Efficacy of pyrvinium pamoate against *Cryptosporidium parvum* infection *in vitro* and in a neonatal mouse model

Running title: Inhibition of *Cryptosporidium* by pyrvinium

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Effective approved drug therapy for *Cryptosporidium* in immunocompromised patients does not exist. Here we investigated the non-absorbed antihelminthic drug, pyrvinium pamoate, for growth-inhibition against the intestinal protozoan parasite, *Cryptosporidium parvum*. Fifty percent growth inhibition in human enterocytic HCT-8 cells by a quantitative alkaline phosphatase immunoassay was 354 nM for pyrvinium. For comparison, in the same assay, fifty percent growth inhibition was 711 µM for paromomycin and 27 µM for chloroquine. We used a neonatal mouse model to measure *in vivo* anti-*Cryptosporidium* activity of pyrvinium pamoate. Beginning three days after infection, pyrvinium at 5 mg/kg/day or 12.5 mg/kg/day was administered to the treatment group mice for four or six consecutive days. Nine days after infection, the mice were sacrificed and drug efficacy was determined by comparing numbers of oocysts present in fecal smears of treated versus untreated mice. Trophozoite infection intensity in the ileocaecal intestinal region was also compared using H&E stained histological slides. We observed >90% reduction in infection intensity in the pyrvinium treated mice compared to the untreated controls, along with a substantial reduction in tissue pathology. Based on these results, pyrvinium pamoate is a potential drug candidate for treatment of cryptosporidiosis in immunocompetent and immunocompromised individuals.
INTRODUCTION

Cryptosporidium is an important apicomplexan protozoan pathogen that contributes significantly to diarrheal disease throughout the world, in both humans and animals (9, 10, 21). Infections in immunocompetent hosts are generally restricted to the intestinal epithelium, causing an acute, self-limiting gastroenteritis. However, in AIDS patients and other immune compromised individuals, infection can result in life-threatening, chronic diarrhea and may spread to extra-intestinal locations (16, 26). Although the efficacy of numerous antimicrobial agents against Cryptosporidium infection has been tested using animal and cell culture models, there is currently no reliably effective therapeutic for the treatment of chronic cryptosporidiosis in immune compromised patients (30).

Recently, nitazoxanide (NTZ), a nitrothiazole benzamide, was FDA approved for the treatment of cryptosporidiosis in immunocompetent adults and children aged >1 year (2). However, while clinical studies are ongoing, the efficacy of NTZ for treatment of Cryptosporidium infection in immune compromised patients has not yet been demonstrated (1). An IC_{50} of 3.8 µM has been reported for nitazoxanide in cell culture (12). In a neonatal mouse model, oral administration of NTZ at 150 mg/kg reduced oocyst output to less than 5% of that seen in controls (6); however, NTZ at 100 mg/kg or 200 mg/kg was ineffective at reducing parasite burdens in a SCID mouse model (24). Prior to FDA approval of NTZ as an anti-Cryptosporidium therapeutic, the glycoside antibiotic paromomycin (PRM) was one of the most widely used agents to treat Cryptosporidium infections but still was not reliably effective or ever approved by the FDA. Though PRM performs well against Cryptosporidium in animal and cell culture models (24), results of this drug in human clinical trials have been equivocal (13, 15).
Reported IC$_{50}$ values for paromomycin have varied, ranging from 83 µM (29) to >100 µM (17). In a neonatal mouse model, PRM at 50 mg/kg reduced oocyst shedding to less than 2% of that seen in controls (6).

Pyrvinium pamoate is a cyanine dye, a substituted quinoline, that has been used to treat pinworm (Enterobius vermicularis) infections (5) and as well as strongyloidiasis in humans (27). In 1955, pyrvinium received FDA approval for enterobiasis treatment in adults and children (NDA-9582). The usual human dosage is 5 mg/kg/day, up to 350 mg; however, pyrvinium has been used safely in humans with doses as high as 35 mg/kg/day for 3-5 days. The drug has no measurable absorption across the gastrointestinal tract and 90% is excreted in feces (23). With the discovery of more effective and broad-spectrum agents for treatment of helminth infections, the drug has been discontinued in the United States, but is still available under the Parke-Davis label in Europe. In a recent screen of FDA approved drugs for anti-malaria activity, pyrvinium was identified with an IC$_{50}$ of 3 nM against the apicomplexan parasite, Plasmodium falciparum (7). Despite potent in vitro activity, the drug was not pursued for malaria treatment since there is no measurable absorption of pyrvinium into the bloodstream. However, because Cryptosporidium infection is generally confined to the gastrointestinal epithelium, we hypothesized that pyrvinium would be effective against this luminal apicomplexan protozoan for which no effective therapy is currently approved for immunocompromised patients. Here we report the efficacy of pyrvinium pamoate against C. parvum in cell culture and using a neonatal mouse model.
MATERIALS AND METHODS

C. parvum oocysts.

The C. parvum (Iowa isolate) oocysts were obtained from experimental infection of a female Holstein calf, extracted from the feces using continuous flow centrifugation, purified by cesium chloride gradient centrifugation, and stored at 4°C in phosphate-buffered saline (PBS) (pH 7.4).

Drugs.

Pyrvinium pamoate and paromomycin were purchased from MP Biomedicals, (Solon, OH) and chloroquine from Sigma (St. Louis, MO). Paromomycin and chloroquine were diluted into water just prior to use. Pyrvinium was first dissolved in dimethyl sulfoxide (DMSO) or ethanol and then diluted in water prior to use.

Pyrvinium activity in cell culture.

HCT-8 cells (CCL-244) were obtained from the American Type Culture Collection (Manassas, VA), and maintained in RPMI 1640 medium supplemented with 10% Opti-MEM (GIBCO-BRL, Grand Island, NY), 2% fetal bovine serum (FBS) and 2 mM L-Glutamine. To determine in vitro drug efficacy, a quantitative alkaline phosphatase immunoassay was used to measure parasite growth inhibition in cell culture as described previously (11, 29). Briefly, 96-well, flat-bottom microtiter plates were seeded with 5 × 10^4 HCT-8 cells 24 hours prior to infection. For infection, maintenance media was removed and 5 × 10^3 oocysts were added to wells in 100 µL of RPMI 1640 supplemented with 10% FBS and 0.05% bile salts. After
incubation at 37°C for 90 min to induce excystation and allow cell invasion, cells were washed once with warm PBS to remove unexcysted oocysts and free sporozoites. Negative control wells to measure background absorbance received $5 \times 10^3$ non-viable oocysts subjected to 5 cycles of freezing in liquid nitrogen and thawing in a 37°C water bath. Drugs were diluted to appropriate concentrations and added to cells in 150 µL of parasite growth medium. For pyrvinium treatment, final DMSO levels were 0.5%. Vehicle control wells for the pyrvinium group received growth medium containing 0.5% DMSO. The glycoside antibiotic, paromomycin, and another quinoline, chloroquine, were used as comparison drugs. Each drug concentration was tested in triplicate wells in 2 independent experiments. Plates were incubated for 48 hours at 37°C in a 5% CO$_2$/95% humidified air incubator, then fixed in 8% formalin for 2 hours at room temperature. After fixation, plates were washed 3× with PBS, then blocked for 1 hour with 300 µL of a blocking solution consisting of 5% bovine serum albumin (BSA) and 0.002% Tween-20 in PBS. Rat anti-Cryptosporidium polyclonal sera raised to washed sporozoite membrane proteins (29) (provided by Steve Upton at Kansas State University) were diluted 1:500 in a solution of 1% BSA/0.002%Tween-20 in PBS and 50 µL was added to each well. After a 30 min incubation at room temperature, the wells were washed 3× with PBS and 50 µL of goat anti-rat horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in 1% BSA/0.002% Tween-20/PBS was added to each well. After 20 min, plates were washed 3× with PBS and 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB, KPL, Gaithersburg, MD) solution was added. After 10 minutes, 100 µL of Stop Solution (KPL, Gaithersburg, MD) was added to each well and plates were read at 450 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA). The background absorbance reading taken from wells receiving freeze-thawed oocysts was subtracted from all drug treated and control well absorbances and the percent growth inhibition was
calculated as 1- (mean A_{450} in infected wells with drug / mean A_{450} in infected wells without drug) × 100.

The cytotoxicity of pyrvinium pamoate to HCT-8 cells was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), which measures the number of viable, metabolically active cells by their ability to convert a tetrazolium compound, MTS, to a colored formazan product. This assay has been described previously (21, 36) for use in determining cytotoxicity of drugs tested against Cryptosporidium using multiple cell lines. Briefly, 96-well microtiter plates were seeded with 10^4 cells/well and incubated at 37°C for 24 hours. Cells were then exposed to various drug concentrations or 0.5% DMSO for 48 hours. Following addition of the CellTiter 96 AQueous One Solution Reagent to wells, plates were incubated for 1.5 hours at 37°C, after which time absorbance was read at 490 nm. The background absorbance reading from wells receiving media but no cells was subtracted from drug treated and solvent control well readings and percent cytotoxicity was calculated as 1 – (mean A_{490} drug treated wells / mean A_{490} solvent control wells) × 100. Chloroquine cytotoxicity was measured by the Neutral Red assay which measures viable cells by accumulation of dye into lysosomes. For chloroquine this gives more sensitive results (28).

Pyrvinium activity in neonatal mice.

The in vivo efficacy of pyrvinium pamoate was tested using a neonatal mouse model for Cryptosporidium infection, which is well characterized and has been described previously (6). Briefly, 3-day old Balb/C mice (National Cancer Institute, Frederick, MD) initially in groups of 4-6, then groups of 8-11 (Table 1) were orally inoculated with 10^5 C. parvum oocysts. Three days post-infection, treatment regimens were initiated. Pyrvinium was administered orally at 5
mg/kg/day or 12.5 mg/kg/day for 4 or 6 consecutive days. Five mg/kg/day is the dose of pyrvinium used for treatment of *Enterobius* in humans. Pyrvinium was initially dissolved in 100% DMSO or ethanol. Prior to administration, drug stocks were diluted to the desired concentration in water so that final solvent levels were 5% for DMSO and 5% or 10% for ethanol. Vehicle treated mice received 5% DMSO in water or 5 or 10% ethanol in water. As a positive control comparison drug, paromomycin was administered at 100 mg/kg/day for 4 or 6 consecutive days. Vehicle control mice received an equal volume of water. All mice were euthanized by cervical dislocation on day 9 post-infection, when one study showed that levels of oocyst shedding peak in infected neonatal mice (20). To quantify oocyst shedding, fecal smears were made from 2-3 microliters of stool removed from the distal colon. Thin smears were methanol-fixed and oocysts were stained with immunofluorescent antibodies (IFA) using a commercially available test kit (MeriFluor *Cryptosporidium/Giardia*, Meridian Bioscience Inc, Cincinnati, OH) as per manufacturer instructions. For each smear, oocyst counts were made from 21 microscopic fields that represented a vertical transect through the center of the IFA slide well at 400x magnification. This enabled an approximate standardization of counts despite slight variation in density of fecal smears across the area of the slide wells. Levels of *C. parvum*, trophozoite stage, in the intestinal epithelium were evaluated for mice treated for 6 days with 5 mg/kg pyrvinium or 100 mg/kg paromomycin, and their respective controls. To quantify trophozoites, 1 cm intestinal sections were excised at points 1 cm, 2 cm, 3 cm, 4 cm and 6 cm proximal to the appendix. The appendix and ileocecal junction with 1 cm of proximal colon was collected separate from the distal 2 cm of colon. Each tissue section was fixed separately in neutral-buffered formalin, prepared by standard histological procedures and stained with hematoxylin-eosin (H&E). Duplicate 1 cm sections from all mice were microscopically
examined in a blinded fashion by two readers, and the number of trophozoites per 1000 epithelial cells was determined, examining multiple fields on both ends of each tissue section. The mean number of trophozoites /1000 cells and standard deviation was determined for each tissue section within each treatment group. An overall histology score for comparing trophozoites in treated versus untreated groups was derived for each mouse by summing the total number of trophozoites per 1000 cells for each of the ileal sections as well as the appendix, cecum and colon.

Statistical analysis.

The statistical analysis was performed using STATA 8.0 (copyright © 1984-2003 Stata Corporation, College Station, TX). A Kruskal-Wallis non-parametric test of equality was used to determine if there were any significant differences in oocyst or trophozoite stage parasite counts between the different treatment groups. Pairwise comparisons using a non-parametric two-sample Mann-Whitney test were used to determine whether reductions in oocysts and intestinal trophozoites for treated versus untreated control mice were statistically significant, and whether levels of oocyst shedding and trophozoites were equivalent for paromomycin and pyrvinium treated mice. Results were considered to be significant at P < 0.05.

RESULTS

Drug activity in vitro.
The effects of pyrvinium, paromomycin and chloroquine treatment on parasite growth in cell culture were investigated by exposure of infected cells to the antimicrobial agents for 48 hours (Fig. 1). Fifty percent inhibitory concentrations (IC_{50}) for pyrvinium, paromomycin and chloroquine were calculated as 354 nM, 711 µM and 27 µM, respectively, indicating that pyrvinium was ~2000 times more potent than paromomycin and ~76 times more potent than chloroquine in vitro. Cytotoxic effects of pyrvinium on HCT-8 cells after 48 hours of exposure were minimal (< 15% reduction in cells) at all dose levels except the highest dose, 1.6 µM, where a 40% reduction in cell numbers was observed. Chloroquine cytotoxicity by the Neutral Red assay was 15% reduction in cells at 100 µM, 12% at 80 µM and <5% at all lower doses. Cytotoxicity of paromomycin in HCT-8 cells has been previously described as negligible by Gargala (12).

**Drug activity in neonatal mice.**

The efficacy of pyrvinium, in comparison to that of paromomycin, was tested in a neonatal mouse model (20). Oocyst shedding by *C. parvum*-infected mice, as one indicator of drug efficacy, was enumerated by IFA on distal colonic fecal smears made at the time of necropsy. The effects of various treatment regimens on oocyst shedding are presented in Table 1. At the 5mg/kg/day dose, >90% reduction in oocyst shedding was observed in the pyrvinium-treated mice compared to the vehicle control mice. Based on the confidence intervals, the level of oocyst shedding in 5 mg/kg/day pyrvinium treated mice was equivalent to that of 100 mg/kg/day paromomycin treated mice, though paromomycin was administered at a 20 fold higher dose. Slightly higher levels of oocyst shedding were observed at the 12.5 mg/kg/day pyrvinium dose, which may be associated with drug-induced toxicity, as 3 mice in this treatment
group died though there were no deaths in any other group. However, after 4 days of pyrvinium
treatment at either dose, there was no significant reduction in mouse weight compared to controls
(data not shown).

In addition to evaluation of oocyst shedding, levels of *C. parvum* trophozoites in the
intestinal epithelium were enumerated by microscopic evaluation of H&E stained histological
sections from treated and untreated mice (Fig. 2). The comparative effects of treatment with
pyrvinium or paromomycin for 6 consecutive days on levels of *C. parvum* trophozoites in
individual intestinal sections are shown in Fig. 3. Parasite densities tended to be highest at the
terminal ileum, adjacent to the appendix. Though parasite densities were generally lower in the
appendix than in the ileum, we observed that low numbers of trophozoites could often be found
there even when the ileum appeared to be clear of infection, indicating that the appendix could
act as a reservoir for *Cryptosporidium* in the GI tract, from which the ileum could be re-
populated. Both drugs showed near 90% or greater reduction in the mean number of trophozoites
in the terminal ileum, appendix and colon.

An overall score for comparing levels of trophozoites in treated versus untreated groups
was derived by summing the total number of trophozoites per 1000 cells over all of the tissue
sections for each individual mouse. Box plots displaying the differences in this summary score
between treatment groups are presented in Fig. 4. Based on mean summary score values, there
was an 85% reduction in trophozoites in the pyrvinium treated group and a 99% reduction in the
paromomycin treated group compared to their respective controls. However, the mean summary
score for the pyrvinium group was skewed by a single outlier mouse that had levels of
trophozoites equivalent to those seen in control mice, though levels of oocyst shedding by this
mouse were much lower than seen in controls. The reduction in trophozoites for both pyrvinium
and paromomycin treatment groups compared to their controls was statistically significant (P < 0.05) but there was no statistical difference in numbers of trophozoites between paromomycin and pyrvinium treated mice (P = 0.2682).

**DISCUSSION**

In light of the severe consequences of *Cryptosporidium* infection in HIV/AIDS patients and other immune compromised individuals, there is an urgent need for an anti-*Cryptosporidium* therapeutic that is reliably effective in these high-risk populations. Although numerous other drugs have demonstrated some activity against *Cryptosporidium* in pre-clinical studies, the safety of many of these drugs for human treatment has not been established. Taking into consideration the urgent need for a therapeutic and the considerable time and cost required to move a drug through the FDA approval process, one strategy to reduce the time required for an effective drug to reach the market is to test drugs that have been previously FDA-approved for other uses for activity against *Cryptosporidium*. In this report, we assessed the *in vitro* and *in vivo* efficacy of pyrvinium pamoate, an antihelminthic drug approved for treatment of enterobiasis.

In this study we demonstrated that pyrvinium pamoate was a potent inhibitor of *C. parvum* growth, both in an *in vitro* cell culture system and in neonatal mice. In *C. parvum*-infected HCT-8 cells, the IC$_{50}$ for pyrvinium (354 nM) was ~2000× lower than the observed IC$_{50}$ for paromomycin and ~76× lower than that of chloroquine. In previous studies, an IC$_{50}$ for paromomycin as low as 83 µM has been reported (29), in contrast to the IC$_{50}$ of 711 µM reported here, but variability in IC$_{50}$ values for paromomycin have been noted in the literature. In one study, paromomycin treatment at 100 µM resulted in only 40% growth inhibition (17) and in
another study, a concentration of 500 µM failed to achieve a measurable decrease in *C. parvum* levels *in vitro* (3). Concentrations of paromomycin as high as 3200 µM have been needed to achieve >80% reduction in parasite numbers (24). Several *in vitro* systems for testing the anti-*Cryptosporidium* activity of drugs have been described in the literature, and variations in the different procedures, along with variability in oocyst infectivity and excystation rates, can make comparison of results between laboratories difficult, as is seen with IC₅₀ results for paromomycin. In contrast to our observations for paromomycin treatment, the *in vitro* activity of chloroquine against *C. parvum* in this study was consistent with previous findings where a 20 µM dose produced 33% growth inhibition (3).

Pyrvinium was also a potent inhibitor of parasite growth in neonatal mice. A dose of 5 mg/day was sufficient to reduce oocyst shedding to 4-7% of that seen in controls. This level of reduction was equivalent to that seen for mice treated with 100 mg/kg/day paromomycin, a 20 fold higher drug dose. A 2.5 fold increase in the pyrvinium dose failed to further reduce oocyst shedding levels below those seen for the 5 mg/kg group. Unexpectedly, oocyst shedding increased slightly at the 12.5 mg/kg dose, possibly caused by drug-induced diarrhea, which was more severe in this treatment group than at the 5 mg/kg dose. Three deaths provided further evidence of drug toxicity at the 12.5 mg/kg dose, which may have resulted from increased absorption of pyrvinium from the gastrointestinal tract in neonatal mice. Acute oral toxicity studies have shown that higher doses of pyrvinium pamoate are tolerated in adult mice. In one report, doses as high as 125 mg/kg were well tolerated (5). In another study, treatment with 128 mg/kg pyrvinium resulted in >50% mortality, though a dose of 64 mg/kg was associated with a 93.5% survival rate (25). Results from drug studies in other animals, including rats, dogs and monkeys, demonstrate the relatively low toxicity of more than 100 mg/kg/day of pyrvinium
pamoate following oral administration (25) and doses as high as 35 mg/kg have been safely used in humans for the treatment of strongyloidiasis (27). However, the observed low dose toxicity of pyrvinium pamoate in neonatal mice prevents dose-escalation studies using this animal model for cryptosporidiosis, which will have to be investigated using an alternative model system such as a piglet diarrhea model (24).

Though intensity of oocyst shedding was equivalent for pyrvinium and paromomycin treated groups, the reduction in mean levels of trophozoites in the intestinal epithelium was greater for paromomycin treated mice than for pyrvinium treated mice, compared to their respective controls (99% and 85% reduction, respectively). However, this difference between the two drug treatment groups was not statistically significant. The mean histology score for the pyrvinium group was significantly skewed by a single outlier mouse that had levels of trophozoites in the ileum equivalent to those seen in controls, though levels were reduced in the appendix, cecum and colon. As a result, the mean histology score for this group was \( \sim 33 \times \) higher than the median value and was associated with very large standard deviations. Surprisingly, the oocyst count from the fecal smear of this outlier mouse was lower than oocyst counts from several other mice in the treatment group. To verify the IFA results, stool remaining in the colon section on the histology slide from the outlier mouse was examined for the presence of oocysts, but no evidence of large numbers of oocysts was seen anywhere in the colon. The reasons for the discrepancy between oocyst shedding levels and the histology results for this mouse are unclear. It is possible that different mechanisms of action are responsible for the effects of pyrvinium on trophozoites and oocyst production. Without a better understanding of the mechanism of Cryptosporidium inhibition by pyrvinium, it is difficult to explain these results. It is also interesting to note that the two littermates of the outlier mouse had the second and third highest
numbers of trophozoites in the group, indicating that some shared exposure, such as the nursing habits of the dam, may have affected the response of these mice to drug treatment. One possible explanation is that the dam was providing a sub-optimal amount of milk, which along with drug-induced diarrhea, could have contributed to the malnourishment of these mice. The effects of malnourishment on severity of *Cryptosporidium* infection are well known (19).

The anti-parasitic mechanism of action of pyrvinium has not been studied in depth and consequently is not well understood. The proposed mechanism of action in intestinal helminths has been inhibition of respiration in aerobes or interference with exogenous glucose utilization (8, 22). Despite mutagenic activity in bacteria and yeast, there has been no evidence of genotoxicity in mammalian cell lines (18) or in the colon of mice administered pyrvinium at doses up to 12.5 times the recommended human dose (14). In fact, anti-tumor activity of pyrvinium has been reported under glucose starvation conditions (8). The lack of pyrvinium absorption probably plays a role in the absence of genotoxicity *in vivo*.

In summary, we found oral administration of pyrvinium pamoate to be highly effective at reducing *C. parvum* growth *in vitro* and at reducing both oocyst shedding and intestinal trophozoites in infected neonatal mice. Based on the efficacy of this drug against *C. parvum* and the distantly related malaria parasite, *P. falciparum*, we postulate that pyrvinium will be effective against other species of *Cryptosporidium* of medical and veterinary importance, including *C. hominis* and *C. andersonii*, respectively. Though trials in humans will be necessary to determine the minimum effective dose and tolerable doses, the safety of pyrvinium for treatment in humans has already been established (4, 27), which will significantly reduce the time and costs required for clinical trials. Based on these results, we believe pyrvinium pamoate is a potential drug
candidate for treatment of cryptosporidiosis in immunocompetent and also immunocompromised individuals, for whom no effective therapy is currently approved.

AKNOWLEDGEMENTS

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REFERENCES


### TABLES AND FIGURE LEGENDS

#### Table 1. Comparative efficacies of pyrvinium and paromomycin for reducing oocyst shedding from the distal colon of *C. parvum*-infected neonatal mice

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<th>Treatment</th>
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* This value represents the mean sum of oocysts counted in 21 microscopic fields at 40x that represented a vertical transect through the center of the IFA slide well.

SD = Standard deviation
FIG. 1. Effects of pyrvinium, chloroquine and paromomycin on *C. parvum* growth in HCT-8 cells. Drugs were added to cultures 1.5 h after the addition of $5 \times 10^3$ oocysts/well and incubated for 48 h. Parasite growth was determined by ELISA using a rat polyclonal anti-*Cryptosporidium* sera. Inhibition percentages of pyrvinium (squares), chloroquine (triangles) and paromomycin (circles) are from biologic replicate experiments. Error (SD) is from triplicate wells with the biologic duplicates.

FIG. 2. Representative intestinal sections from *C. parvum*-infected neonatal mice stained with hematoxylin and eosin (H&E). Trophozoites are shown covering the intestinal epithelial cells in the ileum, appendix and colon in control mice, while only few trophozoites are present in pyrvinium and paromomycin treated mice. Size bar is 50 microns. Images are 400x.

FIG. 3. Effects of drug treatment for 6 consecutive days on levels of *C. parvum* trophozoites in individual intestinal sections. (A) At necropsy, 1 cm intestinal sections were taken from the ileum, appendix, cecum and colon and stained with H&E for histological analysis to determine the extent of mucosal infection in drug treated versus untreated mice. Results are expressed as mean ($\pm$ SD) numbers of trophozoites per 1000 epithelial cells. Experiments testing 100 mg/kg paromomycin, n=9, (light gray) against its no drug controls, n=11, (dark gray) and 5 mg/kg
Pyrvinium, n=10, (white bars) against no drug controls, n=9, (black bars) were performed at different times. Control mice had few trophozoites in the proximal ileum, with levels peaking at the terminal ileum and decreasing again to the colon. Oocysts in fecal matter were not counted.

In paromomycin treated mice, trophozoites were only observed in the terminal ileum, appendix and colon. (B) Pyrvinium inhibition percentages by intestinal sections for each of 10 individual mice (circles) depicting means (horizontal bars). A single pyrvinium-treated mouse had little inhibition in the ileum, while more marked inhibition in the appendix and colon. Paromomycin inhibition percentages were greater than 90% in all sections (not shown).

FIG. 4. Intestinal epithelium infection summary score for treatment groups of *C. parvum*-infected neonatal mice. The cumulative score for extent of intestinal infection was calculated as the sum of the number of trophozoites per 1000 epithelial cells from all of the tissue sections for each mouse. Treatment group data are presented in box plots displaying the median value (21159, 10, 13750, and 63, for paromomycin no drug control, paromomycin, pyrvinium no drug control and pyrvinium, respectively). A Kruskal-Wallis non-parametric test of equality and pairwise comparisons using a non-parametric two-sample Mann-Whitney test showed that levels of trophozoites in both the pyrvinium and paromomycin treatment groups were significantly (P<0.05) lower than their respective controls, but not significantly different from each other (P=0.2682).
% Inhibition

Terminal ileum (-5cm)
Terminal ileum (-3cm)
Terminal ileum (-2cm)
Terminal ileum (-1cm)
Terminal ileum
Appendix
Cecum
Proximal Colon
Distal Colon