

Antifungal Activities of Posaconazole and Granulocyte-Macrophage Colony-Stimulating Factor Ex Vivo and in Mice with Disseminated Infection Due to *Scedosporium prolificans*

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Invasive infection due to *Scedosporium prolificans* is characterized by drug resistance and a high rate of mortality. The effects of posaconazole (POS), an investigational antifungal triazole, murine granulocyte-macrophage colony-stimulating factor (GM-CSF), and their combination against *S. prolificans* were evaluated ex vivo and in a newly developed murine model of disseminated infection due to this organism. When POS was combined with polymorphonuclear leukocytes from untreated or GM-CSF-treated mice ($P < 0.01$) ex vivo, it had increased activity in terms of the percentage of hyphal damage. Immunocompetent BALB/c mice were infected with 4×10^4 conidia of *S. prolificans* via the lateral tail vein. At 24 h postinfection the mice were treated with GM-CSF (5 μ g/kg of body weight/day subcutaneously), POS (50 mg/kg/day by gavage), both agents, or saline only. Half of the brain, lung, liver, and kidney from each animal were cultured; and the other half of each organ was processed for histopathology. The mean survival times were 7.0 ± 0.3 days for the controls, 7.4 ± 0.4 days for POS-treated mice, 8.0 ± 0.3 days for GM-CSF-treated mice ($P = 0.08$ compared with the results for the controls), and 7.3 ± 0.3 days for POS-GM-CSF-treated mice. Fungal burdens (determined as the numbers of CFU per gram of tissue) were found in descending orders of magnitude in the kidneys, brains, livers, and lungs. The burdens were significantly reduced in the brains of GM-CSF-treated mice ($P < 0.05$) and the livers of POS-treated mice ($P < 0.05$). The numbers of lesions in the organs closely corresponded to the fungal burdens. GM-CSF tended to prolong survival ($P = 0.08$ compared with the results for the controls). While the combination of POS and GM-CSF showed enhanced activity ex vivo, it did not increase the activities of the two agents against this highly refractory filamentous fungus in mice.

Scedosporium prolificans is an emerging opportunistic filamentous fungus associated with localized or disseminated infections, particularly in patients with hematologic malignancies or organ transplants (19, 23, 24, 41, 43, 44, 47–49). Systemic infections due to *S. prolificans* are almost always fatal, whereas bone, soft tissue, and joint *S. prolificans* infections occur most frequently in children and young adults (10, 19, 23). Antifungal susceptibility studies of conventional and new antifungal drugs and their activities against clinical *S. prolificans* isolates have demonstrated that the organism has multidrug resistance, indicating an inherently therapy-refractory fungus (6, 26).

Posaconazole (POS), an investigational antifungal triazole (25), inhibits the cytochrome P-450-dependent demethylases of fungi, thus interfering with cell membrane biosynthesis (13, 27, 45). It possesses potent antifungal activity against a broad spectrum of fungal pathogens (5, 9, 11, 12, 21, 22, 31–34, 42). A recent report (16) has demonstrated that POS is active in a murine

model of infection due to *Scedosporium apiospermum*, the second medically important species of the genus *Scedosporium*.

In the last decade, the combined effects of immune modulators and antifungals have been explored in animal models to determine whether treatment with these agents can be used as an alternative approach to the treatment of disseminated fungal infections (37). In murine models of systemic infections due to *Candida* or *Aspergillus* spp., granulocyte colony-stimulating factor (G-CSF) administered in combination with fluconazole or POS was found to improve survival (17, 18; F. Menzel, C. Jackson, A. Patera, J. Halpern, A. Cacciapuoti, R. Hare, and D. Loebenberg, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-858, 2002). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an agent that enhances the antifungal capacities of polymorphonuclear leukocytes (PMNs), monocytes, and macrophages and that has broader immunoenhancing effects than G-CSF (38). Preclinical studies and a few clinical studies have yielded encouraging results when GM-CSF was used in combination with conventional antifungal agents (3, 7, 28, 37, 39, 46). In this study, a murine model of therapy-refractory invasive infection due to *S. prolificans* has been developed, and the combined activities of these agents were examined ex vivo and in vivo.

MATERIALS AND METHODS

Organism. *S. prolificans* isolate CM906 (CBS-467.74) was originally recovered from a patient with osteomyelitis and was kindly donated by Juan Luis Rodri-

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guez-Tudela. The isolate was resistant to both polyenes and triazoles, including POS (MICs $\geq 16 \mu\text{g/ml}$), as determined by the National Committee for Clinical Laboratory Standards M38-P microdilution method (29). This organism was used in all experiments and was maintained as a frozen stock at -20°C . For both the *ex vivo* and the *in vivo* experiments, CM906 was grown by culturing it on potato dextrose agar (Merck, Darmstadt, Germany) at 37°C for 7 days. The inoculum was prepared by flooding the plate with sterile phosphate-buffered saline (PBS; Biochrom KG, Berlin, Germany), scraping the surface to detach the fungal cells, and filtering the suspension through sterile gauze to remove the hyphae and agar particles. The conidia were washed, counted with a hemacytometer, and suspended in PBS at 4×10^5 per ml. The number of conidia was checked by plating serial dilutions of the conidial suspension and recording the CFU counts.

Drugs. POS was provided by the Schering-Plough Research Institute (Kenilworth, N.J.) in powder form. The drug was suspended in 0.4% methylcellulose–0.5% Tween 80–0.9% NaCl and prepared as described previously (21). Recombinant murine GM-CSF was purchased from Sigma Chemical Company (St. Louis, Mo.) as a lyophilized preparation and was dissolved in PBS containing 1% bovine serum albumin at $1 \mu\text{g/ml}$.

Animals. Inbred healthy female and male BALB/c mice (age, 6 to 8 weeks; weight, 20 to 25 g) were used for the studies. Three female or male mice were housed in each cage, which contained wood bedding, and had free access to water and food. All rules and regulations of the Aristotle University Committee for Animal Welfare were observed in the animal facility and for the care procedures.

Ex vivo studies. Sixty-four animals divided into two groups were studied. The first group received 0.1-ml injections of $5 \mu\text{g}$ of GM-CSF/kg of body weight/day subcutaneously (s.c.); and the second group, the control group, received 0.1-ml injections of saline s.c. for four consecutive days. The GM-CSF dosages used were selected on the basis of previously reported data (8, 15) on the activities of murine PMNs pretreated with GM-CSF against *Scedosporium* sp. hyphae. On the fifth day, the animals received an intraperitoneal injection of 1 ml of 10% peptone, and 3 h later PMNs were recovered by peritoneal lavage with 3 to 4 ml of PBS. The cells were resuspended in the same buffer solution and counted on a hemacytometer. Staining with May-Grunwald Giemsa stain showed that $>95\%$ of the cells recovered in this manner were PMNs. PMNs from GM-CSF-treated mice as well as PMNs from saline-treated mice were subsequently tested for their abilities to damage hyphae of *S. prolificans* alone or in combination with $1 \mu\text{g}$ of POS per ml.

XTT assay. PMN-induced hyphal damage was assessed by using the dye 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)2H-tetrazolium-5-carboxanilide sodium salt (XTT; Sigma) and coenzyme Q_0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) (14). Briefly, $200 \mu\text{l}$ of a suspension containing 7.5×10^4 conidia/ml in yeast nitrogen broth (YNB) was seeded in a 96-well flat-bottom microplate, and the conidia were allowed to grow at 32°C for 18 h. The growth medium was replaced with $200 \mu\text{l}$ of fresh YNB containing $1 \mu\text{g}$ of POS per ml, and the hyphae were subsequently incubated at 32°C for 4 h. The medium was then replaced by Hanks balanced salt solution (Gibco), and PMNs were added at a 5:1 effector cell-to-target cell (E:T) ratio. After incubation at 37°C in 5% CO_2 for 2 h, the PMNs were lysed by three washes in $200 \mu\text{l}$ of H_2O and vigorous shaking for 5 min per wash, before the addition of $150 \mu\text{l}$ of PBS containing 0.25 mg of XTT per ml and $40 \mu\text{g}$ of coenzyme Q_0 per ml. After incubation at 37°C in 5% CO_2 for 1 h, $100 \mu\text{l}$ was transferred to a new plate and the absorbance was read at a wavelength of 450 nm with reference to a wavelength of 690 nm. Antihyphal activity was calculated as percent hyphal damage, which was equal to $(1 - X/C) \times 100$, where X is the absorbance of the test wells and C is the absorbance of the control wells, which contained only hyphae. Each set of conditions was tested in quadruplicate, and the results were averaged.

In vivo studies. A total of 168 mice were submitted to the *in vivo* studies. In each experiment, at 24 h after infection, groups of six mice (three males and three females) either were not treated (controls) or were treated with single daily doses of GM-CSF, POS, or the combination. On day 10 after the initiation of treatment, the surviving mice were killed by cervical dislocation. The inoculum and the drugs were administered in 0.1-ml volumes by use of sterile 1-ml syringes with 27-gauge needles. The mice were infected via the lateral tail vein with different concentrations of conidial suspensions ranging from 2×10^4 to 500×10^4 conidia per 0.1 ml. An inoculum of 4×10^4 was chosen for subsequent studies because it resulted in 100% mortality of the control mice by day 10.

The GM-CSF dosage was chosen on the basis of the results of our *ex vivo* studies and previously published data (8). Furthermore, doses of GM-CSF of 5, 12.5, and $25 \mu\text{g/kg/day}$ were administered to the mice s.c. While there was a trend for the fungal burdens to be lower when $12.5 \mu\text{g/kg/day}$ was administered, these were not significantly different from the fungal burdens obtained by administra-

tion of 5 and $25 \mu\text{g/kg/day}$. Therefore, $5 \mu\text{g/kg/day}$ was used in the subsequent experiments. In preliminary experiments 50, 100, or 250 mg of POS/kg/day was administered to the mice by gavage. Since there was no dose-dependent response in the clearance of *S. prolificans* from the organs of the mice and previous pharmacokinetic studies of POS (30) showed that it has good oral bioavailability over a concentration range of 40 to 80 mg/kg , the concentration of 50 mg/kg/day was chosen for use in the survival and tissue burden studies.

Organ fungal cultures. Mice were dissected under aseptic conditions; and the livers, kidneys, lungs, and brains were removed. Half of each organ was weighed and homogenized in 2 ml of saline solution. After dilution of each homogenized tissue 1:10 and 1:100, samples of 0.1 ml were plated on potato dextrose agar plates containing $10 \mu\text{g}$ of chloramphenicol per ml and $25 \mu\text{g}$ of gentamicin per ml. The plates were incubated at 37°C for 3 to 4 days, the numbers of CFU were counted, and the values obtained were converted to log CFU per gram per organ for statistical evaluation.

Histopathology. The other halves of the livers, kidneys, lungs, and brains were fixed in 10% neutral formalin solution, embedded in paraffin, and stained with hematoxylin-eosin and Gomori's methenamine-silver nitrate stains. Tissue sections through the entire hemiorgan were prepared and microscopically inspected by a blinded investigator (S.L., an animal pathologist) for the presence of inflammatory foci and the extent of organ damage caused by *S. prolificans*. Scores of 0 to 3 were assigned as follows: 0, no focal lesions in the total section; 1, one to two focal lesions per section; 2, three to five focal lesions per section; and 3, more than five focal lesions per section.

Statistical evaluation. Statistical assessments were performed with InStat (version 3.0) software (Graphpad Inc., San Diego, Calif.). The *ex vivo* data were evaluated by analysis of variance (ANOVA) with the Tukey test for multiple comparisons. The Kruskal-Wallis test with Dunn's posttest was used for the fungal burdens and histopathological studies. Prior to statistical analysis, all values of fungal burdens were transformed to their corresponding natural logarithms and expressed as the means \pm standard errors of the mean. The log rank test was used to analyze the survival data. A two-sided P value of ≤ 0.05 was taken to denote statistical significance.

RESULTS

Ex vivo studies. Eight experiments with eight mice in each experiment and two treatments were conducted. PMNs isolated from saline-treated (control) or GM-CSF-treated mice were used to evaluate their antifungal activities alone or in combination with POS against *S. prolificans* hyphae. Incubation of the hyphae with the combination of POS and PMNs from saline-treated mice induced damage significantly greater than that induced by each component alone ($P < 0.01$). Furthermore, the incubation of hyphae with the combination of POS plus PMNs from GM-CSF-treated mice showed enhanced activity against *S. prolificans* hyphae ($P < 0.01$). Specifically, POS alone induced $8.2\% \pm 2.0\%$ hyphal damage, PMNs from saline-treated mice alone induced $18.0\% \pm 1.0\%$ hyphal damage, PMNs from GM-CSF-treated mice induced $20.6\% \pm 1.3\%$ hyphal damage, the combination of POS plus PMNs from saline-treated mice induced $27.4\% \pm 1.3\%$ hyphal damage, and the combination of POS plus PMNs from GM-CSF-treated mice induced $30.3\% \pm 2.6\%$ hyphal damage (Fig. 1). There was no significant difference between the percent hyphal damage induced by PMNs isolated from mice treated with four daily s.c. doses of $5 \mu\text{g}$ of GM-CSF per kg and that induced by PMNs from control mice treated with saline (Fig. 1).

In vivo studies. (i) Organ fungal burdens. Seven *in vivo* experiments were conducted. A murine model of disseminated infection due to *S. prolificans* was developed with an inoculum of 4×10^4 conidia/mouse. The infection was characterized by changes in fur consistency and weight loss and the onset of lethargy and neurological symptoms, such as leaning to one side, jumping, and the complete loss of balance. The most

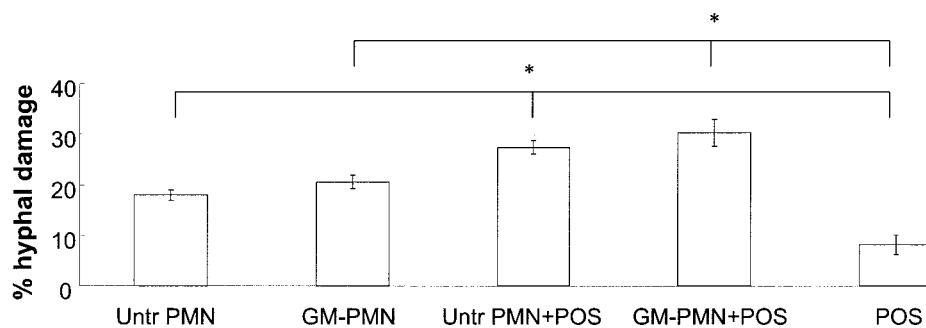


FIG. 1. Percent hyphal damage induced by PMNs from saline-treated mice (Untr PMN) or from mice treated with GM-CSF (GM-PMN) alone, POS alone, or the combination at an effector cell-to-target cell ratio of 5:1. Bars indicate means ± standard errors of the mean of eight experiments. The asterisks indicate statistical differences ($P < 0.01$) found by ANOVA with the Tukey posttest for multiple comparisons between the combination of PMNs from saline-treated mice and mice treated with POS and PMNs from either saline-treated mice or mice treated with POS alone and between the combination of PMNs from mice treated with GM-PMN and POS and PMNs from mice treated with either GM-PMN or POS alone.

infected organ was the kidney, with 3.77 ± 0.19 log CFU/g of organ, followed by the brain, with 3.41 ± 0.10 log CFU/g, and the liver, with 1.99 ± 0.10 log CFU/g. Even though no lung infections were apparent with the inoculum used in this study, conidial suspensions of 7×10^4 to 8×10^4 did cause lung infections in saline-treated mice. However, the lungs had the lowest fungal burden among all organs tested (data not shown).

Mice treated with 50 mg of POS/kg/day demonstrated significantly reduced CFU counts in the livers ($P < 0.05$). GM-CSF treatment produced no significant reductions in the liver and kidney fungal burdens but resulted in decreased brain fungal burdens compared to those in the controls ($P < 0.05$) (Table 1).

(ii) **Histopathology.** Microscopic examination of tissue sections showed various numbers of pyogranulomatous inflammatory foci, depending on the treatment received and the organ examined. The lesions were characterized by focal necrosis of the organ tissue and infiltration by PMNs, monocytes (or neuroglial cells in the brain), and a small number of lymphocytes. *S. prolificans* hyphae were apparent within the inflammatory foci.

The mean lesion scores for control and treated mice are shown in Table 2. While fewer focal lesions were observed in the livers than in the brains and the kidneys, there were no significant differences in the focal lesion scores assigned to any

organ by histopathology between the different treatment groups and the control mice.

(iii) **Survival.** The mean survival times for the four groups of mice are shown in Table 3. The inoculum size used in this study caused 100% mortality of control mice by day 10. Compared to the 0% rate of survival among the 36 saline-treated animals, the rates of survival were 22% among the 36 POS-treated mice, 30% among the 33 GM-CSF-treated mice ($P = 0.08$ compared to the results for the controls), and 15% among the 34 animals treated with the combination of POS and GM-CSF. While GM-CSF-treated mice tended to survive longer, treatment with POS alone or in combination with GM-CSF did not significantly prolong the survival of the mice.

DISCUSSION

This study is the first to examine the activities of POS in combination with GM-CSF, a cytokine with relatively broad activity in enhancing the antifungal capacities of phagocytes (38), against *S. prolificans*, a therapy-refractory filamentous fungus. While the two agents combined showed enhanced activity against *S. prolificans* hyphae ex vivo, they did not show parallel enhanced efficacy in a newly established immunocompetent murine model of disseminated infection due to this organism.

TABLE 1. Fungal burdens in the brains, livers, and kidneys of mice treated with saline (control), POS, GM-CSF, or the combination of POS and GM-CSF

Organ	Fungal burden (log CFU/g) in mice treated with ^a :			
	Saline	POS	GM-CSF	POS-GM-CSF
Brain	3.41 ± 0.10	3.25 ± 0.11	2.97 ± 0.18^b	3.23 ± 0.14
Liver	1.99 ± 0.10	1.70 ± 0.09^b	2.05 ± 0.14	1.78 ± 0.07
Kidneys	3.77 ± 0.19	3.08 ± 0.25	4.08 ± 0.19	3.53 ± 0.19

^a All values are expressed as the means ± standard errors of the means for each organ. The Kruskal-Wallis nonparametric ANOVA with Dunn's posttest was used to evaluate the differences from saline-treated (control) mice.

^b $P < 0.05$.

TABLE 2. Histopathological scores assigned to the focal lesions observed in the brains, livers, and kidneys of mice treated with saline (control), POS, GM-CSF, or the combination of POS and GM-CSF

Organ	Focal lesion score for mice treated with ^a :			
	Saline	POS	GM-CSF	POS-GM-CSF
Brain	1.50 ± 0.22	1.48 ± 0.22	0.84 ± 0.16	1.59 ± 0.28
Liver	0.11 ± 0.06	0.21 ± 0.08	0.30 ± 0.09	0.08 ± 0.05
Kidneys	1.43 ± 0.20	1.56 ± 0.21	1.32 ± 0.23	1.15 ± 0.22

^a All values are expressed as the means ± standard errors of means of the focal lesion scores for each organ. Lesion scores were calculated as described in Materials and Methods. Data were analyzed by the Kruskal-Wallis ANOVA with Dunn's posttest for multiple comparisons. No significant differences were seen among the treatment groups.

TABLE 3. Survival of *S. prolificans*-infected mice treated with saline (control), POS, GM-CSF, or the combination of POS and GM-CSF

Treatment	No. of dead mice/no. of infected mice	Mean survival time (days) ^a	P
Saline (control)	36/36	7.00 ± 0.35	
POS	25/32	7.34 ± 0.35	NS ^b
GM-CSF	23/33	8.00 ± 0.32	0.08
POS-GM-CSF	29/34	7.32 ± 0.33	NS

^a All values are expressed as the means ± standard errors of the means. Differences in mean survival times between POS-, GM-CSF-, or POS-GM-CSF-treated and saline-treated (control) mice were evaluated by the log rank test.

^b NS, no significant difference from the results for saline-treated mice.

Clinical isolates of *S. prolificans* are almost universally resistant to amphotericin B, fluconazole, and ketoconazole and are often resistant to itraconazole and miconazole (2, 4, 20, 35, 40). The activity of POS has been shown to be equivalent or superior to those of amphotericin B, fluconazole, voriconazole, and itraconazole against both common and new emerging pathogens (5, 9, 31, 32, 34). The particular isolate used for this study was resistant to both polyenes and triazoles. Ex vivo, 1 µg of POS per ml was found to exert a low level of activity in damaging *S. prolificans* hyphae. However, it exerted significantly enhanced activity when it was combined with PMNs derived from either saline-treated or GM-CSF-treated mice. This result is in agreement with the findings of previous in vitro studies with human PMNs (14), and its mechanism needs further elucidation. The antihyphal effect of this concentration of POS, as determined by the XTT viability assay, cannot be compared to the MICs obtained by the NCCLS assay, since the methods are totally different.

We also sought to enhance the activity of POS in combination with GM-CSF in a murine model of disseminated scedosporiosis achieved after healthy mice were infected with the conidia of *S. prolificans*. We hypothesized that GM-CSF may enhance the destructive effect of PMN antifungal metabolites on the surface of the fungus in such a way that POS can more efficiently act on its ergosterol synthesis target(s). POS administered 24 h after infection at a concentration of 50 mg/kg/day appears to be selectively effective in reducing fungal burdens in the liver but not in the kidneys or the brain. In contrast to the ex vivo result of enhanced activity, no significant differences in the rates of survival among mice infected with *S. prolificans* were observed between those treated with the combination and those treated with either POS or GM-CSF alone. This discordance may be related to the animal model used as well as to pharmacodynamic and pharmacokinetic factors related to the two agents in mice. Even though the therapeutic regimen used in this study was chosen on the basis of pharmacokinetic studies of POS in mice (1, 16, 17, 21, 30, 31), the resilient nature of the isolate that we used resulted in deep infections that progressively damaged the tissues studied.

By comparison, GM-CSF significantly decreased the fungal burdens in the brains of mice and also tended to prolong the survival of the infected animals. However, it did not affect the fungal burdens in other organs. While brain damage appeared to determine the outcome in this model, the concentrations of either of the two agents in the brain that may have influenced

the superior effect of GM-CSF are unknown. The concentrations in organs or other subtle differences may be responsible for this difference.

On the other hand, histopathologic sections of the organs showed various numbers of pyogranulomatous inflammatory foci, depending on the treatment received and the organ examined. Although pneumonia is a frequent finding of disseminated infection due to *S. prolificans* in immunocompromised patients (2, 36), lung involvement is achieved only after the administration of higher inocula in this model of immunocompetent mice. The kidneys and the brains were the most affected organs. Nevertheless, there were no significant differences in the number of focal lesions between treated and control mice.

In conclusion, this study demonstrated that POS and GM-CSF have a combined effect in damaging *S. prolificans* hyphae ex vivo. When POS and GM-CSF are administered to mice with invasive infection due to *S. prolificans*, they have selective beneficial effects on the burdens in certain organs but offer no additional benefit to survival. These studies need to be extended further by adopting modified therapeutic strategies for invasive infections due to *S. prolificans* in immunocompetent and immunocompromised mice.

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