TNP-470 Is an Effective Antimicrosporidial Agent

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Therapy for microsporidia, which cause diarrhea and a wasting syndrome in persons with AIDS, has had limited success. Fumagillin, a naturally secreted water-insoluble antibiotic, has in vitro activity against microsporidia and has been used successfully in the treatment of superficial keratitis in patients with AIDS, but systemic therapy has been limited by toxicity of the currently available fumagillin salt. TNP-470, a semisynthetic analogue of fumagillin, was studied in vitro and in the athymic nude mouse model of microsporidiosis. RK13 cells were infected with microsporidia of the family Encephalitozoonidae and treated at day 3 with TNP-470. This agent was highly effective, with an ID_{50} (50% inhibitory dose compared with control) of 0.001 μg/mL. TNP-470 also demonstrated in vivo activity against Encephalitozoon cuniculi, with prolonged survival and the prevention of the development of ascites in infected athymic mice. These data suggest that the fumagillin derivative TNP-470 is a promising agent for the treatment of microsporidiosis.

Microsporidia, obligate, intracellular, spore-forming parasites, cause diarrhea and a wasting syndrome in persons with AIDS. Although microsporidia have been described in immunocompetent patients, most cases occur in AIDS patients with suppressed CD4 cell counts [1]. In this setting, microsporidia cause a wasting syndrome associated with diarrhea and malabsorption [2]. Of the microsporidia that infect humans, Enterocytozoon bieneusi causes most microsporidia-related disease in the United States; however, many other microsporidia genera have been described as the etiologic agent in human diseases.

Three Encephalitozoonidae members have been associated with disease in humans: Encephalitozoon cuniculi, Encephalitozoon hellem, and Encephalitozoon intestinalis (previously known as Septata intestinalis) [1]. It appears that these microsporidia can disseminate widely in their hosts, and involvement of most organs by these organisms has now been documented. In addition, microsporidia of the genera Nosema, Vittaforma, Pleistophora, and Trachipleistophora have also been associated with human disease [2]. Therapy with albendazole has eradicated Enc. intestinalis infection and its symptoms [3]. Patients with Ent. bieneusi who have received albendazole treatment have had a limited response, and biopsy has demon-
strated persistence of organisms in treated responders [4]. Additional antemicroridian agents are needed.

Fumagillin is a naturally secreted, water-insoluble antibiotic of *Aspergillus fumigatus*. Fumagillin bicyclohexammonium chloride (Fumadil B; Mid Continent Agrimarketing, Lenexa, KS), a water soluble form of fumagillin, was first recognized as an effective treatment for microsporidiosis of honey bees caused by *Noosema apis* [5]. Shadduck [6] demonstrated that the in vitro replication of *Enc. cuniculi* was inhibited by fumagillin within 48 h, and this effect was maintained as long as the antibiotic remained in the medium. With discontinuation of the drug, the parasite count returned to pretreatment levels [6]. The mechanism of action of fumagillin is not known. Possible explanations from cytochemical evidence are that fumagillin may decrease DNA and inhibit RNA synthesis or that it interferes with fatty acid metabolism and, thus, with spore membranes [5, 6].

*Enc. hellem* keratoconjunctivitis has been successfully treated with topical fumagillin (*fumagillin bicyclohexammonium*) [7]; however, systemic treatment in mammals has been limited due to the toxicity of fumagillin bicyclohexammonium. TNP-470, a potent semisynthetic analogue of fumagillin, has potent antiangiogenic activity and is relatively nontoxic in mammals [8]. Given the activity of fumagillin against microsporidia, we examined the antemicroridian effect of this drug in vitro and in a mouse model of disseminated microsporidiosis.

Materials and Methods

RK13 (rabbit kidney) cells (ATCC CCL37) were maintained in culture in MEM supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin, and 1% streptomycin as previously described [9]. These cells were infected with *Enc. cuniculi*, *Enc. hellem*, or *Enc. intestinalis* spores in microwell plates. TNP-470 (TAP Pharmaceutical, Deerfield, IL) was dissolved in DMSO (Sigma, St. Louis) at a concentration of 1 mg/mL.

**In vitro assay.** For the evaluation of antemicroridianal activity, RK13 cells (5 x 10^5/mL) were plated into each well of a 24-well tissue culture plate (Falcon Multiwell; Becton Dickinson, Lincoln Park, NJ) and allowed to incubate for 3 days until confluent. Tissue culture plates were then infected by replacing the medium with medium containing 10^5 Encephalitozoonidae spores/mL. Spores were counted with a hemocytometer. Medium was changed every 2 days after infection. On day 7, drug treatment was started. TNP-470 was used at 1, 0.1, 0.01, 0.001, and 0.0001 μg/mL. *Enc. intestinalis* was screened at 0.1 and 0.01 μg/mL. Eight duplicate wells were done for each infection condition. In addition, eight wells were maintained without drug, with the medium being replaced every 3 days. Medium containing drugs was replaced every 2 days, and drug efficacy was assessed 9 days after treatment (day 16 after infection) by counting foci of infected cells after Giemsa staining.

**In vivo assay.** Nude mice (BALB/c nu/nu, National Cancer Institute, Bethesda, MD) were infected with 5 x 10^4 to 5 x 10^5 *Enc. cuniculi* by intraperitoneal (ip) injection on day 1. *Enc. cuniculi* was chosen as the reference standard for Encephalitozoonidae because in vitro data demonstrated that the ID_{50} (50% inhibitory dose compared with control) for all members of the family was similar, and human data suggest that members of this family cause overlapping syndromes. In previous animal experiments, we determined that *Enc. cuniculi* was pathogenic for athymic nude mice, causing a reproducible ascites syndrome 3–5 weeks following ip inoculation. This finding was similar to that reported by others [10]. The protocol was designed to determine if TNP-470 eliminated this syndrome. In addition, mortality in the mice was monitored in the initial experiments. Three separate experiments were done, and 5 mice were used for each drug dosage evaluated within each experiment.

In experiment 1, TNP-470 was injected ip starting 3 days after infection. Dosages of 100 mg/kg daily, 100 mg/kg three times a week, or 50 mg/kg three times a week were given for 2 weeks. In this experiment, TNP-470 was utilized by dissolving it in ethyl alcohol and diluting the alcohol in water to allow a 0.3- to 0.5-mL ip injection to provide the desired dose. Each mouse received <0.3% ethyl alcohol/dose. Mortality was used as an end point in experiment 1.

For experiments 2 and 3, the intravenous (iv) formulation that was used consisted of TNP-470 (10 mg/mL), G2 β-cyclodextrin (72.6 mg/mL), and NaOH (33.3 μg/mL) when reconstituted in sterile water. The formulation was diluted in distilled water to provide the appropriate end concentrations of 10, 50, and 100 mg/kg in a volume of 0.3–0.5 mL. The experiments were designed to test suppression of microsporidia, not to cure infection. Uninfected control mice were used to monitor mortality from drug, and infected control mice were used to monitor the rate of ascites development and mortality due to microsporidia.

**Statistical analysis.** Data were analyzed by Student’s *t* test or nonparametric analysis (Mann-Whitney rank sum and Wilcoxon’s signed rank tests), using SigmaStat (version 2.0; Jandel Scientific, San Rafael, CA).

Results

**In vitro experiments.** By day 7 after infection (see table 1), foci of *Enc. cuniculi* and *Enc. hellem* were present in the RK13 cells before the addition of antemicroridianal agents. In the untreated *Enc. hellem* plates, 16.5% of the RK13 cells were infected at day 16 after infection. At an antemicroridianal concentration of 0.01 μg/mL, 0.3% of the RK13 cells were infected, and at 0.001 μg/mL, 6.2% of the RK13 cells were infected (ID_{50} of TNP-470 for *Enc. hellem*). In the untreated plates infected with *Enc. cuniculi*, 10.6% of the RK13 cells were infected, while at an antemicroridianal concentration of 0.01 μg/mL, 0.3% of the cells were infected. At a concentration of 0.001 μg/mL, 5.6% of the RK13 cells were infected (ID_{50} of TNP-470 for *Enc. cuniculi*).

Thus, TNP-470 was highly effective against both *Enc. hellem* and *Enc. cuniculi*, with an ID_{50} of 0.001 μg/mL. *Enc. intestinalis* was tested and completely inhibited by TNP-470 at 0.1 and 0.01 μg/mL. The ID_{50} for albendazole (SmithKline Beecham, London, UK) in the same assay system, using *Enc.
The ID<sub>50</sub> of TNP-470 for <i>Enc. cuniculi</i> and <i>Enc. hellem</i> was 0.001 µg/mL. In our assay, this drug was 10 times more active than its parent compound, fumagillin. Concentrations of TNP-470 that inhibited microsporidia were not toxic for RK13 cells. Recently, in vitro studies of TNP-470 against <i>Enc. intestinalis</i> and <i>Vittaforma corneae</i> demonstrated antimicrosporidial activity of this compound for these microsporidia as well [11].

In previous animal modeling experiments, we had determined that <i>Enc. cuniculi</i> was pathogenic for athymic nude mice, causing a reproducible ascites syndrome 3–5 weeks after ip inoculation. In the present study, we demonstrated that TNP-470 prolongs the survival of athymic nude mice infected with <i>Enc. cuniculi</i> and prevents the development of ascites. At a TNP-470 dosage of 10 mg/kg per day or 50 mg/kg three times a week, mice were protected from the development of ascites, with no clinical evidence of toxicity. Survival was prolonged by ~2 weeks. At a TNP-470 dosage of 100 mg/kg per day or 100 mg/kg three times a week, clinical toxicity (ruffled fur and death) was observed within 2 weeks.

Fumagillin, the parent compound of TNP-470, has clinical effectiveness, but it is limited by its toxicity when administered iv. Fumagillin bicyclohexammonium, an agricultural product used for the treatment of nosematosis in honey bees, has been successfully used in humans to treat <i>Enc. hellem</i> infection of the superficial cornea and conjunctiva (keratoconjunctivitis or punctate epithelial keratopathy) that is seen mainly in patients with AIDS [1, 2]. The clinical response to topical fumagillin for this condition is well described [7]. TNP-470 is a potent angiogenesis inhibitor that is very effective in inhibiting endothelial cell proliferation in both in vitro and in vivo models, although the action of this drug on endothelial cells has not been elucidated [12]. Currently, phase II human clinical trials of this drug as an angiogenesis inhibitor are underway. Given the activity of TNP-470 against <i>Enc. hellem</i>, its minimal in vitro toxicity to mammalian cells, and its antiangiogenic properties (minimizing angiogenesis as a consequence of infection), this drug may be very useful as a topical agent for the treatment of microsporidian keratoconjunctivitis.

Recently, fumagillin has been used to successfully treat microsporidia of the family Enterocytozoonidae: <i>Ent. bieneusi</i> in the super®cial cornea and conjunctiva (keratoconjunctivitis or punctate epithelial keratopathy) that is seen mainly in patients with AIDS [1, 2]. The clinical response to topical fumagillin for this condition is well described [7]. TNP-470 is a potent angiogenesis inhibitor that is very effective in inhibiting endothelial cell proliferation in both in vitro and in vivo models, although the action of this drug on endothelial cells has not been elucidated [12]. Currently, phase II human clinical trials of this drug as an angiogenesis inhibitor are underway. Given the activity of TNP-470 against <i>Enc. hellem</i>, its minimal in vitro toxicity to mammalian cells, and its antiangiogenic properties (minimizing angiogenesis as a consequence of infection), this drug may be very useful as a topical agent for the treatment of microsporidian keratoconjunctivitis.

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<table>
<thead>
<tr>
<th>Dosage</th>
<th>Mean length of survival, days (±SE)</th>
<th>Ascites*</th>
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<tbody>
<tr>
<td>0</td>
<td>37 ± 0.5</td>
<td>5/5</td>
</tr>
<tr>
<td>10 mg/kg/day</td>
<td>44 ± 1†</td>
<td>0/5†</td>
</tr>
<tr>
<td>50 mg/kg/day</td>
<td>&gt;55†</td>
<td>0/5†</td>
</tr>
<tr>
<td>50 mg/kg/3 times a week</td>
<td>ND</td>
<td>0/5†</td>
</tr>
</tbody>
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* No. of mice developing ascites/total no. of mice infected with <i>Enc. cuniculi</i>.
† P < .05 drug treatment vs. no treatment.

### Table 1. Activity of TNP-470 against microsporidia.

<table>
<thead>
<tr>
<th>TNP-470 (µg/mL)</th>
<th>&lt;i&gt;Encephalitozoon hellem&lt;/i&gt;</th>
<th>&lt;i&gt;Encephalitozoon cuniculi&lt;/i&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.5 ± 0.2</td>
<td>11.1 ± 0.9</td>
</tr>
<tr>
<td>1.0</td>
<td>0.3 ± 0.02*</td>
<td>0.1 ± 0.02*</td>
</tr>
<tr>
<td>0.1</td>
<td>0.8 ± 0.02*</td>
<td>0.3 ± 0.02*</td>
</tr>
<tr>
<td>0.01</td>
<td>0.3 ± 0.02*</td>
<td>0.3 ± 0.05*</td>
</tr>
<tr>
<td>0.001</td>
<td>6.2 ± 0.4*</td>
<td>5.6 ± 0.2*</td>
</tr>
<tr>
<td>0.0001</td>
<td>16.5 ± 0.4</td>
<td>10.6 ± 0.2</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean % cells infected ± SE. RK13 (rabbit kidney) cells (ATCC CCL37) were maintained in culture in MEM supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin, and 1% streptomycin and infected with 10<sup>5</sup> <i>Enc. cuniculi</i> or <i>Enc. hellem</i> spores in microwell plates (Falcon Multisol; Becton Dickinson, Lincoln Park, NJ). TNP-470 treatment was started on day 7, and drug efficacy was assessed 9 days after treatment (day 16 after infection) by counting infected cell foci. Eight duplicate wells were done for each infection condition.

* Significant by Student’s t test (<i>P</i> < .05), compared with no drug.

<i>hellem</i>, <i>Enc. cuniculi</i>, or <i>Enc. intestinalis</i>, was 0.0625 µg/mL. We did not examine the ability of the Encephalitozoonidae to regrow after a 9-day exposure to this drug followed by cultivation in the absence of drug. However, at $\geq 0.5$ µg/mL TNP-470, no organisms were evident at the light microscopic level, and no organisms could be found by electron microscopy.

**In vivo experiments.** TNP-470 also demonstrated significant in vivo activity against Encephalitozoonidae (table 2). In experiment 1 (using TNP-470 in ethanol), 100 mg/kg per day was toxic to mice, as evidenced by ruffled fur and premature death. A dosage of 50 mg three times a week was tolerated, and survival was increased in infected mice. The mean length of survival (LOS) (± SE) after infection was 37 ± 0.5 days for infected untreated mice and 44 ± 1 days for mice given TNP-470 (50 mg/kg) ip three times a week (<i>P</i> < .05).

In experiment 2, no toxicity was seen with TNP-470 at 50 mg/kg per day using the iv (i.e., clinical) formulation of TNP-470 (10 mg/mL TNP-470, 72.6 mg/mL G2 β-cyclodextrin, and 33.3 µg/mL NaOH when reconstituted in sterile water). Untreated mice had an LOS of 38 ± 0.5 days after infection. Mice treated with TNP-470 at 10 mg/kg per day had an LOS of 44 ± 1 days, and at 50 mg/kg per day, no mortality was seen by day 55, at which time the experiment was terminated (<i>P</i> < .05 for increased survival with treatment).

In experiment 3, the development of ascites was used as an end point. Of the 5 infected untreated control mice, 2 developed ascites by day 24, 3 by day 30, and all 5 by day 35. Ascitic fluid was positive for <i>Enc. cuniculi</i>. In mice treated with TNP-470 at 10 mg/kg per day, 50 mg/kg per day, or 50 mg/kg three times a week for 2 weeks, no ascites developed over a 42-day follow-up period (<i>P</i> < .05 compared with untreated mice).

### Discussion

In the present study, we demonstrated that TNP-470 is a highly active antimicrosporial agent both in vitro and in vivo.
humans [13] and Nucleospora (Enterocytozoon) salmonis in salmonid fish [14]. *N. salmonis* infections occur in salmonid fishes with a leukemic-like condition; some of the infected fishes may also be infected by a retrovirus [15]. Experimental microsporidia infection in salmon revealed that fumagillin was effective in eradicating *N. salmonis* from salmonid fish [16]. *N. salmonis*-infected fish were also cured by treatment with TNP-470 administered orally in feed (unpublished data). In a presentation at the 4th Conference on Retroviruses and Opportunistic Infections, it was reported that oral fumagillin eradicated *Ent. bieneusi* from the stool of 9 AIDS patients with diarrhea [12]; however, oral therapy was associated with thrombocytopenia. These data suggest that fumagillin and its derivatives are also promising agents for the treatment of Enterocytozoonidae infections. We believe a trial of TNP-470 for diarrhea due to *Ent. bieneusi* is indicated.

### References