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Antigiardial and antiamebic activities of fexinidazole and its metabolites: new drug leads for giardiasis and amebiasis

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ABSTRACT The intestinal parasites *Giardia lamblia* and *Entamoeba histolytica* are major causes of morbidity and mortality associated with diarrheal diseases. Metronidazole is the most common drug used to treat giardiasis and amebiasis. Despite its efficacy, treatment failures in giardiasis occur in up to 5%–40% of cases. Potential resistance of *E. histolytica* to metronidazole is an increasing concern. Therefore, it is critical to search for more effective drugs to treat giardiasis and amebiasis. We identified antigiardial and antiamebic activities of the rediscovered nitroimidazole compound, fexinidazole, and its sulfone and sulfoxide metabolites. Fexinidazole is equally active against *E. histolytica* and *G. lamblia* trophozoites, and both metabolites were 3- to 18-fold more active than the parent drug. Fexinidazole and its metabolites were also active against a metronidazole-resistant strain of *G. lamblia*. *G. lamblia* and *E. histolytica* cell extracts exhibited decreased residual nitroreductase activity when metabolites were used as substrates, indicating nitroreductase may be central to the mechanism of action of fexinidazole. In a cell invasion model, fexinidazole and its metabolites significantly reduced the invasiveness of *E. histolytica* trophozoites through basement membrane matrix. A q.d. oral dose of fexinidazole and its metabolites at 10 mg/kg for 3 days reduced *G. lamblia* infection significantly in mice compared to control. The newly discovered antigiardial and antiamebic activities of fexinidazole, combined with its FDA-approval and inclusion in the WHO Model List of Essential Medicines for the treatment of human African trypanosomiasis, offer decreased risk and a shortened development timeline toward clinical use of fexinidazole for treatment of giardiasis or amebiasis.

KEYWORDS antiparasitic agents, *Giardia*, *Entamoeba histolytica*

Giardiasis has an estimated worldwide annual prevalence of 280 million cases and about 500,000 new cases are reported each year (1). In developed countries, *Giardia lamblia*, the protozoan responsible for giardiasis, infects ~2% of adults and ~6%–8% of children (2, 3). The prevalence of *G. lamblia* infection is higher in developing countries (4, 5). *G. lamblia* was the single most frequently identified pathogen in all drinking water outbreaks reported in the US during 1971–2006 and was responsible for 121 outbreaks during that period (3). In 2010, the total number of reported cases of giardiasis in the US was 19,927 (3). Each year, hospitalizations resulting from giardiasis cost approximately $34 million; additionally, each ambulatory care visit for giardiasis costs $121–$273 (6). Because of its link with poverty, *Giardia* was included in the WHO Neglected Diseases Initiative in 2004 (7).

Invasive amebiasis, caused by the intestinal protozoan parasite *Entamoeba histolytica*, is a major health and social problem in the developing world. It is also a problem in the developed world in travelers, immigrants, and men who have sex with men (8, 9). Approximately 500 million people worldwide are infected annually with *E. histolytica*, resulting in 50 million cases of invasive amebiasis (10) and about 70,000 annual deaths (11). The estimated prevalence of *E. histolytica* infection in the US is 4% (12). The mortality

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from fulminating amebic colitis is almost 70% and that from amebic liver abscess is up to 10% (13). A recent study found that amebiasis produces about 2.2 million DALYs (8), and it accounts for 12.5% of all microbiologically confirmed cases, with an estimated incidence of 14 in every 1,000 returning travelers (9, 14). Both *G. lamblia* and *E. histolytica* have been listed by the NIH as category B priority biodefense pathogens due to their low infectious dose and potential for dissemination through compromised food and water supplies in the United States.

Metronidazole is the most common drug used to treat giardiasis and invasive amebiasis (15), but it has several adverse effects, such as nausea, vomiting, diarrhea, and constipation (13). Standard treatment with metronidazole requires 250 mg 3 times daily dosing for 3–5 days for giardiasis (2) and at least 10 days at high dosage (750 mg t.i.d) to eradicate intestinal amebae (16). Newer metronidazole derivatives such as tinidazole (17) and nitazoxanide (18) have fewer side effects and shorter treatment courses. However, potential resistance of *E. histolytica* to metronidazole is an increasing concern, as *in vitro E. histolytica* trophozoites adapt to therapeutically relevant levels of metronidazole (19). In spite of the efficacy of nitroimidazole drugs, treatment failures in giardiasis occur in up to 40% of cases (20) and cross-resistance occurs to tinidazole and nitazoxanide (21). Benzimidazole drug albendazole has been used, but its efficacy varies considerably (22) and *in vitro* resistance and treatment failure with albendazole have also been reported (2). Given the prevalence of giardiasis and amebiasis, the development of new antimicrobials to treat these infections is a critical unmet need to avert future outbreaks and potential terrorist attacks.

MATERIALS AND METHODS

Chemicals and reagents

White, solid bottom tissue culture-treated 96-well microplates were purchased from E&K Scientific (Santa Clara, CA). CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega (Madison, WI); dimethyl sulfoxide (DMSO) and metronidazole were purchased from Sigma-Aldrich (St. Louis, MO); fexinidazole, fexinidazole sulfoxide, and fexinidazole sulfone were provided by Drugs for Neglected Diseases *initiative* (DND*i*) through Epichem Pty Ltd (Bentley, WA, Australia).

Maintenance of *G. lamblia* and *E. histolytica*

Trophozoites of *G. lamblia* metronidazole-sensitive (MtzS) WB and GS/M and *E. histolytica* HM1:IMSS strains were axenically maintained in TYI-S-33 medium supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) (23, 24). The metronidazole-resistant (MtzR) *G. lamblia* WB-M2 (25) was maintained in 50 µM metronidazole but was grown without the drug for 2–3 days before experiments. All experiments were performed using trophozoites and cells harvested during the logarithmic phase of growth.

In vitro drug testing against *G. lamblia* and *E. histolytica* trophozoites

Candidate compounds were screened and reassayed for EC_{50} determination against *G. lamblia* and *E. histolytica* trophozoites using a final 8-point concentration range. 2.5 µL of 5 mM stock compounds in 100% DMSO was diluted with 17.5 µL sterile water to yield 625 µM working concentration of compounds. A threefold serial dilution was then performed yielding a concentration range of 0.25–625 µM. From this dilution plate, 4 µL was transferred into the 96-well screen plates followed by the addition of 96 µL of trophozoites (5,000 parasites per well) to yield a final 8-point concentration range spanning 0.01–25 µM in final 0.5% DMSO (26, 27). The assays were performed in triplicate, and assay plates were incubated for 48 h at 37°C in the GasPak EZ gas-generating anaerobe pouch system (VWR). At the end of incubation, the assay plates were equilibrated to room temperature for 30 min, and 50 µL of CellTiter-Glo Luminescent Cell Viability Assay (Promega) was added in each well of the 96-well plates. The plates

were then placed on an orbital shaker at room temperature for 10 min to induce cell lysis. After lysis, the plates were again equilibrated at room temperature for 10 min to stabilize luminescent signal (26). The resulting ATP-bioluminescence of the trophozoites was measured at room temperature using an EnVision Multilabel Reader (PerkinElmer, Waltham, MA). Negative controls in the screen plates contained 0.5% DMSO and positive controls contained 30 µM metronidazole (Sigma-Aldrich).

Data analysis and statistics

Percent inhibition relative to maximum and minimum reference signal controls was calculated using the formula:

% Inhibition = [(mean of Maximum Signal Reference Control − Experimental Value)/(mean of Maximum Signal Reference Control − mean of Minimum Signal Reference Control)] ×100 (26)

Visualization and statistical analysis of compound screening data were performed using GraphPad Prism software 10.0.2.

Nitroreductase activity assays

We determined the nitroreductase activity in the cell lysates of *G. lamblia* WB and *E. histolytica* HMI:IMSS strains by measuring oxidation of NADPH in the presence of metronidazole, fexinidazole, and its metabolites as substrates. Briefly, the reaction mixture in 96-well plate contained Tris-acetate (100 mM Tris-HCl, 50 mM acetate buffer, pH 7.0), 5 µg of cell lysate protein, 100 µM of either fexinidazole or 100 µM of fexinidazole sulfoxide or 100 µM of fexinidazole sulfone or 100 µM of metronidazole, and 0.5 mM NADPH. A control reaction was performed with 0.5% DMSO. The assays were performed in triplicate in three independent experiments. Each experiment contained controls of extract lacking NADPH, buffer with NADPH, and buffer only. Reactions were monitored every 15 s at 340 nm for 30 min for the change in the amounts of metronidazole, fexinidazole, and its metabolites as substrates (28). Nitroreductase activity was determined by following the decrease in absorbance at 340 nm generated by the oxidation of NADPH in the presence of metronidazole, fexinidazole, and its metabolites.

Efficacy testing in animal model of *G. lamblia* infection

G. lamblia MtzS GS/M trophozoites were grown to mid-logarithmic phase, and adult C57BL/6 mice (The Jackson Laboratory) were infected by oral gavage with 10^7 trophozoites/mouse in a 50 µL volume in *Giardia* growth medium (16). We selected MtzS GS/M strain because earlier study showed that MtzR strain WB-M2 could not reliably establish infection in mice (25). After 2 days of infection, mice were treated orally by gavage with 10 mg/kg q.d. of fexinidazole, fexinidazole sulfoxide, and fexinidazole sulfone in 0.1% Hypromellose in PBS for 3 days. Control mice received only 0.1% Hypromellose in PBS. At the end of treatment, the entire small intestine was removed, opened longitudinally, placed into 2–5 mL PBS, and cooled on ice for 10 min. Live trophozoites were counted in a hemocytometer (27). All animal studies were reviewed and approved by the UC San Diego Institutional Animal Care and Use Committee. Animal studies were performed under the University of California San Diego Institutional Animal Care and Use Committee (IACUC) approval #S00205 and adhered to guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and the USDA Animal Care Policies. Mice were euthanized by CO_2 inhalation and cervical dislocation in accordance with the recommendations of the American Veterinary Medical Association Guidelines on Euthanasia.

Drug effects on invasion by *E. histolytica*

To test whether fexinidazole and its metabolites influence invasiveness of *E. histolytica*, we used a transwell matrigel invasion assay (29). Briefly, 75,000 *E. histolytica* trophozoites were preincubated for 3 h with 0.5% DMSO, 10 µM of metronidazole, 10 and 20 µM of

fexinidazole, 1.5 and 3 µM of fexinidazole sulfoxide, and 1.3 and 2.5 µM of fexinidazole sulfone, and after 3 h, cells were re-suspended in serum-free TYI medium (23) and loaded in the upper chamber of a BioCoat Matrigel Invasion Chamber (Corning). The lower chamber contained TYI medium supplemented with 10% adult bovine serum. The chamber was incubated at 37°C for 72 h in a GasPak EZ gas-generating anaerobe pouch system (VWR). Trophozoites that had migrated into the lower chamber were counted using a hemocytometer. The data were obtained from three independent experiments each performed in triplicate.

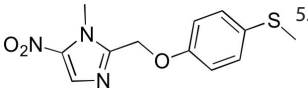
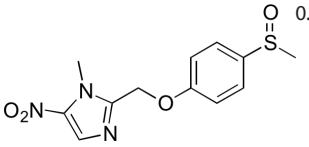
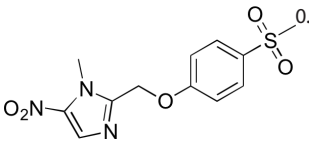
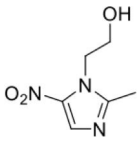
RESULTS

In vitro drug testing against *G. lamblia* and *E. histolytica* trophozoites

Since fexinidazole is a nitroimidazole and is rapidly oxidized *in vivo* to sulfoxide and sulfone metabolites (30), we tested the activity of not only fexinidazole but also its metabolites against *G. lamblia* and *E. histolytica*. Both fexinidazole and its metabolites were active against two MtzS *G. lamblia* strains, WB and GS/M. The parent compound had an EC_{50} of 5.6 µM against the *G. lamblia* WB strain, whereas the metabolites were more than 10-fold more active, with the sulfone metabolite exhibiting an EC_{50} of 0.3 µM and the sulfoxide metabolite an EC_{50} of 0.5 µM (Table 1; Fig. 1). Similarly, both metabolites were more active than the parent compound against the *G. lamblia* GS/M strain. Fexinidazole sulfoxide showed an EC_{50} of 0.4 µM, and the sulfone metabolite had an EC_{50} of 0.2 µM, while fexinidazole EC_{50} was 1.3 µM against the GS/M strain (Table 1). The parent compound and metabolites had EC_{50} better than metronidazole, which were 7.9 and 3.7 µM against the WB and GS/M strains, respectively. Fexinidazole and metabolites were also active against the *G. lamblia* MtzR WB-M2 strain (Table 1). The EC_{50} of fexinidazole and metabolites against the resistant strain was similar to the EC_{50} against the WB parental strain, whereas the EC_{50} of metronidazole was more than 10-fold higher in the resistant strain than the parental strain.

A similar trend was seen with *E. histolytica* trophozoites when tested for EC_{50} determination at 48 h. The parent compound had an EC_{50} of 9.7 µM against *E. histolytica,* while the metabolites were >5-fold more active, with the sulfone metabolite exhibiting

TABLE 1 EC_{50}[a] of fexinidazole, fexinidazole sulfoxide, fexinidazole sulfone, and metronidazole against *G. lamblia*

Compounds	Structure	*G. lamblia* EC_{50} (pEC_{50} ± SE) (µM)			*E. histolytica* EC_{50} (pEC_{50} ± SE) (µM)
		Strain WB MtzS	Strain GS/M MtzS	Strain WB-M2 MtzR	
Fexinidazole		5.6 (5.3 ± 0.01)	1.3 (5.9 ± 0.14)	7.4 (5.1 ± 0.17)	9.7 (5.3 ± 0.01)
Fexinidazole sulfoxide		0.5 (6.3 ± 0.02)	0.4 (6.4 ± 0.07)	0.5 (6.3 ± 0.07)	1.5 (5.8 ± 0.01)
Fexinidazole sulfone		0.3 (6.5 ± 0.01)	0.2 (6.7 ± 0.07)	0.2 (6.7 ± 0.05)	1.3 (5.9 ± 0.01)
Metronidazole		7.9 (5.1 ± 0.05)	3.7 (5.4 ± 0.11)	80.4 (4.1 ± 0.04)	5.3 (5.3 ± 0.05)

[a]EC50 minimum $n = 3$.

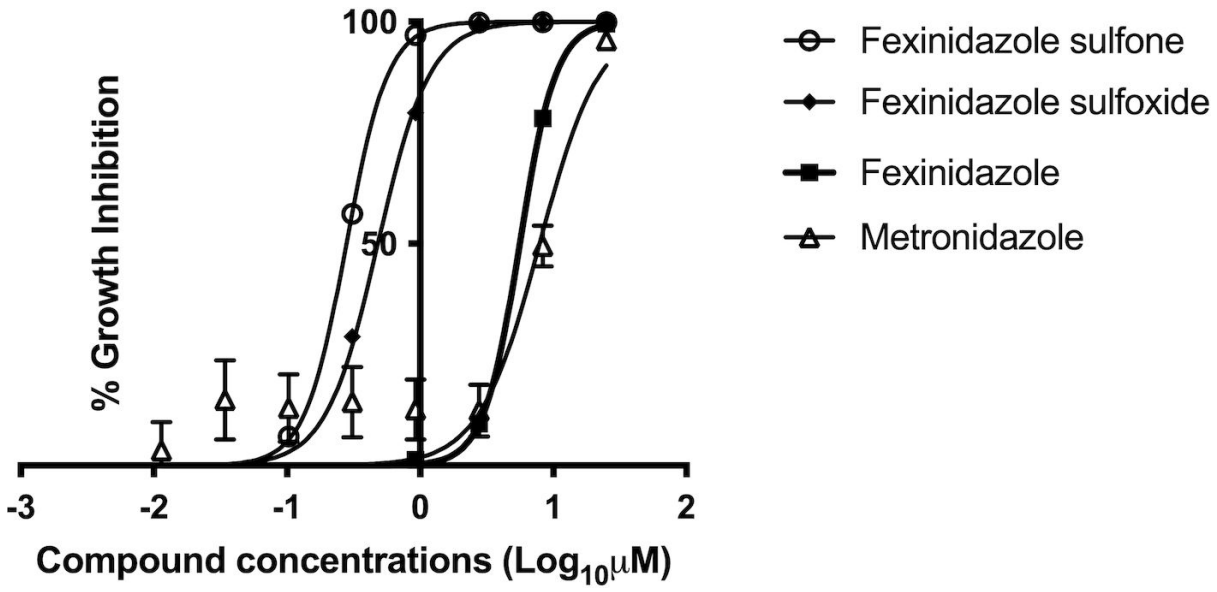


FIG 1 Activity (EC_{50}) of fexinidazole, fexinidazole sulfoxide, fexinidazole sulfone, and metronidazole against *G. lamblia* WB strain trophozoites. Different concentrations of compounds were tested for activity (EC_{50}) against the trophozoites. The data represent the mean ± SE.

an EC_{50} of 1.3 µM and the sulfoxide metabolite an EC_{50} of 1.5 µM against *E. histolytica* (Table 1).

The cytotoxicity (CC_{50}) of fexinidazole and metronidazole against the human hepatic cell line HepG2 are >200 and >100 µM, respectively (31, 32), so the selectivity index (SI) (CC_{50}/EC_{50}) was >36 and >21 for fexinidazole against *G. lamblia* and *E. histolytica*, which compares favorably to the SI of >16 and >19 for metronidazole against *G. lamblia* and *E. histolytica*. Fexinidazole and its metabolites did not have any effects on the cysts of *G. lamblia* (data not shown).

Nitroreductase activity assays

Since several studies have suggested that bioactivation of fexinidazole is mediated by oxygen-insensitive type I nitroreductase (NTR) (33) and *G. lamblia* and *E. histolytica* contain genes encoding NTR I (28, 34), we determined the nitroreductase activity present in the cell lysates of *G. lamblia* and *E. histolytica* when the parent drug fexinidazole and its metabolites were used as substrates. Oxidation of NADPH in the presence of different nitroheterocyclic compounds as substrates showed the highest nitroreductase activity in *G. lamblia* extract with metronidazole followed by fexinidazole and its metabolites as substrates (Fig. 2A). This is consistent with the increased potency of metabolites against *G. lamblia* than metronidazole (Table 1). In case of *E. histolytica*, the highest residual activity obtained with metronidazole and fexinidazole as substrates is consistent with the similar EC_{50} of metronidazole and fexinidazole on whole cell *E. histolytica*. Although *E. histolytica* lysate did not exhibit low nitroreductase activity when fexinidazole sulfone was used as a substrate, the lowest residual activity was detected with the sulfoxide metabolite as a substrate (Fig. 2B). This reduction in the nitroreductase activity with sulfoxide as a substrate matches with the high potency of fexinidazole sulfoxide against *E. histolytica* trophozoites (Table 1).

Efficacy testing in animal model of *G. lamblia* infection

Fexinidazole is orally available and is rapidly converted to two metabolites, the sulfoxide and the sulfone. In the *in vitro* study, both parent compound and metabolites were found to be active against *G. lamblia*. We next tested the *in vivo* efficacy of fexinidazole, fexinidazole sulfoxide, and fexinidazole sulfone in a mouse model of giardiasis with the

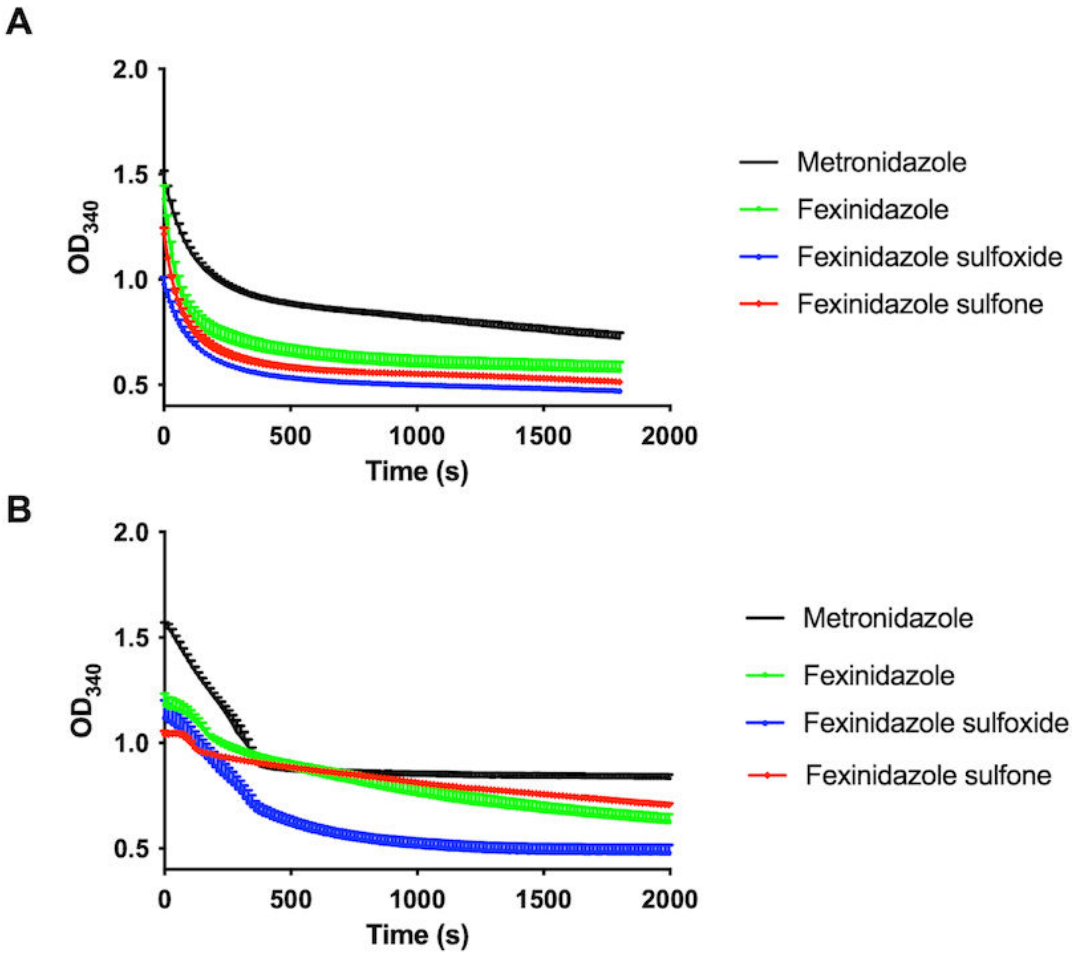


FIG 2 Nitroreductase activity in *G. lamblia* and *E. histolytica* cell lysates. Oxidation of NADPH in the presence of 100 µM each of fexinidazole, fexinidazole sulfoxide or fexinidazole sulfone, or metronidazole as substrates was used to evaluate the nitroreductase activity present in *G. lamblia* (A) and *E. histolytica* (B) cell lysates. Nitroreductase activity was determined by following the decrease in absorbance at 340 nm every 15 s for 30 min. The data represent the mean and standard error of mean for assay performed in triplicate and measured every 15 s.

MtzS GS/M strain. *In vivo* efficacy testing of fexinidazole and its metabolites against metronidazole-resistant *G. lamblia* strain could not be done due to lack of infectivity of MtzR WB-M2 strain in mice (25). In an earlier study (35), the pharmacokinetic profiles of fexinidazole sulfoxide and sulfone metabolites were assessed in mice. The key parameters after an oral dose of 10 mg/kg in female mice were as follows: terminal elimination half-life ($T_{1/2}$) = 0.8 h for fexinidazole sulfoxide and 1.9 h for fexinidazole sulfone; maximum concentration of drug in serum (C_{max}) = 3.56 µg/mL for fexinidazole sulfoxide and 6.88 µg/mL for fexinidazole sulfone; time to C_{max} (T_{max}) = 0.5 h for fexinidazole sulfoxide and 2 h for fexinidazole sulfone; and area under the concentration-time curve to infinity (AUC_{inf}) = 7.68 µg h/mL for fexinidazole sulfoxide and 43.4 µg h/mL for fexinidazole sulfone (35). The absolute bioavailability of fexinidazole after oral administration was 41% in mice (36), and fexinidazole sulfoxide and sulfone can achieve plasma levels of 12 µM and 22 µM, respectively after single oral administration of 10 mg/kg of fexinidazole sulfoxide to uninfected mice (35). These plasma concentrations are well above the EC_{50} we determined *in vitro*. Earlier toxicology studies showed that fexinidazole is well tolerated. The No Observed Adverse Event Level (NOAEL) of fexinidazole in the 28-day repeated dose toxicokinetic study in both rats and beagle dogs was 200 mg/kg/day (36). Considering an excellent safety profile and favorable pharmacokinetic parameters of fexinidazole and its metabolites, we selected a dose of 10 mg/kg. A

q.d. oral dose of fexinidazole, fexinidazole sulfoxide, and fexinidazole sulfone at 10 mg/kg for 3 days significantly reduced the number of *G. lamblia* compared to the number in control mice ($P < 0.05$) (Fig. 3). We did not observe gross pathological changes in the small intestine with drug treatment.

Drug effects on invasion by *E. histolytica*

Since *E. histolytica* invades the large intestine to cause amebic colitis, we determined the effect of fexinidazole and its metabolites on the invasiveness of *E. histolytica* in a controlled *in vitro* model. A 3 h pre-incubation of trophozoites with fexinidazole, fexinidazole sulfoxide or fexinidazole sulfone at their EC_{50} and 2× EC_{50} concentrations decreased the invasion of trophozoites significantly after 72 h compared to 0.5% DMSO-treated trophozoites (Fig. 4). Fexinidazole at 10 µM caused significantly more decrease in invasion than the same concentration of metronidazole (Fig. 4).

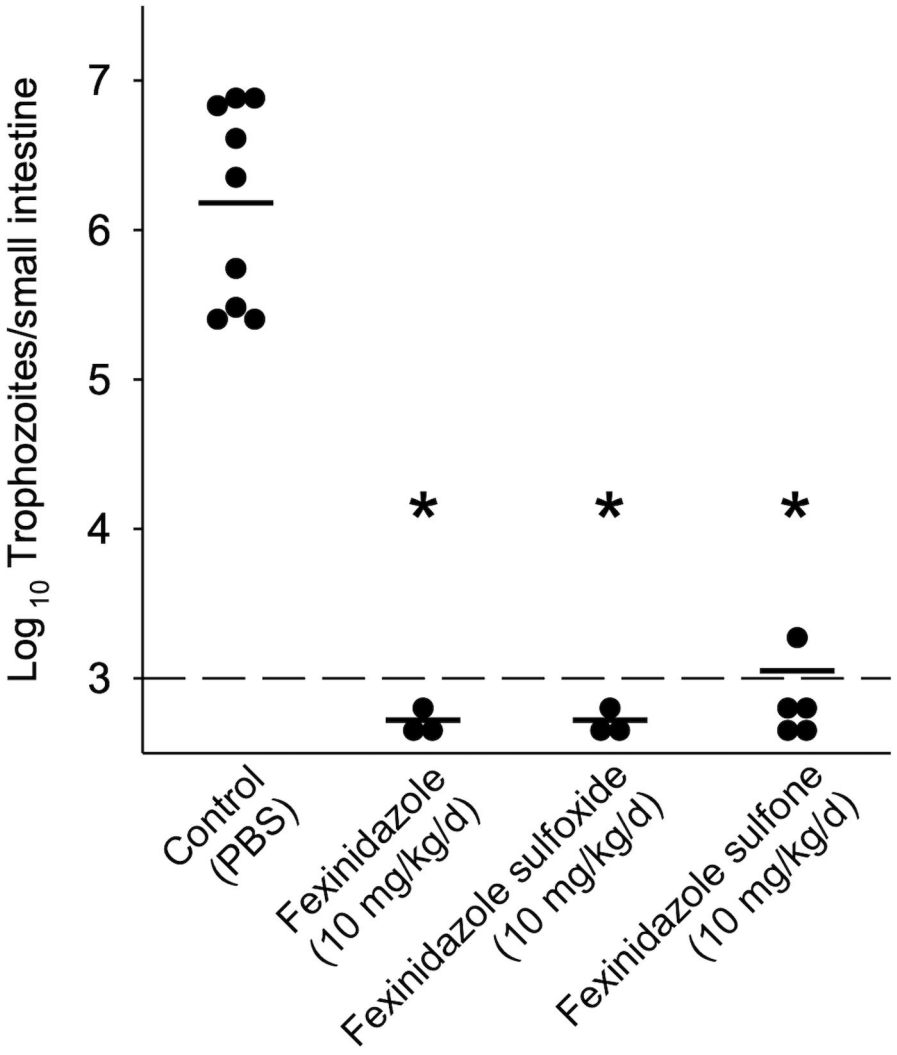


FIG 3 Effect of fexinidazole, fexinidazole sulfoxanide, and fexinidazole sulfone in a mouse model of giardiasis. At 2 days postinfection with *G. lamblia*, mice were treated once daily with 10 mg/kg with fexinidazole, fexinidazole sulfoxide, and fexinidazole sulfone for 3 days. Control mice received 0.1% Hypromellose in PBS. *$P < 0.05$ by Mann-Whitney *U* test compared to PBS-treated control mice. Each black circle indicates Log_{10} number of trophozoites present in the small intestine of each mouse treated with the vehicle control (0.1% Hypromellose in PBS) or a compound. The dashed horizontal line represents the detection limit of the assay.

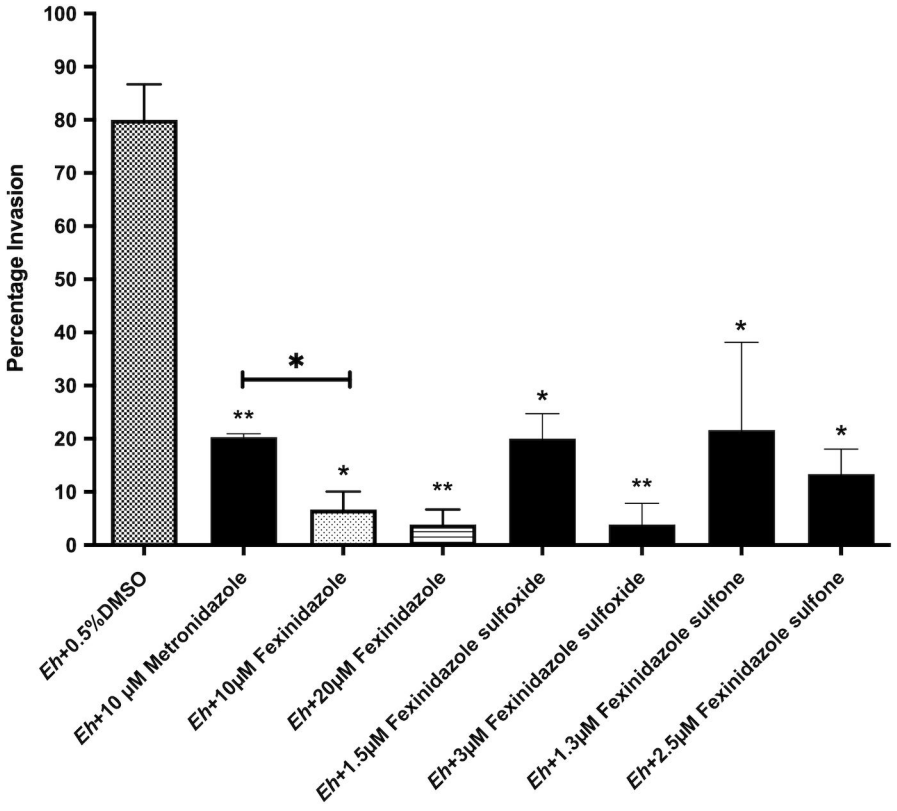


FIG 4 Effect of fexinidazole, fexinidazole sulfoxide, and fexinidazole sulfone on invasion of matrigel by *E. histolytica*. *E. histolytica* trophozoites were incubated for 3 h with 0.5% DMSO, 10 µM of metronidazole, 10 and 20 µM of fexinidazole, 1.5 and 3 µM of fexinidazole sulfoxide, and 1.3 and 2.5 µM of fexinidazole sulfone, and at the end of incubation, trophozoites were allowed to invade the matrigel for 72 h. After 72 h, the migrated cells in the lower chamber were counted and percentage invasion of trophozoites was calculated and plotted. *$P < 0.05$ by student's *t*-test compared to 0.5% DMSO-treated *E. histolytica* trophozoites. The horizontal bar represents the percentage invasion of matrigel in 10 µM metronidazole-treated versus 10 µM fexinidazole-treated trophozoites and *$P < 0.05$ by student's *t*-test compared to 10 µM metronidazole-treated *E. histolytica* trophozoites.

DISCUSSION

The nitroimidazole scaffold has an established history of antiparasitic activity and the nitro group is critical to the mode of action. Despite more than 50 years of use, the full antimicrobial potential of nitroimidazole compounds has not been fully explored. Recent studies strongly suggest that the systematic development of next-generation nitroimidazole drugs can lead to superior antiparasitic agents (16, 32).

In the early 1980s, the nitroimidazole fexinidazole was identified as a broad-spectrum antimicrobial agent with lower toxicity and comparative ease of chemical synthesis (37). In the development of a suckling mouse model of *G. lamblia* infection, a preliminary investigation of the activity of fexinidazole was also conducted (38). However, fexinidazole's clinical development was not pursued, mainly due to the avoidance of the development of nitroaromatic compounds at that time (33). More recently, the compound was "rediscovered" and selected for development by the Drugs for Neglected Diseases *initiative* (DND*i*) as drug lead for human African trypanosomiasis, caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. A detailed analysis of genotoxic potential of fexinidazole was undertaken utilizing different assays such as *in vitro* micronucleus test on human lymphocytes, an *in vivo* mouse bone marrow micronucleus test, and an *ex vivo* unscheduled DNA synthesis test in rats (36). These studies suggested that fexinidazole may not be genotoxic. DND*i* then carried out phase I

clinical trials in 2010 and 2011, assessing the safety and pharmacokinetics of fexinidazole in human volunteers given a single dose or multiple doses, and a phase II/III study of clinical safety and efficacy in sleeping sickness patients was commenced in 2012 (39). The human clinical studies demonstrated that doses of up to 3,600 mg of fexinidazole in fasted conditions were safe and well tolerated in healthy male subjects of sub-Saharan origin. A dosing regimen of fexinidazole 1,800 mg/day for 4 days and 1,200 mg/day for 6 days with concomitant food was shown to meet the required criteria of effective drug concentrations with a good safety profile (39). Free fraction of metabolites in human studies were about 60% and 43% for sulfoxide and sulfone, respectively, indicating that neither metabolite is highly protein bound (39). Based on the clinical data, fexinidazole is now FDA-approved and included in the WHO Model List of Essential Medicines, 21st List (2019) and WHO Model List of Essential Medicines for Children, 7th List (2019) for the treatment of human African trypanosomiasis. Fexinidazole is currently used for the treatment of both first and second stages of human African trypanosomiasis in pediatric patients aged 6 years and older. Since the safety and effectiveness of fexinidazole have not been established for patients younger than 6 years old (40), it is not recommended for patients in that age group.

Given the renewed interest in the chemotherapeutic potential of nitroheterocyclic compounds for giardiasis and amebiasis, we initiated investigations of the drug candidate fexinidazole and its two principal metabolites, fexinidazole sulfoxide and fexinidazole sulfone, for *in vitro* activity against *G. lamblia* and *E. histolytica* and *in vivo* efficacy against *G. lamblia*.

Our *in vitro* studies identified nanomolar and low micromolar potency of the sulfoxide and sulfone metabolites of fexinidazole against *G. lamblia* and *E. histolytica*. Previous studies showed that fexinidazole is rapidly metabolized *in vivo* through oxidation to at least two biologically active sulfoxide and sulfone metabolites and the blood concentrations of these metabolites exceed that of fexinidazole, suggesting that the metabolites are the therapeutically relevant species *in vivo* (36). Once-daily oral dosing of 1,800 mg/day for 4 days led to human plasma concentrations of sulfoxide and sulfone metabolites of 7.768 µg/mL and 18.79 µg/mL, respectively, or 26.3 µM and 60.4 µM, respectively (39), which are about 17- to 40-fold more than the *in vitro* EC_{50} we identified against *E. histolytica* or *G. lamblia*. Based on the *in vitro* activity of the metabolites, we hypothesized that these two metabolites would exert greater efficacy in the animal model of giardiasis.

In our study, fexinidazole and its sulfoxide and sulfone metabolites showed *in vivo* efficacy in a mouse model of giardiasis at single oral daily dose of 10 mg/kg for 3 days. This dose is very low when compared with doses used in the *in vivo* efficacy studies of fexinidazole and metabolites in other parasitic diseases. The effective daily doses were at least 25 mg/kg in case of visceral leishmaniasis (30) and 100 mg/kg for Chagas disease (35) and human African trypanosomiasis (36, 41). Our effective daily dose of 10 mg/kg in mouse represents a human equivalent dose of 0.8 mg/kg, which is 1/25 of the dose that was found well tolerated in human clinical studies (1,200 mg/day human dose of fexinidazole for 6 days with concomitant food, equivalent to ~20 mg/kg) (36). Considering its simple chemistry and shorter steps to synthesize fexinidazole, it is expected to be relatively inexpensive. Fexinidazole was calculated to be not more than US$50 per treatment or perhaps significantly less for the treatment of African sleeping sickness (36). While metronidazole cost varies considerably, a 2018 cost of oral metronidazole ranged from US$4.38 to US$13.14 for 500 mg (42). Based on this price, treatment cost of giardiasis with metronidazole is almost similar to the treatment of African sleeping sickness with fexinidazole. Considering the shorter treatment schedule and low effective total human equivalent dose for giardiasis, which is expected to be at least 1/42 of the total dose that was used in phase II/III clinical study for African sleeping sickness, the total cost for the treatment of giardiasis would be significantly less than the cost of treatment for African sleeping sickness and similarly lower than the cost of metronidazole.

Previous experiments suggested that metronidazole was not a good substrate for *G. lamblia* NTR I (34). Considering the nanomolar potency of fexinidazole metabolites on metronidazole-resistant *G. lamblia* and lower nitroreductase activity with fexinidazole metabolites than metronidazole as substrates, fexinidazole may not exert similar resistance as metronidazole. This will require further investigation on the development of resistance against fexinidazole metabolites and understanding the underlying mechanism that may confer resistance. It will also be important to carry out detailed molecular and biochemical studies on the implication of nitroreductases in the activation of fexinidazole and its metabolites.

In conclusion, we have identified a safe, inexpensive, rediscovered oral antigiardial, and antiamebic drug, fexinidazole. This alternative nitro-based antimicrobial with good efficacy, pharmacokinetic, and toxicity profiles is suitable for further clinical development as oral drug therapy for the treatment of giardiasis and amebiasis. Pediatric use of fexinidazole for patients less than 6 years old will require safety and effectiveness study in that age group.

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Jose Ignacio Escrig, Formal analysis, Investigation | Yukiko Miyamoto, Formal analysis, Investigation | Alejandro Delgado Aznar, Formal analysis, Investigation | Lars Eckmann, Supervision | Anjan Debnath, Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing – original draft

REFERENCES

1. Adam RD. 2001. Biology of *Giardia lamblia*. Clin Microbiol Rev 14:447–475. https://doi.org/10.1128/CMR.14.3.447-475.2001
2. Gardner TB, Hill DR. 2001. Treatment of giardiasis. Clin Microbiol Rev 14:114–128. https://doi.org/10.1128/CMR.14.1.114-128.2001
3. Yoder JS, Harral C, Beach MJ, Centers for Disease Control and Prevention (CDC). 2010. Giardiasis surveillance - United States, 2006-2008. MMWR Surveill Summ 59:15–25.
4. Cifuentes E, Suárez L, Espinosa M, Juárez-Figueroa L, Martínez-Palomo A. 2004. Risk of *Giardia intestinalis* infection in children from an artificially recharged groundwater area in Mexico city. Am J Trop Med Hyg 71:65–70. https://doi.org/10.4269/ajtmh.2004.71.65
5. Granados CE, Reveiz L, Uribe LG, Criollo CP. 2012. Drugs for treating giardiasis. Cochrane Database Syst Rev 12:CD007787. https://doi.org/10.1002/14651858.CD007787.pub2
6. Collier SA, Stockman LJ, Hicks LA, Garrison LE, Zhou FJ, Beach MJ. 2012. Direct healthcare costs of selected diseases primarily or partially transmitted by water. Epidemiol Infect 140:2003–2013. https://doi.org/10.1017/S0950268811002858
7. Savioli L, Smith H, Thompson A. 2006. *Giardia* and *Cryptosporidium* join the 'neglected diseases initiative'. Trends Parasitol 22:203–208. https://doi.org/10.1016/j.pt.2006.02.015

8. Hotez PJ, Alvarado M, Basáñez M-G, Bolliger I, Bourne R, Boussinesq M, Brooker SJ, Brown AS, Buckle G, Budke CM, et al. 2014. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. PLoS Negl Trop Dis 8:e2865. https://doi.org/10.1371/journal.pntd.0002865
9. Shirley D-A, Farr L, Watanabe K, Moonah S. 2018. A review of the global burden, new diagnostics, and current therapeutics for amebiasis. Open Forum Infect Dis 5:ofy161. https://doi.org/10.1093/ofid/ofy161
10. WHO/PAHO/UNESCO report. A consultation with experts on amoebiasis. Mexico city, Mexico 28-29 January, 1997. 1997. Epidemiol Bull 18:13–14.
11. World Health Organization. 1998. The world health report 1998: life in the 21st century: a vision for all. World Health Organization, Geneva.
12. Gunther J, Shafir S, Bristow B, Sorvillo F. 2011. Short report: amebiasis-related mortality among United States residents, 1990-2007. Am J Trop Med Hyg 85:1038–1040. https://doi.org/10.4269/ajtmh.2011.11-0288
13. Sweetman SC. 2002. Martindale: the complete drug reference. 33rd ed. Pharmaceutical Press, London.
14. Swaminathan A, Torresi J, Schlagenhauf P, Thursky K, Wilder-Smith A, Connor BA, Schwartz E, Vonsonnenberg F, Keystone J, O'Brien DP, GeoSentinel Network. 2009. A global study of pathogens and host risk factors associated with infectious gastrointestinal disease in returned international travellers. J Infect 59:19–27. https://doi.org/10.1016/j.jinf.2009.05.008
15. Freeman CD, Klutman NE, Lamp KC. 1997. Metronidazole. A therapeutic review and update. Drugs 54:679–708. https://doi.org/10.2165/00003495-199754050-00003
16. Miyamoto Y, Kalisiak J, Korthals K, Lauwaet T, Cheung DY, Lozano R, Cobo ER, Upcroft P, Upcroft JA, Berg DE, Gillin FD, Fokin VV, Sharpless KB, Eckmann L. 2013. Expanded therapeutic potential in activity space of next-generation 5-nitroimidazole antimicrobials with broad structural diversity. Proc Natl Acad Sci U S A 110:17564–17569. https://doi.org/10.1073/pnas.1302664110
17. Fung HB, Doan T-L. 2005. Tinidazole: a nitroimidazole antiprotozoal agent. Clin Ther 27:1859–1884. https://doi.org/10.1016/j.clinthera.2005.12.012
18. Rossignol JF, Ayoub A, Ayers MS. 2001. Treatment of diarrhea caused by *Giardia intestinalis* and *Entamoeba histolytica* or *E. dispar*: a randomized, double-blind, placebo-controlled study of nitazoxanide. J Infect Dis 184:381–384. https://doi.org/10.1086/322038
19. Wassmann C, Hellberg A, Tannich E, Bruchhaus I. 1999. Metronidazole resistance in the protozoan parasite *Entamoeba histolytica* is associated with increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase. J Biol Chem 274:26051–26056. https://doi.org/10.1074/jbc.274.37.26051
20. Nabarro LEB, Lever RA, Armstrong M, Chiodini PL. 2015. Increased incidence of nitroimidazole-refractory giardiasis at the hospital for tropical diseases, London: 2008-2013. Clin Microbiol Infect 21:791–796. https://doi.org/10.1016/j.cmi.2015.04.019
21. Wright JM, Dunn LA, Upcroft P, Upcroft JA. 2003. Efficacy of antigiardial drugs. Expert Opin Drug Saf 2:529–541. https://doi.org/10.1517/14740338.2.6.529
22. Miyamoto Y, Eckmann L. 2015. Drug development against the major diarrhea-causing parasites of the small intestine, *Cryptosporidium* and *Giardia*. Front Microbiol 6:1208. https://doi.org/10.3389/fmicb.2015.01208
23. Diamond LS, Harlow DR, Cunnick CC. 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans R Soc Trop Med Hyg 72:431–432. https://doi.org/10.1016/0035--9203(78)90144-x
24. Keister DB. 1983. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans R Soc Trop Med Hyg 77:487–488. https://doi.org/10.1016/0035-9203(83)90120-7
25. Tejman-Yarden N, Millman M, Lauwaet T, Davids BJ, Gillin FD, Dunn L, Upcroft JA, Miyamoto Y, Eckmann L. 2011. Impaired parasite attachment as fitness cost of metronidazole resistance in *Giardia lamblia*. Antimicrob Agents Chemother 55:4643–4651. https://doi.org/10.1128/AAC.00384-11
26. Debnath A, Parsonage D, Andrade RM, He C, Cobo ER, Hirata K, Chen S, García-Rivera G, Orozco E, Martínez MB, Gunatilleke SS, Barrios AM, Arkin MR, Poole LB, McKerrow JH, Reed SL. 2012. A high-throughput drug screen for *Entamoeba histolytica* identifies a new lead and target. Nat Med 18:956–960. https://doi.org/10.1038/nm.2758
27. Debnath A, Shahinas D, Bryant C, Hirata K, Miyamoto Y, Hwang G, Gut J, Renslo AR, Pillai DR, Eckmann L, Reed SL, McKerrow JH. 2014. Hsp90 inhibitors as new leads to target parasitic diarrheal diseases. Antimicrob Agents Chemother 58:4138–4144. https://doi.org/10.1128/AAC.02576-14
28. Pal D, Banerjee S, Cui J, Schwartz A, Ghosh SK, Samuelson J. 2009. *Giardia*, *Entamoeba*, and *Trichomonas* enzymes activate metronidazole (nitroreductases) and inactivate metronidazole (nitroimidazole reductases). Antimicrob Agents Chemother 53:458–464. https://doi.org/10.1128/AAC.00909-08
29. Probst A, Nguyen TN, El-Sakkary N, Skinner D, Suzuki BM, Buckner FS, Gelb MH, Caffrey CR, Debnath A. 2019. Bioactivity of farnesyltransferase inhibitors against *Entamoeba histolytica* and *Schistosoma mansoni*. Front Cell Infect Microbiol 9:180. https://doi.org/10.3389/fcimb.2019.00180
30. Wyllie S, Patterson S, Stojanovski L, Simeons FRC, Norval S, Kime R, Read KD, Fairlamb AH. 2012. The anti-trypanosome drug fexinidazole shows potential for treating visceral leishmaniasis. Sci Transl Med 4:119re1. https://doi.org/10.1126/scitranslmed.3003326
31. Fersing C, Basmaciyan L, Boudot C, Pedron J, Hutter S, Cohen A, Castera-Ducros C, Primas N, Laget M, Casanova M, Bourgeade-Delmas S, Piednoel M, Sournia-Saquet A, Belle Mbou V, Courtioux B, Boutet-Robinet É, Since M, Milne R, Wyllie S, Fairlamb AH, Valentin A, Rathelot P, Verhaeghe P, Vanelle P, Azas N. 2019. Nongenotoxic 3-nitroimidazo[1,2-*a*]pyridines are NTR1 substrates that display potent *in vitro* antileishmanial activity. ACS Med Chem Lett 10:34–39. https://doi.org/10.1021/acsmedchemlett.8b00347
32. Jarrad AM, Debnath A, Miyamoto Y, Hansford KA, Pelingon R, Butler MS, Bains T, Karoli T, Blaskovich MAT, Eckmann L, Cooper MA. 2016. Nitroimidazole carboxamides as antiparasitic agents targeting *Giardia lamblia*, *Entamoeba histolytica* and *Trichomonas vaginalis*. Eur J Med Chem 120:353–362. https://doi.org/10.1016/j.ejmech.2016.04.064
33. Patterson S, Wyllie S. 2014. Nitro drugs for the treatment of trypanosomatid diseases: past, present, and future prospects. Trends Parasitol 30:289–298. https://doi.org/10.1016/j.pt.2014.04.003
34. Müller J, Rout S, Leitsch D, Vaithilingam J, Hehl A, Müller N. 2015. Comparative characterisation of two nitroreductases from *Giardia lamblia* as potential activators of nitro compounds. Int J Parasitol Drugs Drug Resist 5:37–43. https://doi.org/10.1016/j.ijpddr.2015.03.001
35. Bahia MT, Nascimento AFS, Mazzeti AL, Marques LF, Gonçalves KR, Mota LWR, Diniz L de F, Caldas IS, Talvani A, Shackleford DM, Koltun M, Saunders J, White KL, Scandale I, Charman SA, Chatelain E. 2014. Antitrypanosomal activity of fexinidazole metabolites, potential new drug candidates for Chagas disease. Antimicrob Agents Chemother 58:4362–4370. https://doi.org/10.1128/AAC.02754-13
36. Torreele E, Bourdin Trunz B, Tweats D, Kaiser M, Brun R, Mazué G, Bray MA, Pécoul B. 2010. Fexinidazole--a new oral nitroimidazole drug candidate entering clinical development for the treatment of sleeping sickness. PLoS Negl Trop Dis 4:e923. https://doi.org/10.1371/journal.pntd.0000923
37. Raether W, Seidenath H. 1983. The activity of fexinidazole (HOE 239) against experimental infections with *Trypanosoma cruzi*, trichomonads and *Entamoeba histolytica*. Ann Trop Med Parasitol 77:13–26. https://doi.org/10.1080/00034983.1983.11811668
38. Boreham PF, Phillips RE, Shepherd RW. 1986. The activity of drugs against *Giardia intestinalis* in neonatal mice. J Antimicrob Chemother 18:393–398. https://doi.org/10.1093/jac/18.3.393
39. Tarral A, Blesson S, Mordt OV, Torreele E, Sassella D, Bray MA, Hovsepian L, Evène E, Gualano V, Felices M, Strub-Wourgaft N. 2014. Determination of an optimal dosing regimen for fexinidazole, a novel oral drug for the treatment of human African trypanosomiasis: first-in-human studies. Clin Pharmacokinet 53:565–580. https://doi.org/10.1007/s40262-014-0136-3
40. Imran M, Khan SA, Alshammari MK, Alqahtani AM, Alanazi TA, Kamal M, Jawaid T, Ghoneim MM, Alshehri S, Shakeel F. 2022. Discovery, development, inventions and patent review of fexinidazole: the first all-oral therapy for human African trypanosomiasis. Pharmaceuticals (Basel) 15:128. https://doi.org/10.3390/ph15020128
41. Kaiser M, Bray MA, Cal M, Bourdin Trunz B, Torreele E, Brun R. 2011. Antitrypanosomal activity of fexinidazole, a new oral nitroimidazole

drug candidate for treatment of sleeping sickness. Antimicrob Agents Chemother 55:5602–5608. https://doi.org/10.1128/AAC.00246-11
42. Rajasingham R, Enns EA, Khoruts A, Vaughn BP. 2020. Cost-effectiveness of treatment regimens for *Clostridioides difficile* infection: an evaluation of the 2018 infectious diseases society of America guidelines. Clin Infect Dis 70:754–762. https://doi.org/10.1093/cid/ciz318