HHS Public Access

Author manuscript

Med Mycol. Author manuscript; available in PMC 2015 May 25.

Published in final edited form as:

Med Mycol. 2011 April ; 49(0 1): S77–S81. doi:10.3109/13693786.2010.499374.

Newer Combination Antifungal Therapies for Invasive Aspergillosis

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Abstract

Optimal therapy for invasive aspergillosis is unknown, and many clinicians have attempted to utilize a combination antifungal approach to improve outcomes. However, while numerous *in vitro* studies, animal models, and clinical reports suggest the possibility that combination antifungal therapy might offer improved results, there is no definitive accepted strategy. The current available antifungals used in various combination approaches have not demonstrated a clear improvement over monotherapy. The current classes of drugs targeting the cell wall and cell membrane may need adjunctive agents focused on separate cellular pathways, such as cell stress response or cellular signaling, to maximize efficacy. The calcineurin pathway and the Hsp90 pathway are two such untouched arenas in which targeted manipulation may lead to great advances against aspergillosis.

Keywords

aspergillosis; Aspergillus; combination; antifungal; calcineurin

Introduction

In the last decade, there has been a surge of development of newer antifungals for invasive aspergillosis (IA), creating new hope for treatment and increasing the permutations of new potential combination therapies. Drawing from other infectious diseases such as HIV, tuberculosis, and cryptococcal meningitis [1], combination therapy for IA seems plausible to optimize therapy. Although no controlled clinical trial supports it use and the efficacy of combination therapy for IA has not been conclusively established [2], clinicians are desperately seeking new strategies to improve outcomes.

However, the existing antifungal classes have not shown tremendous advances beyond the use of voriconazole monotherapy compared to amphotericin B. While the advent of voriconazole [3] therapy has greatly improved survival, it has been difficult to make further advances. Nearly every possible combination of the existing agents, including mixing

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different classes of drugs to better attack diverse targets, have been tested *in vitro* and some *in vivo*. Despite these efforts, little progress has been made on the combination antifungal front to optimize the possible clinical benefit from the combination approach.

There are several foreseeable advantages to combination antifungal therapy: a widened spectrum and potency of drug activity, more rapid antifungal effect, synergy, lowered dosing of toxic drugs, and a reduced risk of antifungal resistance[4]. While each individual antifungal agent has limitations, combinations might prove more effective, as seen with the now standard highly-active anti-retroviral therapy used with HIV patients. Utilizing agents with different mechanisms of action is a hallmark in current medical therapies in numerous medical disciplines, but of course one has to be cautious that some combinations may be antagonistic or clinically indifferent with additive side effects. More is not necessarily better, but the current antifungal approach is clearly not optimal as patients continue to die from IA in increasing numbers.

Brief Review of Important Animal Model Data on Combination Antifungal Therapy

Studying combination antifungal therapy in animal models of IA has historically been problematic for numerous reasons. The very reason for studying infection in animals is to adequately and appropriately mimic human disease. However, many studies have used relatively artificial means of generating invasive disease such as intravenously administering an *Aspergillus* inoculum at high concentrations, which may limit extrapolation to clinical disease. Other issues with antifungal testing in animals center on relevant dosing, as for instance most studies have not measured serum or tissue azole levels to ensure adequate exposure and consistent levels that would be obtained in a patient.

The most often quoted *in vivo* combination antifungal animal model study examined voriconazole + caspofungin [5]. In this immunosuppressed guinea pig model of intravenously inoculated invasive aspergillosis, treatment with amphotericin B or caspofungin (1 mg/kg/d or 2.5 mg/kg/d) resulted in 30–50% mortality. However, treatment with voriconazole or voriconazole plus either caspofungin dose resulted in no mortality (0/12 in all experimental arms). Although this report ignited excitement, this guinea pig model actually showed no difference in mortality or mean times of survival in the combination therapy arm compared to voriconazole monotherapy. Semi-quantitative organ cultures for fungal burden (CFU/gram of tissue) from the liver, lung, kidney, and brain in the combinations were better than untreated controls ($p < 0.0025$), but there is no report of any difference between the combination therapy and voriconazole monotherapy. Unfortunately, on the basis of this study, no definitive role for combination therapy can be established.

A subsequent study of neutropenic guinea pigs inoculated intravenously with *A. fumigatus* used different doses of the same antifungals [6] to further explore the combination approach. In the pilot studies leading up to this work, the investigators found that using voriconazole at the same doses as used before for 7 days led to survival of all animals even with a higher challenge infection dose, while a lower dose of voriconazole (1 mg/kg) led to only marginal

prolongation of survival. Therefore, this study was designed to purposefully minimize the possible therapeutic effects of each drug alone in an effort to demonstrate any enhanced effects of the combination. The infections were initiated with two different challenge doses of *A. fumigatus* as an additional methodology of lessening the impact of the disease and allowing even small combination benefits to shine through. The survival curves for the higher challenge dose analyzed by log rank revealed statistically significant prolongation of survival for caspofungin (1 mg/kg/d) ($p=0.002$) and the combination of voriconazole (1 mg/kg BID) + caspofungin ($p=0.0004$) over untreated controls, but no other comparisons were significant. From a survival benefit, there was no advantage with the combination approach. When analyzing fungal burden with quantitative PCR, the same results appeared. In the end, these investigators confirmed a benefit of the combination of voriconazole + caspofungin over monotherapies, but found the benefit was very small and did not impact survival.

These studies and a collection of other animal model experiments offer no clear-cut benefit to combination antifungal therapy against IA. Numerous studies have consistently revealed the importance of exact experimental conditions in determining outcome, demonstrating that manipulating inoculum or immunosuppression conditions is often more telling than the antifungal agents used themselves. Since animal models serve only as surrogates of clinical medicine, the real test lies in clinical responses to combination therapy. Perhaps the future lies in novel approaches, possibly newer targets such as iron chelation [7] or newer signaling pathways. Regardless, a fresh perspective might be required for real clinical advances.

Brief Review of Important Clinical Data on Combination Antifungal Therapy

The largest analysis of combination therapy for IA reviewed 6,281 total cases of IA management from 1966–2001. The 249 clinical cases reviewed comprised a total of 23 different antifungal combinations, including 16 unique double antifungal and 7 triple antifungal regimens. A total of 64% of patients showed improvement, with mortality from IA at 34%. While a response rate of 64% is encouraging, there are obvious limitations to a retrospective review. Additionally, this exhaustive review is now dated as the older combination used are not employed by contemporary clinicians with a much larger arsenal of available antifungal agents.

In a prospective, open study, patients with proven or probable IA received either a combination of standard dose (3 mg/kg/day) liposomal amphotericin B and caspofungin versus high-dose liposomal amphotericin B (10 mg/kg/day) monotherapy [8]. There were significantly more favorable overall responses (10/15) and survival (100%) in the combination group compared with the high-dose monotherapy group (4/15; 80% survival). This study implied that two agents using different mechanisms of action (cell membrane and cell wall) are preferred over even a larger dose of a proven agent.

However, using the principle of two mechanistically different agents, the antifungal combination of a triazole + echinocandin, most commonly voriconazole + caspofungin, appears to be the most frequently used in the last decade. This is likely due to the hypothesized synergistic interaction due to the simultaneous inhibition of 1,3-β-D-glucan

synthesis in the fungal cell wall and ergosterol synthesis in the fungal cell membrane [5, 9]. One study to help address this issue of voriconazole + caspofungin was a retrospective review of 47 patients with proven or probable IA from 1997–2001 who experienced failure of primary therapy with amphotericin B formulations.[10] Salvage therapy was begun with voriconazole (n=31) versus voriconazole + caspofungin (n=16) after ≥ 7 days of amphotericin B therapy.

The overall survival rate three months after the day of diagnosis of IA was higher among those who received combination therapy (p=0.048). Similarly, three-month survival after the start of salvage therapy was greatest amongst combination therapy patients (HR 0.43; 95% CI 0.17–1.1, p=0.050). However, an accompanying editorial to this article pointed out the preclinical data with combination therapy seem promising, yet inconsistent [11]. The authors then analyzed the patients at one year found there was no difference in overall survival (p=0.26). Other clinical retrospective reviews have yielded generally similar lack of an obvious benefit with combination therapy, focusing instead on the important contribution of underlying disease and immune reconstitution to outcome. Without a large scale clinical trial using combination antifungal therapy, we are relegated to these *in vitro*, *in vivo*, and smaller clinical (often retrospective) reviews of the field to make today's clinical judgments.

The current Infectious Diseases Society of America guidelines for treating IA [12] state that combination therapy does have a potential role in therapy, but the data are obviously unclear. Likely as important is maximizing drug levels of effective azoles through adequate therapeutic drug monitoring and the need for early and accurate diagnosis with non-culture-based methodology. Throughout all IA studies ever performed, immune reconstitution remains of paramount importance to overall host response.

Tackling a Novel Antifungal Approach – The Calcineurin Pathway

The *A. fumigatus* calcineurin pathway is an exciting and currently untapped antifungal target that can be exploited to improve the treatment and prevention of IA. Calcineurin is a conserved serine-threonine-specific Ca2+-calmodulin-activated protein phosphatase important in mediating cell stress responses[13]. It is a heterodimer composed of a catalytic A and a regulatory B subunit and upon mobilization of calcium stores the catalytic subunit is bound by Ca2+-calmodulin [14]. Calcineurin functions in fungi through the transcription factor CRZ1 (calcineurin-responsive zinc finger), while in human T-cells it dephosphorylates the cytoplasmic component of the nuclear factor of activated T-cells (NF-AT), which is necessary for interleukin (IL)-2 transcription and T-cell activation.

Calcineurin also controls a panoply of signaling molecules, is implicated in a myriad of medical conditions. Calcineurin inhibitors have successfully treated patients with a wide spectrum of diseases (reviewed in [15]). Calcineurin is the target of the immunosuppressants cyclosporine A (CsA) and tacrolimus (FK506)[16]. Clinical experience suggests calcineurin inhibition decreases invasive fungal infections and patient responses to CsA and FK506 support the hypothesis that targeting fungal calcineurin may have clinical benefits. For example, CsA therapy was associated with a 54% decrease in the incidence of IA in heart transplant recipients compared to conventional immunosuppression[17]. Similarly, solid

organ transplant recipients receiving FK506 had significantly lower rates of disseminated IA[18].

Calcineurin inhibition has already been clearly shown to affect pathogenicity, suggesting that optimal inhibition is a viable antifungal molecular target. We deleted calcineurin A (*cnaA*) and showed profound defects in hyphal growth and virulence [19], including a significant lack of normally invading hyphae. The Δ*cnaA* strain was then tested in five fundamentally distinct animal models in two independent laboratories, each with purposefully different immunosuppression and infectious delivery to ensure robust *in vivo* findings. Infection with the Δ*cnaA* strain in the animal models led to a significant attenuation of virulence (10% vs. 90% mortality, $p < 0.001$) compared to the wild-type and complemented strains[19]. We subsequently deleted the calcineurin-responsive zinc finger (*crzA*) and again found defects in hyphal growth as well as avirulence. The *A. fumigatus* Δ*crzA* strain has a less severe hyphal phenotype not as the Δ*cnaA* strain [20]. Unlike the *A. fumigatus* Δ*cnaA* strain, Δ*crzA* hyphae do grow and lung histopathology revealed very stunted hyphae.

Calcineurin appears to control *A. fumigatus* cell wall homeostasis, hyphal growth, and virulence, but the real task is developing novel antifungals that overcome the theoretical impediment of not cross-reacting with human calcineurin which would inhibit the vital T cell activity so crucial to combating IA. There is precedent of success here, as the azole antifungals target ergosterol in the fungal cell membrane, and toxicity occurs due to the overlap with the human bilipid cholesterol membrane. There are definite regions of several key calcineurin pathway genes that are unique from the human counterparts (unpublished data), and those are the focus of concerted efforts to uncover fungal-specific targets.

Calcineurin controlling the echinocandin “paradoxical effect”

In *A. fumigatus*, pharmacologic inhibition of calcineurin or deletion of calcineurin pathway components in combination with caspofungin treatment has been shown to attenuate growth and enhance cell wall damage [21, 22]. Previously, we showed that echinocandin treatment of *A. fumigatus* produced an increase in cell wall chitin content and that the calcineurin pathway plays an important role in this response [23]. Attenuated activity of echinocandin antifungals at high concentrations, known as the “paradoxical effect”, is a well-established *in vitro* phenomenon in *Candida albicans* and *Aspergillus fumigatus* with potentially important clinical implications. We showed that treatment of the *A. fumigatus* Δ*cnaA* and Δ*crzA* strains, harboring gene deletions of the calcineurin A subunit and calcineurin-dependent transcription factor respectively, with high concentrations of caspofungin revealed that the *A. fumigatus* paradoxical effect is calcineurin pathway-dependent. We also found that caspofungin treatment resulted in increased chitin synthase gene transcription, leading to a calcineurin-dependent increase in chitin synthase activity [24]. Taken together, our data suggest a regulatory role for *A. fumigatus* calcineurin signaling in the chitin biosynthetic response observed during paradoxical growth in the presence of high-dose caspofungin treatment. We found that regulation of transcriptional levels of chitin synthase genes in response to caspofungin is controlled, at least in part, by the calcineurin pathway. This is supported by the lack of upregulation of *chsA* and *chsC* in the Δ*cnaA* mutant and the

decrease in upregulation by addition of FK506, a specific calcineurin inhibitor. To verify that the calcineurin-dependent transcriptional upregulation of *chsA* and *chsC* produced increased chitin synthase activity, we showed that treatment of *A. fumigatus* with caspofungin (4 μg/ml) caused a calcineurin-dependent increase in chitin synthase activity.

Our work supports a mechanistic role for calcineurin in the development of the paradoxical effect of *A. fumigatus*. A possible regulatory scenario involves the calmodulin-mediated activation of CnaA in response to cell wall damage following caspofungin treatment. Activated CnaA can then dephosphorylate its transcription factor, CrzA, which, in turn, induces transcription of *chsA* and *chsC*. These chitin synthases may be responsible for the chitin biosynthetic response to 1,3-β-D-glucan inhibition.

Hsp90 as a novel combination antifungal approach

Hsp90 is a molecular chaperone that is induced by stress in eukaryotes and regulates the folding and transport of client proteins. Hsp90 directly interacts with calcineurin and keeps it poised for activation, but the complete interplay between these two is not fully understood. Calcineurin inhibitors have been shown to strongly reduce fluconazole resistance in Hsp90-dependent mutants, suggesting that Hsp90 may potentiate resistance through a common calcineurin regulator [25]. Hsp90 has also been shown to potentiate the acquisition of fluconazole resistance in *S. cerevisiae* [25].

Hsp90 and calcineurin inhibitors strongly reduced the resistance of *C. albicans* to fluconazole and voriconazole, but did not affect the resistance of *A. terreus* [25]. Calcineurin inhibitors and Hsp90 inhibitors equally increased the sensitivity of *A. terreus* to an echinocandin (caspofungin). Subsequently, Hsp90 pharmacologic inhibition through geldanamycin reduced *A. fumigatus* resistance to caspofungin. In a wax moth larvae model, combination therapy with geldanamycin + caspofungin improved survival ($p<0.0001$) of the otherwise lethal infections treated with either monotherapy [26].

While more detailed studies in *C. albicans* have shed light on the roles of Hsp90 in antifungal drug resistance as well as possible adjunctive therapeutic strategies, there is a paucity of work completed in *A. fumigatus*. The true role of Hsp90 in virulence is unclear at present. We are currently evaluating this pathway and the connection and potential synergistic benefits with calcineurin inhibition to better treat IA.

Conclusion

Currently there is an ongoing clinical trial investigating the efficacy of primary therapy with voriconazole versus voriconazole + anidulafungin. This multi-national trial is the largest combination therapy clinical trial ever undertaken, and will hopefully answer some questions about the clinical utility of using two drugs versus a single agent. While this current work with our existing antifungals is crucial to advancing the field, manipulating novel pathways such as calcineurin and Hsp90 holds future hope for new combination approaches. It is clear that the benefit from the current antifungals used could be improved , and exploiting the cell signaling and stress response of the fungus offers hope for improved patient survival.

Acknowledgments

This work was supported by WJS through a Basic Science Faculty Development grant from the American Society for Transplantation and a Children's Miracle Network grant.

Conflict of Interest: Advisory board for Merck, Speaker's Bureau for Pfizer, Grant support from Astellas.

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