Inhibition of Isoleucyl-tRNA Synthetase as a Potential Treatment for Human African Trypanosomiasis

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Background: Current drugs against sleeping sickness are limited, toxic, and inefficient.

Results: In vivo genetic and chemical studies identified isoleucyl-tRNA synthetase (IleRS) as a drug target and an IleRS inhibitor that cures mice of infection.

Conclusion: Inhibition of IleRS constitutes a potential treatment for sleeping sickness.

Significance: Identification of drug targets and inhibitors aids in development of drugs against T. brucei and related parasites.

Trypanosoma brucei sp. causes human African trypanosomiasis (HAT; African sleeping sickness). The parasites initially proliferate in the hemolymphatic system and then invade the central nervous system, which is lethal if not treated. New drugs are needed for HAT because the approved drugs are few, toxic, and difficult to administer, and drug resistance is spreading. We showed by RNAi knockdown that T. brucei isoleucyl-tRNA synthetase is essential for the parasites in vitro and in vivo in a mouse model of infection. By structure prediction and experimental analysis, we also identified small molecules that inhibit recombinant isoleucyl-tRNA synthetase and that are lethal to the parasites in vitro and highly selective compared with mammalian cells. One of these molecules acts as a competitive inhibitor of the enzyme and cures mice of the infection. Because members of this class of molecules are known to cross the blood-brain barrier in humans and to be tolerated, they may be attractive as leading candidates for drug development for HAT.

Human African trypanosomiasis (HAT), also known as African sleeping sickness, is caused by brucei-group trypanosomes and is endemic in 36 sub-Saharan countries (1, 2). Two subspecies with different geographic distributions and transmission dynamics cause somewhat different human diseases. Trypanosoma brucei gambiense occurs in West Africa and causes a chronic disease, whereas Trypanosoma brucei rhodesiense occurs in East Africa and produces a more acute disease (1, 2). During acute infection (stage 1), parasites proliferate in the host bloodstream and lymphatics and undergo antigenic variation, thereby evading elimination by the immune system (1, 2). In chronic infections (stage 2), parasites are present in the central nervous system, resulting in multiple clinical sequellae and death if not treated (1–3). Current drug treatments for stage 1 HAT include pentamidine and suramin (for T. brucei gambiense and T. brucei rhodesiense, respectively), whereas those for stage 2 HAT include melarsoprol and eflornithine (for T. brucei rhodesiense and T. brucei gambiense, respectively). More recently, nifurtimox and eflornithine in combination have also been used as a treatment for chronic disease (4, 5). These treatments are highly toxic, require complicated dosing, and must also contend with increasing parasite drug resistance (5–8). Hence, there is a dire need for new effective drugs for HAT, especially for the second stage.

Several approaches have been taken to develop anti-HAT drugs, ranging from large compound library screening against the organisms in vitro (which is target-agnostic) to target-specific structure-based drug design (9, 10). We have taken a hybrid approach to identify potential drugs for HAT. We began by using genetic methods to selectively assess the essentiality in vitro and in vivo of predicted “druggable” enzymes in T. brucei (11). We then applied target-specific chemistry design to identify compounds that inhibited the enzyme activity and parasite growth and confirmed their specific inhibitions using biochemical- and molecular-based approaches. Finally, we tested whether our compounds cured the infection in vivo using a mouse model and validated their potential use for drug development.

Aminoacyl-tRNA synthetases have been identified as possible drug targets for several infectious diseases (12–14). They are responsible for charging a specific tRNA with its cognate amino acid, which is essential for protein synthesis (15). Drugs targeting isoacceptor-tRNA synthetase (IleRS) have been successfully developed against bacterial infections, e.g. mupirocin (16). In T. brucei, IleRS is encoded by one gene that undergoes alternative mRNA trans-splicing, thereby allowing tRNA isoacceptylation to be performed in both the cytoplasm and mitochondrion (17). The amino acid sequence of IleRS is conserved among T. brucei, Trypanosoma cruzi, and Leishmania sp. Because the genomes of these parasites are highly conserved (18), the validation of drug targets and the discovery of inhibitors for T. brucei may also aid in the development of new drugs for leishmaniasis and Chagas disease (19, 20).
Here, we present genetic and chemical validation of *T. brucei* IleRS as a target for drug development. We knocked down the IleRS gene by RNAi and found it to be essential for growth and infection of mice. We also identified small molecule inhibitors that are highly selective to the parasites, including a molecule that acts as a competitive inhibitor of the IleRS enzyme and cures mice of infection. These results may aid in the development of new drugs for HAT.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction for RNAi and Transfection**

The inducible RNAi plasmid for silencing IleRS gene expression was generated using the pQuadra system (21). Briefly, 400 bp of the gene were selected using RNAiT software (22) and amplified by PCR using oligonucleotides with specifically designed BstXI sites (7538- F, ATACCAATGTTAGGTACG-TCACAACCCAACCTGGGA; and 7539-R, ATACCATAGATT-GGGATTTCCTCCCAGATATTTT). Ligation with BstXI-digested pQuadra1 and pQuadra3 plasmids generated the pQ041 vector, containing inverted repeats of the PCR product separated by a spacer region. Transfection of NotI-linearized digested pQuadra1 and pQuadra3 plasmids generated the recombinant IleRS (rIleRS) activity in the presence of compounds was calculated as follows: % remaining rIleRS activity = (rIleRSnc × 100)/rIleRSnc, where rIleRSnc is the average of the rIleRS activity measurements with compounds and rIleRSnc is the average of the rIleRS activity measurements without compounds (vehicle only).

**Cell-based Compound Screen**

Compounds were obtained from the National Cancer Institute. Compounds were searched by similarity to Ile-AMP using a Tanimoto similarity index of 80%. We used the PubChem search at the National Center for Biotechnology Information and the National Cancer Institute database. Compound stocks were prepared at a 10 mM concentration in dimethyl sulfoxide. *T. brucei* bloodstream forms (100 μl at 2.0 × 10^5 parasites/μl) were plated in 96-well plates and mixed with 100 μl of compounds diluted in HMI-9 medium with 10% FBS (Me_2SO at 0.2%). Parasites not treated with compounds were also plated as controls. After 48 h of incubation at 37 °C and 5% CO_2, 20 μl of alamarBlue (Invitrogen) was added, and the assays were developed for 4 h. Fluorescence measurements were obtained using a SpectraMax M2 microplate reader (Molecular Devices) with excitation at 544 nm and emission at 590 nm (590-nm cutoff). Data were analyzed using GraphPad Prism for Windows.

**In Vivo RNAi and Mouse Treatment with Inhibitors**

**RNAi in Vivo**—The *T. brucei* IleRS RNAi line growing at mid-log phase was used to infect BALB/cAnNHsd mice (6–8-week-old males; Harlan Laboratories). 1.0 × 10^7 parasites in 200 μl of HMI-9 medium were injected inpraperitoneally. Doxycycline at 200 μg/ml and 5% sucrose were added to the drinking water either 18 h before infection or 24 or 48 h after infection to induce RNAi expression (replaced daily). For non-induced conditions, mice received drinking water containing 5% sucrose only. Parasitemia was monitored daily starting at day 2 post-infection by tail prick. Mice with parasitemia of >1.0 × 10^8 parasites/ml of blood were killed. All procedures were performed in the vivarium of the Seattle Biomedical Research Institute in compliance with the laws and institutional guidelines (Institutional Animal Care and Use Committee KS-01).

**Molecular Modeling and Docking**

The *T. brucei* IleRS structure was modeled by homology to *Thermus thermophilus* IleRS (Protein Data Bank code 1JZQ) (16) using the SWISS-MODEL workspace (27). Docking was performed with compound NSC70422 and N-[isoleucenyl]-N’-[adenosyl]-diaminosulfone (Ile-AMS; the structure previously determined with *T. thermophilus* IleRS) using AutoDock 4.2 and Vina 1.1.2 (28). Data visualization was performed with PyMOL (Version 1.5.0.4, Schrödinger, LLC).

**Data Presentation and Statistical Analysis**

All data are shown as the mean ± S.D. Comparisons among groups were made by two-tailed *t* test for repeated measures using GraphPad Prism Version 5.00 for Windows. The *p* values <0.05 with a confidence interval of 95% were considered statistically significant unless specified otherwise.

**RESULTS**

IleRS Is Essential for *T. brucei* Growth and Infection—To genetically validate IleRS as a drug target in the *T. brucei* blood-
in vivo in b non-induced.

RNAi against IleRS.

growth curve of RNAi against IleRS. Tetracycline induction of RNAi against IleRS resulted in rapid...

T. brucei bloodstream form, we knocked down its expression by RNAi. A...

parasites could be detected at 72 h after RNAi induction (Fig. 1a). Quantitative RT-PCR analysis showed an 80% decrease in IleRS mRNA at 24 h after tetracycline induction, confirming its knockdown (supplemental Fig. S2a). No inhibition of parasite growth upon tetracycline induction was observed with a T. brucei line transfected with a control vector without RNAi insertions (supplemental Fig. S2b).

To assess IleRS essentiality in vivo, we infected BALB/c mice with the T. brucei IleRS RNAi line. Doxycycline (a stable tetracycline analog) was added to the mouse drinking water to induce RNAi expression, and parasitemia was monitored daily. Doxycycline addition to the water either before or after infection resulted in rapid and complete parasite clearance (Fig. 1b). Mice were monitored for up to 20 days post-infection without any recurrence of infection. Notably, mice that received doxycycline at 48 h post-infection presented high parasitemia levels at 72 h (∼10^7 parasites/ml of blood), but they were still cured upon RNAi induction (Fig. 1b), which shows that knockdown of this gene not only impairs parasite infection but also cures highly infected mice. No effect on parasitemia was observed when the parental cell line (SM427, which does not express an RNAi construct) was infected in the presence of doxycycline (supplemental Fig. S2c), which rules out any effect of doxycycline on the parasite growth. These results show that IleRS is essential for parasite growth and infection in vivo. The rapid cure of highly infected mice after RNAi induction implies that IleRS is a potential target for drug development, suggesting that inhibition of this enzyme could lead to a rapid cure of infection.

Identification of Ile-AMP Analogs That Inhibit T. brucei Growth—During tRNA aminoacylation, IleRS hydrolyzes ATP and conjugates isoleucine to AMP, thereby forming the Ile-AMP intermediate (15). Because Ile-AMP has been shown to strongly interact with IleRS (K_d ∼ 10^−6 M) (16, 29), we reasoned that compounds that mimic its structure could inhibit its enzyme activity. 20 Ile-AMP analogs (>80% similarity to Ile-AMP) were obtained from the National Cancer Institute compound collection and tested against T. brucei bloodstream form growth (Fig. 2, a and b, and supplemental Table S1). We identified 14 Ile-AMP analogs that inhibited T. brucei growth with EC_{50} (effective concentration that inhibits parasite growth by 50%) values between 3.9 nM and 4.4 μM (Fig. 2b and supplemental Table S1). The EC_{50} values of these compounds have also been determined for 59 different mammalian cell lines (named NCI60 cells) (30). A comparison of the EC_{50} values for the most effective compounds revealed six with lower EC_{50} values for T. brucei compared with NCI60 cells (Fig. 2c). Some compounds have been previously tested in vivo for other conditions, and thus, pharmacokinetic and toxicity information is also available (supplemental Table S1), e.g. NSC404241, which has been approved for human use and which crosses the blood-brain barrier (31). The compound NSC63984 has also been shown to cross the blood-brain barrier, although it did not completely cure mice infected with T. brucei (32). Overall, we identified small molecules that inhibit parasite growth and that are selective compared with mammalian cells; thus, they may be feasible leading candidates for drug development for HAT.

Identification of a Competitive Inhibitor of T. brucei IleRS—To evaluate whether these Ile-AMP analogs are targeting the IleRS enzyme, we expressed and purified T. brucei rIleRS from E. coli (Fig. 3a). The enzyme was active as detected by tRNA^Ile aminoacylation in vitro (Fig. 3b). Enzyme inhibition analysis showed that these small molecules reduced rIleRS activity (Fig. 3c), especially NSC70422, which presented an IC_{50} of 14 nM (Fig. 3d). It is noteworthy that this molecule inhibited T. brucei growth with an EC_{50} of 88 nM and ∼900-fold parasite selectivity (Fig. 2, b and c). To investigate its mechanism of inhibition of IleRS, we performed enzymology studies. NSC70422 increased the rIleRS K_m for isoleucine, but no effect was observed on the enzyme V_{max} (Fig. 3e), which is consistent with a mechanism of competitive inhibition. The very low inhibition constant (K_i = 2.7 ± 1.4 nM) resembles the high affinity interaction between IleRS and its reaction intermediate, Ile-AMP (12, 16, 29). Docking analysis of NSC70422 on the IleRS structure predicted its interaction with Gln-578 and Pro-50, two amino acids involved in the active site pocket of IleRS.
in isoleucine interaction (16), and with the AMP-binding site (Fig. 3f). Therefore, NSC70422 seems to interact with the Ile-AMP-binding site of IleRS and to interfere with isoleucine binding, in accordance with its mode of action as a competitive inhibitor.

To assess whether IleRS is the primary target of NSC70422, we overexpressed IleRS minus its mitochondrial targeting signal (IleRS-α-ATG\textsuperscript{3V5}, containing a C-terminal three-V5 tag) in the \textit{T. brucei} bloodstream form and performed growth inhibition assays to determine the EC\textsubscript{50}. Overexpression of IleRS-α-ATG\textsuperscript{3V5} in \textit{T. brucei} conferred a 3.5-fold increase in the EC\textsubscript{50} of NSC70422 (242 nM) compared with non-induced cells (69 nM, which is similar to 88 nM for wild-type cells) (Fig. 3, g-i). Overexpression of a catalytically inactive IleRS mutant (IleRS-α-ATG\textsuperscript{3V5}(D539A/W541A)) (33) by tetracycline induction failed to increase the EC\textsubscript{50} (Fig. 3, g and i). Because Asp-539 and Trp-541 are in the IleRS active site, their mutation may interfere with IleRS binding to its substrates as well as to NSC70422. Because the mutant enzyme is catalytic inactive, its overexpression does not rescue the inhibitory effect of NSC70422. In contrast, overexpression of WT IleRS provides extra enzyme active sites, which partially rescue the inhibitory effect of NSC70422.

**FIGURE 2. Identification of Ile-AMP analogs that inhibit \textit{T. brucei} growth.** a, Ile-AMP structure. Positions 1–5 indicate the locations of chemical groups changed in the analogs analyzed. Comprehensive information on the compounds and their structures is provided in supplemental Table S1. b, structure-activity relationship of the Ile-AMP analogs tested against \textit{T. brucei}. The EC\textsubscript{50} for \textit{T. brucei} is shown under the compound structure. c, comparison of the EC\textsubscript{50} values of selected compounds for \textit{T. brucei} and NCI60 cells. The EC\textsubscript{50} values for NCI60 cells represent the mean of the EC\textsubscript{50} data for 59 different cell lines (30). Compound selectivities were obtained by dividing the mean of the NCI60 EC\textsubscript{50} by the \textit{T. brucei} EC\textsubscript{50} data for each compound. Data shown in b are the mean of at least three experiments performed in triplicate.
FIGURE 3. Identification of a competitive inhibitor of *T. brucei* IleRS. 

a, *T. brucei* IleRS (122 kDa) resolved by 4–20% SDS-PAGE and stained with Imperial Coomassie stain (Pierce). b, IleRS aminoaclylation assay performed with 32P-labeled tRNA<sub>Ile</sub>. P1 nuclease digestion of Ile-tRNA<sub>Ile</sub> resulted in free [32P]AMP and [32P]Ile-AMP, which were separated by TLC. c, inhibition of IleRS activity with 14 Ile-AMP analogs. Assays were performed with compounds at 0.1 and 1 μM as described previously (25). The molecule NSC616354 is a positive control (25). Data are shown as the percentage of remaining IleRS activity (see “Experimental Procedures”). d, dose-response inhibition of IleRS with NSC70422. The experiment was performed with 1–1000 nM NSC70422. e, enzymology analysis of NSC70422 inhibition of IleRS. Assays were performed with 5–50 nM NSC70422 and various concentrations of isoleucine (0.1–20 mM). The Michaelis-Menten constant (K<sub>m</sub>), maximum velocity (V<sub>max</sub>), and inhibition constant (K<sub>i</sub>) were calculated by nonlinear regression with GraphPad Prism software. The table shows the apparent K<sub>m</sub> and V<sub>max</sub> for IleRS (with respect to isoleucine) in the absence or presence of NSC70422. f, docking of NSC70422 (black lines) and Ile-AMS (yellow lines) in the modeled structure of *T. brucei* IleRS. The alignment shows amino acids of the IleRS catalytic site. Pro-50 and Gln-578 (red boxes) are involved in isoleucine binding. His-58–His-61 (HYGH motif; blue box) are involved in AMP binding. Yellow, green, and cyan boxes show amino acids of the binding site highlighted in the model (16, 33). Green boxes also show Asp-539 and Trp-541 mutated to alanine in g and i. Asp-539 is not visible in the IleRS model due to the orientation of the binding site, which favors showing the NSC70422 inhibitor. Ile-AMS is an Ile-AMP analog known to interact with *T. thermophilus* IleRS (16) and was used here for comparison. Tb, *T. brucei*; Tc, *T. cruzi*; Ld, Leishmania donovani; Th, *T. thermophilus*; Hs, Homo sapiens. g, analysis of NSC70422 EC<sub>50</sub> values in *T. brucei* overexpressing cytoplasmic IleRS (IleRS<sup>-α-ATG</sup>-AT). NSC70422 EC<sub>50</sub> values were calculated for *T. brucei* SM427 or cell lines overexpressing WT IleRS<sup>-α-ATG</sup>-ATG<sup>WT</sup> (DS39A/W541A). Tet, 1 μg/ml tetracycline. h, graph showing the growth inhibition curve shift of NSC70422 EC<sub>50</sub> values after overexpression of WT IleRS<sup>-α-ATG</sup>-ATG<sup>WT</sup> in *T. brucei*. i, Western blot analysis of WT IleRS<sup>-α-ATG</sup>-ATG<sup>WT</sup> and mutant IleRS<sup>-α-ATG</sup>-ATG<sup>DS39A/W541A</sup> expression in *T. brucei*. *T. brucei* lysates in the presence or absence of 1 μg/ml tetracycline were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with monoclonal antibodies against the three-V5 tag. The membrane was stripped and reblotted with monoclonal antibodies against HSP70 (mAb78). Data in c, d, g, and h are the mean ± S.D. of at least three experiments performed in triplicate. **, p < 0.007.
observed by an increase in EC_{50}. These results confirm that NSC70422 targets IleRS and inhibits its activity in vivo, consistent with the enzymology analysis (Fig. 3, c–e). These results show that NSC70422 is a potent and specific intracellular inhibitor of T. brucei IleRS.

**IleRS Inhibitor Cures T. brucei Infection in Vivo**—Finally, we sought to evaluate whether these small molecules cure infection in vivo. T. brucei-infected mice were treated with NSC70422, and parasitemia and survival times were monitored daily. A 4-day treatment with dosages as low as 10 mg/kg twice per day was sufficient to suppress parasite growth in vivo (Fig. 4a). Mice presented prolonged life spans upon NSC70422 treatment, and complete cure of infection was observed with 50 mg/kg twice per day (Fig. 4b); cure was determined by the absence of parasitemia in treated mice for >30 days post-infection. Mice were monitored for up to 60 days post-infection without recurrence of infection. No signs of toxicity were observed when mice were treated with 50 mg/kg NSC70422 four times per day for 4 days (data not shown), in agreement with a previous report of no toxicity with daily doses of up to 400 mg/kg for 5 days (30). An increased life span was also observed upon treatment of the mice with compounds NSC404241, NSC7359, and NSC30605 (Fig. 4c and supplemental Fig. S3). NSC404241 crosses the blood-brain barrier in humans (31). Although this has not yet been tested for the other compounds, it implies that this class of compounds may be candidates for drugs against stage 2 HAT. These results show that NSC70422 is effective against *T. brucei in vivo* and therefore a new lead for the development of drugs for treating HAT.

**DISCUSSION**

One of the main factors hampering the development of effective drugs for trypanosomatid parasites is a lack of drug target knowledge (20). Although there is a variety of work that identifies essential genes in *T. brucei in vitro*, there are still limited examples of target validation, including in vivo analysis of gene essentiality and chemical validation with identification of inhibitors as candidates for accelerating the design of new drugs. Here, we have presented the validation of IleRS as a drug target in *T. brucei* and identified new small molecule inhibitors that are potential candidates for the development of new drugs for treatment of HAT. We have shown that IleRS is essential for parasite growth in vitro and for in vivo infection of mice. RNAi knockdown of IleRS resulted in rapid parasite death, despite incomplete knockdown (80%) of its mRNA at 24 h. The rapid cell death upon partial RNA knockdown suggests that the low levels of target mRNA and hence protein may not be sufficient for sustaining cell growth, as also observed for other essential genes in *T. brucei* (34, 35). Because IleRS is involved in protein synthesis, the rapid cell death likely resulted from the parasites’ inability to synthesize new proteins. Infected mice could also be cured after RNAi induction against *T. brucei* IleRS in vivo, which suggests that inhibition of this enzyme could render parasites unable to sustain an infection and thus is a target for drug development.

We found that the small molecule NSC70422 is a specific inhibitor of IleRS and that it is safe and effective against *T. brucei* in a murine model of infection. Although little is known about this molecule’s pharmacokinetics and toxicity in humans, other molecules of this class (e.g. NSC404241) are well tolerated in humans and are known to cross the blood-brain barrier (31, 36), an important prerequisite for treating stage 2 HAT. Besides NSC404241, the molecule NSC63984 has also been shown to cross the blood-brain barrier (32), suggesting that this class of molecules may be explored for the develop-
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ment of drugs against stage 2 HAT. Because this small molecule shows promising results in vivo in a mouse model, pharmacokinetic studies will be performed with this molecule and other analogs to develop new drugs against HAT and related parasites.

T. brucei and human IleRS amino acid sequences have 38% identity, with several amino acid differences encompassing the active site. This indicates that the greater susceptibility of trypanosomes compared with human cells to these inhibitors could be due to differences in IleRS protein structure, its association with other proteins or protein complexes, and inhibitor uptake and metabolism.

Several attempts to purify human IleRS from E. coli (using the pET-28 system and E. coli BL21 Rosetta(DE3)pLysS) failed due to its extensive degradation. Similar results were obtained when human IleRS was coexpressed with the GroES-GroEL-chaperones (37), which indicates that the human protein is unstable in E. coli. Nonetheless, comparison of the human and T. brucei EC50 values for NSC70422 showed a 900-fold parasite selectivity, and no toxicity in mice was observed upon high dose administration, suggesting that NSC70422 may preferentially inhibit the parasite protein.

The discoveries of new drug targets in T. brucei through genetic and chemical validation may also help advance development of drugs against the related parasites T. cruzi and Leishmania sp. because of their genomic similarities (18). The T. brucei IleRS sequence is highly conserved in T. cruzi and Leishmania sp. and is also likely to be essential for their survival. Although genetic manipulation of these parasites is less tractable compared with T. brucei, the inhibitors identified here may be useful for their chemical validation and provide a scaffold for development of new drugs against them.

In summary, we have validated the enzyme IleRS as a target for drug development in T. brucei and identified a selective inhibitor that cures the infection in vivo. This small molecule represents a new lead for drug development against HAT.

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