Antifungal Efficacy of GM237354, a Sordarin Derivative, in Experimental Oral Candidiasis in Immunosuppressed Rats

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GM237354 is a novel sordarin derivative with a broad spectrum of potent activity against a wide range of fungi. The members of this new class of antifungal agents act as potent inhibitors of fungal protein synthesis. In this study, the therapeutic effects of GM237354 were investigated in a novel experimental oral Candida albicans infection model in immunosuppressed rats. The animals were immunosuppressed with dexamethasone in their drinking water and infected on three alternate days. GM237354 was given three times per day for seven consecutive days at 1.25, 2.5, 5, or 10 mg/kg of body weight per dose. In addition, to provide a preliminary idea of the correlation between regimen administration and therapeutic efficacy, GM237354 was administered to two additional groups of rats at 5 mg/kg once or twice a day for 7 days. The drug efficacy was assessed microbiologically, histologically, and by a morphometric study of lesions. Evident agreement was observed among results obtained by the different methods in all of the animals studied. Microbiologically, the efficacy of GM237354 was determined by measuring the number of C. albicans organisms in the oral cavities of rats in the middle (day 4) and at the end (day 7) of the treatment. GM237354 administered at 5, 7.5, 10, 15, or 30 mg/kg/day for 7 days significantly reduced the number of CFU in the oral cavities of treated rats compared with the number of CFU in the oral cavities of the untreated controls. A significant reduction was also observed when GM237354 was administered at 7.5, 15, or 30 mg/kg/day for 4 days. Furthermore, C. albicans was not detected in oral swabs from any infected rats after 1 week of treatment when GM237354 was administered at 15 or 30 mg/kg/day or after 4 days of treatment at 30 mg/kg/day. Histologically, untreated control animals showed extensive colonization of the epithelium of the dorsal tongue by numerous hyphae. Animals treated with GM237354 at 7.5 mg/kg/day showed small areas with superficial hyphal penetration into the epithelium that produced intraepithelial microabscesses. However, animals treated with GM237354 at 15 mg/kg/day showed multiple regenerative areas of the covering epithelium, and only focalized zones of the tongue surface were occupied by hyphae. No hyphal colonization of the epithelium was seen in rats treated with GM237354 at 30 mg/kg/day and which showed extensive areas of epithelial regeneration of the tongue. The histopathology findings were confirmed by morphometry studies, and the percentage of epithelium occupied by C. albicans hyphae decreased from 17.5% in the control group to 4.8 and 0.1% in animals treated with GM237354 at 7.5 and 15 mg/kg/day, respectively. These results demonstrated that the sordarin derivative GM237354 was effective against experimental oral candidiasis in immunosuppressed rats, and further studies are needed to determine the potential of GM237354 for use in the treatment of this infection in humans.

Results of epidemiological surveys indicate that Candida organisms are present as commensals in the oral cavities of approximately 40% of healthy subjects (4) and that Candida albicans specifically is carried as a commensal organism in the mouths of approximately one-third of the population (20). As a consequence of this, the opportunistic fungus C. albicans is a major cause of oral and esophageal infections in immunocompromised patients (8, 9) and affects up to 90% of patients with human immunodeficiency virus infection or AIDS (17). The expression of C. albicans virulence in the oral cavity is strongly correlated with impairment of the immune system (1, 20). Other conditions predisposing individuals to oral C. albicans infection include hyposalivation (16, 19), diabetes mellitus, prolonged use of antibiotics or immunosuppressive drugs, and poor oral hygiene (1, 22).

In recent years, fluconazole has become one of the drugs of choice for treating this fungal infection (21) because of its excellent pharmacokinetic characteristics and low toxicity (10). Resistance of Candida spp. to azole agents has been considered an infrequent event, though recent studies have indicated the possibility of treatment failures associated with Candida resistance to fluconazole (6, 17). In addition, the development of resistance in patients with extensive prior azole use is frequent (24). Therefore, new and effective drugs are needed to treat this fungal infection.

The sordarins are a new class of antifungal compounds that act by inhibiting the protein synthesis elongation cycle (7). Sordarin derivatives have demonstrated a potent and relatively broad-spectrum antifungal activity in several in vitro (14) and in vivo (18) studies. The novel mode of action and potent antifungal activity of sordarins have led to the design of several new compounds for potential clinical development, including GM237354.

The need for an animal model of oral candidiasis arises from the fact that human beings are notoriously variable, and sev-
eral animal models have been developed to study the pathogenesis of *C. albicans* oral infections (1). We have developed an experimental model in rats with impaired immune function and a stable yeast population in the oral cavity. The efficacy of GM237354 against experimental oral *C. albicans* infection in immunosuppressed rats was demonstrated by microbiological and histopathological studies.


**MATERIALS AND METHODS**

**Antifungal agents.** The sordarin derivative GM237354 (Fig. 1) was synthesized at the Glaxo Wellcome Research Centre in Madrid, Spain, and was supplied as a sodium salt powder. Immediately before each experiment, the compound was dissolved in sterile deionized water, and dilutions were made to the desired concentrations.

**Organism.** Therapeutic efficacy studies were performed against *C. albicans* 4711E, a clinical isolate obtained from the Glaxo Wellcome Laboratories (Greenford, United Kingdom) culture collection. This strain was stored at the Glaxo Wellcome Research Centre in Madrid, Spain, and was supplied as a sodium salt powder. Immediately before each experiment, the compound was dissolved in sterile deionized water, and dilutions were made to the desired concentrations.

**Antifungal treatment.** Just before treatment, the animals were sampled to confirm the presence of *C. albicans* and to quantify the number of CFU in the oral cavity. Then, the animals were randomized and assigned to groups of five. Treatment was administered for seven consecutive days (from day 0 to day 6). GM237354 was administered every 8 h (TID) by subcutaneous injection (0.5 ml) at doses of 1.25, 2.5, 5, and 10 mg/kg of body weight. Two additional groups treated at 5 mg/kg once or twice a day were added to the experiment to study the impact of the regimen administration on the therapeutic efficacy. The control group (*n* = 13) received sterile saline by the subcutaneous route.

**Microbiology.** The drug efficacy was assessed microbiologically by measuring the number of *C. albicans* organisms in oral swabs obtained in the middle (day 4) and 24 h after the end (day 7) of treatment. Oral samples were collected by rolling a sterile cotton swab over the oral cavity and suspending it in 1.0 ml of sterile saline. The oral samples were cultured in duplicate onto Bengal Rose chloramphenicol agar (Microkit Iberica, S. A., Barcelona, Spain) by using an autoplate (Spiral Biotech, Aplicaciones Analiticas, Barcelona, Spain). The plates were incubated at 37°C for 24 to 48 h, the CFU were counted, and the totals per swab were calculated. Plates with less than two colonies were considered negative cultures (the detection threshold was 40 CFU/swab).

**Pathology.** Gross observations, histological findings, and morphometry studies were performed in untreated animals and animals treated with GM237354 at 1.25, 2.5, 5, and 10 mg/kg TID for 7 days.

(i) **Gross observations.** At the end of the experiment (24 h after the last administration of GM237354), the animals were sacrificed by an overdose of pentobarbital (Eutalinder, Normor, Spain). The tongues were removed by an incision at the base, and gross observations and photographs were made immediately, following gentle rinsing of the tongue.

(ii) **Histological findings.** Tongues were routinely processed for light microscopy. Briefly, the tongues were fixed in toto by immersion in neutral buffered formalin solution for 48 h, and serial transverse sections were made of the whole tongue. These sections were again fixed in formalin for 12 h, followed by embedding in paraffin. Finally, 5-μm-thick sections were obtained from the paraffin blocks and stained with hematoxylin and eosin (HE), as well as with periodic acid-Schiff (PAS) stain, for histological findings and fungal visualization.

(iii) **Morphometric study.** In order to quantify the extent of the oral candidiasis and the evolution of the infection with and without antifungal treatment, several histological sections of each tongue were selected. In these sections, the length of the surface of the epithelium occupied by hyphae (LV.h) was measured; this value, as a percentage, represents the proportion of epithelium occupied by hyphae with respect to the total epithelial surface on the dorsum of the tongue. In those areas occupied by hyphae, the following volume density (VD) parameters were also determined. (i) **VDh** expressed as a percentage, represents the VD of the epithelium with hyphae, considering all epithelial layers (from basal to superficial layers). This parameter represents the extension and range of hyphae in the superficial layers. (ii) **VDh** corresponds to the number and size of the hyphae. This parameter is expressed as a percentage with respect to the total epithelial surface of the tongue. These parameters were also determined. (i) **VDh** expressed as a percentage, represents the VD of the epithelium with hyphae, considering all epithelial layers (from basal to superficial layers). This parameter represents the extension and range of hyphae in the superficial layers. (ii) **VDh** corresponds to the number and size of the hyphae. This parameter is expressed as a percentage with respect to the total epithelial surface of the tongue.
Gross observations, histological findings, and morphometry studies were performed in untreated and treated animals administered GM237354 at 1.25, 2.5, 5, and 10 mg/kg TID for 7 days.

FIG. 3. Oral candidiasis in immunosuppressed rats; untreated control group. (a and b) Macroscopic (a) and panoramic (b) histological lingual candidiasis; the epithelium of the tongue shows irregular thickness and hyperkeratotic areas with plentiful hyphae (arrowheads). PAS; magnification, ×600. (c) Diffuse abundant Candida mycelial elements affecting the superficial layers of the epithelium. PAS; magnification, ×125. (d) Large focal group of hyphae penetrating into the deeper layers of the epithelium. PAS; magnification, ×125. (e) Magnification of panel d. Note the abundant hyphae that have destroyed the keratinocytes, associated with small leukocyte infiltrates. PAS; magnification, ×600.

FIG. 4. Oral candidiasis in immunosuppressed rats; animals treated with GM237354 at 1.25 mg/kg TID for 7 days. (a and b) Macroscopic (a) and panoramic (b) histological lingual candidiasis; the transverse sections show important focal thickening of the epithelium (arrowhead). HE; magnification, ×5. (c) Multifocal hyperplasia of the epithelium, associated with irregular accumulations of hyphae (arrowheads). PAS; magnification, ×600. (d) Intraepithelial microabscess containing numerous leukocytes, neutrophils, and partially degenerated hyphae. PAS; magnification, ×600. (e) Destruction of several epithelial layers, with extensive neutrophil infiltration in relation to the presence of hyphae in the epithelium. PAS; magnification, ×125.

FIG. 5. Oral candidiasis in immunosuppressed rats; animals treated with GM237354 at 10 mg/kg TID for 7 days. (a and b) Macroscopic (a) and panoramic (b) histological lingual candidiasis; note the different coloration of the dorsal surface of the tongue, due to the alternation of hyperplastic and normal epithelial areas. PAS; magnification, ×5. (c) Basal cell hyperplasia, with scant differentiation of squamous cells and horny layer dyskeratosis. There are no C. albicans hyphae in the epithelial surface layer. PAS; magnification, ×125. (d) Focal alteration of the epithelial maturation of the tongue, reflected by intense dyskeratosis alternation associated with normal epithelium. A small increase of lymphocytes in the lamina propria was observed. HE; magnification, ×125. (e) Atrophy of the epithelium associated with intense regenerative changes. HE; magnification, ×125.

TABLE 1. Microbiological study of therapeutic efficacy of GM237354 against oral candidiasis in rats

<table>
<thead>
<tr>
<th>Regimen (mg/kg)</th>
<th>Log CFU/swab (mean ± SD)</th>
<th>No. of infected animals/total (%)</th>
<th>Log CFU/swab (mean ± SD)</th>
<th>No. of infected animals/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.0 ± 0.7</td>
<td>13/13 (100)</td>
<td>3.9 ± 0.6</td>
<td>13/13 (100)</td>
</tr>
<tr>
<td>3.75 5 TID</td>
<td>3.6 ± 0.9</td>
<td>5/5 (100)</td>
<td>4.5 ± 0.8</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>5 TID</td>
<td>3.7 ± 0.8</td>
<td>5/5 (100)</td>
<td>2.4 ± 0.4*</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>7.5 5 TID</td>
<td>2.8 ± 0.3*</td>
<td>5/5 (100)</td>
<td>2.5 ± 0.3*</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>10 5 TID</td>
<td>3.3 ± 0.5*</td>
<td>5/5 (100)</td>
<td>2.6 ± 0.4*</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>15 5 TID</td>
<td>2.7 ± 0.3*</td>
<td>5/5 (100)</td>
<td>&lt;1.6**</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>30 10 TID</td>
<td>0/5 (0)</td>
<td>1/13 (77)</td>
<td>&lt;1.6**</td>
<td>0/5 (0)</td>
</tr>
</tbody>
</table>

* Rats were orally infected three times at 24-h intervals with 3 × 10^6 C. albicans 4711E cells/ml. GM237354 was administered subcutaneously once a day (OD), twice a day (BID), or TID for seven consecutive days (day 0 to day 6). The mean log CFU/swab just before treatment was 5.0 ± 0.7 (n = 43). +, P < 0.05; **, P < 0.01 (versus the control treatment). The limit of detection was 40 CFU/swab.
and superficial hyperkeratosis. Only focalized zones of the epithelium, characterized by basal keratinocyte hyperplasia and superficial hyperkeratosis, as well as deep infection into the lamina propria. Multifocal leukodiapedesis phenomenon was associated with an increased presence of inflammatory cells in the lamina propria. Multifocal leukodiapedesis into the epithelium was observed in association with these inflammatory infiltrates in the lamina propria. In multiple areas, the intraepithelial hyphae caused keratinocyte destruction and the formation of intraperiapithelial microabscesses. All animals treated with GM237354 at 3.75 or 7.5 mg/kg/day showed hyphal colonization even deeper, reaching the parabasal cells of the epithelium. In these cases, the phenomenon was associated with an increased presence of inflammatory cells in the lamina propria. Multifocal leukodiapedesis into the epithelium was observed in association with these inflammatory infiltrates in the lamina propria. In multiple areas, the intraepithelial hyphae caused keratinocyte destruction and the formation of intraperiapithelial microabscesses. All animals treated with GM237354 at 3.75 or 7.5 mg/kg/day showed hyphal colonization on the tongue surface, with focal hyphal penetration that produced intraepithelial microabscesses. Animals treated with GM237354 at 15 mg/kg/day showed hyphae on the tongue surface, with focal hyphal penetration that produced intraepithelial microabscesses. All animals treated with GM237354 at 3.75 or 7.5 mg/kg/day showed hyphae on the tongue surface, with focal hyphal penetration that produced intraepithelial microabscesses. Animals treated with GM237354 at 15 mg/kg/day showed hyphae in the epithelium of the dorsum of the tongue. Animals treated with GM237354 at a dose of 30 mg/kg/day showed no areas of hyphal colonization, and the morphometric study was consequently not performed in such cases. The VD$_h$ values in animals treated with GM237354 at 3.75 mg/kg/day were similar to those of the controls (30.9 versus 25.6% in the controls). The VD$_h$ values were in turn significantly reduced ($P < 0.05$) to 13.5 and 0.9% in the groups treated at 7.5 and 15 mg/kg/day, respectively. Statistically significant differences ($P < 0.05$) in VD$_h$ values were also observed between the group treated at 15 mg/kg/day and the control group.

### DISCUSSION

The growing population of immunocompromised patients receiving immunosuppressive or anticancer therapy has led to an increased incidence of opportunistic mycoses. Although oral candidiasis is not a life-threatening disease, the sustained immunosuppression in these patients facilitates the recurrence of infection. Children and adolescents with a compromised or suppressed immune status are particularly susceptible to the development of oral candidiasis (8). We have therefore developed a standardized experimental model of oral *C. albicans* infection in immunocompromised rats. Systemic corticosteroid dosing in drinking water 1 week before the challenge significantly decreased the white blood cell count. This decrease remained constant throughout the experiment (data not shown). The present immunosuppressed-animal model appears to more closely mimic the situation seen in clinical settings. In addition, the administration of systemic antimicrobials, particularly tetracycline, is widely used to facilitate the development of candidiasis in the rat oral cavity (15, 23). On the other hand, Allen and Beck (2) have described strain-related differences in *C. albicans* pathogenicity in the rat oral mucosa; consequently, we used our well-characterized strain of *C. albicans*, which has been widely used for therapeutic studies in rodents with systemic candidiasis, demonstrating its pathogenic properties (3, 18).

Sordarin derivatives are a novel class of antifungals with potent broad-spectrum antifungal activity in several in vitro studies (14), and earlier research indicates that sordarin derivatives possess promising activity in several animal models of infection (18). Clemons and Stevens have recently demonstrated that sordarin (GM193663, GM211676, and GM237354) are effective in the treatment of experimental systemic coccidioidomycosis in mice (5), and Graybill et al. demonstrated that sordarin was effective in a murine model of histoplasmosis (11).

This study has shown that the sordarin derivative GM237354 administered therapeutically to immunosuppressed rats with oral candidiasis effectively reduces organism-mediated oral cavity injury, as measured by colony counts, gross pathology, and histological examination. Microbiologically, GM237354 has shown a good dose-dependent therapeutic effect in the oral-candidiasis model. Thus, the therapeutic efficacy of GM237354 against oral candidiasis was observed when at least 5 mg of the compound per kg per day was administered to rats for 7 days, significantly decreasing the load of *C. albicans* in the tongue. Animals treated with GM237354 at 15 mg/kg/day showed *C. albicans* hyphae in the epithelium of the dorsum of the tongue. Animals treated with GM237354 at a dose of 30 mg/kg/day showed no areas of hyphal colonization, and the morphometric study was consequently not performed in such cases. The VD$_h$ values in animals treated with GM237354 at 3.75 mg/kg/day were similar to those of the controls (30.9 versus 25.6% in the controls). The VD$_h$ values were in turn significantly reduced ($P < 0.05$) to 13.5 and 0.9% in the groups treated at 7.5 and 15 mg/kg/day, respectively. Statistically significant differences ($P < 0.05$) in VD$_h$ values were also observed between the group treated at 15 mg/kg/day and the control group.

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mouths of infected animals compared with that in the controls. However, when the compound was administered every 24, 12, or 8 h, the eradication of microorganisms after 7 days of treatment was observed in 20, 40, or 100% of infected animals, respectively.

Evident agreement between cultures from the oral cavity and the clinical and histological evidence of infection was also observed. Animals treated with lower doses of GM237354 (3.75 mg/kg/day) showed persistent C. albicans culture positivity, as well as abundant mycelial penetration into the epithelium of the tongue. However, in animals treated with GM237354 at 7.5 mg/kg/day, the number CFU of C. albicans obtained from the mouths of infected animals decreased significantly. In these animals, the histological study demonstrated Candida to have disappeared from the surface of the tongue; however, some hyphae remained within the most superficial keratinocyte layers. The viability of these few hyphae within the epithelium is not known. Although the pathogenicity of the mycelia cannot be defined, this small population of hyphae apparently did not have the same morphological features as those in the infected and untreated animals. At a dose of 15 mg/kg/day, the presence of immunocompetent cells with exocytosis and the complete absence of C. albicans on the dorsal surface of the tongue suggest that the sordarin derivative was extremely effective at this dose. These results are completely consistent with the 100% microbiological eradication of C. albicans previously observed in animals treated at 15 mg/kg/day. As expected, the histological study of the group, treated at 30 mg/kg/day showed no hyphae in either the surface or epithelium of the tongue. In this group, the morphology of the lingual epithelium was normal, though in some areas regenerative transformation was frequently seen, reflected by a proliferation of basal cells and an altered maturation of the keratinocytes. These regenerative changes may be directly related to the lesion induced by Candida or to the action of the exudation of immunocompetent cells into the epithelium. It is well established that these changes are completely regressive. The sordarin derivatives eliminate candidal organisms, and the inflammatory response consequently decreases; as a result, the regenerative process is delayed, giving rise to normal proliferation. Therefore, GM237354 eradicated the fungal load from the mouths of infected animals and produced no important or irreversible lesions of the oral mucosa.

The morphometric study clearly demonstrated the therapeutic effect of sordarins, indicating an excellent correlation among the microbiological, histological, and morphometric findings. This kind of study can thus be a useful tool for the in vivo evaluation of new antifungal agents.

In conclusion, the results of the present study are encouraging, although further investigations and comparative toxicity profile studies are needed to confirm sordarin derivatives as very promising and effective antifungal agents in human candidal infections.

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REFERENCES