Evaluation of Maduramicin and Alborixin in a SCID Mouse Model of Chronic Cryptosporidiosis

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Two polyether ionophores, maduramicin and alborixin, were evaluated for anticryptosporidial activity in a severe combined immune deficient (SCID) mouse model of cryptosporidiosis. Groups of SCID mice were inoculated with 10⁶ oocysts of bovine origin by oral gavage. Maduramicin or alborixin was administered beginning 4 weeks postinfection at 3 mg/kg of body weight per day. Maduramicin treatment resulted in a 96% reduction in fecal parasite load over the 3-week treatment period (P < 0.003). This reduction correlated with decreases in tissue parasite loads observed in histological sections of the small intestine (P < 0.000002) and the colon (P < 0.000006). A significant decrease in oocyst shedding was also observed after a 3-week treatment with alborixin (71% reduction, P < 0.01). Maduramicin was also evaluated in a relapsing model of cryptosporidiosis in which the infection was observed to recur after treatments were discontinued. Some toxicity, as demonstrated by weight loss, was observed with both maduramicin and alborixin. Both drugs exhibited significant anticryptosporidial activities with concomitant moderate toxicity. These polyether ionophores should be valuable as positive controls in compound evaluation studies and as lead compounds for chemical optimization (modification).

Cryptosporidium parvum is a protozoan organism that infects the epithelial cells of the small intestine, causing diarrheal illness in animals and humans. In immunocompetent individuals it causes a flu-like illness that is usually resolved in 2 to 3 weeks. In immunodeficient individuals, especially those with AIDS, the disease is often protracted and severe and may become life threatening (10). Although many drugs have been tested clinically, an effective treatment for C. parvum infections has yet to be found (11).

A comprehensive review of current cryptosporidial research recommended further testing of five agents: alborixin, arprinocid, cyclosporin A, lasalocid, and maduramicin (8). Interestingly, alborixin, maduramicin, and lasalocid are membrane-active polyether ionophores which have been effective in the control of coccidiosis in poultry and cattle (7). These same ionophores have been evaluated against C. parvum in vivo and have demonstrated some efficacy. The outcome of the experiment and the efficacy of the drug being evaluated differed depending on the animal models employed (15). For example, lasalocid was shown to be effective in immunosuppressed rat models but not in an immunodeficient model (6, 15, 24). Additionally, the majority of these studies have been carried out on a short-term basis, with treatment beginning soon after inoculation.

The absence of a reliable cell-based assay for determining anticryptosporidial activity has led to the development of several animal models of cryptosporidiosis. These include neonatal mice (9, 13, 22, 27), chemically immunosuppressed rodents (5, 24–26), and immunodeficient mice (13, 14, 18, 19, 29). Given the potentially severe outcome of cryptosporidial infections in immunosuppressed and immunocompromised patients, models of chronic cryptosporidiosis most representative of human disease are needed for evaluating potential therapeutic agents. These models should reflect the range of disease states observed in human immunodeficiency virus-infected individuals and in particular disseminated stages that may lead to mortality.

In a previous study, an examination of two of these agents in a neonatal mouse model revealed that alborixin and maduramicin were effective in reducing oocyst shedding by 99 and 98%, respectively (4). However, treatment was performed for a relatively short time and chronic infections in these mice are not possible since they recover spontaneously. In the current study, maduramicin and alborixin were evaluated in our models of chronic cryptosporidiosis employing severe combined immunodeficient mice (SCID) mice. Infections in SCID mice produce a disease state similar to that observed in AIDS patients in that the infections become chronic and colonization outside the intestinal tract is observed (19, 20). Compounds were administered for 4 weeks prophylactically or for 3 weeks therapeutically after infections had become well established (4 weeks postinfection).

MATERIALS AND METHODS

Isolate. The C. parvum isolate used for this study was the IOWA bovine isolate originally obtained from Harley Moon (National Animal Disease Center, Ames, Iowa). Oocysts of the isolate were generated in newborn holstein bull calves as previously described (2). Newborn calves were placed in isolation quarters in customized pens and infected at 2 days of age with approximately 10⁹ oocysts. All fecal material passed during peak oocyst shedding (days 5 to 12 postinfection) was collected and stored at 4°C in 2.5% potassium dichromate.

Purification of oocysts was performed by sieving feces sequentially through stainless steel screens with diminishing pore sizes; the final mesh size was 230 (63 μm). Further oocyst purification was achieved by sequential centrifugation procedures involving discontinuous sucrose (1.064 to 1.103 g/ml) gradients as previously described (2).

Animals. Female C.B-17 scid/scid (SCID) mice, ages 6 to 8 weeks, were...
purchased from the University of Wisconsin Gnotobiotic Laboratory (Madison). Mice were maintained at the VA Medical Center in an isolation room under pathogen-free conditions. Mice were housed in microisolator cages (Nalgene Labware, Rochester, N.Y.) in high-efficiency particulate-air-filtered laminar flow units (Labline, Maywood, N.J.). All cages, food, water, and bedding were sterilized before use. Sterilized water (e.g., masks, gowns, and gloves) was worn when mice were handled. All manipulations were done on a high-efficiency particulate-air-filtered laboratory bench.

Compounds. The polyether ionophores were obtained as technical powders from industrial sources. Maduramicin was obtained from American Cyanamid Company (Princeton, N.J.), and alborixin was kindly supplied by Byron Blagburn (Auburn University, Auburn, Ala.; originally obtained from The Upjohn Company, Kalamazoo, Mich.). These compounds were dissolved in water containing dimethyl sulfoxide (final concentration, 1%).

Experimental design. Studies were performed twice with maduramicin prophylactically or therapeutically as described below. In all studies, mice (12 per group) were inoculated with 10⁶ oocysts of bovine origin by oral gavage. Control mice received sterile drinking water with 1% dimethyl sulfoxide. Water bottles were weighed every other day, and the amount of drug administered was adjusted according to the average amount of water consumed per mouse (three mice per bottle). In all experiments, the level of infection was monitored at least once a week by flow-cytometric assays for the presence of the parasite in the feces. Mice were weighed weekly in all experiments to determine weight loss or failure to gain weight due to drug toxicity. At necropsy, the intestinal tract, liver, and gallbladder were removed for histological examination.

For the prophylactic studies, maduramicin was administered 24 h before oocyst inoculation (10⁶ oocysts), given ad libitum for 21 days in water at 3 mg/kg of body weight per day, and reduced to 1 mg/kg for an additional 7 days because of the overt toxicity of the drug. Mice were euthanized on day 28 for histological analyses.

For the therapeutic studies, maduramicin or alborixin administration began 4 weeks postinfection at 3 mg/kg/day. Mice were euthanized for histological analyses after 3 weeks of treatment (49 days after oocyst inoculation).

Additionally, studies were performed to determine if infections in mice would recur after maduramicin treatment was discontinued. For these studies, maduramicin was administered at 3 mg/kg/day as before beginning 4 weeks postinfection and continuing for 3 weeks. The compound was then removed, and mice were given sterile drinking water for an additional 2 weeks. Mice were necropsied 63 days after oocyst inoculation, and the intestinal tract, liver, and gallbladder were removed for histological examination.

Flow-cytometric enumeration of fecal oocysts. Mouse fecal samples were collected weekly and subjected to a microscopic variation of the discontinuous sucrose gradient method reported for the purification of oocysts from calf stool (2). Briefly, fecal pellets were homogenized in adjusted volumes of 2.5% potassium phosphate-buffered saline (PBS; 0.01 M, pH 7.2) supplemented with 0.1% bovine serum albumin. The partially purified stool concentrate was incubated for 30 min at 37°C with 5 µl of an oocyst-specific monoclonal antibody conjugated with fluorescein isothiocyanate (OW50-FITC, 1:50 dilution in PBS). Samples were adjusted to 600 µl with PBS, stored at 4°C, and protected from light until analyzed by flow cytometry with a FACScan (Becton Dickinson, Mountain View, Calif.).

Samples were evaluated with a 102-s sampling interval (100 µl) with logical gating of forward to side scatter and OW50-FITC fluorescence signals on the flow cytometer (1). Data files were collected and stored on a floppy disk with a flow cytometer (Becton Dickinson) and subsequently analyzed with software provided with the FACScan (Lysis II; Becton Dickinson). Each experimental run included positive and negative controls which were used to calibrate the region settings necessary to discriminate the labeled oocyst population from the background debris. Absolute counts were calculated from the data files for oocysts per 100 µl of sample suspension. Numbers were analyzed statistically as described below.

Histological enumeration. Tissue samples were removed from euthanized mice and fixed in 10% buffered formalin. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Small and large intestines and gallbladder and liver tissue sections were examined at a magnification of ×400, and the number of parasites per field was determined. A total of five random fields were counted per sample and then averaged.

Statistical analyses. Microscopic counts and flow cytometry data were evaluated by analysis of variance, correlation, and regression (Microsoft Excel; Microsoft Corporation, Redmond, Wash.).

RESULTS

Results of the prophylactic treatment regimen using maduramicin are presented in Fig. 1. A significant decrease in oocyst shedding in fecal samples was observed (P < 0.0001) at 3 mg/kg/day and even after the dose was reduced to 1 mg/kg/day (P < 0.0003) (Fig. 1a). Histological data are shown in Fig. 1b. Significant decreases (98%) were observed in the number of organisms found in the small intestine (P < 0.00000003) and colon (P < 0.000004), and biliary system. The data are means ± standard errors.

![Graph showing changes in oocyst shedding and tissue colonization](http://aac.asm.org/)

Fig. 1. (a) Effects on oocyst excretion in SCID mice of prophylactic treatment with 3 mg of maduramicin per kg per day for 3 weeks followed by 1 mg/kg/day for 1 week (P < 0.0003). The arrow indicates the time of drug reduction. (b) Numbers of parasites in the small intestine (P < 0.00000003), colon (P < 0.000004), and biliary system. The data are means ± standard errors.
asites in tissue sections. Histologically, a 98% reduction in the parasite load was observed in the tissue of the treated mice compared with the parasite load in the tissue of control mice. This reduction was significant compared with the parasite load in the small intestine (P < 0.000002) and the colon (P < 0.000006) samples of the control mice (Fig. 2b). In repeated experiments significant decreases were again observed in both the oocyst shedding and the tissue colonization. Reductions in the parasite load of 75, 85, and 60% were observed in the stool, ileum, and colon (data not shown). Although parasites were not observed in the gallbladder or hepatic ducts in the first experiment (Fig. 2b), small numbers were detected in the subsequent study (data not shown). A 50% reduction of the parasite load was observed in the gallbladder and hepatic ducts compared with that of the controls.

In a separate experiment, mice treated therapeutically with maduramicin exhibited an 84% reduction in fecal parasite load after 3 weeks of treatment (7 weeks after oocyst inoculation). However, when the treatment was discontinued for 2 weeks, the infection recurred and was not statistically different from that of controls (P < 0.08 at 9 weeks postinfection) (Fig. 3a). An increased parasite load in the ileum and colon was apparent 2 weeks after treatment was discontinued. Differences in numbers of parasites in tissue sections between control and treated groups were not significant (Fig. 3b).

Alborixin was also evaluated in the SCID mouse model by using a therapeutic oral administration schedule (Fig. 4). A significant decrease in oocyst shedding was observed after 3 weeks of treatment with alborixin (71% reduction, P < 0.01). Reductions in parasite load in the ileum (65%, P < 0.003), colon (35%, P < 0.02), and biliary tract were observed.

Weight loss was not observed until 1 week posttreatment in the mice treated prophylactically with maduramicin (Fig. 5a). After 3 weeks of treatment, the majority of the mice had ruffled fur and had lost weight (the mean weight loss compared with the weight of controls was 4.0 g). After the maduramicin dosage was reduced (from 3 to 1 mg/kg/day), the average weight of the mice increased during the last week (Fig. 5a). This corresponded to an increase in the parasite load in the same study (Fig. 1).

Toxicity was also observed in the therapeutic trials with both maduramicin and alborixin. Weight loss was apparent at 1 week posttreatment. However, the average weight loss over the 4-week period was approximately 3 g in the mice treated with maduramicin (3 mg/kg/day [Fig. 5b] and 4 mg/kg/day [data not shown]) and 2 g in mice treated with alborixin (data not shown). No deaths were observed at any of the dosages used. Thus, the polyether ionophores were tolerated in mice at 3 mg/kg/day and at smaller dosages over the 4-week treatment period.

FIG. 2. (a) Effects on oocyst excretion in SCID mice of therapeutic treatment with 3 mg of maduramicin per kg per day for 3 weeks administered 4 weeks postinfection (P < 0.003). (b) Numbers of parasites in the small intestine (P < 0.000002), colon (P < 0.000006), and biliary system. The data are means ± standard errors.

FIG. 3. (a) Effects on oocyst excretion in SCID mice of therapeutic treatment with 3 mg of maduramicin per kg per day for 3 weeks, after which treatment was discontinued (P < 0.08). (b) Numbers of parasites in the small intestine (P < 0.4), colon (P < 0.2), and biliary system. The data are means ± standard errors.
Polyether ionophores have been used as anticoccidial drugs in poultry and cattle (21). These compounds are thought to work by affecting the movement of cations across membranes, causing an influx of sodium and calcium and an efflux of potassium. This in turn results in pH changes within the cell, affecting metabolic processes and damaging organelles. Coccidia are reportedly more sensitive to these changes (30) or may have a greater affinity for binding ionophores than host cells. Although some toxicity was evident in vivo as demonstrated by weight loss in the mice, we have observed relatively low toxicity to host cells at concentrations that inhibit C. parvum growth in vitro (unpublished observation).

At least three ionophores have been evaluated in cell culture against Cryptosporidium spp. (3, 17). Monensin reduced parasite development in vitro by more than 90% but had little activity in vivo (21, 28). Lasalocid was shown to eliminate established infections in immunosuppressed rats treated therapeutically or prophylactically (23), but it failed to demonstrate activity in an immunodeficient model (15). Maduramicin and alborixin demonstrated efficacy between 1 to 2.5 mg/kg in a neonatal mouse model when administered prophylactically. Both ionophores were shown to decrease the parasite load by >98% (4).

In the current study, for the first time, maduramicin and alborixin were evaluated in an immunodeficient murine model of chronic cryptosporidiosis. These compounds markedly decreased parasite load in the feces. Overall, the histology data correlated with the oocyst shedding data. However, a few individual mice were found to be negative for cryptosporidial infection by histological examination but were positive by detection of oocysts in the feces. This is not surprising since stools represent the sampling of the entire gastrointestinal tract and therefore should be unaffected by the shifts of parasite localization on the epithelial surfaces often seen in histologic specimens.

Toxicity was noted in both maduramicin- and alborixin-treated groups as indicated by weight loss. This weight loss might have been due to alterations of the gut epithelium, since cells in treated mice appeared enlarged and distorted. Weight loss was apparent at 1 week posttreatment. However, average weight loss over a 4-week period was approximately 3.0 g in the treated mice, corresponding to a 10 to 15% loss in total body weight. Thus, these compounds exhibited significant anticryptosporidial activities and were sufficiently well tolerated for our assay purposes. No adverse reactions were found when neonatal BALB/c mice (4) or nude mice (15) were treated with these compounds. However, treatment in these models was limited to 7 and 8 days, respectively. Although in their current formulations maduramicin and alborixin are too toxic for human use, the possibility of using them in combination with newly developed anticryptosporidial agents is a reasonable next step.
Chemical modification of these promising compounds to reduce toxicity should be undertaken. Approximately half of individuals infected with the human immunodeficiency virus who recover from cryptosporidial infections develop recurrent cryptosporidiosis. It is not known if these are reinfections or if a biliary or other extraintestinal reservoir contributes to the chronicity of the infection. Clinical deterioration (including relapse) occurred in 70% of patients with bile duct involvement (12). We have observed that SCID mice tolerate intestinal infections but deteriorate and die after hepatobiliary colonization.

Of significance was the finding that discontinuation of treatment with the polyether ionophores led to recurrent infections. This was observed even in a prophylactic study in which >99.0% reduction of fecal oocysts was observed (data not shown). While complete elimination of the coccidia has been demonstrated after a 10-week treatment with anticoxidial drugs, longer treatments or combination therapies may be necessary to eradicate the parasite in immunocompromised hosts (16). Recurrence of experimental infections has been demonstrated in rats even when the parasite could not be detected in the tissue or feces for 10 weeks (25). Relapse may be due in part to parasites sequestered in the biliary tract or disseminated to other sites. If so, the extraintestinal sites of cryptosporidial colonization complicate treatment strategies because drugs will need to be systematically redistributed to these sites if anticoxidial activity is to be effective. Extension of this model to evaluate drugs that would have an effect on the late (lethal) stages of cryptosporidiosis in SCID mice is currently under way.

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REFERENCES