* spp., *Aspergillus* spp. and *C. neoformans*.<sup>82–85</sup> Combinations of antifungal agents with proton pump inhibitors, antiarrhythmic agents, cholesterol-lowering agents, immunomodulators, antineoplastic compounds and antiparasitic drugs have also been explored.<sup>2,86–88</sup> Several of these combinations have exhibited synergy against fungal pathogens, but their potential for treatment needs further evaluation. In this regard, a recent study has indicated synergy *in vitro* between itraconazole and amiodarone, lansoprazole or nifedipine against isolates of *A. fumigatus*. The combination of itraconazole with calcium pump blockers showed synergy *in vitro*, even for itraconazole-resistant strains.<sup>89</sup>

### Animal studies on combination antifungal agents

In contrast with susceptibility testing *in vitro*, which determines the inherent susceptibilities of organisms to antimicrobial agents, testing *in vivo* using experimental models may allow clinical effectiveness to be predicted.<sup>13</sup>

Several animal models have been developed to screen for synergy between antifungal compounds.<sup>4</sup> Comparing the results

of different animal studies is complicated because of divergent methodology and differences in animal species, infection location and immune status. Many animal models rely on intravenous inoculation of yeasts or conidia that does not mimic the initiation and progress of the majority of fungal infections in humans. Discriminative animal models are technically more complicated, but by mimicking infection in humans more closely, they allow efficacy to be measured in several ways. These models are considered more reliable for ascertaining whether an antimicrobial agent is suitable for treating a human infection, but have not yet been employed for assessing combinations of antifungal agents. Many animal studies lack the statistical power to detect significant differences in efficacy of different therapies. In addition, some animal models raise doubts about the validity of their results because they employ lower doses of antifungal agents than are recommended for clinical use, the definitions of synergy or antagonism are not consistent and adequate pharmacokinetic data are lacking.

Despite this, animal models have contributed data that can help to predict the efficacy of antifungal compounds (Table 2).

### Drug combinations

**Amphotericin B plus flucytosine.** Except for cryptococcal infections, this combination is not clearly superior to monotherapy with amphotericin B.<sup>23,90,91</sup> A study published in 1978 indicated synergy *in vivo* between amphotericin B and flucytosine in a mouse model of systemic candidiasis.<sup>24</sup> Similar results were reported for this combination even when the *C. albicans* strains were resistant *in vitro* to flucytosine.<sup>91</sup> However, rat and rabbit models showed that this combination was no better than amphotericin B alone for treating aspergillosis.<sup>92,93</sup> Monotherapy and combination antifungal therapy were similar in terms of survival, and antagonism was not found. This combination has been recently shown to be ineffective for treating murine disseminated fusariosis.<sup>73</sup>

**Amphotericin B plus azole agents.** Combination therapy with amphotericin B and fluconazole was tested in rabbit models of endocarditis, pyelonephritis and endophthalmitis, and in a mouse model of disseminated candidiasis.<sup>94–97</sup> Combinations were less effective than amphotericin B alone in decreasing the fungal

**Table 2.** Summary of interactions between antifungal agents described in animal models of fungal infections. The table displays the interactions most frequently reported

Combination antifungal agents	Efficacy <i>in vivo</i> by fungal species		
	<i>Candida</i> spp.	<i>C. neoformans</i>	<i>Aspergillus</i> spp.
AMB + FC	similar to AMB monotherapy	superior to AMB monotherapy	similar to AMB monotherapy
AMB + azole agents	inferior to AMB monotherapy, but superior to azole monotherapy	similar to AMB monotherapy, but superior to azole monotherapy	similar to AMB monotherapy, but superior to azole monotherapy
Azole agents + FC	similar to azole monotherapy	superior to azole monotherapy	similar to azole monotherapy
AMB + TBF	ND	ND	ND
Azoles + TBF	ND	ND	ND
AMB + echinocandins	similar to monotherapies	ND	superior to monotherapies
Azole agents + echinocandins	similar to monotherapies	ND	superior to monotherapies

AMB, amphotericin B; FC, flucytosine; TBF, terbinafine; ND, no data.

load in the organs of infected animals and in sterilizing infected tissues. Overall, survival was not significantly different. A combination of fluconazole and amphotericin B showed slightly better activity *in vivo* than did fluconazole alone. A study in mice with systemic infection due to both fluconazole-resistant and -susceptible isolates of *C. albicans*, showed that therapy with amphotericin B plus fluconazole was effective against resistant strains and antagonistic for susceptible isolates.<sup>94</sup> Similarly, the majority of studies *in vivo* of amphotericin B combined with ketoconazole or itraconazole showed no interactions against *Candida*.<sup>90,98</sup>

Results in animal models of invasive aspergillosis varied from antagonism to no interaction for combinations of amphotericin B with either ketoconazole or fluconazole.<sup>91,92</sup> In a murine model of cerebral aspergillosis, combination therapy with amphotericin B plus itraconazole resulted in better survival than was found for either drug alone. Mice treated with amphotericin B had a 40% survival rate, and only 10% of those treated with itraconazole survived, whereas treatment with the combination resulted in a survival rate of 70%.<sup>17</sup>

A murine model of cryptococcosis showed that combination therapy with fluconazole and amphotericin B was more effective than fluconazole alone, and at least as effective as amphotericin B monotherapy, in improving survival and lowering tissue burden.<sup>41</sup> A murine model of histoplasmosis showed no interaction between itraconazole and amphotericin B and antagonism between fluconazole and the polyene.<sup>49</sup>

Sequential therapy with an azole agent followed by amphotericin B has been the focus of some animal models. A rabbit model of endocarditis and pyelonephritis caused by *C. albicans* showed that pre-exposure to fluconazole reduced fungal susceptibility to amphotericin B.<sup>48</sup> In a murine model of acute invasive pulmonary aspergillosis, pre-exposure to itraconazole lowered the efficacy of amphotericin B monotherapy given later. The authors stressed that the fungal lung load was consistently higher in animals pre-exposed to the azole.<sup>99</sup>

**Azole agents plus flucytosine.** Animal studies of candidiasis and aspergillosis have found these combinations as effective as monotherapy.<sup>91,92,95</sup> Studies in rabbit models of deep candidiasis demonstrated that fluconazole given in combination with flucytosine resulted neither in enhanced killing in infected tissues nor in higher survival rates than was observed when fluconazole was given alone. No interactions *in vivo* were observed for fluconazole plus flucytosine or ketoconazole plus flucytosine in murine models of aspergillosis. Several studies with murine models of infections due to *C. neoformans* have indicated that combined therapy with fluconazole and flucytosine was superior to single-drug treatment.<sup>100,101</sup> Notably, the enhanced therapeutic effect was found even when the doses of flucytosine were far lower than those commonly employed (40–100 µg/g/daily instead of 150 µg/g/daily).<sup>102</sup> Flucytosine given in combination with the newer azole, posaconazole, was also found to be superior to monotherapy in a murine model of cryptococcosis. The combination was not significantly more effective in terms of survival, but was more effective in reducing tissue burden.<sup>52</sup>

**Combinations with terbinafine.** To date, the synergy that has been described *in vitro* between terbinafine and other antifungal agents has not been seen in animal models of infection.

Terbinafine appears to be inactive when given alone to rodents because of a rapid hepatic first-pass effect.<sup>103,104</sup> Some studies have demonstrated poor penetration of terbinafine into the lung and other tissues (0.4% of the plasma concentration).<sup>105</sup> Understandably, the efficacy of terbinafine for treating invasive mycoses has been questioned.

**Combinations with echinocandins.** Most of the studies in animal models reported the activity of caspofungin combined with other antifungal agents for treating *Aspergillus* infections. A murine model of disseminated aspergillosis showed that caspofungin plus amphotericin B reduced the kidney burden in 60% of animals (as measured by real-time PCR) to levels less than those of the single agents.<sup>17</sup> Caspofungin in combination with voriconazole resulted in similar mortality rates as did voriconazole monotherapy in a Guinea pig model of invasive aspergillosis.<sup>106</sup> However, the combination reduced tissue burden 1000-fold compared with those for the control groups, and prolonged survival time. Therapy with caspofungin and fluconazole did not show any benefit over individual antifungal agents in a murine model of candidiasis.<sup>107</sup>

Combinations of micafungin and amphotericin B for treating murine aspergillosis showed either synergy or no interaction. A survival rate of 100% was found for a combination of micafungin plus amphotericin B, compared with survival rates of 62% and 54%, respectively, for the drugs alone.<sup>17</sup> A combination of micafungin with ravuconazole in neutropenic rabbits with pulmonary aspergillosis led to significant reductions in mortality, fungal burden and serum galactomannan antigenaemia, compared with either drug alone.<sup>108</sup> However, others have found no interactions *in vivo* for other murine models of aspergillosis for combinations of micafungin with either amphotericin B or itraconazole.<sup>109,110</sup>

**Other combinations.** The combination of rifampicin and amphotericin B was not clearly superior to monotherapy for treating murine pulmonary aspergillosis.<sup>93,111</sup> This combination was also tested in a murine model of fusariosis, but was as ineffective as amphotericin given alone.<sup>73</sup> However, amphotericin B plus rifampicin was more effective than the polyene alone in a model of experimental keratitis due to *C. albicans*.<sup>112</sup>

The activity of fluconazole plus ofloxacin was tested in a murine model of disseminated candidosis, in which the animals were infected with a fluconazole-resistant *C. albicans* strain.<sup>77</sup> The survival of the mice was not prolonged, but the burden of yeast in the kidney and spleen was reduced significantly in groups treated with fluconazole plus ofloxacin. Another study showed that fluconazole, in combination with either trovafloxacin or ciprofloxacin, was effective in treating murine mucormycosis,<sup>113</sup> but was not clearly superior to monotherapy with amphotericin B. Nikkomycin Z and fluconazole or micafungin have been found to be synergistic, respectively, in experimental histoplasmosis<sup>114</sup> and in murine aspergillosis.<sup>110</sup>

Combinations of antifungal agents with non-antimicrobial drugs have shown synergy, for example, fluconazole given with immunomodulators in murine models of systemic candidiasis,<sup>77,115–117</sup> or with cyclosporin in experimental endocarditis due to *C. albicans*.<sup>118</sup>

## Clinical reports on combination of antifungal agents

Many factors have an influence on clinical efficacy, such as the difficulty in diagnosing deep mycosis, the heterogeneity of the patients affected, host immunity, pharmacokinetics and the availability of antifungal compounds at the infection site. Hence, few clinical trials have been conducted and clinical experience is mainly based on individual case reports.

### Drug combinations

**Amphotericin B plus flucytosine.** This combination is considered the standard treatment for cryptococcal meningitis.<sup>119,120</sup> Double-blind multicentre trials showed that treatment with amphotericin B (0.7 mg/kg/day) plus flucytosine (100 mg/kg/day) resulted in an increased rate of cerebrospinal fluid sterilization and decreased mortality at 2 weeks, compared with monotherapy.

However, the combination is not clearly superior for treating other fungal infections. There are reports of clinical improvement or recovery after combination therapy with amphotericin B plus flucytosine in fungal infections that were notoriously difficult to treat. In cases of cerebral aspergillosis, mycotic sinusitis and arteritis due to *Aspergillus* spp. and periprosthetic infection due to *C. glabrata*, clinical resolution or dramatic improvement were obtained when combination therapy was given. However, there is likely to be a publication bias in favour of reports of success. Moreover, surgery played an essential role in patient survival.<sup>121–124</sup>

There have been several clinical trials to evaluate the combination of amphotericin B plus flucytosine for treating candidiasis, aspergillosis and other mycoses. A review of their results shows that the combination was similar to, or somewhat better than, monotherapy in terms of efficacy. The adverse effects of combination therapy were similar to those reported for amphotericin B monotherapy. A summary of findings of clinical case series that have evaluated amphotericin B in combination with flucytosine is shown in Table 3.<sup>125–129</sup> Finally, a detailed meta-analysis has been published recently on combination antifungal therapy for invasive aspergillosis involving a total of 249 cases treated with 23 different antifungal combinations.<sup>17</sup> Amphotericin B in combination with flucytosine was used in 49% of cases and resulted in improvement in the majority of patients treated with this combination. Some patients were treated with a triple therapy of amphotericin B plus flucytosine and rifampicin. The authors concluded that it is premature to recommend combinations for general use and each case needs to be addressed individually.

**Amphotericin B plus azole agents.** Successful outcomes with treatments including amphotericin B in combination with an azole agent have been documented in case reports. Therapy with amphotericin B plus fluconazole was used successfully to treat prosthetic valve endocarditis due to *Candida* spp.<sup>130</sup> This combination was also useful for treating systemic infections due to *Trichosporon beigelii* in a patient with burns, and a bone marrow transplant recipient.<sup>131,132</sup> In addition, liposomal amphotericin B plus fluconazole was effective in treating bilateral renal fungal balls due to *C. albicans* in an extremely low birth weight infant.<sup>133</sup> Invasive sinonasal disease due to *Scopulariopsis* and a case of abdominal wall mucormycosis were successfully treated with amphotericin B and

itraconazole.<sup>134,135</sup> This combination was particularly useful in treating cases of aspergillosis.<sup>17,136</sup> The evaluation of the role of antifungal combinations is confounded by many factors, including surgical debridement, variable immune status, different drug doses and the use of immunomodulators. There are also case reports of invasive aspergillosis that failed to respond to this regimen.<sup>137</sup>

Therapy with amphotericin B plus azole agents has been extensively reviewed.<sup>39</sup> In general terms, combinations were not superior to monotherapy.<sup>138</sup> One retrospective study included patients treated for haematological malignancies who had developed invasive pulmonary aspergillosis and were treated with amphotericin B plus itraconazole.<sup>139</sup> Eight of the patients failed to respond to the combination. Notably, 22 patients who received itraconazole with or without amphotericin B were cured or showed improvement. However, surgical resection was performed in 15 cases. Another case series compared 11 patients treated with the combination with 10 patients who had received the polyene alone.<sup>140</sup> Of the patients who received the combination therapy, nine (82%) were cured or improved, and of those who received the monotherapy, five (50%) were cured or improved.

The most convincing clinical trial of combination therapy in *Candida* infections has been published recently.<sup>141</sup> A total of 219 adult non-neutropenic patients were randomized to receive either amphotericin B (0.7 mg/kg/day) plus fluconazole (800 mg/day) or fluconazole (800 mg/day) plus placebo. The overall response rates were 68% for patients receiving combination therapy and 56% for those receiving fluconazole and placebo ( $P=0.043$ ). *Candida* persisted in 6% of subjects treated with the combination and 17% of cases treated with fluconazole alone. Although patients receiving monotherapy had significantly higher Acute Physiology and Chronic Health Evaluation (APACHE) scores, the combination was not antagonistic, the success rate was slightly better and there was more rapid clearance from the bloodstream than was achieved with fluconazole alone.

The potential for antagonism between antifungal agents given sequentially has been an object of concern to some experts. The sequence of amphotericin B followed by itraconazole in treating aspergillosis has been the most studied,<sup>142,143</sup> and appears safe and is recommended in recent therapeutic guidelines.<sup>144,145</sup> Other sequential therapies, such as itraconazole followed by polyenes, have not been analysed extensively. A case series reported seven heart-transplant recipients suffering from invasive aspergillosis.<sup>146</sup> Four patients were treated with itraconazole given parenterally, but amphotericin B was started after 12–26 days of itraconazole therapy, when clinical or radiographic deterioration was observed. Subsequent treatment with the polyene resulted in improvement in every case. No antagonism was noted, but the contribution of the itraconazole therapy to the therapeutic success could not be assessed. Other case reports have evaluated different sequential treatments, but they are too limited to draw any meaningful conclusions.<sup>17</sup>

**Azole agents plus flucytosine.** A regimen combining an azole, particularly fluconazole, with flucytosine is considered a suitable alternative for treating cases of cryptococcosis that fail to respond to conventional therapies.<sup>147–150</sup> Some clinical trials have shown such combinations to be effective and safe,<sup>151,152</sup> and to have the advantage of obviating the need for parenteral access required for amphotericin B infusion and lowering the risk of



**Table 3.** Summary of findings of clinical case series of amphotericin B in combination with flucytosine

Variable analysed	Clinical trial and reference				
	Smego <i>et al.</i> <sup>126</sup>	Goldman <i>et al.</i> <sup>125</sup>	Verweij <i>et al.</i> <sup>124</sup>	Abele-Horn <i>et al.</i> <sup>123</sup>	Silling <i>et al.</i> <sup>122</sup>
Year	1984	1993	1994	1996	1999
Type of study	retrospective	retrospective	prospective	prospective	prospective
Number of patients	17	62	28	72	98
Underlying conditions	neonates	neutropenic	neutropenic	intensive care	neutropenic
Type of mycoses	<i>Candida</i> meningitis	infection by <i>Candida krusei</i>	invasive mycosis, including 18 aspergillosis	systemic candidiasis	febrile neutropenia
Combination therapy	several doses of AMB plus FC	several doses of AMB plus FC	AMB 0.5 mg/kg/day plus FC 150 mg/kg/day	AMB 1–1.5 mg/kg/day plus FC 100 mg/kg/day	AMB 0.5–1 mg/kg/day plus FC 150 mg/kg/day
Monotherapy	several doses of AMB	AMB 0.5 mg/kg/day or AMB 1 mg/kg/day	AMB 0.5 mg/kg/day	FLZ 400 mg/day	FLZ 400 mg/day or FLZ 800 mg/day
Response rate with combination versus monotherapy	combined superior <sup>a</sup>	combined superior to low-dose AMB	similar	similar <sup>b</sup>	similar <sup>c</sup>
Adverse events with combination versus monotherapy	similar	similar	similar	superior with combination	superior with combination

AMB, amphotericin B; FC, flucytosine; FLZ, fluconazole.

<sup>a</sup>Not statistically significant.

<sup>b</sup>Combination was significantly better in cases of *Candida* peritonitis.

<sup>c</sup>Combination was significantly better in cases with radiological signs of pneumonia.

nephrotoxicity, since fluconazole and flucytosine can be administered orally. However, the clinical efficacy of azole agents plus flucytosine for other fungal infections has not been properly evaluated and there are only case reports available. A patient with non-Hodgkin lymphoma suffering from pulmonary aspergillosis was successfully treated with amphotericin B followed by a combination of oral itraconazole plus flucytosine.<sup>122</sup> In addition, fluconazole in combination with flucytosine was used successfully for treating patients with candidaemia and renal failure, and in some cases of candidaemia due to fluconazole-resistant isolates.<sup>153,154</sup> A double-blind, randomized, controlled trial compared the efficacy of fluconazole with that of itraconazole plus flucytosine for the treatment of oesophageal candidiasis in patients with AIDS.<sup>155</sup> The trial included 85 patients who were evaluated, respectively, after 2 weeks and 3 months of treatment by endoscopic and clinical examination. Both therapeutic regimens demonstrated a similar efficacy (>90% of rate of cure), but itraconazole plus flucytosine was better than monotherapy for patients with fluconazole-resistant *Candida* oesophagitis.

**Combinations with terbinafine.** Terbinafine penetrates deep tissues poorly, with most of the administered dose being found in adipose and skin tissues.<sup>105</sup> Hence, there are doubts about using this drug for treating invasive fungal infections. On the other hand, small clinical case series demonstrated the efficacy of the monotherapy with terbinafine in the treatment of refractory pulmonary aspergillosis in patients who were not immunocompromised.<sup>103,104</sup> There are also data to suggest that this drug was effective for treating subcutaneous and systemic mycoses.<sup>156</sup>

A limited, randomized study compared amphotericin B plus placebo with amphotericin B plus terbinafine (750 mg/day) for the treatment of invasive aspergillosis.<sup>17</sup> This showed significantly higher mortality in the combination group. By contrast, a patient with oropharyngeal candidiasis due to a fluconazole-resistant strain of *C. albicans* responded to treatment with a combination of fluconazole plus terbinafine.<sup>157</sup> A case of invasive facial infection due to *Pythium insidiosum* and another of refractory chromoblastomycosis due to *Fonsecaea pedrosoi* were successfully treated with itraconazole plus terbinafine.<sup>158,159</sup> Notably, combinations of voriconazole plus terbinafine with or without aggressive surgical debridement have resulted in the cure or control of deep infections due to *Scedosporium proliferans*.<sup>160–163</sup> This species is resistant to all currently available systemic antifungal agents,<sup>164</sup> and disseminated infections are almost uniformly fatal.

**Combinations with echinocandins.** There are reports of cases of invasive aspergillosis that have responded to caspofungin in combination with either itraconazole or lipid formulations of amphotericin B.<sup>165–168</sup> Breakthrough or successful therapy has also been reported for other mycoses. Caspofungin plus liposomal amphotericin B was useful in treating a visceral mucormycosis.<sup>169</sup> Combined therapy with the echinocandin and itraconazole cured a case of progressive hyalohyphomycosis due to *Paecilomyces lilacinus*.<sup>170</sup> An immune-deficient child with inoperable cerebral phaeohyphomycosis due to *Cladophialophora bantiana* was successfully treated with voriconazole plus caspofungin.<sup>171</sup> Although the patient died, the natural rapid progression of the infection was altered by the

combination therapy, in the absence of surgery. A case of *S. prolificans*-associated osteomyelitis was successfully treated with debridement, local irrigation with polyhexamethylene biguanide, and the systemic administration of voriconazole and caspofungin.<sup>172</sup>

A retrospective study included 48 patients with proven, probable or possible invasive aspergillosis.<sup>173,174</sup> The majority of patients (65%) received caspofungin plus liposomal amphotericin B as salvage therapy for progressive infection after at least 7 days of monotherapy. The response rate was 42% and no significant toxic effects were described, but the response rate for patients with documented infections was dramatically lower (18%).

Sequential therapy with echinocandins has not been analysed *in vivo*, but there is some evidence to support this approach since sequential exposure of *A. fumigatus* to itraconazole followed by caspofungin resulted in enhanced activity of the echinocandin against the isolates.<sup>175</sup>

**Other combinations.** Amphotericin B or azole agents in combination with rifampicin was used some years ago to treat aspergillosis.<sup>17,176</sup> Combination therapy resulted in improvement in most cases, but the varying degree of immune suppression and differences in types of infection makes evaluation difficult. Clinical reports of combinations with other antibacterial agents and other classes of compounds are too scarce to make generalizations,<sup>78,86,177</sup> although there are some promising data about combinations of cytokines and other immunomodulators with antifungal agents.<sup>135,178–182</sup> An extensive review on immunotherapy for treating invasive aspergillosis has been recently reported, to which the interested reader can refer.<sup>115</sup>

## Conclusion

What value are combinations of antifungal agents for therapy? Our understanding of the efficacy of combination therapy is based largely on the results of studies conducted *in vitro* and in experimental animal models. *In vitro* studies have yielded controversial results that are highly dependent on the criteria used to evaluate the antifungal interaction and vary from strong synergy to overt antagonism. Antagonism has been seldom described for some combinations such as amphotericin plus flucytosine, azole agents plus flucytosine, azoles plus terbinafine and combinations with echinocandins. However, overt antagonism has been frequently observed for amphotericin B in combination with either azole agents or terbinafine.

Laboratory results need to be correlated with clinical outcomes, and experimental animal models can bridge the gap between *in vitro* and clinical evaluation of antimicrobial agents. Notably, the synergy observed *in vitro* for several combinations was not found *in vivo*. The majority of studies in animal models found no interactions highlighting the difficulty in determining synergy *in vivo*. In addition, conflicting results may be attributed to the method used to evaluate the interaction. The enhanced activity *in vivo* of combinations has usually been defined by lower tissue burden rather than by better survival. Moreover, the majority of synergistic interactions defined by significant decreases in organ burden were classified as no interactions in terms of survival rates.

The clinical efficacy of combination therapy relies heavily on case reports; series with clinical trials are too scarce to draw any

firm conclusions. However, some trends can be detected. Amphotericin B plus flucytosine is superior to single-agent therapy with the polyene for treating cryptococcal infections but not for other fungal disease. Overall, amphotericin B plus azole agents have not been found superior to monotherapy with the polyene, and antagonism has been described *in vitro* and in animal models. Azole agents plus flucytosine are similar to azole monotherapy in term of clinical efficacy, but these combinations may provide an alternative for treating patients suffering from cryptococcal infections and infections due to azole-resistant *Candida* spp. **Amphotericin B plus terbinafine is not effective in combination against *Aspergillus* spp. and antagonism has been described. Azole agents plus terbinafine and combinations with echinocandins have been shown to be effective in some cases of deep mycoses, although the literature is probably biased towards reports of success.**

There is insufficient evidence to make any recommendations for combination therapy and it is premature to use it for the majority of cases. As Johnson *et al.*<sup>4</sup> have reported recently, the use of combination therapy will be considered in unique settings. Combination therapy could provide an alternative to monotherapy for patients with invasive infections that are difficult to treat due to multiresistant species such as *S. prolificans*, *Fusarium* spp., *Scopulariopsis brevicaulis*, *P. lilacinus* and *Rhodotorula* spp. **In addition, combination therapy, for instance with azole agents plus either flucytosine or terbinafine, could be used for patients who fail to respond to standard treatment.** However, there is a pressing need for the conduction of randomized controlled trials and detailed prospective observation studies to determine the efficacy and toxicity of combinations.

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## Review

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