Discovery of Novel Antigiardiasis Drug Candidates

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Giardiasis is a severe intestinal parasitic disease caused by *Giardia lamblia*, which inflicts many people in poor regions and is the most common parasitic infection in the United States. Current standard care drugs are associated with undesirable side effects, treatment failures, and an increasing incidence of drug resistance. As follow-up to a high-throughput screening of an approved drug library, which identified compounds lethal to *G. lamblia* trophozoites, we have determined the minimum lethal concentrations of 28 drugs and advanced 10 of them to *in vivo* studies in mice. The results were compared to treatment with the standard care drug, metronidazole, in order to identify drugs with equal or better anti-*Giardia* activities. Three drugs, fumagillin, carbadox, and tioxidazole, were identified. These compounds were also potent against metronidazole-resistant human *G. lamblia* isolates (assemblages A and B), as determined in *in vitro* assays. Of these three compounds, fumagillin is currently an orphan drug used within the European Union to treat microsporidiosis in immunocompromised individuals, whereas carbadox and tioxidazole are used in veterinary medicine. A dose-dependent study of fumagillin in a giardiasis mouse model revealed that the effective dose of fumagillin was ~100-fold lower than the metronidazole dose. Therefore, fumagillin may be advanced to further studies as an alternative treatment for giardiasis when metronidazole fails.

The enteric protozoan parasite *Giardia lamblia* causes giardiasis, a severe diarrheal disease, and it is commonly acquired from contaminated freshwater and public water supplies. The disease is highly prevalent in regions that lack adequate water management. In regions where giardiasis is endemic and where environmental contamination is high, the reinfection rate might reach 90%. Children chronically infected with *Giardia* suffer from malnutrition, growth retardation, poor cognitive function, and death. Because giardiasis impairs development and socioeconomic improvements, the World Health Organization has included the disease in its Neglected Diseases Initiative (1). Treatments with standard care drugs, such as metronidazole, tinidazole, and albendazole, fail at a rate of ~20% (2–6). The unpleasant side effects of these drugs lead to patient noncompliance, compounding the growing concerns about the spread of drug-resistant strains. *G. lamblia* evades the host immune system by frequently switching its variant-specific surface proteins; therefore, vaccine development has proven to be a challenge. For these reasons, we have undertaken the discovery of alternative anti-*Giardia* drugs that are not subject to current resistance mechanisms.

To enable high-throughput compound library screening (HTS), we developed a bioluminescence-based assay that monitors the cellular ATP contents in *G. lamblia* trophozoite, which correlate with the viability of the organism (7). We used this assay to identify compounds that kill the trophozoites by screening a Library of Pharmaceutical Active Compounds (LOPAC¹²⁸⁰) (Sigma-Aldrich, St. Louis, MO) and the NIH Chemical Genomics Center Pharmaceutical collection library (NPC) of approved drugs, which consisted of 2,816 compounds at the time. The screen revealed 43 drugs lethal to *G. lamblia* trophozoites and nontoxic to mammalian CHO cells, of which 11 were novel anti-*Giardia* agents (7). The HTS was performed with the assemblage A *G. lamblia* WB isolate, and we confirmed that the novel anti-*Giardia* drugs were also potent against the assemblage B *G. lamblia* GS isolate using both the ATP content assay and a trophozoite enumeration method. Drugs already approved for use with other

indications are attractive anti-*Giardia* drug candidates because the cost and duration of the preclinical phase are greatly reduced and, depending on the compound, the clinical trial phase may be shorter and less costly. We have therefore undertaken the pursuit of further preclinical studies of a selected set of the newly discovered drugs, triaging compounds that proved inadequate along the way.

While the ATP content assay is amenable to high-throughput screening, it requires confirmation by a secondary assay. Specifically, the ATP-dependent luciferase bioluminescence signal is abolished when a compound inhibits the metabolic activity of the trophozoite, but the organisms might remain viable and resume proliferation upon the completion of treatment. High-throughput trophozoite enumeration is also limited in scope because it does not reliably detect morphological changes associated with unviable trophozoites. Although more laborious, the determination of the minimum lethal concentration (MLC) followed by a proliferation assay in the absence of the compound remains the most reliable method for evaluating *G. lamblia* trophozoite viability and the potential for the recurrence of infection.

Here, we report on follow-up studies of drugs discovered by the previously reported HTS campaign (7). We have determined the MLCs of 28 selected drugs, based on criteria discussed later, and advanced 10 of them to *in vivo* mice studies. We also confirmed that the most effective drugs emerging from the studies in the mouse giardiasis model exhibit excellent potencies against

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metronidazole-resistant *G. lamblia* isolates and thus are promising candidates for further development as alternative anti-giardial therapeutic agents.

MATERIALS AND METHODS

***G. lamblia* cultures.** Trophozoites of the *G. lamblia* assemblage B isolate GS/H7 (8) were grown anaerobically in borosilicate glass screw-cap culture tubes (Fisher Scientific) at pH 7.0 in modified TYI-S-33 medium (7, 9). The medium was supplemented with 10% heat-inactivated bovine serum (Sigma-Aldrich) and 0.05% bovine bile (Sigma-Aldrich). To attain low-oxygen-tension conditions, the tubes were filled to 85 to 90% of their total volume capacity and incubated without shaking at 37°C. Subcultures (2×10^5 trophozoites per tube) were made three times a week. For *in vitro* assays and mouse infection, the trophozoites were detached from the wall of the tube by chilling the cultures on ice for 20 min. The metronidazole-resistant *G. lamblia* assemblage A 713M3 (10) and assemblage B 1279-M1 (11) isolates were kindly provided by Lars Eckmann at the University of California, San Diego. These isolates were cultured in the above medium, which was supplemented with gradually increasing metronidazole concentrations up to 50 μ M.

Minimum lethal concentration determinations. MLCs were determined by incubating *G. lamblia* trophozoites with the drugs in 96-well culture plates (Corning Incorporated), followed by transferring the trophozoites to 8-ml tubes (Fisher Scientific) for proliferation. The assays were performed in duplicate. Dry compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma) at a stock concentration of 10 mM and then diluted 1:100 in growth medium to a final compound concentration of 100 μ M. One-hundred-microliter aliquots of compound solutions were prepared by 2-fold serial dilutions (12 concentrations) in medium. This was followed by the addition of 10 μ l of *G. lamblia* culture containing 10,000 organisms into medium with the compound to be tested in 96-well plates. Metronidazole dissolved in DMSO and diluted to a final concentration of 100 μ M with the growth medium served as a positive control, and the medium-diluted DMSO alone served as a negative control. The plates were incubated under anaerobic conditions in sealed bags (Becton Dickinson and Company) at 37°C for 3 days and surveyed visually under the microscope to check trophozoite survival, mobility, and attachment. The plates were chilled on ice for 30 min, and the entire contents of each of 4 wells in the growth/death transition were transferred into the 8-ml tubes containing growth medium and no drug. The tubes were incubated under anaerobic conditions for 3 days at 37°C and checked under a microscope. The MLC was attributed to the lowest concentration without any live organisms.

ATP content assay. The ATP content assay was described in detail previously (7). The assays were performed in duplicate. Tubes containing *G. lamblia* trophozoites were placed on ice for 30 min. One-hundred-microliter aliquots of each tube were transferred into 96-well black clear-bottom assay plates, followed by the addition of 70 μ l/well of the ATPlite reagent (PerkinElmer) to initiate one-step cell lysis and detection of the ATP levels. The luminescence signals were measured on an EnSpire 2300 plate reader (PerkinElmer).

Giardiasis animal model and drug treatment. Nash and colleagues reported the adult mouse model used in the current study (12). Fifteen 4-week-old C57BL/6J 15-g female mice (Jackson Laboratories, Bar Harbor, ME) were infected with *G. lamblia* GS/H7 trophozoites. The trophozoites (500,000 suspended in 200 μ l of TYI-S-33 medium) were administered by oral gavage. On days 3 through 6 after infection, 10 mice were treated once daily with 5 mg of a drug (~ 333 mg/kg of body weight), except for fumagillin and riboflavin butyrate, which were administered at 2-mg and 10-mg doses, respectively. The remaining untreated five mice served as controls. The drugs were suspended in 200 μ l of corn oil and were administered by oral gavage. Each mouse was euthanized on day 7. *G. lamblia* cultures from the small intestine were harvested and axenized, as recently published (13). Two inches of the upper small intestine was dissected and washed with 2 ml of medium supplemented with antibiotics

(1 mg/ml each piperacillin and moxalactam). The harvested small intestines were opened longitudinally and minced in a petri dish containing 10 ml of ice-chilled medium. The plates were placed on ice for 30 min to allow the trophozoites to detach from the intestine and were surveyed under the microscope to estimate the trophozoite population by the plate survey method described previously (14). The trophozoites were enumerated in several random fields at all depths at a magnification of $\times 20$ with the Axiovert 40 C microscope (Zeiss). The University of Maryland College Park Institutional Animal Care and Use Committee (IACUC) approved the animal studies.

Mouse trophozoite load quantification by proliferation of axenic cultures. The entire content of each petri dish was transferred to a 15-ml glass tube, the volume was adjusted to 14 ml by adding medium containing 1 mg/ml each piperacillin and moxalactam, and the tube was vigorously vortexed to separate the trophozoites from the intestine debris. For the following 2 h, the tubes were kept at 37°C to allow trophozoite attachment to the wall of the glass tube, after which the medium containing the intestine debris was decanted and replaced by fresh medium. The tubes were kept at 37°C for another 15 min, followed by a second medium replacement, but this time the medium was supplemented with 1 mg/ml piperacillin, 1 mg/ml moxalactam, 5 μ g/ml amoxicillin-clavulanic acid, and 10 μ g/ml nalidixic acid. The tubes were kept at 37°C for 6 days for proliferation. Trophozoite growth was determined once daily by direct cell counting using a Beckman Coulter Z1 counter (Beckman Coulter, Inc.) and by monitoring the ATP content using the ATPlite reagent.

The proliferation measurements were used to calculate growth curves and the trophozoite load of drug-treated mice compared with that of the untreated mice. Trophozoite load was quantified by calculating the initial population from the growth curves, fitting the luminescence or the trophozoite-counting data to the Malthusian model of exponential growth: $N_t = N_0 \times e^{rt}$, where N_t is the signal of the population at time t , r is the growth rate, and N_0 is the signal of the initial population at the end of treatment, i.e., the value we seek to determine. The percent load was determined relative to the N_0 of the untreated mice samples.

RESULTS AND DISCUSSION

Confirmation of drug potency *in vitro* by MLC determination.

We determined the MLCs of 27 selected drugs approved for other diseases or in clinical trials but not yet approved for the treatment of giardiasis (in addition to 1 drug analog and the comparator drug, metronidazole). The MLCs are provided in Table 1. These experiments were performed with the *G. lamblia* GS isolate that was later used in the *in vivo* adult mouse model studies. Previously, we demonstrated consistent potencies of drugs against the assemblage A *G. lamblia* WB and assemblage B *G. lamblia* GS using the ATP content and trophozoite enumeration assays (7). Table 1 also shows the 50% inhibitory concentrations (IC₅₀s) for the GS trophozoite viabilities of 16 compounds, which confirmed consistency between the potencies in the WB and GS strains, as determined by the ATP content assay. Thus, the relationship between the IC₅₀ and MLC is independent of the *G. lamblia* strain.

The selection of compounds for MLC determinations was based on IC₅₀s that were comparable to or better than that of the standard care drug, metronidazole. Hydroxycobalamin (vitamin B₁₂) was the only exception, with a higher IC₅₀, which was examined because it is a safe natural product that is not well absorbed through the gut. Indeed, because of its low toxicity, the Food and Nutrition Board has not set an upper intake level for its consumption (15). In general, drugs/vitamins that do not cross the intestinal epithelial barrier but kill the trophozoites are extremely attractive candidates for anti-giardial therapy agents because *G. lamblia* trophozoites attach to the gut epithelial cells but do not invade them.

TABLE 1 Comparison between IC₅₀s and MLCs of selected anti-*Giardia* drugs

Compound	IC ₅₀ (μM) for <i>G. lamblia</i> strain:		MLC (μM) for <i>G. lamblia</i> GS
	WB	GS	
Fumagillin	0.01	0.002	0.26
17-Allylamino-geldanamycin ^{a,b}	0.05		13.9
Bortezomib	0.07	0.06	0.51
CB1954 ^b	0.08		0.5
Carboquone ^a	0.12		1.54
Nocodazole ^a	0.12		0.15
Acivicin	0.17		13.9
Tenonitroazole	0.19		1.54
Nifuroxime	0.31	1.0	1.54
Nithiamide	0.39	0.32	4.63
Nitarosone	0.43	0.9	2.6
Decitabine	0.97	0.15	2.6
Tioxidazole	0.97	0.63	1.54
Deserpidine ^a	1.09	0.28	1.54
Riboflavin butyrate (vitamin B ₂)	1.09	1.13	41.7
Amlexanox ^a	1.73		>41.7
BTO-1 ^b	1.73	2.0	41.7
Idarubicin ^a	2.17		>41.7
Piceatannol	2.17	3.6	13.4
Disulfiram ^a	2.74	1.6	1.5 ^c
Resveratrol	3.29		>208
Thiram ^a	3.45		0.8 ^c
Auranofin ^a	5.46		41.7
Carbadox	5.46	0.24	4.6
Mitoxantrone ^a	5.46		>41.7
GW9662	6.87	12.7	208
Metronidazole ^d	8.65	1.6	7.7
Hydroxycobalamin (vitamin B ₁₂)	13.7	78.3	>208

^a These compounds were cytotoxic to CHO cells.^b 17-Allylamino-geldanamycin and CB1954 are in clinical trials; BTO-1 and GW9662 are in preclinical development.^c Disulfiram and thiram MLC determinations used growth medium containing a reduced cysteine concentration (2.9 mM cysteine), because the usual concentration (11.6 mM) blunts the activities of thiram compounds (13, 14).^d The comparative drug whose IC₅₀ was used as criterion to select compounds for MLC determination.

None of the selected drugs belonged to the nitroimidazole compound class, as they have already been extensively developed. Our aim was to identify less frequently used classes of compounds which would not be subject to the nitroimidazole resistance mechanism. We also examined the MLC of a nondrug analog of the approved drug disulfiram, thiram, because both compounds inactivated a novel *G. lamblia* drug target, carbamate kinase (13, 16). Ten of the selected drugs were not listed in our previous publication because they exhibited cytotoxicity in CHO cells (7). Nevertheless, these 10 approved drugs were advanced to the MLC determination stage because the cytotoxicity determined *in vitro* does not always recapitulate the *in vivo* toxicity.

Table 1 provides the viability data using nonpharmaceutical-grade pure compounds. The majority of compounds (23 out of 28) selected based on the IC₅₀s also exhibited potency using the MLC assay with *G. lamblia* GS. The remaining five drugs (amlexanox, idarubicin, resveratrol, mitoxantrone, and vitamin B₁₂) had no detectable potency at the maximum concentration tested, and GW9662 (not an approved drug) exhibited very low potency. The

discrepancy in the potencies between the two assays might be due to the mechanism of action of the drugs, which temporarily halts the metabolic activity of trophozoites without killing them. In these cases, transferring the organisms to medium lacking the drug allows recovery of metabolic activity and enables proliferation. In the clinic, such drugs might result in the recurrence of giardiasis upon treatment completion; thus, these compounds were eliminated.

Some of the remaining 23 drugs considered to be potent against *G. lamblia* GS exhibited MLCs substantially higher than IC₅₀s but that were still comparable to or better than the MLC of metronidazole. Although MLCs are expected to be somewhat elevated compared with IC₅₀s, large discrepancies were viewed with caution because *G. lamblia* WB and GS are major isolates in humans, and the desired drugs should be effective against both. Auranofin is an example of such a compound. The compound exhibited 8-fold-lower potency in the GS isolate compared with that of the WB isolate.

The drugs chosen to be advanced to the animal studies exhibited potencies against both WB and GS isolates. Riboflavin butyrate (a vitamin B₂ derivative used widely in Japan) was selected for further study because it exhibited significant potency in the MLC assay and is known to be a safe natural product, whereas vitamin B₁₂ was excluded because of a lack of detectable potency in the MLC assay. In addition, we eliminated some compounds based on documented toxicity data. The animal studies of disulfiram and thiram have been reported earlier, together with structural studies of the enzyme these compounds target, carbamate kinase (13). The remaining 10 compounds that were advanced to animal studies are listed in Table 2.

Drug treatments of *Giardia*-infected adult mice. Adult, weanling, and neonatal mice have been reported in the literature as giardiasis animal models. Human *G. lamblia* GS/H7 is the only known isolate that also infects adult mice (12). Other human *G. lamblia* isolates infect neonatal and weanling mice (17–21), which are more delicate and prone to accidental injuries during oral gavage. For easier and safer handling of the multiple compounds tested, we elected to evaluate the efficacy of the anti-giardial drugs in adult mice infected with *G. lamblia* GS/H7 trophozoites, using a new axenization protocol developed in our laboratory that enables quantification of the results by coupling the *in vivo* studies to an *in vitro* proliferation assay (13).

The adult mouse model we used couples infection and treatment to the proliferation of cultures harvested from the small intestine and axenized by using an antibiotic cocktail that includes the β-lactamase inhibitor clavulanic acid. This protocol facilitates accurate quantification of the results as we discussed earlier (13). We adopted a triage procedure to screen all 10 compounds for efficacy in the mouse model by first using relatively high drug doses (5 mg/day), with two exceptions, fumagillin (2 mg/day) and riboflavin butyrate (10 mg/day). The initial high doses facilitated quicker elimination of ineffective drugs as well as early indication of any potential toxic effects. The standard care drug, metronidazole, was used at the same dose as a control. As can be seen in Table 3, three of the drugs were highly toxic at the 5-mg/day dose, which necessitated euthanizing the mice (deserpidine, nifuroxime, and nitarosone). Nithiamide was lethal in three mice, whereas the remaining seven mice appeared unaffected. As a precaution, these seven mice were treated with a reduced dose of 1 mg/day after the first day.

TABLE 2 Drugs that advanced to animal studies

Drug	Indication	Reported mechanism of action (reference)
Carbadox	Swine dysentery (feed additive)	Unknown
Decitabine	Myelodysplastic syndromes	DNA methyltransferase inhibitor (44)
Deserpidine	Hypertension and psychotic relief	Reserpine derivative, inhibits vesicular neurotransmitter transporter VMAT2 (45)
Fumagillin	Intestinal and eye microsporidiosis in immunocompromised patients (orphan)	Methionine aminopeptidase 2 inhibitor (35)
Nifuroxime	Antifungal and antiprotozoal (topical)	Free radical generation (46)
Nitarzone	Poultry blackhead disease (feed additive)	Inhibits mitochondrial protein that regulates reactive oxygen species (47)
Nithiamide	Trichomoniasis	By analogy to nitazoxanide, a <i>G. lamblia</i> pyruvate/ferredoxin oxidoreductase inhibitor (48)
Piceatannol	Purported to have multiple health benefits (natural compound but not an approved drug)	Protein-tyrosine kinase inhibitor (49)
Riboflavin butyrate	Vitamin B ₂ ; multiple health benefits	Unknown
Tioxidazole	Parasitic worms in horses	By analogy to benzimidazoles, <i>G. lamblia</i> tubulin polymerization inhibitors (50)

Posttreatment proliferation assays. The gut flora is populated by anaerobic microbial species that thrive on the medium required for optimal anaerobic trophozoite growth *in vitro*. Until recently, bacterial contamination impeded the axenization of the *G. lamblia* cultures, which prevented proliferation experiments to evaluate the outcome of *in vivo* studies. We have developed a simple axenization protocol by identifying an antibiotic cocktail that does not interfere with *G. lamblia* growth (13). This cocktail includes the standard antibiotics used to avoid bacterial contamination in *in vitro* studies (piperacillin and moxalactam), as well as the combination drug amoxicillin-clavulanic acid and the quinolone nalidixic acid. The requirement for clavulanic acid, a class A β -lactamase inhibitor, suggests that in most cases, the commensal bacteria in the gut of the Jackson Laboratories C57BL/6J mice we used have acquired the class A β -lactamase; this is not a surprising finding considering the wide use of β -lactam antibiotics. We have used this mouse model and axenization protocol extensively for 2 years and encountered microbial contamination in only 7% of the experiments. This infrequent contamination was due to rod-

shaped Gram-positive bacteria. We have subsequently identified the organism as *Erysipelotrichaceae bacterium*, which is an abundant organism in mouse and human intestinal microbiota. Thus, other antibiotics that do not impair *Giardia* growth, for example, vancomycin, may help eliminate any remaining contamination.

Table 3 summarizes the results of the animal studies with the 10 drugs using high once-daily treatments for 4 days. As can be seen, the three evaluation methods of trophozoite load yielded consistent results. The agreement between the visual enumeration immediately after harvesting the small intestine and the two proliferation measurements is notable because scanning an entire plate is impractical, and only a few fields can be inspected under the microscope. Therefore, for drugs that appear to cure the infection, it is possible to miss the remaining trophozoites altogether. The proliferation assays are crucial for eliminating false-positive drugs.

The mouse studies eliminated the highly toxic drugs deserpidine, nifuroxime, and nitarzone. Nithiamide was also eliminated because of its lethal effect at a high dose (5 mg) and poor efficacy at

TABLE 3 *In vivo* adult mice studies of trophozoite load following high-dose once-daily drug treatment for 4 days

Drug	Trophozoite load (%) (<i>P</i> value) based on ^a :			
	Visual enumeration	Proliferation assay ^b		Dose/comments
		Trophozoite count	ATP content	
Metronidazole	1.9 (0.003)	0.01 $\pm$ 0.02 (2.3E-04)	0.002 $\pm$ 0.0052 (6E-06)	5 mg/day
Carbadox	4.6 $\pm$ 6.1 (2E-08)	NC ^c	0.00 $\pm$ 0.08 (5E-05)	5 mg/day
Fumagillin	0.7 $\pm$ 2.1 (5.1E-11)	0.04 $\pm$ 0.05 (1.5E-05)	0.03 $\pm$ 0.06 (1.1E-07)	2 mg/day
Tioxidazole	1.4 $\pm$ 2.0 (0.000006)	0.08 $\pm$ 0.22 (1.1E-11)	0.07 $\pm$ 0.20 (2.5E-09)	5 mg/day
Decitabine	10.6 $\pm$ 15.8 (4E-05)	34.0 $\pm$ 66.1 (0.150)	31.4 $\pm$ 54.5 (0.065)	5 mg/day
Riboflavin butyrate	74 $\pm$ 132 (0.57)	62 $\pm$ 31 (0.18)	63 $\pm$ 32 (0.18)	10 mg/day
Nithiamide	33 $\pm$ 27 (0.02)	57 $\pm$ 52 (0.46)	56.5 $\pm$ 39 (0.09)	5 mg on day 1; 3 mice dead/euthanized next day, 7 treated with 1 mg/day on days 2-4
Piceatannol	834 $\pm$ 1,720 (0.13)	923 $\pm$ 346 (0.0003)	914 $\pm$ 220 (0.00001)	5 mg/day; enhanced proliferation
Deserpidine	NC	NC	NC	5 mg/day; mice euthanized due to toxicity
Nifuroxime	NC	NC	NC	5 mg/day; mice euthanized due to toxicity
Nitarzone	NC	NC	NC	5 mg/day; mice euthanized due to toxicity

^a Trophozoite load was calculated relative to levels in the untreated mice. The *P* value was calculated based on a *t* test relative to the trophozoite load of the control untreated mice.

^b The trophozoite load calculated from the growth curves at time zero, i.e., the end of animal treatment, either by cell counting or ATP luminescence (see Materials and Methods for details).

^c NC, not counted because no proliferation was observed during the duration of the experiment.

TABLE 4 Drug potencies for metronidazole-resistant and metronidazole-sensitive *G. lamblia* strains

Drug	MLC (μ M) for strain (metronidazole resistance):		
	1279-M1 (resistant)	713M3 (resistant)	GS (susceptible)
Carbadox	12.5	12.5	4.6
Fumagillin	0.19	0.09	0.01
Tioxidazole	0.75	0.38	1.45
Metronidazole	62.5	125	7.7

a lower dose (1 mg), as it reduced the trophozoite load by only 50% compared with that of the untreated mice. Similarly, decitabine reduced the trophozoite load by 70% and did not clear the infection. Riboflavin butyrate was even less effective despite the high dose used (10 mg/day). The least effective compound was piceatannol, which unexpectedly enhanced trophozoite colonization of the intestine by nearly 10-fold compared with that of the control mice. We note that in order to run the *in vivo* studies, the selected drugs were repurchased in large quantities as pharmaceutical-grade material. All MLCs of the pharmaceutical-grade drugs were redetermined prior to animal dosing. Unlike all other drugs, the MLC of the pharmaceutical-grade piceatannol increased by 10-fold, suggesting that much of the potency measured using the nonpharmaceutical-grade samples might have been due to contaminants or degradation products. Nevertheless, this does not explain the enhancement of *G. lamblia* proliferation compared with that in the untreated mice, and it suggests a complex host-microbiome response to piceatannol.

Among the 10 compounds studied in the mouse model, fumagillin, carbadox, and tioxidazole were as effective as or better than metronidazole in curing giardiasis. Fumagillin required a lower dose than metronidazole to clear the parasite (120 and 300 mg/kg, respectively). Carbadox showed only a few trophozoites left, and these exhibited unhealthy morphology and were unable to proliferate (Table 3). These 3 compounds were then advanced to determine their *in vitro* potencies in metronidazole-resistant *G. lamblia* strains.

Fumagillin, carbadox, and tioxidazole retain potency in metronidazole-resistant *G. lamblia* isolates. The goal of the current study was to identify alternative anti giardiasis drugs in cases of treatment failure and drug resistance to the most commonly used drug, metronidazole. Such alternative drugs should act by a different mechanism than that of metronidazole and potentially could be administered in combination with standard care drugs to minimize the emergence of resistance. To demonstrate the potential utility of fumagillin, carbadox, and tioxidazole in addressing metronidazole treatment failures, we determined the MLCs of these drugs in metronidazole-resistant *G. lamblia* isolates. The 713M3 (10) and 1279-M1 (11) isolates were derived from the assemblage A (713) and assemblage B (1279) strains that infect humans. Prior to the determination of the MLCs, these *G. lamblia* isolates reached confluence in medium supplemented by 50 μ M metronidazole (recall that the MLC value for metronidazole in *G. lamblia* GS is 7.7 μ M; Table 1). Table 4 provides the MLCs of the three drugs and of metronidazole in *G. lamblia* 713M3 and 1279-M1, together with that of the comparative metronidazole-sensitive strain, *G. lamblia* GS. These data confirm that the three drugs exhibit comparable potencies in both metronidazole-sensitive and metronidazole-resistant *G. lamblia* strains. Based on the chemical nature of the compounds and their potencies in metronidazole-resistant *G. lamblia* strains, it may be concluded that fumagillin, carbadox, and tioxidazole act by different mechanisms from that of metronidazole.

TABLE 5 Drug efficacy in adult mice infected with *G. lamblia* GS after 4-day once-daily treatment

Dose (mg/kg/day)	Drug parameter values with:					
	Fumagillin ^a			Metronidazole ^b		
	Load (%) ^c	SD (%)	<i>P</i> (<i>t</i> test) ^d	Load (%) ^c	SD (%)	<i>P</i> (<i>t</i> test) ^d
120	0	0.05	1.5 E−05	0	2.3	2.2E−02
12	7	14	0.003	88	53	0.28
0.6	11	13	0.007	ND ^e	ND	ND

^a For fumagillin, the estimated ED₅₀ was <0.6 mg/kg, the LD₅₀ was 2,000 mg/kg, and the therapeutic index (LD₅₀/ED₅₀) was >3,000.

^b For metronidazole, the estimated ED₅₀ was ~50 mg/kg, the LD₅₀ was 3,000 mg/kg, and the therapeutic index (LD₅₀/ED₅₀) was >100.

^c Trophozoite load is calculated relative to the untreated mice.

^d The *P* value is calculated based on a *t* test relative to the trophozoite load of the control untreated mice.

^e ND, not determined.

Dose dependence in *in vivo* studies revealed that the efficacy of fumagillin in the mouse giardiasis model is superior to that with metronidazole. Carbadox and tioxidazole are currently used in veterinary medicine. These drugs may be repurposed to treat non-food-chain domestic animals infected with metronidazole-resistant *Giardia*, such as dogs and cats, but it cannot be easily adopted to treat human disease. In contrast, fumagillin is indicated in human disease and may be a useful alternative for treating giardiasis patients that do not respond to standard care drugs. Therefore, we performed an initial dose-dependent treatment study comparing fumagillin and metronidazole. Three once-daily doses were tested over 4-day treatments at 0.6, 12, and 120 mg/kg. As shown in Table 5, fumagillin cured giardiasis in the adult mouse model at a much lower dose than metronidazole. Although the exact required dose in humans cannot be projected from the mice studies, the >0.6-mg/kg daily dose is comparable with the fumagillin dose used to treat humans with amebiasis or microsporidiosis, which is three daily doses of 20 mg over 1 to 2 weeks (22, 23). Moreover, for most drugs, there is a clear reduction in dose translation from mice to humans (24). According to this trend, the recommended dose to treat giardiasis may be lower than that used to treat amebiasis and microsporidiosis patients. In addition, the 50% lethal dose (LD₅₀) of fumagillin for administration to mice via the oral route is 2,000 mg/kg (25), suggesting an excellent therapeutic index that is >3,000. In comparison, patients with giardiasis are treated with 250 mg metronidazole twice daily for 5 to 7 days (26), and treatment failures call for an increased dose, which is accompanied by increased adverse effects.

Fumagillin as a potential anti-giardial drug. We previously reported that disulfiram, a drug used to treat chronic alcoholism, exhibited efficacy in curing giardiasis in mice, and we discovered that it inhibits an essential *Giardia* enzyme, carbamate kinase (13). In addition, auranofin, a gold-containing drug used to alleviate rheumatism, has been shown to cure giardiasis and inhibit *Giardia* thioredoxin oxidoreductase activity (11). We have now discovered a third drug indicated for human use, fumagillin, which shows promise as an anti-giardial agent. Fumagillin is used to cure intestinal microsporidiosis caused by the protozoan *Enterocytozoon bieneusi* (23, 27) and has been approved by the European Union (EU) as an orphan drug to treat HIV and immunocompromised organ transplant patients with this infection (European Medicines Agency document reference EMEA/COMP/82/02 Rev. 2). Fumagillin has been used to treat microsporidiosis in honeybees for many years (28), and it is effective when used topically in the treatment of microsporidial keratoconjunctivitis in humans (29).

Moreover, >50 years ago, a U.S. Navy study by Killough and colleagues (22) showed that fumagillin was effective in curing intestinal amebiasis. They reported that the amebiasis patients were often suffering from multiple parasitic diseases, including giardiasis, and that fumagillin exhibited efficacy in curing these infections as well. However, the literature contains no follow-up studies that examined the drug as an anti-giardial agent. Thus, the results of the animal studies reported here substantiate the earlier, somewhat anecdotal, clinical studies.

Fumagillin drew renewed attention when it was discovered that it inhibited angiogenesis in mice (30). No data are available in the literature regarding bioavailability and plasma levels with oral administration of fumagillin; however, the drug is an ineffective antiangiogenesis agent when taken orally. The mouse studies showed that systemic administration was required, which resulted in severe weight loss (31). Subsequent antiangiogenesis drug development efforts have focused on compound toxicity and oral availability. For killing the *Giardia* trophozoites that attach to the intestinal lumen and do not invade cells, the lack of oral availability is advantageous because the high drug concentration in the intestinal lumen can combat the parasite locally, whereas the severe systemic toxic effects associated with injection or infusion may be avoided. More recently, fumagillin has been shown to impair diet-induced obesity in mice treated by oral gavage with doses of 1 mg/kg/day (32), and an orally administered fumagillin derivative, beloranib, has reached human clinical trials as treatment for obesity (33).

Amebiasis clinical studies used oral administration of fumagillin, and Killough and colleagues (22) did not detect adverse side effects in the otherwise-healthy patients. In contrast, Molina and colleagues (23) reported that although 100% of the immunocompromised patients treated for intestinal microsporidiosis were cured, some exhibited a toxic effect on bone marrow (primarily thrombocytopenia). This toxicity was not severe, because the platelet counts of these patients recovered spontaneously 1 to 2 weeks after the discontinuation of the fumagillin therapy. Nevertheless, platelet counts should be monitored when treating immunocompromised patients (27).

Fumagillin inhibits methionine aminopeptidase-2 (MetAP2) of humans and *Plasmodium falciparum* (34, 35) and has been shown by X-ray crystallography to form a covalent bond with an active site histidine residue of both the MetAP2 of humans and the microsporidium *Encephalitozoon cuniculi* (36, 37). MetAP enzymes, which are present in all organisms (except viruses), excise the amino-terminal methionine of proteins. Two identified

classes of MetAPs, MetAP1 and MetAP2, exhibit little amino acid sequence identity (38, 39), yet both are metalloproteases that share the same overall folding and key catalytic residues (40, 41). Fumagillin selectively inhibits MetAP2 and not MetAP1, and the structural basis for this difference has been discussed (40). An exception to this rule was reported recently, as fumagillin inhibited MetAP2 and, to a lesser extent, MetAP1, of the intestinal parasite *Cryptosporidium parvum* (42). Prokaryotes produce only one MetAP, eubacteria produce MetAP1, and archaea produce MetAP2. In contrast, the genomes of eukaryotes, including the human genome, encode both MetAP1 and MetAP2. The *Giardia* genome is an exception, because despite being a eukaryote, its genome encodes only MetAP2. Although the target of fumagillin in *Giardia* has not been confirmed experimentally, its MetAP2 shares 38% amino acid sequence identity and invariant key active site residues with both the human and *E. cuniculi* MetAP2 enzymes. The same homology level exists between the human and *E. cuniculi* enzyme, both of which are inhibited by fumagillin. Therefore, it is likely that fumagillin targets the *Giardia* MetAP2 as well. No other fumagillin targets have been identified so far, although as with any drug, binding to other proteins cannot be excluded. Yet, the essential function of MetAP2, encoded by the only MetAP gene in the *Giardia* genome, is consistent with the lethal effect that fumagillin exerts on the organism.

The designation of fumagillin as an orphan drug for treating HIV and renal transplant patients involved clinical trials that included a total of 22 patients (23, 27). Large-scale clinical trials will be required to approve fumagillin as a standard care anti-giardial drug. Repurposing fumagillin is an attractive proposition, yet the outcome of clinical trials cannot be predicted. It might be necessary to modify the compound to reduce platelet toxicity in immunocompromised patients, for example by reducing compound transport across the intestinal epithelial cell barrier. The drug development process would benefit from accompanying structural studies that reveal the effect of compound modification at the atomic level. Comparative modeling shows that fumagillin is not only likely to bind to the *Giardia* MetAP2 with the same mode as it binds to the human and *E. cuniculi* MetAP2 enzymes but also that structural differences in loops surrounding the active site offer opportunities to enhance drug selectivity vis-à-vis the human enzyme. The SWISS-MODEL server (43) was used to build a comparative model using the human enzyme structure in complex with fumagillin (37) (PDB entry code 1BOA). The amino acid sequence alignment shows that there are a number of loops that contain insertions or deletions. Accordingly, the two structures are expected to differ in these regions. Two *G. lamblia* MetAP2 loops in the vicinity of the fumagillin binding site contain 2- and 3-residue insertions (within loops comprising residues 258 to 263 and 321 to 327) compared with the human MetAP2. The conformations of these loops as generated by the SWISS-MODEL program differ from the template (Fig. 1, in magenta). Although the model reliability in these regions is limited, it is obvious that the inserted residues must alter the loop structure, which in turn might affect the conformations of the adjacent regions. Thus, the active site structural differences provide opportunities for developing fumagillin derivatives that inhibit the *Giardia* MetAP2 selectively and do not interfere with the human MetAP2.

Conclusions. The preclinical studies reported here have shown that fumagillin is a promising and efficacious anti-giardial drug. Fumagillin exhibits potency against both metronidazole-

FIG 1 Depiction of differences between the human MetAP2 crystal structure and a homology model of the *G. lamblia* MetAP2, focusing on the vicinity of the fumagillin binding site. The *G. lamblia* MetAP2 model was generated using the SWISS-MODEL server (43) and was superpositioned on the structure of human MetAP2 determined in complex with fumagillin (37) (PDB entry code 1BOA). A partially transparent surface of human MetAP2 is shown with the atomic color scheme as follows: gray, carbon; blue, nitrogen; red, oxygen; yellow, sulfur. The active site histidine that forms a covalent bond with fumagillin is shown as a stick model with the same color scheme. Two cobalt ions are highlighted as cyan spheres. Fumagillin is shown as a stick model with green carbon atoms and red oxygen atoms. The traces of two *G. lamblia* MetAP2 loops adjacent to the fumagillin (residues 258 to 263 and 321 to 327) containing insertions with respect to the human MetAP2 structure are modeled differently than the template protein, as highlighted in magenta.

sensitive and metronidazole-resistant *G. lamblia* isolates; hence, it offers an alternative treatment for giardiasis that is refractive to standard care drugs. Moreover, it would be worthwhile to investigate whether combining fumagillin with standard care drugs prevents or slows down the spread of resistance. The comparative three-dimensional model suggests that the key residues that surround the bound fumagillin in the human MetAP2 are conserved in the *G. lamblia* MetAP2. There are also significant structural changes in the vicinity of the active site and, if necessary, these could be exploited to increase drug selectivity toward the *G. lamblia* MetAP2 and avoid unwanted inhibition of the human MetAP2.

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