FOLIA PARASITOLOGICA 45: 129-139, 1998.

Screening of compounds for antimicrosporidial activity *in vitro*

Elizabeth S. Didier[1], **Joseph A. Maddry**[2], **Cecil D. Kwong**[2], **Linda C. Green**[1], **Karen F. Snowden**[3] and **John A. Shadduck**[4]

[1]Department of Microbiology, Tulane Regional Primate Research Center, Covington, LA 70433, U.S.A.;

[2]Department of Organic Chemistry, Southern Research Institute, Birmingham, AL 35255, U.S.A.;

[3]Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77843, U.S.A.;

[4]Heska Corporation, Fort Collins, CO 80525, U.S.A.

Key words: microsporidia, chemotherapy, opportunistic protozoa

Aabstract. Relatively few effective compounds are available for treating microsporidiosis in humans. In this study, several compounds were assayed for activity against *Encephalitozoon intestinalis* (Cali, Kotler et Orenstein, 1993) and *Vittaforma corneae* Shadduck, Meccoli, Davis et Font, 1990 *in vitro*. Of the benzimidazoles tested, albendazole was most effective and the MIC_{50} values were 8.0 ng/ml and 55.0 ng/ml for *E. intestinalis* and *V. corneae*, respectively. Fumagillin and its analogue, TNP-470 were nearly equally effective against both *E. intestinalis* and *V. corneae*. The MIC_{50} values of fumagillin were 0.52 ng/ml and 0.81 ng/ml, and the MIC_{50} values of TNP-470 were 0.35 ng/ml and 0.38 ng/ml for *E. intestinalis* and *V. corneae*, respectively. In addition, 12 of 44 purines and pteridines with putative tubulin binding activity that were synthesized at Southern Research Institute (SRI), inhibited microsporidial replication by more than 50% at concentrations that were not toxic to the host cells. Several chitin synthesis/assembly inhibitors inhibited growth of the microsporidia *in vitro* but were toxic for the host cells making it difficult to interpret the results. One exception was lufenuron, which caused no significant toxicity to the host cells and expressed approximate MIC_{50} values of 2.95 µg/ml and 6.3 µg/ml against *E. intestinalis* and *V. corneae*, respectively. These results warrant further studies on albendazole, fumagillin, TNP-470, lufenuron, and the selected SRI purines and pteridines for developing therapeutic strategies for microsporidiosis.

Microsporidia (phylum Microspora) are protozoan parasites that cause opportunistic infections in persons who are immunodeficient (e.g., AIDS patients) or immunosuppressed (e.g., organ transplant recipients) (Orenstein 1991, Canning and Hollister 1992, Sax et al. 1995, Rabodonirina et al. 1996, Kelkar et al. 1997). Clinical signs of disease include chronic diarrhea, conjunctivitis, sinusitis, myositis, nephritis, peritonitis, and hepatitis such that virtually all organs can become infected with microsporidia (Orenstein et al. 1997). At least twelve species of microsporidia have been reported to infect man (Didier et al. 1998). Currently, no universally effective therapy is available for treatment of all species of microsporidia. Two drugs that have been used clinically, however, include albendazole, which is effective for treatment of infections due to *Encephalitozoon* species (Blanshard et al. 1992, 1993, Wanke et al. 1993, Aarons et al. 1994, Dieterich et al. 1994, Franzen et al. 1994, Weber et al. 1994a,b, DeGroote et al. 1995, Dore et al. 1995, Franssen et al. 1995, Sobottka et al. 1995, Joste et al. 1996, Lowder et al. 1996) and fumagillin, which can be used topically (e.g., keratitis), but is too toxic for systemic use (Diesenhouse et al. 1993, Rosberger et al. 1993, Wilkins et al. 1994, Garvey et al. 1995, Shah et al. 1995, Didier et al. 1996a, Lowder et al. 1996).

The purpose of this study was to screen compounds for antimicrosporidial activity *in vitro*. *Enterocytozoon bieneusi* is the most commonly-reported microsporidian species to infect humans, but it cannot be grown in long-term culture. Therefore, *Vittaforma corneae* (Shadduck, Meccoli, Davis et Font, 1990), which can be grown in culture and grows in direct contact with the host cell cytoplasm, similar to *E. bieneusi*, was used as a surrogate in these studies (Silveira and Canning 1995). In addition, *Encephalitozoon intestinalis* Cali, Kotler, et Orenstein 1993 (syn. *Septata intestinalis*), was used in these *in vitro* drug screenings because it is the second most commonly-reported microsporidian found to infect humans and can be grown in culture.

MATERIALS AND METHODS

Organisms. Microsporidia were grown in RK-13 cells using procedures previously described (Didier et al. 1991, 1996b). The human isolate of Vittaforma corneae (syn. Nosema corneum) was cultured from corneal tissue of an individual with stromal keratitis and iritis (Davis et al. 1990, Shadduck et al. 1990, Silveira and Canning 1995). Encephalitozoon intestinalis was isolated from a bronchoalveolar lavage specimen as previously described

Address for correspondence: E.S. Didier, Department of Microbiology, Tulane Regional Primate Research Center, 18703 Three Rivers Road, Covington, LA 70433, U.S.A. Phone: ++1 504 892-2040; Fax: ++1 504 893-1352; E-mail: esdid@tpc.tulane.edu

(Hartskeerl et al. 1995, Didier et al. 1996b). 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, NJ; 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. Compounds were purchased from Sigma Chemical Co. (St. Louis, MO) or were provided from the sources listed in Table 1. Stock solutions of compounds were dissolved in DMSO 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 2.5-5 × 10^5 cells/ml RPMI 1640 (containing 2 mM L-glutamine and 5% fetal bovine serum). The plates were incubated overnight at 37°C with 5% CO_2 to allow the cells to reach confluency. Microsporidia were added in 0.5 ml volumes at a concentration of 3.0 × 10^6/ml (i.e. a final ratio of 3 : 1 organisms/cell). Three hours later, non-internalized or non-adherent parasites were washed off and fresh media, with or without drugs, were added as described for each experiment. Control cultures that were not given drugs received medium with equivalent amounts of DMSO used in preparing drug stock solutions. Media were replaced every three-to-four days and care was taken not to remove organisms from the bottom of the wells. On day ten, 100 µl of 10% (w/v) SDS was added to each of the wells to release organisms from host cells and the total numbers of organisms in the wells were counted using 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 non-treated 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. In preliminary experiments, RK-13 cells were incubated with the test compounds without microsporidia as described above, and examined by light microscopy using an inverted microscope. Drug toxicity was noted if the host cells became subconfluent or changed in morphology when compared with the non-treated cells. In some experiments, the effects of the test compounds on host cell viability were measured using an MTT [3(4,5- dimethyl-thiazol-2-yl-)2,5-diphenyl tetrazolium bromide] colorimetric assay as described by Mossman and Fong (1989) and as applied for testing antimicrosporidial drug activity by Beauvais et al. (1994). On day 10 of the drug assays, replicate wells of host cells treated with test drugs were incubated with 50 µl of the MTT stock solution (5 mg/ml in Tris-buffered saline, pH 7.4) and the culture plates were incubated for an additional four hrs 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 wave length of 570 nm and a reference wave length of 630 nm. The percent of host cell viability was calculated as the OD values of treated cells divided by the mean OD of non-treated cells × 100. Three replicates were used for each group of treatments.

Focus-forming assay. To determine if microsporidia harvested after drug treatments were infectious, a modified focus-forming assay was used (Schmidt and Shadduck 1984). Organisms were recovered from culture wells after drug treatments, washed with TBS (10 min at 400 g), and resuspended in tissue culture medium at a concentration of 1 × 10^4 organisms per ml. One-ml volumes of spores from treated and non-treated cultures were added to new 24-well culture dishes containing confluent monolayers of RK-13 cells. Media changes (without drugs) were made on day four and the monolayers were fixed with methanol (10 min at laboratory temperature) on day eight. The monolayers were stained with Calcofluor White 2R (0.3 ml of a 0.5% solution per well) for five 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 microsporidia infections. At least 50 fields were viewed per well and each assay was repeated three times.

Statistical analysis. Student's t-test (two-sided) was used when comparing results between any two treatment groups, and KWIKSTAT software (TexaSoft Products, Cedar Hill, TX) was used to calculate the results.

RESULTS

Several groups of compounds initially were assayed for antimicrosporidial activity and these included several benzimidazoles, fumagillins, and chitin synthesis/assembly inhibitors (Table 1). Of the benzimidazoles tested, albendazole, mebendazole, and thiabendazole inhibited replication of *Encephalitozoon intestinalis* by more than 50% at doses that did not appear toxic to the host cells. Furthermore, at higher concentrations, albendazole inhibited replication of *Vittaforma corneae* as well. Fumagillin, and its analogue TNP-470 inhibited both *E. intestinalis* and *V. corneae* at concentrations that were not toxic for the host cells. Many of the chitin synthesis/assembly inhibitors were toxic for the host cells with the exception of lufenuron which inhibited both microsporidian species by more than 50% at concentrations that were not toxic to the host cells.

Additional compounds that were assayed and which demonstrated moderate activity in inhibiting the growth of the microsporidia, included berberine, nitazoxanide, desacetylnitazoxanide, and sodium nitroprusside (Table 1). Several antineoplastic compounds were tested which included colchicine, demecolcine, and vinblastine. These compounds, however, caused too much host cell toxicity to ascertain efficacy in inhibiting microsporidial growth. Forty-four purines and pteridines also were screened for antimicrosporidial activity (Table 2). These compounds were chosen for putative tubulin binding

Table 1. Screening of compounds for antimicrosporidial activity

		Inhibition of microsporidian growth		Toxicity	
Group Compound Source Therapy category[b]	Concetration	*E. intestinalis*	*V. corneae*	Visual[c]	MTT assay (% +/- st. dev.)[d]
Benzimidazoles:					
Albendazole	0.001 µg/ml	2.0 ± 10.4	4.1 ± 4.3	no	90.5 ± 4.8
Sigma	0.010 µg/ml	66.0 ± 25.7	14.0 ± 6.6	no	97.8 ± 6.1
Anthelmintic	0.100 µg/ml	85.2 ± 28.1	86.7 ± 25.5	no	57.5 ± 4.9
	1.000 µg/ml	87.4 ± 10.1	89.2 ± 22.4	yes	39.2 ± 4.2
	10.000 µg/ml	88.2 ± 26.8	93.8 ± 14.2	yes	27.8 ± 11.8
Mebendazole	0.010 µg/ml	64.8 ± 6.6	–[e]	no	–
Sigma	0.100 µg/ml	69.2 ± 6.4	–	yes	–
Anthelmintic	1.000 µg/ml	74.5 ± 5.1	–	yes	–
Metronidazole	0.010 µg/ml	13.4 ± 5.2	–	no	–
Sigma	0.100 µg/ml	17.1 ± 7.0	–	no	–
Antiprotozoal, Antiamebic	1.000 µg/ml	36.2 ± 5.9	–	no	–
Quinacrine	0.010 µg/ml	-9.3 ± 6.1	–	no	–
Sigma	0.100 µg/ml	11.2 ± 11.3	–	no	–
Anthelmintic	1.000 µg/ml	36.7 ± 7.9	–	no	–
Thiabendazole	0.010 µg/ml	34.9 ± 6.2	–	no	–
Sigma	0.100 µg/ml	47.1 ± 10.4	–	no	–
Anthelmintic	1.000 µg/ml	88.4 ± 2.0	–	no	–
Fumagillins:					
Fumagillin	0.00001 µg/ml	33.2 ± 18.8	–	no	¨94.5 ± 2.8
Sanofi Santé Animale	0.00005 µg/ml	50.3 ± 11.6	–	no	98.5 ± 9.1
Antiprotozoal, Antiamoebic	0.0001 µg/ml	64.3 ± 2.9	24.5 ± 13.9	no	87.8 ± 1.5
	0.0005 µg/ml	62.9 ± 16.1	38.4 ± 10.0	no	103.2 ± 3.1
	0.001 µg/ml	68.9 ± 8.7	56.7 ± 15.5	no	116.9 ± 2.3
	0.010 µg/ml	85.9 ± 1.4	–	no	83.1 ± 13.0
	0.100 µg/ml	85.4 ± 2.3	–	no	77.3 ± 5.7
	1.000 µg/ml	86.5 ± 1.9	–	no	72.3 ± 9.0
TNP-470	0.00001 µg/ml	39.4 ± 15.6	–	no	80.2 ± 3.5
Takeda	0.00005 µg/ml	47.6 ± 16.1	–	no	80.1 ± 5.9
Pharmaceutical	0.0001 µg/ml	44.5 ± 16.1	35.8 ± 26.2	no	76.1 ± 8.0
(not listed)	0.0005 µg/ml	71.2 ± 23.2	54.4 ± 10.4	no	81.2 ± 7.7
	0.001 µg/ml	83.6 ± 17.6	75.1 ± 4.4	no	97.5 ± 4.3
	0.010 µg/ml	81.5 ± 1.9	–	no	86.5 ± 2.5
	0.100 µg/ml	78.7 ± 3.3	–	no	87.6 ± 12.4
	1.000 µg/ml	84.1 ± 2.0	–	no	87.1 ± 1.3
Chitin synthesis/assembly inhibitors:					
Ivermection	0.010 µg/ml	30.5 ± 6.0	–	no	–
Sigma	0.100 µg/ml	41.1 ± 4.9	–	no	–
Anthelmintic	1.000 µg/ml	67.6 ± 5.1	–	yes	–
Lufenuron	1.250 µg/ml	3.5 ± 11.1	36.9 ± 8.3	no	102.8 ± 14.7
Ciba-Geigy	2.500 µg/ml	32.2 ± 6.9	30.3 ± 3.4	no	103.3 ± 4.5
Ectoparasiticide	5.000 µg/ml	54.1 ± 7.1	40.9 ± 11.7	no	113.8 ± 4.4
	10.000 µg/ml	61.8 ± 3.2	62.5 ± 3.1	no	101.2 ± 5.3

(Table 1 – continued)

Nikkomycin	0.001 µg/ml	19.1 ± 12.7	–	no	–
Calbiochem	0.010 µg/ml	7.4 ± 27.4	–	no	–
(not listed)	0.100 µg/ml	47.5 ± 10.1	–	yes	–
	1.000 µg/ml	38.9 ± 17.3	–	yes	–
	5.000 µg/ml	75.3 ± 5.6	–	yes	–
Plumbagin	0.010 µg/ml	6.4 ± 16.6	–	no	–
Sigma	0.100 µg/ml	37.9 ± 10.1	–	no	–
(none given)	1.000 µg/ml	64.9 ± 7.1	–	yes	–
Tunicamycin	0.010 µg/ml	54.5 ± 3.7	–	no	–
Sigma	0.100 µg/ml	56.9 ± 3.8	–	yes	–
(none given)	1.000 µg/ml	82.0 ± 4.1	–	yes	–
Miscellaneous:					
Berberine	0.001 µg/ml	-10.5 ± 8.2	219 ± 9.9	no	100.0 ± 9.8
PRM Pharmaceuticals	0.010 µg/ml	41.0 ± 6.9	20.3 ± 13.9	no	125.9 ± 23.1
Antibacterial, Antimalarial	0.100 µg/ml	47.0 ± 12.3	42.2 ± 8.1	no	122.8 ± 20.1
	1.000 µg/ml	62.0 ± 3.7	55.2 ± 2.9	no	122.4 ± 29.9
	10.000 µg/ml	56.4 ± 3.4	63.1 ± 3.3	yes	32.3 ± 6.3
Colchicine	0.010 µg/ml	43.1 ± 9.4	–	no	–
Sigma	0.100 µg/ml	69.0 ± 6.9	–	yes	–
Antineoplastic	1.000 µg/ml	84.7 ± 4.8	–	yes	–
Demecolcine	0.010 µg/ml	34.4 ± 10.6	–	no	–
Sigma	0.100 µg/ml	59.2 ± 6.7	–	yes	–
Antineoplastic	1.000 µg/ml	43.76 ± 9.7	–	yes	–
Desacetyl nitazoxanide	0.001 µg/ml	27.2 ± 5.1	31.7 ± 16.3	no	91.3 ± 1.2
Romark Laboratories	0.010 µg/ml	30.0 ± 6.7	57.1 ± 5.5	no	97.9 ± 2.5
(not listed)	0.100 µg/ml	40.4 ± 12.5	63.6 ± 24.1	no	94.1 ± 2.1
	1.000 µg/ml	64.0 ± 23.8	66.1 ± 15.7	no	103.6 ± 2.5
	10.000 µg/ml	86.2 ± 21.5	89.7 ± 20.7	yes	3.7 ± 5.9
Nitazoxanide	0.001 µg/ml	19.4 ± 12.5	22.3 ± 19.6	no	85.5 ± 6.4
Romark Laboratories	0.010 µg/ml	33.7 ± 14.8	25.7 ± 13.1	no	96.9 ± 0.6
(not listed)	0.100 µg/ml	48.5 ± 11.9	38.6 ± 17.1	no	88.4 ± 1.7
	1.000 µg/ml	57.5 ± 27.2	42.3 ± 19.3	no	98.4 ± 3.7
	10.000 µg/ml	83.1 ± 18.4	83.0 ± 44.6	yes	3.2 ± 5.1
Sodium nitroprusside	0.001 µg/ml	2.8 ± 9.1	35.7 ± 7.6	no	100.0 ± 12.3
Sigma	0.010 µg/ml	42.5 ± 5.9	47.2 ± 3.5	no	111.5 ± 4.1
Antihypertesive	0.100 µg/ml	62.3 ± 8.5	55.2 ± 5.4	no	99.3 ± 0.9
	1.000 µg/ml	57.9 ± 3.8	60.7 ± 3.9	no	100.9 ± 5.2
	10.000 µg/ml	60.3 ± 3.9	67.9 ± 4.3	no	85.2 ± 3.7
Vinblastine	0.010 µg/ml	54.3 ± 5.0	–	yes	–
	0.100 µg/ml	66.8 ± 9.2	–	yes	–
	1.000 µg/ml	71.5 ± 5.5	–	yes	–

[a]The percent inhibition was determined as 100 – [(mean number of parasites recovered from treated cultures – mean number of parasites recovered from non-treated cultures) × 100].
[b]Therapy category as defined by the Merck Index (1996).
[c]Determination of toxicity based on visual examination through an inverted light microscopy. Toxicity (ie. "yes") was defined as changes in host cell confluency or morphology compared with non-treated host cells.
[d]Host cell viability was measured by the MTT assay in cultures incubated with the test compounds and compared with absorbance values of host cells cultures without the compounds.
[e]–; indicates not tested.

Table 2. Inhibition of *Encephalitozoon intestinalis* growth by SRI compounds.

Compound designation	Concentration	Parasite inhibition (% ± st. dev.)[a]	Toxicity[b]
SRI 6252	0.001 µg/ml	17.4 ± 2.6	no
	0.010 µg/ml	0.0 ± 10.4	no
	0.100 µg/ml	12.1 ± 0.9	no
	1.000 µg/ml	17.4 ± 3.0	no
	10.00 µg/ml	45.6 ± 9.9	yes
SRI 6262	0.001 µg/m	11.1 ± 1.3	no
	0.010 µg/m	6.4 ± 0.7	no
	0.100 µg/ml	26.5 ± 1.4	no
	1.000 µg/ml	37.2 ± 6.8	no
	10.00 µg/ml	57.1 ± 12.2	no
SRI 6270	0.001 µg/ml	14.3 ± 5.1	no
	0.010 µg/ml	0.0 ± 60.6	no
	0.100 µg/ml	75.7 ± 30.5	yes
	1.000 µg/ml	82.9 ± 39.4	yes
	10.00 µg/ml	87.1 ± 18.0	yes
SRI 6272	0.001 µg/ml	10.0 ± 3.4	no
	0.010 µg/ml	16.7 ± 4.7	no
	0.100 µg/ml	20.0 ± 7.9	no
	1.000 µg/ml	70.0 ± 62.9	yes
	10.00 µg/ml	88.3 ± 48.3	yes
SRI 6274	0.01 µg/ml	39.5 ± 8.3	no
	0.10 µg/ml	59.1 ± 8.2	no
	1.00 µg/ml	70.6 ± 5.1	yes
SRI 6281	0.001 µg/ml	12.6 ± 1.5	no
	0.010 µg/ml	9.4 ± 1.7	no
	0.100 µg/ml	28.3 ± 6.2	yes
	1.000 µg/ml	58.3 ± 8.3	yes
	10.00 µg/ml	61.4 ± 9.5	yes
SRI 6308	0.001 µg/ml	19.7 ± 9.6	no
	0.010 µg/ml	14.5 ± 0.5	no
	0.100 µg/ml	21.7 ± 6.8	no
	1.000 µg/ml	32.5 ± 12.3	yes
	10.00 µg/ml	42.2 ± 23.1	yes
SRI 6309	0.001 µg/ml	31.9 ± 0.8	no
	0.010 µg/ml	9.1 ± 1.3	no
	0.100 µg/ml	31.9 ± 20.6	no
	1.000 µg/ml	23.9 ± 12.8	yes
	10.00 µg/ml	74.3 ± 38.7	yes
SRI 6310	0.001 µg/ml	6.5 ± 0.5	no
	0.010 µg/ml	1.8 ± 0.4	no
	0.100 µg/ml	29.6 ± 5.4	no
	1.000 µg/ml	74.1 ± 51.8	yes
	10.00 µg/ml	74.1 ± 62.3	yes
SRI 6312	0.001 µg/ml	-1.4 ± 0.2	no
	0.010 µg/ml	-17.7 ± 2.5	no
	0.100 µg/ml	-2.0 ± 0.5	no
	1.000 µg/ml	21.1 ± 3.7	yes
	10.00 µg/ml	29.9 ± 3.8	yes
SRI 6455	0.01 µg/ml	45.1 ± 19.7	no
	0.10 µg/ml	70.8 ± 3.7	no
	1.00 µg/ml	85.5 ± 3.4	yes
SRI 6463	0.01 µg/ml	17.9 ± 15.6	no
	0.10 µg/ml	80.9 ± 3.3	no
	1.00 µg/ml	84.8 ± 3.7	yes
SRI 6476	0.001 µg/ml	2.9 ± 1.1	no
	0.010 µg/ml	10.0 ± 2.2	no
	0.100 µg/ml	5.7 ± 1.3	no
	1.000 µg/ml	55.0 ± 25.1	yes
	10.00 µg/ml	77.5 ± 74.7	yes

Compound designation	Concentration	Parasite inhibition (% ± st. dev.)[a]	Toxicity[b]
SRI 6512	0.01 µg/ml	-13.5 ± 14.9	no
	0.10 µg/ml	-9.8 ± 17.5	no
	1.00 µg/ml	13.2 ± 7.6	no
SRI 6592	0.01 µg/ml	3.2 ± 7.2	no
	0.10 µg/ml	-0.7 ± 16.2	no
	1.00 µg/ml	79.4 ± 6.0	yes
SRI 6606	0.01 µg/ml	11.8 ± 20.8	no
	0.10 µg/ml	19.6 ± 14.3	no
	1.00 µg/ml	45.0 ± 7.8	no
SRI 6607	0.01 µg/ml	4.2 ± 13.6	no
	0.10 µg/ml	58.6 ± 14.1	no
	1.00 µg/ml	74.1 ± 8.3	no
SRI 6650	0.01 µg/ml	-8.2 ± 7.9	no
	0.10 µg/ml	-8.9 ± 7.6	no
	1.00 µg/ml	11.46 ± 3.5	no
SRI 6707	0.01 µg/ml	-14.4 ± 5.2	no
	0.10 µg/ml	-5.2 ± 15.3	no
	1.00 µg/ml	-0.8 ± 8.4	no
SRI 6782	0.01 µg/ml	10.8 ± 17.2	no
	0.10 µg/ml	20.9 ± 19.4	no
	1.00 µg/ml	51.5 ± 3.7	no
SRI 6798	0.01 µg/ml	57.5 ± 5.4	no
	0.10 µg/ml	75.8 ± 6.0	no
	1.00 µg/ml	78.8 ± 4.6	no
SRI 6804	0.01 µg/ml	32.4 ± 15.6	no
	0.10 µg/ml	71.0 ± 8.2	no
	1.00 µg/ml	61.4 ± 5.4	yes
SRI 6809	0.01 µg/ml	7.3 ± 8.2	no
	0.10 µg/ml	0.0 ± 4.2	no
	1.00 µg/ml	26.5 ± 8.8	no
SRI 6839	0.01 µg/ml	26.8 ± 21.9	no
	0.10 µg/ml	53.9 ± 18.2	no
	1.00 µg/ml	59.0 ± 12.2	no
SRI 6894	0.01 µg/ml	6.5 ± 5.2	no
	0.10 µg/ml	7.1 ± 7.6	no
	1.00 µg/ml	3.9 ± 4.6	no
SRI 7109	0.01 µg/ml	-6.5 ± 7.1	no
	0.10 µg/ml	12.6 ± 12.4	no
	1.00 µg/ml	-2.5 ± 9.5	no
SRI 7175	0.01 µg/ml	59.5 ± 4.6	no
	0.10 µg/ml	52.5 ± 13.5	no
	1.00 µg/ml	64.6 ± 7.1	no
SRI 7462	0.01 µg/ml	19.1 ± 7.9	no
	0.10 µg/ml	64.9 ± 8.6	yes
	1.00 µg/ml	77.3 ± 7.5	yes
SRI 7563	0.01 µg/ml	71.9 ± 7.9	no
	0.10 µg/ml	58.4 ± 11.6	yes
	1.00 µg/ml	69.7 ± 8.9	yes
SRI 7614	0.01 µg/ml	73.2 ± 5.0	no
	0.10 µg/ml	73.2 ± 6.6	no
	1.00 µg/ml	83.1 ± 4.1	yes
SRI 7714	0.01 µg/ml	1.0 ± 6.6	no
	0.10 µg/ml	38.2 ± 7.6	yes
	1.00 µg/ml	81.8 ± 4.9	yes
SRI 7896	0.01 µg/ml	12.7 ± 7.3	no
	0.10 µg/ml	4.1 ± 5.0	no
	1.00 µg/ml	77.5 ± 3.7	yes

(Table 2 – continued)

Compound designation	Concentration	Parasite inhibition (% ± st. dev.)[a]	Toxicity[b]
SRI 8117	0.01 µg/ml	4.8 ± 8.2	no
	0.10 µg/ml	28.3 ± 12.9	yes
	1.00 µg/ml	65.8 ± 3.6	yes
SRI 8202	0.01 µg/ml	22.6 ± 2.8	no
	0.10 µg/ml	69.8 ± 6.3	yes
	1.00 µg/ml	75.7 ± 3.1	yes
SRI 8228	0.01 µg/ml	56.3 ± 5.1	yes
	0.10 µg/ml	65.4 ± 5.9	yes
	1.00 µg/ml	78.0 ± 5.9	yes
SRI 8229	0.01 µg/ml	41.6 ± 10.2	yes
	0.10 µg/ml	72.2 ± 7.7	yes
	1.00 µg/ml	68.7 ± 7.8	yes
SRI 8582	0.01 µg/ml	15.3 ± 6.2	no
	0.10 µg/ml	74.8 ± 3.1	yes
	1.00 µg/ml	75.4 ± 4.8	yes
SRI 8654	0.01 µg/ml	8.9 ± 3.5	no
	0.10 µg/ml	16.8 ± 10.1	no
	1.00 µg/ml	8.4 ± 8.4	no
SRI 8686	0.01 µg/ml	-1.1 ± 11.3	no
	0.10 µg/ml	6.0 ± 17.6	no
	1.00 µg/ml	68.3 ± 18.3	yes
SRI 8691	0.01 µg/ml	0.0 ± 13.6	no
	0.10 µg/ml	67.2 ± 5.3	yes
	1.00 µg/ml	74.6 ± 3.0	yes
SRI 8708	0.01 µg/ml	21.3 ± 11.4	no
	0.10 µg/ml	69.5 ± 4.9	yes
	1.00 µg/ml	75.7 ± 4.4	yes
SRI 8709	0.01 µg/ml	56.6 ± 6.1	yes
	0.10 µg/ml	57.2 ± 6.9	yes
	1.00 µg/ml	70.4 ± 3.5	yes
SRI 8710	0.01 µg/ml	40.6 ± 7.2	yes
	0.10 µg/ml	53.6 ± 11.9	yes
	1.00 µg/ml	72.5 ± 7.7	yes
SRI 8829	0.01 µg/ml	9.9 ± 13.0	no
	0.10 µg/ml	1.1 ± 13.3	no
	1.00 µg/ml	-2.0 ± 3.4	no

[a]The percent inhibition was determined as 100 – [(mean number of parasites recovered from treated cultures – mean number of parasites recovered from non-treated cultures) × 100].

[b]Toxicity was based on visual examination as described in Table 1 and Materials and Methods.

properties and 12 of these compounds were found to inhibit *E. intestinalis* growth by more than 50% at concentrations that were not toxic to the host cells as determined by visual inspection.

In additional experiments, albendazole, fumagillin, TNP-470, and lufenuron were added to RK-13 cells which had been infected with *E. intestinalis* seven days earlier to determine if these compounds could inhibit microsporidial growth after the establishment of the infection. Concentrations of albendazole (0.1 µg/ml), fumagillin (0.01 µg/ml), and TNP-470 (0.01 µg/ml) used in these experiments were based on the concentrations that inhibited microsporidian growth by approximately 80% and were not toxic by visual examinations in the screening experiments. Lufenuron was used at 10.0 µg/ml which was the highest dose tested in the screening experiments and was not toxic to the host cells. The numbers of parasites released into the culture medium were counted every three to four days, and the results in Figure 1 show the percent of organisms released into the supernatants of treated cultures compared with non-treated cultures over time. Parasite shedding was reduced to 50% of that from non-treated cultures approximately 2.8 days after initiating treatment with fumagillin (0.01 µg/ml) and by approximately 3.6 days after beginning treatments with albendazole (0.1µg/ml) or TNP- 470 (0.01 µg/ml). It took approximately 5.4 days before lufenuron (10.0 µg/ml) treatment inhibited replication of *E. intestinalis* to 50% of that seen in the non-treated cultures.

Table 3. Infectivity of *Encephalitozoon intestinalis* recovered after treatment with test compounds.

Treatment	Number of Infected Cell (Foci) per 10 Fields (± st. dev.)	Percent of Control (± st. dev.)	P[a]
Albendazole (0.1 µg/ml)	0.07 ± 0.02	0.11 ± 0.003	< 0.001
Fumagillin (0.01 µg/ml)	0.14 ± 0.09	0.21 ± 0.135	< 0.001
TNP-470 (0.01 µg/ml)	0.18 ± 0.25	0.28 ± 0.040	< 0.001
Lufenuron (10.0 µg/ml)	15.30 ± 1.13	23.36 ± 17.30	< 0.001
None	65.50 ± 5.60	100 ± 8.50	< 0.001[b]

[a] Comparisons were made with parasites recovered from non-treated cultures and assayed for infectivity.

[b] The P value when compared with with the number of infectious parasites recovered after treatment with albendazole, fumagillin, or TNP-470.

Microsporidia were still seen in the cultures after treatment with albendazole, fumagillin, TNP-470, or lufenuron as shown in Figure 1. Since it was not possible to determine if these organisms were mature or infectious by light microscopic examination, the recovered parasites were assayed in the focus-forming assay to measure relative infectivity. RK-13 cells were infected with *E. intestinalis* and treated with albendazole (0.1 µg/ml), fumagillin (0.01 µg/ml), TNP-470 (0.01 µg/ml), and lufenuron (10.0 µg/ml) from day 7 through day 14. Parasites recovered from the medium on day 14 were adjusted to 1×10^4 organisms and assayed for infectivity in the focus-forming assay. The results in Table 3 indicate that significantly fewer infectious microsporidia were recovered after treatments with albendazole, fumagillin, TNP-470, or lufenuron compared with the number of infectious organisms recovered from non-treated cultures ($P < 0.001$). Further, treatments with albendazole, fumagillin, and TNP-470 generated significantly fewer infectious organisms than generated after treatment with lufenuron ($P < 0.001$).

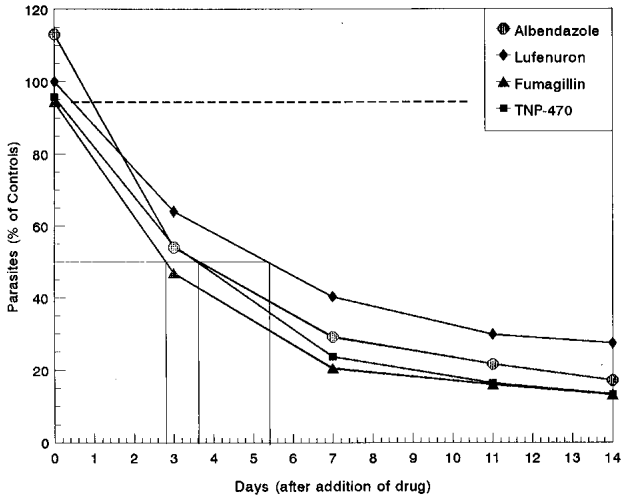

Fig. 1. Effects of drugs on established *Encephalitozoon intestinalis* infections *in vitro*. RK-13 cells were infected with *E. intestinalis*. Seven days later, and with each subsequent medium change, albendazole (0.1 µg/ml), fumagillin (0.01 µg/ml), TNP-470 (0.01 µg/ml), or lufenuron (10.0 µg/ml) were added to the cultures. Parasites released into the supernatants were counted after each medium change. Values on the *y* axis represent the percent of spores shed from treated cultures relative to non-treated cultures. Assays were run in triplicate and values represent the mean numbers of accumulated organisms at each time point.

DISCUSSION

Currently, the two most effective drugs for treating microsporidiosis are albendazole and fumagillin. Albendazole is a broad spectrum anti-protozoal benzimidazole which binds to the colchicine binding site of b-tubulin thereby inhibiting microtubule polymerization (Lacey 1985). Albendazole has shown variable levels of clinical efficacy against *Enterocytozoon bieneusi*, but organisms were rarely cleared from the stool (Blanshard et al. 1992, Wanke et al. 1993, Dieterich et al. 1994, Franzen et al. 1994). However, albendazole appears to be more effective against the *Encephalitozoon* species. Although relapses have occurred in patients with *E. intestinalis* or *Encephalitozoon hellem*, after discontinuation of albendazole, patients responded well to a second course of treatment resulting in relief of symptoms. Furthermore, *Encephalitozoon* spp. spores often were cleared from stool, urine, and other tissue sites after treatment with albendazole (Blanshard et al. 1992, 1993, Wanke et al. 1993, Aarons et al. 1994, Franzen et al. 1994, Weber et al. 1994a,b, DeGroote et al. 1995, Dore et al. 1995, Franssen et al. 1995, Sobottka et al. 1995, Joste et al. 1996, Lowder et al. 1996). In the study presented here, albendazole treatment for ten days *in vitro* significantly inhibited replication of *E. intestinalis*, and at higher concentrations, inhibited *V. corneae*, as well. Negligible numbers of infectious organisms were recovered after treatment with 0.10 µg/ml albendazole for one week suggesting that *in vitro* albendazole can be parasiticidal if treatment is continued for a sufficient period of time, as well.

Several purines and pteridines were assayed for antimicrosporidial activity and were selected for their putative tubulin binding abilities. These compounds were synthesized at Southern Research Institute and expressed a wide range of inhibitory effects. Of the 44 compounds that were screened, 12 inhibited replication of *E. intestinalis* by more than 50% at concentrations that were not toxic for the host cells.

Fumagillin, an antibiotic produced by *Aspergillus fumigatus*, is used for treating insects with microsporidiosis (Ketznelson and Jamieson 1952, Hartwig and Przelecka 1971, Armstrong 1976) and historically, was used for treating patients with amebiasis (McCowen et al. 1951, Killough et al. 1952). *In vitro*, fumagillin inhibits *E. cuniculi* replication (Shadduck 1980), and clinically, fumagillin has been used topically to treat *E. hellem* and *E. intestinalis* infections of the eye (Diesenhouse et al. 1993, Rosberger et al. 1993, Wilkins et al. 1994, Garvey et al. 1995, Shah et al. 1995, Didier et al. 1996a, Lowder et al. 1996). A limitation of fumagillin, however, is that typically it cannot be given systemically to patients with *E. bieneusi* or disseminated *Encephalitozoon* infections due to high toxicity. In one report, oral administration of fumagillin cleared *E. bieneusi* in several AIDS patients, but did cause thrombocytopenia (Molina et al. 1997).

In other studies, fumagillin was found to inhibit endothelial cell development and was studied for its tumor inhibition. Due to its high toxicity, analogues were synthesized and the most effective of these was TNP-470 (Ingber et al. 1990, Kusaka et al. 1991, Pluda et al. 1993, Yamaoka et al. 1993a,b, Yanase et al. 1993, Tsujimoto et al. 1995, Yanai et al. 1995). In the studies presented here and previously (Didier 1997), fumagillin and TNP-470 were seen to inhibit both *E. intestinalis* and *V. corneae* by more than 50% at non-toxic concentrations. In addition, few infectious organisms could be recovered after treatment for one week with either fumagillin or TNP-470. These results also were consistent with those of Beauvais et al. (1994) and Franssen et al. (1995) who found that fumagillin was among the most effective compounds assayed against *E. cuniculi in vitro*.

Chitin is a component of the microsporidian spore wall (Vávra et al. 1993, Bigliardi et al. 1996). Compounds that target chitin synthesis or assembly therefore were screened for their abilities to inhibit replication of microsporidia *in vitro*. Lufenuron, which acts as a chitin synthesis inhibitor, was found to be most effective and least toxic of this group of compounds in the initial assays. Lufenuron has been found to control cat fleas (*Ctenocephalides felis*) in cats and dogs (Blagburn et al. 1994, 1995, Hink et al. 1994, Nishida et al. 1995, Smith et al. 1996, Franc and Cadiergues 1997) and is the active component in the flea-control drug

named Program^{R} (Ciba-Geigy Corp., Greensboro, NC, USA). No toxicity was reported in any of the dogs or cats treated with lufenuron. In studies presented here, lufenuron inhibited *E. intestinalis* and *V. corneae* at 5.0 and 10.0 µg/ml, respectively, and caused no detectable toxicity. It appeared that lufenuron acted to inhibit the later stages of development. The number of infected cells (i.e. infectious foci) in the treated and non-treated cultures appeared to be similar (data not presented), but the size of each infectious focus, appeared smaller in the treated cultures than in the non-treated cultures. This was borne out by the significantly lower number of parasites recovered from treated versus non-treated cultures. However, there were significantly more infectious (i.e. focus-forming) organisms recovered from the lufenuron-treated cultures than from the cultures treated with albendazole, fumagillin, or TNP-470 suggesting that higher concentrations of lufenuron may be required and/or that treatments may need to be prolonged.

Additional compounds were screened for antimicrosporidial activities. Berberine was assayed because of reported anti-protozoal activities (Ghosh et al. 1985, Kaneda et al. 1990), and anti-microsporidial activity, specifically (McDevitt et al. 1996). Berberine is a plant alkaloid that has been used in China and India as an antidiarrheal agent (Schiller 1995). The results generated in this study corroborated the findings of McDevitt et al. (1996) in demonstrating berberine activity for inhibiting replication of *E. intestinalis* and *V. corneae in vitro*.

Nitrogen intermediates have been shown to be mediators of antiparasitic activities expressed by macrophages (James 1995) and were found to be involved in macrophage-mediated killing of *E. cuniculi* (Didier et al. 1994, Didier 1995). In addition, He et al. (1996) demonstrated that nitric oxide donors inhibited germination of *E. intestinalis*. In the screening studies presented here, sodium nitroprusside was found to inhibit both *E. intestinalis* and *V. corneae* at doses above 0.1 µg/ml without any discernible toxicity for the host cells.

Two additional antiparasitic compounds that were assayed were nitazoxanide and desacetylnitazoxanide (Rossignol and Maisonneuve 1984, Murphy and Friedman 1985, Dubreuil et al. 1996). Nitazoxanide has shown efficacy in a clinical trial for treatment of *Cryptosporidium parvum* and is currently being studied in the AIDS Clinical Trials Groups protocol 336 for efficacy against *C. parvum* (Framm and Soave 1997). In these preliminary *in vitro* screening assays, deacetyl nitazoxanide inhibited both *E. intestinalis* and *V. corneae* at concentrations that were not toxic to the host cells while nitazoxanide appeared to be more effective against *E. intestinalis*.

Until *E. bieneusi* can be grown in culture, the *in vitro* model utilizing *E. intestinalis* and *V. corneae* provides a means of screening compounds for antimicrosporidial activities. *E. intestinalis* is the most commonly-reported microsporidian that infects humans and that can be grown in culture, and *V. corneae* appears to represent the best microsporidian surrogate for *E. bieneusi*. Both *V. corneae* and *E. bieneusi* replicate in direct contact with the host cell cytoplasm, although *V. corneae* is also intimately associated with endoplasmic reticulum (Desportes-Livage et al. 1985, Silveira and Canning, 1995). *V. corneae* and *E. bieneusi* are relatively less susceptible to albendazole than the *Encephalitozoon* species, but both organisms are very susceptible to fumagillin *in vitro* and *in vivo*, respectively (Didier 1997, Molina et al. 1997), but fumagillin is highly toxic *in vivo*. These results support further studies on the fumagillin analogue, TNP-470, along with lufenuron, several purines and pteridine derivatives, berberine, and nitazoxanide and its analogue, desacetylnitazoxanide.

Acknowledgements. The excellent technical assistance of Ms. Donna Bertucci and Ms. Linda Rogers is gratefully acknowledged. We also thank Dr. René Desmarais of Sanofi Santé Animale (Victoriaville, QC, Canada) for providing fumagillin (bicycloheximide fumagillin), Dr. Hajime Toguchi of Takeda Chemical Industries, LTD for providing TNP-470 [O-(chloroacetyl-carbamoyl) fumagillol; AGM-1470], Dr. William Campbell of Ciba-Geigy Corporation (Greensboro, NC, U.S.A.), Dr. J. Francois Rossignol of Romark Laboratories (Tampa, FL, USA) for providing nitazoxanide and deacetyl nitazoxanide, Dr. Diane Schneider of PRM Pharmaceuticals (Ardmore, PA, U.S.A) for providing berberine. This work was supported by grants RR00164, AI40323, and AI40324 from the National Institutes of Health (Bethesda, MD, U.S.A.).

REFERENCES

AARONS E., WOODROW D., HOLLISTER W.S., CANNING E.U., FRANCIS N., GAZZARD B.G. 1994: Reversible renal failure caused by a microsporidian infection. AIDS 8: 1119-1121.

ARMSTRONG E. 1976: Fumidil B and benomyl: chemical control of *Nosema kingi* in *Drosophila willistoni*. J. Invert. Pathol. 27: 363-366.BEAUVAIS B., SARFATI C., CHALLIER S., DEROUIN F. 1994: *In vitro* model to assess effect of antimicrobial agents on *Encephalitozoon cuniculi.* Antimicrob. Agents Chemother. 38: 2440-2448.

BIGLIARDI E., SELMI M.G., LUPETTI P., CORONA S., GATTI S., SCAGLIA M., SACCHI L. 1996: Microsporidian spore wall: ultrastructural findings on *Encephalitozoon hellem* exospore. J. Euk. Microbiol. 43: 181-186.

BLAGBURN B.L., HENDRIX C.M., VAUGHAN J.L.,LINDSAY D.S., BARNETT S.H. 1995: Efficacy of lufenuron against developmental stages of fleas (*Ctenocephalides felis felis*) in dogs housed in simulated home environments. Am. J. Vet. Res. 56: 464-467.

BLAGBURN B.L., VAUGHAN J.L., LINDSAY D.S.,

TEBBITT G.L. 1994: Efficacy dosage titration of lufenuron against developmental stages of fleas (*Ctenocephalides felis felis*) in cats. Am. J. Vet. Res. 55: 98-101.

BLANSHARD C., ELLIS D.S., DOWELL S.P., TOVEY G., GAZZARD B.G. 1993: Electron microscopic changes in *Enterocytozoon bieneusi* following treatment with albendazole. J. Clin. Pathol. 46: 898-902.

BLANSHARD C., ELLIS D.S., TOVEY D.G., DOWELL S., GAZZARD B.G. 1992: Treatment of intestinal microsporidiosis with albendazole in patients with AIDS. AIDS 6: 311-313.

CALI A., KOTLER D.P., ORENSTEIN J.M. 1993: *Septata intestinalis*, N. G., N. Sp., an intestinal microsporidian associated with chronic diarrhea and dissemination in AIDS patients. J. Euk. Microbiol. 40: 101-112.

CANNING E.U., HOLLISTER W.S. 1992: Human infections with microsporidia. Rev. Med. Microbiol. 3: 35-42.

DAVIS R.M., FONT R.L., KEISLER M.S., SHADDUCK J.A. 1990: Corneal microsporidiosis. A case report including ultrastructural observations. Ophthalmology. 97: 953-957.

DEGROOTE M.A., VISVESVARA G.S., WILSON M.L., PIENIAZEK N.J., SLEMENDA S.B., DASILVA A.J., LEITCH G.J., BRYAN R.T., REVES R. 1995: Polymerase chain reaction and culture confirmation of disseminated *Encephalitozoon cuniculi* in a patient with AIDS. Successful treatment with albendazole. J. Infect. Dis. 171: 1375-1378.

DESPORTES I., LE CHARPENTIER Y., GALIAN A., BERNARD F., COCHAND-PRIOLLET B., LAVERGNE A., RAVISSE P., MODIGLIANI R. 1985: Occurrence of a new microsporidian, *Enterocytozoon bieneusi* n. g., n. sp., in the enterocytes of a human patient with AIDS. J. Protozool. 32: 250-254.

DIDIER E.S. 1995: Reactive nitrogen intermediates implicated in the inhibition of *Encephalitozoon cuniculi* (phylum Microspora) replication in murine peritoneal macrophages. Parasite Immunol. 17: 405-412.

DIDIER, E.S. 1997: Effects of albendazole, fumagillin, and TNP-470 on microsporidial replication *in vitro*. Antimicrob. Agents Chemother. 41: 1541-1546.

DIDIER E.S., DIDIER P.J., FRIEDBERG D.N., STENSON S.A., ORENSTEIN J.M., YEE R.W., TIO F.O., DAVIS R.M., VOSSBRINCK C., MILLICHAMP N., SHADDUCK J.A. 1991: Isolation and characterization of a new microsporidian, *Encephalitozoon hellem* (n. sp.) from three AIDS patients with keratoconjunctivitis. J. Infect. Dis. 163: 617-621.

DIDIER E.S., ROGERS L.B., BRUSH A.D., WONG S., BERTUCCI D.C. 1996a: Diagnosis of disseminated microsporidian *Encephalitozoon hellem* infection by PCR-Southern analysis and successful treatment with albendazole and fumagillin. J. Clin. Microbiol. 34: 947-952.

DIDIER E.S., ROGERS L.B., ORENSTEIN J.M., BAKER M.D., VOSSBRINCK C.R., VAN GOOL T., HARTSKEERL R., SOAVE R., BEAUDET L.M. 1996b: Characterization of *Encephalitozoon* (*Septata*) *intestinalis* isolates cultured from nasal mucosa and bronchoalveolar lavage fluids of two AIDS patients. J. Euk. Microbiol. 43: 34-43.

DIDIER E.S., SNOWDEN K.F., SHADDUCK J.A. 1998: Biology of microsporidian species infecting mammals. Adv. Parasitol. (in press).

DIDIER E.S., VARNER P.W., DIDIER P.J., ALDRAS A.M., MILLICHAMP N.J., MURPHEY-CORB M., BOHM R., SHADDUCK J.A. 1994: Experimental microsporidiosis in immunocompetent and immunodeficient mice and monkeys. Folia Parasitol 41: 1-11.

DIESENHOUSE M.C., WILSON L.A., CORRENT G.F., VISVESVERA G.S., GROSSNIKLAUS H.E., BRYAN R.T. 1993: Treatment of microsporidial kerato-conjunctivitis with topical fumagillin. Am. J. Ophthalmol. 115: 293-298.

DIETERICH D.T., LEW E., KOTLER D.P., POLES M., ORENSTEIN J.M. 1994: Treatment with albendazole for intestinal disease due to *Enterocytozoon bieneusi* in patients with AIDS. J. Inf. Dis. 169: 178-183.

DORE G.J., MARRIOTT D.J., HING M.C., HARKNESS J.L., FIELD A.S. 1995: Disseminated microsporidiosis due to *Septata intestinalis* in nine patients infected with the human immunodeficiency virus: response to therapy with albendazole. Clin. Infect. Dis. 21: 70-76.

DUBREUIL L., HOUCKE, I., MOUTON, Y., ROSSIGNOL, J.F. 1996: *In vitro* evaluation of activities of nitazoxanide and tizoxanide against anaerobes and aerobic organisms. Antimicrob. Agents Chemother. 40: 2266-2270.

FRAMM S.R., SOAVE R. 1997: Agents of diarrhea. Med. Clin. N. Am. 81: 427-447.

FRANC M., CADIERGUES M.C. 1997: Use of injectable lufenuron for treatment of infestations of *Ctenocephalides felis* in cats. Am. J. Vet. Res. 58: 140-142.

FRANSSEN F.F.J., LUMEIJ J.T., VAN KNAPEN F. 1995: Susceptibility of *Encephalitozoon* cuniculi to several drugs *in vitro*. Antimicrob. Agents Chemother. 39: 1265-1268.

FRANZEN C., FAETKENHEUER G., SALZBERGER B., MUELLER A., MAHRLE G., DIEHL V., SCHRAPPE M. 1994: Intestinal microsporidiosis in patients with acquired immunodeficiency syndrome - report of three more German cases. Infection 22: 417-419.

GARVEY M.J., AMBROSE P.G., ULMER J.L. 1995: Topical fumagillin in the treatment of microsporidial keratoconjunctivitis in AIDS. Ann. Pharmacother. 29: 872-874.

GHOSH A.K., BHATTACHARYYA F.K., GHOSH D.K. 1985: *Leishmania donovani*: amastigote inhibition and mode of action of berberine. Exp. Parasitol. 60: 404-413.

HARTSKEERL R.A., VAN GOOL T., SCHUITEMA A.R.J., DIDIER E.S., TERPSTRA W.J. 1995: Genetic and immunological characterization of the microsporidian *Septata intestinalis* Cali, Kotler, and Orenstein, 1993: reclassification to *Encephalitozoon intestinalis*. Parasitology 110: 277-285.

HARTWIG A., PRZELECKA A. 1971: Nucleic acids in intestine of *Apis mellifica* infected with *Nosema apis* and treated with fumagillin DCH. Cytochemical and autoradiographic studies. J. Invertebr. Pathol. 18: 331-336.

HE Q., LEITCH G.J., VISVESVARA G.S., WALLACE S. 1996: Effects of nifedipine, metronidazole, and nitric oxide donors on spore germination and cell culture infection of the microsporidia, *Encephalitozoon hellem* and *Encephalitozoon intestinalis*. Antimicrob. Agents Chemother. 40: 179-185.

HINK W.F., ZAKSON M., BARNETT S. 1994: Evaluation of a single oral dose of lufenuron to control flea infestations in dogs. Am. J. Vet. Res. 55: 822-844.

INGBER D., FUJITA T., KISHIMOTO S., SUDO K., KANAMARU T., BREM H., FOLKMAN J. 1990: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 348: 555-557.

JAMES S.L. 1995: Role of nitric oxide in parasitic infections. Microbiol. Rev. 29: 533-547.

JOSTE N.E., RICH J.D., BUSAM K.J., SCHWARTZ D.A. 1996: Autopsy verification of *Encephalitozoon intestinalis* (microsporidiosis) eradication following albendazole therapy. Arch. Pathol. Lab. Med. 120: 199-203.

KANEDA Y., TANAKA T., SAW T. 1990: Effects of berberine, a plant alkaloid, on the growth of anaerobic protozoa in axenic culture. Tokai. J. Exp. Clin. Med. 15: 417-423.

KELKAR R., SASTRY P.S., KULKARNI S.S., SAIKIA T.K., PARIKH P.M., ADVANI S.H. 1997: Pulmonary microsporidial infection in a patient with CML undergoing allogeneic marrow transplant. Bone Mar. Transplant. 19: 179-182.

KETZNELSON H., JAMIESON C.A. 1952: Control of *Nosema* disease of honey bees with fumagillin. Science 115: 70.

KILLOUGH J.H., MAGILL G.B., SMITH R.C. 1952: The treatment of amebiasis with fumagillin. Science 115: 71-72.

KUSAKA M., SUDO K., FUJITA T., MARUE S., ITOH F., INGBER D., FOLKMAN J. 1991: Potent antiangiogenic action of AGM-1470: comparison to the fumagillin parent. Biochem. Biophys. Res. Commun. 174: 1070-1076.

LACEY E. 1985: The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. Int. J. Parasitol. 18: 885-936.

LOWDER C.Y., MCMAHON J.T., MEISLER D.M., DODDS E.M., CALABRESE L.H., DIDIER E.S., CALI A. 1996: Microsporidial keratoconjunctivitis cause by *Septata intestinalis* in a patient with acquired immunodeficiency syndrome. Am. J. Ophthalmol. 121: 715-717.

MCCOWEN M.C., CALLENDER M.E., LAWLES J.F., Jr. 1951: Fumagillin (H-3), a new antibiotic with amebicidal properties. Science 113: 202-203.

MCDEVITT J.T., SCHNEIDER D.M., KATIYAR S.K., EDLIND T.D. 1996: Berberine: a candidate for the treatment of diarrhea in AIDS patients. Intersci. Conf. Antimicrob. Agents Chemother. F172: 130.

MERCK INDEX 1996: An Encyclopedia of Chemicals, Drugs, and Biologicals. 12th Edition. Merck & Co., Inc., Whitehouse Station, NJ, USA, 1741 pp.

MOLINA J.M., GOGUEL J., SARFATI C., CHASTANG C.I., DESPORTES I., MICHIELS J.F., MASLO C., KATLAMA C., COTTE L., LEPORT C., RAFFI F., DEROUIN F., MODAI J. 1997: Drug screening for the treatment of *Enterocytozoon bieneusi* infections in patients with HIV-infections. 4th Conf. Retrov. Oppor. Infec., Abstract No. 687: 191.

MOSSMAN T.R., FONG T.A.T. 1989: Specific assays for cytokine production by T cells. J. Immunol. Meth. 116: 151-158.

MURPHY J.R., FRIEDMANN J.C. 1985: Pre-clinical toxicology of nitazoxanide – a new antiparasitic compound. J. Appl. Toxicol. 5: 49-52.

NISHIDA Y., HAGA C., ODA K., HAYAMA T. 1995: Disinfestation of experimentally infested cat fleas, *Ctenocephalides felis*, on cats and dogs by oral lufenuron. J. Vet. Med. Sci. 57: 655-658.

ORENSTEIN J.M. 1991: Microsporidiosis in the acquired immunodeficiency syndrome. J. Parasitol. 77: 843-864.

ORENSTEIN J.M., GAETZ H.P., YACHNIS A.T., FRANKEL S.S., MERTENS R.B., DIDIER E.S. 1997: Disseminated microsporidiosis in AIDS: are any organs spared? AIDS 11: 385-386.

PLUDA J.M., WYVILL K., LIETZAU J., FIGG D., SAVILLE M.W., NGUYEN B.Y., FOLI A., BAILEY J., COOPER M., BRODER S., YARCHOAN R. 1993: A phase I trial of TNP-470 (AGM-1470) administered to patients with HIV-associated Kaposi's sarcoma (KS). First Nat. Conf. Hum. Retrovir. Relat. Infect. No. 61.

RABODONIRINA M., BERTOCCHI M., DESPORTES-LIVAGE I., COTTE L., LEVREY H., PIENS M.A., MONNERET G., CELARD M., MORNEX J.F., MOJON M. 1996: *Enterocytozoon bieneusi* as a cause of chronic diarrhea in a heart-lung transplant recipient who was seronegative for human immunodeficiency virus. Clin. Infect. Dis. 23: 114-117.

ROSBERGER D.F., SERAREVIC O.N., ERLANDSON R.A., BRYAN R.T., SCHWARTZ D.A., VISVESVARA G.S. 1993: Successful treatment of microsporidial keratoconjunctivitis with topical fumagillin in a patient with AIDS. Cornea 12: 261-265.

ROSSIGNOL J.F., MAISONNEUVE H. 1984: Nitazoxanide in the treatment of *Taenia saginata* and *Hymenolepis nana* infections. Am. J. Trop. Med. Hyg. 33: 511-512.

SAX P.E., RICH J.D., PIECIAK W.S., TRNKA Y.M. 1995: Intestinal microsporidiosis occurring in a liver transplant recipient. Transplantation 60: 617-618.

SCHILLER L.R. 1995: Review article: anti-diarrhoeal pharmacology and therapeutics. Aliment Pharmacol. Ther. 9: 87-106.

SCHMIDT E.C., SHADDUCK J.A. 1984: Mechanisms of resistance to the intracellular protozoan parasite, *Encephalitozoon* cuniculi, in mice. J. Immunol. 133: 2712-2719.

SHADDUCK J.A. 1980: Effect of fumagillin on *in vitro* multiplication of *Encephalitozoon* cuniculi. J. Protozool. 27: 202-208.

SHADDUCK J.A., MECCOLI R.A., DAVIS R., FONT R.L. 1990: First isolation of a microsporidian from a human patient. J. Infect. Dis. 162: 773-776.

SHAH G.K., PFISTER D., PROBST L.E., FERRIERI P., HOLLAND E. 1995: Diagnosis of microsporidial keratitis by confocal microscopy and the chromatrope stain. Am. J. Ophthalmol. 121: 89-91.

SILVEIRA H., CANNING E.U. 1995: *Vittaforma corneae*, N. Comb. for the human microsporidium *Nosema corneum* Shadduck, Meccoli, Davis & Font, 1990, based on its ultrastructure in the liver of experimentally infected athymic mice. J. Euk. Microbiol. 42: 158-165.

SMITH R.D., PAUL A.J., KITRON U.D., PHILIP J.R., BARNETT S., PIEL M.J., NESS R.W., EVILSIZER M. 1996: Impact of an orally administered insect growth regulator (lufenuron) on flea infestations of dogs in a controlled simulated home environment. Am. J. Vet. Res. 57: 502-504.

SOBOTTKA I. ALBRECHT H., SCHAEFER H., SCHOTTELIUS J., VISVESVARA G.S., LAUFS R., SCHWARTZ D.A. 1995: Disseminated *Encephalitozoon* (*Septata*) *intestinalis* infection in a patient with AIDS: novel diagnostic approaches and autopsy-confirmed parasitological cure following treatment with albendazole. J. Clin. Microbiol. 33: 2948-2952.

TSUJIMOTO H., HAGIWARA A., OSAKI K., OHYAMA T., SAKAKIBARA T., SAKUYAMA A., OHGAKI M., IMANISHI T., WATANABE N., YAMAZAKI J., SHIRASU M., SAKAKURA C., SHIMOTSUMA M., TAKAHASHI T. 1995: Therapeutic effects of the angiogenesis inhibitor TNP-470 against carcinomatous peritonitis in mice. Anti-Cancer Drugs 6: 438-442.

VÁVRA J., DAHBIOVÁ R., HOLLISTER W.S., CANNING E.U. 1993: Staining of microsporidian spores by optical brighteners with remarks on the use of brighteners for the diagnosis of AIDS associated human microsporidioses. Folia Parasitol 40: 267-272.

WANKE C.A., BHALLA M., MATTIA A.R., HELLER H.M. 1993: Case records of the Massachusetts General Hospital (Case report 51-1993). N. Eng. J. Med. 329: 1946-1954.

WEBER R., BRYAN R.T., SCHWARTZ D.A., OWEN R.I. 1994a: Human microsporidial infections. Clin. Microbiol. Rev. 7: 426-461.

WEBER R., SAUER B., SPYCHER M.A., DEPLAZES P., KELLER R., AMMANN R., BRINER J., LUETHY R. 1994b: Detection of *Septata intestinalis* in stool specimens and coprodiagnostic monitoring of successful treatment with albendazole. Clin. Infect. Dis. 19: 342-345.

WILKINS J.H., JOSHI N., MARGOLIS T.P., CEVALLOS V., DAWSON C.R. 1994: Microsporidial kerato-conjunctivitis treated successfully with a short course of fumagillin. Eye 8: 703-704.

YAMAOKA M., YAMAMOTO T., IKEYAMA S., SUDO K., FUJITA T. 1993a: Angiogenesis inhibitor TNP-47 (AGM-1470) potently inhibits the tumor growth of hormone-independent human breast and prostate carcinoma cell lines. Cancer Res. 53: 5233-5236.

YAMAOKA M., YAMAMOTO T., MASAKI T., IKEYAMA S., SUDO K., FUJITA T. 1993b: Inhibition of tumor growth and metastasis of rodent tumors by the angiogenesis inhibitor O-(chloroacetyl-carbamoyl)-fumagillol (TNP-470; AGM-1470). Cancer Res. 53: 4262-4267.

YANAI S. H., OKADA S.H., SAITO K., KUGE Y., MISAKI M., OGAWA Y., TOGUCHI H. 1995: Antitumor effect of arterial administration of a medium-chain triglyceride solution of an angiogenesis inhibitor, TNP-470 in rabbits bearing VX-2 carcinoma. Pharm. Res. 12: 653-657.

YANASE T., TAMURA M., FUJITA K., KODAMA S., TANAKA K. 1993: Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines *in vitro* and *in vivo*. Cancer Res. 53: 2566-2570.

Received 16 July 1997

Accepted 17 November 1997