Combination Antifungal Therapy: From Bench to Bedside

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Invasive fungal infections are major causes of mortality in immunocompromised patients. Despite improved outcomes with new antifungals, there remains a pressing need to further improve outcomes, especially with invasive aspergillosis and other invasive mold infections. Combination antifungal therapy is an attractive option that offers the prospect for improved efficacy, decreased toxicity, reduced likelihood for the emergence of resistance, and shorter courses of therapy. The current available evidence regarding the role of combination antifungal therapy for invasive fungal infections is discussed in this article, including data from in vitro studies, animal models, and human clinical trials to try to clarify this important issue. Randomized, prospective clinical trials are urgently needed, especially for invasive aspergillosis.

Introduction

In immunocompromised patients, invasive fungal infections (IFI) are major causes of mortality. Invasive *Aspergillus* (IA) and non-*albicans Candida* infections are gaining importance as causes of IFI and response rates are suboptimal. Invasive zygomycosis is likewise a growing fungal threat with suboptimal treatment prospects. Combinations of drugs have been found to be more useful and more effective than monotherapies in the treatment of certain neoplastic diseases, certain severe bacterial infections, and HIV infection. Accordingly, combination antifungal therapy seems to be an appealing option.

The rationale for combination antifungal therapy is that treatment outcomes may be improved in several ways. First, therapeutic efficacy may be improved by the use of two or more active drugs than by any one drug alone. This can happen by more complete or more rapid eradication of organisms or differential penetration of drugs into various infected tissues. Second, there may be a lower risk for the emergence of resistance. If a pathogen becomes resistant to one drug, the other drug would prevent it from emerging as a cause of clinical treatment. Third, there may be less toxicity with shorter courses of treatment or lower doses of individual drugs. Fourth, one can achieve broader coverage when the target pathogen may not be known for certain or where mixed infection is possible.

The mechanisms by which drug combinations can increase pathogen kill are either by 1) each drug simultaneously attacking the organism at two independent biochemical targets or metabolic pathways or 2) each drug aiming at different points in the same metabolic pathway. For fungi, most drugs that have been studied in combination act on one of several fungal targets: ergosterol in the cell membrane (eg, from binding by polyenes or inhibition of biosynthesis by azoles or allylamines), (1,3)-β-D-glucan in the cell wall (eg, by inhibition of biosynthesis by echinocandins), or DNA synthesis (eg, by inhibition by flucytosine, a pyrimidine analog).

Although the benefits of combination therapy are appealing, it is important to note that there are risks that may offset the potential value. Efficacy may be attenuated due to antagonistic effects of the drug combination. Toxicity may be greater, due to drug interactions. The cost would certainly be greater. These considerations make it imperative that careful evaluation of drug combinations be undertaken.

What is the nature of the evidence at present regarding the role of combination antifungal therapy for IFI? In this article, data from in vitro studies, animal models, and human clinical trials are reviewed to clarify this important issue. In vitro studies and animal models currently provide much of the support for combination antifungal therapy. However, the methods used in these studies are not fully standardized and may not be entirely relevant to humans. The design and conduct of clinical trials of combination antifungal therapy for IFI is challenging. In this discussion, we emphasize findings concerning *Aspergillus* and other mold organisms as these are the most challenging IFI today.

In Vitro Studies of Antifungal Combinations

The aim of in vitro testing is to identify whether drugs exert synergistic or antagonistic effects rather than additive

or indifferent effects [1]. Several methods have been used to determine the effects of drug combinations in vitro. The most common laboratory tests are the checkerboard dilution method, time-kill method, and Epsilometer test [2••]. By far, the checkerboard method is the most commonly used. In this method, synergism exists if there is a decrease in the minimum inhibitory concentration (MIC) of either drug in the presence of the second drug. An antagonistic effect is defined as an increase in MIC of either drug in the presence of a second drug. The checkerboard dilution method has varying MIC endpoints for different drugs. For example, the amphotericin B MIC is read as 100% reduction in turbidity. On the other hand, MICs for azoles are defined by 80% reduction in turbidity. These assessments are made visually [3]. This can falsely reduce the MIC of azoles when tested with amphotericin B [4].

Time-kill methods can detect variations in antifungal activity over time. When time-kill data are added to sigmoidal dose-response models, fungal pharmacodynamics are found to be species-specific [5,6]. For example, amphotericin B has rapid fungicidal activity in vitro against *Candida albicans* and some *Aspergillus* species as exemplified by a steep dose-response curve. In contrast, azoles, such as voriconazole, have steep dose-response curves for molds but a shallow dose-response curve for *C. albicans* [7].

The advantages and drawbacks of these various methods to assess antifungal drug combinations have been reviewed [2••,8–13]. The major limitations of in vitro assays are that in vitro and in vivo correlations have yet to be well established, standardization of echinocandin testing has not been achieved, and filamentous organisms are more problematic to assess than yeast organisms. Moreover, different results have been seen using different fungal species and strains and varying antifungal drug concentrations.

Candida

Results have been variably synergistic, indifferent, and occasionally antagonistic with various combinations such as amphotericin B + fluconazole, fluconazole + flucytosine, and amphotericin B + flucytosine [2••]. Combinations of voriconazole + fluconazole, micafungin, and amphotericin B have demonstrated indifference to synergistic effects but no antagonism. The combination of amphotericin B and caspofungin demonstrated additive effects. A human recombinant monoclonal antibody against heat shock protein 90 found in fungal cell walls (Mycograb [NeuTec Pharma; Manchester, UK]) was noted to be synergistic in vitro with amphotericin B against *Candida* species [14•].

Cryptococcus

Several combinations—such as amphotericin + fluconazole, itraconazole, or posaconazole and flucytosine + itraconazole or posaconazole—have been tested. Studies of these combinations have mostly demonstrated additive or synergistic effects [2••].

Aspergillus

Several studies (but not all) demonstrated synergy with voriconazole or ravuconazole or posaconazole plus an echinocandin [2••,15–17]. Similarly, amphotericin B plus an echinocandin demonstrated additive or synergistic effects [18]. The combination of terbinafine with several different third-generation azoles has been generally associated with synergy [19,20].

To summarize, the value of in vitro assays is that information can be gained to guide additional testing in vivo. Findings to date have been quite variable with conflicting signals between different methods of in vitro assessments. Such variations appear to be attributable to a number of reasons, such as different types of fungal strains and species, different inoculation size, different drug concentrations, varying definitions of endpoints, and variations in measurements. Furthermore, correlations between animal model data and clinical trial findings have not adequately been studied, and what little data exist often do not show consistency between the in vitro data and the in vivo findings. Clearly, standardization of methodologies, uniformity of definition of endpoints, and correlational studies with animal models (and clinical trials) are needed.

Animal Models to Assess Combination Antifungals

Animal studies are necessary for screening combination antifungal therapy because of methodologic limitations of in vitro studies as detailed above. In addition, animal models allow the exploration of drug absorption, metabolism, and tissue distribution, pharmacodynamics, and toxicities that cannot be examined by in vitro testing.

Candida

The combinations of fluconazole + amphotericin B or flucytosine have had variable findings in animal models, including some data in one model suggesting antagonism [2••]. Caspofungin and amphotericin B demonstrated synergistic activity in a murine model [21•].

Cryptococcus

The combination of amphotericin B and flucytosine was found to be synergistic against cryptococcosis [22]. Similarly, azoles plus flucytosine have demonstrated synergy.

Aspergillus

Considerable attention has been devoted to *Aspergillus* in recent years. The combination of caspofungin and voriconazole demonstrated synergy against IA in a guinea pig model with respect to reductions in tissue colony counts, but survival was no different compared with monotherapy [23]. Micafungin and ravuconazole combination therapy showed synergistic action in a neutropenic rabbit model with IA [15]. The combination of amphotericin B plus an echinocandin has also been found to reduce tissue levels

of organisms to lower levels than either single drug alone in a chronically immunosuppressed murine model [24].

Zygomycetes

Combination therapy with amphotericin B lipid complex and caspofungin was compared with amphotericin B lipid complex alone for disseminated zygomycosis in diabetic ketoacidotic mice [25]. Even though caspofungin has no clinically relevant activity against zygomycetes, the combination therapy arm significantly increased the time to death compared with single-agent therapy with amphotericin B lipid complex. Deferasirox, an iron chelator that deprives fungi of iron, has been found to have synergistic activity with liposomal amphotericin B in a murine model [26•].

Animal studies have shortcomings, and these have been reviewed [2••,8–13,27]. Animal models lack standardization of methods for assessing combined antifungal effects, making direct comparisons between these studies difficult. Variations in study design, study endpoints, durations of antifungal therapy, and the sequence of antifungal therapy, make the precise interpretation of antifungal combination therapy efficacy challenging. In addition, antifungal pharmacokinetics have not been entirely documented in animal models and may vary from one animal species to another and may differ from humans. Subtle changes in antifungal efficacy are difficult to detect because many animal studies are underpowered. Animal models typically involve inducing hyperacute fungal infections in an immunosuppressed state by injecting a large inoculum of a single fungus, which results in a high death rate within a matter of days. In contrast, in human fungal infections, there is usually a slower course of action with a complex interplay of host factors contributing to immunosuppression. Colony counts are used to assess antifungal efficacy and this can lead to higher response rates than are seen in human studies; moreover, for certain antifungal drugs that cause fragmentation of organisms, colony counts may not correlate with fungal biomass. Finally, drugs are often administered in subtherapeutic doses (so that not all the animals are cured by either of the individual drugs) by either of the individual drugs; whether this is relevant to the human situation is questionable, and whether interactions at subtherapeutic doses would also be present at therapeutic doses is not clear.

Human Clinical Trials of Combination Antifungals

Assessment of antifungal efficacy in human clinical trials is difficult. Success of a therapy is typically made by subjective assessments of clinical outcomes because surrogate markers—which can more precisely and reliably estimate fungal burden, such as enzyme-linked immunosorbent assay for fungal antigens or polymerase chain reaction for fungal DNA—have not been validated for assessing therapeutic antifungal efficacy in humans. The efficacy of antifungal drugs is also influenced by the interplay of complex host factors, such as changes in immunosuppression, sites of infection, and drug pharmacokinetics, which cannot be predicted by in vitro studies or animal models. These considerations make interpretation of clinical trial results difficult.

Candida

In a randomized, blinded, multicenter trial, 236 non-neutropenic patients with candidemia due to species other than *Candida krusei* were given either high-dose fluconazole (800 mg/d) plus placebo (for an average of 5–6 days with therapy continued with fluconazole) or high-dose fluconazole + amphotericin B (0.7 mg/kg/d for an average of 5–6 days with continuation of fluconazole therapy) [28]. At day 30, 57% versus 69% (P = 0.08) in the fluconazole + placebo arm versus fluconazole + amphotericin B arm had successful sterilization of the bloodstream, but this was not statistically significant. The combination therapy arm (fluconazole and amphotericin B) had faster clearance of fungi from the bloodstream (94% vs 83%; P = 0.02). On further analysis, there were higher Acute Physiology and Chronic Health Evaluation II (APACHE II) scores in the fluconazole and placebo arm. Response rates decreased as APACHE II scores increased. Combination therapy with fluconazole and amphotericin B was better for those patients with intermediate APACHE II scores [28]. Even the short exposure to amphotericin B in the combination arm was associated with higher nephrotoxicity (23% vs 3%) than in the placebo arm. Based on this trial, the Infectious Diseases Society of America *Candida* consensus guidelines state that the fluconazole and amphotericin B combination is an option for the treatment of invasive candidiasis [29].

Most clinical trials of antifungal therapy for invasive candidiasis have been conducted in non-neutropenic patients with less severe forms of infection. There are also case reports of successful antifungal combination therapy after failure of single-agent fungal therapy in immunosuppressed patients with severe candidiasis (for example, meningitis, hepatosplenic candidiasis, and endocarditis) [30]. These suggest that the combination improved antifungal activity, but these observations need to be validated in controlled clinical trials of invasive candidiasis in immunosuppressed patients.

In a multicenter, blinded, randomized study in patients with culture-confirmed invasive candidiasis, a complete response was present in 48% of patients given amphotericin B alone versus 84% in the Mycograb + amphotericin B combination group ($P < 0.001$). *Candida* attributable mortality was also reduced (18% vs 4%). Mycograb was well tolerated [14•]. Novel agents, such as Mycograb, may in the future be an adjunct to IFI therapy. A variety of immunotherapies incorporated as adjuncts to antifungal agents also are promising [24].

Cryptococcus

In a randomized, double-blind, multicenter trial, patients with a first episode of AIDS-associated cryptococcal meningitis were randomized to 2 weeks of amphotericin B (0.7 mg/kg/d) with or without flucytosine (100 mg/kg/d) followed either by itraconazole 400 mg/d or fluconazole 400 mg/d for a further 8 weeks [31]. Successful therapy was defined as clearance of cerebrospinal fluid (CSF) cultures by 2 weeks and 10 weeks or clinical stability at 2 weeks and absence of symptoms at 10 weeks. CSF cultures were negative at 2 weeks in 60% of those receiving amphotericin B and flucytosine combination therapy and in 51% of the single-agent amphotericin B arm ($P = 0.06$). At 10 weeks, 72% of patients given fluconazole and 60% of those given itraconazole had sterilization of the CSF. Clinical outcomes including clinical responses and overall mortality were similar in the two groups. However, the addition of flucytosine in the first 2 weeks and fluconazole therapy for a further 8 weeks was independently associated with clearance of the CSF. Therefore, the amphotericin B and flucytosine combination has been recommended as induction therapy in cryptococcal meningitis. In another randomized prospective clinical trial, 64 AIDS patients with their first episode of cryptococcal meningitis were randomized to amphotericin B (0.7 mg/kg/d) or amphotericin B + flucytosine (100 mg/kg/d) or amphotericin B with fluconazole (400 mg/d) or triple therapy with amphotericin B, flucytosine, and fluconazole [32]. The primary endpoint was fungicidal activity as assessed by the rate of clearance of cryptococcal colony forming units in serial quantitative CSF cultures measured at day 3, 7, and 14 of therapy. CSF cryptococcal colony forming unit clearance was exponential and significantly faster with the amphotericin B and flucytosine combination. Triple therapy was not better than the combination of amphotericin B and flucytosine. The decision to use the amphotericin B + flucytosine combination for cryptococcal meningitis is now the standard of care in this disease.

Aspergillus

Most IA infection studies assessing antifungal therapy are small, uncontrolled, retrospective efforts involving single institutions without a comparative group. These studies seldom distinguish between documented (proven or probable) and possible IA infection, which is important as documented IFI generally have a worse prognosis [33]. Patients with persistent neutropenia and IA infection have a poorer response to combination antifungal therapy, but only a few reports account for neutrophil recovery when analyzing response to antifungal therapy [34]. Similar to invasive candidiasis, the complex interplay of host factors, such as comorbidities, sites of infection, and immunosuppression, can impact the efficacy of antifungal therapy in invasive mold infections.

In a retrospective study of patients with hematologic malignancies, patients with definite or probable (23 patients) or possible (25 patients) IA infection were given either salvage caspofungin + liposomal amphotericin B after failure of 7 days of liposomal amphotericin B or primary caspofungin + liposomal amphotericin B [35]. The combination of caspofungin and liposomal amphotericin B was better when used for primary rather than salvage therapy in documented IA infection (response rates of 53% vs 35%). No clinically significant toxicity was observed. The response rate in patients with progressive, documented IA infection was only 18%. Treatment failure occurred more in those patients with documented IA infection who had received steroids before the study or those patients who received less than 14 days of combination antifungal therapy. Only 38% of those with persistent neutropenia responded to the caspofungin and liposomal amphotericin B combination [35]. In another retrospective study of 30 patients with acute leukemia, IA infection was treated with caspofungin plus an amphotericin B formulation [36]. Favorable responses were seen in 60% of patients. Only 33% of patients were proven or probable IA; unfortunately, most patients were only "possible" cases. In a retrospective survey of the combination of itraconazole + lipid amphotericin B versus lipid amphotericin B alone given as primary therapy in patients with hematologic malignancies, there did not appear to be any differences in response at end of therapy or survival [37].

In another retrospective study, hematopoietic stem cell transplant or chemotherapy patients with IA infection who had failed primary therapy with an amphotericin B formulation received either voriconazole alone ($n = 31$) or a combination of voriconazole and caspofungin ($n = 16$) as salvage therapy. The voriconazole and caspofungin combination was associated with an improved 3-month survival rate in comparison to single-agent voriconazole (hazard ratio, 0.42; 95% CI, 0.17–1.1; $P = 0.048$). Multivariable models showed that the voriconazole and caspofungin combination was associated with reduced mortality independent of other prognostic factors. The lowest probability of death from IA was in those who received the combination antifungal regimen [38]. A follow-up analysis of these cohorts showed no difference in survival at 1 year [39]. The strengths of this study are the fact that only documented cases of IA infection (proven or probable) were included and the endpoint of death is indisputable. Its shortcomings are the retrospective design, the small sample size, the possibility of changes in the manner in which clinicians declared "failure" of first line therapy, and an earlier switch to salvage that may have occurred in more recently treated patients (the group that got the combination therapy), which may have given a more favorable predisposition to the combination regimen.

In a prospective, multicenter observational study, 40 solid organ transplant recipients with IA infection (proven or probable) were given front-line combination therapy with voriconazole and caspofungin. This cohort was compared with a historical control group of 47 solid organ

transplant recipients who had received a lipid formulation of amphotericin B for IA infection. Overall survival at 90 days was not different (67% vs 51%; $P = 0.11$). However, combination antifungal therapy was independently associated with an improved 90-day survival in a multivariate analysis in those with renal failure and in the subset of patients with *Aspergillus fumigatus* infection (adjusted hazard ratio 0.37; 95% CI, 0.16–0.84; $P = 0.019$) [40•]. Of interest, in this study, no correlation was found between clinical outcome and the in vitro antifungal interactions of the *Aspergillus* isolates to the combination of voriconazole and caspofungin.

Although the clinical experience appears to support some optimism for added benefits with combination therapy, clearly the data are not sufficient to allow definitive conclusions. Only randomized, prospective studies can determine if the combination of voriconazole and caspofungin should move to the front line in the therapy of IA infection.

Zygomycetes

The efficacy of combination antifungal therapy using liposomal amphotericin B + caspofungin for zygomycosis has been explored only in case reports. For example, a patient with acute myelogenous leukemia and rhinocerebral zygomycosis unresponsive to liposomal amphotericin B responded to the addition of caspofungin [41]. In an open-label study of 91 patients, posaconazole was started in those with proven (76%) or probable (24%) zygomycosis who were refractory to 7 or more days of previous antifungal therapy [42]. Thirteen of the 91 patients received combination therapy with amphotericin B and posaconazole. After 12 weeks, there was a 60% overall response rate (46% partial response and 14% complete response) with a 17% failure rate in the posaconazole arm. In the amphotericin B and posaconazole combination therapy arm, there was a partial response of 46% and a failure rate of 31%. However, combination antifungal therapy was preferentially administered to patients with more serious infection with poorer performance status. Furthermore, this was not a randomized study, so no firm conclusions can be drawn about the role of combination antifungal therapy in zygomycosis. A randomized phase 2 trial to evaluate the combination of liposomal amphotericin B with deferasirox (an iron chelator that like deferiprone and in contrast to deferoxamine deprives zygomycetes of the ability to uptake iron needed to support its growth) is now underway.

Conclusions

The data accumulated so far from in vitro, animal models, and clinical trial experience indicate that the decision to use combination antifungal therapy for cryptococcal meningitis is evidence based. For non-neutropenic patients with invasive candidiasis, single-agent antifungal therapy remains the primary treatment. However, for more severe cases of invasive candidiasis (such as meningitis, endophthalmitis, endocarditis, or hepatosplenic disease) or refractory invasive candidiasis, combination antifungal therapy (amphotericin B + fluconazole) is an option. The aggregate of current data does not support the primary use of combination antifungal therapy for IA infections. However, for patients who develop breakthrough infections while receiving first-line monotherapy or have more resistant molds, combination antifungal therapy may provide additional antifungal activity and may be warranted. Nonetheless, large, multicenter, randomized, prospective trials comparing combination antifungal therapy to single-agent antifungal therapy for IFI as first-line therapy are needed to achieve the power required to detect a difference between the two treatments. Randomized, prospective clinical trials are especially needed for IA. Better diagnostic tests need to be developed to rapidly and accurately diagnose fungal infections and thereby allow faster enrollment and completion of clinical trials on a smaller scale. Combination antifungal therapy assessments also need to be standardized. The development of valid surrogate markers is also needed to allow more reliable assessment of clinical responses.

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No further conflict of interest information relevant to this article was reported.

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