Amphotericin B+ and voriconazole+echinocandin combinations against Aspergillus spp.: Effect of serum on inhibitory and fungicidal interactions

Antigoni Elefanti1, Johan W. Mouton2, Paul E. Verweij2, Athanasios Takris3, Loukia Zerva1, Joseph Meletiadis1

Short title: Antifungal combinations against Aspergillus in serum

1 Clinical Microbiology Laboratory, Attikon University Hospital, Medical School, National and Kapodistrian University of Athens, Greece

2 Department of Medical Microbiology, University Medical Center Nijmegen, The Netherlands

3 Department of Microbiology Medical School, National and Kapodistrian University of Athens, Greece

Keywords: amphotericin B, voriconazole, echinocandins, synergy, Aspergillus

Correspondence: Joseph Meletiadis, Ph. D.

Asst. Prof. of Mycology,
Clinical Microbiology Laboratory,
Attikon University Hospital
Rimini 1, Haidari, 124 62 Athens
Tel: 210-583-1909, Fax: 210-532-6421
Email: jmeletiadis@med.uoa.gr
Antifungal combination therapy with voriconazole or amphotericin B and an echinocandin is often employed as primary or salvage therapy for the management particularly of refractory aspergillosis. The pharmacodynamic interactions of amphotericin B- and voriconazole-based combinations with the three echinocandins caspofungin, micafungin and anidulafungin in the presence of serum were tested against 15 A. fumigatus, A. flavus and A. terreus isolates assessing both their growth inhibitory and fungicidal activity. In vitro activity of each drug alone and in combination at 1:1 fixed concentration ratio was tested with a broth microdilution colorimetric method and interactions were assessed with isobolographic analysis. Synergy was found for all amphotericin B- and voriconazole-based combinations with amphotericin B-based combinations showing strong inhibitory synergistic interactions (interaction indices 0.20-0.52) and voriconazole-based combinations demonstrating strong fungicidal synergistic interactions (0.10-0.29) (p<0.001). Drug- and species specific differences were found with caspofungin and A. fumigatus exhibiting the weakest synergistic interactions. In the presence of serum, the synergistic interactions were reduced in the following order (from largest to smallest decrease) micafungin>anidulafungin>caspofungin and A. flavus>A. fumigatus>A. terreus resulting in additive interactions particularly for inhibitory activities of amphotericin B+echinocandin combinations and fungicidal activities of voriconazole+echinocandin combinations. Drug- and species specific differences were found in the presence of serum for inhibitory activities of antifungal drugs with lowest interaction indices observed for amphotericin B+caspofungin (median 0.77) and for A. terreus (median 0.56). The present in vitro data showed that serum had a major impact on synergistic interactions of amphotericin B/voriconazole+echinocandin combinations resulting in additive interactions explaining the indifferent outcome usually observed in vivo.
INTRODUCTION

Invasive aspergillosis is a life-threatening disease in immunocompromised patients associated with high mortality, despite antifungal therapy (1). Although voriconazole is the first choice for treatment for invasive aspergillosis, amphotericin B and echinocandins (mainly caspofungin) are also used to treat this infection (2, 3). Alternatively and in particular for refractory aspergillosis, antifungal combination therapy is often employed as primary or salvage therapy for the management of this infection with the hope to improve monotherapy outcome (4-6). Amongst the most commonly used antifungal combination regimens is voriconazole or amphotericin B with an echinocandin given the potential for synergistic interactions because of the distinct mechanism of action of these drugs; voriconazole and amphotericin B alters cell membrane function whereas echinocandins alters cell wall function.

Few comparative clinical trials assessed the efficacy of antifungal combinations as primary therapy of invasive aspergillosis showing an insignificant advantage of voriconazole+echinocandin compared to voriconazole monotherapy (7, 8) and favorable response of liposomal amphotericin B+echinocandin combination therapy compared to the mootherapy with a higher dose of liposomal amphotericin B (9). However, pharmacodynamic interactions in clinical trials may be obscured by confounding factors like underlying disease, concomitant therapy, and toxicity and because combination regimens were compared with one of the two monotherapy regimens without taking into account the effect of the second drug. Most of the available information concerning the efficacy of antifungal combinational therapy is coming from preclinical studies with in vitro studies demonstrating mostly synergistic to additive/indifferent interactions (10-15) and in vivo showing mostly no significant improvement compared to monotherapy (16-25).

Although standardized antifungal susceptibility testing is now available (26, 27), the information gleaned from in vitro susceptibility testing is characterized by limitations, as the MIC provides only a static measurement of antimicrobial effect in a defined medium (28). Biological
fluids such as human serum and urine can have profound effects on antimicrobial pharmacodynamics (29, 30) with numerous studies demonstrating that only the free or unbound fraction of drug is available for antimicrobial activity (31). However, the MICs of antifungal drugs are usually increased in the presence of serum but the increase cannot be predicted based on the free drug concentrations (32-36). We also recently investigated the effect of serum on antifungal drugs against *Aspergillus* spp. showing a differential effect with increased activity of voriconazole and echinocandins (at supra-MEC concentrations) and decreased activity of amphotericin B and echinocandins (at sub-MEC concentrations) in the presence of serum not predicted by % protein binding (37). Although the impact of human serum on in vitro activities of single drugs has been previously investigated, the effect of serum on antifungal combinations is largely unknown.

Serum may influence the in vitro activity of antifungal drugs directly by decreasing the free fraction of drugs and indirectly affecting fungal growth (38, 39). Since antifungal agents are extensively bound to serum proteins with rates of 96% for caspofungin, 99.8% for micafungin (14), ~99% for anidulafungin, ~60% for voriconazole and >95% for amphotericin B (40), the impact of serum on the nature and/or magnitude of pharmacodynamic interactions is expected to be large.

Given that MIC determination in the presence of serum may be a better predictor of in vivo outcome (33), pharmacodynamics interactions in the presence of serum may be clinically relevant.

A comparative in vitro study assessing antifungal combinations of the three echinocandins with amphotericin B or voriconazole against different *Aspergillus* species is missing. Therefore, the aim of the present study was to characterize the pharmacodynamic interactions of amphotericin B- and voriconazole-based combinations with the three echinocandins caspofungin, micafungin and anidulafungin in the presence of serum against *A. fumigatus*, *A. flavus* and *A. terreus* isolates assessing both their growth inhibitory and fungicidal activity of the combinations.
MATERIALS AND METHODS

Isolates and inoculum. Fifteen Aspergillus clinical isolates (5 Aspergillus fumigatus (AFM), 5 Aspergillus flavus (AFL) and 5 Aspergillus terreus (AT)) were tested. Species were identified morphologically. Isolates were kept frozen in 10% glycerol at -70°C and revived by subculturing twice onto Sabouraud Dextrose Agar (SDA) plates containing chloramphenicol for 7 to 10 days at 35°C. Conidia were collected with a wet cotton swab and suspended in sterile normal saline containing 0.005% Tween20. The conidial suspensions were adjusted using the Neubauer counting chamber to 4 x 10^4 conidia/ml corresponded to two times the final inoculum in saline or human serum. The concentration and viability of each suspension was confirmed by culturing on SDA plates for 24h at 35°C. The Candida krusei (ATCC 6258), Candida parapsilosis (ATCC 22019) and Aspergillus fumigatus (ATCC MYA-3626) were used as quality control strains.

Growth medium and human serum. RPMI 1640 medium (with L-glutamine and without bicarbonate), buffered with 0.165 M 3-N-morpholinepropanesulfonic acid (MOPS) (AppliChem, Darmstadt, Germany) to pH 7.0 was used as growth medium. Human serum was pooled from outpatients, heat inactivated at 56°C for 30 minutes and stored at -70°C for 7-10 days until used.

Antifungal agents and drug dilutions. Stock solutions of pure powders were prepared in dimethyl sulfoxide (voriconazole and anidulafungin and amphotericin B) or water (caspofungin, micafungin) and were stored at -70°C until used. Following the principles of CLSI M38-A2 (27), serial twofold dilutions at 2x the final concentration were prepared in 8 wells containing 100 μl of double strength test medium (RPMI 1640 medium with MOPS) in 96-well flat-bottom microtitration plates (Costar 3596; Corning Inc., Antisel, Athens, Greece). The final concentrations of antifungal agents, after the addition of conidial inocula, ranged from 8 to 0.06 mg/L. For combination studies a 1:1 fixed concentration ratio design was followed where 50μl of the first drug was combined with 50μl of the second drug at 4x the final concentration which ranged from 8 to
0.06 mg/L for each drug. The 1:1 ratio was chosen in order to approximate the ratio of trough levels attained with standard dosing of antifungal drugs (41).

**XTT and Menadione.** The XTT sodium salt, (Applichem, Bioline, Athens, Greece) was dissolved in sterile distilled water before use. Menadione (Applichem, Bioline, Athens, Greece) was used as electron coupling agent and was initially dissolved in absolute ethanol at a concentration of 58 x 10^{-3} M. A working solution of 0.5 mg/ml of XTT with 31.25 x 10^{-6} M of menadione was prepared in sterile distilled water and it was filter before use.

**Susceptibility testing.** The *in vitro* susceptibilities of all 15 *Aspergillus* isolates to amphotericin B, voriconazole and all three echinocandins alone and in combination were tested following the principles of CLSI M38-A2 method using the standard medium without and with 50% (v/v) human serum. To achieve this, 100 µl of conidial suspensions in sterile saline or in 100% human serum were inoculated into 100 µL of double strength growth medium containing twofold serial dilutions of antifungal drugs at 2x (for drugs alone) or 4x (for combinations) the final concentrations in 96-well flat-bottom microtitration plates. Plates were then incubated at 35°C for 48h and fungal growth in each well was quantified visually with the aid of a magnifying mirror and with the XTT methodology in order to assess growth inhibitory and fungicidal activities as described below based on previously published studies (42, 43).

All experiments were performed in triplicates. The minimum effective concentrations (MEC) of caspofungin, micafungin and anidulafungin were macroscopically and microscopically determined as the lowest drug concentration with prominent growth reduction and short, stubby and aberrant hyphae, respectively whereas the minimum inhibitory concentration (MIC) of amphotericin B and voriconazole was determined as the lowest drug concentration showing complete inhibition of growth (27).

**Inhibitory activity.** Inhibitory activities of antifungal drugs were assessed using the XTT methodology previously described (44, 45). Briefly, after the 48h of incubation, 50 µl of the XTT-menadione working solution were added to each well, yielding a final concentration of 0.1 mg/ml of
XTT and 6.25x10^{-6} M of menadione. Subsequently, the microtitration plates were incubated at 37°C for 2 h. Plates were shaken for 1 to 2 min (Wallac Plate Shake 1296-004; Wallac OY, Turku, Finland) until the formazan derivatives were completely dissolved and the color absorbance was measured spectrophotometrically at 450 nm with reference wavelength at 630 nm (Infinite F200, Tecan, Austria).

The background absorbance $A_{\text{background}}$ (absorbance of conidia-free plates processed in the same way as the inoculated plates) was subtracted and the % of growth at each drug concentration ($A_{\text{well}}$) with and without serum was calculated based on the absorbance of the drug-free control ($A_{\text{drug free}}$) with and without serum, respectively as $100\% \times \frac{(A_{\text{well}} - A_{\text{well background}})}{(A_{\text{drug free well}} - A_{\text{drug free well background}})}$.

**Fungicidal activity.** After determination of growth inhibitory activities, fungicidal activities of all antifungal combinations were assessed as previously described (43). Briefly, the contents of all clear wells and the well at 0.5xMIC were carefully aspirated, and washed twice with 200 μl of normal saline prewarmed at 37°C. After gentle agitation, 200 μl of fresh growth medium were added to each well and microtitration plates were incubated at 37°C for 24 h. Subsequently, 50 μl of the XTT-menadione working solution were added to each well and further incubated at 37°C for 2 h. After plates were shaken for 1 to 2 min, the color absorbance was measured spectrophotometrically at 450 nm with reference wavelength at 630 nm (Infinite F200, Tecan, Austria) and % of fungal growth was calculated described above.

**Growth endpoint determination.** The % of fungal growth estimated from the growth inhibitory and fungicidal experiments, were further analyzed with nonlinear regression analysis based on the sigmoidal $E_{\text{max}}$ model with variable slope, described by the equation $E = E_{\text{min}} + (E_{\text{max}} - E_{\text{min}}) \times C^n/(C^n + IC_{50}^n)$, where $E_{\text{max}}$ is maximal growth at the drug-free control for the growth inhibitory experiments and the maximal XTT absorbance for the fungicidal experiments, $E_{\text{min}}$ is the minimal growth observed at high drug concentrations, C is the drug concentration, IC_{50} is the drug concentration corresponding to 50% of $E_{\text{max}} - E_{\text{min}}$ and $n$ is the Hillslope (GraphPad Prism 5.0).
Software, San Diego, CA). Based on this equation, the near-maximum inhibitory (IC₉₀) and fungicidal (FC₉₀) concentration was calculated as the concentration corresponding to 10% growth (Eₘₐₓ-Eₐₘₜ) from the growth inhibitory and fungicidal experiments, respectively.

As previously shown, the MIC of amphotericin B and voriconazole and the MEC of echinocandins corresponds to the lowest drug concentration with 10% (IC₉₀) and 50% (IC₅₀) of growth, respectively with the XTT methodology (44, 45) whereas the minimal fungicidal concentration of amphotericin B and voriconazole corresponds to the lowest drug concentration with 10% of growth (FC₉₀) with the XTT methodology (43).

**Interaction analysis.** Pharmacodynamic interactions based on inhibitory and fungicidal activities were assessed at 10% (IC₉₀ and FC₉₀) and 50% growth endpoints (IC₅₀ and FC₅₀) with the Lowe additivity-based isobolographic analysis as previously described (46). Briefly, for the combination of amphotericin (AMB) or voriconazole (VOR) with each echinocandin (ECH) the interaction index (Iₗ) was calculated for each X% growth endpoint based on the following equation

\[ Iₗ = \frac{Cₗ,AMB/VOR_{comb}}{ICₗ,AMB/VOR_{alone}} + \frac{Cₗ,ECH_{comb}}{ICₗ,ECH_{alone}} \]

where \( ICₗ,AMB/VOR_{alone} \) and \( ICₗ,ECH_{alone} \) are the concentration of amphotericin B/voriconazole and the echinocandin alone and \( Cₗ,AMB/VOR_{comb} \) and \( Cₗ,ECH_{comb} \) are the concentrations of amphotericin B/voriconazole and the echinocandin in combination corresponding to X% (10% or 50%) growth endpoint, respectively. The \( ICₗ,AMB/VOR_{alone} \) and \( ICₗ,ECH_{alone} \) were determined from the Emax modeling of the single drug concentration-effect curves from all three replicates. Whenever echinocandins did not exhibit a 10% growth endpoint (e.g. in absence of serum) at concentrations tested, the \( ICₗ,ECH_{alone} \) was considered as the next highest twofold concentration tested i.e. 16 mg/l. This approximation has a minimal impact on the Iₗs which are mainly determined by the first term of the interaction index equation.

\[ Cₗ,AMB/VOR_{comb}/ICₗ,AMB/VOR_{alone} \] since the second term \( Cₗ,ECH_{comb}/ICₗ,ECH_{alone} \) is much smaller than the first term because \( Cₗ,ECH_{comb} ≪ ICₗ,ECH_{alone} \). The \( Cₗ,AMB/VOR_{comb} \) and \( Cₗ,ECH_{comb} \) were determined from the Emax modeling of the concentration-effect curves of the 1:1 fixed-ratio combinations from all three replicates where the total concentration \( C_{TOT} = Cₗ,AMB/VOR_{alone} + Cₗ,ECH_{alone} \) was used as the
independent variable for the regression analysis (47). Individual $C_{X,AMB/VOR}$ and $C_{X,ECH}$ were calculated from $C_{TOT}$ obtained from regression analysis as $C_{TOT}/2$ since both drugs in the 1:1 fixed-ratio combination are at equal concentrations. Synergy and antagonism was concluded when the $I_i$ was statistically significantly lower than 1 and higher than 1.25, respectively, based on t test as previously described (48). In any other case additivity was concluded.

In order to compare the interactions 1) among the three echinocandins for amphotericin B-based and voriconazole-based combinations, 2) between amphotericin B and voriconazole for each echinocandin, 3) the interactions of each combination with and without serum, and 4) all combinations among the three species, the $I_i$s were analyzed with repeated measures analysis of variance followed by Tukey’s multiple comparison test (Graphpad Prism 5.0). A $P$ value lower than 0.05 (two-tailed) was considered as statistically significant.

RESULTS

Concentration effect relationship for single drugs. The MICs of the QC strains were within the reference ranges (27). In particular for *A. fumigatus* QC strain, the CLSI and XTT MICs, MECs and MFCs of amphotericin B, voriconazole and echinocandins were within the previously determined ranges (42, 43). The MEC of caspofungin, micafungin and anidulafungin against all *Aspergillus* isolates were 0.5-1 mg/L, 0.06-0.12 mg/L and 0.03-0.06 mg/L, respectively and the MICs amphotericin B and voriconazole were 0.5-4 mg/L and 0.25-2 mg/L, respectively. Similarly, the XTT IC_{50}s of caspofungin, micafungin and anidulafungin were 0.07-1 mg/l, 0.01-0.09 mg/l and 0.01-0.06 mg/l, respectively (data not shown) and the XTT IC_{90}s of amphotericin B and voriconazole were 0.71-2.99 mg/l and 0.14-5.28 mg/l, respectively for all strains and species (Table 1).

Pharmacodynamic interactions of combinations. The results of interaction analysis for amphotericin B- and voriconazole-based combinations with the three echinocandins are shown in Table 1 for the inhibitory and in Table 2 for fungicidal activities for the 10% growth endpoint.
Similar results but weaker interactions were found for both inhibitory and fungicidal effects for the 50% growth endpoint (data not presented).

**Pharmacodynamic interactions in absence of serum.** Synergy was found for all amphotericin B- and voriconazole-based combinations for both inhibitory and fungicidal activities (Ii<1, p<0.05) (Tables 1 and 2). Overall, voriconazole-based combinations showed 2.13-7.4-fold stronger synergistic fungicidal interactions compared to inhibitory interactions particularly with micafungin and anidulafungin (p<0.001). The synergistic interactions of amphotericin B-based echinocandin combinations (Iis 0.20-0.52) were stronger than the voriconazole-based echinocandin combinations (0.43-0.84) for inhibitory effects whereas the opposite was observed for fungicidal effects (0.14-0.58 vs. 0.10-0.29, respectively) (p<0.001) (Table 1 and 2).

Significant differences were found among the three echinocandins with caspofungin-based combinations showing weaker synergy compared to micafungin and anidulafungin-based combinations for inhibitory (median Ii 0.34 vs. 0.26 vs. 0.24 for amphotericin B and 0.60 vs. 0.55 for voriconazole, respectively) and fungicidal activity (0.31 vs. 0.26 vs. 0.19 and 0.37 vs. 0.14 vs. 0.12, respectively) (p<0.0001). Among the three species, significant differences were found only for inhibitory effects with weaker synergy observed for *A. fumigatus* (median Ii=0.60) compared to *A. flavus* (median Ii=0.35) and *A. terreus* (median Ii=0.44) isolates (p=0.0005).

**Pharmacodynamic interactions in the presence of serum.** The interaction indices of all amphotericin B- and voriconazole-based echinocandin combinations increased in the presence of serum reversing the synergy detected in absence of serum to additivity in the presence of serum for inhibitory activities (ANOVA p<0.001) (Table 1). This phenomenon was also observed for the fungicidal activities of voriconazole+echinocandins combinations. Overall, the largest reduction of synergistic inhibitory interactions in the presence of serum was found for amphotericin B+echinocandin (average increase of 3.3-fold) compared to voriconazole+echinocandin (average increase of 2-fold) combinations and among echinocandins’ combinations for micafungin- (3.6-fold) and anidulafungin-based (2.9-fold) combinations compared to caspofungin-based (2-fold)
combinations (Table 1) (p<0.0001). Of note, the reduction of synergistic inhibitory interactions in
the presence of serum resulted in antagonism for amphotericin B+micafungin combination against
A. flavus. Regarding the fungicidal effects, the largest reduction of synergistic interactions was
found for voriconazole+echinocandin combinations and particularly with micafungin (2.5-8.1 fold)
and anidulafungin (1.7-5.5-fold) (p<0.0001) (Table 2). Significant differences among the three
Aspergillus species were found only for growth inhibitory effects with A. terreus demonstrating the
smallest increase of lis in the presence of serum (mean increase 1.7-fold) compared to the A.
fumigatus (4.4-fold) and A. flavus (6.7-fold) (p=0.011).

Drug specific differences were found in the presence of serum, based on inhibitory effects
where overall amphotericin B+caspofungin showed the lowest (median li=0.77) and amphotericin
B+micafungin the highest lis (median li=2.19) among all combinations; no significant differences
were found among voriconazole-based combinations (median li 1.01-1.033). Fungicidal interactions
did not differ significantly among all combinations (median li 0.40-0.63) in the presence of serum.
Species specific differences were also found for inhibitory effects with the lowest lis observed with
A. terreus (median li=0.56) compared to A. fumigatus (median li=1.43) and A. flavus (median
li=1.15) (p=0.019) and for the fungicidal effects with lowest lis observed for A. fumigatus (median
li=0.31) compared to A. flavus (median li=0.62) and A. terreus (median li=0.48) (p=0.0031).

**DISCUSSION**

Synergy in vitro was found in absence of serum for all amphotericin B+ and
voriconazole+echinocandin combinations with amphotericin B-based combinations exhibiting
strong inhibitory and voriconazole-based combinations exhibiting strong fungicidal synergistic
interactions. Drug- and species-specific differences were found with caspofungin and A. fumigatus
exhibiting the weakest synergistic interactions. In the presence of serum, the synergistic interactions
were reduced resulting in additive interactions particularly for inhibitory effects. The largest
decrease of synergistic interactions was observed for inhibitory activities of amphotericin B+echinocandin combinations and for fungicidal activities of voriconazole+echinocandin combinations. Among echinocandins and *Aspergillus* species, the synergistic interactions were reduced in the presence of serum in the following order from largest to smallest decrease micafungin>anidulafungin>caspofungin and *A. flavus>*A. fumigatus>*A. terreus*. In the presence of serum most combinations were additive based on inhibitory effects, with amphotericin B+caspofungin having the smaller interaction indices and amphotericin B+micafungin having the largest, whereas based on fungicidal effects most combinations remain synergistic with no significant differences among them. Among *Aspergillus* species, the weakest synergistic interactions were found with *A. flavus* for both inhibitory and fungicidal effects.

In most previously published studies, amphotericin B or voriconazole combinations with an echinocandin were tested with a microdilution assays and assessed based on the fractional concentration inhibitory (FIC) indices resulting in additive/indifferent interactions (FIC indices 0.5-4) and few times in synergistic interactions (FIC index<0.5) but never in antagonistic interactions (FIC indices >4) (10, 49-52). The discrepancy with the findings of the present study where most combinations were synergistic was due to the different cutoffs used to assess in vitro interactions. Although the FIC index range 0.5-4 was proposed to define additive/indifferent interactions (53), this range is very broad to detect significant pharmacodynamic interactions and combinations with FIC index <1 and >1.25 were found to be in vivo synergistic and antagonistic, respectively (48, 54, 55). These cutoffs may also be reliable for voriconazole+echinocandin combinations since it was previously found that the voriconazole+anidulafungin combination prolonged survival compared to monotherapy when in vitro the FIC index was 0.5-1 (16). Using the cutoff of 1 most of in vitro combinations was synergistic rather than additive as found in the present study (15, 56).

Another source of discrepancy may be the different growth endpoints for amphotericin B/voriconazole (MIC or complete growth inhibition) and echinocandins (MEC or prominent growth inhibition) (11). FIC indices should always be calculated based on iso-effective concentrations i.e.
drug concentrations that produce the same effect at a certain level e.g. 10% growth inhibition, fungicidal action, or metabolic inhibition (57). Thus, the MIC which corresponds to complete (100%) growth inhibition and the MEC which corresponds to lowest concentration with aberrant hyphae and correlate with prominent (50%) growth inhibition are not iso-effective (45). Using the same endpoint for both drugs alone and in combination overcome the dilemma which endpoint should we use for the combination of amphotericin B/voriconazole and an echinocandin. In the present study, pharmacodynamic interactions were assessed based on two different effects i.e. inhibitory and fungicidal effects, at two different effect levels (10% and 50%) with stronger interactions observed at 10% level as also found previously (58). Effective concentrations were calculated with nonlinear regression analysis providing a greater precision and accuracy than the approximate and variable visually determined MICs and MECs.

The synergistic interaction between amphotericin B/voriconazole and an echinocandin could be explained by the different mechanism of actions targeting cell membrane and wall, respectively. The reduction of synergy in the presence of serum could be explained by the effect of serum on each drug alone and in combination. Serum increased the ICs and FCs of amphotericin B and amphotericin B+echinocandin particularly for fungicidal effects. By contrast, voriconazole ICs and FCs were reduced in the presence of serum whereas voriconazole+echinocandin concentrations increased or remained the same. The most prominent effect of serum that led to reduction of synergistic interactions was the marked decrease of ICs of echinocandins observed in the presence of serum resulting to higher interaction indices. The reduction of synergistic fungicidal interactions was due to higher increase of total concentration of combinations in the presence of serum compared to increase of drugs alone. This differential effect may be related to the molecular interactions for binding sites since amphotericin B, voriconazole and echinocandins are highly bound to albumin (41). The increase of ICs and FCs could be due to protein binding, decreasing the amount of free drug available to exert the inhibitory or fungicidal effect. The reduction of echinocandins’ ICs may be due to glycerol response, calcineurin stress response pathways and
chitin synthesis which are synergistically inhibited by serum and echinocandins in a similar way like the synergistic interaction between the chitin synthase inhibitor nikkomycin Z, or the calcineurin inhibitors tacrolimus and cyclosporine and echinocandins (59, 60).

Most in vivo studies of amphotericin B/voriconazole+echinocandin combinations showed an indifferent outcome (i.e. combination therapy outcome similar to monotherapy) (11, 16, 17, 19-22, 25, 61, 62) and few demonstrated an enhanced outcome compared to monotherapy (18, 23, 24, 63). However, in most of the latter studies single doses of combined drugs were used making impossible the determination of a synergistic interactions since both additive and synergistic interactions would have an effect better than each drug alone. In one study where synergy was found between voriconazole and anidulafungin in mice dosed once daily, the ratio of voriconazole:anidulafungin trough levels were <1:8 that rarely observed in humans with bid dosing of voriconazole (63). This in agreement with a recent clinical trial of antifungal combination therapy with the same drugs where no significant improvement was found as observed in the present study in the presence of serum (8). Finally, the unexpected antagonism between micafungin and amphotericin B in the presence of serum against A. flavus is in line with the in vivo antagonism when micafungin was sequentially combined with low dose of liposomal amphotericin B and requires further investigation to elucidate whether this effect is because of immunological and/or pharmacodynamic interactions (64). In vivo extrapolation of in vitro data would require experiments with multiple doses, determination of dose-response curves of drugs alone and in combination and pharmacokinetic data correlating in vitro concentrations with in vivo drug levels.

In conclusion, the present in vitro data showed that serum had a major impact on synergistic interactions of amphotericin B/voriconazole+echinocandin combinations resulting in additive interactions explaining the indifferent outcome usually observed in vivo.

**ACKNOWLEDGMENT**

This study was supported by an unrestricted grant from Pfizer Hellas.
REFERENCES


Table 1. Inhibitory pharmacodynamic interactions between amphotericin B (AMB) and voriconazole (VOR) with each of the three echinocandins caspofungin (CAS), micafungin (MIC) and anidulafungin (ANI) with and without serum. Median and range of drug concentrations and interaction indices are presented for each species corresponding to a 10% growth endpoint.

<table>
<thead>
<tr>
<th>Species (isolates)</th>
<th>Drugs without serum</th>
<th></th>
<th>Drugs with serum</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AMB</td>
<td>Drug IC₉₀s of drugs alone</td>
<td>Total IC₉₀s concentration</td>
<td>Interaction indices</td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td>AMB+ VOR+ AMB+ VOR+</td>
<td>AMB+ VOR+ AMB+ VOR+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMB</td>
<td>1.01 (0.71-1.63)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td>0.29 (0.14-0.53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>&gt;8 (&gt;8-&gt;8) 1.01 (0.35-1.16) 0.41 (0.28-0.75) 0.40 (0.23-0.85) s 0.84 (0.61-1.02) d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC</td>
<td>&gt;8 (&gt;8-&gt;8) 0.90 (0.75-1.38) 0.39 (0.20-0.58) 0.52 (0.27-0.74) s 0.70 (0.50-0.95) s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANI</td>
<td>&gt;8 (&gt;8-&gt;8) 0.74 (0.50-1.42) 0.38 (0.24-0.65) 0.40 (0.26-0.74) s 0.69 (0.60-0.92) s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. fumigatus (N=5)</td>
<td>1.35 (1.24-2.25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMB</td>
<td>1.35 (1.24-2.25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td>0.17 (0.14-0.29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>&gt;8 (&gt;8-&gt;8) 0.68 (0.2-1.29) 0.22 (0.14-0.32) 0.25 (0.08-0.49) s 0.56 (0.41-0.77) s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC</td>
<td>&gt;8 (&gt;8-&gt;8) 0.71 (0.16-0.87) 0.16 (0.13-0.26) 0.20 (0.06-0.27) s 0.45 (0.38-0.58) s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANI</td>
<td>&gt;8 (&gt;8-&gt;8) 0.69 (0.16-0.87) 0.16 (0.13-0.26) 0.20 (0.06-0.27) s 0.44 (0.37-0.56) s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. flavus (N=5)</td>
<td>4.6 (3.9-5.67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMB</td>
<td>4.6 (3.9-5.67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td>0.07 (0.02-0.12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>1.52 (0.52-6.72) 2.3 (1.58-4.29) 0.15 (0.12-0.21) 1.07 (0.63-1.69) d 1.28 (0.91-2.46) d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC</td>
<td>0.36 (0.26-0.59) 2.86 (1.67-4.12) 0.14 (0.02-0.19) 4.09 (2.72-5.05) a 0.95 (0.35-1.19) d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANI</td>
<td>1.78 (0.86-4.78) 2.5 (2.12-3.98) 0.16 (0.12-0.22) 1.13 (0.91-1.51) d 1.34 (0.99-2.29) d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. terreus (N=5)</td>
<td>3.58 (2.56-4.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMB</td>
<td>3.58 (2.56-4.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td>0.19 (0.14-2.86)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>16 (0.94-16) 0.8 (0.76-3.52) 0.27 (0.23-0.65) 0.33 (0.13-0.6) s 0.69 (0.13-0.98) d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC</td>
<td>16 (1.22-16) 2.2 (1.43-2.77) 0.34 (0.23-1.28) 0.65 (0.31-1.24) d 0.89 (0.71-1.25) d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANI</td>
<td>16 (16-16) 1.83 (1.29-2.72) 0.34 (0.26-1.25) 0.34 (0.22-0.39) s 0.81 (0.26-1.27) d</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Total concentration represents the sum of the concentration of the two drugs in combination at a ratio of 1:1
2. Superscripts s, d and a indicate synergistic (I<1), additive and antagonistic (I>1.25) interaction, respectively.
Table 2. Fungicidal pharmacodynamic interactions between amphotericin B (AMB) and voriconazole (VOR) with each of the three echinocandins caspofungin (CAS), micafungin (MIC) and anidulafungin (ANI) with and without serum. Median and range of drug concentrations and interaction indices are presented for each species corresponding to a 10% growth endpoint.

<table>
<thead>
<tr>
<th>Species (isolates)</th>
<th>Drugs</th>
<th>IC₉₀ of drugs alone</th>
<th>without serum</th>
<th>IC₉₀ concentration¹</th>
<th>interaction indices²</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fumigatus (N=5)</td>
<td>AMB</td>
<td>2.1 (1.25-4.38)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td></td>
<td>1.66 (0.67-86.17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>1.33 (0.97-1.97)</td>
<td>0.86 (0.43-3.24)</td>
<td>0.33 (0.16-0.53)³</td>
<td>0.29 (0.01-0.52)³</td>
</tr>
<tr>
<td>MIC</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>0.98 (0.69-1.48)</td>
<td>0.33 (0.14-0.99)</td>
<td>0.25 (0.14-0.39)³</td>
<td>0.10 (0.01-0.17)³</td>
</tr>
<tr>
<td>ANI</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>0.97 (0.58-1.1)</td>
<td>0.52 (0.19-1.04)</td>
<td>0.23 (0.07-0.39)³</td>
<td>0.16 (0.01-0.39)³</td>
</tr>
<tr>
<td>A. flavus (N=5)</td>
<td>AMB</td>
<td>2.22 (1.26-6.34)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td></td>
<td>1.26 (0.41-6.86)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>3.4 (1.3-4.32)</td>
<td>0.55 (0.43-1.09)</td>
<td>0.58 (0.19-1.00)³</td>
<td>0.25 (0.04-0.52)³</td>
</tr>
<tr>
<td>MIC</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>1.86 (1.25-2.92)</td>
<td>0.48 (0.35-0.61)</td>
<td>0.46 (0.13-0.82)³</td>
<td>0.19 (0.04-0.43)³</td>
</tr>
<tr>
<td>ANI</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>1.59 (1.15-2.78)</td>
<td>0.41 (0.24-0.45)</td>
<td>0.44 (0.11-0.66)³</td>
<td>0.17 (0.03-0.42)³</td>
</tr>
<tr>
<td>A. terreus (N=5)</td>
<td>AMB</td>
<td>6.39 (2.22-88.85)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td></td>
<td>9.15 (4.86-12.48)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>2.34 (1.23-7.64)</td>
<td>4.34 (1.8-6.35)</td>
<td>0.17 (0.04-0.28)³</td>
<td>0.25 (0.17-0.35)³</td>
</tr>
<tr>
<td>MIC</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>2.16 (1.08-5.31)</td>
<td>1.21 (0.62-5.9)</td>
<td>0.23 (0.03-0.73)³</td>
<td>0.12 (0.03-0.26)³</td>
</tr>
<tr>
<td>ANI</td>
<td>&gt;8 (&gt;8–&gt;8)</td>
<td>1.41 (1.05-1.6)</td>
<td>1.01 (0.83-11.6)</td>
<td>0.14 (0.01-0.36)³</td>
<td>0.17 (0.05-0.52)³</td>
</tr>
</tbody>
</table>

1 total concentration represents the sum of the concentration of the two drugs in combination at a ratio of 1:1
2 Superscripts s, d and a indicate synergistic (I<1), additive and antagonistic (I>1.25) interaction, respectively.