Curative and Preventive Anticryptosporidium Activities of Sinefungin in an Immunosuppressed Adult Rat Model

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An immunosuppressed rat model was used to investigate the anti-Cryptosporidium parvum activity of sinefungin. In infected animals, oral sinefungin therapy resulted in a dose-related suppression of oocyst shedding, which correlated with oocyst disappearance from ileal sections. When administered prior to or on the day of oocyst challenge, sinefungin successfully prevented infection. These data suggest that sinefungin could be considered as a candidate molecule in the treatment of human cryptosporidiosis, considered to be the most significant enteric opportunistic infection in AIDS.

Cryptosporidium parvum is a coccidial protozoan that primarily inhabits the brush border of enterocytes and causes diarrheal disease in various mammalian host species (8). In immunocompetent humans, C. parvum infection may produce a self-resolving diarrhea (6, 7, 23). In immunocompromised patients, it causes severe and prolonged diarrhea, and it is considered one of the most important enteric opportunistic infections in AIDS patients. So far, on the basis of therapeutic outcomes, limited or no effect of proposed anti-C. parvum therapies has been observed (8).

In vitro models provide oocysts for only a short period of time and drug screening is not easily performed (13). Thus, immunocompromised adult animal models with chronic, life-threatening infections which mimic the status of patients are needed to properly evaluate candidate therapies. We have developed an animal model with immunosuppressed adult rats, and several drugs have been tested as both curative and preventive agents (5). The present report shows that sinefungin exhibits a unique curative and preventive activity against C. parvum infection in this model.

The anticryptosporidial activity of sinefungin in immunosuppressed Sprague-Dawley rats was evaluated. As described earlier, animals used in these experiments weighed 200 to 250 g and their feces were free of C. parvum oocysts before the study (4). Immunosuppression was induced with a regimen of 25 mg of hydrocortisone acetate (Roussel, Paris, France) injected subcutaneously twice weekly, starting 5 weeks before and ending 3 weeks after C. parvum challenge. During the same period of time, the animals were fed a regular low-protein (7%) diet (bread exclusively). Rats were housed one per cage, and each cage was sterilized twice a week to avoid possible reinfestations. Feeding bottles were heat sterilized every day.

Challenge (inoculation by oral gavage on day 0) consisted of 105 C. parvum oocysts per animal. C. parvum infection was assessed as the shedding of C. parvum oocysts in the feces as described in a previous study (4). Briefly, at 24-h, feces were collected and suspended in a 10% (wt/vol) formalin solution and homogenized. Oocysts were counted by utilizing a phase-contrast microscopy examination of smears prepared by mixing fecal suspensions with a carbolfuschine solution (17). The number of oocysts per microscopic field (MF [×400 magnification]) was counted. For each sample, a total of 10 to 100 MFs were counted, depending on the density of parasites. For each animal, 24-h excretion was calculated as the arithmetic mean oocyst number per MF. Each group of animals was studied for several days, and the results are given as the arithmetic mean 24-h excretion (oocysts per MF per 24 h). Statistical comparisons between groups were performed with the Fisher and Yates χ2 test and the nonparametric Mann and Whitney test. In addition, the number of rats shedding oocysts at the end of the experiment in each group is given as the ratio of the number of shedder rats to the total number of animals studied in the group.

Histologic examination was performed on three rats in each experimental group. The distal segment of ileus was cut, fixed, and embedded in paraffin, and 4-μm sections were either stained with hematoxylin-eosin or revealed by using an immunofluorescent anticytosporidial antibody (18). To correct for the patchy ileal localization of the parasite, 10

<table>
<thead>
<tr>
<th>Dose regimen from day 0 to day 10 (mg/kg of body wt/day)</th>
<th>No. of rats</th>
<th>Mean ± SD excretion from day 14 to day 21 (oocysts/MF/24 h)</th>
<th>% Inhibitiona from day 14 to day 21</th>
<th>No. of shedder rats at day 21 (estimated %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control untreated group)</td>
<td>5</td>
<td>8.3 ± 5.2</td>
<td></td>
<td>5 (100)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.45 ± 0.79b</td>
<td>94.5</td>
<td>3 (60)</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0.03 ± 0.03b</td>
<td>99.6</td>
<td>2 (40)</td>
</tr>
</tbody>
</table>

a Inhibition = 1 - (mean in the treated group)/(mean in the control untreated group) × 100.
b Statistically different from the mean in the control untreated group (P = 0.002).

* Corresponding author.
TABLE 2. Dose dependence of the curative anti-C. parvum activity of sinefungin

<table>
<thead>
<tr>
<th>Dose regimen from day 7 to day 21 (mg/kg of body wt/day)</th>
<th>No. of rats</th>
<th>Mean ± SD excretion from day 14 to day 21 (oocysts/MF/24 h)*</th>
<th>% Inhibitiona from day 14 to day 21</th>
<th>No. of shedder rats at day 21 (estimated %)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>16</td>
<td>2.64 ± 2.2</td>
<td>75.5</td>
<td>16 (100)</td>
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<tr>
<td>0.05</td>
<td>17</td>
<td>1.15 ± 1.4</td>
<td>89.3</td>
<td>6 (86)</td>
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<tr>
<td>0.25</td>
<td>12</td>
<td>0.72 ± 0.51</td>
<td>93.3</td>
<td>17 (100)</td>
</tr>
<tr>
<td>0.5</td>
<td>15</td>
<td>0.15 ± 0.23</td>
<td>96.6</td>
<td>11 (92)</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>0.14 ± 0.18</td>
<td>98.7</td>
<td>14 (93)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.16 ± 0.24</td>
<td>98.5</td>
<td>4 (80)</td>
</tr>
<tr>
<td>7.5</td>
<td>5</td>
<td>0.09 ± 0.12</td>
<td>99.1</td>
<td>4 (80)</td>
</tr>
<tr>
<td>10</td>
<td>14</td>
<td>0.04 ± 0.08</td>
<td>99.6</td>
<td>8 (57)</td>
</tr>
</tbody>
</table>

* Mean excretion in the control untreated group was 10.8 ± 7.2 (n = 21). In treated groups, means are statistically different from the mean in the control untreated group (P < 0.01), except for groups treated with 0.01 and 0.05 mg/kg of body weight per day (P < 0.05).

b Inhibition = 1 - (mean in the treated group)/mean in the control untreated group) × 100.

c Number of shedder rats in the control untreated group, 21 (100%).

ileal sections were examined for each animal. Oocysts fixed to enterocytes of the intestinal villi were microscopically counted.

Sinefungin produced by Streptomyces incarnatus was obtained from Rhône-Poulenc Santé, Vitry-sur-Seine, France. The curative and preventive activities of sinefungin were established simultaneously by studying three groups of rats in each series of experiments.

In group 1, rats, immunosuppressed as above, were challenged with C. parvum oocysts (day 0) and did not receive sinefungin (control untreated group). In all experiments, mean excretion averaged 8.48 ± 3.6 oocysts per MF per 24 h.

In group 2, rats were immunosuppressed, challenged with C. parvum oocysts (day 0), and treated with sinefungin easily solubilized in water (10 ml per rat per 24 h). In all experiments, it was verified that the drug had taken effect within 6 h.

In group 3, rats were immunosuppressed but not challenged, and no drugs were administered. Controls were established so that no spontaneous infection occurred in this group of rats during the time of the experiments.

Because immunosuppressed rats are susceptible to opportunistic infections such as Pneumocystis carinii pneumonia or aspergillosis, only rats surviving 7 days or more after challenge were included in the study. On day 7, the overall mortality rate of immunosuppressed animals was not significantly different between sinefungin-treated (mean, 16% of the animals) and nontreated (mean, 12% of the animals) (P > 0.5). In C. parvum-challenged animals, chronic oocyst shedding was ascertained from day 7 (in separate experiments, all immunocompetent rats did not shed oocysts over 4 to 7 days after challenge).

In the first series of experiments, the anticryptosporidial curative activity of sinefungin was demonstrated. Sinefungin was given from days 7 to 21, and oocyst excretion was measured from day 14 to day 21. As shown in Table 1, there was a dramatic decrease in oocyst excretion under sinefungin therapy.

The activity of sinefungin was found to be dose related in the range of 0.01 to 10.0 mg/kg of body weight per 24 h (Table 2).

The preventive activity of sinefungin was ascertained in two series of experiments. As shown in Tables 3 and 4, preventive therapy with sinefungin was effective, and efficiency was dependent on the duration of treatment prior to C. parvum challenge.

Three to 10 days after cessation of curative therapy with 5 to 10 mg/kg/day, a reappearance of oocyst shedding was observed in 4 of 11 of animals which did not shed oocysts on day 21. With the preventive protocol, with sinefungin at a dose regimen of from 0.01 to 0.5 mg/kg/day, shedding observed on the seventh day after discontinuation of therapy ranged from 285 to 5% of the mean excretion in control untreated animals. At preventive-dose regimens of from 0.5 to 2 mg/kg/day, no oocyst shedding was observed up to 20 days after the end of therapy.

In pooled results from histologic studies of untreated as well as sinefungin-treated infected animals, it was observed that a shedding range of 2 to 30 oocysts per MF per 24 h in feces corresponded to a range of 0.5 to 15 oocysts per microvillus present in intestinal mucosa. A direct correlation

TABLE 3. Preventive anti-C. parvum activity of sinefungin

<table>
<thead>
<tr>
<th>Dose regimen from day 0 to day 10 (mg/kg of body wt/day)</th>
<th>No. of rats</th>
<th>Mean ± SD excretion from day 4 to day 10 (oocysts/MF/24 h)*</th>
<th>% Inhibitionb from day 4 to day 10</th>
<th>No. of shedder rats at day 21 (estimated %)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>10</td>
<td>1.02 ± 1.29</td>
<td>77.3</td>
<td>10 (100)</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0.04 ± 0.05</td>
<td>99.1</td>
<td>7 (70)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0.03 ± 0.06</td>
<td>99.3</td>
<td>5 (50)</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0.00c</td>
<td>99.8</td>
<td>4 (40)</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>0.00c</td>
<td>99.9</td>
<td>1 (30)</td>
</tr>
</tbody>
</table>

* Mean excretion in the control untreated group, 4.49 ± 6.51 (n = 10). In treated groups, means are statistically different from the mean in the control untreated group (P < 0.01), except for the group treated with 0.25 mg/kg of body weight per day (P = 0.1).

b Inhibition = 1 - (mean in the treated group)/mean in the control untreated group) × 100.

c Number of shedder rats in the control untreated group, 10 (100%).

d Mean and standard deviation lower than 0.01.
was found between both counting methods ($r = 0.86$ and $r = 0.83$ in sinfungin-treated and nontreated groups, respectively).

Present data confirm the validity of the hydrocortisone-treated, protein-deprived rat model as a tool to identify drugs with in vivo anticytosporidial activity (4, 5). Animals mimic features of cytosporidiosis reported in AIDS patients, including biliary localizations which may constitute a remote parasite reservoir (3a). However, similarly to dexamethasone-treated rats and unlike T-lymphocyte-depleted or -deficient mice, which may represent a closer AIDS cryptosporidiosis model, they do not develop diarrhea but exhibit a decreased volume of feces (4, 24, 28).

Sinfungin is a natural nucleoside antibiotic produced by Streptomyces griseolus and S. incarnatus (15, 16). It was found to be effective as an antiprotozoal drug against Plasmodium falciparum (21, 27), Leishmania spp. (1), American and African Trypanosoma spp. (9, 22), Toxoplasma gondii, and Entamoeba histolytica (10). The biochemical mechanism of the antiparasitic activity of sinfungin possibly involves transmethylation reactions or enzymes of polyamine biosynthesis (10). It was shown that sinfungin has an inhibitory activity in vitro against the transmethylase of T. gondii (11). Both natural and synthetic sinfungins exhibit an antileishmanial activity, and the results of several analogous studies with derivative drugs have provided additional information on the minimal active structure for antiprotozoal activity (2, 3, 26).

Compared with other drugs, the activity of sinfungin seems very significant. Indeed, the list of inconclusive attempts to treat C. parvum in immunocompromised patients is rapidly growing and includes the use of more than 90 different therapeutic and preventive modalities (8). The limited efficiency of immunotherapy observed in some patients and animal models remains to be confirmed (12, 29). With animal models, few drugs have been reported as exhibiting an anticytosporidial activity. It was observed that arprinocid and some sulfamides reduce the number of oocysts in feces or the number of parasites in intestinal tissue sections (19, 25). Azithromycin and other macrolides were recently shown to reduce the severity of the ileal infection (20, 24). We have confirmed in our model the activity of lasalocid previously reported in calves (5, 14). Although it is difficult to extrapolate between different models, the overall activity of sinfungin appears to exceed that of other drugs tested (5). Studies are in progress to determine optimal curative and preventive regimens of sinfungin in immunosuppressed rats.

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#### REFERENCES


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**TABLE 4. Preventive anticytosporidial activity of sinfungin**

<table>
<thead>
<tr>
<th>Dose regimen from day −35 to day 14 (mg/kg of body w/day)</th>
<th>No. of rats</th>
<th>Mean ± SD excretion from day 4 to day 10 (oocysts/MF/24 h)*</th>
<th>% Inhibition* from day 4 to day 10</th>
<th>No. of shedder rats at day 21 (estimated %)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>8</td>
<td>1.59 ± 2.03</td>
<td>79.0</td>
<td>8 (100)</td>
</tr>
<tr>
<td>0.05</td>
<td>8</td>
<td>1.27 ± 2.56</td>
<td>83.2</td>
<td>8 (100)</td>
</tr>
<tr>
<td>0.1</td>
<td>8</td>
<td>0.22 ± 0.33</td>
<td>97.0</td>
<td>6 (75)</td>
</tr>
<tr>
<td>0.25</td>
<td>8</td>
<td>0.04 ± 0.04</td>
<td>&gt;99.9</td>
<td>5 (63)</td>
</tr>
<tr>
<td>0.5</td>
<td>8</td>
<td>0.009a</td>
<td>&gt;99.9</td>
<td>1 (13)</td>
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<tr>
<td>1.0</td>
<td>8</td>
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<td>100.0</td>
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<tr>
<td>2.0</td>
<td>8</td>
<td>0.00</td>
<td>0.0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

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* Mean excretion in the control untreated group, 7.58 ± 5.29 (n = 8). In treated groups, means are statistically different from the mean in the control untreated group ($P < 0.01$).

* Inhibition = 1 − (mean in the treated group)/(mean in the control untreated group) × 100.

* Number of shedder rats in the control untreated group, 8 (100%).

* Mean and standard deviation lower than 0.01.