In Vitro Combination of Isavuconazole with Echinocandins against Azole-Susceptible and -Resistant Aspergillus spp

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SUSCEPTIBILITY

In Vitro Combination of Isavuconazole with Echinocandins against Azole-Susceptible and -Resistant *Aspergillus* spp.

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ABSTRACT *In vitro* combinations of isavuconazole with echinocandins were evaluated against 30 *Aspergillus* strains with a two-dimensional checkerboard microdilution method and an agar-based diffusion method. With the checkerboard method, the three combinations showed indifferent interactions for all strains. With the agar-based method, indifferent interactions were found for all strains for isavuconazole-micafungin and isavuconazole-anidulafungin. For the isavuconazole-caspofungin combination, indifference was found in 24/30 strains, synergism in 4/30 strains, and antagonism in 2/30 strains.

KEYWORDS isavuconazole, echinocandins, *Aspergillus* spp., EUCAST, gradient strips, MIC, combination

Although azoles are very active against aspergillosis (1–6), acquired azole resistance in Europe and other parts of the world has been reported in recent years (7–10). It has been shown that isolates that are resistant to itraconazole and/or voriconazole may be cross resistant to isavuconazole (11, 12). Therefore, we might expect the emergence of isavuconazole-resistant isolates in the same way as occurred for the other azole drugs. The main mechanisms of azole resistance in *Aspergillus fumigatus* are mutations in the *cyp51A* gene, encoding 14α-demethylase (8, 10).

Antifungal combination is a therapeutic strategy that can be beneficial when drug interactions are synergistic, as well as in cases of infection with a resistant organism (13, 14). Indeed, it was recently proposed that a combination of voriconazole with an echinocandin may be used in clinical practice in cases of invasive aspergillosis due to azole-resistant isolates (15). *In vitro* and animal findings regarding voriconazole-echinocandin combinations mainly show indifferent to synergistic effects (16–24). Whether combinations of isavuconazole with echinocandins possess synergistic activity against filamentous fungi is still poorly studied. Here, we chose to examine the *in vitro* combinations of isavuconazole with caspofungin, anidulafungin, and micafungin against azole-susceptible and azole-resistant *A. fumigatus* isolates and against *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus terreus*, and *Aspergillus niger* isolates.

(This study was presented in part at the 27th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, Austria, 22 to 25 April 2017.)

Thirty isolates were tested, including 5 azole-susceptible *A. fumigatus* isolates, 5 azole-resistant *A. fumigatus* isolates, 5 *A. flavus* isolates, 5 *A. nidulans* isolates, 5 *A. terreus* isolates, and 5 *A. niger* isolates. An isolate was considered azole resistant when a MIC for itraconazole, posaconazole, or voriconazole was above the current clinical breakpoints determined by EUCAST (http://www.eucast.org/clinical_breakpoints). The 5

Received 6 July 2017 **Returned for modification** 10 August 2017 **Accepted** 9 October 2017

Accepted manuscript posted online 16 October 2017

Citation Raffetin A, Courbin V, Jullien V, Dannaoui E. 2018. *In vitro* combination of isavuconazole with echinocandins against azole-susceptible and -resistant *Aspergillus* spp. Antimicrob Agents Chemother 62:e01382-17. https://doi.org/10.1128/AAC.01382-17.

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azole-resistant *A. fumigatus* isolates were resistant to itraconazole and to posaconazole. Cyp51A mutations were a 34-bp tandem repeat and L98H for 3 isolates (2 of which were resistant to voriconazole), G54W for 1 isolate, and P216L for 1 isolate (the latter 2 isolates were susceptible to voriconazole). The testing of combinations of isavuconazole and each of the echinocandins (caspofungin, anidulafungin, and micafungin) was performed using two techniques.

The first technique was a two-dimensional checkerboard microdilution method based on the EUCAST reference method (25). The final concentrations were 0.125 to 8 μg/ml for isavuconazole (Basilea, Basel, Switzerland), 0.008 to 4 μg/ml for caspofungin (MSD, Kenilworth, NJ), 0.0005 to 0.25 μg/ml for micafungin (Astellas, Tokyo, Japan), and 0.0005 to 0.25 μg/ml for anidulafungin (Pfizer, New York, NY). MICs were determined visually after 48 h of incubation at 35°C. MICs were first determined with complete inhibition endpoints (100% inhibition) for the drugs alone and in combination, and partial inhibition endpoints (50% inhibition for isavuconazole and minimal effective concentrations [MECs] for the echinocandins alone and for the combinations) were also used. Plates were also read spectrophotometrically (26). This technique was repeated twice, in two independent experiments.

For the gradient concentration strip method, RPMI agar plates were inoculated with the same spore suspension as used for the checkerboard microdilution method, already adjusted to 10^6 CFU/ml. Three plates were used for each isolate, i.e., one with an isavuconazole MIC test strip (Liofilchem, Roseto degli Abruzzi, Italy), one with an echinocandin MIC test strip (bioMérieux, Craponne, France), and one with the combination. Experiments were performed as described previously (23, 27). MICs were determined visually after 48 h of incubation at 35°C, using complete inhibition (100%) for isavuconazole and partial inhibition (50%) for the echinocandins and the combinations. MICs were also determined with complete inhibition endpoints for the combinations. For both techniques, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, and *Aspergillus fumigatus* ATCC 204305 were used for quality control. Fractional inhibitory concentration index (FICI) values were calculated to determine whether the drug interactions were synergistic (FICI values of $\leq$0.5), indifferent (FICI values of $>$0.5 to $<$4), or antagonistic (FICI values of $\geq$4) (28).

The activities of isavuconazole and echinocandins, either alone or in combination, were first determined by checkerboard microdilution (Table 1). Isavuconazole MICs ranged from 0.5 to 8 μg/ml against azole-resistant *A. fumigatus*, from 0.25 to 4 μg/ml against azole-susceptible *A. fumigatus*, from 0.25 to 4 μg/ml against *A. flavus*, *A. terreus*, and *A. nidulans*, and from 4 to 8 μg/ml against *A. niger*. Caspofungin MICs were $>$4 μg/ml against all isolates from all species, and MECs ranged from 0.125 to 1 μg/ml. Micafungin MICs were $>$0.25 μg/ml (the highest concentration tested) against all isolates, and MECs ranged from 0.004 to 0.06 μg/ml. Anidulafungin MICs were $>$0.25 μg/ml against all isolates, and MECs ranged from 0.004 to 0.12 μg/ml.

Using complete inhibition endpoints, we found indifferent effects for all strains with the EUCAST-based checkerboard method. The median FICI values ranged from 0.625 to 2.001 for the isavuconazole-caspofungin combination, from 1.001 to 2.001 for the isavuconazole-micafungin combination, and from 0.501 to 2.001 for the isavuconazole-anidulafungin combination. With partial inhibition endpoints, mainly indifferent effects were found but some synergistic interactions were observed (Table 2).

We checked our findings with spectrophotometric readings, which confirmed the indifferent effects of most of the combinations, with FICI values ranging from 0.501 to 1.001 for the isavuconazole-caspofungin combination in 26/30 strains (86.7%), for the isavuconazole-micafungin combination in 28/30 strains (93.3%), and for the isavuconazole-anidulafungin combination in 29/30 strains (96.7%). The results of agar diffusion tests are summarized in Table 3. Isavuconazole, caspofungin, micafungin, and anidulafungin MICs against all isolates ranged from 0.032 to 2 μg/ml, from 0.003 to 0.38 μg/ml, from 0.002 to 0.012 μg/ml, and from 0.002 to 0.006 μg/ml, respectively. Using complete inhibition endpoints, we found indifferent effects for all strains for the isavuconazole-micafungin combination and the isavuconazole-

TABLE 1 Interactions of isavuconazole with echinocandins against *Aspergillus* spp., determined with the checkerboard broth microdilution technique[a]

Species	FICI (mean [range])[b]					
	Isavuconazole with caspofungin[c]		Isavuconazole with micafungin[c]		Isavuconazole with anidulafungin[c]	
	MIC	MEC	MIC	MEC	MIC	MEC
Azole-resistant *A. fumigatus* (5 isolates)	1.025 (1–1.125)	0.928 (0.5–2)	1.201 (1.001–2.001)	0.71 (0.312–1.5)	1.201 (1.001–2.001)	0.732 (0.531–1.25)
Azole-susceptible *A. fumigatus* (5 isolates)	1.201 (1.001–2.001)	0.577 (0.31–1)	1.001	0.37 (0.317–0.517)	1.401 (1.001–2.001)	0.643 (0.5–1.033)
A. niger (5 isolates)	1.126 (0.625–2.001)	0.409 (0.059–1.062)	1.001	0.621 (0.164–2.033)	1.104 (0.516–1.001)	0.663 (0.158–2.033)
A. terreus (5 isolates)	1.001	0.875 (0.37–1.007)	1.001	0.907 (0.375–1.062)	1.001	0.873 (0.5–1.033)
A. flavus (5 isolates)	0.926 (0.625–1.001)	0.832 (0.528–1.014)	1.201 (1.001–2.001)	0.633 (0.317–1.017)	1.101 (0.501–2.001)	0.662 (0.508–1.017)
A. nidulans (5 isolates)	1.001	1.034 (1.028–1.056)	1.001	0.937 (0.562–1.033)	1.001	1.031 (1.008–1.062)

[a]The effects of isavuconazole in combination with echinocandins against 30 *Aspergillus* isolates were determined with the checkerboard broth microdilution technique, using two different endpoints.
[b]Corresponding to the lowest FICI values.
[c]MIC corresponds to complete inhibition of growth for both drugs, and MEC corresponds to the lowest drug concentrations resulting in aberrant hyphal growth for both drugs.

TABLE 2 Interactions of isavuconazole with caspofungin, micafungin, and anidulafungin against *Aspergillus* spp., determined with the checkerboard broth microdilution technique using two inhibition endpoints[a]

Inhibition endpoint and species	No. of isolates								
	Isavuconazole with caspofungin			Isavuconazole with micafungin			Isavuconazole with anidulafungin		
	S	I	A	S	I	A	S	I	A
Complete inhibition endpoint									
Azole-resistant *A. fumigatus* (5 isolates)	0	5	0	0	5	0	0	5	0
Azole-susceptible *A. fumigatus* (5 isolates)	0	5	0	0	5	0	0	5	0
A. niger (5 isolates)	0	5	0	0	5	0	0	5	0
A. terreus (5 isolates)	0	5	0	0	5	0	0	5	0
A. flavus (5 isolates)	0	5	0	0	5	0	0	5	0
A. nidulans (5 isolates)	0	5	0	0	5	0	0	5	0
Total (30 isolates)	0	30	0	0	30	0	0	30	0
Partial inhibition endpoint									
Azole-resistant *A. fumigatus* (5 isolates)	1	4	0	1	4	0	0	5	0
Azole-susceptible *A. fumigatus* (5 isolates)	2	3	0	4	1	0	1	4	0
A. niger (5 isolates)	3	2	0	4	1	0	3	2	0
A. terreus (5 isolates)	1	4	0	1	4	0	1	4	0
A. flavus (5 isolates)	0	5	0	1	4	0	0	5	0
A. nidulans (5 isolates)	0	5	0	0	5	0	0	5	0
Total (30 isolates)	7	23	0	11	19	0	5	25	0

[a]For isavuconazole, MICs were defined as the lowest concentrations of the antifungal agents that completely inhibited fungal growth. For the echinocandins, two different visual determinations of the endpoint were performed, i.e., complete inhibition of growth (MIC) and partial inhibition of growth defined as the lowest drug concentration resulting in aberrant hyphal growth (MEC), by examination with an inverted microscope. S, synergism; I, indifference; A, antagonism.

anidulafungin combination with the gradient concentration strip method. For the isavuconazole-caspofungin combination, the FICI values ranged from 0.630 to 3.940 (indifferent) in 24/30 strains (80%), from 0.084 to 0.424 (synergistic) in 4/30 strains (13.3%) (3 *A. terreus* strains and 1 azole-susceptible *A. fumigatus* strain), and from 4.149 to 5.601 (antagonistic) in 2/30 strains (6.7%) (2 *A. niger* strains). These findings were similar when interpreted with partial endpoints. Typical patterns observed by using gradient concentration strips are presented in Fig. 1.

Because azole resistance in *Aspergillus* is an emerging problem in Europe (7–10, 29) and in other parts of the world (9, 11, 30), therapeutic strategy alternatives to azole monotherapy are urgently needed (15). In this study, combinations of isavuconazole with echinocandins showed mainly indifferent effects against *Aspergillus* spp., and antagonism was almost never observed. On the whole, these results are in accordance with those of previous studies on azole-echinocandin interactions.

Although a number of studies have reported data on the efficacy of combinations of azoles (itraconazole, voriconazole, and posaconazole) and echinocandins (caspofungin, micafungin, and anidulafungin) (18, 20, 22, 24, 31–33), whether isavuconazole possesses synergistic activity when combined with echinocandins has been poorly

TABLE 3 Interactions of isavuconazole with echinocandins against *Aspergillus* spp., determined with gradient concentration strips[a]

Species (no. of isolates)	Isavuconazole with caspofungin				Isavuconazole with micafungin				Isavuconazole with anidulafungin			
		No. of isolates				No. of isolates				No. of isolates		
	FICI range	S	I	A	FICI range	S	I	A	FICI range	S	I	A
Azole-resistant *A. fumigatus* (5)	1.011–3.294	0	5	0	1.001–1.024	0	5	0	0.688–1.016	0	5	0
Azole-susceptible *A. fumigatus* (5)	0.425–2.291	1	4	0	1.008–1.024	0	5	0	0.774–1.024	0	5	0
A. niger (5)	1.016–5.601	0	3	2	1.002–1.341	0	5	0	1.001–1.502	0	5	0
A. terreus (5)	0.084–1.046	3	2	0	1.005–1.008	0	5	0	1.005–1.010	0	5	0
A. flavus (5)	1.042–2.084	0	5	0	1.005–1.349	0	5	0	0.508–1.024	0	5	0
A. nidulans (5)	0.630–3.061	0	5	0	0.917–1.170	0	5	0	1.004–1.042	0	5	0
Total (30)	0.084–5.601	4	24	2	0.917–1.349	0	30	0	0.508–1.502	0	30	0

[a]Interactions between the antifungal agents were analyzed using FICI values. MICs were determined with partial inhibition endpoints. S, synergism; I, indifference; A, antagonism.

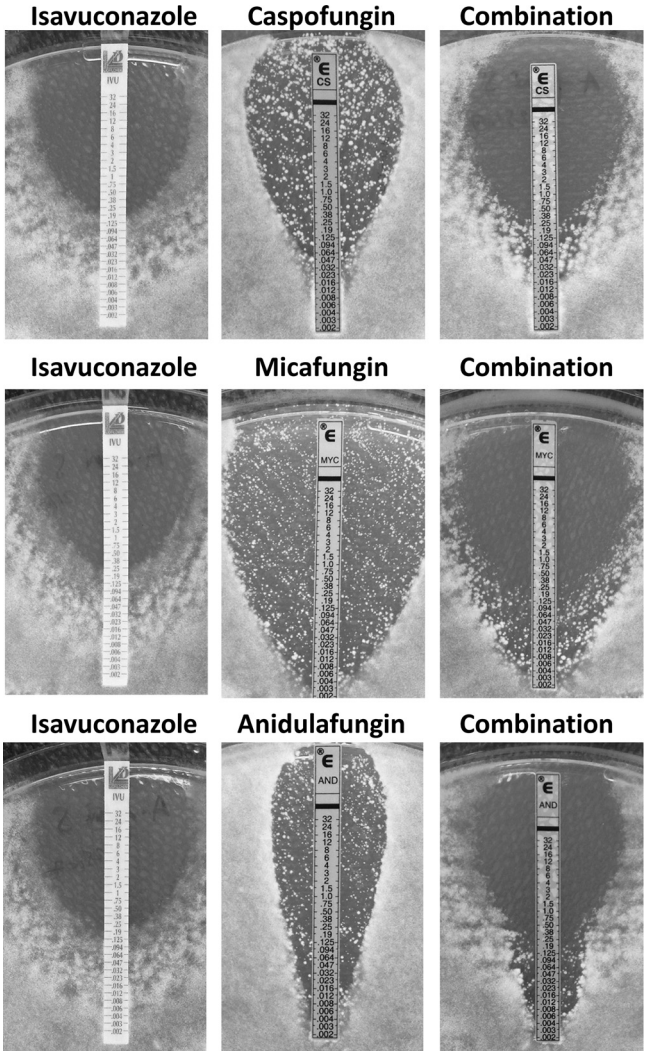

FIG 1 Agar diffusion test results for the combinations of isavuconazole with caspofungin, micafungin, and anidulafungin against azole-susceptible *A. fumigatus* strain 292.

studied so far. Katragkou et al. showed that the interactions of isavuconazole with micafungin against *A. fumigatus*, *A. flavus*, and *A. terreus* were synergistic *in vitro* (34); in that study, however, synergism was considered when the FICI value was <1. Previous studies chose this definition to obtain a more symmetrical indifference range (FICI values of 1 to 1.25) than the conventional one (FICI values of 0.5 to 4) (24, 35). According to this definition, we would have found that the combinations were synergistic against most of the strains. Gebremariam et al. did not demonstrate any synergism of the isavuconazole-micafungin combination in treating pulmonary murine mucormycosis (36). To our knowledge, no clinical trials have been performed to study the combination of isavuconazole with echinocandins.

Because antifungal combinations are difficult to test against filamentous fungi, we used two methods, based on different principles (a checkerboard microdilution broth technique and an agar diffusion technique), and we chose to interpret the results with complete inhibition endpoints. We also used two reading methods and showed, as reported previously (26, 37–40), that spectrophotometric reading is a good alternative to visual reading for MIC determinations with the EUCAST-based technique.

Because we included azole-susceptible and azole-resistant *Aspergillus* isolates, we could demonstrate that the interactions between isavuconazole and echinocandins

were not linked to the azole susceptibility of the isolates. In the literature, studies indicate controversy regarding this point (20, 24, 39).

In conclusion, combinations of isavuconazole and echinocandins mainly showed indifferent interactions against *Aspergillus* spp., and antagonism was almost never observed. Further *in vivo* evaluation of these combinations is warranted especially for difficult-to-treat invasive aspergillosis.

ACKNOWLEDGMENT

We thank Basilea for providing the isavuconazole gradient concentration strips.

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