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In Vitro Activities of Pentamidine, Pyrimethamine, Trimethoprim, and Sulfonamides against *Aspergillus* Species

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The susceptibilities of 70 strains of *Aspergillus* species were tested against seven different sulfa drugs and pentamidine by a microdilution method with RPMI 1640 and yeast nitrogen base media. Sulfamethoxazole, sulfadiazine, and pentamidine were active in vitro. The MICs obtained with RPMI 1640 were significantly higher than those with yeast nitrogen base. More studies are needed to further elucidate the action of these drugs.

Invasive aspergillosis is now one of the most common invasive fungal infections in immunocompromised patients and carries high mortality rates (6). Invasive aspergillosis is a rare complication of end-stage AIDS despite the immunocompromised status of the host. Although invasive aspergillosis in AIDS is associated with neutropenia and corticosteroid therapy, other factors might contribute to the low incidence. Sulfonamides, especially trimethoprim-sulfamethoxazole (SXT), are antimicrobial agents frequently employed for prophylaxis in AIDS patients to prevent *Pneumocystis carinii* pneumonia. Sulfa drugs are active against *Paracoccidioides brasiliensis* (16, 17), and pentamidine (PNT) has some activity against yeast (2, 5, 13). We have recently shown that sulfamethoxazole (SMX) is active in vitro against *Aspergillus fumigatus* and therefore might help to prevent invasive aspergillosis in AIDS patients receiving SXT prophylaxis (1). The aim of this study was to further evaluate the in vitro activities of seven different sulfa compounds and PNT against *Aspergillus* isolates comprising six different species in two different media.

Seventy clinical isolates of *Aspergillus* were tested: 20 isolates of *A. fumigatus* and 10 isolates each of the following species: *A. flavus*, *A. niger*, *A. nidulans*, *A. ustus*, and *A. terreus.* Isolates were passaged twice in PDA at an interval of 5 to 7 days at 37°C. All isolates were tested in duplicate on 2 different days. A broth microdilution method was performed according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (M38-P) (14). The drugs used in this study were trimethoprim (TMP), SMX, SXT, pyrimethamine (PMT), dapsone (DAP), sulfamethizole (SMT), sulfisoxazole (SSX), sulfadiazine (SDZ), sulfamethoxypyridazine (SMP), and PNT. All drugs were obtained as standard powders from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. The final concentrations of the drugs ranged from 16 to 0.01 μg/ml for TMP and DAP, 320 to 0.31 μg/ml for SMX, and 16/320 to 0.01/0.31 μg/ml for SXT (1:20 dilution ratio), 4 to 0.004 μg/ml for PMT, 500 to 0.4 μg/ml for SMT, 128 to 0.12 μg/ml for PNT, and 400 to 0.3 μg/ml for SSX, SDZ, and SMP. Dimethyl sulfoxide was used to dissolve all drugs except for PNT, which was dissolved in water.

The drug dilutions were made in RPMI 1640 medium (with L-glutamine, without bicarbonate) (GIBCO BRL, Life Technologies, Woerden, The Netherlands) and in yeast nitrogen base (YNB) (Difco Laboratories, Sparks, Md.) and were prepared according to the manufacturer's instructions. Both media were buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

The tests were performed in 96-well flat-bottom microtitration plates (Corning, Inc., New York, N.Y.). Conidia were collected with a cotton stick and suspended in sterile water. After the heavy particles were allowed to settle, the turbidities of the supernatants were measured spectrophotometrically (Spectronic 20D; Milton Roy, Rochester, N.Y.) at 530 nm, and transmission was adjusted to 80 to 82% and diluted to obtain a final inoculum of 0.5×10^4 to 5×10^4 CFU/ml. The inoculum size was verified by determination of the number of viable CFU per milliliter after plating serial dilutions of the inoculum onto Sabouraud dextrose agar (SDA).

Each suspension was diluted 1:50 in RPMI 1640 and in YNB to obtain two times the final inoculum.

After agitation, the plates were incubated at 35°C for 48 h. The visual MIC was defined as the lowest concentration showing prominent growth inhibition (MIC-2, approximately ≥50% inhibition). For spectrophotometric endpoint determination, the optical density (OD) was measured with a spectrophotometer (MS2 reader, Titertek-Plus; ICN Biomedical, Ltd., Basingstoke, United Kingdom) at 405 nm. The OD of the blank, a microtitration plate to which a conidium-free inoculum had been added and incubated, was subtracted from the OD values. The percentage of growth for each well was calculated by comparing the OD of the well with that of the drug-free control. The in vitro fungicidal activity (minimal fungicidal concentration [MFC]) of each agent was determined by streaking 100 μl from each well that showed complete inhibition onto SDA plates. The plates were incubated at 35°C. The MFC was

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TABLE 1. Comparison of the MICs of TMP, PMT, PNT, and sulfonamides in RPMI and YNB media for all of the *Aspergillus* species

MIC parameter[a]	MIC (μg/ml)[b]																	
	TMP		SMX		SXT		PMT		DAP		SMT		SMP		SSX		SDZ		PNT	
	RPMI	YNB	RPMI	YNB	RPMI	YNB	RPMI	YNB	RPMI	YNB	RPMI	YNB	RPMI	YNB	RPMI	YNB	RPMI	YNB	RPMI	YNB
GM	>16	>16	102.4*	61.2	116.5*	57.1	4.5	>4	>16	>16	>500	336	>200	108.2	>400	>400	321.6*	123.8	37.1*	13.2
Range	16–>16	8–>16	40–>320	2.5–>320	20–>320	2.5–>320	0.25–>4	0.25–>4	>16	16–>16	125–>500	0.9–>500	50–>400	6.2–>400	200–>400	25–>400	12.5–>400	0.3–>400	1–>128	0.25–>128
MIC_{50}	>16	>16	80	40	80	40	4	>4	>16	>16	>500	>500	200	100	>400	>400	>400	100	32	8
MIC_{90}	>16	>16	320	>320	320	>320	>4	>4	>16	>16	>500	>500	>200	>200	>400	>400	>400	>400	>128	>128

[a] GM, geometric mean of MIC; MIC_{50} and MIC_{90}, MICs at which 50 and 90% of the isolates tested, respectively, are inhibited.
[b] *, statistically significant difference ($P < 0.05$) between the GMs in RPMI and YNB.

defined as the lowest drug concentration at which growth of less than one colony was observed, which corresponds to 99.9% killing.

The results were analyzed by the Mann-Whitney test, and differences were considered statistically significant at $P < 0.05$.

The inoculum size was checked by enumeration of colonies per milliliter of serial dilutions on SDA plates. These cultures showed that the final inoculum varied between 1×10^4 and 4×10^4 CFU/ml, which is within the recommended range (14). The MICs of all of the drugs in both media tested are summarized in Table 1. All sulfonamides and PNT showed higher MICs in RPMI medium than in YNB medium ($P < 0.0001$). The lowest MICs in YNB were obtained for SMX, SXT, SMP, SDZ, and PNT.

SSX and SMT were inactive against most of the strains. TMP, PMT, and DAP were inactive against all of the isolates. SMX, SXT, SMT, SMP, and SDZ were active against the 20 *A. fumigatus* isolates (Table 2). In vitro, lower MICs were observed for *A. niger* for SMX, SXT, SDZ, SMP, and PNT and for SMX, SXT, SMT, SMP, SDZ, and PNT against *A. nidulans*. SXT and PNT showed the highest activity for this fungus. All of the drugs were less active against *A. ustus* and *A. terreus*, except for PNT.

No statistically significant difference in the MICs between SMX alone or in combination with TMP (SXT) was found ($P > 0.05$).

Considering the MIC/MFC ratios, fungistatic activity was observed for all drugs tested, except for PNT, which was fungicidal for *A. nidulans.* Nevertheless, despite the low MIC and the volume plated for MFC determination, carryover of drug could be present. More studies by other methods are needed in order to confirm this observation.

Sulfonamides act as a competitive antagonist of *p*-aminobenzoic acid (PABA), which is an integral component of the structure of folic acid. The result of decreased folic acid synthesis is a decrease in nucleotides, with subsequent inhibition of growth (21). Sulfonamides exhibit in vitro activity against a broad spectrum of gram-positive and gram-negative bacteria, parasites, and fungi (21), and the combination SXT acts synergistically against *Paracoccidioides brasiliensis* (3, 9, 16, 17, 20). The in vitro antimicrobial activity of sulfonamides is strongly influenced by the composition of the test medium, since the presence of PABA and folic acid in the medium inhibits their activity (21). RPMI is the standard medium recommended by the NCCLS (14) for in vitro susceptibility testing of yeast and molds, but contains four times more PABA than YNB (according to the manufacturer's manual). Consequently, MICs were found to be higher with RPMI than those obtained with YNB with the same inocula. Furthermore, RPMI poorly supports the growth of *Aspergillus* species compared with the richer medium, YNB (12). These factors probably contribute to the discrepancies found between the two media. There are no MIC endpoints for sulfa drugs against molds, but in previous studies, MIC-2 was used as endpoint against *P. brasiliensis* and *C. albicans* (4, 16, 17). Therefore, we chose the same endpoint in this study. The concentrations of sulfa drugs in plasma in general are between 100 and 200 μg/ml. For SMX, levels of 40 to 160 μg/ml are generally found (8, 21), which is near or above the MICs found for *A. fumigatus*, *A. flavus*, *A. niger*, and *A. nidulans*.

TABLE 2. In vitro activities of TMP, PMT, PNT, and sulfonamides against *Aspergillus* species in YNB

Species (*n*)	In vitro activity (μg/ml) of[a]:																			
	TMP		SMX		SXT		PMT		DAP		SSX		SMT		SDZ		SMP		PNT	
	GM	MFC	GM	MFC	GM	MFC	GM	MFC	GM	MFC	GM	MFC	GM	MFC	GM	MFC	GM	MFC	GM	MFC
A. fumigatus (20)	>16	>16	19.3	>320	19.3	>320	>4	>4	>16	>16	>400	>400	105	>500	39.2	>400	59.4	>400	>128	>128
A. flavus (10)	>16	>16	64.9	>320	64.9	>320	>4	>4	>16	>16	>400	>400	1,000	>500	303.1	>400	114.8	>400	>128	>128
A. niger (10)	>16	>16	49.2	>320	74.6	>320	2.6	>4	>16	>16	>400	>400	>500	>500	81.2	>400	70.6	>400	4.92	>128
A. nidulans (10)	>16	>16	22.9	>320	16.2	>320	1.8	>4	>16	>16	214.3	>400	53.9	>500	24.3	>400	40.6	>400	0.4	6.5[b]
A. ustus (10)	>16	>16	>320	>320	>320	>320	>4	>4	>16	>16	303.1	>400	>500	>500	>400	>400	373.2	>400	1.1	128
A. terreus (10)	>16	>16	226	>320	184	>320	>4	>4	>16	>16	>400	>400	>500	>500	>400	>400	400	>400	2.14	>128

[a] GM, geometric mean of the MIC.
[b] GM range, 2 to 16 μg/ml. MFCs at which 50 and 90% of the isolates are tested, 8 and 16 μg/ml, respectively.

The mechanism of action for PNT is unknown, and the compound displays multiple effects in extracts of *P. carinii* (19). In vitro, PNT is active against a number of different protozoa and fungi (10, 11, 13, 19). In vivo, patients who received aerosolized PNT had less oral colonization with *Candida* species (15). Patients who receive 4 mg/kg of body weight daily by slow intravenous infusion can achieve a concentration of 0.5 to 3.2 μg/ml in blood (5). However, much higher levels are found in tissues, with concentrations up to 56 μg/g in lung, 300 μg/g in liver, and 123 μg/g in the kidney (5). When achievable drug levels are related to MICs, *A. nidulans*, *A. niger*, *A. ustus*, and *A. terreus* have MICs below the plasma drug concentration and tissue drug levels. These species are often clinically refractory to standard treatment with amphotericin B (7, 18), and therefore PNT might be useful as a treatment for infections due to these *Aspergillus* species.

More studies with different media and animal models and clinical studies are necessary to elucidate the potential of these drugs for the treatment of *Aspergillus* infections.

APPENDIX

The Eurofung Network consists of the following participants: Emmanuel Roilides, coordinator, and Nicos Maglaveras, Aristotle University, Thessaloniki, Greece; Tore Abrahamsen and Peter Gaustad, Rikshospitalet National Hospital, Oslo, Norway; David W. Denning, University of Manchester, Manchester, United Kingdom; Paul E. Verweij and Jacques F. G. M. Meis, University of Nijmegen, Nijmegen, The Netherlands; Juan L. Rodriguez-Tudela, Instituto de Salud Carlos III, Madrid, Spain; George Petrikkos, Athens University, Athens, Greece.

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