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INTRODUCTION

*Giardia lamblia* is a protozoa which causes giardiasis, and is a major cause of parasitic diarrheal disease worldwide. Its methods of contamination and spreading are fecal-contaminated surfaces or materials, through ingestion of highly infectious cysts. Disease symptoms present in about half of infected individuals, and while typically transient, some patients remain symptomatic for extended periods. (6) In the setting of immunodeficiency, chronic giardiasis can cause weight loss, malabsorption and increased mortality. (7) Giardial infections are treated with metronidizole (Mz) which is a nitroimidazole compound, dosed 250mg three times a day for 5-10 days. Metronidazole is active against several anaerobic protozoa and bacteria, and operates by entering the cells as a prodrug via passive diffusion, later activated in the cytoplasm or an organelle within the different microbes. (8) Metronidazole’s mechanism of action in giardia required reduction of the 5-nitro group of the imidazole ring by ferredoxin leading to formation of toxic radicals that damage and inactivate critical molecules. (9) Various strains of giardia lamblia exist, some only in a laboratory setting and others naturally occurring, that display variable degrees of resistance to metronidazole. Mz resistance in Giardia occurs in the laboratory (10) and the mechanism of Mz resistance appear to be diverse. In our research, three Mz sensitive (Mzs) strains, C6, 106, and 713, and six Mz resistant (Mzr) strains, C6a!, C6 0.5, 106c17, 106-2ID10, 713M3, 713c17 were used, along with a library of ~1,000 newly synthesized nitro compounds, to explore questions of resistance similarities between different strains, as well as the genetic basis of these resistance pathways.
To address those questions, the degree of resistance by each strain toward each compound was first measured and the strains were analyzed to form a pattern, according to similar susceptibility to the same compounds.

**BACKGROUND**

*Giardia lamblia* is a major cause of parasitic diarrheal illness worldwide. Even in the United States, *Giardia* is the most common cause of outbreaks of parasitic disease, with prevalence rates of 1-7% based on population sampled. Infections are particularly frequent and severe in young children in day-care centers, travelers, hikers, and military personnel in the field (1-5). Infection is initiated by ingestion of cysts, which are shed in feces, can survive in water for months, and are resistant to many disinfectants (6). Fewer than ten cysts can establish human infection, making the parasite highly infectious and a credible accidental and bioterrorism threat to the safety of public water supplies (7). Once ingested, flagellated, non-invasive trophozoites emerge from the cysts and colonize the upper small intestine where they attach to the epithelial lining, causing villus and brush border microvillus atrophy, and digestive enzyme deficiencies (8). Half of all stool-positive *Giardia* infections are symptomatic with diarrhea, epigastric pain, nausea, and vomiting, which can cause malabsorption and malnutrition, especially in children. Acute giardiasis may disable patients for extended periods and elicit protracted post-infectious syndromes, while chronic infection can lead to delayed development and impairment of cognitive skills in children.

Synthetic 5-nitroimidazole, metronidazole, is highly active in vitro against *Giardia*. Mtz has a long record of efficacy and safety in humans and is typically given three daily
250mg oral doses for 5-10 days with effectiveness in 80-95% of cases (9). Adverse effects include headache, nausea, unpleasant metallic taste which contribute to poor compliance. Non-5NI nitro drugs have been used against giardiasis but they generally have lower efficacy. Nitazoxanide given 500mg BID for 3 days had efficacy of 70-80%, and is also impacted by Mtz resistance (9). Other drugs used to treat giardiasis with mixed efficacy include albendazole and antimalarial drug, quinacrine which is 90% effective but not widely used as it has serious adverse effects (10).

Nitro drugs are prodrugs whose microbial specificity is due to the strict requirement for reduction to toxic free radical intermediates by low redox potential reactions present only in the anaerobic target microbes. Giardia metabolism is fermentative with electron transport proceeding in the absence of mitochondrial oxidative phosphorylation. However, the parasite is microaerotolerant and can reduce O2 and thus protect the highly oxygen-sensitive, central metabolic enzyme, pyruvate:ferredoxin oxidoreductase (PFOR), and iron-containing ferredoxins. PFOR decarboxylates pyruvate and donates electrons to ferredoxin, which in turn reduces other components in the electron transport chain and leads to ATP generation. Reduced ferredoxin can reduce the critical nitro group of Mz to toxic radicals which kill the parasite (11).

Despite the overall efficacy of 5NI drugs, treatment failures in giardiasis happen up to 20% of cases, proving clinical resistance. In vitro resistance can be induced so that parasites grow in clinically relevant levels of Mtz. Additionally, Mtz-resistant Giardia exhibit cross-resistance to other prescribed 5NI drugs and nitazoxanide. Prior studies
indicate that side chain modifications of 5nitro heterocyclic compounds can markedly improve antigiardial activity and overcome Mtz resistance (12).
MATERIALS AND METHODS

Materials. The compounds used in the first part of this study were synthesized by collaborating chemistry lab. They were all soluble at 20mM in dimethyl sulfoxide (DMSO) and were used for activity screens.

Giardia lamblia isolates and culture. The following G. lamblia assemblage A isolates were used. WB (ATCC 50803) (C6), BRIS/83/HEPU/106 (106), and BRIS/83/HEPU/713 (713), 106-2ID10, 106C17, 713M3, 713c17, C6a!, and C6 0.5.

All cell lines were grown in TYDK medium with bovine calf serum and bovine bile under anaerobic conditions (Anaerobic system; Remel). The Mz⁺ strains were grown in 50 μM Mz but were grown free of the drug 2 days prior to the experiments.

Nitro Drug susceptibility assays and screening. Susceptibility of 9 different Giardia lamblia isolates to 657 nitro drugs was tested in quantitative 48-hr growth and survival assays. Serial dilutions of test compounds (from 20 μM to 1 nM), or Mz as a control, was made in Giardia Lamblia growth medium in 96-well plates, using our robotic liquid handling system (Beckman Biomek 3000). Solvent alone served as a control.

Trophozoites (2,000/well) were added, and plates were incubated anaerobically (Anaeropack system, Remel) at 37°C for 48 h. Growth and viability were determined at the end of the incubation period by assaying ATP levels in live cells with the BacTiter Microbial Cell Viability Assay (Promega), a luminescence assay based on firefly luciferase-catalyzed reaction of ATP with luciferin. The assay was not affected by
components in the growth media or by nitro drugs, and thus permitted rapid single-step analysis of ATP directly in the microtiter wells (11). Luminescence is plotted against drug concentrations, and EC50 values (effective concentrations that inhibit growth by 50%) are derived by numeric interpolation. The MzR lines exhibit stable Mz resistance and display distinct forms of resistance, as indicated by differential responses to different nitro drugs (15) and implicated mechanisms of resistance (13,14,16).

Activity testing will be done as described above, using all MzR and MzS lines: C6, 106, 713, 106-2ID10, 106C17, 713M3, 713c17, C6a!, and C6 0.5. For each compound, we determined the residual activity in MzR cells relative to the parental MzS cells in the syngeneic pairs. Compounds that exhibit a >2-fold improvement compared to Mz, in residual activity against the resistant lines, were selected.

RESULTS

Screening of 4 Giardia lamblia isolates’ (106Sensitive, 106Resistant, 713Sensitive, 713Resistant) susceptibility to 657 nitro drugs displayed an expected variety of susceptibility of individual isolates to individual nitro compounds. Additionally, the two resistance strains (106R and 713R) had variable susceptibility to the same compound, with one having markedly higher susceptibility to the compound than another. This implied that resistance mechanisms in two resistant isolates were different, and hence that resistance is not homogenous. For compound screening against a higher number of Giardia lamblia isolate, we selected 46 compounds that displayed efficacy against both original resistant strains (106R and 713R), by excluding compounds
with pEC50 of <5. Selected compounds were generally representative of the entire 657 library of nitro drugs, with the aforementioned exclusion of ineffective compounds.

With further susceptibility screen, 46 compounds were tested against 9 *Giardia lamblia* isolates. The Mz\(^{+}\) strains, C6, 106, and 713, had the highest pEC50 values (highest susceptibility) and 106\(_{2ID10}\) and 713\(_{M3}\) had the lowest pEC50 values (most resistance) with respect to Mz. Furthermore, some strains displayed higher susceptibility to most compounds, while others, such as C6\(_{0.5}\), showed higher resistance to most compounds. C6\(_{a!}\) showed a wide spreading of pEC50 values for the compounds, meaning that while some compounds effectively inhibited its growth, to others, this strain was highly resistant. Most strains, however, show higher susceptibility to some compounds than others. Figure 1B shows pEC50 each of 46 compounds, and Metronidazole, for each strain.

As shown in figure 2, the Mz-sensitive parental lines C6 and 106 have a high correlation coefficient of 0.91. A correlation coefficient of above 0.90 was also observed between any pair of the Mz-sensitive parental lines (C6, 106, 713). The correlation of Mz-resistant lines with each other was highly variable within and among resistant strains from different families. In figure 2, C6 \(_{0.5}\) and 2ID10 are shown to have a correlation coefficient of 0.67, and overall lower pEC50 values compared with parental lines. A high r-value indicates that the two strains are affected by compounds similarly, while a low r-value indicates that the two strains show different susceptibilities to the same compound. Similar susceptibility to the same compounds suggests that the pathway that
the compounds use to inhibit growth of the strains, are similar or the same. In contrast, different susceptibilities to the same compounds suggest that the two strains do not share the same pathway by which the drugs act or fail to act as a result of resistance pathways. Hence, these findings suggest that multiple resistance pathways exist, leading to our next visualization of the same data, shown as a heatmap, in figure 3. The compounds are organized in order of decreased pEC50 averages across all strains per compound, and the strains are organized based on similar activity profile.

**DISCUSSION**

While *Giardia lamblia* infection is routinely treated with 5-nitroimidazole (5-NI) drugs, namely Metronidazole, this treatment is ineffective in up to 20% of cases (3,4). Furthermore, reinfections and chronic infections occur in endemic areas, making treatment with 5-NI more challenging (5). Identification of resistance pathways, and discovery of compounds that circumvent the resistance pathways in resistant strains, meaning they effectively eliminate resistant strains via other pathways, could be beneficial in finding alternative treatment options. Screening our library of 567 compounds showed a wide spectrum of activity against sensitive and resistant strains. Further screening against 9 strains (3 Mz-sensitive and 6 Mz-resistant) showed similar activity to the same compounds amongst the sensitive strains, suggesting similar underlying susceptibility mechanism to these compounds. In contrast, differential susceptibility to the same compounds between Mz-resistants of the same original family or of different original families suggests that resistance is not explained by family origin. As such, there may be new groupings made based on susceptibility or resistance patterns to compounds. The heatmap shows while compounds like Jar-3-206a have essentially
uniform activity throughout all strains, compounds such as Jar-3-180h have distinctly different activity levels between groups of strains. Phenotypically variable activity of strains, suggest genotypic differences underlying the resistance mechanisms. Future work should be done to elucidate the genetic basis for the diverse resistance mechanisms. Additional future work also includes identifying the most efficacious compounds for further in vitro experiments in mice.

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Figure 1. Variable resistance. Out of library of 567 compounds, 48 representative compounds were selected and tested against all 9 strains of Giardia. Variable resistance to metronidazole was observed across the 9 strains, organized by family in Fig 1. Furthermore, variable resistance is observed for the compounds used across the strains, with some strains having a higher susceptibility to some all compounds compared with other strains, displaying a phenotypic difference in resistance, suggesting genotypic different mechanisms of resistance.
Figure 2. (Bottom) Mz-sensitive strains, C6 and 106 are shown to have similar level of growth inhibition to the same compounds, with an r-value of 0.91 while (Top) Mz-resistant strains C6 0.5 and 2ID10 derived from C6 and 106, respectively, have overall lower pEC50 values and a lower correlation coefficient of 0.67.
Figure 3. Heatmap of compound effectivity pattern per strain. Shows a pattern of effectivity of compounds across strains. The compounds were arranged top-bottom based on the decreasing pEC50 average within all lines for each compound. The strains were arranged from right to left based on similar activity.