Impact of *in vivo* Triazole and Echinocandin Combination Therapy for Invasive Pulmonary Aspergillosis: Enhanced Efficacy against *Cyp51* Mutant Isolates

Running Title: Combination Therapy for Invasive Aspergillosis

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ABSTRACT

Previous studies examining combination therapy for invasive pulmonary aspergillosis (IPA) have revealed conflicting results including antagonism, indifference, and enhanced effects. The most commonly strategy employed combination for this infection includes a mold-active triazole and echinocandin. Few studies have evaluated combination therapy from a pharmacodynamic (PD) perspective and even fewer have examined combination therapy against both wild-type and azole-resistant Cyp51 mutant isolates. The current studies aim to fill this gap in knowledge. Four A. fumigatus isolates were utilized including a wild-type, an Fks1 mutant (posaconazole susceptible, caspofungin resistant), and two Cyp51 mutants (posaconazole resistant). A neutropenic murine model of IPA was used for the treatment studies. Dosing design included monotherapy posaconazole, monotherapy caspofungin, and combination therapy of both. Efficacy was determined using quantitative PCR and normalized to known quantities of conidia (conidial equivalents, C.E.). The static dose, 1-log kill dose, and associated PD target AUC/MIC were determined for mono- and combination therapy. Monotherapy experiments revealed potent activity for posaconazole, with 3-4 log_{10} Aspergillus C.E/ml reduction with the two 'low' MIC isolates. Posaconazole alone was less effective for the two isolates with higher MICs. Caspofungin monotherapy did not produce a significant decrease in fungal burden for any strain. Combination therapy with the two antifungals did not enhance efficacy for the two posaconazole susceptible isolates. However, the drug combination produced synergistic activity against both posaconazole resistant isolates. Specifically, the combination resulted in a 1-2 log_{10} decline in burden that would not have been predicted based on the monotherapy results.
for each drug. This corresponded to a reduction in the free drug posaconazole AUC/MIC needed for stasis of up to 17-fold. The data suggest that combination therapy using a triazole and echinocandin may be a beneficial treatment strategy for triazole resistant isolates.
INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is a leading cause of morbidity and mortality in immunosuppressed patients (1, 2). Despite advances in the antifungal armamentarium, including the development of new generation triazoles with potent Aspergillus activity and echinocandins, outcomes remain suboptimal with mortality rates near 50% (3). One treatment strategy that has been proposed to improve outcomes is combination of two or more antifungals with distinct mechanisms of action. This approach has proven useful for a number of other infectious diseases such as HIV, tuberculosis, gram-negative bacterial sepsis, enterococcal endocarditis and cryptococcal meningitis (4-8). However, combination studies against Aspergillus, both in vitro and in vivo, have produced conflicting results for different infection models and drug combinations (9-12). One combination of interest is a mold active triazole and echinocandin.

Aspergillus active triazoles are considered first line therapy for IPA and have proven efficacy in initial and salvage therapy (13-16). However, the recent emergence of A. fumigatus isolates exhibiting reduced susceptibility to triazoles is a threat to this class (17-21). We theorized that consideration of the pharmacokinetics and pharmacodynamics of the triazole and echinocandin interaction would advance our understanding of the utility of this combination strategy. We specifically posited that [1] the in vivo potency of the mold active triazoles makes identification of synergistic interactions between a triazole and echinocandin difficult to demonstrate for Cyp51 wild-type organisms and [2] when there is inadequate triazole drug exposures or triazole drug resistance that beneficial interactions would be observed.
MATERIALS AND METHODS

Organisms. Four *A. fumigatus* isolates (DPL, EMFR S678P, F16216, and F11628) were chosen for the current study including two wild-type at *Cyp51* (one wild-type and one *Fks1* mutant) and two *Cyp51* mutants. Isolates DPL, F16216, and F11628 are clinical isolates, whereas EMFR S678P is a laboratory engineered mutant. The two *Cyp51* mutants were chosen based upon varying posaconazole MIC. A moderately elevated MIC isolate F16216 (posaconazole MIC 2 mg/L) and a highly elevated MIC isolate F11628 (posaconazole MIC 8 mg/L) were utilized. Organisms were grown and subcultured on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI). The organisms were chosen to include similar fitness as determined by growth in lungs and mortality in untreated mice over 7 d (*Table 1*).

Drugs. Posaconazole solution and caspofungin powder were obtained from the University of Wisconsin Hospital and Clinics pharmacy. Posaconazole drug solutions were prepared on the day of use with sterile saline as the diluent and vortexed vigorously prior to administration by oral-gastric (OG) gavage. Caspofungin was similarly prepared on the day of use with sterile saline as the diluent and was administered by intraperitoneal injection.

*In vitro Susceptibility.* Posaconazole MIC and Caspofungin MEC were determined for both drugs by broth microdilution using the CLSI M38-A2 method (22). MICs were performed in duplicate three times; the median value is reported in *Table 1*.
Animals. Six-week-old Swiss/I CR specific-pathogen-free female mice weighing 23-27 g were used for all studies (Harlan Sprague-Dawley, Indianapolis, IN). Animals were housed in groups of five and allowed access to food and water ad libitum. Animals were maintained in accordance with the American Association for Accreditation of Laboratory Care criteria (23). The Animal Research Committee of the William S. Middleton Memorial VA Hospital and University of Wisconsin-Madison approved the animal studies.

Infection Model. Mice were rendered neutropenic (polymorphonuclear cells <100/mm^3) by injection of 150 mg/kg cyclophosphamide subcutaneously (SC) on days -4 and -1 before and +3 days after infection. Prior studies have shown this protocol sustains neutropenia (CFU < 100 mm^3) for the 7 d experiment (24-26). Additionally, cortisone acetate (250 mg/kg SC) was administered on day -1 as previously described (24, 27, 28). Mice were also given ceftazidime 50 mg/kg/d SC to prevent opportunistic bacterial infection.

Organisms were subcultured on PDA 5 days prior to infection and incubated at 37° C. On the day of infection, the inoculum was prepared by flooding the culture plate with 5 ml of normal saline with 0.05% Tween-20. The conidial suspension was collected and quantitated by hemacytometer (Bright-Line, Hausser Scientific, Horsham PA). The suspension was diluted to a final concentration of 1-2 x 10^7. Viability was assessed by viable plate counts on PDA.

Prior to induction of infection, mice were anesthetized with 50 μl IM injection of ketamine (37.5 mg/ml) and xylazine (5 mg/ml). Fifty μl of a 1-2 x10^7 conidial suspension
was pipetted into the anterior nares and aspirated into the lungs. This procedure produced invasive aspergillosis in over 90% of animals and 100% mortality in untreated infected mice.

**Antifungal Dosing Design.** Each antifungal drug was administered alone and in combination in the *in vivo* model. Posaconazole was administered by OG route using five 4-fold increasing doses from 0.156 mg/kg to 40 mg/kg once daily. Similarly, caspofungin was administered by IP route using five 4-fold increasing doses from 0.156 mg/kg to 40 mg/kg once daily. A checkerboard design of combination therapy was utilized resulting in 25 different combination regimens (*Table 2*). The doses were selected to vary the expected result from zero effect to maximal effect or the highest tolerated dose. Controls were utilized for each experiment and included a start and end of therapy. Four mice were used for each treatment regimen and control group. Therapy was initiated 2 hours after infection. At the time of sacrifice of moribund animals or at study endpoint (7 d), lungs were aseptically harvested and processed for quantitative PCR (qPCR) as described below.

**Lung Processing and Organism Quantitation.** Processing and quantitation of lung burden was performed based upon previously described protocols (29, 30). Briefly, at the time of sacrifice for moribund animals or at the end of therapy (7 d), lungs were aseptically harvested and placed in 2 ml of sterile saline in a 2-ounce sterile polyethylene Whirl-Pak bag (Nasco, Fort Atkinson, WI). The lungs were manually homogenized using direct pressure (31). One ml of the primary homogenate was placed
in a sterile bead beating tube (Sarstedt, Newton, NC) with 700 μl of 425-600 μm acid-washed glass beads (Sigma-Aldrich, St. Louis, MO). The primary homogenate was bead beaten in a Bio-spec mini bead beater (Bartlesville, OK) for 90 s at 4200 rpm to yield a secondary homogenate. One hundred μl of the secondary homogenate was mixed with 100 μl of buffer ATL and 20 μl of Proteinase K (Qiagen, Valencia, CA) and incubated overnight at 56° C with gentle agitation. DNA was then isolated following the DNEasy Blood and Tissue protocol (Qiagen, Valencia, CA). A final elution step was carried out with 100 μl of Elution Buffer AE (Qiagen, Valencia, CA) placed over the column twice to maximize DNA isolation. The DNA was stored at -20° C until day of qPCR.

qPCR plates were prepared on the day of assay. Defined quantities of conidia (conidial equivalents) were used for standard curves. Samples were assayed in triplicate using a Bio-Rad CFX96 Real-Time System (Hercules, CA). A single copy gene, Fks1, was chosen for quantitation (32). Primer sequences included: forward primer 5’GCCTGGTAGTGAAGCTGAGGT-3’, reverse primer 5’CGGTGAATGTAGGCATGTTGTCC-3’, and probe 6-FAM-AGCCAGCGCCGAAATG-MGB-3’ (Integrated DNA Technologies, Coralville, IA).

The Fks1 mutation (S678P) was not located in the primer:probe area of the genome. The primer:probe set was validated for all isolates by comparing the kinetics and quantitative results for known quantities of conidia over the dynamic range (10^2 - 10^8) (data not shown). Prior studies in our lab have also shown that there is an absence of inhibitors that may adversely affect the qPCR reaction by spiking lung homogenate with
known quantities of conidia. Organism burden was reported as conidial equivalents (C.E.) per ml of primary lung homogenate (log$_{10}$ C.E./ml lung homogenate).

**Pharmacokinetics.** Murine posaconazole and caspofungin pharmacokinetic data including AUC and protein binding was derived from our previous studies published in this journal (33, 34).

**Outcome Measure and Pharmacodynamic Index Exploration.** The effect of a particular dosing regimen (monotherapy or combination therapy) was determined by comparing the mean change in log$_{10}$ C.E./ml lung homogenate at the end of therapy or time of sacrifice to the initial starting log$_{10}$ C.E./ml lung homogenate at time zero. The dose-response graphs were fit to a sigmoid Hill-type dose response curve. The AUC/MIC was used as the PD index for exploration of exposure response relationships based upon previous PK/PD investigations for posaconazole and caspofungin (33-37).

Both total and free (non-protein bound) drug concentrations were considered.

**Monotherapy Analysis.** The qPCR data was modeled according to a Hill-type dose response equation: log$_{10} D = \log_{10} \left( \frac{E}{E_{\text{max}}} - E \right)/N + \log_{10} ED_{50}$, where $D$ is the drug dose, $E$ is the growth (as measured by qPCR and represented as C.E./ml of lung homogenate) in untreated control mice, $E_{\text{max}}$ is the maximal effect, $N$ is the slope of the dose-response relationship, and $ED_{50}$ is the dose needed to achieve 50% of the maximal effect. The posaconazole and caspofungin static dose (i.e. no change in fungal burden from the start of therapy) and dose associated with a net 1-log decrease
in burden (1-log kill), when achieved, were determined for all isolates. The PD target
total and free drug AUC/MIC for each endpoint was also calculated. The co-efficient of
determination ($R^2$) was used to estimate the percent variance in the change of log$_{10}$
C.E./ml of lung homogenate over the treatment period for the different dosing regimens
that could be attributed to the PD index AUC/MIC. The static dose and 1-log kill dose
and associated free drug AUC/MIC for the two Cyp51 wild-type isolates and the Cyp51
mutants were compared by Mann-Whitney rank sum test.

Combination Therapy Analysis. The potential for combination therapy to confer
beneficial microbiological effects compared to monotherapy were explored in two
manners. First, we compared the drug dose associated with two endpoints (static dose
and 1 log kill dose) for each drug and isolate in monotherapy to that for the drug in
combination therapy using the Student’s $t$-test. When monotherapy resulted in a static
dose above the highest dose used (40 mg/kg/24 h), the static dose was set to 40
mg/kg/24 h for comparison against combination therapy using the Student’s $t$-test.

The second analysis was employed to determine the presence of traditionally
defined antagonism, indifference, or synergy by Bliss interaction analysis (38).
Specifically, Bliss independence is described by the formula: $E_C = (E_A + E_B) - (E_A \times E_B)$
(Equation 1), where $E_C$ is the expected (i.e. calculated) fractional effect of a particular
combination therapy regimen consisting of drug A and drug B, $E_A$ is the fractional effect
of monotherapy with drug A, and $E_B$ is the fractional effect of monotherapy with drug B.
Fractional effects for monotherapy were determined by comparing the observed
monotherapy effect of a particular dosing regimen to maximal effect, and is represented
by the equation: $EA = (E_{\text{max}} - E_{\text{mono A}})/E_{\text{max}}$ (Equation 2), where $E_{\text{mono A}}$ is the observed monotherapy effect in relation to no effect for drug A and $E_{\text{max}}$ is the maximum effect. The same equation was used to calculate $EB$: $EB = (E_{\text{max}} - E_{\text{mono B}})/E_{\text{max}}$ (Equation 3).

Therefore, for each monotherapy dosing regimen a fractional (or percent) effect compared to maximal effect was determined and then utilized to calculate the $E_c$ for each combination dosing regimen using Equation 1. Observed combination effects ($E_{\text{obs}}$) were then compared to the calculated effect ($E_c$) for each isolate at each combination dosing regimen. The difference between observed combination effect and calculated combination effect, including 95% confidence intervals (C.I.), was calculated by the following equation: $\Delta E = E_{\text{obs}} - E_c$, where $E_{\text{obs}}$ is the observed combination effect in relation to no effect and $E_c$ is the calculated (i.e. predicted) combination effect.

Enhanced effect or synergy was suggested, if $\Delta E$, including the 95% C.I. for $E_{\text{obs}}$ and $E_c$, was greater than 0. Antagonism was concluded if $\Delta E$, including the 95% C.I. for $E_{\text{obs}}$ and $E_c$, was less than 0. All other cases where the 95% C.I. for $\Delta E$ would include 0, the conclusion was indifference (Bliss independence).

To account for the potential for small and likely clinically insignificant synergistic or antagonistic interactions to be found mathematically based on the Bliss interaction analysis, we defined a biologically meaningful change a priori. Specifically, a fractional change leading to a $1 \log_{10}$ increase or decrease in fungal burden or approximately 0.2 (or 20%) was considered relevant. Therefore, only observed effects (including 95% C.I.) that did not overlap with calculated effects (including 95% C.I.) and the difference between the two was 20% or more were deemed significantly synergistic or antagonistic.
RESULTS

Antifungal Susceptibility and In vivo Fitness. Posaconazole and caspofungin in vitro susceptibility testing (each drug alone), genetic mutations where applicable, and the relative fitness in the in vivo murine model of each isolate are shown in Table 1. Posaconazole MIC was elevated for strain F16216 at 2 mg/L and further elevated for strain F11628 at 8 mg/L, whereas the MIC was lower (0.25 mg/L) for the two organisms that did not have Cyp51 mutations. The isolate containing an Fks1 hot spot mutation (EMFR S678P) exhibited higher MEC for caspofungin (16 mg/L) compared to the three isolates without a mutation (range 0.25 – 0.5 mg/L). The organisms exhibited similar in vivo fitness exhibited by growth and mortality in untreated animals. At the start of therapy mice had 5.60 ± 0.4 log$_{10}$ C.E./ml of lung homogenate and the burden increased to 7.41 ± 0.44 log$_{10}$ C.E./ml of lung homogenate in untreated animals. Each isolate produced 100% mortality prior to end of study in untreated animals.

Pharmacokinetics. Data from our previous pharmacokinetic studies of posaconazole and caspofungin in this mouse model were used for the current study (33, 34). The AUC over the dose range was linear for both drugs. Thus, for dose levels that were not directly measured, the AUC was estimated using linear extrapolation or interpolation. The posaconazole total drug AUC range was 1.78 – 351 mg*h/L over the dose range of 0.156 – 40 mg/kg/24 h. The caspofungin total drug AUC range was 5.21 – 452 mg*h/L over the dose range of 0.156 – 40 mg/kg/24 h. Protein binding was 99% and 97% for posaconazole and caspofungin, respectively.
**Monotherapy Analysis.** A sigmoid dose-response relationship for posaconazole was observed for each isolate studied. As expected, higher doses were necessary to achieve similar microbiologic outcomes against organisms with elevated posaconazole MICs (Figure 1). Posaconazole treatment against the more susceptible strains resulted in more than a 3-log_{10} C.E./ml reduction in lung burden. However, as shown in Figure 2, caspofungin monotherapy exhibited only modest *in vivo* effect in this animal model for each of the strains. The posaconazole static dose and 1-log kill dose (when achieved) was determined for each isolate and is shown in Table 3. A dose of 1.87 and 1.09 mg/kg/24 h of posaconazole associated net stasis for Cyp51 wild-type strains DPL and EMFR S678P, respectively. Comparatively, a similar level of efficacy for the Cyp51 mutant strains required more than 30-fold more drug (static dose >40 mg/kg/24 h). The dose response curve was steep for Cyp51 wild-type isolates and only 1.19 to 1.26 mg/kg/24 h was needed to produce a 1-log reduction in burden compared to that at the start of therapy. Caspofungin therapy did not produce the stasis or 1 log reduction endpoints for any of the four isolates.

The posaconazole free drug AUC/MIC exposures were determined for each isolate and fit to a Hill sigmoid dose-response model. The posaconazole exposure response data fit the model well (Figure 3) with an *R*² of 0.79. Taking into account free drug concentrations, the posaconazole AUC/MIC values associated with the stasis endpoints for the susceptible strains DPL and EMFR S678P were 0.85 and 0.50, respectively. The static dose AUC/MIC targets could not be determined for the two Cyp51 mutants, F16216 and F11628, as net stasis was not achieved over the dose range studied. The posaconazole free drug AUC/MIC associated with the 1 log
reduction endpoint was only slightly higher for the Cyp51 wild-type isolates. As noted above caspofungin monotherapy pharmacodynamic targets could not be determined as the stasis and 1 log reduction endpoints were not achieved in the monotherapy.

**Combination Analysis.** The dose-response results for combination therapy are shown in Figure 4. The impact of the addition of caspofungin to the posaconazole stasis and 1 log reduction dose levels are shown in Table 4. There were no significant changes in the posaconazole static dose or 1-log reduction dose for the Cyp51 wild-type DPL isolate. However, the posaconazole dose endpoints were somewhat higher for the wild-type EMFR S678P strain in combination with two caspofungin dose levels, 40 and 10 mg/kg/24 h. Conversely, the posaconazole dose response curves were distinctly shifted to the left for the Cyp51 mutants across many of the caspofungin exposures (Figure 4, panels C & D). The enhancement in efficacy was most evident for the more highly resistant F11628 isolate (Table 4). For instance, the posaconazole static dose for the three highest caspofungin additions was 7- to 13-fold lower in combination therapy than monotherapy ($p \leq 0.004$). If one examines the posaconazole monotherapy free drug AUC/MIC associated with net stasis for the susceptible isolates (mean = 0.68), the posaconazole free drug AUC/MIC target associated with net stasis in these three combination therapy regimens against the azole resistant isolate F11628 was 8.5- to 17-fold lower ($p < 0.001$).

Bliss independence analysis found no synergistic or antagonistic combinations against the wild-type isolate DPL. There were also no synergistic combinations for Cyp51 wild-type, Fks1 mutant isolate EMFR S678P. However, similar to the static dose
analysis, there were three antagonistic combinations (Table 5). Synergistic combinations were noted for both Cyp51 mutants. Three combination regimens exhibited 22 to 25% more effect in combination than would have been expected if the two drugs were acting independently for the resistant F16216. Seven combination regimens exhibited synergy against the isolate with the highest posaconazole MIC (F11628). The seven synergistic combinations (see Table 5) exhibited 22 to 49% more microbiological effect than would have been expected if the two drugs were acting independently. These combinations produced a statistically significant $1 - 2.5 \log_{10}$ decrease in observed infectious burden compared to the predicted combination effect. Surface-response three-dimensional plots for the two posaconazole resistant isolates are shown in Figures 5 and 6.

**DISCUSSION.**

Combination anti-infective therapy with two or more drugs that act at different sites has been considered in situations when outcomes in monotherapy are suboptimal. This strategy has been recently popular in the study of therapy for invasive *Aspergillus* infections. The mold active triazoles and echinocandins are two of the commonly studied classes due to their efficacy and relative safety (13, 14, 16, 39, 40). Many *in vitro* assays have demonstrated additive or synergistic interactions for the two drug classes. However, evidence from *in vivo* models has been conflicting (9-12). Clinically, a number of small, non-randomized trials have suggested potential benefit of this drug class combination (11, 40-44). One potential explanation for differences among previous *in vivo* study results relates to variation in drug exposure. Most *in vivo*
combination studies have utilized minimal if any dose-ranging and often only a single regimen with each antifungal. This is understandable given the large number of animals and cost of these studies. However, it is possible or even likely that not all dosing regimen combinations will be optimal for detection of enhanced efficacy. For example, the few or single dose levels chosen are often based upon optimal efficacy in the infection model or the maximally tolerated dose. This may be particularly problematic for the echinocandin class given the possibility of a paradoxical effect. We attempted to overcome this limitation by examination of a wide dose range (256-fold for each compound) to include a full sigmoid effect (no effect to maximal effect) concentration range from monotherapy experiments. This approach results in a checkerboard design similar to most in vitro studies, but was costly and did utilize a large number of mice.

The in vivo efficacy of posaconazole monotherapy against Cyp51 wild-type strains was marked with a maximal kill of $3-4 \log_{10} \text{C.E./ml}$. This potency has been confirmed in other in vivo studies (35, 36, 45, 46). Caspofungin monotherapy, however, was less effective in this model. The reasons for this are not clear; however, previous in vivo studies have shown limited microbiological effectiveness in terms of reducing fungal burden (26, 47). A recent in vitro micro-colony study observed slowing but not the halt of Aspergillus growth in the presence of echinocandins, and this phenomenon may be reflected in our in vivo study (48). Additionally, a previous study has suggested the primary means of echinocandin effectiveness may be related to beta-glucan unmasking and resultant increase recognition and killing by polymorphonuclear cells (49). We utilized a neutropenic model and therefore this could explain why only modest microbiological effect was noted in the absence of polymorphonuclear cells.
Another goal of the present study was to consider the impact of MIC variation and drug resistance on the drug class interaction. While echinocandin resistance in *Aspergillus* is at this point a laboratory phenomenon, triazole resistance is an emerging clinical threat in many regions of the world (17-21). To our knowledge, this is the first *in vivo*, dose-ranging pharmacodynamic study examining the effects of combination triazole and echinocandin therapy against Cyp51 wild-type and mutant isolates.

An additional study factor that can impact interpretation of drug interaction experiments is the analysis model. Among the numerous potential approaches we chose two analyses. The first was a simple and practical analysis of the impact of combination on the antifungal dose associated with meaningful treatment endpoints, in this case both stasis and killing. The second method utilized the Bliss independence model (50). This model operates on the assumption that two drugs act at different, independent, and mutually non-exclusive sites. Given the distinct sites of drug action and more importantly the relatively modest activity of the echinocandins in this infection model we felt this was the most biologically relevant. We were encouraged to observe congruence with the two analytical approaches.

Similar to previous *in vivo* studies, our results were in some manner, conflicting in that the interactions were not consistent for each of the four *Aspergillus* strains. However, we were not entirely surprised to find difficulty in demonstrating beneficial combination effects against Cyp51 wild-type isolates given the extreme potency of posaconazole in previous monotherapy experiments (46). This is similar to results from clinical study of this combination in which most patients were likely to be infected with wild-type, triazole susceptible isolates and enhanced effect and improvement in patient
outcomes was not observed (51). Unfortunately, susceptibility data was not tracked in this clinical study and therefore it is unknown whether enhanced effects would be observed based on triazole susceptibility. It is interesting to note the observation of statistically antagonistic against the echinocandin-resistant strain EMFR S678P. A significantly higher posaconazole static dose was observed in combination therapy and specifically three combinations exhibited antagonistic effects based on Bliss analysis. The basis for this antagonistic interaction is unclear but an area for future mechanistic investigation. The clinical relevance of this observation is unclear since this isolate was a laboratory engineered mutant strain and echinocandin resistance in Aspergillus appears to be an incredibly rare clinical event. Further studies, especially with a clinical echinocandin resistant isolate, will be important to further understand this finding.

In contrast, we were intrigued to find a quite large enhancement of efficacy of the drug combination for Cyp51 mutants. For both Cyp51 mutant isolates, the effect of combinations resulted in 1 to 2.5 log₁₀ enhanced microbiological effect compared to monotherapy. Based on our previous demonstration of a strong correlation between qPCR results and animal mortality (46), this enhanced effect would correspond to an increase in survival of 17 - 43%. For each mutant isolate there was at least one combination regimen that resulted in net cidal activity, whereas in posaconazole monotherapy stasis was not achieved. The addition of the echinocandin seemed to restore the cidal activity for the triazole. It is further interesting that the impact of this combination was most profound for the least triazole susceptible isolate, where the observed fractional effect was over 200% greater than predicted by monotherapy. This corresponds to an approximate 2 log₁₀ increase in microbiological activity over what
would have been predicted. The posaconazole static dose for this combination was reduced by almost 13-fold compared to monotherapy, both highly statistically significant and one might expect clinically important ($p \leq 0.001$). Previous in vivo studies have demonstrated similar enhanced effects with voriconazole and each of the three licensed echinocandins (52-57), although this is the first to utilize posaconazole and examine the results from a pharmacodynamic perspective.

There are limitations to the current study that deserve consideration. The complexity and size of the experiment using a checkerboard technique made it difficult to study a larger number of isolates. Secondly, we did not consider sequential combination therapy which is commonly used as salvage therapy for patients failing monotherapy. Study with the triazole/polyene combination has identified differences when this approach has been explored. Finally, we did not evaluate other triazole/echinocandin combinations. While one might expect similar results for drugs with similar mechanisms of action, a previous in vitro pharmacodynamic study against Cyp51 mutants did not demonstrate significant enhancement with combination voriconazole and anidulafungin (58).

In summary, we did not observe enhanced in vivo effect for combination posaconazole and caspofungin therapy against Cyp51 wild-type organisms. This suggests that combination therapy may not offer further benefit over triazole monotherapy as long as drug concentrations are sufficient against a triazole susceptible isolate. In contrast, treatment efficacy was enhanced for Aspergillus isolates with elevated posaconazole MICs. The mechanisms that underlie this phenomenon are unknown but an intriguing area for further research. These findings challenge our
therapeutic strategy when dealing with a drug-resistant isolate. In many situations, when drug resistance is encountered the approach employed is to abandon the drug the organism is resistant to and use an alternative class to which the organism is susceptible. However, our in vivo results contest this paradigm. We found the combination of a posaconazole and caspofungin in the setting of posaconazole resistance can not only outperform echinocandin monotherapy, but can rescue cidal activity that is typical for the triazoles against susceptible strains. This finding suggests the optimal strategy when encountering triazole resistance in IPA may be combination therapy with a triazole and echinocandin. However, it will be important to verify these observations with a larger set of triazole resistant isolates. These results, though, provide a basis for further study of combination therapy, with the focus on triazole resistant isolates.

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Figure Legends

Figure 1. Posaconazole monotherapy dose-response curves for each isolate. Open symbols represent wild-type Cyp51 organisms and closed symbols represent Cyp51 mutants. Each data point is the mean ± SD log_{10} C.E./ml of lung homogenate for four mice. The horizontal dashed line represents net stasis or infectious burden at the start.
of therapy. Points above the line represent an increase in burden (i.e. net growth) whereas those below the line represent decrease in burden.

**Figure 2.** Caspofungin monotherapy dose-response curves for each isolate. Open symbols represent wild-type Cyp51 organisms and closed symbols represent Cyp51 mutants. Each data point is the mean ± SD log_{10} C.E./ml of lung homogenate for four mice. The horizontal dashed line represents net stasis or infectious burden at the start of therapy. Points above the line represent an increase in burden (i.e. net growth) whereas those below the line represent decrease in burden.

**Figure 3.** The relationship between posaconazole monotherapy AUC/MIC and microbiological effect is plotted for each of the 4 A. fumigatus isolates. Free (not protein bound concentrations) were used. Open symbols denote results from wild-type Cyp51 organisms and closed symbols Cyp51 mutants. The horizontal dashed line represents net stasis or infectious burden at the start of therapy. Points above the line represent an increase in burden (i.e. net growth) whereas those below the line represent decrease in burden. The coefficient of determination (R^2) based on the Hill equation is shown in the upper corner, with associated PD parameters including E_{max}, ED_{50}, and slope (N).

**Figure 4.** Dose-response curves for combination posaconazole and caspofungin therapy against isolate (A) DPL (wild-type), (B) EMFR S678P (Cyp51 wild-type, Fks mutant), (C) F16216 (Cyp51 mutant), and (D) F11628 (Cyp51 mutant). Each graph represents the microbiological effect of varied posaconazole doses (shown on x-axis)
with addition of each of the 5 dosing regimens of caspofungin (represented by each of the 5 curves). In total there are 25 different combination data points on each graph. Each data point represents the mean ± SD in log$_{10}$ C.E./ml of lung homogenate for four mice. The horizontal dashed line represents net stasis or infectious burden from the start of therapy. Points above the line represent an increase in burden (i.e. net growth) whereas those below the line represent decrease in burden.

**Figure 5.** Three-dimensional surface-response plot of combination posaconazole and caspofungin therapy and microbiological effect against F16216 (Cyp51 mutant, Posaconazole MIC = 2 mg/L). The vertical axis represents change in burden from the start of therapy. Each data point is the mean change in log$_{10}$ C.E./ml of lung homogenate from four mice. Areas above zero (green, yellow and orange) represent an increase in burden (i.e. net growth). Areas below zero (blue and dark blue) represent decrease in burden.

**Figure 6.** Three-dimensional surface-response plot of combination posaconazole and caspofungin therapy and microbiological effect against F11628 (Cyp51 mutant, Posaconazole MIC = 8 mg/L). The vertical axis represents change in burden from the start of therapy. Each data point is the mean change in log$_{10}$ C.E./ml of lung homogenate from four mice. Areas above zero (green, yellow and orange) represent an increase in burden (i.e. net growth). Areas below zero (blue and dark blue) represent decrease in burden.
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pharmacokinetic equivalent doses of voriconazole and anidulafungin.


Table 1. *In vitro* susceptibility and *in vivo* fitness of select *A. fumigatus* isolates.

<table>
<thead>
<tr>
<th><em>A. fumigatus</em> Isolate</th>
<th>Caspofungin MEC (mg/L)</th>
<th>Posaconazole MIC (mg/L)</th>
<th><em>In vivo</em> Growth (log₁₀ C.E./ml lung homogenate) in untreated control</th>
<th>Mortality in untreated control at day 7</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPL</td>
<td>0.25</td>
<td>0.25</td>
<td>1.7 ± 0.4</td>
<td>100%</td>
<td>Wild-type</td>
</tr>
<tr>
<td>EMFR S678P</td>
<td>16</td>
<td>0.25</td>
<td>1.6 ± 0.4</td>
<td>100%</td>
<td>Fks1 S678P</td>
</tr>
<tr>
<td>F16216</td>
<td>0.5</td>
<td>2</td>
<td>2.0 ± 0.6</td>
<td>100%</td>
<td>Cyp51 L98H+TR</td>
</tr>
<tr>
<td>F11628</td>
<td>0.5</td>
<td>8</td>
<td>1.9 ± 0.5</td>
<td>100%</td>
<td>Cyp51 G138C</td>
</tr>
</tbody>
</table>
Table 2. Checkerboard dosing design for mono- and combination therapy.

<table>
<thead>
<tr>
<th>Caspofungin Dose (mg/kg/d)</th>
<th>40</th>
<th>10</th>
<th>2.5</th>
<th>0.625</th>
<th>0.156</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2.5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.625</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.156</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

The numbers in each cell represent the number of mice included in each treatment group.
Table 3. Posaconazole monotherapy dose, total and free drug AUC/MIC needed to achieve a net stasis and 1-log kill endpoints (when achieved) for each isolate.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>SD (mg/kg/24h)</th>
<th>MIC (mg/L)</th>
<th>24h tAUC/MIC</th>
<th>24h fAUC/MIC</th>
<th>1-log kill (mg/kg/24h)</th>
<th>24h tAUC/MIC</th>
<th>24h fAUC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPL</td>
<td>1.87</td>
<td>0.25</td>
<td>85.1</td>
<td>0.85</td>
<td>3.12</td>
<td>142</td>
<td>1.42</td>
</tr>
<tr>
<td>EMFR S678P</td>
<td>1.09</td>
<td>0.25</td>
<td>49.7</td>
<td>0.50</td>
<td>2.28</td>
<td>104</td>
<td>1.04</td>
</tr>
<tr>
<td>F16216</td>
<td>&gt;40</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td>&gt;40</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>F11628</td>
<td>&gt;40</td>
<td>8</td>
<td>X</td>
<td>X</td>
<td>&gt;40</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

X= not attainable
Table 4. Posaconazole combination therapy static dose, 1-log kill dose, and associated AUC/MIC PD targets (when achieved) in combination therapy with five different doses of caspofungin against two Cyp51 wild-type and two Cyp51 mutant A. fumigatus isolates.

<table>
<thead>
<tr>
<th></th>
<th>Caspofungin Dose (mg/kg/24 h)</th>
<th>Posaconazole Static Dose (mg/kg/24 h)</th>
<th>MIC (mg/L)</th>
<th>Posaconazole Static Dose 24 h Total Drug AUC/MIC</th>
<th>Posaconazole Static Dose 24 h Free Drug AUC/MIC</th>
<th>Posaconazole 1-log Kill Dose (mg/kg/24 h) Total Drug AUC/MIC</th>
<th>Posaconazole 1-log Kill Dose (mg/kg/24 h) Free Drug AUC/MIC</th>
<th>Posaconazole 1-log Kill Total Drug 24 h AUC/MIC</th>
<th>Posaconazole 1-log Kill Free Drug 24 h AUC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPL</td>
<td>40</td>
<td>3.00</td>
<td>0.25</td>
<td>137</td>
<td>1.37</td>
<td>3.27</td>
<td>149</td>
<td>1.49</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.00</td>
<td>0.25</td>
<td>137</td>
<td>1.37</td>
<td>3.29</td>
<td>150</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.86</td>
<td>0.25</td>
<td>130</td>
<td>1.30</td>
<td>3.14</td>
<td>143</td>
<td>1.43</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>3.98</td>
<td>0.25</td>
<td>182</td>
<td>1.82</td>
<td>4.79</td>
<td>218</td>
<td>2.18</td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td>0.156</td>
<td>3.07</td>
<td>0.25</td>
<td>140</td>
<td>1.40</td>
<td>3.38</td>
<td>154</td>
<td>1.54</td>
<td>1.54</td>
</tr>
<tr>
<td>EMFR S678P</td>
<td>40</td>
<td>3.88</td>
<td>0.25</td>
<td>177</td>
<td>1.77</td>
<td>6.44</td>
<td>294</td>
<td>2.94</td>
<td>2.94</td>
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<tr>
<td></td>
<td>10</td>
<td>2.85</td>
<td>0.25</td>
<td>130</td>
<td>1.30</td>
<td>5.51</td>
<td>251</td>
<td>2.51</td>
<td>2.51</td>
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<tr>
<td></td>
<td>2.5</td>
<td>1.55</td>
<td>0.25</td>
<td>70.1</td>
<td>0.70</td>
<td>3.47</td>
<td>158</td>
<td>1.56</td>
<td>1.56</td>
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<tr>
<td></td>
<td>0.625</td>
<td>1.36</td>
<td>0.25</td>
<td>52.2</td>
<td>0.62</td>
<td>3.98</td>
<td>182</td>
<td>1.82</td>
<td>1.82</td>
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<tr>
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<td>0.156</td>
<td>1.03</td>
<td>0.25</td>
<td>47.3</td>
<td>0.47</td>
<td>3.57</td>
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<td>1.63</td>
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<tr>
<td>F1621B</td>
<td>40</td>
<td>&gt;40</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt;40</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>&gt;40</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>24.5†</td>
<td>2</td>
<td>128</td>
<td>1.28†</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>0.156</td>
<td>28.6†</td>
<td>2</td>
<td>140</td>
<td>1.40†</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>F11628</td>
<td>40</td>
<td>3.11†</td>
<td>8</td>
<td>4.43</td>
<td>0.04†</td>
<td>8.48</td>
<td>12.1</td>
<td>0.12†</td>
<td>0.12†</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.07†</td>
<td>8</td>
<td>5.80</td>
<td>0.06†</td>
<td>13.8</td>
<td>19.6</td>
<td>0.20†</td>
<td>0.20†</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>5.75†</td>
<td>8</td>
<td>8.19</td>
<td>0.08†</td>
<td>21.0</td>
<td>29.2</td>
<td>0.29†</td>
<td>0.29†</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>11.0†</td>
<td>8</td>
<td>15.7</td>
<td>0.16†</td>
<td>&gt;40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.156</td>
<td>17.6†</td>
<td>8</td>
<td>25.1</td>
<td>0.25†</td>
<td>&gt;40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Static dose, 1-log kill dose, and associated PD targets total and free drug AUC/MIC were significantly higher (p < 0.001) for these combinations than for monotherapy.

† Static dose, 1-log kill dose, and associated PD targets total and free drug AUC/MIC were significantly lower (p < 0.001) for these combinations than for monotherapy.

X = not attained
Table 5. Posaconazole and caspofungin combination regimens exhibiting synergy or antagonism by Bliss independence analysis.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Posaconazole Dose (mg/kg/24 h)</th>
<th>Caspofungin Dose (mg/kg/24 h)</th>
<th>Ec (95% C.I.)</th>
<th>Eobs (95% C.I.)</th>
<th>ΔE</th>
<th>Synergy or Antagonism</th>
</tr>
</thead>
<tbody>
<tr>
<td>F16216</td>
<td>40</td>
<td>0.625</td>
<td>0.40 (0.32-0.49)</td>
<td>0.65 (0.56-0.71)</td>
<td>0.25</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>40</td>
<td>0.13 (0.09-0.16)</td>
<td>0.35 (0.29-0.41)</td>
<td>0.22</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>40</td>
<td>0.13 (0.09-0.17)</td>
<td>0.38 (0.29-0.47)</td>
<td>0.25</td>
<td>Synergy</td>
</tr>
<tr>
<td>F11628</td>
<td>40</td>
<td>40</td>
<td>0.37 (0.21-0.53)</td>
<td>0.86 (0.81-0.91)</td>
<td>0.49</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>10</td>
<td>0.43 (0.28-0.58)</td>
<td>0.80 (0.76-0.84)</td>
<td>0.37</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>40</td>
<td>0.38 (0.25-0.52)</td>
<td>0.67 (0.54-0.79)</td>
<td>0.29</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.625</td>
<td>0.27 (0.19-0.35)</td>
<td>0.61 (0.48-0.75)</td>
<td>0.34</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.156</td>
<td>0.19 (0.13-0.24)</td>
<td>0.55 (0.43-0.67)</td>
<td>0.36</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40</td>
<td>0.31 (0.12-0.50)</td>
<td>0.81 (0.59-0.83)</td>
<td>0.30</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.5</td>
<td>0.32 (0.15-0.49)</td>
<td>0.54 (0.51-0.56)</td>
<td>0.22</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>40</td>
<td>0.49 (0.46-0.52)</td>
<td>0.14 (0.11-0.17)</td>
<td>-0.35</td>
<td>Antagonism</td>
</tr>
<tr>
<td>EMFR S678P</td>
<td>2.5</td>
<td>10</td>
<td>0.50 (0.45-0.55)</td>
<td>0.24 (0.18-0.29)</td>
<td>-0.26</td>
<td>Antagonism</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.625</td>
<td>0.53 (0.48-0.60)</td>
<td>0.20 (0.16-0.24)</td>
<td>-0.33</td>
<td>Antagonism</td>
</tr>
</tbody>
</table>

Ec, predicted fractional effect based in Bliss independence equation (Equation 1)
Eobs, observed fractional effect in the combination dosing experiment
ΔE, difference between Ec and Eobs (Eobs - Ec)