Inhibition of *Cryptosporidium parvum* in Neonatal Hsd:(ICR)BR Swiss Mice by Polyether Ionophores and Aromatic Amidines†

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Cryptosporidicidal effects of two polyether ionophores (maduramicin and alborixin), a fluorinated 4-quinolone (enrofloxacin), and three analogs of pentamidine were evaluated in a suckling mouse bioassay. Treatment with all compounds except enrofloxacin and one of the pentamidine analogs [1,3-di(4-imidazolino-phenoxy)propane] resulted in significant ($P < 0.05$) reductions in oocyst excretion.

Cryptosporidium spp. are small (2 to 6 μm, depending on the stage in the life cycle) protozoans that infect and develop within the microvillous border of epithelial surfaces of various organ systems of vertebrates (5). Several *Cryptosporidium* species have been named; to date, only two species, *Cryptosporidium parvum* and *Cryptosporidium muris*, are known to parasitize mammals (19). *C. parvum* develops principally in the small intestine and is the species responsible for diarrhea in many animals including humans (19). Prior to the discovery of the human immunodeficiency virus and AIDS, parasites within the genus *Cryptosporidium* engendered little more than academic interest. It is now known that *C. parvum* may cause a transient gastrointestinal flu-like illness in humans and that immunocompromised humans, especially those with AIDS, can develop severe, protracted cryptosporidial infections resulting in severe diarrhea and perhaps death (3). *C. parvum* is now included in the AIDS-defining complex of opportunistic agents. Although numerous therapeutic and preventive modalities have been evaluated, none have consistently eliminated the parasite or cured clinical cryptosporidiosis in immunologically normal humans and humans with AIDS (7).

Several assays have been proposed for evaluation of cryptosporidicidal compounds in vivo and in vitro. Each possesses inherent difficulties or deficiencies. Adult mice (15) are less susceptible than suckling mice to cryptosporidial infections and usually excrete too few oocysts to allow their use as model hosts in chemotherapy studies. A model utilizing chemically immunocompromised rats presents similar problems regarding inconsistencies in excretion rates and numbers of oocysts excreted (14). Infections in vitro and in ovo also yield suboptimal numbers of parasites and are unlikely to identify drugs requiring metabolism to active products (4). At present, the suckling mouse assay appears to provide reliable results with the fewest deficiencies or difficulties (18).

The polyether ionophores are monovalent and divalent cation-specific anticoccidial compounds effective in the prevention and control of *Eimeria*-induced coccidiosis of cattle and poultry (2). The extremely high anticoccidial activity of the ionophores has led to the discovery and development of many such compounds.

The quinolones (e.g., decoquinate and buquinolate) are effective as anticoccidial agents against *Eimeria* spp. and are currently used as control agents in cattle or poultry (2). A new generation of fluorinated 4-quinolones, with a broad spectrum of antibacterial activity (e.g., enrofloxacin), are currently under development (6). The cryptosporidicidal activities of the fluorinated 4-quinolones are unknown.

The bisamidines (pentamidine and related compounds) are a diverse group of agents with demonstrated parasiticidal activity (2). Pentamidine and its analogs possess a broad spectrum of activity against intracellular and extracellular protozoa and against *Pneumocystis carinii* (1, 9, 17). When pentamidine was administered parenterally to humans with AIDS and concurrent cryptosporidiosis, no effect was observed on either diarrhea or excretion of oocysts (16). However, lack of efficacy could be attributed to the route of administration. Parenteral dosing may not allow optimum contact between *Cryptosporidium* stages and pentamidine. Neither pentamidine nor its analogs have been evaluated against *Cryptosporidium* species following oral administration.

In this report, we describe our murine model for the evaluation of potential cryptosporidicidal compounds and report the results of evaluations of the cryptosporidicidal effects of two polyether ionophores (maduramicin and alborixin), a fluorinated 4-quinolone (enrofloxacin), and three analogs of pentamidine (Fig. 1 and 2).

C. parvum (AUCp-1 isolate), originally obtained from a naturally infected dairy calf, is maintained in the laboratories of Byron L. Blagburn and David S. Lindsay by periodic passage in male donor holstein calves. Oocysts are isolated from feces and concentrated by flotation in Sheather's sucrose solution (8) (specific gravity = 1.18), enumerated with the aid of a hemacytometer, and stored in Hanks' balanced salt solution at 4°C for not more than 6 weeks prior to inoculation into suckling mice.

The polyether ionophores and enrofloxacin used in this study were obtained as technical powders (maduramicin and alborixin) or an injectable solution (enrofloxacin) from industrial sources as follows: maduramicin, American Cyanamid Corporation (Princeton, N.J.); alborixin, The Upjohn

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Compound Structure   Compound Name

Maduramycin

Alborixin
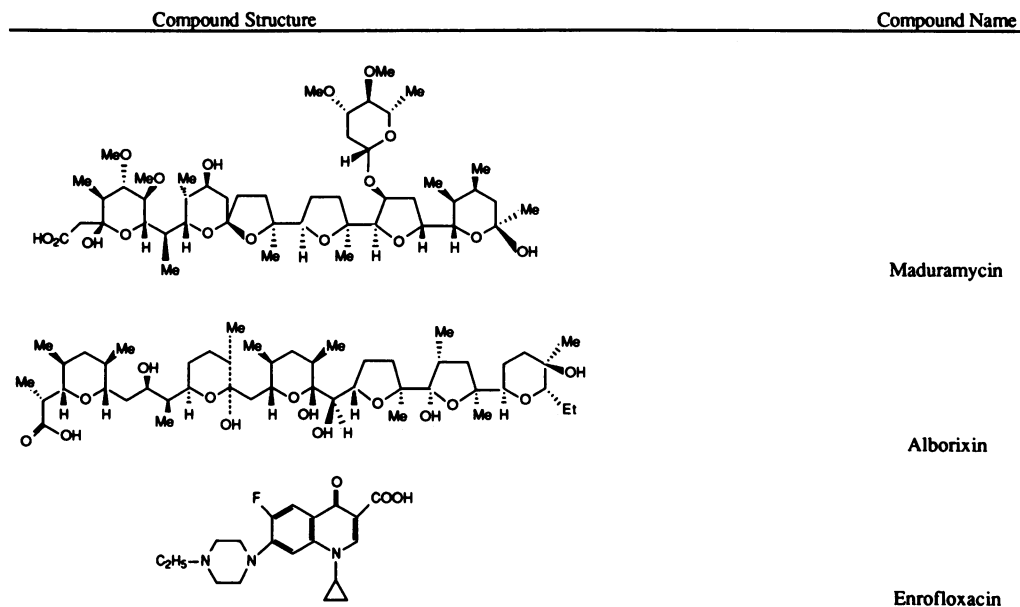
Enrofloxacin

FIG. 1. Polyether ionophores and quinolones evaluated against *C. parvum*.

Company (Kalamazoo, Mich.); and enrofloxacin, Mobay Animal Health (Shawnee Mission, Kans.). Pentamidine analogs were synthesized in the laboratory of Richard R. Tidwell, using the methods described previously (17). Immediately prior to administration, compounds were mixed with 90% (vol/vol) propylene glycol in deionized water or deionized water alone (pentamidine analogs) at the appropriate concentrations to achieve the desired dosages when administering a volume of 10 μl to a 3-g mouse (Tables 1 and 2). Control mice in each experiment received the same vehicle without the test compound.

Evaluations were conducted as follows using our suckling mouse bioassay. One-day-old Hsd:(ICR)BR outbred Swiss Webster mice were obtained from commercial sources. Mice were shipped to Auburn University as individual females with litters. Upon arrival, pups were cross-fostered into litters on the basis of body weight. This procedure created litters of six 1-day-old mice with approximately equal mean body weights. Litters were allocated to treatment and control groups, using a random number table. Treated mice received test compounds once daily (see below) at dosages recommended by the supplier (Tables 1 and 2). Mice in each

treatment and control group were treated using a Tracor Atlas microdoser and syringe pump equipped with a tuberculin syringe and a 25-gauge, 1.59-cm-long needle, to which a small piece of microbore tubing, (outer diameter, 0.076 cm; inner diameter, 0.025 cm) was fitted (Tracor Atlas, Houston, Tex.). Treatments with test compounds and infection with *C. parvum* oocysts were performed by inserting the microbore tubing into the mouse's esophagus to a point approximately midway between the pharanyx and the stomach. The syringe pump and microdoser were precalibrated so that numerical increases on the microdoser corresponded to known volumetric increases in solubilized compound or vehicle. Mice were weighed prior to each treatment so that an increased volume of compound could be given as the weight of the mice increased (constant dose rate). Control groups received the same amount of vehicle without the candidate drug. Treatments were initiated when mice in each group had achieved a weight of approximately 3.0 g. Mice in each litter (treatment group) were treated daily with test compound on day −1 (day prior to infection) through day 6. Mice in each group were infected with approximately 10^5 oocysts of *C. parvum* on day 0, at least 2 h after treatment on

Compound No.   Compound Structure   Compound Name

1   1,4-Di(4-imidazolino-2-methoxyphenoxy)butane

2   1,3-Di(4-imidazolinophenoxy)propane

3   1,3-Di(4-amidino-2-methoxyphenoxy)propane
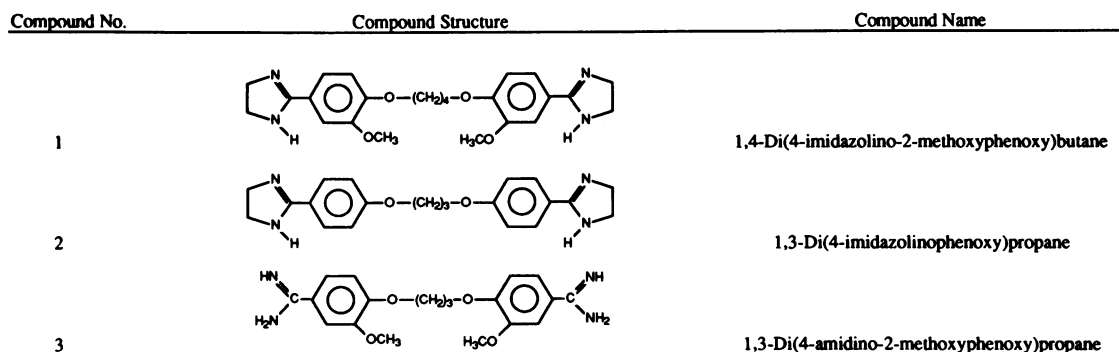
FIG. 2. Pentamidine analogs evaluated against *C. parvum*.

TABLE 1. Activities of maduramicin, alborixin, and enrofloxacin against experimentally induced *C. parvum* infections in Hsd:(ICR)BR Swiss mice[a]

Expt. no. and treatment group	No. of mice	Dosage[b] (mg/kg of body weight)	No. (mean[c] ± SD) of *C. parvum* oocysts recovered/mouse
1a			
Maduramicin	39	1	29 ± 28[d]
Control	31		194 ± 143[e]
1b			
Maduramicin	33	2.5	2 ± 2[d]
Control	29		87 ± 44[e]
2a			
Alborixin	19	1.5	0.9 ± 1.1[d]
Control	24		122 ± 65[e]
2b			
Alborixin	12	2.5	1.5 ± 2.5[d]
Control	15		161 ± 127[e]
3a			
Enrofloxacin	24	3	171 ± 68[d]
Control	24		120 ± 83[d]
3b			
Enrofloxacin	15	1	148 ± 103[d]
Control	10		109 ± 57[d]

[a] See Fig. 1 for chemical structures of the three compounds.

[b] Drugs were mixed in 90% propylene glycol–10% deionized water; control mice received propylene glycol-water only.

[c] Means represent actual hemacytometer counts. Mean total oocysts recovered from mice in each group can be approximated by multiplying by 25,000.

[d,e] Means within each experiment with different superscripts are significantly different ($P < 0.05$).

TABLE 2. Activities of pentamidine analogs against experimentally induced *C. parvum* infections in Hsd:(ICR)BR Swiss mice[a]

Expt. no. and treatment group	No. of mice	Dosage[b] (mg/kg)	No. (mean[c] ± SD) of *C. parvum* oocysts recovered/mouse
4			
Compound 1	19	2.8	4.5 ± 2.9[d]
Control	19		49.8 ± 47[e]
5			
Compound 2	18	11.3	13.8 ± 11.5[d]
Control	14		10.3 ± 7.3[d]
6			
Compound 3	32	11.3	28.6 ± 37.7[d]
Control	24		69.3 ± 71.7[e]

[a] See Fig. 2 for chemical structures of compounds 1 to 3.

[b] Drugs were mixed with deionized water; control mice received deionized water only.

[c] Means represent actual hemacytometer counts. Mean total oocysts recovered from mice in each group can be approximated by multiplying by 25,000.

[d,e] Means in each experiment with different superscripts are significantly different.

that day. Mice in all treatment and control groups were euthanatized by cervical dislocation on day 7. The portion of the alimentary tract from the pylorus to the rectum was removed from each mouse and separately placed in 10 ml of 2.5% (wt/vol) potassium dichromate solution. The alimentary tract from each mouse was then ground by using a Tekmar Tissuemizer (model SDI-1810; Tekmar Company, Cincinnati, Ohio) to free oocysts. This was accomplished by inserting the grinder rotor into the potassium dichromate solution containing the alimentary tract and by slowly increasing the rotor speed to a maximum rate of 24,000 rpm over a period of approximately 15 s. This procedure was repeated until a homogeneous mixture was achieved. Grinding pulses did not exceed 15 s to avoid overheating the specimen and possibly damaging oocysts. The numbers of oocysts per milliliter of the 10-ml homogenates were determined with the aid of a hemacytometer. Oocysts were not stained to enhance identification of oocysts prior to enumerations. Mean numbers of oocysts recovered from treated and control mice were compared by using the nonparametric Wilcoxon Mann-Whitney test. A probability level of $P < 0.05$ was considered significant.

Adverse reactions resulting from treatment with the test compounds were not observed. Daily weight gains for mice treated with each test compound were similar to mice in the control groups (detailed data not presented). Of the six compounds evaluated, four significantly reduced the numbers of oocysts recovered from treated mice (Tables 1 and 2). Excellent efficacy was observed for the polyethers maduramicin and alborixin (Table 1). Neither of these com-

pounds have been evaluated against *C. parvum* previously. Studies with other polyethers in both in vivo and in vitro assays have yielded variable results (11, 13). For example, lasalocid appeared to have activity against *C. parvum*, but this activity was observed only at a dosage that was toxic to infected calves (12). Monensin reduced development of *C. parvum* in cell cultures by more than 90% compared with that of controls (11) but had little apparent activity in vivo (12, 18). Our results indicate the need to further evaluate alborixin and maduramicin against *C. parvum*. It would be especially interesting to examine the effects of these compounds in a clinical model of cryptosporidiosis.

Among the pentamidine analogs, compounds 1 and 3 demonstrated activity at the dosages tested (Table 2). These results are noteworthy because they demonstrate the activity of certain pentamidine analogs against *C. parvum* and imply an even broader spectrum of activity for these compounds than originally indicated (1, 9, 17). Their broad spectrum of activity is also supported by the demonstration of activity of certain pentamidine analogs against *Toxoplasma gondii* in cell cultures (10). Numerous additional analogs of pentamidine have been synthesized and are available from Richard Tidwell's laboratory. Additional detailed structure-activity studies are needed to identify those compounds with maximum efficacy against *C. parvum* and minimum host toxicity.

It is important to note that the evaluation regimens used in these experiments were prophylactic, because test compounds were administered to mice prior to infection with *C. parvum*. Further evaluations of these compounds utilizing a therapeutic regimen are desirable.

REFERENCES

1. **Bell, C. A., J. E. Hall, D. E. Kyle, M. Grogi, K. A. Ohement, M. A. Allen, and R. R. Tidwell.** 1990. Structure-activity relationships of analogs of pentamidine against *Plasmodium falciparum* and *Leishmania mexicana amazonensis*. Antimicrob. Agents Chemother. **34:**1381–1386.

2. **Chabala, J. C., and M. W. Miller.** 1986. Chemistry of antipro-

tozoal agents, p. 25–85. *In* W. C. Campbell and R. S. Rew (ed.), Chemotherapy of parasitic diseases. Plenum Press, New York.

3. **Current, W. L.** 1989. *Cryptosporidium* spp., p. 282–341. *In* P. D. Walzer and R. M. Genta (ed.), Parasitic infections in the immunocompromised host. Marcel Dekker, Inc., New York.

4. **Current, W. L.** 1990. Techniques and laboratory maintenance of *Cryptosporidium*, p. 31–49. *In* J. P. Dubey, C. A. Speer, and R. Fayer (ed.), Cryptosporidiosis of man and animals. CRC Press, Inc., Boca Raton, Fla.

5. **Current, W. L., and B. L. Blagburn.** 1990. *Cryptosporidium*: infections in man and domestic animals, p. 155–185. *In* P. L. Long (ed.), Coccidiosis of man and domestic animals. CRC Press, Inc., Boca Raton, Fla.

6. **Dougherty, T. J., and J. J. Saukkonen.** 1985. Membrane permeability changes associated with DNA gyrase inhibitors in *Escherichia coli*. Antimicrob. Agents Chemother. **28:**200–206.

7. **Fayer, R., C. A. Speer, and J. P. Dubey.** 1990. General biology of *Cryptosporidium*, p. 1–29. *In* J. P. Dubey, C. A. Speer, and R. Fayer (ed.), Cryptosporidiosis of man and animals. CRC Press, Inc., Boca Raton, Fla.

8. **Ivens, V. R., D. L. Mark, and N. D. Levine.** 1978. Principal parasites of domesticated animals in the United States, p. 253. Colleges of Agriculture and Veterinary Medicine special publication 52. Colleges of Agriculture and Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana-Champaign.

9. **Jones, S. K., J. E. Hall, M. A. Allen, S. D. Morrison, K. A. Ohemeng, V. V. Reddy, J. D. Geratz, and R. R. Tidwell.** 1990. Novel pentamidine analogs in the treatment of experimental *Pneumocystis carinii* pneumonia. Antimicrob. Agents Chemother. **34:**1026–1030.

10. **Lindsay, D. S., and B. L. Blagburn.** Unpublished data.

11. **McDonald, V., R. Stables, D. C. Warhurst, M. R. Barer, D. A. Blewett, H. D. Chapman, G. M. Connally, P. L. Chiodini, and K. P. W. J. McAdam.** 1990. In vitro cultivation of *Cryptosporidium parvum* and screening for anticryptosporidial drugs. Antimicrob. Agents Chemother. **34:**1498–1500.

12. **Moon, H. W., G. N. Woode, and F. A. Ahrens.** 1982. Attempted chemoprophylaxis of cryptosporidiosis in calves. Vet. Rec. **110:**181.

13. **Moore, J. A., B. L. Blagburn, and D. S. Lindsay.** 1988. Cryptosporidiosis in animals including humans: a review. Compend. Cont. Educ. Pract. Vet. **10:**275–287.

14. **Rehg, J. E., M. L. Hancock, and D. B. Woodmansee.** 1987. Characterization of a cyclophosphamide-rat model of cryptosporidiosis. Infect. Immun. **55:**2669–2674.

15. **Sherwood, D., K. W. Angus, D. R. Snodgrass, and S. T. Tzipori.** 1982. Experimental cryptosporidiosis in laboratory mice. Infect. Immun. **38:**471–475.

16. **Soave, R., R. L. Danner, C. L. Honig, P. Ma, C. C. Hart, T. Nash, and R. B. Roberts.** 1984. Cryptosporidiosis in homosexual men. Ann. Intern. Med. **100:**504–511.

17. **Tidwell, R. R., S. G. Kilgore, J. D. Geratz, K. A. Ohemeng, M. Cory, and J. E. Hall.** 1990. Analogues of 1,5-bis(4-amidophenoxy)pentane (pentamidine) in the treatment of experimental *Pneumocystis carinii* pneumonia. J. Med. Chem. **33:**1252–1257.

18. **Tzipori, S. T., I. Campbell, and K. W. Angus.** 1982. The therapeutic effect of 16 antimicrobial agents on *Cryptosporidium* infection in mice. Aust. J. Exp. Biol. Med. Sci. **60:**187–190.

19. **Upton, S. J., and W. L. Current.** 1985. The species of *Cryptosporidium* (Apicomplexa, Cryptosporidiidae) infecting mammals. J. Parasitol. **71:**625–629.