ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2005, p. 1232–1235 Vol. 49, No. 3
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.3.1232–1235.2005

Combined Activity In Vitro of Caspofungin, Amphotericin B, and Azole Agents against Itraconazole-Resistant Clinical Isolates of *Aspergillus fumigatus*

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Received 16 July 2004/Returned for modification 15 September 2004/Accepted 21 November 2004

Interactions in vitro between amphotericin B, itraconazole, voriconazole, and caspofungin against itraconazole-resistant *Aspergillus fumigatus* clinical strains were determined. Differential results were obtained depending on the criteria (MIC or minimal effective concentration) used. Caspofungin and voriconazole exhibited the most potent interactions, with synergy against at least 50% of isolates, and the average fractional concentration index was 0.38. Antagonism was not found for any combination.

To date, itraconazole resistance in *Aspergillus fumigatus* is an uncommon phenomenon (12). Several recently reported studies have indicated that the rate of itraconazole resistance in vitro (MIC of >8 μg/ml) is lower than 5% among clinical strains of *A. fumigatus* (1, 16, 18, 22, 33). Resistant clinical isolates have been isolated largely from patients receiving prolonged itraconazole therapy, who usually suffer from difficult-to-treat aspergillosis (6, 34). In addition, these data in vitro have correlated with results of studies of animal models of infection (10, 11).

In contrast to clinical isolates, *A. fumigatus* mutants that are highly resistant to itraconazole are easily selected in vitro (24). Several resistance mechanisms have been described, and azole cross-resistance has been observed (14, 15, 23, 31). These data suggest that itraconazole resistance among clinical strains may become more common in the future, associated with the spread of antifungal therapies.

Combination therapy could be an alternative to monotherapy for patients with invasive infections due to resistant organisms and for some patients who failed to respond to standard treatment (7). The increase in available antifungal compounds has raised the number of potential combinations, a therapeutic resource which could be exploited clinically (19, 32).

We have analyzed the combined activity in vitro of several antifungal agents against a collection of 14 itraconazole-resistant (MICs of >8.0 μg/ml) clinical isolates of *A. fumigatus*.

(This work was presented in part at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 2003.)

Fungi. A panel of 14 clinical isolates was tested. Strains were labeledCNM-CM (for the Spanish Centro Nacional de Microbiología molds culture collection) and given a number of identification. Table 1 displays the identification of strains. CNM-CM-1244 (original strain identification AF-72), CNM-CM-2158 (AF-1422), CNM-CM-2159 (F/6919), CNM-CM-2160 (F/7075), CNM-CM-2161 (Br130), CNM-CM-2162 (Br181), CNM-CM-2163 (SO/3827), and CNM-CM-2164 (SO/3829) were kindly provided by D. W. Denning. Strain CNM-CM-2097 (AF1237) was provided by E. Dannaoui. *A. fumigatus* ATCC 204305 and *Aspergillus flavus* ATCC 204304 were included as quality control organisms in each set of experiments.

Antifungal agents. The antifungal agents used in the study were as follows: amphotericin B (Sigma Aldrich Quimica S.A., Madrid, Spain), itraconazole (Janssen S.A., Madrid, Spain), voriconazole (Pfizer S.A., Madrid, Spain), and caspofungin (Merck & Co., Inc., Rahway N.J.).

Antifungal susceptibility testing. The individual MICs were determined by following the National Committee for Clinical Laboratory Standards (NCCLS) reference method (25), with minor modifications. The modifications included the use of RPMI 1640 with L-glutamine buffered to pH 7 with 0.165 M MOPS (morpholinepropanesulfonic acid) and 1 M NaOH supplemented with 18 g of glucose per liter (RPMI–2% glucose; OXOID, Madrid, Spain) and inoculum preparation by microscopic enumeration with a cell-counting hemocytometer (Neubauer chamber; Merck, S.A., Madrid, Spain). Some reports have demonstrated that these modifications generate reproducible in vitro susceptibility data and that hemocytometer counting is the most reliable and accurate method for inoculum preparation (8, 27). All inoculum suspensions were quantified by plating on Sabouraud agar plates.

Sterile plastic microtitration plates with 96 flat-bottomed wells each were employed. The trays were inoculated with 0.100 ml of the inoculum suspensions in each well. The plates were incubated at 35°C for 48 h in a humid atmosphere. Visual readings were performed with the help of a mirror. For amphotericin B, itraconazole, and voriconazole, MICs were defined as the lowest concentration of the antifungal agent that completely inhibited fungal growth. For caspofungin, two different visual determinations of the endpoint were performed: (i) complete inhibition of growth (MIC) and (ii) the lowest

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TABLE 1. FICi's of 14 clinical isolates per antifungal combination[a]

Strain	FICi for the combination:							
	AMB-ITC (MIC)	AMB-VRC (MIC)	AMB-CPF		ITC-CPF		VRC-CPF	
			MIC	MEC	MIC	MEC	MIC	MEC
CNM-CM-21	2.0	1.0	0.75	0.56	2.0	1.5	0.18	0.14
CNM-CM-22	2.0	1.0	0.75	0.50	2.0	0.28	0.26	0.19
CNM-CM-796	0.75	0.75	1.0	1.0	2.0	0.04	0.50	0.19
CNM-CM-1244	1.0	0.50	0.55	0.55	2.0	0.37	0.62	0.62
CNM-CM-1910	2.0	0.37	0.50	0.50	2.0	0.75	1.50	0.62
CNM-CM-2097	2.0	2.0	1.0	1.0	2.0	0.26	0.55	0.55
CNM-CM-2158	0.75	1.0	0.55	0.41	2.0	0.31	0.55	0.37
CNM-CM-2159	1.0	0.50	1.0	1.0	2.0	0.28	0.62	0.62
CNM-CM-2160	3.0	0.25	0.75	0.26	2.0	0.19	0.28	0.25
CNM-CM-2161	3.0	0.25	0.56	0.50	2.0	0.19	0.55	0.50
CNM-CM-2162	0.75	0.75	1.0	1.0	2.0	0.19	0.18	0.18
CNM-CM-2163	0.55	1.0	1.0	0.55	1.5	1.5	0.50	0.50
CNM-CM-2164	0.75	0.50	1.0	0.55	0.55	0.37	0.26	0.14
CNM-CM-2266	1.0	1.0	1.0	1.0	2.0	1.5	0.55	0.50
Average FICi	1.46	0.77	0.81	0.67	1.86	0.55	0.50	0.38
Number and % synergy[b]	0/14 (0)	6/14 (42.8)	1/14 (7.1)	5/14 (35.7)	0/14 (0)	10/14 (64.3)	7/14 (50)	10/14 (64.3)

[a] FICi values are arithmetic means of six repetitions. AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; CPF, caspofungin.
[b] The numbers of strains for which the combination showed synergy out of the total number of strains are shown, and the percentage is given in parentheses.

drug concentration resulting in aberrant hyphal growth by examination with an inverted microscope (3, 30), or the minimum effective concentration (MEC).

Interaction of drugs in vitro. Drug interaction was evaluated in a checkerboard microdilution design. The combined effects were analyzed by the summation of the fractional concentration index (FICi). For combinations including caspofungin, the FICi was also calculated by taking into account both the MIC and the MEC of the echinocandin. The interactions were defined as synergistic when the FICi was ≤ 0.5 and as antagonistic if FICi was >4, and indifference or no interaction was defined by a FICi that was >0.5 but ≤ 4. Duplicate testing on three separate days was performed.

Analysis of data. A descriptive statistical analysis of the MIC, the MEC, and FICi values was done with Statistical Package for the Social Sciences (version 12.0) (SPSS S.L., Madrid, Spain).

MICs and MECs. For the 14 isolates tested, the MIC of amphotericin B was ≤ 0.5 μg/ml; all isolates were resistant to itraconazole in vitro (MICs of >8.0 μg/ml). For 12 strains, the MIC of voriconazole was ≤ 2.0 μg/ml, and for two organisms, the MIC of voriconazole was ≥ 4.0 μg/ml. MICs of caspofungin were consistently over 16.0 μg/ml. In contrast, caspofungin exhibited a good activity in vitro when MECs were determined. The geometric mean of the caspofungin MEC was 1.66 μg/ml, and MECs ranged from 0.50 to 4.0 μg/ml. The MICs of the four antifungal agents for the quality control organisms agreed with those depicted in NCCLS document M38-A (25).

With regard to the combined effects of antifungal agents in vitro, Table 1 shows arithmetic means of FICi values after six repetitions per combination of compounds and per isolate. The table also displays the number and percentage of strains for which synergy was described. The average FICi of the amphotericin B-itraconazole combination for the 14 clinical strains was 1.46, and neither synergistic nor antagonistic effects were described for any isolate. The amphotericin B-voriconazole combination exhibited an indifferent effect, with FICi values averaging 0.77. The combination showed a synergistic effect against 6 of 14 strains (42.8%), and antagonism was not described. Notably, synergy was noticed for the two strains that had voriconazole MICs of ≥ 4 μg/ml (CNM-CM-1910 and CNM-CM-2159).

When analyzing combinations with caspofungin, significant differences were found between FICi's obtained by using MICs and those calculated with MECs. Indifference was found for the amphotericin B-caspofungin combination against the majority of clinical isolates. Average FICi's with MICs and MECs were 0.81 and 0.67, respectively. However, synergy was described for 1 of 14 isolates (7.1%) with MICs and for 5 of 14 strains (35.7%) if the FICi was calculated by using MECs. Antagonism was not observed. The combined effect of the itraconazole-caspofungin combination was classified as indifference regardless of the values used for FICi calculation. However, the average FICi with MECs was 0.55, an index close to synergy. In addition, a synergistic effect was observed in 10 of 14 (64.3%) strains, and antagonism was not found. Regarding the voriconazole-caspofungin combination, synergistic interaction was noticed, with the average FICi's with MICs and MECs being 0.50 and 0.38, respectively. Antagonism was absent, and synergy was described for 7 of 14 (50%) isolates if the FICi included MICs and for 10 of 14 (64.3%) organisms if the MEC was used for FICi calculation. Unlike the amphotericin B-voriconazole combination, voriconazole-caspofungin did not exhibit synergy against the two strains with voriconazole MICs of ≥ 4 μg/ml, and the combination showed an indifferent interaction for the two isolates.

A number of works have reported data on the efficacy of combination therapy against *A. fumigatus*. In the case of amphotericin B and azole agents, the majority of works found that antifungal combinations were indifferent in vitro against this

species (13). Combinations with echinocandins have shown largely to be synergistic against *Aspergillus* spp. Studies in vitro of the interaction between amphotericin B and caspofungin have indicated an indifferent to synergistic effect for most of the *Aspergillus* strains tested (3, 4), and antagonism was not reported. Clinical reports have described cases of invasive aspergillosis that responded to this combination (2, 5, 17, 21, 29). Regarding combinations of caspofungin and azole agents, studies in vitro have demonstrated synergy against *Aspergillus* species, varying from 38 to 100% of isolates, depending on the combination and interaction definitions (26, 30). Notably, synergy was documented for the majority of isolates when susceptibility testing endpoints were defined as substantial inhibition of growth. Lower rates of synergy were found if the endpoint was defined as the lowest concentration of the antifungal agent that completely inhibited fungal growth or when the MEC was chosen for evaluating interactions. Caspofungin in combination with either itraconazole or voriconazole has been shown to be efficient in animal models of aspergillosis and in treating some difficult-to-treat human infections caused by species of *Aspergillus* (9, 20, 28).

In our study, an indifferent effect was observed for combinations of amphotericin B and azole agents. Combinations with caspofungin provided a different effect, depending on the antifungal agent and MIC or MEC endpoint determination, but antagonism was absent. Amphotericin B-caspofungin and itraconazole-caspofungin combinations showed an indifferent effect when the MIC was used, although the combinations were synergistic against a number of strains if the MEC was taken as the visual endpoint. It should be noted that other authors have noticed synergy between these antifungal agents when using the MIC as the endpoint criterion (30). The conflicting results could be explained largely by the criteria used for evaluating antifungal interaction. Caspofungin plus voriconazole exhibited a synergistic effect regardless of the endpoint used. These results in vitro should be confirmed by studies in vivo or clinical evidence.

L.A.-F. is a fellow of the Instituto de Salud Carlos III (grant 02/2002).

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