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In Vitro Synergy Testing of Anidulafungin with Itraconazole, Voriconazole, and Amphotericin B against *Aspergillus* spp. and *Fusarium* spp.

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The in vitro interactions of anidulafungin with itraconazole, voriconazole, and amphotericin B were evaluated by using the checkerboard method. For *Aspergillus* spp., anidulafungin with amphotericin B showed indifference for 16/26 isolates, while anidulafungin with either azole showed a synergy trend for 18/26 isolates. All drug combinations showed indifference for 7/7 *Fusarium* sp. isolates.

Invasive fungal infections due to molds are becoming more prevalent in immunocompromised patients (21). Among the invasive mold infections, *Aspergillus* spp. and *Fusarium* spp. are particularly challenging to manage, due to aggressive courses and high mortality (11, 20).

Since 1959, amphotericin B deoxycholate (AMBD) had been considered the "gold standard" for the treatment of fungal infections. However, due to high failure rates and significant toxicity (6), other agents are being explored today both singly and in combination therapy. Among the azoles, itraconazole (ITR) continues to show some promise against *Aspergillus* spp. (3). Voriconazole (VOR), a novel azole (11), is perhaps the current "gold standard" for the treatment of invasive aspergillosis, although success rates are still less than optimal (5, 8). The echinocandins (caspofungin, anidulafungin [ANID], and micafungin) inhibit 1,3-β-D-glucan synthesis and have in vitro and in vivo activity against *Candida* and *Aspergillus* spp. (4, 16, 17). In the clinical setting, caspofungin appears to be at least as effective as AMBD for salvage therapy of invasive aspergillosis compared to historical controls (13).

Due to the high mortality and lack of an ideal drug for these diseases, combination therapy has been an attractive possibility that has recently received much attention in medical mycology. Early studies have shown in vitro and in vivo advantages of several combinations. Arikan et al. (2) showed additive to synergistic effects of caspofungin with AMBD in vitro against *Aspergillus* and *Fusarium* spp. In a guinea pig model, colony counts of *Aspergillus* spp. and the number of culture-positive tissues were reduced after treatment with VOR and caspofungin compared with either of the agents alone (10). Similarly, Petraitis et al. showed that the combination of micafungin and ravuconazole in a rabbit model had synergistic effects against invasive aspergillosis (18).

The purpose of this study was to evaluate the in vitro interactions of ITR, VOR, and AMBD with ANID against *Aspergillus* spp. and *Fusarium* spp. as preliminary work to support further in vivo and clinical research on these combinations.

Isolates. Twenty-six clinical isolates of *Aspergillus* spp. and seven clinical isolates of *Fusarium* spp. were used. The species distribution was as follows: eight isolates of *Aspergillus flavus*,

TABLE 1. Mean (range) MIC-0 and MIC-2 FICI values at 48 hours for 26 *Aspergillus* sp. and 7 *Fusarium* sp. isolates

Species (*n*)	Mean FICI value (range)					
	Anidulafungin plus itraconazole		Anidulafungin plus voriconazole		Anidulafungin plus amphotericin B	
	MIC-0	MIC-2	MIC-0	MIC-2	MIC-0	MIC-2
A. flavus (8)	0.82 (0.50–1.00)	0.42 (0.25–0.50)	1.00 (1.00–1.02)	0.57 (0.50–1.00)	2.04 (0.56–4.33)	2.31 (0.63–5.00)
A. fumigatus (8)	0.75 (0.50–1.00)	0.47 (0.27–0.52)	0.56 (0.50–1.00)	0.37 (0.26–0.51)	0.52 (0.52–1.02)	0.63 (0.27–1.50)
A. niger (5)	0.90 (0.50–1.00)	2.58 (2.12–4.42)	1.00 (1.00–1.00)	2.24 (2.12–2.48)	0.70 (0.49–1.03)	1.22 (0.61–2.00)
A. terreus (5)	0.70 (0.50–1.00)	1.26 (0.48–2.50)	0.8 (0.50–1.00)	1.14 (0.48–2.24)	1.52 (0.51–2.02)	31.22 (1.48–134.00)
F. oxysporum (2)	2.00 (2.00–2.00)	2.00 (2.00–2.00)	2.00 (2.00–2.00)	1.00 (1.00–1.00)	2.04 (2.02–2.06)	2.14 (2.03–2.25)
F. solani (5)	1.81 (1.06–2.00)	2.00 (2.00–2.00)	2.00 (2.00–2.00)	2.00 (1.00–3.00)	1.54 (0.63–2.00)	1.90 (0.75–2.50)

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TABLE 2. Categorical interpretation of FICI results for 26 *Aspergillus* sp. isolates and 7 *Fusarium* sp. isolates by using MIC-2 at 48 h

Species (*n*)	No. of isolates with given FICI result								
	Anidulafungin plus itraconazole			Anidulafungin plus voriconazole			Anidulafungin plus amphotericin B		
	Synergy	Indifference	Antagonism	Synergy	Indifference	Antagonism	Synergy	Indifference	Antagonism
A. flavus (8)	8	0	0	7	1	0	0	5	3
A. fumigatus (8)	8	0	0	8	0	0	5	3	0
A. niger (5)	0	4	1	0	5	0	0	5	0
A. terreus (5)	2	3	0	3	2	0	0	3	2
F. oxysporum (2)	0	2	0	0	2	0	0	2	0
F. solani (5)	0	5	0	0	5	0	0	5	0

eight isolates of *A. fumigatus*, five isolates of *A. niger*, five isolates of *A. terreus*, two isolates of *Fusarium oxysporum*, and five isolates of *F. solani*.

Drugs and synergy testing. Pure powders of ITR (Janssen), VOR (Pfizer), ANID (Vicuron), and AMBD (Bristol-Myers Squibb) were dissolved to obtain stock concentrations of 6,400 mg/ml. Serial dilutions were made to 6.25 mg/ml. Checkerboard testing was carried out in RPMI 2% glucose in microdilution plates by using elements from the CLSI (formerly NCCLS) M-38A and M-27A2 methods (14, 15). Drug dilutions in twofold increments were prepared at fourfold levels above the desired final concentration for each drug tested. Each of the wells contained combination drug dispensed at 50 μl each, effectively creating a 2× concentration of each drug. Plates were stored at −70°C until inoculation. Conidia of mold isolates were harvested, and the suspension was spectrophotometrically adjusted to 0.5 McFarland turbidity standard. A total of 0.1 ml of each mold suspension was dispensed into serially diluted wells containing the drugs, reaching the final targeted drug concentration. The potency and concentration of the drugs in the final plates were verified by testing the single drug row and column with quality control strains as outlined in the CLSI methods, and the experiment was performed only once.

Plates were incubated at 35°C and read at 24 and 48 h. MICs and fractional inhibitory concentration indices (FICIs) were visually read and determined at the optically clear (MIC-0) and prominent growth reduction (MIC-2) endpoints. The FICI was then calculated and interpreted according to standard procedures (1, 9): FICIs of ≤0.5 signified synergy, and FICIs of >4.0 signified antagonism. Values between 0.5 and 4 were considered indifferent.

Table 1 shows the mean (range) FICI values for the different drug combinations at 48 h, and Table 2 shows categorical interpretations using the MIC-2 endpoint (which correlates well with the minimal fungicidal concentration, the suggested endpoint for echinocandins). Synergy between ANID and both azoles was observed in 18 of 26 isolates of *Aspergillus* spp. Synergy was most often observed with *A. fumigatus* and *A. flavus*. With ANID and AMBD, indifference was most often seen; synergy and antagonism were seen with five strains each of *Aspergillus* spp. at 48 h and MIC-2 endpoints. For *Fusarium* spp., all drug combinations suggested indifference. Antagonism between ANID and azoles was rare. This was seen with ANID and ITR against a single *Aspergillus* strain under the test conditions of Table 2 and was not consistently seen with any strain under all four test conditions (MIC-0 and MIC-2 at 24 and 48 h). Paradoxically, increased growth (7) was observed at the highest concentration of ANID and AMBD for 90% of *Fusarium* sp. isolates and 28% of *Aspergillus* sp. isolates.

Although with limited numbers and having a moderate effect at most, this in vitro study presents data showing that ANID frequently exhibits in vitro synergy with VOR and ITR when tested against *Aspergillus* spp. Synergism in vitro has been reported for *Aspergillus* spp. with triazoles used in combination with caspofungin (E. K. Manavathu, G. J. Alangaden, and P. H. Chandrasekar, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-854, 2002), and a previous in vitro study suggested potential synergistic to additive effects of caspofungin in combination with AMBD against *Fusarium* spp. (2). In the present study, activity against *Fusarium* was limited, as shown by the findings of indifference for all drugs in all seven isolates.

The significance of the paradoxical "Eagle-like" effect we observed with the AMBD combination is unknown. This was observed frequently for *Fusarium* spp. (against which ANID alone is inactive) and much less frequently for *Aspergillus* spp. and only at the highest concentrations of the drugs. This effect has been previous described for echinocandins and *Candida albicans* (19).

Although the checkerboard method has not been standardized for testing molds, it has the advantage of simplicity in performance and interpretation (12). The lack of correlation between this method and Etest, time-kill curves, or in vivo outcomes makes its usefulness for determining definitive synergy open to debate (9). Nevertheless, the potential synergy of azoles and echinocandins has been supported by an in vivo guinea pig model of disseminated aspergillosis with reduced colony counts in liver, lung, kidney, or brain tissues (10). Similarly, results with an experimental rabbit model of invasive pulmonary aspergillosis suggested decreasing serum galactomannan levels, burden of organisms, and overall mortality with such combinations (18). Both in vitro and animal model data suggest that further in vivo evaluation of ANID combinations, particularly with the azoles and against *Aspergillus* spp., is warranted.

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