Inhibitors of Methionyl-tRNA Synthetase Have Potent Activity against Giardia intestinalis Trophozoites

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The methionyl-tRNA synthetase (MetRS) is a novel drug target for the protozoan pathogen Giardia intestinalis. This protist contains a single MetRS that is distinct from the human cytoplasmic MetRS. A panel of MetRS inhibitors was tested against recombinant Giardia MetRS, Giardia trophozoites, and mammalian cell lines. The best compounds inhibited trophozoite growth at 500 nM (metronidazole did so at ~5,000 nM) and had low cytotoxicity against mammalian cells, indicating excellent potential for further development as anti-Giardia drugs.

Giardiasis is an important international health problem responsible for ~280 million symptomatic infections worldwide each year (1). Transmitted by the fecal-oral route, Giardia intestinalis causes diarrhea, stomach cramps, and weight loss. The illness can be self-limited in 2 to 4 weeks but is often persistent. The impact in children is particularly profound, where giardiasis contributes to malnutrition, growth retardation, and poor cognitive function (2, 3). Vaccines have not been developed for clinical use, and case management depends on antimicrobial chemotherapy. The main drugs used are nitroimidazoles (metronidazole and tinidazole) with efficacies of 80% to 95%, but they also have high rates of side effects (4). Approximately 20% of cases involve clinical metronidazole-resistant Giardia (1). Second-line drugs such as albendazole, nitazoxanide, furazolidone, and paromomycin have somewhat lower efficacy rates than nitroimidazoles and/or potentially dangerous side effects (4). Thus, new and safer drugs, acting by novel mechanisms, are needed to combat the spread of giardiasis particularly due to nitroimidazole-resistant strains.

Translating nucleotide-containing gene sequences into proteins is a core process in all biological organisms. Aminoacyl-tRNA synthetases (aaRS) are required enzymes for protein translation and have been shown to be essential in genetic knockout or knockdown studies of various organisms, including yeast and protozoa (5–7). Inhibitors of aaRS enzymes have potent antibiotic, antifungal, and antiprotozoal activities with candidate compounds in preclinical and clinical development (7–11). Previous research by this group has led to the discovery of potent inhibitors of methionyl-tRNA synthetases (MetRS) of trypanosomatid parasites (8, 12, 13) and has demonstrated activity in animal models of Trypanosoma brucei infection (8, 12).

Higher eukaryotic organisms typically have separate sets of aaRSs for the cytoplasmic and the mitochondrial compartments. In the situation of MetRS, humans have a type 1 MetRS for the mitochondrion and a type 2 MetRS for the cytoplasm. Giardia intestinalis, which does not contain mitochondria, only has a type 1 MetRS enzyme (14). Alignments of the amino acid sequences of the Giardia, human, and Trypanosoma brucei MetRS enzymes are provided for comparison (Table 1). The table shows the amino acids that form a binding site for inhibitors as revealed by the crystal structure of T. brucei MetRS complexed with inhibitor 1312 (PDB accession number 4EG5) (15). The Giardia MetRS (GL50803_22204 from strain WB) has high sequence conservation with the T. brucei MetRS in this region (20/25, 80% identical) (Table 1), suggesting that many inhibitors developed for the T. brucei MetRS will likely inhibit the Giardia MetRS. Importantly, Giardia MetRS has lower sequence identity than the human cytoplasmic MetRS (13/25, 52%) (Table 1), allowing plenty of opportunity for selective inhibitors. Of note, Giardia MetRS has comparable sequence identity (19/25, 76%) to the human mitochondrial MetRS, the significance of which is discussed later.

The full-length open reading frame for Giardia MetRS (GL50803_22204) was cloned into the bacterial expression plasmid AVA0421, incorporating a 6-histidine tag onto the N terminus of the protein (16). The enzyme was purified by metal affinity chromatography with a yield of 17 mg/liter. An aminoacylation assay using [3H]-methionine was developed for functional experiments following procedures used to analyze the Trypanosoma brucei MetRS and human mitochondrial MetRS (8, 12, 13). The optimized assay (conditions were the same as those for the Trypanosoma brucei MetRS aminoacylation assay previously described [13], except for a 20 nM Giardia MetRS and a 60-min incubation time) had an average signal to background ratio of 49 ± 12 and an average Z’ score of 0.79 ± 0.03. The assay was applied for measuring the 50% inhibitory concentrations (IC50) of compounds from an in-house collection of MetRS inhibitors (Fig. 1). The syntheses or vendors of the compounds are described elsewhere or are to be published separately (8, 12, 13). The activity of inhibitors on the growth of Giardia trophozoites was quantified using a bioluminescence readout with a 48-h incubation at 35°C (17). A Giardia intestinalis isolate (ATCC 50580) was cultured according to ATCC instructions with the addition of Diamond vitamin Tween 80 solution (58980C; Sigma-Aldrich) (18).
compound 1312 is representative of the aminquinolone series
derived from compounds originally reported by scientists at Glaxo-
SmithKline (GSK) about a decade ago (19). Compound 1312 has
remarkable potency against the *Giardia* MetRS (IC$_{50}$/H11005 7 nM),
although the potency against *Giardia* GS/M strain trophozoites
was much lower (50% effective concentration [EC$_{50}$/H11011 7 µM]),
suggesting a poor ability to permeate membranes, a feature that
was previously reported with this series (20). A urea-based MetRS
inhibitor, 1356 (12), had comparatively poor inhibition of the
*Giardia* MetRS (IC$_{50}$/H11005 3,011 nM), and relatively weak activity
against trophozoites (EC$_{50}$/H11005 16,833 nM). Compounds 1331,
1575, and 1614 are derivatives with the amino-benzimidazole or
amino-imidazopyridine moiety on the right side of the molecules.
Compound 1710 is similar to 1575 but has a closed ring system on the left side and retains
excellent enzyme activity (IC$_{50}$/H11005 23 nM). We observed a substan-
tial gain in trophozoite potency when fluorine was added to the
imidazopyridine (e.g., 1709 and 1717) with EC$_{50}$/s in the 500 nM
range. These compounds are about 10 times as potent as metro-
nidazole (EC$_{50}$/H11011 5,000 nM). Compound 1683 has changes to the
middle (linker) portion of the scaffold and, interestingly, it com-

![Chemical structures of MetRS inhibitors and metronidazole.](image-url)
completely lost activity against the *Giardia* MetRS (>10,000 nM) despite being a potent inhibitor of the *T. brucei* MetRS (95 nM). This observation underscores the need to screen compounds against the *Giardia* MetRS instead of relying on the *T. brucei* MetRS as a surrogate.

In a related work, high-throughput screening for inhibitors of the *T. brucei* MetRS enzyme was conducted through the NIH-supported molecular libraries screening program. The screening of 364,131 compounds yielded 1,370 confirmed hits and ultimately 54 compounds representing 12 different scaffolds with growth inhibitory activity against the *T. brucei* cultures (13). From this screening, compounds 99356418 and 3718852 were tested against the *Giardia* MetRS with *IC₅₀* of 445 nM and 2,135 nM, respectively (Table 2). Compound 3718852 and some others have no detectable inhibition of the human mitochondrial MetRS (>10,000 nM), illustrating that selective inhibition of the *Giardia* MetRS enzyme over the human type 1 MetRS is achievable should it be necessary. These compounds (Fig. 1) represent entirely different structural scaffolds from previous MetRS inhibitors and have potential to optimize anti-*Giardia* activity.

Finally, Met-SA1 mimics methionyl-adenylate and is a potent nonspecific inhibitor of all MetRS enzymes (20). It has strong activity against *Giardia* trophozoites, and not surprisingly, it has no therapeutic window on mammalian cells (ATCC CRL-8155 and HepG2), presumably due to inhibiting the human cytoplasmic MetRS enzyme. In contrast, most of the other compounds have little cytotoxicity against mammalian cells. For example, the most potent compound against trophozoites, 1717, has a therapeutic index of ~100 against mammalian cells, suggesting that it does not inhibit the human cytoplasmic (type 2) MetRS. Inhibition of the human mitochondrial (type 1) MetRS does not appear to be correlated with cytotoxicity against mammalian cells (Table 2). This could be due to its poor ability to permeate the mitochondrial compartment or to other biological reasons.

As our most potent compound against *Giardia* trophozoites, 1717 was tested for its minimum lethal concentration (MLC) using published procedures (21). The technique involves incubating trophozoites in compounds at various dilutions for 3 days, washing off the drug, incubating for another 3 days, and making observations of trophozoite outgrowth. The MLC for 1717 was 2.8 μM, lower than the 5.6 μM measured for metronidazole. These results demonstrate that MetRS inhibitors have "cidal" (as opposed to "static") anti-*Giardia* activity.

The data from Table 2 were plotted to evaluate the correlation between the MetRS enzyme and trophozoite activity (Fig. 2). We observed a strong correlation (*R² = 0.81*), indicating that the target of biological activity for the compounds in *Giardia* was likely the MetRS enzyme (Fig. 2). These experiments serve as preliminary data for the chemical validation of MetRS as a drug target for *Giardia*. Further chemical optimization of MetRS inhibitors and proof-of-concept studies with animal models of giardiasis are planned.

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