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Chemical Validation of Methionyl-tRNA Synthetase as a Druggable Target in *Leishmania donovani*

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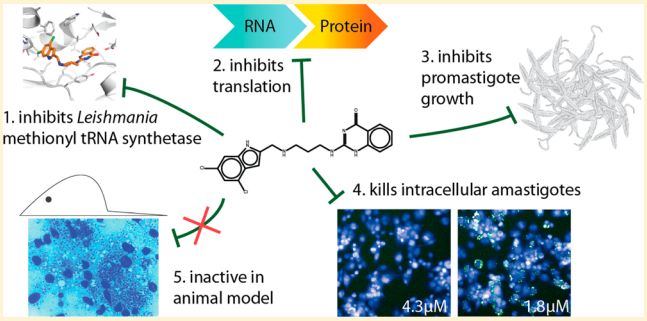
Supporting Information

ABSTRACT: Methionyl-tRNA synthetase (MetRS) has been chemically validated as a drug target in the kinetoplastid parasite *Trypanosoma brucei*. In the present study, we investigate the validity of this target in the related trypanosomatid *Leishmania donovani*. Following development of a robust high-throughput compatible biochemical assay, a compound screen identified DDD806905 as a highly potent inhibitor of *Ld*MetRS (K_i of 18 nM). Crystallography revealed this compound binds to the methionine pocket of MetRS with enzymatic studies confirming DDD806905 displays competitive inhibition with respect to methionine and mixed inhibition with respect to ATP binding. DDD806905 showed activity, albeit with different levels of potency, in various *Leishmania* cell-based viability assays, with on-target activity observed in both *Leishmania* promastigote cell assays and a *Leishmania tarentolae in vitro* translation assay. Unfortunately, this compound failed to show efficacy in an animal model of leishmaniasis. We investigated the potential causes for the discrepancies in activity observed in different *Leishmania* cell assays and the lack of efficacy in the animal model and found that high protein binding as well as sequestration of this dibasic compound into acidic compartments may play a role. Despite medicinal chemistry efforts to address the dibasic nature of DDD806905 and analogues, no progress could be achieved with the current chemical series. Although DDD806905 is not a developable antileishmanial compound, MetRS remains an attractive antileishmanial drug target.



KEYWORDS: *drug discovery, kinetoplastids, translation, tRNA synthetase, parasite*

Kinetoplastid parasites of the *Leishmania* species are responsible for leishmaniasis, a neglected tropical disease prevalent in 98 countries, with 350 million people at risk.[1] These protozoan parasites are transmitted by the bite of phlebotomine sandflies leading to the development of visceral, cutaneous, or mucocutaneous leishmaniasis; the former of which is fatal if untreated.[1] As with most neglected diseases, the treatment options currently available suffer from limitations, including high cost, host toxicity, emerging drug resistance, and suboptimal dosing regimens, leading to a pressing need to discover new therapeutics.[2,3]

In the search for new antileishmanial therapeutics, we have adopted a balanced approach to drug discovery, with both phenotypic screening campaigns and target-based approaches providing the best opportunity to discover a range of new chemical matter.[4,5] A particular challenge associated with target-based approaches is a lack of fully validated drug targets in *Leishmania*.[1] Targets are therefore selected on the basis of those involved in highly essential biological pathways and extrapolation of validation data from related trypanosomatid parasites. Methionyl-tRNA synthetase (MetRS; EC 6.1.1.10) meets both criteria as this enzyme plays a crucial role in protein synthesis,[6] a fundamental pathway in all organisms, and has also been validated as a druggable target in the closely related *Trypanosoma brucei* parasite, with inhibitors of *T. brucei* MetRS shown to cure *T. brucei* bloodstream infections in a mouse model of human African trypanosomiasis.[7−10] As a family, tRNA synthetases have also been shown to be good targets in the anti-infectives space.[11−13] MetRS was therefore prioritized as a target for entry into a *Leishmania* drug discovery program.

MetRS catalyzes the synthesis of methionyl-tRNA in a two-step reaction; the first step results in the production of a methionyl-AMP intermediate and pyrophosphate, with the second step resulting in the production of methionyl-tRNA and AMP.[14] This enzyme is highly conserved among kinetoplastid parasites (both *T. brucei* and *Trypanosoma cruzi* MetRS are 76%

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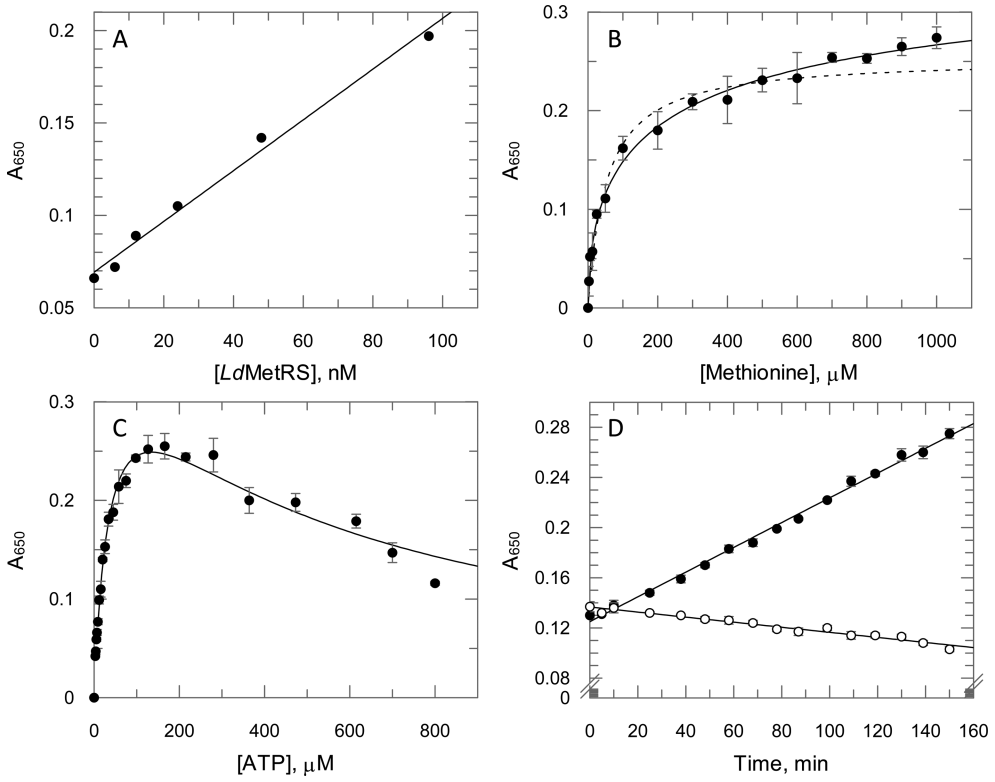

Figure 1. *Ld*MetRS assay development summary. (A) Linearity of the assay with respect to enzyme concentration. (B) Methionine K_m determination in the presence of a saturating concentration of ATP (200 μM). Solid line is best fit to Hill equation and dotted line to Michaelis–Menten equation. (C) ATP K_m determination in the presence of a saturating concentration of methionine (1 mM). (D) Assay linearity with respect to time under the final assay screening conditions of 50 μM methionine and 100 μM ATP either with (closed circles) or without (open circles) 50 nM *Ld*MetRS enzyme. Data are shown as mean ± SD (n = 3 technical replicates).

similar to the *Leishmania* enzyme at the amino acid level). A key challenge in therapeutically targeting a well conserved enzyme is achieving selectivity over the homologous human protein. As *Leishmania* MetRS is more closely related to the human mitochondrial MetRS (81% sequence identity in catalytic pocket) compared to the human cytoplasmic enzyme (41% identity in catalytic pocket), assessing selectivity over the human mitochondrial form, as well as human protein synthesis, will be important in a drug discovery program.

In the present study, the validity of MetRS as a target in *Leishmania donovani* was investigated. Extrapolation of data from *T. brucei* suggests the *L. donovani* enzyme will also be an essential and druggable target, with development of a biochemical, high-throughput compatible screening assay possible.[7,8,15,16] In addition, the availability of several downstream tools provides an effective route for characterizing and progressing any MetRS inhibitors identified. These include *T. brucei* and *Leishmania major* MetRS crystal structures[17,18] which provide powerful tools for cocrystallizing any inhibitors identified, thus providing insight into the binding mode with the target. In addition, several phenotypic cell-based screens are available and are routinely used as part of the *Leishmania* drug discovery pipeline.[19] Such assays include the use of free-living promastigote or axenic amastigote parasites (from the insect stage and mammalian stage of the *Leishmania* life cycle, respectively) and the more complex, but more physiologically relevant, intracellular amastigote assay.[20−24] Furthermore, an *in vivo* model of leishmaniasis allows progression of molecules through to a recognized animal model of this neglected disease.[25]

Here, we describe the identification of *L. donovani* MetRS (*Ld*MetRS) inhibitors, characterize the binding mode of our lead molecule using crystallographic and biochemical methods, and confirm on-target activity in cells. Unfortunately, our lead molecule did not show efficacy in our leishmaniasis animal model; we include discussions around potential explanations for this failure to translate.

RESULTS AND DISCUSSION

Identification of the *Ld*MetRS Inhibitor DDD806905. Recombinant *Ld*MetRS was produced, and a biochemical enzyme assay, using the BIOMOL Green assay platform, was developed to monitor the first step of the MetRS reaction, namely, the reaction of methionine and ATP to produce methionyl-AMP and pyrophosphate. Assay conditions were refined to ensure optimal screening conditions were employed. This involved assessing buffer conditions and determining the optimal enzyme concentration, assay linearity with respect to time, and Michaelis constants for methionine and ATP (Figure 1A–D). The apparent K_m for methionine was determined to be 173 ± 83 μM (with a Hill slope of 0.57 ± 0.06) and 37 ± 3 μM for ATP (with ATP also displaying substrate inhibition with an apparent K_i of 482 ± 44 μM)). The Hill slope of 0.57 observed with methionine can be indicative of negative cooperativity between two subunits of a dimeric protein as observed for MetRS from *Bacillus stearothermophilus*.[26] However, analysis of the oligomeric state of the protein using SEC-MALS (size exclusion chromatography–multi-angle light scattering) suggests the protein is monomeric (Figure S1), supporting previously published crystallography data showing that the

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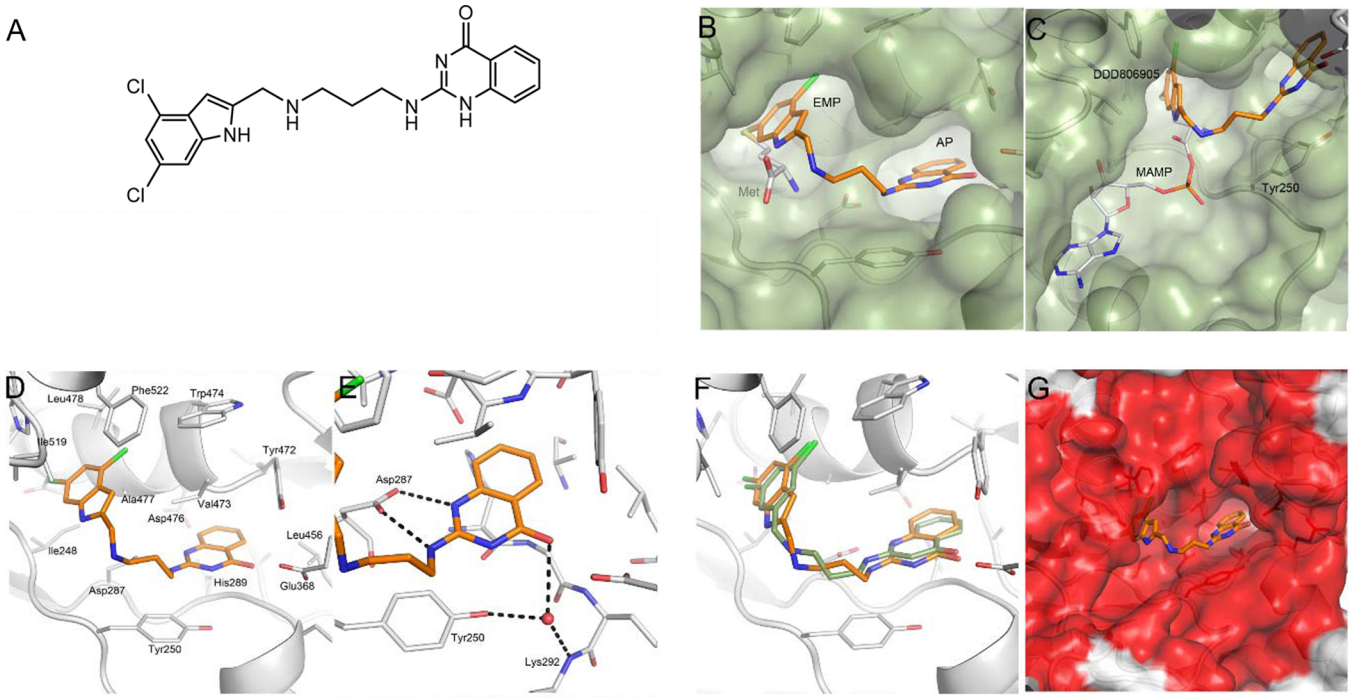

Figure 2. DDD806905 binding mode. (A) DDD806905 was identified as our lead *Ld*MetRS inhibitor, with an IC_{50} of 94 nM. (B) Crystal structure of *Tb*MetRS:DDD806905 (PDB 5NFH). DDD806905 bridges the expanded methionine pocket (EMP) and the ligand stabilized auxiliary pocket (AP). The dichloroindole moiety occupies the same site as methionine (C atoms gray). The solvent accessible surface of *Tb*MetRS:DDD806905 is shown in dark green. (C) Comparison of the binding mode of DDD806905 (C atoms gold) compared to *Tb*MetRS:methionyl adenylate (MAMP, C atoms gray, PDB 4EG3). (D) Binding mode of *Tb*MetRS:DDD806905 showing protein side chains that line the binding site. (E) H-bond interactions between quinazolinone moiety of DDD806905 and residues lining the auxiliary pocket. H-bond interactions are shown as dashed lines and water molecules as red spheres, and key residues are labeled. (F) The binding modes of DDD806905 (C atoms gold) compared with aminoquinolone ligand Chem 1312 PDB 4EG5 (C atoms green). (G) Sequence conservation is high between *Tb*MetRS and *Ld*MetRS around the DDD806905 binding site with identical residues colored red and nonidentical colored gray.

L. major crystal structure is monomeric.[18] An alternative explanation for the low Hill slope could be conformational selection as previously observed for inhibitors binding to the *T. brucei* MetRS enzyme.[27] In this model, two conformations of the ligand-free enzyme exist, with differing affinities for ligands and different k_{cat} values.[28]

Using final assay conditions of 50 nM *Ld*MetRS, 50 μM methionine, and 100 μM ATP, a panel of compounds closely related to known inhibitors of bacterial MetRS[29] (24 members of a 2-amino benzimidazole series, along with a singleton 2-aminoquinazolin-4-one) was screened. These compounds inhibited the *Ld*MetRS enzyme with a range of potencies (IC_{50} values of 94 nM to 100 μM) (Table S1), the most active compound being the 2-aminoquinazolin-4-one singleton DDD806905 (IC_{50} of 94 nM (95% CI, 57−156 nM)) (Figure 2A).

The IC_{50} value for DDD806905 determined in this screening assay was close to the enzyme concentration used in the reaction, meaning DDD806905 was potentially displaying tight binding inhibition under the assay conditions employed. To more accurately define the potency of DDD806905, it is more appropriate to fit the dose response data to the Morrison equation[30] (eq 3 in the Supporting Information). Prior to fitting data to the Morrison equation, an accurate determination of the active enzyme concentration is required. This was achieved by titrating the *Ld*MetRS enzyme in the presence of 1 μM DDD806905 (Figure S2A), revealing that 78% of the *Ld*MetRS protein sample is catalytically active. This resulted in an *Ld*MetRS active enzyme concentration of 39 nM. Using this value as a constant and fitting dose response data to the Morrison equation revealed a DDD806905 K_i^{app} of 41 nM (Figure S2B).

Characterization of DDD806905 Binding and Mode of Inhibition. Determination of the binding mode of the aminoquinazolinone inhibitor DDD806905 was carried out using both crystallographic and biochemical methods. For crystallographic determination of the binding mode, *T. brucei* MetRS (*Tb*MetRS) protein was used as a structural surrogate (Table S2). Despite an overall sequence identity of only 76% between *Ld*MetRS and the *Tb*MetRS, the residues lining and surrounding the ligand binding site are highly conserved (Figure S3) with a sequence identity of 95%. The only sequence difference within the site is a conservative valine to leucine substitution (*Tb*MetRS Leu 456); therefore, *Tb*MetRS is a valid structural system to understand the mode of action for DDD806905 against *Ld*MetRS.

Previous studies have shown that the binding of aminoquinolone inhibitors stabilize an open conformation of *Tb*MetRS with expansion of the methionine binding pocket and opening of an auxiliary binding pocket.[17] The *Tb*MetRS:DDD806905 complex shows the ligand to bridge the auxiliary pocket (AP) and expanded methionine pocket (EMP) of the enzyme (Figure 2B). Overlaying the binding mode of DDD806905 with the intermediate methionyl adenylate (PDB 4EG3)[27] shows the linker between the dichloro indole and quinazolinone groups would block the binding of the beta phosphate group of ATP. The ligand stabilized opening of the AP involves a change in rotomer of

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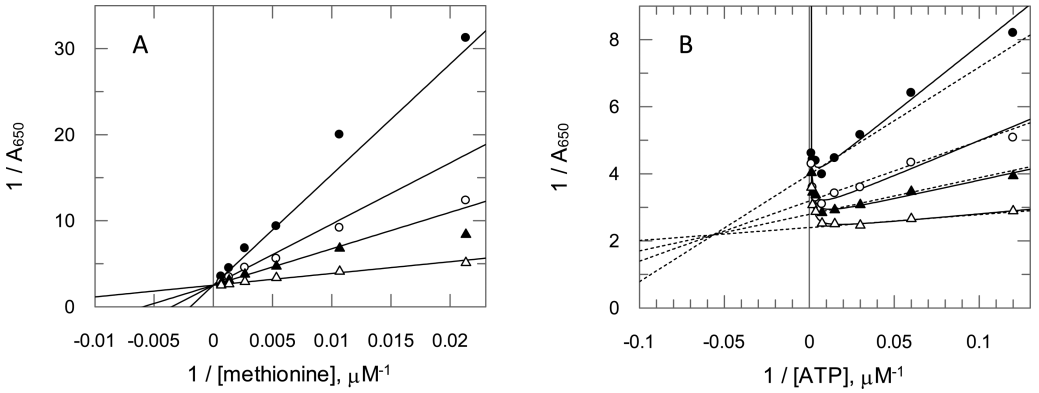

Figure 3. DDD806905 mode of inhibition. (A) *Ld*MetRS biochemical mode of inhibition studies were carried out with reciprocal plots of A_{650} and substrate concentration revealing that DDD806905 displays competitive inhibition with respect to methionine with a K_i of 18 nM calculated. (B) Mode of inhibition studies also revealed that DDD806905 displays mixed inhibition with respect to ATP with a K_i of 21 nM calculated. Inhibitor concentrations used were as follows: 2 × IC_{50} (closed circles), 1 × IC_{50} (open circles), 0.5 × IC_{50} (closed triangles), and 0 × IC_{50} (open triangles).

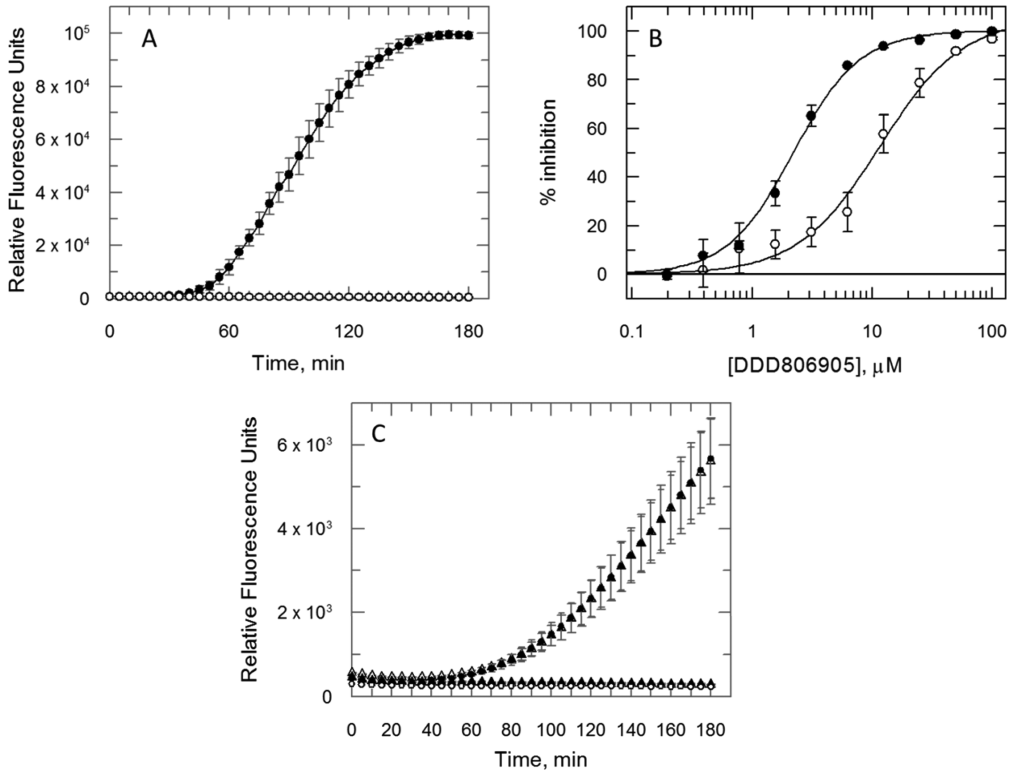

Figure 4. DDD806905 inhibits *Leishmania in vitro* translation. (A) *In vitro* translation in a *Leishmania tarentolae* extract was monitored over time by tracking expression of an eGFP construct (closed circles), with a "minus construct" negative control included (open circles). (B) The ability of DDD806905 to inhibit expression of eGFP in the *L. tarentolae* extract was investigated with this *Ld*MetRS inhibitor inhibiting protein synthesis with an EC_{50} of 2.2 μM (closed circles). In the presence of an additional 1.5 mM methionine, the EC_{50} was shifted to 12 μM (open circles), indicative of on-target activity, as DDD806905 is a known methionine competitive inhibitor of *Ld*MetRS. (C) *In vitro* translation in a HeLa cell extract was also monitored by tracking expression of a GFP construct (closed circles) over time, with "minus construct" (closed triangles) and cycloheximide (protein synthesis inhibitor) controls (open circles) included. When DDD806905 was included at a concentration of 100 μM (open triangles), no inhibition of *in vitro* translation was observed in this human cell extract. Data are shown as mean ± SD (n = 3 technical replicates (cycloheximide data, n = 2 technical replicates)).

Tyr250, a consequence of which is the widening of the ATP binding cleft which may reduce the affinity for ATP (Figure 2C). The side chains lining the DDD806905 binding site are largely hydrophobic in nature (Figure 2D) with limited polar interactions. The quinazolinone moiety occupies the AP pocket retaining key bidentate H-bonds between the quinazolinone NH and the amino NH of Asp287 (Figure 2E). The carbonyl moiety forms an H-bond to a water molecule coordinated by the side chain of Tyr250 and the backbone NH of Lys292. The 4,6-dichloro indole moiety occupies the EMP pocket with the 6-chloro atom overlaying with the substrate methionine S atom (Figure 2C). The indole NH does not make any specific interactions with protein. The binding mode of DDD806905 is similar to the published aminoquinolone complex (PDB 4EG5) (Figure 2F) with a root-mean-square deviation of 0.22 Å for the position of all residues within 5 Å of the ligand between

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*Tb*MetRS:DDD806905 and the aminoquinolone complex 4EG5. The residues that form the ligand binding site and those that facilitate the conformational change in the CP domain are highly conserved between *Tb*MetRS and *Ld*MetRS (Figures 2G and S3), confirming that *Tb*MetRS is a valid model system to understand the binding mode of DDD806905.

Further characterization of the mode of inhibition of DDD806905 was carried out biochemically by varying each substrate concentration at various inhibitor concentrations. Each data set was individually fitted to the Michaelis−Menten equation or a modified high-substrate inhibition Michaelis−Menten equation, and the resulting Lineweaver−Burk plots were examined for diagnostic patterns of competitive, mixed, or uncompetitive inhibition.

Data from these analyses indicate that DDD806905 displays competitive inhibition with respect to methionine binding (Figure 3A), and further fitting to a global competitive inhibition model (eq 4 in the Supporting Information) resulted in a DDD806905 K_i of 18 ± 2 nM (K_i ± SE) being returned. This biochemically determined methionine competitive behavior confirmed the crystallographic data which revealed DDD806905 binds to the methionine pocket of MetRS.

This DDD806905 mode of inhibition study was repeated using ATP as the variable substrate. Fitting these data sets to the modified high-substrate inhibition Michaelis−Menten equation and plotting a double-reciprocal plot of the data revealed the characteristic substrate inhibition profile shown in Figure 3B (solid lines). To allow global fitting, we excluded the substrate concentrations at which substrate inhibition was observed. The remaining data were fitted to a mixed inhibition model (eq 5 in Supporting Information). It can be seen that DDD806905 displays characteristic mixed inhibition with respect to ATP binding (Figure 3B, dashed lines). This ATP mode of inhibition study returned a DDD806905 K_i of 21 ± 5 nM (K_i ± SE), which is highly comparable to the K_i calculated from the methionine competitive inhibition experiment previously described.

From the ATP study, it should also be noted that the K_i' value of 227 ± 23 nM indicates DDD806905 binds preferentially to the free enzyme and the inhibitor profile is more closely related to competitive inhibition. This supports kinetic data which reveals there is positive cooperativity between the methionine and ATP pockets (methionine K_m shifts from 170 to 1500 μM when either 400 μM ATP (10 × K_m) or 80 μM ATP (2 × K_m) is used, respectively (Figure S4)), a feature also observed in MetRS from *E. coli*, *B. stearothermophilus*, and *S. aureus*.[31−33] Consequently, alterations in ATP concentration can impact both binding of methionine and DDD806905 in the methionine pocket. This interaction between ATP and methionine sites is further confirmed from inhibitor studies using different substrate concentrations. These show that the IC_{50} of DDD806905 increases when either methionine or ATP concentrations are increased suggesting that both substrates have "competitive like" profiles (Figure S5A−C).

DDD806905 Inhibits *in Vitro* Translation. Inhibitors of the *Ld*MetRS enzyme would be expected to inhibit protein synthesis. To confirm whether this was the case, DDD806905 was tested for its ability to inhibit protein synthesis in a commercially available *Leishmania tarentolae* cell extract.[34−36] Initial experiments revealed that production of an eGFP protein from a plasmid containing the eGFP gene can be monitored in this system using a 384-well assay format and a standard plate-based reader (Figure 4A).

Subsequently, the ability of DDD806905 to inhibit production of this eGFP protein was determined, with DDD806905 shown to inhibit protein synthesis with an EC_{50} of 2.2 μM (Figure 4B, closed circles). This potency is considerably weaker than that observed in the biochemical enzyme assay, where DDD806905 returned an IC_{50} of 94 nM. One possible reason for this 23-fold drop off in potency is the difference in substrate concentrations present in the respective assays (the biochemical assay was run using methionine and ATP concentrations of 50 and 100 μM, respectively, whereas the *L. tarentolae* cell extracts were supplemented with 136 μM methionine and 1200 μM ATP in addition to their endogenous concentrations). As described above, the potency of DDD806905 is seen to shift in the biochemical assay when substrate concentrations are increased, accounting, at least in part, for the different potencies observed in the biochemical assay and *L. tarentolae* cell extract.

To demonstrate that inhibition of translation in the *L. tarentolae* cell extract was indeed through inhibition of MetRS, the potency of DDD806905 in the protein synthesis assay was determined in the presence and absence of an additional 1.5 mM methionine. When extra methionine is present in the assay, there is a 5-fold shift in DDD806905 potency (EC_{50} shift from 2.2 to 12 μM), highly indicative of on-target activity against MetRS (Figure 4B).

It was interesting to note that, when DDD806905 was tested in a similar *in vitro* translation assay using a human HeLa cell extract, no inhibition of protein synthesis was observed (Figure 4C). Although this observation does not prove selectivity over the more closely related human mitochondrial MetRS enzyme, it does indicate that DDD806905 fails to inhibit the human cytoplasmic MetRS enzyme when tested at a concentration of 100 μM. To investigate potential mitochondrial toxicity, we carried out a mitochondrial protein synthesis assay which revealed some inhibition of mitochondrial protein synthesis with an IC_{50} of 1.7 μM (95% CI, 1.0−2.8 μM) (Figure S6). In addition, DDD806905 showed toxicity against *Leishmania* infected, PMA-differentiated THP-1 cells (THP-1 EC_{50}, 10 μM). Reducing mammalian cell toxicity and the level of mitochondrial protein synthesis inhibition would therefore have to be an important goal of a drug development program.

Cellular and *in Vivo* Efficacy of DDD806905. To investigate whether DDD806905 has antileishmanial activity, it was tested in a *L. donovani* intracellular amastigote assay, where an EC_{50} of 2.9 μM (95% CI, 2.2−3.8 μM; n = 8) was determined (Figure S7). Next, we progressed DDD806905 to an *in vivo* mouse visceral leishmaniasis efficacy model. Unfortunately, no efficacy was observed (Figure 5), with only a 19% knock-down of liver parasite counts observed after 50 mg/kg, twice daily oral dosing for 10 days, compared to vehicle dosed control animals. In contrast, the clinically used control compound miltefosine displayed 99.6% parasite knock-down following 30 mg/kg, once daily oral dosing for 10 days. Blood levels of DDD806905 were measured during the efficacy study and showed that drug total levels were maintained above the MIC (minimum inhibitory concentration = 4.3 μM) measured in the intracellular assay for at least 8 h after dosing (Figure S8A,B). However, due to very high plasma protein binding (fu = 0.006 in mouse plasma and 0.007 in human plasma), blood free levels of DDD806905 were significantly below the *in vitro* MIC. To gain insight into the importance of protein binding,

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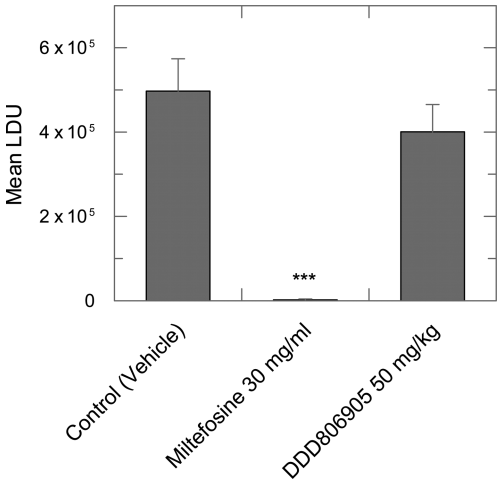

Figure 5. DDD806905 mouse visceral leishmaniasis efficacy study with integrated pharmacokinetics. Efficacy of DDD806905 was assessed in a mouse model of visceral leishmaniasis at 50 mg/kg b.i.d. for 10 days, along with vehicle (b.i.d. for 10 days) and miltefosine (30 mg/kg q.d. for 10 days) controls. Mean reduction in liver parasite burden expressed as Leishman Donovan Units (LDU). Miltefosine revealed a 99.6% reduction in liver LDU compared to vehicle control (***p < 0.001), with DDD806905 showing only a 19.4% reduction in liver LDU (integrated pharmacokinetics results shown in Figure S8).

we performed an *in vitro* serum shift assay with DDD806905 against *Leishmania* promastigotes which revealed the expected linear correlation between promastigote EC_{50} and amount of protein in the media (Figure S9).[37] Since *in vivo* efficacy is usually also driven by the drug free level, the very high plasma protein binding is likely a key reason for the lack of activity for DDD806905 in our visceral leishmaniasis model. Interestingly, a related MetRS inhibitor was also shown not to be curative in an animal model of human African sleeping sickness in spite of having promising *in vitro* cell activity.[7]

Efficacy of MetRS Inhibition in Promastigotes and Axenic Amastigotes and On-Target Activity. The above experiments showed that DDD806905 was around 10-fold more potent against *Leishmania* promastigotes compared to intracellular amastigotes ($EC_{50\text{-pro}}$ of 0.27 μM (95% CI, 0.25−0.29 μM) (in 10% serum), $EC_{50\text{-intracellular}}$ of 2.9 μM (95% CI, 2.2−3.8 μM)). The compound was also found to be 50-fold less active against axenic amastigotes relative to promastigotes ($EC_{50\text{-axam}}$ of 13.7 μM (95% CI, 10.5−17.6 μM)). To assess on-target activity and further explore potential life-cycle stage differences in susceptibility, a series of related 2-amino benzimidazoles with varying degrees of *Ld*MetRS potency were tested for their ability to inhibit *Leishmania* parasite growth, using both promastigote and axenic amastigote *Leishmania* viability assays (Table S1). As for DDD806905, the compounds in this panel showed a large drop in potency from promastigotes to amastigotes. A reasonable correlation is

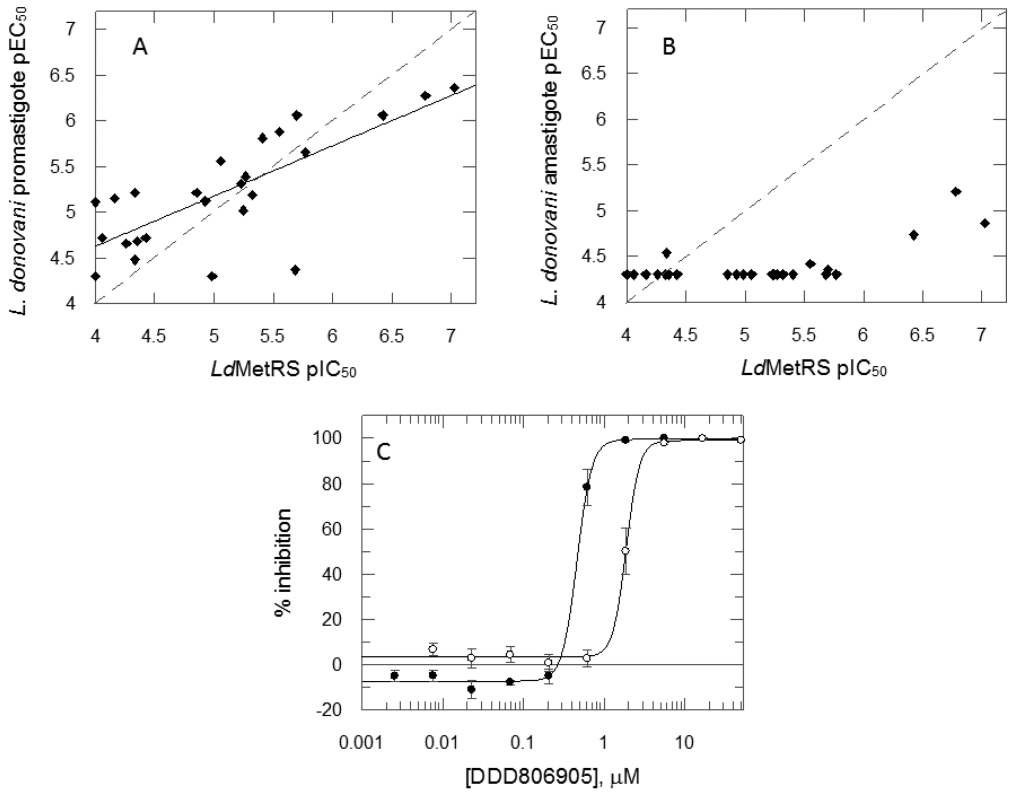

Figure 6. Cellular efficacy of *Ld*MetRS inhibition. (A) A panel of *Ld*MetRS inhibitors show a range of potencies (IC_{50} 94 nM to 100 μM) in the *Ld*MetRS enzymatic assay. Plotting the −log IC_{50} (pIC_{50}) of this enzymatic data against −log EC_{50} (pEC_{50}) data from the *L. donovani* promastigote assay reveals these potencies correlate well. Solid line represents linear regression with a correlation coefficient of 0.76. (B) When the same compounds are tested in the *L. donovani* axenic amastigote assay, most are inactive. In both (A) and (B), dashed lines represent equipotency in the *Ld*MetRS enzyme assay and the *Leishmania* phenotypic assay. (C) Confirmation of on-target activity of DDD806905 in *L. donovani* promastigotes was carried out by testing this compound in the absence (closed circles) and presence (open circles) of excess methionine (2 mM). The EC_{50} shifts from 0.46 to 1.9 μM in the absence and presence of excess methionine, respectively. Data presented as mean ± SD (n = 3 biological replicates).

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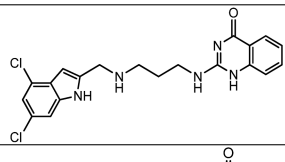
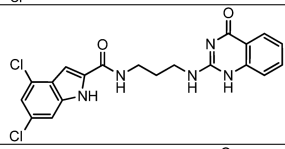
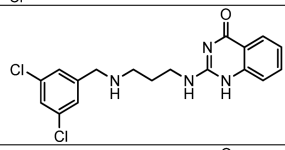
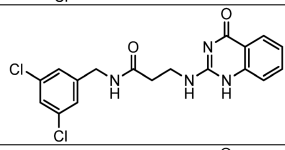
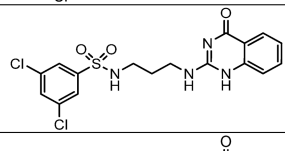
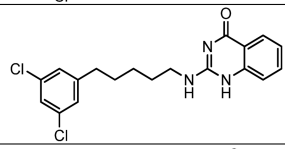
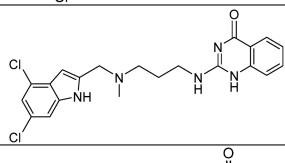
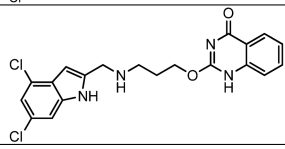
Table 1. Enzyme Inhibition and Cellular Data for DDD806905 and Analogues

Compound ID	Structure	*Ld*MetRS IC_{50} (μM)	*Leishmania* Axenic Amastigote EC_{50} (μM)
DDD806905		0.09	14
25		3.3	>50
26		0.74	38
27		2.8	22
28		1.9	19
29		>100	>50
30		0.03	18
31		22	>50

observed between enzyme data and *L. donovani* promastigote data (Figure 6A), suggestive of on-target activity, whereas these same compounds showed low, or no, inhibition in the *L. donovani* axenic amastigote assay (Figure 6B).

While the correlation between enzyme data and promastigote data is indicative of on-target activity, further studies attempted to confirm whether this was the case. Initial efforts to confirm the on-target effect of DDD806905 were explored by attempting to generate MetRS overexpressing and knockout *Leishmania* cell lines. All efforts to modulate MetRS expression levels in *L. donovani* proved to be toxic to the parasite, suggesting that tight regulation of this enzyme is important to parasite survival, supporting the validation of MetRS as a highly essential target in *Leishmania*.

In the absence of MetRS overexpressing or knockout cell lines, alternative methods were used to determine on-target inhibition of MetRS in the parasite. As with the *in vitro* translation system, DDD806905 was tested in a *Leishmania* promastigote assay in both the presence and absence of extra methionine. The EC_{50} of this compound shifted from 0.46 to 1.9 μM after addition of extra methionine, highly indicative of on-target activity (Figure 6C).

Properties of DDD806905 Accounting for Potency Differences between Life-Cycle Stages. To account for the discrepancy in data from the different *Leishmania* parasite assays, it is noteworthy that these cell assays were performed in media at different pH values (with promastigote and axenic amastigote assays run in media at pH 7.3 and pH 5.6, respectively) and that in the intracellular amastigote assay the parasites reside inside the acidic parasitophorous vacuole. We experimentally determined the pK_a's for DDD806905 (Figure S10A) (pK_a of 3.4, 7.5 and 11), and the data shows that, at pH 7.3, 0.01% of the compound is present in an un-ionized state. This falls to essentially 0% at pH 5.6. In addition, the lipophilicity of the compound was significantly reduced at pH 5.6 (measured log $D_{5.6}$ of 0.3) compared with the lipophilicity at pH 7.3 (measured log $D_{7.3}$ of 2.6) (Figure S10B). The lower total level of uncharged, membrane permeable, compound in acidic axenic amastigote media compared to neutral promastigote media will result in a lower intracellular compound

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concentration, providing a potential explanation for the reduced potency in this assay. In the intracellular assay, the parasites also reside in an acidic compartment, but the total acidic volume is much smaller in this setting (volume of acidic organelles only versus volume of media and acidic organelles in the axenic assay), resulting in a higher fraction of uncharged, membrane permeable compound under intracellular conditions.

Attempts were made to increase the fraction of nonionized species by chemically modifying DDD806905 to reduce its basicity. Unfortunately, all attempts to test this hypothesis by modifying the benzylic amine resulted in significant loss of activity against the enzyme (Table 1). In particular, converting the pendant amine to the corresponding amide, i.e., DDD806905 cf. compound **25** (also compound **26** cf. compound **27**) or sulphonamide, i.e., compound **26** cf. compound **28** reduced enzyme activity. Similarly, removal of the basic nitrogen by replacement with methylene, i.e., compound **29** cf. compound **26**, removed enzyme activity. In addition, substitution of the 2-nitrogen for an oxygen (i.e., DDD806905 cf. compound **31**) was not tolerated. Methylating the benzylic amine (compound **30**), which is not expected to change basicity, was tolerated, but it did not improve cellular activity.

Therefore, despite medicinal chemistry efforts to reduce the basicity of DDD806905, the SAR suggests that the basic nitrogen is required for activity and DDD806905 remains our lead *Ld*MetRS inhibitor. These results support the hypothesis that the lack of activity seen in the axenic amastigote assay is not due to MetRS being a poor drug target but rather due to poor drug partitioning into axenic amastigotes resulting from the highly protonated state of the active compounds in acidic media.

DDD806905 is Lysosomotropic. Due to its highly protonated state under acidic conditions, it is conceivable that DDD806905 is accumulating in acidic cellular compartments, which could help explain the lower potency in the intracellular assay relative to the promastigote assay and may contribute to the lack of activity in the animal efficacy study. To assess this, we carried out a lysosomal sequestration assay.[38,39] The results in Figure 7 reveal that DDD806905 indeed shows the hallmarks of lysosomal accumulation/trapping, whereas, as expected, the nonbasic sulphonamide analogue compound **28** does not. We also confirmed the well-known lysosomal accumulation of the antimalarial drug chloroquine.[40] For chloroquine, this is essential as its mode of action depends on accumulation in the acidic food vacuoles of the malaria parasite. In our intracellular *Leishmania* assay, the host cells present a relatively large lysosomal compartment,[41,42] and on the basis of the data presented here, we propose that the THP-1 lysosomes act as a sink for DDD806905, thus reducing the amount of free, membrane permeable compound available to reach the parasites, hence contributing to the potency drop-off seen between promastigotes and intracellular amastigotes. We also measured the volume of distribution (Vdss) for DDD806905 in mice following a single intravenous dose and found it to be high (6 L/kg), indicating accumulation of the compound in tissues, which is in line with the lysosomotropism observed *in vitro*.

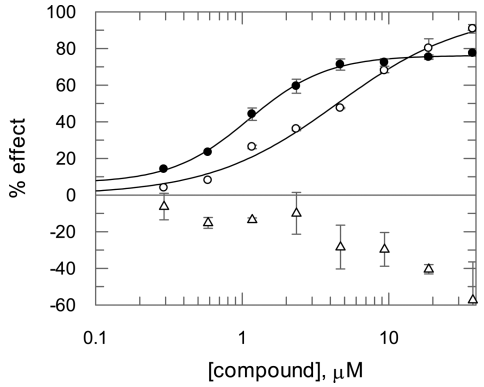

Figure 7. DDD806905 accumulates in acidic compartments. Ability of compounds to compete with lysotracker red accumulation in THP-1 lysosomes was assessed and revealed that both DDD806905 (open circles) and a positive control compound chloroquine (closed circles) did compete with lysotracker red accumulation in this acidic compartment. Representative data shown (mean ± SD, n = 3 biological replicates). In contrast to DDD806905 and chloroquine, compound **28** (open triangles) does not compete with lysotracker red for accumulation in THP-1 lysosomes. Representative data shown (mean ± SD, n = 2 biological replicates).

CONCLUSIONS

We have successfully set up an assay for *Ld*MetRS and established that analogues of bacterial and *T. brucei* MetRS inhibitors inhibit the *Leishmania* enzyme. These compounds almost certainly bind in a very similar manner to the *Leishmania* enzyme as they do to the *T. brucei* enzyme. We have also demonstrated that these compounds are active against *L. donovani* promastigotes. Taken together, the correlation between enzyme activity and promastigote activity (Figure 6A), on-target activity in an *in vitro* protein translation assay (Figure 4B), the cocrystal structure of DDD806905 with MetRS, and the competition experiment with methionine (Figure 6C) provide compelling evidence that the compounds are working on target in promastigotes. However, the compounds showed lower activity against the intramacrophage form of the parasite and no *in vivo* efficacy. On the basis of the results presented here, the most likely reason for the lack of *in vivo* efficacy of DDD806905 is its very small free and membrane permeable fraction due to a combination of high protein binding, ionization, and accumulation in acidic compartments. Although this compound has failed to translate into *in vivo* efficacy, this does not invalidate MetRS as a potential drug target in *Leishmania*. Indeed, MetRS remains an attractive drug target in this parasite, and further efforts to identify novel starting chemical matter against this enzyme target are underway.

MATERIALS AND METHODS

Materials and methods can be found in the Supporting Information. These include experimental procedures for *Ld*MetRS and *Tb*MetRS expression and purification; *Ld*MetRS biochemical assays; *Tb*MetRS crystallography; *Leishmania* and human *in vitro* translation assays; *in vitro Leishmania* assays; mouse efficacy study and integrated PK; *in vitro* physicochemical measurements; chemical synthesis of compounds.

All human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00047.

Determination of the oligomeric state of *Ld*MetRS; DDD806905 K_i^{app} determination; sequence alignment of *Tb*MetRS and *Ld*MetRS; co-operativity between ATP and methionine binding pockets; DDD806905 IC_{50} under different substrate conditions; DDD806905 inhibiting mitochondrial protein synthesis; DDD806905 *Leishmania* intracellular amastigote EC_{50} determination; DDD806905 integrated pharmacokinetics for mouse visceral leishmaniasis efficacy study; DDD806905 EC_{50} in *Leishmania* promastigotes using varying serum concentrations; pH effect of ionization and lipophilicity of DDD806905; enzymatic and phenotypic potencies of *Ld*MetRS inhibitors; data measurement and refinement statistics; materials and methods with associated references (PDF)

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Notes

The authors declare no competing financial interest.

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