

Antifungal Triazoles and Polymorphonuclear Leukocytes Synergize To Cause Increased Hyphal Damage to *Scedosporium prolificans* and *Scedosporium apiospermum*

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***Scedosporium prolificans* and *Scedosporium apiospermum* (*Pseudallescheria boydii*) cause pulmonary and disseminated infections refractory to most currently used antifungal agents in immunocompromised patients. We therefore investigated the potential antifungal activities of the triazoles itraconazole (ITC), voriconazole (VRC), and posaconazole (PSC) in combination with human polymorphonuclear leukocytes (PMNs) against the hyphae of these fungal pathogens. A colorimetric assay with (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide) sodium salt was used for the measurement of hyphal damage as an indicator of antifungal activity. We found that the newer triazoles VRC and PSC displayed synergistic effects with PMNs against *S. prolificans* hyphae after 24 h ($P < 0.05$), whereas the effect of ITC in combination with PMNs was additive ($P < 0.01$). All three triazoles displayed additive antifungal activities in combination with PMNs against *S. apiospermum* hyphae ($P < 0.05$). The synergistic or additive effects that these triazoles exhibited, combined with the antifungal activities of human PMNs, may have important therapeutic implications for the management of infections due to *S. prolificans* and *S. apiospermum*.**

Scedosporium prolificans and *Scedosporium apiospermum* (*Pseudallescheria boydii*) are emerging filamentous fungi that cause fatal pulmonary or disseminated infections in immunocompromised patients and localized infections following penetrating trauma, near drowning, or intravenous drug abuse (2, 3, 13, 14, 17, 18, 20, 21, 23, 26, 29, 30). Clinical isolates of *S. prolificans* possess in vitro resistance to flucytosine and amphotericin B, as well as itraconazole (ITC) and the newer triazoles voriconazole (VRC) and posaconazole (PSC) (2, 3, 7, 17, 21, 26, 37). Similarly, *S. apiospermum* isolates are frequently resistant to amphotericin B and flucytosine and have variable susceptibilities to ITC, VRC, and PSC (7, 10, 13, 18, 20, 29, 35).

The innate host defenses against filamentous fungi consist of the actions of macrophages, polymorphonuclear leukocytes (PMNs), and monocytes. PMNs are the most numerous circulating phagocytes. Most studies of filamentous fungi have investigated the interactions between host phagocytes and *Aspergillus fumigatus* and to a lesser degree *Rhizopus oryzae* (8, 27, 36). Mechanisms of host defenses against other filamentous fungi, such as *Scedosporium* spp., are largely unknown.

At therapeutically relevant concentrations, ITC has no effect on either superoxide or hydrogen peroxide production by PMNs stimulated with phorbol myristate acetate or on phagocytosis of *Candida albicans* (1, 19, 32). However, it has been found to increase the levels of phagocytosis and intracellular killing of *Candida glabrata* by PMNs (38). Synergy of ITC with

macrophages in the killing of *Blastomyces dermatitidis* has also been demonstrated (5).

Little is known, however, about the effects of ITC or the newer triazoles (VRC and PSC) on phagocytic cell response, particularly against resistant filamentous fungi. We therefore investigated the effects of ITC, VRC, and PSC on the antifungal functions of human PMNs against the hyphae of *S. prolificans* and *S. apiospermum*, two medically important multidrug-resistant fungi, to explore the potential synergistic, additive, or antagonistic effects of these antifungal agents in combination with these phagocytes.

MATERIALS AND METHODS

PMN preparation. Whole blood was obtained from healthy adult volunteers. PMNs were purified by dextran sedimentation followed by Ficoll centrifugation (12). The cells were resuspended in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} and were counted on a hemocytometer.

Fungi. The strain of *S. prolificans* used in these studies, strain CM 906, is type strain CBS 465.74 and was kindly donated by Juan Luis Rodriguez-Tudela (Instituto de Salud Carlos III, Madrid, Spain). The strain of *S. apiospermum* used in these studies, strain SA1216, was from a leg skin biopsy specimen (Roilides et al., Trends Invasive Fungal Infect. 4, abstr. P104, 1997). Both isolates caused fatal infections in experimental animals.

Fungi were seeded from frozen stocks on potato dextrose agar (Merck, Darmstadt, Germany) plates and grown for 7 days. The conidia were then harvested by scraping the surfaces of the plates, suspending the scrapings in HBSS (Gibco BRL, Life Technologies Ltd.), and filtering the mixture through sterile gauze. After centrifugation at $1,300 \times g$ for 10 min, the pellet was resuspended in HBSS and the cells were counted on a hemocytometer. The conidial suspension was maintained at 4°C for no longer than 3 weeks, as described previously (25).

Drugs. The antifungal drugs ITC (Janssen Cilag, Beerse, Belgium), VRC (Pfizer Inc., Groton, Conn.), and PSC (Schering Plough Research Institute, Kenilworth, N.J.) were used for these studies. The optimal concentrations of the three triazoles achievable in serum (see Tables 1 and 2) were selected from preliminary dose-response experiments. The concentrations of triazoles selected

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TABLE 1. Hyphal damage induced by triazoles and/or PMNs after coinubation for 3 h (short incubation)

Triazole (concn [$\mu\text{g/ml}$])	% Hyphal damage ^a		
	Triazole	PMNs	PMNs + triazole
<i>S. prolificans</i>			
ITC (2)	22.8 \pm 7.5	28.7 \pm 2.8	27.6 \pm 3.8
VRC (5)	25.7 \pm 8.8	28.7 \pm 2.8	32.4 \pm 3.9
PSC (5)	35.8 \pm 4.4	38.0 \pm 3.2	47.0 \pm 3.6
<i>S. apiospermum</i>			
ITC (1)	57.3 \pm 6.4	40.0 \pm 4.3 ^b	68.5 \pm 6.1
VRC (2.5)	70.5 \pm 6.8	40.0 \pm 4.3 ^b	76.5 \pm 3.6
PSC (0.5)	45.9 \pm 10.8	38.9 \pm 5.9 ^c	60.9 \pm 9.1

^a The values are means \pm SEMs for 6 to 12 experiments. The result for each triazole alone or PMNs alone was compared to the result for the combination of PMNs and a triazole by repeated-measures ANOVA with Dunnett's correction.

^b $P < 0.01$.

^c $P < 0.05$.

were chosen to permit only low to moderate activities of the triazoles in most cases to allow assessment of a potential additive or synergistic effect with PMNs.

Hyphal damage. Damage of hyphae was assessed by a colorimetric assay with the dye (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide) sodium salt (XTT; Sigma, St. Louis, Mo.) plus coenzyme Q (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma). Two hundred microliters of a suspension containing 7.5×10^4 conidia (for the experiments with 3 h of incubation) or 3.75×10^4 conidia (for the experiments with longer incubation periods) of either of the two fungi per ml of yeast nitrogen base supplemented with 2% glucose (YNB) was plated in each well of a 96-well flat-bottom cell culture cluster (Corning Inc., New York, N.Y.). The plate was incubated for 18 h at 32°C to allow germination of the conidia. The YNB was then replaced by RPMI 1640 supplemented with 10% pooled human serum; when necessary, PMNs were added at an effector-to-target cell (E:T) ratio of 5:1 (for the experiments with 3 h of incubation) or 10:1 (for the experiments with longer incubation periods) and the antifungal agents were added at the specified concentrations. Control wells containing hyphae only were treated under the same conditions. After incubation at 37°C with 5% CO₂ for the specified times, the plates were washed three times with H₂O and shaking for 5 min at room temperature in order to lyse the PMNs, as described previously (33). Preliminary experiments in which 6×10^4 PMNs were added to 1.5×10^4 hyphae 30 min before the washing step showed that this thorough washing is sufficient to minimize the conversion of XTT by the remaining PMNs to undetectable levels. Then, 150 μl of phosphate-buffered saline (Biochrom KG, Berlin, Germany) containing 0.25 mg of XTT per ml plus 40 μg of coenzyme Q per ml was added. After incubation at 37°C with 5% CO₂ for 1 h, 100 μl was transferred to a spectrophotometer and the optical density was read at a wavelength of 450 nm by using a reference filter with a wavelength of 690 nm. Antifungal activity was calculated by the following formula: percent hyphal damage = $(1 - X/C) \times 100$, where X is the optical density of the test well and C is the optical density of the control well with hyphae only.

Statistical analysis. Each experiment was performed with the PMNs of one donor and by use of duplicate or quadruplicate wells for each condition. The average value for these replicate wells was taken as the value for that particular donor and experiment. The averages for the replicate wells of each experiment were then used in the data analysis to calculate the mean \pm standard error of mean (SEM) for all experiments conducted under the same conditions. Differences between mean values were statistically evaluated by repeated-measures analysis of variance (ANOVA) followed by Dunnett's correction for multiple comparisons. A two-sided P value of <0.05 indicated statistical significance.

Synergism was calculated as follows: for each experiment, the sum of the hyphal damage produced by the PMNs alone and the drug alone was calculated and compared with the effect of treatment with a combination of PMNs and drug. Synergism was defined as an antifungal effect of the combination which was greater than the effect of PMNs alone plus the effect of the drug alone. An additive effect was defined as an antifungal effect of the combination which was greater than the effect produced by either PMNs or drug alone but which did not reach synergism.

RESULTS

The effects of the three triazoles and the PMNs were first studied by incubating hyphae with or without triazoles and with or without PMNs at an E:T ratio of 5:1 for a relatively short time of 3 h (Table 1). The damage induced against *S. prolificans* hyphae by the combination of ITC, VRC, or PSC with PMNs was similar to that induced by PMNs or triazoles alone. The combination of triazoles plus PMNs induced significantly greater damage than PMNs alone against *S. apiospermum* hyphae. However, since there was no difference between the effects of triazoles plus PMNs versus the effects of triazoles alone, this interaction was not additive.

The possibility that the antifungal activity observed in the experiments described above was time or concentration dependent was further assessed. Time course experiments were performed by incubating 7.5×10^3 germinated conidia with or without PMNs at an E:T ratio of 10:1 and triazoles at two different concentrations achievable in vivo. These experiments showed that the effects of triazoles alone were lower at 24 h than at 3 or 6 h. In addition, the PMN-induced hyphal damage decreased over time (data not shown). However, the time course experiments suggested that longer incubation times may enhance the effects of triazoles and PMNs used in combination. Therefore, these effects were further examined after 24 h of incubation.

When *S. prolificans* hyphae were incubated with or without PMNs in the presence or absence of triazoles for 24 h (Table 2), allazole-PMN combinations exhibited additive effects. Moreover, the effects of both concentrations of PSC in combination with PMNs against *S. prolificans* hyphae attained significant synergism. The treatment of *S. apiospermum* hyphae with the combinations of PMNs and ITC (0.005 and 0.05 $\mu\text{g/ml}$) as well as PMNs and 0.005 μg of VRC or PSC per ml had additive effects but did not produce synergistic effects. However, while the combination of PMNs with 0.05 μg of VRC or

TABLE 2. Hyphal damage induced by triazoles and/or PMNs after coinubation for 24 h (long incubation)

Triazole (concn [$\mu\text{g/ml}$])	% Hyphal damage ^a		
	Triazole	PMNs	PMNs + triazole
<i>S. prolificans</i>			
ITC (0.1)	5.9 \pm 2.7 ^b	12.6 \pm 2.5 ^b	23.1 \pm 3.5
ITC (1)	6.0 \pm 2.4 ^b	12.6 \pm 2.5 ^b	24.1 \pm 3.7
VRC (0.1)	-0.7 \pm 3.3 ^c	12.6 \pm 2.5 ^c	20.3 \pm 4.3
VRC (1)	10.2 \pm 2.4 ^c	12.6 \pm 2.5 ^c	23.7 \pm 4.0
PSC (0.1)	6.0 \pm 2.2 ^b	12.6 \pm 2.5 ^b	24.3 \pm 3.6
PSC (1)	11.9 \pm 2.2 ^b	12.6 \pm 2.5 ^b	30.8 \pm 3.9
<i>S. apiospermum</i>			
ITC (0.005)	10.3 \pm 1.8 ^b	14.5 \pm 2.9 ^c	32.7 \pm 2.2
ITC (0.05)	35.9 \pm 2.4 ^b	14.5 \pm 2.9 ^c	49.3 \pm 3.0
VRC (0.005)	2.8 \pm 3.2 ^b	16.1 \pm 3.0 ^c	22.1 \pm 3.2
VRC (0.05)	40.5 \pm 4.7	16.1 \pm 3.0 ^c	38.1 \pm 3.8
PSC (0.005)	8.8 \pm 2.4 ^b	16.1 \pm 3.0 ^c	29.8 \pm 3.4
PSC (0.05)	52.3 \pm 4.4	16.1 \pm 3.0 ^c	58.6 \pm 5.7

^a The values are mean \pm SEMs for eight or nine experiments. The result for each triazole alone or PMNs alone was compared to the result for the combination of PMNs and a triazole by repeated-measures ANOVA with Dunnett's correction.

^b $P < 0.01$.

^c $P < 0.05$.

PSC per ml damaged the hyphae of *S. apiospermum* significantly more than PMNs alone did, the damage induced by such combinations was similar to that induced by each triazole alone.

DISCUSSION

In this study, the triazoles that are already in clinical use or in advanced clinical development were studied for their effects on the hyphae of *S. prolificans* and *S. apiospermum* with PMN-induced damage. When used in combination with PMNs, all three antifungal triazoles caused significant additive increases in the damage of the hyphae of both fungi. Furthermore, under some of the conditions studied there was synergism between the investigational triazoles and PMNs against *S. prolificans* hyphae.

Additive or synergistic effects of the antifungal triazoles in combination with PMNs were observed with both high and low concentrations of the triazoles. The fact that the combination has synergistic activity when the low concentrations of drug are used may be particularly important in immunocompromised patients when a triazole reaches its trough level in plasma because such synergy may prevent organism regrowth.

The mechanism of antifungal action of triazoles is via the inhibition of lanosterol C₁₄- α -demethylase and ergosterol biosynthesis (11, 16, 22, 31). This inhibition leads to an accumulation of methylated precursors in the fungal membrane. This accumulation of methylated sterols in the membrane of *C. albicans* has been shown to induce an increased sensitivity of the fungus to oxygen-dependent microbicidal systems of the host (11, 28). If antifungal triazoles have the same effect on the cell membranes of *Scedosporium* spp., the increased sensitivity to the PMN microbicidal system could account for the synergistic effects found at 24 h. This may also explain why the triazoles had a combined effect with PMNs after a relatively long period of incubation (24 h) since the accumulation of triazole-induced methylated sterols is a slow process. This delay of the combined effect of triazoles and PMNs contrasts with the short time (3 h) needed for amphotericin B formulations to exhibit additive effects with PMNs against these organisms (C. Gil-Lamaignere, E. Roilides, A. Maloukou, I. Georgopoulou, G. Petrikos, and T. J. Walsh, 38th Annu. Meet. Infect. Dis. Soc. Am., abstr. 112, 2000). Amphotericin B exerts its antifungal activity by binding to preexisting ergosterol and by forming ionic channels in the fungal membrane (4) and thus has a faster mode of action.

The additive and synergistic activities between antifungal triazoles and PMNs against *Scedosporium* spp. are similar to the properties of VRC and PMNs against *A. fumigatus* at 24 h (34). However, to our knowledge this is the first time that such additive and synergistic effects have been reported for ITC, VRC, and PSC against life-threatening drug-resistant filamentous fungi such as *Scedosporium* spp. Moreover, this is the first in vitro description of interactions between PSC and human phagocytic effector cells.

During the course of these studies, a decrease in the level of hyphal damage caused by PMNs was observed over time. This finding could be explained by the functional depletion of oxidative metabolism of PMNs, the induction of apoptosis, and a loss of viability over time. Since PMNs were strongly attached

to the hyphae and any attempt to separate them would severely damage them, we could not assess these hypotheses. However, notably, even after 24 h of incubation, PMNs exhibited some antifungal activity (Table 2). Administration of recombinant cytokines such as granulocyte colony-stimulating factor may delay the development of PMN-induced apoptosis (24). This mechanism may also contribute to the enhanced effects of granulocyte colony-stimulating factor plus triazoles observed in neutropenic mice (15).

Cytokine-elicited granulocyte transfusions are a potentially valuable mode of adjunctive therapy for the treatment of infections caused by drug-resistant fungi (6, 9). The findings from the present study would support the concomitant administration of antifungal triazoles and PMN transfusions to persistently neutropenic patients with invasive scedosporiosis.

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