ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2009, p. 788–790 Vol. 53, No. 2
0066-4804/09/$08.00+0 doi:10.1128/AAC.01131-08
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Antifungal Activity of Caspofungin in Combination with Amphotericin B against *Candida glabrata*: Comparison of Disk Diffusion, Etest, and Time-Kill Methods▽

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Received 22 August 2008/Returned for modification 29 September 2008/Accepted 15 November 2008

The in vitro activities of caspofungin plus amphotericin B against 50 *Candida glabrata* isolates were evaluated by the time-kill, disk diffusion, and Etest methods. In vitro experiments showed a positive interaction. Even though each of these methods uses different conditions and endpoints, the results of the different methods frequently agreed.

Candida glabrata is an opportunistic pathogen that mainly affects severely immunocompromised patients, causing disseminated and frequently fatal infections (9). Many isolates of *C. glabrata* have shown innate resistance to fluconazole, and treatment often fails. Combined therapy could be a therapeutic alternative, but it has been poorly explored (7).

Caspofungin (CAS), an echinocandin, inhibits fungal cell wall synthesis. Amphotericin B (AMB) targets fungal ergosterol, the main component of the fungal cell membrane (5). With their different mechanisms of action, these two drugs could be effective in combination. In this study, we hypothesized that the combination of CAS with AMB could have an advantage against *C. glabrata* over monotherapy with either drug.

Fifty strains of *C. glabrata* were isolated from clinical samples at our laboratory. *Candida parapsilosis* ATCC 22019 was included for quality control (4). Antifungal susceptibility testing was performed, following both the broth microdilution (4) and Etest (Etest technical guide 4; AB Biodisk, Solna, Sweden) methods. The final concentrations were 0.03 to 2.0 μg/ml of AMB and 0.0625 to 64 μg/ml of CAS. MICs were read after 48 h of incubation. The Etest was performed on RPMI 1640 agar plates as recommended (Etest technical guide 4) (1). For CAS, an 80% inhibition in growth was used as the MIC endpoint (microcolonies were ignored), and for AMB, the MIC endpoint was defined as the lowest concentration with complete (100%) growth inhibition (1).

For the time-kill studies, the drugs alone and in combination were used at 1× MIC (1.0 μg/ml for both drugs). The numbers of CFU were determined at 0, 2, 6, and 24 h. The limit of detection was 50 CFU/ml. Fungicidal activity was considered to have been achieved when the number of CFU per milliliter was <99.9% compared with the initial inoculum size. Synergy and antagonism were defined, respectively, as a ≥100-fold increase or decrease in killing compared with that achieved with the most active single agent. If there was less than a 100-fold change, the interaction was considered indifferent (3). For the antifungal combination studies, two types of Etest methods were used. For the first method (Etest-1; described in reference 5), synergy was defined as a decrease of ≥3 dilutions, indifference as a decrease of <2 dilutions, and antagonism as an increase of ≥3 dilutions, respectively, in the resultant MIC. The second method (Etest-2) was carried out as described in a previous study (10). The fractional inhibitory concentration (FIC) index was calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of the combination/the MIC of drug A alone, and FIC B is the MIC of the combination/the MIC of drug B alone. An FIC of ≤0.5 indicated a synergic effect, an FIC of >0.5 to 4.0 an indifferent effect, and an FIC of >4.0 an antagonistic effect (1, 5, 10).

The disk diffusion method was used according to National Committee for Clinical Laboratory Standards guideline M44-A (8). Disks were embedded in the drug alone or in the drugs in combination. The final concentrations for AMB and CAS were 10 μg/disk and 2.5 μg/disk, respectively. The plates were incubated at 35°C, and inhibition zone diameters were measured at 24 to 48 h (6).

TABLE 1. In vitro susceptibilities of 50 isolates to two antifungal agents as determined by Etest and broth microdilution methods

Antifungal agent	Method[a]	MIC range (μg/ml)	% of agreement[b] (± 2 $\log_2$ dilutions)
AMB	BMD	0.25–2 (μg/ml)	86
	ET	0.0020–25 (μg/ml)	
CAS	BMD	0.125–2 (μg/ml)	90
	ET	0.125–1.5(μg/ml)	

[a] BMD, broth microdilution; ET, Etest.
[b] Percentage of agreement between the results is defined as the proportion of Etest MIC results that were within ± 2 $\log_2$ dilutions of the broth microdilution MIC results.

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▽ Published ahead of print on 24 November 2008.

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TABLE 2. Comparison of results of Etest and time-kill methods for the antifungal combination CAS plus AMB

Isolate	FIC index for indicated test:		Interpretation of time-kill test
	Etest-1 (interpretation)	Etest-2 (interpretation)	
1	1.1 (I)	7.18 (A)	I
2	2.52 (I)	1.01 (I)	I
3	3.66 (I)	2.34 (I)	I
4	1.97 (I)	1.16 (I)	I
5	2.28 (I)	1.25 (I)	I
6	0.92 (I)	0.74 (I)	I
7	0.62 (I)	0.83 (I)	I
8	0.13 (S)	1.48 (I)	S
9	0.83 (I)	0.88 (I)	S
10	0.54 (I)	1.16 (I)	I
11	0.17 (S)	0.7 (I)	S
12	1.33 (I)	0.58 (I)	S
13	0.99 (I)	0.49 (S)	I
14	0.51 (I)	0.67 (I)	I
15	0.83 (I)	0.86 (I)	I
16	0.42 (S)	0.83 (I)	S
17	0.56 (I)	1.01 (I)	I
18	1.19 (I)	0.88 (I)	I
19	0.34 (S)	0.58 (I)	S
20	0.42 (S)	0.57 (I)	S
21	0.27 (S)	0.37 (S)	S
22	0.47 (S)	0.89 (I)	S
23	0.47 (S)	0.43 (S)	S
24	0.03 (S)	0.38 (S)	S
25	0.08 (S)	0.83 (I)	S
26	0.36 (S)	1 (I)	S
27	0.06 (S)	1.23 (I)	S
28	0.28 (S)	0.75 (I)	S
29	0.17 (S)	1 (I)	S
30	0.02 (S)	0.11 (S)	S
31	0.42 (S)	0.83 (I)	S
32	0.55 (I)	0.68 (I)	I
33	0.06 (S)	0.82 (I)	S
34	0.59 (I)	0.38 (S)	I
35	2.23 (I)	4.34 (A)	I
36	0.1 (S)	2.26 (I)	S
37	0.42 (S)	1.26 (I)	S
38	1.7 (I)	1.34 (I)	S
39	0.85 (I)	0.77 (I)	I
40	0.63 (I)	1.23 (I)	I
41	0.34 (S)	0.99 (I)	S
42	8.01 (A)	8.17 (A)	I
43	0.68 (I)	0.51 (I)	I
44	0.83 (I)	0.7 (I)	I
45	1.26 (I)	1.01 (I)	I
46	3 (I)	1.42 (I)	I
47	1.19 (I)	1.18 (I)	I
48	1.56 (I)	1 (I)	I
49	3.66 (I)	1.43 (I)	I
50	2.19 (I)	1.18 (I)	I

[a] S, synergy; I, indifference; A, antagonism.

Statistical analysis. The in vitro results were analyzed by Student's *t* test, and a value of <0.05 was considered significant.

The median MICs are presented in Table 1. In general, there was good agreement between the Etest and the broth microdilution test.

As seen in Table 2, time-kill studies for the *C. glabrata* isolates using CAS plus AMB revealed synergy in 23 of 50 (46%) isolates and indifference in 27 of 50 (54%) isolates, whereas antagonism was not detected. The Etest-1 method revealed synergy in 20 of 50 (40%) isolates, indifference in 29 of 50 (58%) isolates, and antagonism in 1 of 50 (2%) isolates. The Etest-2 method revealed synergy in 6 of 50 (12%) isolates, indifference in 41 of 50 (82%) isolates, and antagonism in 3 of 50 (6%) isolates.

TABLE 3. In vitro activities against 50 *C. glabrata* isolates of CAS and AMB alone and in combination as determined by disk diffusion assay[a]

Drug	Halo diam[b] (mean ± SD)
CAS alone	24 ± 2.7[c]
AMB alone	22 ± 4.5[d]
CAS + AMB	29 ± 4.7

[a] Each isolate was tested in duplicate.
[b] With treatment of 2.5 μg/disk of CAS or 10 μg/disk of AMB. SD, standard deviation.
[c] $P < 0.05$ versus results for CAS alone.
[d] $P < 0.05$ versus results for AMB alone.

Concordance of the Etest-1 synergy method and the time-kill assay was demonstrated for 44 of 50 (92%) isolates. Concordance of the Etest-2 synergy method and the time-kill assay was found for 26 of 50 (52%) isolates. However, 26 of 50 (52%) isolates gave the same result for each of three tests (indifference, 22; synergy, 4).

For the Etest-2 method, we opted to place the Etest strips simultaneously on the agar at 90° angles. CAS and AMB exhibited different diffusion characteristics through the agar, resulting in dissimilar ellipse patterns. However, the growth endpoints used to measure the MIC by Etest are different for the two agents. Therefore, we felt that the interpretation of separate ellipses at 90° angles would be difficult.

The disk diffusion assay results are reported in Table 3. The zone diameters of each drug combination were never smaller than those produced by each drug alone, and antagonism was never observed.

Our findings are similar to the results of other investigators who reported that generally, synergistic and indifferent effects against *Candida* spp. are observed, whereas antagonism is rarely detected (2, 3). However, the results of the Etest-1 study indicate that this method could be an acceptable alternative to time-kill studies with antifungal agents. It is possible that preexposure for 1 h with an echinocandin in agar-based medium is sufficient to detect an echinocandin-polyene interaction. The positive interaction between an echinocandin compound and a polyene can be explained by the fact that both drug families possess unique mechanisms of action. It can be postulated that the candins, which inhibit cell wall synthesis, may enhance the activity of AMB by increasing the rate or degree of their access to the cell membrane (3).

Combination therapy with these two drugs may be advantageous against *C. glabrata*, since synergy was seen with some of the isolates (46% for the time-kill method) and antagonism was not seen. Animal studies are warranted to elucidate the potential utility of this combination therapy.

This work was supported by a grant from Eskişehir Osmangazi University (project no. 200511014).

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