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Antifungal interactions within the triple combination of amphotericin B, caspofungin and voriconazole against *Aspergillus* species

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***Objectives*: The *in vitro* effects of caspofungin combined with voriconazole and amphotericin B were tested in triplicate experiments against nine clinical isolates of *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus terreus*.**

***Methods*: The isolates were tested against a range of concentrations of voriconazole (0.015–1.0 mg/L), caspofungin (0.125–256 mg/L) and five concentrations of amphotericin B (0.1–0.5 mg/L) with a microdilution chequerboard method based on the CLSI M38-A reference method and the results were analysed with the fractional inhibitory concentration (FIC) index. The effect of individual drugs on the FIC index of each of the double combinations was also evaluated.**

***Results*: The triple combination of voriconazole, caspofungin and amphotericin B against all *Aspergillus* spp. was synergistic (FIC index 0.49–0.57) at low median concentrations of amphotericin B (0.10–0.22 mg/L) and voriconazole (0.07–0.15 mg/L) over a wide range of caspofungin concentrations (4.32–17.28 mg/L). Antagonistic interactions (FIC index 1.65–2.15) were found at higher median concentrations of amphotericin B (0.3–0.5 mg/L) and voriconazole (0.23–0.68 mg/L) over a similarly wide range of caspofungin concentrations (1.47–32 mg/L).**

***Conclusions*: These concentration-dependent interactions may have important clinical implications, which require further evaluation in animal models of invasive aspergillosis.**

Keywords: azoles, polyenes, echinocandins, synergy, antagonism, fractional inhibitory concentration index

Introduction

The introduction of newer antifungal agents with different mechanisms of action has made combination therapy a possibility and an area of compelling investigational interest. Because of their different mechanisms of action, triazoles, echinocandins and polyenes are potential candidates for combination therapy. Echinocandins inhibit the synthesis of 1,3-β-D-glucan, a key component of the cell walls of most fungi; triazoles inhibit the synthesis of ergosterol by inhibiting the enzyme lanosterol 14α-demethylase; and polyenes act directly at the fungal cell membrane to alter its integrity.[1]

Two-drug combinations such as amphotericin B plus an echinocandin or triazole and triazole plus an echinocandin have been investigated *in vitro* against *Aspergillus fumigatus*,[2–4] and *in vivo* against experimental invasive aspergillosis.[5,6] Two-drug combination therapy has also been used in clinical practice.[7,8] The double combination of triazoles with echinocandins was found to be synergistic *in vitro* and *in vivo* raising questions about the effect of adding a third antifungal agent (e.g. amphotericin B). Triple combination therapy with a triazole, an echinocandin and amphotericin B is sometimes used in clinical practice for the management of refractory invasive aspergillosis infection.[9,10] Typically, the third drug is added sequentially to the two-drug

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combination with the hope of improving antifungal efficacy. However, additional benefit arising from triple combination therapy cannot be assumed and must be established.

Little is known about the *in vitro* antifungal interaction of the triple combination of triazole/echinocandin/polyene. We therefore investigated the *in vitro* combination of voriconazole, caspofungin and amphotericin B and all the interactions among these drugs against *A. fumigatus*, *Aspergillus terreus* and *Aspergillus flavus* with a microdilution chequerboard method. In particular, the effect of different concentrations of amphotericin B on the double combination of voriconazole with caspofungin was explored in detail in order to determine whether the apparent synergistic interaction between voriconazole and caspofungin is enhanced or diminished by the addition of amphotericin B.

Materials and methods

Isolates

Three clinical isolates each of *A. fumigatus* (4215, 2025 and 2350), *A. flavus* (50, 8B and 10B) and *A. terreus* (644, 1290 and 1548) were grown on potato dextrose agar (PDA) slants at 30°C for 5–7 days. Conidia were obtained by scraping agar slants with a sterile pipette to achieve a suspension in sterile normal saline. The densities of the conidial suspensions were measured and adjusted on a spectrophotometer (80–82% transmittance for all species) to yield a 10^6 cfu/mL suspension of each isolate. Each suspension of conidia was diluted 1:25 in the medium in order to obtain four times the final inoculum size, which ranged from 0.5×10^4 to 4.0×10^4 cfu/mL in each well. Inoculum preparation, broth inoculation and incubation time were based on the CLSI (formerly NCCLS) M38-A broth microdilution guidelines for mould susceptibility testing.[11] *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control purposes.

Medium

RPMI 1640 with L-glutamine and without bicarbonate (BioWhittaker™ Cambrex Bio Science, Walkersville, MD, USA) buffered at pH 7.0 with 0.165 M MOPS (Sigma-Aldrich, St Louis, MO, USA) was used through all experiments.

Antifungal drugs

Caspofungin (Merck and Company, Rahway, NJ, USA) was obtained as reagent grade powder from the manufacturer and dissolved in medium in order to obtain an initial solution of 1024 mg/L. Voriconazole (Pfizer Pharmaceuticals, New York, NY, USA) was obtained in a 10 000 mg/L vial for injection and diluted in sterile saline in order to obtain a stock solution of 1000 mg/L. Amphotericin B (Apothecon® Ben Venue Laboratories, Inc., Bedford, OH, USA) at a stock concentration of 5000 mg/L was prepared in sterile water. Since we wanted to study the effect of subinhibitory concentrations of amphotericin B on the double combination of voriconazole + caspofungin at a complete growth inhibition MIC endpoint, we chose a range of voriconazole and caspofungin concentrations that included the MIC of these agents and a range of amphotericin B concentrations ≤MIC of amphotericin B. The range of concentrations chosen for these agents encompasses those that are safely achievable in patients.[12–14]

Voriconazole and caspofungin were 2-fold serially diluted in the medium in order to obtain four times the strength of the final concentrations in the microtitration wells, which ranged from 0.015 to 1.0 mg/L and 0.125 to 256 mg/L, respectively. A 50 μL aliquot of each concentration of voriconazole was combined with 50 μL of each concentration of caspofungin including the drug-free controls in six 96-well flat bottom microtitration plates (Corning Inc., Corning, NY, USA) in order to obtain 11×8 chequerboards as shown in Figure 1. The wells in the last column of each plate contained only medium. A 50 μL aliquot of medium containing four times the final concentrations of amphotericin B (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L), was added to each of the six microtitration plates containing the voriconazole + caspofungin chequerboards. Microdilution plates were stored at –70°C for <1 month prior to the start of testing. In separate plates, amphotericin B was serially diluted in medium in order to obtain final concentrations ranging from 0.1 to 2 mg/L.

Susceptibility testing

Microtitration plates were thawed on the day of testing and inoculated with 50 μL of conidia inoculum. Plates were incubated at 37°C in a 95% humidified environment (Steri-Cult 200 Incubator) for a total of 48 h. After 48 h of incubation, plates were assessed visually, with the aid of a reading mirror. The amount of growth in each microtitration well of all six plates was assessed visually by assigning numerical scores from 0 to 4: 0, optically clear wells; 1, slight growth; 2, prominent reduction in growth; 3, slight reduction growth; and 4, no reduction of growth compared with the drug-free growth control of the plate without amphotericin B. The MIC was defined as the lowest drug concentration that provided no visible growth in the wells. Growth was considered any form of hyphal growth inside the well and was confirmed with observation under an inverted microscope when there was doubt about the presence or not of fungal growth.

The use of a complete growth inhibition endpoint for the analysis of pharmacodynamic interactions within the triple combination led us to use high concentrations of caspofungin in the present study. As described below, pharmacodynamic interactions were assessed with the Loewe additivity theory, which is based on the comparison of concentrations of the drugs, which alone and in combination produced the same effect. A complete growth inhibition endpoint was used in the present study because such an endpoint can be determined for all three drugs tested in this study and it is easier and less variable for visual determination than other endpoints such as minimal effective concentrations or 50% growth inhibition.

High off-scale MIC values were converted to the 2-fold dilution just above the highest concentration tested, and low off-scale MIC values were left unchanged. All tests were repeated three times on different days.

Drug interaction and statistical analysis

Antifungal drug interactions were analysed based on the fractional inhibitory concentration (FIC) index.[15] For all wells of the microtitre plates that corresponded to an MIC, the sum of the fractional inhibitory concentrations (ΣFIC) was calculated for each well with the following equation: $\Sigma FIC = FIC_{CAS} + FIC_{VOR} + FIC_{AMB} = (C_{CAS}/MIC_{CAS}) + (C_{VOR}/MIC_{VOR}) + (C_{AMB}/MIC_{AMB})$, where MIC_{CAS}, MIC_{VOR} and MIC_{AMB} are the MICs of caspofungin, voriconazole and amphotericin B alone, respectively, and C_{CAS}, C_{VOR} and C_{AMB} are the concentrations of caspofungin, voriconazole and amphotericin B in combination, respectively, at all wells with no visible growth which were adjacent to wells with growth (isoeffective combinations). Among all ΣFICs calculated for all isoeffective combinations, we estimated the minimum ΣFIC (ΣFIC_{min}) and the maximum ΣFIC (ΣFIC_{max}).[16–19] Because both synergistic and antagonistic interactions can be present within a drug combination,[20] the FIC index which captures only one type of interaction, may not adequately describe the interactions between the three drugs. Therefore, the

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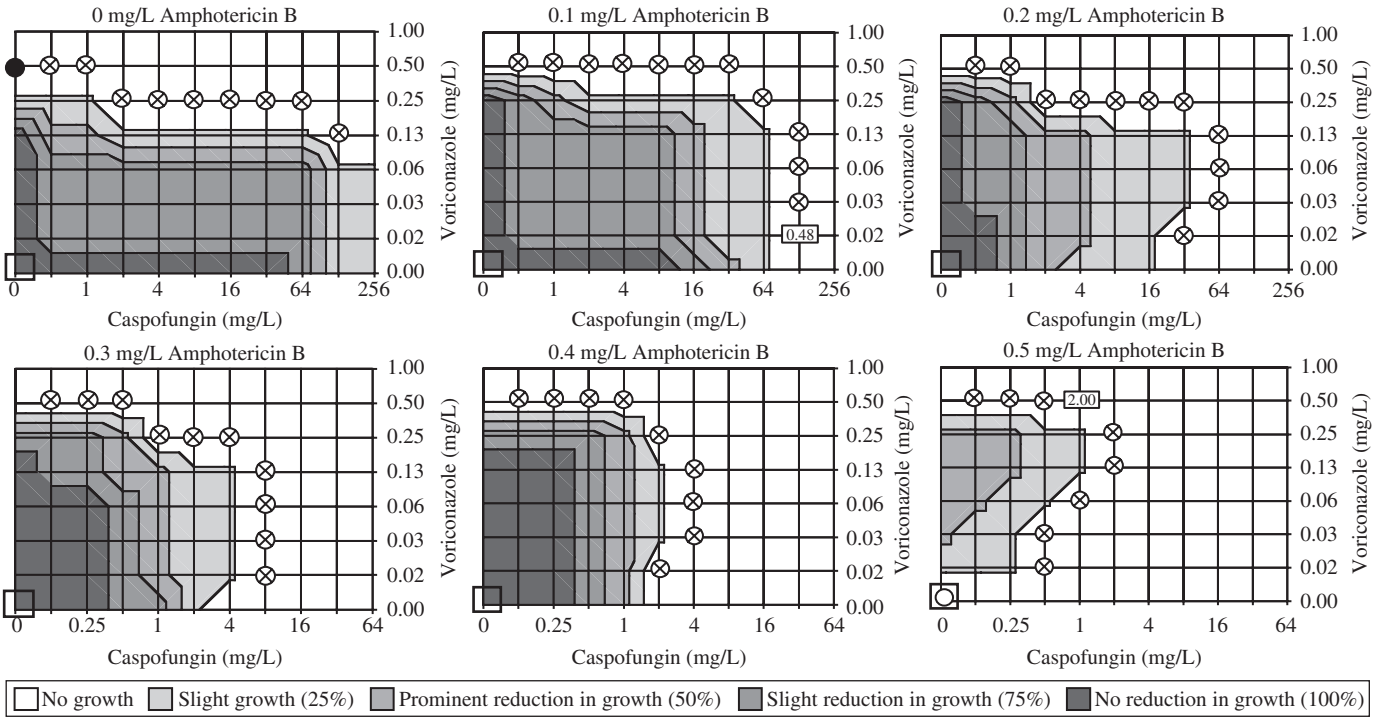

Figure 1. Schematic representation of voriconazole + caspofungin chequerboards in the presence of increasing concentrations of amphotericin B for an *A. fumigatus* isolate (AF4215). The white-to-black gradient corresponds to different levels of fungal growth. Note the synergistic interactions at low concentrations of amphotericin B and the antagonistic interactions at high amphotericin B concentrations. The growth pattern of voriconazole and caspofungin alone is depicted on vertical and horizontal axis of the first plot (0 mg/L amphotericin B). The growth pattern of amphotericin B alone is shown in the bottom left-hand corner of each plot (squares). The growth pattern of amphotericin B + caspofungin combination can be visualized by the growth scores on the horizontal axes of the six contour plots, whereas the growth pattern of the amphotericin B + voriconazole combination can be visualized by the growth scores on the vertical axes of the six contour plots. The MIC of voriconazole alone (black circle), caspofungin alone and amphotericin B alone (white circle) was 0.5, >256 and 0.5 mg/L, respectively. The ΣFIC was calculated for all marked combinations (crossed circles) as $C_{CAS}/MIC_{CAS} + C_{VOR}/MIC_{VOR} + C_{AMB}/MIC_{AMB}$. The numbers inside the white boxes correspond to wells with the $\Sigma FIC_{min} = 0.49 = 128/512 + 0.02/0.5 + 0.1/0.5$ and $\Sigma FIC_{max} = 2.00 = 1/512 + 0.5/0.5 + 0.5/0.5$ of the triple combination.

ΣFIC_{min} and ΣFIC_{max} were used in order to capture the synergistic and antagonistic interactions, respectively, within the triple combination. Thus, among all ΣFICs calculated for a triple combination dataset, the ΣFIC_{min} and ΣFIC_{max} were reported in order to detect synergistic and antagonistic interactions, respectively, as diagrammatically shown in Figure 1.

The cut-offs of 0.5 and 4 for synergy and antagonism, respectively, were originally proposed for two-drug combinations in order take into account interexperimental variation (±1 dilution) of the single-drug antifungal susceptibility testing using geometrically increased drug dilutions.[21,22] As most of the FIC indices of *in vitro* antifungal drug combination studies are within the range of 0.5–4, additivity/indifference was most commonly concluded. Thus, most of the information about drug interactions within this range was lost due to the assumed ±1 dilution error. In the present study, instead of assuming one dilution error for our *in vitro* combination studies, the magnitude of experimental variation was assessed using three replicates.[20] Replication allowed detection of statistically significant deviations of ΣFICs from 1, which is the appropriate cut-off, based on the Loewe additivity theory (combination of 1 mg/L with 1 mg/L of the same drug would result in the same effect as 2 mg/L, i.e. FIC index = 1/2 + 1/2 = 1).[23] A consistent reduction of the concentration of one drug in the combination from 1 to 0.5 mg/L would result in an ΣFIC of 0.75 (0.5/2 + 1/2), which indicates synergy since less drug is needed to produce the same effect. Hence, synergy was concluded when the 95% confidence interval of the ΣFIC_{min}s of all replicates were lower than 1.0, whereas antagonism was concluded when the 95% confidence interval of the ΣFIC_{max}s of all replicates were higher than 1. If the 95% confidence interval included 1.0, additivity was claimed.[15,20,24] Furthermore, the drug concentrations of the combinations corresponding to the ΣFIC_{min} and the ΣFIC_{max} were reported and compared using Student's *t*-test. As further support of this analysis, the cut-off of 1 was previously used to analyse double and triple antimicrobial combinations providing detailed information about drug interactions.[15,20,24–26]

In order to assess the benefit of the triple combination over the double combinations, the ΣFICs of the double combination of drug A and drug B were calculated in the presence of increasing concentrations of the third drug C based on the following general equation, $\Sigma FIC = (C_A/MIC_{A+C}) + (C_B/MIC_{B+C})$, where MIC_{A+C} and MIC_{B+C} are the MICs of drug A and drug B in the presence of a series of concentrations of drug C, and where C_A and C_B are the concentrations of drugs A and B at all isoeffective combinations that corresponded to an MIC in the presence of increasing concentrations of the third drug C. The FIC index of the double combination was then reported as the ΣFIC_{min} or the ΣFIC_{max} depending on which was further from 1.0. These FIC indices were then plotted against the concentrations of the third drug and analysed with one-way analysis of variance (ANOVA) followed by a post-test for linear trend. Finally, in order to adjust for the different MICs, the FIC indices were plotted against the $C_{drug\,C}/MIC_{drug\,C}$ ratio of the third drug.

The three *Aspergillus* species differ in various growth characteristics (e.g. *A. terreus* has longer germination periods than the other species), which may affect the pharmacological actions of

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the antifungal agents in different ways. However, in combination studies these differences are adjusted since the pharmacological actions of the single drugs are compared with those in combination.

Results

MIC results

The geometric mean MIC of voriconazole for *A. fumigatus*, *A. flavus* and *A. terreus* was 0.63, 0.50 and 0.40 mg/L, respectively (Table 1). The geometric mean MIC of caspofungin was 219, 512 and 174 mg/L for *A. fumigatus*, *A. flavus* and *A. terreus*, respectively. The geometric mean MIC of amphotericin B was 0.47, 0.86 and 1.26 mg/L for *A. fumigatus*, *A. flavus* and *A. terreus,* respectively (Table 1).

Triple combination

Table 1 summarizes the results of the FIC index analysis for the triple combination of voriconazole, caspofungin and amphotericin B tested against *A. fumigatus, A. flavus and A. terreus.* The ΣFIC_{min} and ΣFIC_{max} were significantly lower and higher than 1.0, which indicate the presence of both synergistic and antagonistic interactions among the three drugs at different drug concentrations as shown in chequerboards in Figure 1. The median ΣFIC_{min} was 0.49, 0.50 and 0.57, whereas the ΣFIC_{max} was 1.80, 2.15 and 1.65 for *A. fumigatus*, *A. flavus* and *A. terreus*, respectively. The concentrations of voriconazole (0.02–0.5 mg/L) and amphotericin B (0.1–0.2 mg/L) at the synergistic combinations (ΣFIC_{min}) were statistically significantly lower than the concentrations of voriconazole (0.25–1.0 mg/L) and amphotericin B (0.2–0.5 mg/L) at the antagonistic combinations (ΣFIC_{max}) for *A. fumigatus* and *A. flavus*. For *A. terreus*, voriconazole and amphotericin B concentrations at synergistic combinations were not significantly different than the corresponding concentrations at antagonistic combinations (median voriconazole concentrations 0.10 versus 0.23 and median amphotericin B concentrations 0.22 versus 0.3).

Triple versus double combinations

Figure 2 describes the interaction of escalating concentrations of voriconazole, caspofungin and amphotericin B on the double combinations of caspofungin plus amphotericin B, amphotericin B plus voriconazole, and voriconazole plus caspofungin, respectively.

(i) *Voriconazole + caspofungin in the presence of amphotericin B*. The median FIC index for the voriconazole + caspofungin combination was significantly lower than 1, which indicates synergistic interaction for all *Aspergillus* species (0.52, 0.5 and 0.75 for *A. fumigatus*, *A. flavus* and *A. terreus*, respectively) (Figure 2a–c at 0 mg/L of amphotericin B, see highlighted point on each graph). In the presence of increasing concentrations of amphotericin B (0.1–0.5 mg/L), the FIC index of the double combination increased more than two times for all *A. fumigatus* isolates, and two isolates of *A. flavus* and none of the *A. terreus* isolates ($P < 0.05$). This increase was observed at 0.2 mg/L amphotericin B for *A. fumigatus* (Figure 2a) and at 0.4 mg/L amphotericin B for *A. flavus* (Figure 2b) and was due to the decrease of the MIC of caspofungin in the presence of amphotericin B alone.

(ii) *Amphotericin B + caspofungin in the presence of voriconazole*. The median FIC index of the double combination of amphotericin B + caspofungin was significantly lower than 1, which indicates a synergistic interaction for all *Aspergillus* species (0.46, 0.43 and 0.8 for *A. fumigatus*, *A. flavus* and *A. terreus,* respectively) (Figure 2d–f at 0 mg/L of voriconazole, see highlighted point on each graph). The FIC index of the double combination of amphotericin B + caspofungin was increased in the presence of high voriconazole concentrations (0.25 and 0.5 mg/L) for all isolates of *A. fumigatus* (Figure 1d) and two isolates of

Table 1. Interactions among voriconazole, caspofungin and amphotericin B within triple combination against *Aspergillus* spp. assessed with the fractional inhibitory concentration (FIC) index analysis

Endpoint	Species (no. of isolates)	Median (range) ΣFIC	Geometric mean (range) of the MICs (mg/L) of		
			voriconazole	caspofungin	amphotericin B
MIC (drugs alone)	*A. fumigatus* (3)		0.63 (0.5–1)	219 (128–512)[a]	0.47 (0.4–0.6)
	A. flavus (3)		0.50 (0.25–1)	512 (512–512)	0.86 (0.8–1)
	A. terreus (3)		0.40 (0.25–1)	174 (128–256)	1.26 (1–2)
ΣFIC_{min}[b] (synergistic combinations)	*A. fumigatus* (3)	0.49 (0.46–0.51)	0.07 (0.02–0.5)[c]	4.32 (0.5–128)	0.17 (0.1–0.2)[d]
	A. flavus (3)	0.50 (0.40–0.81)	0.15 (0.02–0.5)[e]	14.81 (1–256)	0.10 (0.1–0.1)[f]
	A. terreus (3)	0.57 (0.43–0.86)	0.10 (0.02–0.5)	17.28 (0.5–64)	0.22 (0.1–0.4)
ΣFIC_{max}[g] (antagonistic combinations)	*A. fumigatus* (3)	1.80 (1.60–2.63)	0.68 (0.5–1)[c]	1.47 (0.5–4)	0.3 (0.3–0.5)[d]
	A. flavus (3)	2.15 (1.50–2.62)	0.54 (0.25–1)[e]	5.04 (0.25–128)	0.5 (0.2–0.5)[f]
	A. terreus (3)	1.65 (1.20–2.50)	0.23 (0.02–1)	32 (0.5–256)	0.3 (0.2–0.5)

[a]The MIC for one replicate of an *Aspergillus* isolate was off-scale.
[b]For each isolate the ΣFIC_{min} (minimum sum of the fractional inhibitory concentrations of the three compounds) and its 95% confidence interval for all replicates was lower than 1 indicating synergy.
[c–f]*t*-test $P < 0.05$ compared with concentrations of ΣFIC_{min}.
[g]For each isolate the ΣFIC_{max} (maximum sum of the fractional inhibitory concentrations of the three compounds) and its 95% confidence interval for all replicate was higher than 1 indicating antagonism.

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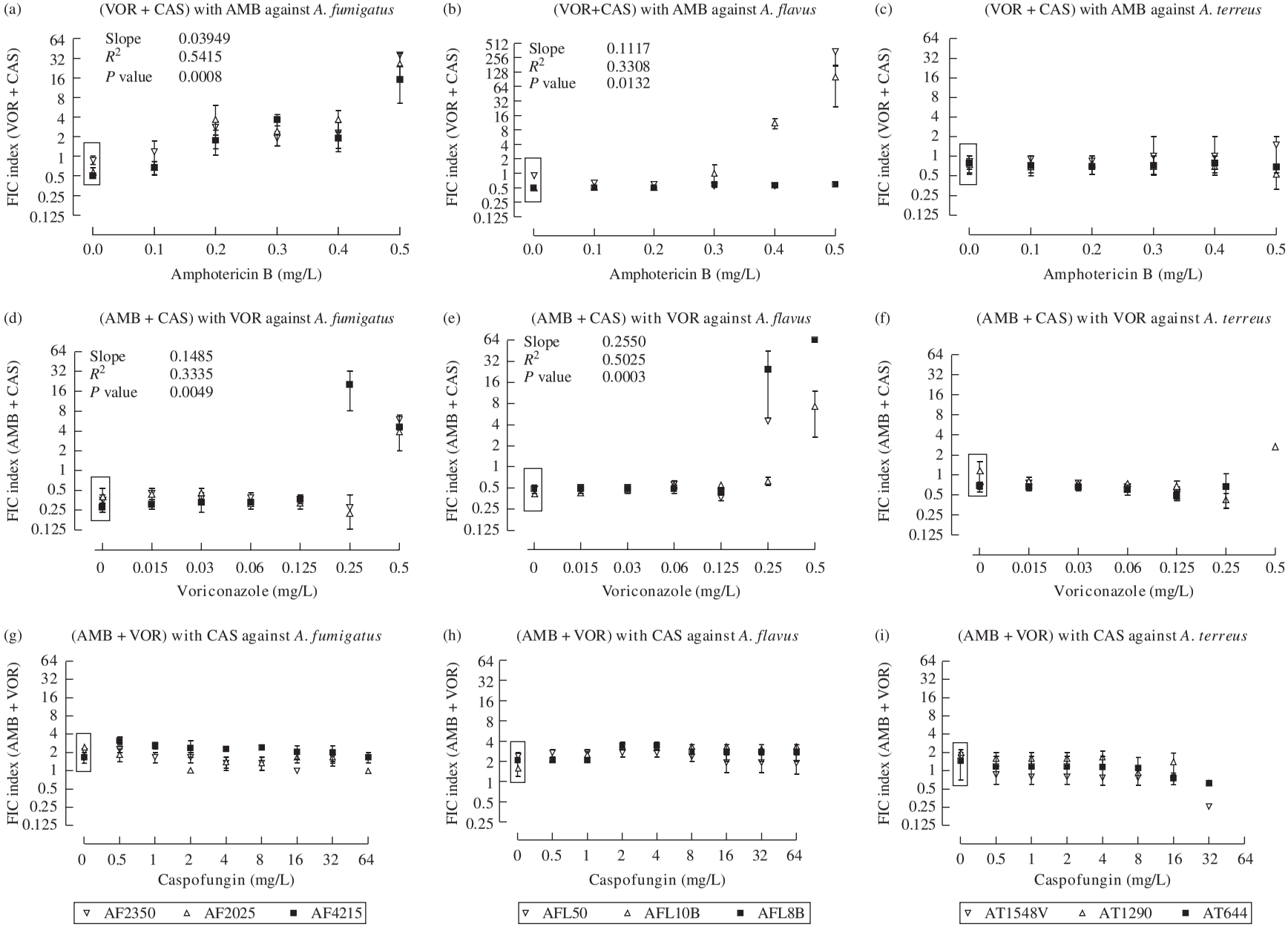

Figure 2. Effect of the third drug on the double combinations. Fractional inhibitory concentration (FIC) indices of double combinations amphotericin B + caspofungin, amphotericin B + voriconazole and voriconazole + caspofungin are presented in the presence of increasing concentrations of voriconazole (VOR), caspofungin (CAS) and amphotericin B (AMB) for each *Aspergillus* isolate. The results of the post-test from linear trend are presented for combinations for which the *P* value of ANOVA was lower than 0.05. Error bars represent range of FIC indices among replicates.

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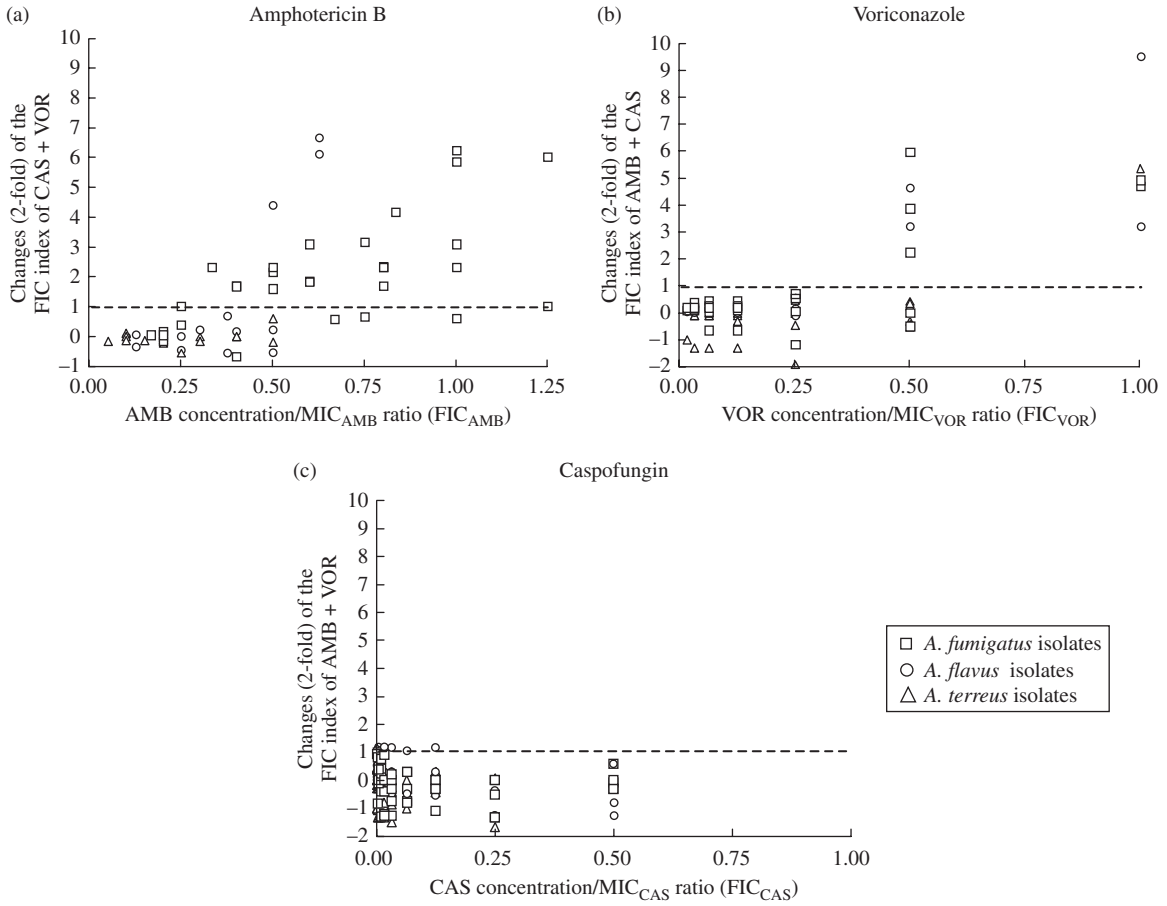

Figure 3. Relationship between the fractional inhibitory concentration (FIC) index of the double combinations caspofungin + voriconazole (a), amphotericin B + caspofungin (b) and amphotericin B + voriconazole (c) and the FIC of amphotericin B (FIC_{AMB}), voriconazole (FIC_{VOR}) and caspofungin (FIC_{CAS}), respectively. As the FIC_{AMB} and FIC_{VOR} approach 1, the FIC index of caspofungin + voriconazole and amphotericin B + caspofungin increases, respectively. AMB, amphotericin B; CAS, caspofungin; VOR, voriconazole.

A. flavus (Figure 2e) for which the FIC index was increased more than 16 times (ANOVA post-test for linear trend $P < 0.05$). This increase was due to the reduction of the MIC of caspofungin in the presence of voriconazole.

(iii) *Amphotericin B + voriconazole in the presence of caspofungin.* The median FIC index of the double combination amphotericin B + voriconazole was significantly higher than 1, which indicates an antagonistic interaction for all *Aspergillus* species (2.5, 2.0 and 2.5 for *A. fumigatus*, *A. flavus* and *A. terreus,* respectively) (Figure 2g–i at 0 mg/L of caspofungin, see highlighted point on each graph). Increasing concentrations of caspofungin from 0.25 to 512 mg/L did not have any significant effect on the FIC index of the double combination amphotericin B + voriconazole. However, a statistically significant 2- to 4-fold reduction of the FIC index was detected at high caspofungin concentrations for two *A. terreus* isolates caused by a reduction of the MIC of voriconazole when all three drugs were combined (Figure 2i).

In order to explain the apparent isolate- and concentration-dependent increase in the FIC index of the double combinations in the presence of voriconazole and amphotericin B but not caspofungin, an extra analysis was performed. Since the actual concentration of the third drug may not be as important to the interaction as the ratio of the concentration of drug C/MIC of the third drug C (FIC_C), the relationship between the FIC index of the double combinations and the FIC_C was explored. As the concentration of amphotericin B or voriconazole approaches its respective MIC (i.e. the FIC approaches 1), the FIC index of the other two compounds increases (Figure 3a and b). This was not observed for caspofungin (Figure 3c).

Discussion

Complex interactions occurred between the three drugs in the triple combination, with both synergistic and antagonistic interactions occurring at different drug concentrations. Synergy in the triple combination was observed at low concentrations of amphotericin B (<0.2 mg/L) and voriconazole (<0.5 mg/L). Antagonism was found at higher concentrations of amphotericin B (0.3–0.5 mg/L) and voriconazole (>0.25 mg/L). This is in concordance with the increase observed in the FIC index of the double combinations voriconazole + caspofungin and amphotericin B + caspofungin with increasing concentrations of amphotericin B and voriconazole, respectively. The synergistic effects of voriconazole + caspofungin and amphotericin B + caspofungin disappear when amphotericin B or voriconazole, respectively, was added to the double combinations resulting in

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antagonism. These effects were mainly observed for *A. fumigatus* and *A. flavus* isolates but not for *A. terreus* probably due to the high amphotericin B MICs for the latter isolates. The double combination of amphotericin B + voriconazole was antagonistic and the addition of caspofungin did not change the interaction.

There are little *in vitro* and *in vivo* data available on triple antifungal combinations. Dannaoui *et al.*[27] reported the *in vitro* evaluation of double and triple combinations of caspofungin, voriconazole, flucytosine and amphotericin B against clinical isolates of *A. fumigatus* and *A. terreus*. Additive interactions (FIC indices ranged between 0.5 and 1) between caspofungin and either amphotericin B or voriconazole were observed for all isolates of *Aspergillus* tested; similar to results in our study. The triple combination of caspofungin + flucytosine + amphotericin B was mostly synergistic. Complex interactions were observed for caspofungin + voriconazole + flucytosine. Synergy or antagonism were found for some isolates depending on the concentrations of voriconazole or caspofungin.[27] In the latter study, the antagonistic interactions were observed at high concentrations of voriconazole, which is in agreement with the results of the present study. Odds[25] previously reported *in vitro* susceptibility results for the triple combination of amphotericin B plus flucytosine plus miconazole or ketoconazole for *Candida* spp. and *A. fumigatus*. The amphotericin B plus flucytosine plus ketoconazole combination was mostly synergistic, and was additive when miconazole was substituted for ketoconazole against three isolates of *A. fumigatus*.

Although triple antifungal combination therapy is often used in a salvage setting, there are few case reports of successful outcome with triple antifungal therapy in humans. In these cases, antifungal drugs are added sequentially in response to a worsening clinical picture. Tascini *et al.*[9] reported a patient with acute myeloid leukaemia and pulmonary cavitary aspergillosis successfully treated with a three drug regimen, which included amphotericin B 3 mg/kg, itraconazole 200 mg twice daily and caspofungin 50 mg iv once daily. Sims-McCallum[10] described the successful treatment of invasive pulmonary aspergillosis in a 12-year-old neutropenic patient with a combination of amphotericin B lipid complex, caspofungin and voriconazole.

Echinocandin/azole *in vitro* combinations have shown mostly additive and synergistic effects,[4] and sometimes indifferent interactions but not antagonism. Using a panel of five markers of antifungal efficacy there was a synergistic interaction between ravuconazole and micafungin in the treatment of experimental invasive pulmonary aspergillosis.[6] Voriconazole combined with caspofungin has also been shown to significantly reduce tissue fungal burden in a guinea pig model over that of controls in a immunocompromised guinea pig of disseminated aspergillosis.[28] These previous *in vitro* and *in vivo* findings are consistent with the present study where the combination of voriconazole + caspofungin was synergistic.

Interactions between caspofungin and amphotericin B *in vitro* have demonstrated synergy, and sometimes indifference but not antagonism against *Aspergillus* species. Synergy between caspofungin and amphotericin B was observed against 50% of *Aspergillus* isolates tested.[2] Cuenca-Estrella *et al.*[29] studied double combinations of amphotericin B, voriconazole, itraconazole and caspofungin against itraconazole-resistant isolates of *A. fumigatus*. No antagonism was found for any combination. The synergistic interaction between amphotericin B and caspofungin was also demonstrated in animal models of invasive aspergillosis where combination therapy resulted in prolonged survival and reduce fungal burden compared with monotherapy groups.[30,31] These previous *in vitro* and *in vivo* findings are also consistent with the present study where the combination of amphotericin B + caspofungin was synergistic.

Amphotericin B/azole combinations have shown variable *in vitro* interactions from additive/indifferent to antagonism.[22] The polyene/azole antagonism was recently demonstrated for the *in vitro* and *in vivo* combination of amphotericin B with ravuconazole in the treatment of experimental invasive pulmonary aspergillosis.[32] However, a concentration-dependent interaction between amphotericin B and itraconazole was found with synergy mainly observed at low concentrations of amphotericin B and antagonism at higher concentrations.[33] This is in agreement with the present study where antagonism was found between amphotericin B with voriconazole using a complete growth inhibition endpoint, which is observed at high concentrations of amphotericin B. A concentration-dependent interaction was also found in the present study for the triple combination, which may be due to the concentration-dependent nature of polyene/azole interaction.

The complexity of the interaction can be explained by the fact that each of the three drugs has different mechanisms of action and each of the possible double combinations has different interactions. Both synergistic and antagonistic interactions occur at different concentrations of each drug. Antagonism was observed at high concentrations of voriconazole and amphotericin B (close to or at MIC), whereas synergy was observed at lower subinhibitory concentrations. These interactions were observed at a wide range of caspofungin concentrations. Because synergistic interactions also occurred at high concentrations of voriconazole, we believe that amphotericin B concentrations may determine the nature of these interactions. Low subinhibitory concentrations of amphotericin B minimize the antagonistic effects of the double amphotericin B + voriconazole combination and maximize the synergistic effects of amphotericin B + caspofungin and voriconazole + caspofungin double combinations.

Among the proposed mechanisms of polyene/azole antagonism is the reduction of amphotericin B binding to depleted fungal membrane ergosterol due to inhibition of the ergosterol biosynthetic pathway by the azole,[22,34,35] the accumulation of azole in the cell membrane that competitively inhibits binding of amphotericin B to bind to ergosterol,[36,37] the interference of amphotericin B with a cell-membrane-associated permease probably involved in azole entry into the cell[38] and reduced azole influx by amphotericin B membrane damage.[39] Amphotericin B at low concentrations (0.2–0.8 μM) forms non-aqueous prepore structures (ionic channels) without the direct participation of ergosterol molecules making the membranes more permeable to urea and glucose.[40] At higher concentrations (>1.2 μM) the initially formed structures interact subsequently with ergosterol in the membrane and form aqueous pores with enlarged diameter. Thus, the synergistic interactions at low subinhibitory amphotericin B concentrations could be explained by increased influx and/or inefficient efflux of voriconazole as a result of permeability changes in the fungal cell membrane caused by amphotericin B. Since ergosterol is not necessary at this stage,[40] inhibition of ergosterol biosynthesis by azoles may not antagonize polyene action. At high amphotericin B concentrations, the drug exerts its antifungal activity by forming aqueous pores, a process that requires ergosterol. Thus, at this stage inhibition of ergosterol biosynthesis by an azole may antagonize polyene action. Finally,

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the synergistic interaction between amphotericin B and caspofungin and between caspofungin and voriconazole may be due to action at different targets since caspofungin inhibits cell wall synthesis; whereas amphotericin B and voriconazole alter membrane integrity.

Antifungal interactions within the triple combination occurred at clinically achievable concentrations,[12–14] which emphasizes the potential clinical importance of these interactions. Note, that the range of safely achievable concentrations of caspofungin in children and adults (0.45–21 mg/L)[14] is encompassed within the range of concentrations that were found in the present study to contribute to synergy and antagonism (1.47–32 mg/L). Subtherapeutic doses of amphotericin combined with high doses of caspofungin and voriconazole may increase antifungal efficacy and decrease potential toxic effects. However, it must be emphasized that the relevance of *in vitro* data for *Aspergillus* species and other invasive moulds to *in vivo* outcomes is unknown. Animal studies are needed to elucidate the clinical implications of these complex interactions observed *in vitro*. Such *in vitro* and *in vivo* correlation studies may form the basis for prospective clinical trials.

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Transparency declarations

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