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Effects of Albendazole, Fumagillin, and TNP-470 on Microsporidial Replication In Vitro

ELIZABETH S. DIDIER*

Department of Microbiology, Tulane Regional Primate Research Center, Covington, Louisiana 70433

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Presently, the two most commonly used drugs for treating microsporidiosis in persons with AIDS are albendazole and fumagillin. Albendazole is effective for treating disseminated infections due to *Encephalitozoon* spp. but is variably effective against *Enterocytozoon bieneusi* infections. Fumagillin is highly effective when used topically to treat ocular infections with *Encephalitozoon hellem* or *Encephalitozoon intestinalis* but is too toxic for systemic use. In this study, the fumagillin analog TNP-470 was assayed for antimicrosporidial activity in vitro. The MICs of TNP-470 at which 50% of isolates were killed (MIC_{50}s) were 0.35 ± 0.21 and 0.38 ± 0.11 ng/ml for *E. intestinalis* and *Vittaforma corneae*, respectively, and were similar to the MIC_{50}s of fumagillin for these organisms, which were 0.515 ± 0.002 and 0.81 ± 0.014 ng/ml, respectively. The MIC_{50} of albendazole for *E. intestinalis* was 8.0 ± 4.23 ng/ml, significantly less ($P < 0.01$) than its MIC_{50} for *V. corneae*, which was 55.0 ± 7.07 ng/ml. TNP-470 inhibited replication of *E. intestinalis* in RK-13 cells if it was given at the same time as infection or if treatment was initiated 7 days later. In addition, treatment of the infected cultures with TNP-470 at a dose of 10 ng/ml for 2 weeks, followed by discontinuation of the drug treatment, resulted in no significant increase in *E. intestinalis* shedding during the following 3 weeks in culture. Because TNP-470 acts against both *E. intestinalis* and *V. corneae*, and because TNP-470 was found by others to be less toxic in vivo, TNP-470 may be a promising new drug for the treatment of microsporidiosis.

Microsporidia are obligately intracellular protozoan parasites which infect arthropods and all classes of vertebrates (10). During the past 10 years, several species of microsporidia have been emerging as agents of opportunistic infections in persons with AIDS (9, 40, 54). Presently, no universally effective drug treatment exists for all the microsporidian species that infect humans. Albendazole has been found to be effective against the *Encephalitozoon* species but is less effective against *Enterocytozoon bieneusi*, the most commonly reported microsporidian in persons with AIDS (1, 6, 7, 13, 15, 18, 19, 24, 25, 31, 37, 48, 53–55). Fumagillin, an angiogenesis inhibitor, also inhibits replication of *Encephalitozoon cuniculi* in vitro (44) and has been used topically to treat ocular infections due to *Encephalitozoon hellem* or *Encephalitozoon intestinalis* (15, 17, 26, 37, 42, 47, 56). Fumagillin, however, is very toxic and has not been used to treat systemic infections with *E. bieneusi* or *Encephalitozoon* species. Analogs of fumagillin have recently been produced, and TNP-470 was found to be as active as fumagillin in inhibiting tumor growth and was less toxic in laboratory animals when used in vivo (29). The purpose of this study was to determine if TNP-470 also expresses antimicrosporidial activity in vitro.

MATERIALS AND METHODS

Organisms. Microsporidia were grown in RK-13 cells by procedures described previously (14). The human isolate of *Vittaforma corneae* (previously named *Nosema corneum*) was cultured from corneal tissue of an individual with stromal keratitis and iritis (12, 45, 47). *E. intestinalis* (previously named *Septata intestinalis*) was isolated from a bronchoalveolar lavage specimen as previously described (8, 16). For drug assays, microsporidia were collected from tissue culture supernatants, washed three times by centrifugation at 400 × *g* for 15 min with Tris-buffered saline (TBS) containing 0.3% Tween 20 (TBS-Tween), and centrifuged over 50% Percoll (Pharmacia, Piscataway, N.J.) at 400 × *g* for 30 min to remove host cell debris. The pellets containing the purified microsporidia were washed with TBS, counted on a hemacytometer, and adjusted to the appropriate concentrations in RPMI 1640 supplemented with 5% fetal bovine serum and 2 mM L-glutamine.

Drugs. Albendazole was purchased from Sigma Chemical Co. (St. Louis, Mo.). Fumagillin (bicycloheximide fumagillin) was kindly provided by René Desmarais of Sanofi Santé Animale (Victoriaville, Quebec, Canada). TNP-470 [*O*-(chloroacetyl-carbamoyl) fumagillol; AGM-1470] was kindly provided by Hajime Toguchi of Takeda Chemical Industries, Ltd. (Osaka, Japan). Stock solutions of compounds were dissolved in dimethyl sulfoxide at a final concentration of 10.0 mg/ml and diluted in tissue culture medium for use in the assays.

Assays for measuring antimicrosporidial activities. RK-13 cells were plated onto 24-well culture plates at a concentration of 5×10^5 cells/ml of RPMI 1640 (containing 2 mM L-glutamine and 5% fetal bovine serum). The plates were incubated overnight at 37°C to reach confluency. Microsporidia were added in 0.5-ml volumes at a concentration of 3.0×10^6 organisms/ml (i.e., a final organism/host cell ratio of 3:1). This ratio of spores to host cells results in approximately 50% of the host cells becoming infected within 3 h of incubation at 37°C. Three hours later, noninternalized or nonadherent parasites were washed off and fresh media, with or without drugs, were added as described for each experiment. Control cultures not given drugs received medium with amounts of dimethyl sulfoxide equivalent to those used in preparing drug stock solutions. Media were replaced every 3 to 4 days, and care was taken not to remove organisms from the bottoms of the wells. On day 10, 100 μl of 10% (wt/vol) sodium dodecyl sulfate was added to each of the wells to release organisms from host cells and the total numbers of organisms in the wells were counted with a hemacytometer. Each treatment was assayed in triplicate, and the percent inhibition of *E. intestinalis* replication was calculated at 100 − [(number of organisms counted in treatment cultures/mean number of organisms in nontreated cultures) × 100]. In some experiments, the cumulative numbers of parasites were determined by adding the number of organisms released into the culture supernatant at a given time point to the number of organisms from the previous time point.

Measurement of drug toxicity. To assess drug toxicity to host cells, host cell viability was measured by using an MTT [3(4,5-dimethyl-thiazol-2-yl-)2,5-diphenyl tetrazolium bromide] colorimetric assay as described by Mossman and Fong (39) and as applied for testing antimicrosporidial drug activity by Beauvais et al. (5). On day 10 of the drug assays, replicate wells of host cells treated with test drugs were incubated with 50 μl of MTT (5 mg/ml in TBS, pH 7.4) and the culture plates were incubated for an additional 4 h at 37°C. The supernatants were then removed, and 0.5 ml of 0.4 N HCl in isopropanol was added to each well to dissolve the formazan. The absorbance values were read on a spectrophotometer at a test wavelength of 570 nm and a reference wavelength of 630 nm. The percent of host cell viability was calculated as the optical density values

* Mailing address: Department of Microbiology, Tulane Regional Primate Research Center, 18703 Three Rivers Rd., Covington, LA 70433. Phone: (504) 892-2040. Fax: (504) 893-1352. E-mail: esdid@tpc.tulane.edu.

of treated cells/the mean optical density of nontreated cells × 100. Three replicates were used for each group of treatments.

Focus-forming assay. To assess the infectivity of microsporidia after drug treatment, a modified focus-forming assay was used (43). Briefly, organisms were recovered from culture wells after drug treatments, washed with TBS (for 10 min at 400 × *g*), and resuspended in tissue culture medium at a concentration of 10^4 organisms per ml. One-milliliter volumes of spores from treated and nontreated cultures were added to new 24-well culture dishes containing confluent monolayers of RK-13 cells. Medium changes (without drugs) were made on day 4, and the monolayers were fixed with methanol (for 10 min at room temperature) on day 8. The monolayers were stained with Calcofluor white 2R (0.3 ml of a 0.5% solution per well) for 5 min, washed with TBS two times, counterstained with 0.1% Evan's blue, washed several times with TBS, and viewed with a fluorescent inverted microscope at a final magnification of ×320 to count foci of microsporidial infections. At least 50 fields per well were viewed, and each assay was repeated three times.

Statistical analysis. Student's *t* test (two-sided) was used to compare results between any two treatment groups, and KWIKSTAT (TexaSoft, Cedar Hill, Tex.) was used to calculate the results.

RESULTS

Antimicrosporidial activities of albendazole, fumagillin, and TNP-470. *E. intestinalis* spores and drugs at various concentrations were added to confluent monolayers of RK-13 cells on day 0, and the total number of organisms and host cell viability were measured on day 10. Results in Fig. 1 demonstrated that albendazole displayed significant ($P < 0.001$) antimicrosporidial activities at doses of 10 ng/ml and above, and statistically significant ($P < 0.05$) host cell toxicity was observed in cultures of cells treated for 10 days at concentrations of 100 ng/ml and above. Significant antimicrosporidial activities were observed in cultures treated with fumagillin ($P < 0.001$) or TNP-470 ($P < 0.001$) at concentrations of 0.1 ng/ml and above. Significant toxicity was observed in cultures treated with fumagillin at concentrations of 100 ng/ml ($P < 0.01$) or 1.0 μg/ml ($P < 0.005$), whereas no statistically significant host cell toxicity was observed after treatment of host cells with TNP-470 for 10 days at any concentration tested.

To determine the MIC_{50} (MIC at which 50% of isolates are killed) of each drug against *E. intestinalis* and *V. corneae* (used as a genus outlier control), drugs were diluted twofold in the concentration range near the expected MIC_{50} values shown in Fig. 1. Results in Table 1 indicate that albendazole and fumagillin expressed significantly higher MIC_{50}s for *V. corneae* than for *E. intestinalis*. No significant difference was seen when the MIC_{50} of TNP-470 for *E. intestinalis* was compared to that for *V. corneae*.

To determine if the three drugs could inhibit microsporidial growth after infections were established, the drugs were added to cultures at a concentration of 10.0 ng/ml 7 days after RK-13 cells were infected with *E. intestinalis*. This dose was chosen as the highest concentration of albendazole that did not cause statistically significant host cell toxicity over 10 days of treatment, as shown in Fig. 1. Organisms released into the culture supernatants were recovered every 3 or 4 days and counted. Results in Fig. 2 represent the accumulated or total numbers of organisms recovered up to each time point. All three drugs, albendazole, fumagillin, and TNP-470, were able to inhibit replication of *E. intestinalis* so that by day 10 (or 3 days after addition of the drug) there were significantly fewer organisms released from the treated cells ($P < 0.005$ for each drug) than from the nontreated cells. The time required to inhibit *E. intestinalis* replication to 50% of that in the controls in this experiment was 2.8 days after addition of fumagillin and 3.65 days after addition of TNP-470 or albendazole (data not shown).

Cultures of RK-13 cells infected with *E. intestinalis* then were treated with each drug (10.0 ng/ml) for 1 week or 2 weeks, after which the cultures were continued without drugs. Negative- and positive-control cultures received no drug or received fresh drug, respectively, with each medium change throughout the course of the experiments. Parasites were harvested from the culture supernatants every 3 or 4 days, and the accumulated numbers of parasites over the course of the experiments were determined (Fig. 3). In cultures incubated in the absence of drugs, the accumulated numbers of spores were statistically significantly higher than those from cultures treated with al-

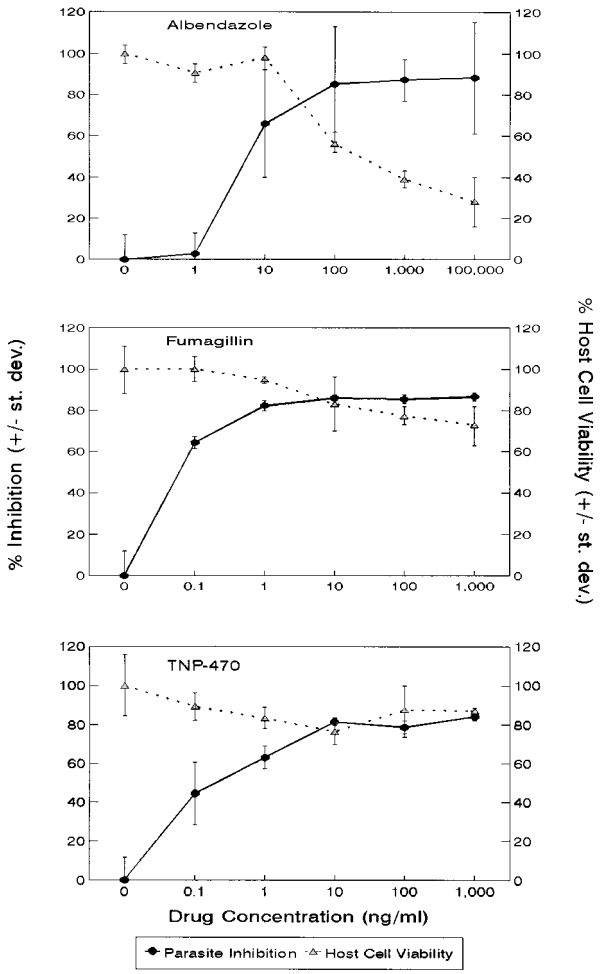

FIG. 1. Dose-response curves for albendazole, fumagillin, and TNP-470. Drugs were added to cultures of RK-13 cells 3 h after addition of *E. intestinalis* and at each medium change every 3 or 4 days. Parasites were counted on day 10, and the values for percent inhibition of parasite replication were determined. Viability levels of host cells incubated with drugs only were determined on day 10 by the MTT assay. Values are the means of three replicates.

TABLE 1. MIC_{50}s of antimicrosporidial drugs

Drug	MIC_{50} (ng/ml)[a]		P^b
	E. intestinalis	*V. corneae*	
Albendazole	8.0 ± 4.23	55.0 ± 7.07	<0.01
Fumagillin	0.515 ± 0.002	0.81 ± 0.014	<0.001
TNP-470	0.35 ± 0.212	0.375 ± 0.106	=0.722

[a] Values are the means of three determinations ± standard deviations.
[b] Comparisons were made between the MIC_{50}s for *E. intestinalis* and those for *V. corneae*.

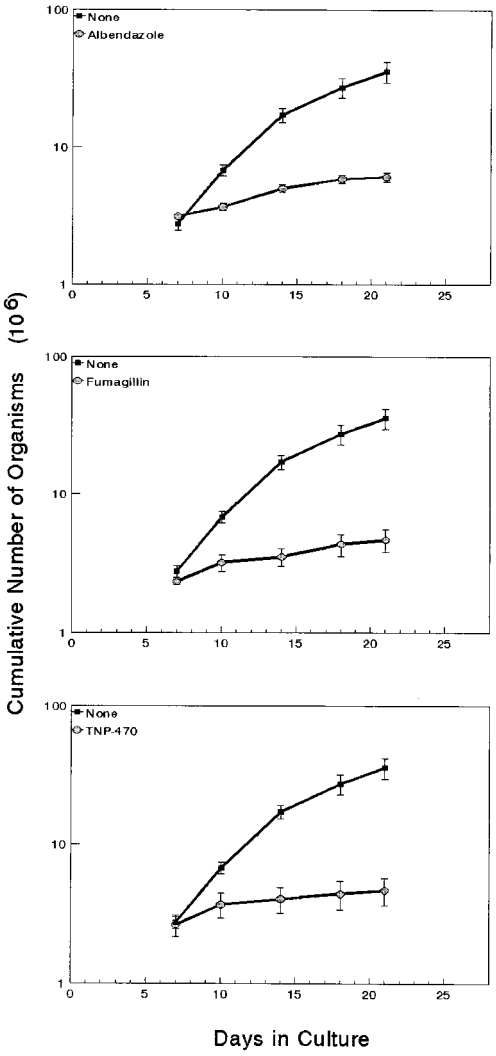

FIG. 2. Effects of drugs on established *E. intestinalis* infections in vitro. RK-13 cells were infected with *E. intestinalis*. Seven days later, and with each subsequent medium change, albendazole, fumagillin, or TNP-470 was added to the cultures at 10 ng/ml. Parasites released into the supernatants were counted after each medium change. Assays were run in triplicate, and values are the mean numbers of accumulated organisms at each time point.

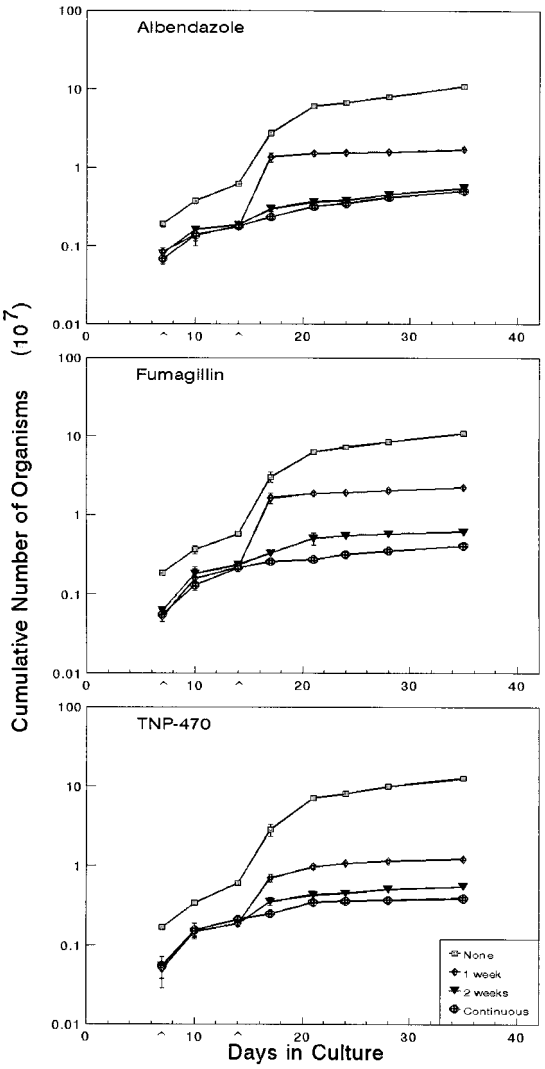

FIG. 3. *E. intestinalis* growth after removal of drugs. Albendazole, fumagillin, or TNP-470 was added to *E. intestinalis*-infected RK-13 cells at a concentration of 10 ng/ml at each medium change for 1 or 2 weeks, after which drug treatments were discontinued. Positive and negative controls received drugs or no drugs throughout the course of this experiment. Assays were run in triplicate, and values are the mean numbers of accumulated parasites at each time point.

bendazole ($P < 0.05$), fumagillin ($P < 0.05$), or TNP-470 ($P < 0.05$) at all time points tested (beginning on day 7).

In cultures treated with albendazole or fumagillin for 1 week only, a significantly higher number of parasites were released in the supernatants by day 17 ($P < 0.001$ for both drugs) than in cultures treated with drug continuously. In cultures treated with TNP-470 for 1 week only, a significantly higher number of spores, compared with continuously treated cultures, did not accumulate until day 21 ($P < 0.001$). The numbers of spores generated in cultures treated with albendazole, fumagillin, or TNP-470 for 2 weeks only were not significantly higher than the numbers of spores released from continuously treated cultures at any of the time points at which assays were performed during the subsequent 3 weeks.

A focus-forming assay was then performed to determine if those parasites that were generated after drug treatment were still infectious. Cultures of *E. intestinalis*-infected RK-13 cells were treated with 10 ng of albendazole, fumagillin, or TNP-470/ml from day 7 through day 14. Control cultures received no drug. On day 14, parasites were harvested, adjusted to 10^4 organisms/ml, and added to fresh RK-13 cells to assess infectivity in a focus-forming assay. Results in Fig. 4 indicate that, compared with nontreated culture, significantly fewer infectious parasites were harvested from cultures treated with albendazole ($P < 0.001$), fumagillin ($P < 0.001$), or TNP-470 ($P < 0.001$).

DISCUSSION

Albendazole used systemically, and fumagillin used topically, currently are the two most effective antimicrosporidial drugs used for persons with AIDS. Albendazole is a broad-spectrum antiprotozoal benzimidazole which binds to the colchicine binding site of β-tubulin, thereby inhibiting microtubule polymerization (36). Albendazole has shown efficacy

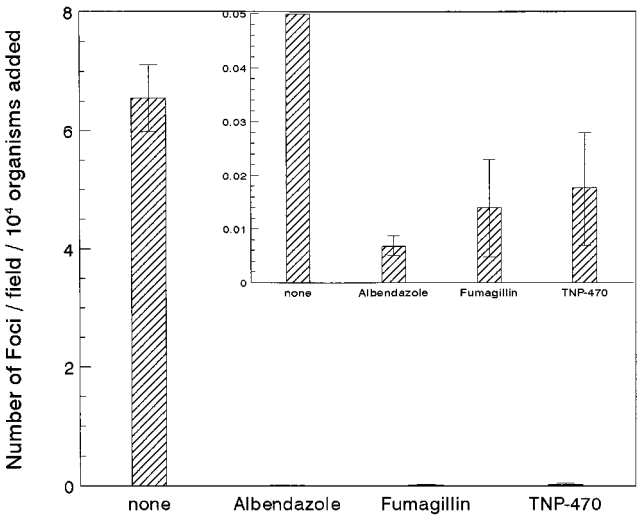

FIG. 4. Recovery of infectious microsporidia after treatment with drugs. Cultures of *E. intestinalis*-infected RK-13 cells were treated with albendazole, fumagillin, or TNP-470 at 10 ng/ml from day 7 through day 14. Control cultures infected with parasites received no drug. On day 14, parasites were recovered and 10^4 of these organisms were added to fresh cultures of confluent RK-13 cells. Calcofluor-stained foci of infected cells were counted on day 8 with an inverted fluorescent light microscope at a final magnification of ×320. Values are the means of three replicates. The inset (upper right corner) shows a closer comparison between the infectivities of parasites harvested from the three drug treatments.

against *Enterocytozoon bieneusi* to varying degrees but is considered clinically more effective against the *Encephalitozoon* spp. of microsporidia (1, 6, 7, 13, 15, 18, 19, 24, 25, 31, 37, 48, 49, 53–55). In studies presented here, the MIC_{50} of albendazole in vitro was significantly lower (approximately sevenfold) for *E. intestinalis* than for *V. corneae*, which was used as a genus outlier control.

Clinical results in persons infected with *E. bieneusi* and treated with albendazole have been variable, and organisms were rarely cleared from the stool (7, 18, 24, 53). Although relapses have occurred in patients with *E. intestinalis* or *E. hellem* after discontinuation of albendazole, patients responded well to a second course of treatment resulting in relief of symptoms. Furthermore, *Encephalitozoon* spp. spores are often cleared from stool, urine, and other tissue sites after treatment with albendazole (1, 6, 7, 13, 15, 19, 24, 25, 31, 37, 48, 53–55). In the context of the tissue culture system presented here, albendazole treatment for 1 week significantly reduced, but did not completely abrogate, the development of infectious organisms. After 2 weeks of treatment, however, no significant increase was observed in spore shedding after discontinuation of the drug and insufficient numbers of spores were released to allow for detection of infectious organisms by the focus-forming assay (unpublished data).

The inhibitory mechanism of albendazole was not examined in this study, but in ultrastructural studies. Canning and colleagues demonstrated that albendazole inhibited *E. cuniculi* replication in vitro and caused development of malformed giant meronts and sporonts containing bundles of 35-nm tubules (11). Furthermore, Blanshard and colleagues reported that in patients with *E. bieneusi* who responded to albendazole treatment, all stages of organisms were abnormal, particularly the proliferative stages, which contained ballooned plasmalemmas and irregularly shaped nuclei (6). These results suggest that albendazole acts by inhibiting karyokinesis and cytokinesis of the microsporidia by virtue of inhibiting microtubule polymerization.

One limitation of albendazole, however, is that it is not uniformly effective for treating *E. bieneusi* infections, and in studies presented here, albendazole was less effective against *V. corneae* than against *E. intestinalis*, based on the approximately sevenfold-higher MIC_{50}. One explanation may lie in the β-tubulin amino acid sequence. Edlind and colleagues have reported that benzimidazole-sensitive organisms express Glu198 and Phe200, and generally Val268, which are found in the β-tubulin sequences of *E. hellem*, *E. cuniculi*, and *E. intestinalis* (20–22, 32). The β-tubulin sequences for *E. bieneusi* and *V. corneae* have not been published but may provide insight as to the sensitivity of these microsporidia to the benzimidazoles.

Fumagillin, an antibiotic produced by *Aspergillus fumigatus*, is a second compound shown to be highly effective against microsporidia. Historically, fumagillin was used for treating insects with microsporidiosis (4, 27, 33) and for treating patients with amebiasis (34, 38). In vitro, fumagillin inhibits *E. cuniculi* replication (44), and clinically, fumagillin has been used topically to treat *E. hellem* and *E. intestinalis* infections of the eye (15, 17, 26, 37, 42, 46, 56). A limitation of fumagillin, however, is that it cannot be given systemically to patients with *E. bieneusi* or disseminated *Encephalitozoon* infections due to high toxicity.

In independent studies, fumagillin was found to inhibit replication of endothelial cells in vitro, and interest developed in using this drug for controlling tumor growth via inhibition of neovascularization (29). Because of the toxicity of fumagillin, analogs were synthesized. TNP-470 was found to inhibit endothelium growth in vitro, to inhibit tumor growth in vivo (29), and to be substantially less toxic than fumagillin (34, 41, 51, 57–60).

The mechanisms by which fumagillin and TNP-470 inhibit microsporidial replication are poorly understood. In in vitro ultrastructural studies, *E. cuniculi* organisms treated with fumagillin were irregularly shaped. Proliferative-stage organisms were typically swollen and contained irregularly shaped cytoplasmic vesicles (44). In cytochemical studies using the microsporidian *Octosporea muscaedomesticae*, fumagillin treatment caused a decrease in total RNA, suggesting that fumagillin inhibited RNA synthesis (30). In tumor cell cultures treated with TNP-470, levels of mRNA encoding for cyclin D1, which plays a role in regulating cell division at the mid-G_1 phase, were significantly lower than in nontreated human umbilical endothelial cells (28). The targets of fumagillin and TNP-470 activities in the microsporidia, however, still need to be determined. It is also possible that fumagillin and TNP-470 act indirectly on microsporidian growth by affecting host cell function or growth.

The results of this study suggest that TNP-470 may show broad-spectrum efficacy against the microsporidia because it inhibited replication of both *E. intestinalis* and *V. corneae* in vitro. In addition, TNP-470 was found to inhibit the replication of *Nucleospora salmonis* in salmonid fish (11a). TNP-470 appears to be well tolerated in persons with AIDS. For example, in AIDS patients with Kaposi's sarcoma, TNP-470 treatments did not accelerate $CD4^+$ T-cell decline and did not cause human immunodeficiency virus p24 levels to increase (41). Furthermore, TNP-470 appears to increase the proliferation of B lymphocytes but not that of other nontransformed cells (2, 3). Taken together, the efficacy of TNP-470 for two different species of microsporidia and the relatively low toxicity described in in vivo studies suggest that TNP-470 may be a promising compound for systemic treatment of microsporidiosis in

humans with or without concomitant human immunodeficiency virus infection.

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