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In Vitro Activity of Caspofungin Combined with Sulfamethoxazole against Clinical Isolates of *Aspergillus* spp.

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Caspofungin (CAS) inhibits fungal cell wall synthesis. Sulfamethoxazole (SMX) inhibits folate biosynthesis and is active in vitro against *Aspergillus* spp. We studied the activities of the combination of CAS and SMX against 31 *Aspergillus* isolates and compared them with that of SMX combined with amphotericin B (AMB) or itraconazole (ITC). MICs and minimal effective concentrations (MECs) were determined by the NCCLS broth microdilution method. With MIC endpoints, the combination of SMX and CAS showed synergy or synergy to additivity against 29 of 31 isolates. With MEC endpoints, synergy to additivity was found against 12 of 31 isolates and indifference was displayed against the rest of them. SMX in combination with AMB or ITC was not truly synergistic, while synergy to additivity was found for SMX-AMB and SMX-ITC against 17 of 31 and 3 of 12 isolates, respectively. No antagonism was found with any of the drug combinations. Further analysis of the synergy of CAS and SMX was performed by detailed measurement of hyphal length by microscopy and time-dependent 2,3-bis(2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT)-based hyphal damage experiments. With MEC endpoints, the combination of CAS and SMX was characterized by a greater than 50% decrease in hyphal length compared to the hyphal lengths achieved with double the concentration of each drug alone. The XTT-based hyphal damage studies showed a statistically significant ($P < 0.05$) reduction in viability with CAS and SMX in combination compared to the viabilities achieved with double the concentration of each drug alone. These findings support the synergy results found by using MIC endpoints and suggest that visual MEC measurements may not be sufficient to identify the synergistic interactions seen by more sensitive, quantitative methods. Animal models are required to validate the significance of the synergy of CAS and SMX against *Aspergillus* spp. observed in vitro.

Invasive aspergillosis is one of the most common invasive fungal infections in immunocompromised patients and carries high mortality rates (10).

Current therapeutic agents such as amphotericin B (AMB) and its liposomal formulations, azoles such as itraconazole (ITC) and voriconazole (VRC), and echinocandins such as caspofungin (CAS) are only partially effective for the treatment of this disease; therefore, more effective drugs and drug combinations are needed (8).

Sulfamethoxazole (SMX) is an antimicrobial drug which inhibits the biosynthesis of folates (4, 6). SMX is moderately active in vitro against *Aspergillus* spp. (2), and *Aspergillus fumigatus* mutants defective in the folate biosynthesis pathway are avirulent (5). SMX is frequently administered in combination with trimethoprim to immunocompromised patients for prophylaxis for *Pneumocystis carinii* pneumonia (PCP). Interestingly, PCP prophylaxis in AIDS patients is associated with a decreased probability of development of invasive aspergillosis (1).

We examined whether the combination of SMX with AMB, ITC, or CAS in vitro exerts synergistic inhibitory effects against 31 clinical isolates of *Aspergillus* spp., possibly providing insight into more effective treatments for invasive aspergillosis. We further elucidated the microscopic changes and the time-dependent hyphal damage which occurs after exposure of *Aspergillus* spp. to the combination of SMX and CAS. Our results suggest that use of the combination of SMX and CAS represents a potentially promising approach for combination drug therapy against *Aspergillus* spp.

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MATERIALS AND METHODS

Strains and inoculum preparation. A total of 31 clinical isolates of *Aspergillus* (*A. fumigatus* [$n = 13$], *A. niger* [$n = 6$], *A. flavus* [$n = 6$], and *A. terreus* [$n = 6$]) (16) were used in this study. *A. fumigatus* strain AF293 (http://www.tigr.org/tdb/) and *A. niger* strain ATCC 16404 were tested in every experiment as internal controls. To prepare conidial inocula, strains were grown for 3 to 4 days on Sabouraud dextrose agar plates at 37°C. Five milliliters of distilled sterile water containing 0.2% (vol/vol) Tween 20 was added, and the conidia were gently harvested by rubbing with a spreader. The conidia were washed three times in distilled sterile water and resuspended in phosphate-buffered saline. The conidia were counted with a hemocytometer and diluted to a final concentration of 2.5×10^4 conidia/ml in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) containing 0.165 M morpholinepropanesulfonic acid MOPS buffer at pH 7.0 (RPMI-MOPS).

Drugs. SMX and AMB (Sigma, St. Louis, Mo.) were dissolved in dimethyl sulfoxide at 40 and 5 mg/ml, respectively, and were further diluted in RPMI-MOPS. CAS (Merck, Rahway, N.J.) was dissolved in sterile distilled water. ITC (Jansen, Piscataway, N.J.) was dissolved in 50% polyethylene glycol 400 at 5 mg/ml.

Checkerboard synergy assay. Drug interactions were assessed by checkerboard assays by the NCCLS M38-P microdilution methodology (13) after 24 h of incubation in standard 96-well plates (Costar; Corning, Corning, N.Y.) (2, 16). The final concentrations of the antifungal agents ranged from 15 to 2,000 μg/ml for SMX, 0.062 to 256 μg/ml for CAS, 0.002 to 2 μg/ml for ITC, and 0.06 to 4

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μg/ml for AMB. Each well received 100 μl of drug at the diluted concentrations. Dilutions were made in RPMI-MOPS. Conidial inocula (2.5×10^4 conidia/ml) in RPMI-MOPS were added at a volume of 100 μl/well. The final volume of each well containing conidia and drugs was 200 μl. The MIC was the lowest drug concentration resulting in complete inhibition of hyphal growth (3). The minimal effective concentration (MEC) was the lowest drug concentration resulting in reduced hyphal growth, as described previously (3). The plates were scanned microscopically with an inverted microscope at low (×40) magnification. The results were used to determine the fractional inhibitory concentration (FIC) of the combination of SMX and CAS, AMB, or ITC for each clinical isolate. FICs were calculated for both MIC and MEC endpoint measurements taken from the well with the lowest drug concentrations needed to achieve these endpoints. FIC indices (FICIs) were calculated as described previously (3).

Calcofluor staining and microscopy. Conidia from selected *Aspergillus* strains were incubated for 24 h at 37°C on glass coverslips in six-well plates (Nunclon; Nalge Nunc, Roskilde, Denmark) containing 4 ml of RPMI-MOPS/well in the presence of different concentrations of SMX (15.5 to 250 μg/ml), CAS (0.06 to 2 μg/ml), and SMX plus CAS (in all 42 pairwise combinations possible) After 24 h, the MECs of CAS and SMX were determined. Coverslips from those wells containing the MECs of each drug alone and in combination were selected for microscopic analysis. Germlings were stained for 15 min at room temperature with calcofluor (0.5 mg/ml in phosphate-buffered saline) to enhance fungal visibility during microscopy. Hyphal length was measured by microscopy with a micrometer. For each germling, measurements were taken from the spore to the tip of the longest germ tube emerging from the spore, but the measurements did not include the lengths of the branch points emanating from this germ tube. For CAS-treated fungi, the measurement was taken from the center of the colony outward to its widest point. Each datum point represents the average measurements for 50 randomly selected germlings. Microscopy was performed with an Olympus BX-40 microscope (equipped for fluorescence with a fluorescein isothiocyanate filter) at ×400 magnification. Images were recorded on an Olympus C-200 digital camera.

Time-dependent hyphal damage assay with XTT. Conidia from selected strains were incubated for between 18 and 30 h in the presence of different concentrations of SMX (15.5 to 125 μg/ml) and CAS (0.06 to 8 μg/ml) in all pairwise combinations of SMX and CAS by the NCCLS M38-P microdilution methodology in standard 96-well plates, as described above. After incubation for the desired time at 37°C, readings from the 2,3-bis(2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT)-based colorimetric assay were performed. The XTT tetrazolium salt colorimetric assay, which measures viability and hyphal damage, was performed as described previously (11). Briefly, at each time point 50 μl of XTT (Sigma) at 1 mg/ml supplemented with 25 μM freshly added menadione (Sigma) was added to each well containing *Aspergillus* grown in 200 μl of RPMI-MOPS in the presence or absence of drug. To ensure that all readings were taken during the linear phase of the colorimetric assay, plates containing XTT were incubated at 37°C for up to 120 min, until the control well containing *Aspergillus* in the absence of drug(s) reached an optical density at 450 nm of 1.0, at which point readings for the entire plate were performed.

Statistical analysis. The XTT hyphal damage assay and microscopy experiments were independently performed three times. The results of representative experiments are displayed. Error bars denote standard deviations. *P* values were calculated by the Student coupled two-tailed *t* test. Differences were considered significant if the *P* value was <0.05.

RESULTS

In vitro activities of SMX and CAS combinations. The combination of SMX and CAS was tested against 31 *Aspergillus* clinical isolates by the checkerboard assay. The MICs, MECs, and FICIs obtained for four representative isolates (*A. fumigatus* 7, *A. niger* 6, *A. terreus* 5, and *A. flavus* 1) at 24 h are shown in Table 1. The MICs and MECs of SMX for *A. fumigatus* ($n = 13$), *A. flavus* ($n = 6$), *A. niger* ($n = 6$), and *A. terreus* ($n = 6$) were between 250 and 2,000 and 31 and 250 μg/ml, respectively. The MICs and MECs of CAS for *A. fumigatus* ($n = 13$), *A. flavus* ($n = 6$), *A. niger* ($n = 6$), and *A. terreus* ($n = 6$) were between 16 and 256 and 0.06 and 0.25 μg/ml, respectively. With MIC endpoints, SMX and CAS exhibited synergy against 15 of 31 isolates (Table 2). Synergy to additivity was found

TABLE 1. MIC, MEC, and FICI results for selected *Aspergillus* spp. at 24 h for combinations of SMX and CAS, SMX and AMB, and SMX and ITC

Isolate	Endpoint	SMX-CAS						SMX-AMB						SMX-ITC					
		Concn (μg/ml)						Concn (μg/ml)						Concn (μg/ml)					
		SMX (alone)	SMX (with CAS)	CAS (alone)	CAS (with SMX)	FICI	Result[a]	SMX (alone)	SMX (with AMB)	AMB (alone)	AMB (with SMX)	FICI	Result	SMX (alone)	SMX (with ITC)	ITC (alone)	ITC (with SMX)	FICI	Result
A. fumigatus 7	MIC	500	125	64	8	0.38	S	500	250	0.16	0.08	1	Ad	250	250	0.5	0.25	1.5	I
	MEC	62.5	31.25	0.125	0.06	1	Ad	125	16	0.08	0.08	1.13	I	62.5	31	0.25	0.25	1.5	I
A. niger 6	MIC	2,000	62.5	128	64	0.53	Ad	1,000	1,000	0.16	0.156	2	I	1,000	500	1.0	1.0	1.5	I
	MEC	250	15.6	0.25	0.125	0.56	Ad	250	16	0.08	0.08	1.06	I	250	125	0.25	0.25	1.5	I
A. terreus 5	MIC	1,000	250	128	16	0.38	S	1,000	1,000	0.62	0.312	1.5	I	500	250	0.5	0.25	1	Ad
	MEC	62.5	15.6	0.06	0.06	1.25	I	250	16	0.16	0.16	1.06	I	250	16	0.125	0.25	2.06	I
A. flavus 1	MIC	1,000	125	256	16	0.19	S	2,000	1,000	0.31	0.625	2.5	I	500	500	0.5	0.25	1.5	I
	MEC	62.5	15.6	0.06	0.06	1.25	I	125	16	0.31	0.31	1.13	I	125	16	0.125	0.25	2.13	I

[a] S, synergistic (FICI ≤ 0.5); Ad, synergistic to additive ($0.5 <$ FICI ≤ 1); I, indifferent ($1 <$ FICI ≤ 4).

TABLE 2. Overall results of checkerboard tests obtained for *Aspergillus* spp at 24 h with combinations of SMX and CAS, SMX and AMB, and SMX and ITC

Drug combination and isolate	MIC endpoint (MEC endpoint)[a]			
	S	Ad	I	An
SMX-CAS				
A. fumigatus (n = 13)	3 (0)	10 (5)	0 (8)	0 (0)
A. niger (n = 6)	0 (0)	4 (4)	2 (2)	0 (0)
A. terreus (n = 6)	6 (0)	0 (3)	0 (3)	0 (0)
A. flavus (n = 6)	6 (0)	0 (0)	0 (6)	0 (0)
SMX-AMB				
A. fumigatus (n = 13)	0 (0)	9 (3)	4 (10)	0 (0)
A. niger (n = 6)	0 (0)	3 (1)	3 (5)	0 (0)
A. terreus (n = 6)	0 (0)	4 (3)	2 (3)	0 (0)
A. flavus (n = 6)	0 (0)	1 (0)	5 (6)	0 (0)
SMX-ITC				
A. fumigatus (n = 4)	0 (0)	0 (3)	4 (1)	0 (0)
A. niger (n = 2)	0 (0)	0 (0)	2 (2)	0 (0)
A. terreus (n = 3)	0 (0)	2 (0)	1 (3)	0 (0)
A. flavus (n = 3)	0 (0)	1 (0)	2 (3)	0 (0)

[a] S, synergistic; Ad, synergistic to additive; I, indifferent; An, antagonistic.

against 14 of 31 isolates, and indifference was found against 2 isolates. With MEC endpoints, SMX and CAS showed synergy to additivity against 12 of 31 isolates and indifference against the rest (Table 2). Notably, no antagonism was found by using MIC or MEC endpoints.

In vitro activities of SMX and ITC or AMB combinations. The combination of SMX and ITC or AMB was tested by the checkerboard assay. The MICs, MECs, and FICIs obtained for four representative isolates (*A. fumigatus* 7, *A. niger* 6, *A. terreus* 5, and *A. flavus* 1) at 24 h are shown in Table 1 (SMX-AMB and SMX-ITC). With MIC endpoints, no synergy between SMX and AMB was found. SMX and AMB showed synergy to additivity against 17 of 31 isolates and indifference against the rest (Table 2). MEC endpoints indicated synergy to additivity against 7 of 31 isolates and indifference against the rest (Table 2).

With MIC and MEC endpoints, no synergy between SMX and ITC was found. Synergy to additivity was found against 3 of 12 isolates tested, and indifference was found against the rest of them (Table 2).

Microscopic analysis of *Aspergillus* spp. in the presence of SMX, CAS, and combinations of the two drugs. Our results obtained by the checkerboard assay indicate that CAS-SMX had synergy or synergy to additivity against 94% of the strains when MIC measurements were used but against only 39% of the strains when MEC measurements were used. To try and resolve this discrepancy, we undertook a detailed microscopic examination of four *Aspergillus* isolates (*A. fumigatus* 7, *A. niger* 6, *A. terreus* 5, and *A. flavus* 1) in the presence of SMX and CAS at the MECs. These strains were selected to represent each of the *Aspergillus* subgroups and because they showed either discrepancies in the synergy testing results by use of MEC endpoints (no true synergy) compared to those obtained by use of MIC endpoints (synergy or synergy to additivity) (*A. fumigatus* 7, *A. terreus* 5, and *A. flavus* 1) or equivalent values (additivity) for both endpoints (*A. niger* 6). Microscopic hyphal length measurements were taken at the MECs of each drug alone or in combination. Surprisingly, the microscopic analysis of calcofluor-stained mycelia demonstrated a greater than 50% decrease in hyphal length when both CAS and SMX were administered in combination compared to the lengths when each drug was administered alone at double the concentration. This effect was seen for all four *Aspergillus* strains, including strains *A. terreus* 5 and *A. flavus* 1, for which indifference was shown with the standard broth microdilution MEC readings (Fig. 1). These results suggest that when SMX and CAS are used in combination at their MECs, there is a synergistic effect, as measured by hyphal length ($P < 0.01$), which is not noticeable by the use of standard MEC measurements.

Time-dependent hyphal damage study of *Aspergillus* spp. in the presence of SMX, CAS, and combinations of the two drugs. To further analyze the discrepancy between the results obtained by the checkerboard assay with MIC and MEC endpoints, we performed a detailed time course analysis with strains *A. fumigatus* 7, *A. niger* 6, *A. terreus* 5, and *A. flavus* 1, which represent each of the *Aspergillus* subgroups. We used the XTT colorimetric assay to assess fungal metabolism and growth over time. The results indicate that the combination of SMX and CAS at their MECs results in a statistically significant decrease in XTT activity ($P < 0.05$) compared to that achieved with double the concentration of each drug alone (Table 3). This effect persisted throughout the time course of the experiment. Taken together, our results indicate that FICIs derived from MEC endpoints can be either additive (*A. flavus* 7, *A. niger* 6) or indifferent (*A. terreus* 5, *A. flavus* 1) (the FICIs derived from MEC endpoints did not indicate synergism or antagonism against any of the strains with any combination), yet the XTT assay and hyphal length measurements at these drug concentrations still indicated synergy, in line with the FICIs derived from the MIC endpoints. These results suggest that when SMX and CAS are combined at their MECs, there

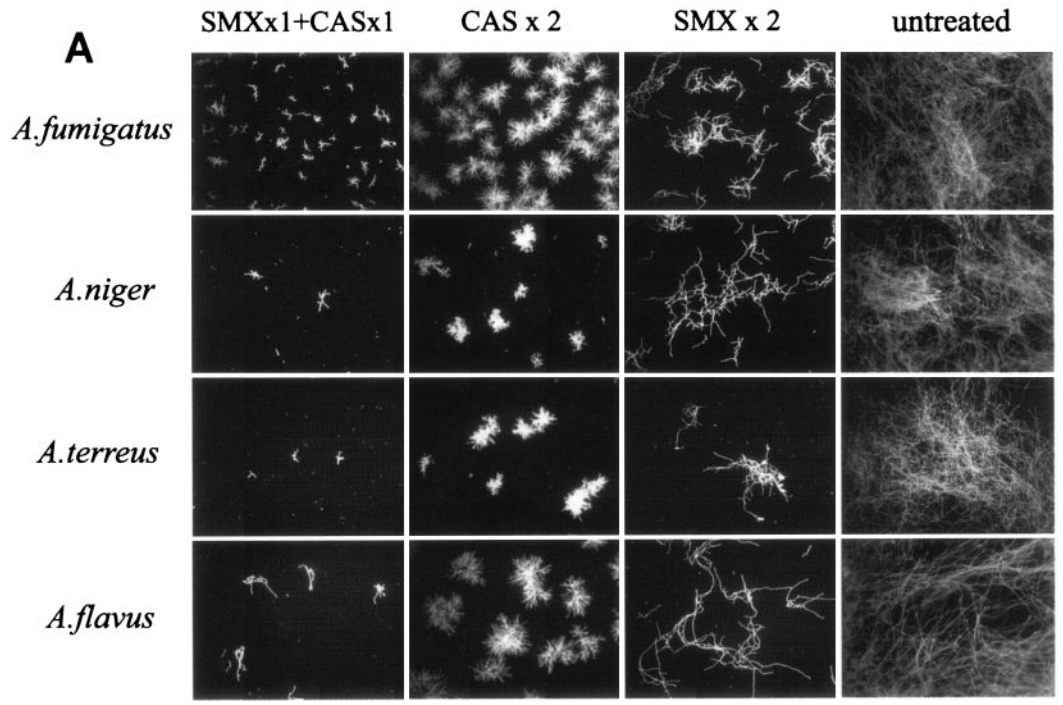
TABLE 3. Time-dependent hyphal damage measurement by XTT viability staining for four isolates[a]

Isolate	Time (h)	% XTT activity[b]		
		SMX ×1 + CAS ×1	SMX ×2	CAS ×2
A. niger 6	18	3 ± 2[c]	27 ± 1	14 ± 4
	24	2 ± 2[c]	61 ± 7	11 ± 4
	30	82 ± 4	89 ± 11	100 ± 4
A. flavus 1	18	46 ± 2[c]	86 ± 8	57 ± 2
	24	18 ± 3[c]	33 ± 8	28 ± 2
	30	14 ± 4[c]	55 ± 7	26 ± 3
A. fumigatus 7	18	8 ± 1[c]	26 ± 7	18 ± 1
	24	16 ± 1	46 ± 7	20 ± 1
	30	24 ± 6[c]	72 ± 12	45 ± 4
A. terreus 5	18	0 ± 3[c]	27 ± 3	22 ± 5
	24	0 ± 0[c]	48 ± 8	12 ± 2
	30	2 ± 0[c]	22 ± 3	10 ± 0

[a] Data for all time points are normalized to the optical density readings for internal control wells containing the strain grown in the absence of drug(s). Values are means ± standard deviations of results for four wells.

[b] ×1, the MEC; ×2, two times the MEC.

[c] $P < 0.05$ compared to the results for both SMX and CAS at two times the MEC.



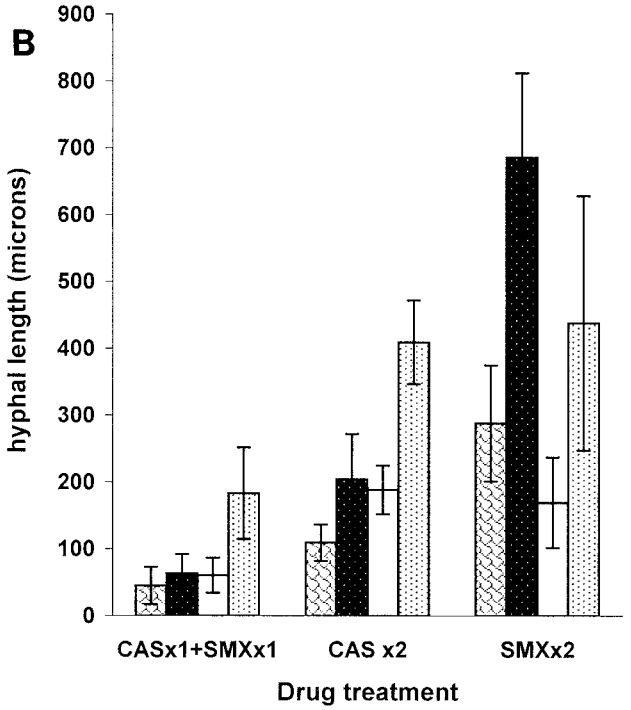


FIG. 1. (A) Microscopic analysis of *A. fumigatus* 7, *A. niger* 6, *A. terreus* 5, and *A. flavus* 1 in the presence of the MECs of CAS (CASx1; *A. terreus* 5 = 1 μg/ml, *A. fumigatus* 7, *A. niger* 6 = 0.25 μg/ml, *A. flavus* 1 = 0.06 μg/ml), the MECs of SMX (SMXx1; *A. niger* 6 = 62 μg/ml, *A. terreus* 5, *A. flavus* 1 = 62 μg/ml, *A. fumigatus* 7 = 31 μg/ml); and SMX and CAS together at the concentrations indicated above and at double the concentrations indicated above (CAS x 2 and SMX x 2, respectively). The experiments were repeated three times, with similar results each time. (B) Mean hyphal length measurements (in micrometers) taken from the experiment described for panel A. Each column represents average measurements for approximately 50 randomly selected germlings. Error bars denote standard deviations. *P* values calculated by comparison between *Aspergillus* strains treated with both drugs combined at the MEC and the same strains receiving each drug alone at two times the MEC were all statistically significant ($P < 0.01$). The bars represent *A. niger* 6, *A. terreus* 5, *A. fumigatus* 7, and *A. flavus* 1 from left to right, respectively.

is a synergistic effect, as measured by fungal metabolic activity and growth which is not apparent by standard MEC tests.

DISCUSSION

Present therapeutic modalities for invasive aspergillosis, including the introduction of new agents such as CAS and VRC, are still associated with significant mortality (14). Thus, any combination therapy which may enhance antifungal activity should be actively pursued. Preliminary studies have demonstrated in vitro synergy or additivity between CAS and AMB, ITC, and VRC (3, 15, 16) for *Aspergillus* spp. In the present study we tested the in vitro activities of the antimicrobial drug SMX in combination with the antifungal drugs CAS, AMB, and ITC against 31 clinical *Aspergillus* isolates obtained from immunocompromised patients with invasive aspergillosis. Our rationale for combining SMX with these drugs was based on three previous findings: (i) SMX alone is moderately active against *A. fumigatus* in vitro within the concentration range reached in serum in vivo (2); (ii) SMX inhibits folate biosynthesis, a pathway unique for bacteria and fungi alike, and *A. fumigatus p*-aminobenzoate auxotrophs, blocked in folate biosynthesis, are avirulent in a mouse model of disseminated aspergillosis (5); and (iii) SMX is frequently administered (as SMX-trimethoprim) to immunocompromised patients for PCP prophylaxis (9).

We demonstrate that the combination of SMX and CAS

displays true synergy (FICI $\leq$ 0.5) against 48% of strains tested and synergy to additivity (0.5 $<$ FICI $\leq$ 1) against an additional 45% of strains when MIC endpoints are used. In contrast, the combinations of SMX and AMB and SMX and ITC are not truly synergistic against any of the strains and show synergy to additivity (0.5 $<$ FICI $\leq$ 1) against 55 and 31% of the strains, respectively. One possible explanation for the higher level of synergy between SMX and CAS is that the entry of SMX into the cell may be impeded by the fungal cell wall, and CAS, by damaging the cell wall, improves the ability of SMX to enter the cell.

It is noteworthy that the trailing effect observed for the growth of all *Aspergillus* spp. in the presence of CAS is significantly diminished when low doses of SMX are added, leading to a marked reduction in MICs when both drugs are used in combination. A similar effect was observed for *Candida albicans* when a fluoroquinolone was used in combination with fluconazole (12).

SMX alone is modestly effective in vitro against *Aspergillus* spp. However, the combination of SMX and CAS reduces the need to administer very high doses of SMX to attain drug efficacy. In addition, the activities of very few of the approximately 15,000 sulfa drugs synthesized thus far have been tested against fungi (9, 4). Therefore, it is possible that other sulfa drugs might be more effective than SMX in inhibiting fungal growth, while still displaying synergy with CAS.

We found a puzzling discrepancy between the results of the MEC and MIC checkerboard assays: the combination of SMX and CAS showed synergy or synergy to additivity against 94% of the strains tested when the MIC endpoint measurements were used but against only 39% of the strains tested when MEC endpoint measurements were used. To try and resolve this discrepancy we performed detailed microscopic and time-dependent hyphal damage analyses with SMX, CAS, and combinations of the two drugs at their MECs. Time-dependant hyphal damage studies with the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide have been used in the past to demonstrate synergy between nikkomycin Z and the echinocandin FK463 against *A. fumigatus* (7). We found that when SMX and CAS are combined at their MECs, there is a synergistic decrease in both hyphal lengths and fungal metabolic activity which is not apparent when MEC measurements are used. We suggest that the use of MEC measurements may be a less sensitive method to depict certain synergistic effects, while the use of MIC measurements may prove to be more indicative of synergism. This assumption needs to be validated, however, by in vivo synergy studies to elucidate whether measurements of hyphal length and metabolic activity, which CAS plus SMX affected synergistically, correlate with fungal killing in vivo and the resolution of infection.

In conclusion, the results of our in vitro assays suggest that a combination of CAS and SMX may act synergistically against *Aspergillus* sp. infections. Methodological improvements, especially regarding the interpretation of MECs, are required. Studies with animal models are needed to validate the in vivo significance of these in vitro results.

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