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Effects of Antifungal Agent Combinations Administered Simultaneously and Sequentially against *Aspergillus fumigatus*

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The in vitro effects of antifungal agent combinations administered simultaneously and sequentially against 15 strains of *Aspergillus fumigatus* were tested by the yeast-malt broth method. The synergistic effect of the combination of amphotericin B (AMPH) and miconazole was observed in nine strains (60%). However, the combinations of AMPH and fluconazole, AMPH and ketoconazole, and AMPH and itraconazole administered simultaneously showed antagonistic effects against three (20%), five (34%), and four (26%) strains, respectively. The effects of combinations of azole antifungal agents administered simultaneously were indifferent or antagonistic against *A. fumigatus*. In experiments measuring the effects of sequentially administered antifungal agents, however, pretreatment with AMPH and then azole antifungal agents exhibited better in vitro efficacy than that found in experiments measuring the effects of simultaneously administered AMPH and azole compounds.

There has been a recent interest in the use of combinations of antifungal agents for chemotherapy. The combination of amphotericin B (AMPH) and flucytosine for the treatment of cryptococcal meningitis indicates that the duration of the treatment could be shorter than that required for AMPH monotherapy (2). However, very few studies have examined the effects of combinations of antifungal agents against *Aspergillus* species in vitro or in vivo (1, 5, 7). In vivo assays are more reliable than in vitro assays, but the former are not suitable for clinical laboratories. In vitro assays are able to deal with large numbers of isolates. However, in vitro susceptibility tests are unreliable in predicting the in vivo activities of antifungal agents. For this reason, animal models of fungal infection have been used to evaluate the potential utility of combination therapy.

We previously reported the results of a susceptibility test of antifungal agents against *Aspergillus fumigatus* by the macrodilution method in yeast-malt (YM) broth (8). In the present study, we examined the in vitro effects of antifungal agent combinations administered simultaneously and sequentially against *A. fumigatus* in order to develop improved clinical combination therapies.

MATERIALS AND METHODS

Fungal strains. Fifteen strains of *A. fumigatus* were isolated from 15 patients with pulmonary aspergillosis (11 with pulmonary aspergilloma, 3 with pyothorax caused by *Aspergillus* spp. and 1 with allergic bronchopulmonary aspergillosis) at Nagasaki University Hospital. All strains were isolated from clinical specimens (nine from sputum, three from bronchial aspirates, two from bronchial lavage, and one from a pleural effusion).

Drugs. Five antifungal agents were used in the study: AMPH (Lot 29670; Bristol-Myers Squibb Co.), miconazole (MCZ; Lot S423502; Janssen Pharmaceutical Ltd.), fluconazole (FLCZ; Lot OA0912; Pfizer Pharmaceutical Inc.), ketoconazole (KTZ; Lot 6013; Janssen Pharmaceutical Ltd.), and itraconazole (ITR; Lot A3201; Janssen Pharmaceutical Ltd.). These drugs were dissolved with dimethyl sulfoxide (DMSO).

Susceptibility testing method and combination effects. The susceptibility testing method was described in our previous report (12). The medium used was YM broth, which consisted of 0.3% malt extract (Difco), 0.3% yeast extract (Difco), 0.3% polypeptone (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 2% glucose (pH 7.0). Tubes containing 5 ml of YM broth were prepared, inoculated with 100 μl of a spore suspension (1.0×10^7 cells per ml), and incubated at 30°C for 24 h on a reciprocal shaker. The final concentration of DMSO was 0.02%.

The MIC of each drug pair was determined to be the lowest concentration at which no significant mycelial growth was observed visually in the YM broth. The combination effect was assessed by checkerboard titration and was expressed as a fractional inhibitory concentration (FIC) index (3). The FIC index was the degree of dilution required, which is equal to the sum of the FIC (concentration of each agent in combination/concentration of each agent alone). A FIC index of <0.5 represents marked synergy, a FIC index of between 0.5 and 1.0 indicates synergy, a FIC index of between 1.0 and 2.0 indicates a subadditive effect, and a FIC index of >2.0 indicates antagonism.

The effects of sequentially administered antifungal agents were examined by preculture in YM broth with a first antifungal agent for 1 h and then the addition of another antifungal agent for 24 h. The total effect was assessed by checkerboard titration and was expressed as a FIC index.

RESULTS

In vitro susceptibility test and combination effects of simultaneously administered antifungal agents. The MICs of the tested antifungal agents are given in Table 1. Of the five drugs

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TABLE 1. MICs of antifungal agents for 15 strains of *A. fumigatus*[a]

Drug	MIC (μg/ml)[a]		
	Range	50%	90%
AMPH	0.78–12.5	3.13	12.5
MCZ	6.25–25	12.5	25
FLCZ	50–100	100	100
KTZ	6.25–25	12.5	25
ITR	0.025–0.39	0.05	0.1

[a] MICs were determined by the YM broth macrodilution method. 50% and 90%, MICs at which 50 and 90% of isolates were inhibited, respectively.

tested, ITR exhibited the most potent antifungal activity. FLCZ generally had high MICs (in excess of 100 μg/ml).

The combination effects of simultaneously administered antifungal agents are summarized in Table 2. With the combination of AMPH and azole antifungal agents, AMPH and MCZ had the greatest synergistic effect against *A. fumigatus*. Pairs of azole compounds showed no marked synergistic effect. MCZ and ITR or KTZ and ITR used together had antagonistic effects against 10 of 15 fungal strains (67%).

Effects of antifungal agents used sequentially. The combination effects of AMPH and azoles not administered simultaneously are given in Table 3. Pretreatment with AMPH and then MCZ or FLCZ resulted in greater synergistic effects than those obtained when the drugs were given simultaneously. Pretreatment with FLCZ, KTZ, or ITR and then AMPH increased the number of strains against which the treatment was antagonistic.

DISCUSSION

The in vitro susceptibility assay is dependent on such factors as the strains of the organisms, exposure time, incubation temperature, and medium composition (6, 10). Denning et al. (5) reported in vitro susceptibility data obtained by a macrodilution broth method for more than 100 isolates of *Aspergillus* species and recommended the clinical use of in vitro susceptibility testing.

We also reported the results of a macrodilution test for susceptibility testing of several antifungal agents against *A. fumigatus* which was simple and rapid and which was characterized by a clear end point (8, 12). We used YM broth as a testing medium because it had the advantage of supporting good cell growth and of being easy and economical to prepare. This assay was useful for examining the effects of combinations of antifungal agents. However, YM broth is inappropriate for use in the measurement of flucytosine activity because it contains antagonists to flucytosine such as purines, pyrimidines, and other nucleosides.

TABLE 2. Effects of antifungal agent combinations administered simultaneously against *A. fumigatus*[a]

Drug	No. (%) of isolates			
	Marked synergy	Synergy	Subadditive effect	Antagonism
AMPH + MCZ	9 (60)	4 (26)	1 (7)	1 (7)
AMPH + FLCZ	1 (7)	4 (26)	7 (47)	3 (20)
AMPH + KTZ	1 (7)	1 (7)	8 (52)	5 (34)
AMPH + ITR	2 (14)	4 (26)	5 (34)	4 (26)
FLCZ + MCZ	0 (0)	2 (13)	8 (53)	5 (34)
FLCZ + ITR	0 (0)	0 (0)	11 (73)	4 (27)
FLCZ + KTZ	0 (0)	0 (0)	10 (66)	5 (34)
MCZ + KTZ	0 (0)	0 (0)	8 (53)	7 (47)
MCZ + ITR	0 (0)	0 (0)	5 (34)	10 (66)
KTZ + ITR	0 (0)	0 (0)	5 (34)	10 (66)

[a] The combination effect was assessed by checkerboard titration and was expressed as a FIC index. An index of <0.5 implied marked synergy, an index of between 0.5 and 1.0 implied synergy, an index of between 1.0 and 2.0 implied a subadditive effect, and an index of >2.0 implied antagonism.

TABLE 3. Effects of combinations of AMPH and azole antifungal agents administered sequentially against *A. fumigatus*

Drugs (first/second)	No. (%) of isolates			
	Marked synergy	Synergy	Subadditive effect	Antagonism
AMPH/MCZ	11 (73)	4 (27)	0 (0)	0 (0)
AMPH/FLCZ	7 (46)	4 (27)	4 (27)	0 (0)
AMPH/KTZ	1 (7)	4 (27)	10 (66)	0 (0)
AMPH/ITR	2 (14)	5 (34)	5 (34)	3 (20)
MCZ/AMPH	8 (52)	5 (34)	1 (7)	1 (7)
FLCZ/AMPH	0 (0)	1 (7)	3 (20)	11 (73)
KTZ/AMPH	0 (0)	2 (14)	3 (20)	10 (66)
ITR/AMPH	0 (0)	1 (7)	3 (20)	11 (73)

[a] The organisms were precultured with the first drug and incubated for 1 h, and then the other antifungal agent was added. The combination effect was assessed by checkerboard titration and was expressed as a FIC index. An index of <0.5 implied marked synergy, an index of between 0.5 and 1.0 implied synergy, an index of between 1.0 and 2.0 implied a subadditive effect, and an index of >2.0 implied antagonism.

The combination of AMPH with an azole antifungal agent was equally or more potent than a single agent against *A. fumigatus*. Odds (9) reported that AMPH and MCZ could be synergistic, indifferent, or antagonistic. Similarly, it has also been reported that the combination of ITR and AMPH can be synergistic, additive, or indifferent (5). A few investigators reported the effects of combinations of azole compounds against filamentous fungi. Odds (9) reported that MCZ and KTZ were antagonistic in vitro against one *A. fumigatus* isolate, and our data also showed that combinations of azole antifungal agents were not synergistic but antagonistic.

Brajtburg et al. (4) investigated the in vitro antifungal action of AMPH used alone or in combination with a second polyene antibiotic. The addition of AMPH to other polyene antibiotics resulted in antagonism against *Candida albicans*. In contrast, potentiation occurred when other polyene antibiotics were added to cultures pretreated with AMPH.

Our results indicated that pretreatment with AMPH, except for the combination of AMPH and ITR, and then the addition of azole antifungal agents potentiated their antifungal activities in comparison with the effects of the combination administered simultaneously. The simplest explanation for this observation is that the low concentration of AMPH damages the cell membranes and azole compounds enter into the cytoplasm of the fungal cells to exercise their fungicidal effects. It suggests that prophylactic administration of AMPH could synergistically promote the activities of azole antifungal agents and their effectiveness in the treatment of aspergillosis.

Schaffner and Frick (11) reported that KTZ prophylaxis antagonized the activity of AMPH against aspergillosis in vitro and in vivo. Our result also showed that pretreatment with an azole and then the addition of AMPH increased the antagonistic effects of the drug. The antagonistic effects found in the in vitro study might be relevant to the clinical outcome.

In consideration of the available clinical experience with low-dose AMPH prophylaxis, our data suggest that subsequent

therapy with azole compounds might show better clinical efficacy against aspergillosis than that obtained by currently used regimens.

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