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In Vitro Combination of Voriconazole and Miltefosine against Clinically Relevant Molds

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Invasive infections caused by filamentous fungi are a major threat for immunocompromised patients. Innate/acquired resistance to antifungal drugs might necessitate combination therapies. We assessed the potential combination of voriconazole with miltefosine, an original drug with antifungal activity against 33 clinically relevant mold isolates, including both azole-susceptible and -resistant *Aspergillus*. Using complete inhibition as an endpoint, interactions were indifferent for 32/33 isolates. An alternative 50% inhibition endpoint showed synergistic interactions for 14/33 isolates. Antagonism was absent.

Invasive fungal infections (IFI) due to filamentous fungi are a major threat for immunocompromised patients. *Aspergillus fumigatus* is the most common IFI, but other species, such as *Aspergillus flavus*, *Aspergillus niger*, or the naturally azole-resistant *Aspergillus ustus*, are also frequently retrieved (1). Currently, the antifungal armamentarium for systemic filamentous infection is restricted to 3 classes: azoles, echinocandins, and polyenes. Echinocandins are only fungistatic against filamentous fungi, and breakthrough of mold IFI during echinocandin treatment has been reported (2). Broad-spectrum antifungal polyene is associated with frequent adverse effects. Moreover, the recent emergence of azole-resistant *Aspergillus* strains is disquieting and potentially a threat for human health (3, 4). Finally, genera such as *Scedosporium* or *Fusarium* exhibit low susceptibility to all antifungals. To overcome acquired or innate antifungal drug resistance and improve IFI management, combinations of drugs belonging to different classes have been tested and may be useful (5–7).

Miltefosine is an alkylphosphocholine with antineoplastic and especially antiparasitic properties. Despite its very frequent gastrointestinal side effects and a strict contraindication in pregnant women, the drug is now widely used for leishmaniasis treatment (8). The activity of miltefosine against fungi has also been demonstrated, both *in vitro* and in a mouse model of disseminated cryptococcosis (9). However, its use for fungal infections in humans is extremely rare. In the present *in vitro* study, we investigated the potential synergy of a combination of voriconazole and miltefosine against different clinically relevant molds.

We used 33 clinical isolates collected in two French hospitals (Pitié Salpêtrière and Hôpital Européen Georges Pompidou, Paris) (Table 1): 12 *A. fumigatus* isolates with wild-type *cyp51A*, 5 *cyp51A*-mutated *A. fumigatus* isolates (4 with the TR_{34}/L98H alteration and one with the newly described sole Y121F alteration [10]), 3 *A. ustus* isolates, 3 *A. flavus* isolates, 3 *Aspergillus* section *Nigri* isolates (2 *A. niger* and 1 *Aspergillus tubingensis*), 4 *Scedosporium apiospermum* isolates, and 3 *Fusarium solani* isolates. Identification was confirmed by molecular sequencing (internal transcribed spacer [ITS] region, beta-tubulin, and calmodulin genes). For miltefosine, MICs were determined using the EUCAST method. A complete inhibition endpoint was determined visually and by spectrophotometric analysis. Alternatively, as previously reported by Widmer et al. (9), the MIC for miltefosine was defined as the concentration producing at least 50% inhibition after 48 h of incubation at 35°C for *Aspergillus* (72 h for *Fusarium* and *Scedosporium*). This alternative endpoint was determined uniquely by spectrophotometric analysis. We used the checkerboard method to test combinations of voriconazole and miltefosine. The MICs of each drug alone and the combinations of the two were determined concomitantly on the same plate. The volume of each drug dispensed was 50 μl, to reach a volume of 100 μl per well. Each well was then inoculated with 100 μl of a suspension containing 2×10^5 to 5×10^5 CFU/ml, yielding a final inoculum of 1×10^5 to 2.5×10^5 CFU/ml per well and a final concentration between 0.5 and 32 mg/liter for miltefosine and between 0.008 and 4 mg/liter for voriconazole. Interaction was determined by calculating the fractional inhibitory concentration index (FICI) as follows: FICI = (MIC of voriconazole combination/MIC of voriconazole alone) + (MIC of miltefosine combination/MIC of miltefosine alone). A FICI value of ≤0.5 indicated synergy between the two drugs, whereas a value of >4 indicated antagonism. Values between 0.5 and 4 indicated indifference (11). Each isolate was tested at least two times. The *Candida parapsilosis* strain ATCC 22019 was used as a quality control.

The duplicates gave similar results (i.e., with ≤1 2-fold dilution difference, except for 2 isolates with two 2-fold dilution differences and one isolate with five 2-fold differences) and identical FICI interpretations (except for one isolate) (see the supplemental material for detailed per-isolate data). The results of antifungal synergy testing are summarized in Table 1. When the 100% inhibition endpoint was used, for the non-*cyp51A*-mutated *A. fumigatus* isolates, the geometric mean MICs for voriconazole and miltefosine were 0.73 mg/liter (range, 0.25 to 1 mg/liter) and 10.7 mg/

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liter (range, 4 to 32 mg/liter), respectively. As expected, the MIC of voriconazole for *A. ustus* was higher (4 to 8 mg/liter). In accordance with a previous work (1), *A. flavus* isolates had higher MICs for miltefosine (>32 mg/liter) than other species. *Scedosporium* and *Fusarium* isolates also had high MICs for both voriconazole and miltefosine. For all isolates except one, the combination of voriconazole and miltefosine had only an indifferent effect. Interestingly, the MICs of miltefosine were in the range of the achievable plasma concentrations (12).

When the less stringent 50% inhibition endpoint was used, we observed synergy (FICI ≤ 0.5) between voriconazole and miltefosine for 5 non-*cyp51A*-mutated *A. fumigatus* isolates and indifference for the 7 others. Interestingly, synergy was observed for one *A. ustus* isolate among three. No synergistic effect was observed for the five *A. fumigatus cyp51A*-mutated isolates, although for one isolate the duplicates gave distinct results, i.e., "synergy" and "indifference," with FICI values of 0.375 and 0.625, respectively. Finally, synergistic effect was observed in the 3 *Aspergillus* section *Nigri* isolates, in 3 of 4 *Scedosporium* isolates, and in 2 of 3 *Fusarium* isolates. Importantly, for all fungi, antagonism was not detected.

Very few case reports have described the use of miltefosine as an antifungal in humans. Miltefosine has been used successfully in a combination therapy with voriconazole and terbinafine against *Scedosporium prolificans* (13) and in a case of *S. prolificans* mediastinitis (14). In animal models against disseminated cryptococcosis or candidiasis, some authors found that miltefosine was effective (9), whereas others reported that it provided only limited effectiveness (15, 16). The drug's potential effect against molds, either alone or in combination, has not yet been tested.

It is important to note that no recommendations exist for the reading and the determination of miltefosine MICs against molds. In 2006, Widmer et al. used a 50% inhibition endpoint (9), while more recently, other authors chose a 100% inhibition endpoint (17). For our study, we determined interactions using both of these endpoints.

Recently, Biswas et al. reported an *in vitro* synergistic effect for miltefosine in combination with azoles against some *Fusarium* or *Scedosporium* isolates as well as several mucormycete strains but decided not to test the combinations against azole-susceptible strains (17). However, even when a strain is susceptible to both drugs independently, a combination may still be useful to potentially clear the pathogen more quickly and stave off the emergence of resistance.

As for our study, when using a 50% inhibition endpoint, synergy between miltefosine and voriconazole was observed for 5/12 *A. fumigatus* and 1/3 *A. ustus* isolates but not for the five *cyp51A*-mutated isolates. However, it should be noted that the MIC geometric mean for miltefosine was reduced more than 7-fold when in combination with voriconazole (4.6 versus 0.62 mg/liter). We observed a similar trend with *A. flavus* isolates. In both cases, a lack of synergistic effect was due to the only one-dilution difference of voriconazole MIC between the drug alone and the drug used in combination. Finally, no antagonism was detected. Taking these data into account, the use of miltefosine, either alone or in combination with voriconazole, to treat aspergillosis or other mold infections may be of interest. Results of the present study should be further assessed in *Galleria mellonella* and murine and/or other animal models.

TABLE 1 Combination of miltefosine and voriconazole against 33 clinically relevant mold isolates[a]

Organism (no. of isolates)	MIC range (mg/liter) (geometric mean)						FICI determination					
	MIC-0				MIC-2			MIC-0			MIC-2	
	Voriconazole		Miltefosine		Voriconazole		Miltefosine			Interpretation		Interpretation
	Alone	Combined	Alone	Combined	Alone	Combined	Alone	Combined	Range (mean)	S	I	Range (mean)	S	I
A. fumigatus stricto sensu (12)	0.25–1 (0.73)	0.5–1 (0.73)	4–32 (10.7)	1–32 (14.7)	0.125–0.5 (0.25)	0.03–0.25 (0.083)	4–16 (8.5)	0.5–4 (0.69)	1.125–4 (2.78)	0	12	0.31–0.625 (0.476)	5	7
cyp51A-mutated *A. fumigatus* (5)[b]	4–8 (4.29)	4–8 (4.59)	4–8 (4.59)	0.5–8 (3.73)	2–4 (3.25)	1–2 (1.62)	4–8 (4.6)	0.5–1 (0.62)	0.625–3 (2)	0	5	0.375–0.75 (0.64)	0	5
A. ustus (3)	4–8	2–4	8–>32	2–16	4–8	0.125–2	8–16	0.5–4	0.56–1	0	3	0.27–0.56	1	2
A. flavus (3)	0.5–2	0.5–2	>32	0.5–>32	0.25–0.5	0.125–0.25	16–>32	0.5–1	1.5–3	0	3	0.51–0.56	0	3
Aspergillus section *Nigri* (3)[c]	0.5–2	0.125–1	16–>32	4–32	0.5–2	0.06–0.25	8–32	2–4	0.3–2	1	2	0.09–0.5	3	0
Scedosporium apiospermum (4)	1–8	1–8	32–>32	32–>32	0.5–2	0.125–0.5	16–32	1–4	2	0	4	0.37–0.62	3	1
Fusarium solani (3)	8	8	8–16	8–16	2–8	0.25–1	4–8	2	1.5–3	0	3	0.375–0.75	2	1

[a] MIC-2 (50% inhibition endpoint) was determined by spectrophotometric analysis (reading at 450-nm wavelength). MIC-0 (100% inhibition endpoint) was determined both visually and by spectrophotometric analysis. Both methods gave similar results. For miltefosine, when MIC was >32, a value of 64 was arbitrarily chosen to calculate the FICI (see the text for the equation). S, synergic effect; I, indifferent effect. A FICI value of ≤0.5 indicates synergy between the two drugs, whereas a FICI value of >4 indicates antagonism. Values between 0.5 and 4 indicate indifference. Each isolate was tested at least two times. Detailed data for all isolates are available in the supplemental material. All isolates are defined at the species level.

[b] *cyp51A*-mutated isolates consisted of 4 isolates harboring the TR_{34}/L98H alterations and one harboring a sole Y121F substitution. For one of these isolates, the duplicates gave discordant results for the MIC-2 reading, with one FICI indicative of synergy (0.375) and the second indicative of indifference (0.625); we chose to indicate it as indifferent in the table.

[c] Two *A. niger* isolates and one *A. tubingensis* isolate.

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S.I. performed the experiments and participated in the writing of the manuscript. M.P. performed several experiments. I.M. performed molecular analyses. E.D. furnished several fungal isolates, participated in scientific discussions, and participated in the writing of the manuscript. D.M. and A.D. participated in the writing of the manuscript. A.F. designed the study and wrote the article.

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