Efficacy of Antiamebic Drugs in a Mouse Model

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Abstract. Nitroimidazole antibiotics are the mainstay of treatment of invasive amebiasis; however, few comparative studies of applicable antibiotics are available. Evidence of sporadic clinical failure and rare reports of metronidazole resistance have led to the investigation of novel antiamebic therapeutics. The goal of this study was to examine drug efficacy in both *in vitro* and *in vivo* models of intestinal amebiasis. We studied six current and three novel drugs. Many drugs, including metronidazole, nitazoxanide, and nitazoxanide derivatives, were shown to be potently inhibitory *in vitro*. However, metronidazole remained the most effective *in vivo*, both in preventative and curative regimens, underscoring the value of animal models in evaluating future therapies.

INTRODUCTION

Intestinal amebiasis, the disease caused by the parasite *Entamoeba histolytica*, is a significant source of morbidity and mortality in developing countries and remains a threat to travelers.^{1,2} Estimates of the global burden range from 50 to 480 million cases per year.³ Treatment with antibiotics is generally highly effective; however, delays or failures in diagnosis or treatment lead to an estimated 40,000–100,000 deaths per year.

Oral metronidazole, a member of the 5-nitroimidazole family, is the standard therapy for treating intestinal amebiasis. Several related compounds, including tinidazole, are efficacious as well. Beyond nitroimidazoles, several alternative drug therapies have been used for invasive amebiasis, including emetine and dehydroemetine, which remain available in the United States for use by patients after failed metronidazole treatment. Chloroquine has also been used for extraintestinal abscess and has shown similar cure rates to metronidazole.⁴ More recently, nitazoxanide, a thiazolide derivative with a broad spectrum of activity against anaerobic bacteria and parasites, has also been shown to be an effective treatment in amebic diarrhea.⁵ After therapy with metronidazole, there can be a high rate of parasite persistence in the gut,⁶ which was recently observed in an outbreak in Japan.⁷ Hence, a treatment course with a luminal agent such as iodoquinol is recommended after metronidazole therapy.

There have been few comparisons of these drugs in humans. We have developed a mouse model of amebiasis whereby mice develop persistent infection with intestinal inflammation after injection of ameba. In this work, we use this model to examine the comparative efficacy of existing and novel drug compounds.

MATERIALS AND METHODS

Ameba. Laboratory strain (American Type Culture Collection, Manassas, VA) *E. histolytica* trophozoites HM1:IMSS were cultured axenically in complete TYI-S-33 medium for *in vitro* assays. *E. histolytica* trophozoites for *in vivo* experiments

were derived from HM1:IMSS sequentially passaged through the mouse cecum and were cultivated xenically.

Antiamebic drugs. For those drugs in clinical use (metronidazole, tinidazole, nitazoxanide, iodoquinol, emetine, and chloroquine), commercial pharmaceutical formulations were obtained from the University of Virginia or nearby pharmacies. Novel therapeutics (AMIX, VPC16a1006, and pyrrvinium) were obtained as pure preparations. Drugs were dissolved in 100% dimethyl sulfoxide (DMSO) at 10× concentration and stored as stock solutions at –20°C. Stock solutions were diluted 1:10 in phosphate-buffered saline (PBS) immediately before use.

***In vitro* susceptibility assays.** *In vitro* drug susceptibility assays were performed using a variation on normal trophozoite culture. Briefly, 5×10^4 *E. histolytica* trophozoites were incubated for 72 hours at 37°C in 1 mL TYI-S-33. Antiamebic drugs were serially diluted in 5 µL DMSO. Negative controls for growth (time = 0 and 72 hours refrigerated cultures) and positive controls (culture medium plus DMSO) were included. After incubation, trophozoites were detached by chilling and counted on a hemocytometer. Raw counts were converted to a percentage growth scale, where the negative control equaled zero and the positive control equaled 100%. All cultures were performed in duplicate.

Mice and *in vivo* infection. Six-week-old CBA/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were maintained under specific pathogen-free conditions at the University of Virginia and were infected in accordance with Institutional Animal Care and Use Committee approved protocols.⁸ Briefly, animals were anesthetized, laparotomized, and intracinally injected with 2×10^6 *E. histolytica* trophozoites. Antiamebic drugs were administered in 100 µL of 10% DMSO or PBS/DMSO control. For initial drug treatment studies (drug regimen 1), drugs were administered by oral gavage or intraperitoneal injection to male CBA/J mice one time before surgery (day –1) and three times after surgery (days +1, +3, and +5), and infection status was assessed by sacrifice on day +7. For post-infection studies (drug regimen 2), mice were infected as described and assessed for established infection after > 7 days. Established infection was determined by In Vivo Imaging System (IVIS) luminescent visualization as described previously⁸ and/or laparotomy (visual inspection and cecal culture). Mice with confirmed infection were orally gavaged daily with drug for 3 consecutive days, and infection status was assessed after mice were euthanized on the fourth day. Amebic antigen in cecal contents was determined by enzyme-linked immunosorbent

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assay (ELISA) according to the manufacturer's instructions (TechLab, Blacksburg, VA). Additionally, cecal contents were used to inoculate complete TYI-S-33 medium to determine the presence of viable ameba.

Pathology and scoring of amebic colitis. After infection and drug treatment, ceca were bisected longitudinally and preserved using Bouin's fixative. Tissue sections were paraffin-embedded, and 4- μ m sections were stained with hematoxylin and eosin. Histopathological assessment of ameba score was determined in blinded fashion for each mouse as previously described.⁹

Statistics. Statistical significance for means was determined using *t* test. Proportions were determined by χ^2 analysis. Clinical scoring was analyzed using the Mann-Whitney *U* test.

RESULTS

We first examined *in vitro* the effect of several drugs currently in use to treat amebiasis, including metronidazole, tinidazole, nitazoxanide, iodoquinol, emetine, and chloroquine (Figure 1). Axenic HM1:IMSS was cultured in the presence of dilutions of drug for 72 hours and counted manually. Metronidazole and tinidazole exhibited potent efficacy *in vitro*, with no observable growth even at a concentration of 0.005 μ g/mL. Nitazoxanide, iodoquinol, and emetine exhibited growth only at the lower concentrations (0.005, 0.1, and 0.05 μ g/mL, respectively). Chloroquine could not inhibit growth even at the highest concentration of 5 μ g/mL.

We then determined a dosing regimen for mice considering the *in vitro* potency as well as human dosing and likely achievable serum concentrations (Table 1). For most drugs, we

TABLE 1
Clinical and experimental drug dosages

Treatment	Lowest inhibitory dose (μ g/mL)	Clinical dose for amebiasis (mg/kg per day)	Murine dosage (mg/kg)
Metronidazole	< 0.005	32.1	10.0
Tinidazole	< 0.005	28.6	10.0
Nitazoxanide	0.02	14.3	10.0/20.0
Iodoquinol	0.435	27.9	55.7
Chloroquine (base)	> 2.143	8.6	17.1
Emetine	0.058	0.09	1.9
Pyriminium pamoate	0.117	7.5*	15.0
AMIX	< 0.005	N/A	10.0
VPC16a1006	0.02	N/A	10.0

*Antihelminthic dose in micrograms per kilogram.

used 10 mg/kg per day, because this has been commonly used for respective mouse studies. For iodoquinol, chloroquine, and emetine, we administered 55.9, 17.1, and 1.9 mg/kg per day, respectively, for proof of principle, because this was double the human dose. For initial experiments, groups of mice were administered the respective drug on day -1 relative to challenge and for three doses thereafter (drug regimen 1). Mice were then killed on day +7 and examined for evidence of infection. Culture positive rate, cecal parasite ELISA, and histologic ameba score showed similar findings, revealing potent efficacy (e.g., 0% culture positive rate) with metronidazole, tinidazole, and iodoquinol (Figure 2). In contrast, nitazoxanide, chloroquine, and emetine were statistically more efficacious than PBS, but a sizeable minority of mice (25%, 16%, and 16%, respectively) remained infected, with similar ameba scores to control mice. Most groups were not powered to discern small differences in efficacy between drug treatments; however, metronidazole was observed to be statistically superior

FIGURE 1. *In vitro* antiamebic efficacy of clinically relevant drugs. HM1:IMSS *E. histolytica* trophozoites (10^4 /mL) were cultured in complete supplemented with drug for 72 hours. Cell numbers were counted on a hemocytometer and scored as the percentage growth relative to ameba grown in unsupplemented media. Data represent three counts of duplicated cultures $\pm$ standard deviation (SD). Isolated error bars indicate positive control (initial count) and negative control (no drug) $\pm$ SD. **P* ≤ 0.05.

FIGURE 2. Efficacy of antiamebic drugs *in vivo*: regimen 1. (A) Drugs were administered by oral gavage or intraperitoneal injection before and after intracecal injection of *E. histolytica* trophozoites (days -1, +1, +3, and +5 relative to challenge). Mice were then killed for evaluation of infection by culture (B), cecal parasite antigen (C), and histology (D). Antigen data represent average OD value $\pm$ standard error of the mean (SEM). * $P \leq 0.05$ between drug and PBS. ** $P \leq 0.05$ between metronidazole and PBS.

to nitazoxanide in these experiments ($P < 0.05$). In addition, we examined parenteral metronidazole and emetine and found that both were efficacious versus PBS; again, 0% culture positivity was observed with metronidazole.

We then examined these drugs in the setting of established infection. Mice were challenged with *E. histolytica*, and infection was confirmed after 7 days by laparotomy or imaging. Mice were then administered three doses of either metronidazole (10 mg/kg per day) or nitazoxanide (20 mg/kg per day; drug regimen 2). Culture positive rate, cecal parasite ELISA, and histologic ameba score revealed that both drugs exhibited efficacy compared with PBS controls (Figure 3) but complete and superior efficacy with metronidazole versus nitazoxanide ($P < 0.05$).

Lastly, we examined novel drugs both *in vitro* and *in vivo* (Figure 4). AMIX and VPC16a1006 are hydrophilic nitazoxanide-related compounds¹⁰; pyrvinium is an antiparasitic medication that has been shown to possess antiamebic properties *in vitro*.¹¹ AMIX and VPC16a1006 exhibited comparable potency with nitazoxanide *in vitro*. Pyrvinium had modest potency *in vitro* (lowest inhibitory dose of 0.05 μ g/mL) but reasonable efficacy *in vivo* in regimen 1. AMIX showed some efficacy with drug regimen 1, but neither AMIX nor VPC16a1006 showed a measurable effect compared with control using drug regimen 2.

DISCUSSION

The most important finding of this work is the demonstration of a mouse model that can be used to compare drug efficacies in intestinal amebiasis. There have been limited comparisons in humans to understand relative drug efficacy. Here, we show that metronidazole and likely, tinidazole have superior efficacy in this model. These drugs are the mainstay of treatment of amebiasis; thus, although our findings are unsurprising, they are important for confirmation.

Although metronidazole therapy is very effective in resolving infections, issues of incomplete clearance against intestinal

ameba and tolerability by patients remain. Additionally, metronidazole resistance has been documented in clinical isolates of *Giardia intestinalis* and *Trichomonas vaginalis* and has been artificially induced in a laboratory strain of *E. histolytica*,^{12,13} although resistance has not been seen clinically.¹⁴ Furthermore, transmission of metronidazole-refractory amebiasis has been reported.⁷ These data have spurred the development of novel antiamebic therapies; there have been many recent reports of novel potentially antiamebic compounds produced by *de novo* synthesis and screening of plant-derived compounds.^{15–22}

Nitazoxanide is a drug recently approved in the United States for cryptosporidiosis. It has been reported to treat intestinal amebiasis both clinically and microbiologically,⁵ but no comparative trials with metronidazole have been performed. Our data would argue against its use over the nitroimidazoles. Because of its poor systemic bioavailability, nitazoxanide concentrates in the gut lumen. Because our model of amebiasis reveals significant tissue destruction and inflammation, we speculated that a drug with improved bioavailability may be superior. Therefore, we evaluated AMIX and VPC16a1006, which are nitazoxanide-related compounds with improved solubility and serum-binding characteristics that should enhance bioavailability. These drugs retained similar efficacy *in vitro* but exhibited unchanged or worse efficacy *in vivo*. To us, this suggests that the more bioavailable derivatives, because of improved absorption, may not achieve sufficiently high luminal concentrations as noted with nitazoxanide.

This *in vitro* to *in vivo* disconnect was a common finding for other drugs as well. Chloroquine, for example, was entirely ineffective *in vitro*, even at concentrations of 5 μ g/mL, but it exhibited reasonable efficacy *in vivo*. The lack of *in vitro* effect has been shown before,²³ and the mechanism of action *in vivo* is unclear.⁴

Emetine has long been used for severe amebiasis, both hepatic and intestinal, and dehydroemetine remains available in the United States through the Centers for Disease Control and Prevention. In our studies, we found emetine to be of moderate efficacy both *in vitro* and *in vivo*. It is only available

FIGURE 3. Efficacy of antiamebic drugs to cure established infection: regimen 2. (A) Drugs were administered by oral gavage or intraperitoneal injection for 3 consecutive days at least 7 days after infection was confirmed by laparotomy with visual inspection and culture or imaging. Mice were then killed for evaluation of infection by culture (B), cecal parasite antigen (C), and histology (D). Antigen data represent average optical density value $\pm$ SEM. * $P \leq 0.05$.

as a parenteral agent, and we tested it against parenteral metronidazole, because the use of parenteral therapies for severe amebiasis is an area of surprisingly little data, essentially only one case series that showed utility with intravenous metronidazole.²⁴ Our results again showed perfect efficacy with par-

enteral metronidazole; thus, our data further support this regimen in severe disease.

Iodoquinol has been widely used for amebiasis, typically after a treatment course with metronidazole, to prevent relapse. Additionally, a large study in India found it to be

FIGURE 4. Assessment of novel antiamebic drugs. *In vitro* efficacy of novel drugs AMIX, VPC16a1006, and pyrinium were examined (A). Drugs were then examined *in vivo* according to drug regimen 1 (B) and 2 (C) *vide supra*. * $P \leq 0.05$.

effective in symptomatic *E. histolytica*- and *E. dispar*-infected patients.²⁵ Iodoquinol showed efficacy in our model, which was one of invasive disease; however, we studied it in the setting of regimen 1, which included a pre-challenge dose (where a luminal agent could presumably work). We also used a rather high dose, double that of humans, which would be a concern because of its known optic neuropathy side effects. Lastly, pyrinium has been used as an antihelminthic for *Enterobius* infection but has broad activity against protozoan parasites, including *Cryptosporidium parvum* and *Giardia intestinalis*.^{11,26} In our model, it exhibited modest efficacy in regimen 1.

In summary, this mouse model of amebiasis is a tractable model to examine drug efficacy, particularly because *in vitro* and *in vivo* efficacies correlate rather poorly. For this reason, we would caution drug screening solely on the basis of *in vitro* efficacy. Our results support metronidazole as the first-line agent, and this model can be used to examine novel drugs in the future.

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