

Susceptibility of *Encephalitozoon cuniculi* to Several Drugs In Vitro

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In the light of the increased incidence of human *Encephalitozoon* infections and the absence of an established treatment protocol, a simple in vitro testing method to compare activities of drugs against *Encephalitozoon cuniculi* was developed. With this in vitro method, the 50% inhibitory concentrations of fumagillin, thiabendazole, albendazole, oxibendazole, and propamidine isethionate for *E. cuniculi* in rabbit kidney cells were determined. Itraconazole, toltrazuril, metronidazole, ronidazole, and ganciclovir were ineffective in this testing system.

A number of drugs have been used to treat microsporidial infections, often on an empirical basis, in mammalian and nonmammalian hosts (2-6, 8-14, 17, 19), but few in vitro studies have evaluated the drug susceptibility of microsporidia (1, 3, 15, 18).

In the light of the increased incidence of human *Encephalitozoon* infections and the absence of an established treatment protocol, the aim of the present study was to compare the activities of drugs used against *Encephalitozoon cuniculi* and to develop a simple in vitro testing system which allows objective evaluation.

MATERIALS AND METHODS

Culture system. *E. cuniculi* was isolated from a naturally infected rabbit at the Laboratory for Parasitology and Mycology of the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands) in 1984 and kept in culture since then. Rabbit kidney (RK13) cells, from a cell line isolated at this institute, were grown in 75-cm² tissue culture flasks (Greiner 658190; Greiner BV, Alphen aan den Rijn, The Netherlands). Experiments were done with eight-well tissue culture (TC) chamber slides (Lab Tek 177402; Nunc Inc., Naperville, Ill.) and 25-cm² TC flasks (Greiner 690160). Prior to each experiment, monolayers of RK13 cells were trypsinized and a cell suspension containing 4×10^5 cells per ml of culture medium was prepared. The wells of the TC chamber slides were filled with 0.4 ml of the cell suspension, and 8.5 ml was placed in the 25-cm² TC flasks. Monolayers became confluent in 1 or 2 days.

To initiate experiments, *E. cuniculi* spores were collected from spore-producing cultures and stored at 5°C. Later experiments were initiated with spores collected from parallel spore-producing cultures. *E. cuniculi* spores were counted in a hemocytometer, and the number was adjusted to 10^6 spores per ml of culture medium. This suspension was used to inoculate the confluent monolayers, giving 4×10^5 spores per well (2.5 spores per cell) and 5×10^6 spores per 25-cm² flask, respectively. At 24 h after infection, the remaining extracellular spores were removed. Culture media were replaced three times per week.

Culture medium and drug solutions. The culture medium consisted of M199 medium with Earle's salts (supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 5% fetal calf serum, and 50 µg of gentamicin per ml) with and without drugs or diluents.

Stock solutions of drugs, at a concentration of 1 or 5 mg/ml, were prepared in demineralized water (metronidazole, ronidazole, and ganciclovir [Cymevene; Syntex BV, Rijswijk, The Netherlands]), in methanol (fumagillin), or in dimethyl sulfoxide (DMSO) (thiabendazole, albendazole, and oxibendazole). Itraconazole (Trisporal; 100 mg of itraconazole per capsule; Janssen Pharmaceutica BV, Tilburg, The Netherlands) was brought into suspension in 4 ml of a 2 N aqueous

HCl solution, and after addition of 16 ml of DMSO, a transparent gel was formed, which dissolved readily in the culture medium. The stock solutions were diluted (1,000 or 200 times) in culture medium to give the working solutions of the drugs, which were stored at 5°C. Toltrazuril (Baycox; 2.5% [wt/vol]; Bayer, Leverkusen, Germany) and propamidine isethionate (Brolene eyedrops; 0.1% [wt/vol]; Rhône-Poulenc Rorer, Amstelveen, The Netherlands) were diluted (5,000 and 200 times, respectively) in culture medium and stored at 5°C. Since benzalkonium chloride is used as a preservative in Brolene eyedrops, our source for propamidine isethionate, and a number of drugs were dissolved in DMSO or methanol, these compounds were tested in extra control cultures at concentrations identical to the concentrations in the working solutions of the corresponding drugs.

Evaluation of drug effect. Drugs were first screened in duplicate and triplicate cell cultures on TC slides at a concentration of 5 µg/ml of culture medium (a concentration which was also used in other in vitro studies [3, 15]) in two or three (fumagillin and metronidazole) independent experiments. In these experiments, drugs were added to 1-day-old cultures to establish the drug effect on proliferation of the parasite. To evaluate the ability of drugs to prevent host cell infection, drugs were also added at a concentration of 5 µg/ml simultaneously with inoculation (in duplicate cultures) in one experiment. The culture media with and without drugs were replaced every 3 days.

After 8 days of drug treatment, the chambers and the silicone seal were removed from the TC chamber slides, which were then fixed in methanol and Giemsa stained. The effects of drugs were determined by counting the intracellular clusters of proliferating parasites in control and drug-treated cultures. The 8-day incubation period allowed the individual parasites which invaded the host cells to develop to clusters of parasites measuring 4 to 25 µm in diameter in control cultures, making microscopic evaluation easier and thus more reliable. Moreover, spore formation and subsequent reinfection of RK13 cells was not seen before approximately 14 days after inoculation, making sure that proliferation was measured within one generation of parasites.

To determine the 50% inhibitory concentration (IC₅₀), serial two- and fivefold dilutions of the effective drugs were made and applied to duplicate and triplicate 1-day-old *E. cuniculi* cultures on TC slides in two independent experiments. Eight days later, growth inhibition was determined as mentioned before. Culture media with and without drugs were replaced every 3 days. To investigate whether the active drugs possess lethal or growth-inhibiting properties, triplicate 1-day-old cultures of *E. cuniculi* were incubated with drugs (5 µg/ml) for 48 h, after which the parasites were allowed to multiply in the absence of drugs. Moreover, these drugs were tested at 5 µg/ml on 8-day-old cultures of *E. cuniculi* on TC slides with four to six cultures per drug in a single experiment. Media with and without drugs were replaced every 3 days between days 8 and 16 after inoculation in the latter experiment.

Spore formation in drug-treated cultures was determined in single TC flask cultures which were incubated for 3 weeks. Culture media with and without drugs were replaced every 3 days. Spores which were formed in the presence of (inactive) drugs were applied to fresh monolayers to see whether these spores were able to infect new RK13 cells.

The effect of each drug and the influence of the preservative and solvents were evaluated by examination of each culture in a standardized manner. First, areas were identified by microscopy where the monolayer was confluent in methanol-fixed and Giemsa-stained cultures. Second, 50 to 150 colonies were counted; this means that 30 to 50 microscopic views were examined for each culture. This method guarantees maximum comparability between cultures. The mean num-

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ber of cells was determined to be 31.2 ± 4.1 per microscopic field at a magnification of $\times 1,000$.

Because of variation in the number of infective spores (which are morphologically indistinguishable from noninfective spores [16]), the level of infection varied between experiments. Control cultures of three independent experiments ($n = 35$), which were initiated with spores that had been stored at 5°C for several weeks, had an average of 0.045 ± 0.006 parasite colony per cell. Control cultures of five independent experiments ($n = 33$) that were started with fresh spores from a spore-producing parallel culture had 0.20 ± 0.03 parasite colony per cell. The ratio of colonies of proliferating parasites to added spores varied from 1.8% in control cultures started with stored spores to 8% in control cultures started with fresh spores.

Percent parasite growth inhibition of each single culture was calculated as follows: $[1 - (\text{no. of parasite colonies in drug-treated cultures}/\text{no. of parasite colonies in control cultures without drugs})] \times 100\%$. Subsequently, the mean growth inhibition rate \pm the standard error of the mean was calculated. The statistical computer program NCSS (NCSS, Kaysville, Utah) was used for data analysis (analysis of variance) and graphic determination of IC_{50} s (Curve Fitter).

RESULTS

In our culture system, proliferating *E. cuniculi* clusters (ranging in size from 4 to 25 μm) were clearly visible inside the monolayer cells within 8 days after inoculation. We first examined the possible adverse effect of solute and drugs on the monolayer. Benzalkonium chloride, DMSO, and methanol, used at concentrations which are also present in the drug dilutions (5×10^{-5} , 0.5, and 0.1%, respectively), had no influence on the growth of *E. cuniculi* (results not shown). Propamidine isethionate, oxibendazole, and albendazole, at concentrations of 5, 10, and 50 $\mu\text{g}/\text{ml}$, had toxic effects on 1-week-old monolayer cells in our experiments. The monolayer cells became vacuolized and the monolayer broke up after 3 and 2 weeks of culture, respectively. This is in agreement with the observations for albendazole made by Beauvais et al. (1). More importantly, the IC_{50} s reported below are all far below the toxic values, and the toxic effect on monolayer cells therefore has minimal effect on the IC_{50} determination. The toxic effect was less (propamidine isethionate) or absent (oxibendazole and albendazole) at a concentration of 5 $\mu\text{g}/\text{ml}$ when 2-day-old monolayers were used. Propamidine isethionate concentrations of 10 and 50 $\mu\text{g}/\text{ml}$ of culture medium were also toxic to 2-day-old monolayers.

At 5 $\mu\text{g}/\text{ml}$, the following five drugs showed a strong inhibitory effect on the growth of *E. cuniculi*: fumagillin (83.8% \pm 2.2%), thiabendazole (89% \pm 2.3%), albendazole (78.4% \pm 5.2%), oxibendazole (90.4% \pm 3.3%), and propamidine isethionate (44.0% \pm 6.9%). Cultures treated with itraconazole, toltrazuril, metronidazole, ronidazole, and ganciclovir showed no significant difference from control cultures at a concentration of 5 $\mu\text{g}/\text{ml}$ (overall analysis of variance significance, $P < 0.001$ by Scheffe's procedure at $\alpha = 0.05$).

There was no difference between cultures which had received drug treatment simultaneously with inoculation and cultures in which treatment was started 24 h after inoculation (overall analysis of variance significance, $P < 0.001$ by Scheffe's procedure at $\alpha = 0.05$). The IC_{50} s of the active compounds varied between 8.6×10^{-4} and 5 $\mu\text{g}/\text{ml}$ (Table 1). Maximum inhibition levels were already reached at 0.005, 0.5, 0.01, and 0.01 $\mu\text{g}/\text{ml}$ with fumagillin, thiabendazole, albendazole, and oxibendazole, respectively. Even at the highest concentration tested (50 $\mu\text{g}/\text{ml}$), some pycnotic parasites were visible in all of the drug-treated cultures.

When *E. cuniculi* cultures were treated with fumagillin, thiabendazole, oxibendazole, albendazole (5 $\mu\text{g}/\text{ml}$), and propamidine isethionate (50 $\mu\text{g}/\text{ml}$) during the 48 h after infection, the cultures treated with thiabendazole, albendazole, and oxibendazole did not recover within 14 days after infection, although sporadically clusters of small numbers of individual

TABLE 1. IC_{50} s of drugs effective against *E. cuniculi* determined in two independent experiments in duplicate and triplicate cultures

Drug	Mean IC_{50} ($\mu\text{g}/\text{ml}$) \pm SEM
Fumagillin	0.00086 \pm 0.00015
Itraconazole	>5
Ronidazole	>5
Metronidazole	>5
Toltrazuril	>5
Thiabendazole	0.30 \pm 0.04
Albendazole	0.0044 \pm 0.0008
Oxibendazole	0.0015 \pm 0.00015
Ganciclovir	>5
Propamidine isethionate	$\pm 5^a$

^a Exact determination of the IC_{50} of propamidine isethionate was not possible because of the toxic effect of this drug on the monolayer cells.

parasites were seen. The cultures treated with fumagillin had few proliferating *E. cuniculi* colonies. In cultures treated with propamidine isethionate, the monolayer cells were destroyed within 14 days after infection, but large numbers of intact parasites were found at that time.

Treatment of 8-day-old *E. cuniculi* cultures with these drugs did not result in complete clearance of parasites from the monolayer cells (Table 2) and could not prevent spore formation in these cultures.

In the presence of fumagillin, thiabendazole, albendazole, oxibendazole, and propamidine isethionate, no spores were formed after 3 weeks of treatment at a concentration of 5 $\mu\text{g}/\text{ml}$, whereas the control cultures had approximately 10^4 spores per ml of supernatant at that time.

Spores formed in the presence of the inactive drugs itraconazole, toltrazuril, metronidazole, and ganciclovir were able to infect fresh RK13 cells, resulting in infection levels comparable to those of control cultures and subsequent spore formation 2 weeks after inoculation (results not shown). Spores formed in the presence of ronidazole were not tested.

DISCUSSION

The in vitro method reported here, using *E. cuniculi* in rabbit kidney cells on TC slides, is a simple method for evaluation of drugs in the search for more active compounds against microsporidia. Fixed and stained TC slides are easily conserved by use of a mounted coverslip and can be conveniently stored for future use. When fresh spores were used, the infection rate was considerably higher than that obtained with spores that had been stored at 5°C , but this did not influence the mean coefficient of variation between experiments (16.6% \pm 6% and 16.6% \pm 2.2%, respectively), which is slightly higher

TABLE 2. Growth inhibition in *E. cuniculi* cultures after application of drugs at day 8 after inoculation at a concentration of 5 $\mu\text{g}/\text{ml}$ ^a

Drug	Mean % growth inhibition \pm SEM	No. of cultures
Fumagillin	67.9 \pm 1.8	6
Thiabendazole	71.4 \pm 2.1	6
Albendazole	63.0 \pm 3.1	6
Oxibendazole	21.8 \pm 4.1	4
Ganciclovir	0 \pm 5	6

^a Between the first application of drugs and day 16 after inoculation, when the cultures were fixed and stained, the culture medium was replaced three times per week with fresh medium containing drugs.

than that found by Beauvais et al. (1) in their experiments. In those studies, drugs were applied 5 h after inoculation, whereas in our system, the parasites were allowed to proliferate for 24 h before they were exposed to the drugs tested. Moreover, drugs were also tested in our experiments with parasites which had been growing without drugs for 8 days, resembling the *in vivo* situation in which, among others, older parasites are subjected to drug treatment.

Most of the drugs claimed to be effective against a number of microsporidial parasites were tested with this *in vitro* system. Our finding that fumagillin, albendazole, and propamidine isethionate have a strong inhibitory effect is in accordance with those of others (1–5, 9, 12, 16).

The *in vitro* concentration we used for primary drug screening in our experiments (5 µg/ml) is similar to the concentrations used *in vitro* by others (3, 16). Moreover, at a concentration of 5 µg/ml, cytotoxic effects were seen in some cases, which made even higher drug concentrations undesirable.

Although benzalkonium chloride at a concentration of $5 \times 10^{-5}\%$ did not inhibit the growth of *E. cuniculi* in our experiments, it is very possible that *in vivo* this compound enhances the action of propamidine isethionate. Waller (18) found an inhibitory effect of 0.1% benzalkonium chloride on *E. cuniculi* *in vitro*, while the concentration of this compound used as preservative in Brolene eyedrops is 0.01%. In our *in vitro* experiments, host cell infection was not prevented by any of the drugs tested. Fumagillin, thiabendazole, oxibendazole, and albendazole seemed very efficient in prevention of *E. cuniculi* proliferation at a concentration of 5 µg/ml. After 8 days of treatment, no proliferating colonies were seen in these cultures, but merely empty vacuoles with some individual parasites were found and no spores were formed. Treatment of *E. cuniculi* cultures with thiabendazole, oxibendazole, and albendazole (5 µg/ml) for 48 h after infection was enough to decimate the parasites. Treatment with fumagillin for 48 h after infection resulted in a decrease in the number of viable parasites, but the remaining parasites resumed proliferation to form normal colonies when the drug was removed, in contrast to the cultures treated with thiabendazole, oxibendazole, and albendazole. Treatment of 8-day-old cultures with these drugs did not clear the parasites from their host cells, and there were remaining colonies (Table 2) and few spores were being formed. This is in contrast to the results of Shaddock (15), who found no parasite growth in both rabbit kidney and canine embryo cells upon treatment with 5 µg fumagillin per ml from days 4 to 16 after inoculation.

Even at a drug concentration of 50 µg/ml (150 to 60,000 times the IC_{50}), some individual parasites were seen at day 6 after inoculation in our experiments. This is not in agreement with the results of Beauvais et al. (1), who found no parasites at fumagillin concentrations higher than 0.05 µg/ml and reported 100% effectiveness of albendazole at a concentration of 0.01 µg/ml, although degenerated parasites were found at that concentration. Since parasite viability cannot be evaluated microscopically, we also included the degenerated parasites in the parasite counts in our experiments, which in fact decreased the calculated effectiveness of fumagillin, thiabendazole, oxibendazole, and albendazole in our studies. A possible explanation for the apparently higher effectiveness of fumagillin found by Beauvais et al. (1) could be the fact that in their experiments, drug treatment was started 5 h after inoculation, whereas in our experiments, the time between inoculation and subsequent treatment was 24 h.

Propamidine isethionate, applied simultaneously with or 1 day after infection of monolayer cells, was effective in preventing parasite proliferation: after 8 days of treatment, no prolif-

erating colonies of *E. cuniculi* were seen. Treatment of 8-day-old cultures with 5 µg/ml (approximately the IC_{50}) was unsuccessful: although the number of spores produced was slightly decreased compared with that of the controls, the morphology of the parasite and the estimated number of colonies were equal to the control values. A serious drawback of propamidine isethionate is the extensive cytotoxic effect that was seen in all treated cultures, even at the IC_{50} .

We were not able to confirm the antimicrosporidial action of itraconazole, toltrazuril, and metronidazole at the concentrations used in our *in vitro* system. This is in accordance with the results of Canning and Hollister (3), who found no effect of these drugs *in vitro* on *Nosema bombysis* in *Spodoptera frugiperda* cells at a concentration of 5.3 µg/ml; Beauvais et al. (1) also found no inhibition by metronidazole at concentrations of up to 10 µg/ml. However, itraconazole was reported to exhibit moderate inhibition of *E. cuniculi* growth in MRC5 cells at a concentration of 1 µg/ml, which was also toxic to the host cells (1). Ronidazole, which was more effective than metronidazole on nitroimidazole-resistant *Trichomonas gallinae* (7), was ineffective in our study. The use of metronidazole, however, has been associated with rapid clinical improvement of *Enterocytozoon bienersi*-associated diarrhea in a number of AIDS patients (6, 14).

Ganciclovir, at a concentration of 5 µg/ml, did not inhibit *E. cuniculi* growth in our experiments. *In vivo* use did not clearly indicate any action of this drug against *E. bienersi*, since it was administered simultaneously with metronidazole, implying that either of these drugs could be responsible for the clinical improvement of diarrhea in one AIDS patient (14).

The drugs found most effective in our study are fumagillin, albendazole, oxibendazole, and thiabendazole. Treatment of keratoconjunctivitis caused in AIDS patients by *E. hellem* with topical metronidazole or thiabendazole or oral itraconazole, among other drugs, resulted in no improvement (4). In that study, water-insoluble thiabendazole was used in a suspension, whereas in our *in vitro* experiments, we used a solution of thiabendazole in DMSO, which was diluted further in culture medium. Fumagillin, topically administered in water-soluble form (Fumidil B; Mid-Continent Agrimarketing, Overland Park, Kans.), was used to reduce clinical symptoms markedly in two cases of keratoconjunctivitis (4). Albendazole has been used in human AIDS patients for treatment of intestinal microsporidiosis with various results (2, 5). To our knowledge, oxibendazole has never been used to treat human microsporidiosis.

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