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Chemical Validation of Trypanothione Synthetase

A POTENTIAL DRUG TARGET FOR HUMAN TRYPANOSOMIASIS[*S]

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In the search for new therapeutics for the treatment of human African trypanosomiasis, many potential drug targets in *Trypanosoma brucei* have been validated by genetic means, but very few have been chemically validated. Trypanothione synthetase (TryS; EC 6.3.1.9; spermidine/glutathionylspermidine:glutathione ligase (ADP-forming)) is one such target. To identify novel inhibitors of *T. brucei* TryS, we developed an *in vitro* enzyme assay, which was amenable to high throughput screening. The subsequent screen of a diverse compound library resulted in the identification of three novel series of TryS inhibitors. Further chemical exploration resulted in leads with nanomolar potency, which displayed mixed, uncompetitive, and allosteric-type inhibition with respect to spermidine, ATP, and glutathione, respectively. Representatives of all three series inhibited growth of bloodstream *T. brucei in vitro*. Exposure to one of our lead compounds (DDD86243; 2 × EC_{50} for 72 h) decreased intracellular trypanothione levels to <10% of wild type. In addition, there was a corresponding 5-fold increase in the precursor metabolite, glutathione, providing strong evidence that DDD86243 was acting on target to inhibit TryS. This was confirmed with wild-type, TryS single knock-out, and TryS-overexpressing cell lines showing expected changes in potency to DDD86243. Taken together, these data provide initial chemical validation of TryS as a drug target in *T. brucei*.

Human African trypanosomiasis (HAT)[2] is endemic in sub-Saharan Africa and is estimated to be responsible for more than 30,000 deaths/year (1). The causative agent of HAT is the protozoan parasite *Trypanosoma brucei*, and, if left untreated, the disease is fatal. Despite the death toll and disease burden of HAT, there is an acute lack of therapies available. Treatment is solely dependent upon a small repertoire of drugs that suffer from serious limitations, including high cost (1), unacceptable host toxicity (2), poor efficacy, lack of availability, and acquired parasite drug resistance (3). As a result, there is a pressing need to discover new therapeutics. Research has revealed a wealth of information regarding enzymes and pathways that are crucial to the survival of *T. brucei*, and, based on these studies, many anti-parasitic drug targets have been proposed. Efforts are therefore under way to translate this parasitic knowledge into the identification of new therapeutics for HAT.

Metabolic pathways that are both essential for parasite survival and absent from the host provide hugely attractive starting points for drug discovery (4). One such pathway is the thiol metabolism of *T. brucei* (5–8). These parasites are uniquely dependent upon trypanothione (N^1,N^8-bis(glutathionyl)spermidine) as their principal thiol, in contrast to most other organisms (including their mammalian hosts), which utilize glutathione (γ-L-glutamyl-L-cysteinylglycine (GSH)) (9). All trypanothione-dependent enzymes studied to date, including trypanothione reductase (10), tryparedoxin (11), and tryparedoxin peroxidase (11), have been validated as drug targets in *T. brucei* using genetic techniques, such as classical gene knock-out and RNA interference, thus highlighting the essentiality of the trypanothione pathway in *T. brucei* function.

In *T. brucei*, trypanothione is synthesized from GSH and spermidine (Spd) by an ATP-dependent C-N ligase, trypanothione synthetase (TryS; EC 6.3.1.9), with N^1- and N^8-glutathionylspermidine as intermediates (12, 13). Despite a lack of chemical validation, TryS has been genetically validated as a drug target, with RNA interference and gene knock-out studies confirming that TryS is essential for *T. brucei* growth in both bloodstream and procyclic forms (14–16). As well as this genetic validation, *T. brucei* TryS also represents a highly attractive drug target in other categories assessed in our standard assessment profile (17). TryS is unlikely to have resistance or toxicity issues because it is a single copy gene in *T. brucei* (12), there is no alternative bypass mechanism, and there is no equivalent enzyme in humans. In addition, the kinetic mechanism is known, and potent mechanism-based inhibitors have been identified (18). Furthermore, TryS from *Leishmania major* has recently been crystallized (19), providing an opportunity to co-crystallize any TryS inhibitors identified. Based on this attractive target assessment profile, TryS became a high priority target for entry into a hit discovery program.

To perform a successful high throughput screen, a fit-for-purpose enzyme assay is required. A spectrophotometric assay has previously been described in which TryS activity is measured by coupling ADP formed in the reaction to pyruvate kinase/lactate dehydrogenase and monitoring oxidation of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures".

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[2] The abbreviations used are: HAT, human African trypanosomiasis; Spd, spermidine; SKO, single knock-out; TryS, trypanothione synthetase.

NADH (20). Although this cuvette-based assay has been successfully used to characterize the TryS enzyme (12), this assay platform is not amenable to high throughput screening. Here we describe the development of a novel TryS assay suitable for high throughput screening, the identification and characterization of TryS inhibitors, and, most importantly, the chemical validation of TryS as a drug target in *T. brucei*.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant TryS—The expression of recombinant *T. brucei* TryS utilized the construct pET15b-*TbTRYS* (12); however, expression conditions were adapted to ensure elevated levels of protein sufficient for high throughput screening. In this study, freshly transformed pET15b-*TbTRY*_ArcticExpress(DE3)RIL competent cells (Stratagene) were used to inoculate a 100-ml starter culture that was grown overnight at 37 °C in LB medium, containing 50 μg/ml carbenicillin and 20 μg/ml gentamycin, with agitation (250 rpm). Transformed cells were centrifuged briefly (4000 × *g*, 10 min, 4 °C) and used to inoculate 500 ml of LB medium and grown at 37 °C until A_{600} was >0.6. Cells were harvested by centrifugation (4000 × *g*, 30 min, 4 °C) and subsequently used to equally inoculate 8 × 1 liter of autoinduction growth medium (no antibiotic selection) to bulk up cell mass (30 °C, 250 rpm) until A_{600} was ~0.6. The cultures were cooled to 12 °C and incubated for 48 h, and the cells containing the expressed recombinant protein were harvested as described previously.

Cells were resuspended in buffer (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, DNase, 1 mM tris(2-carboxyethyl)phosphine plus one complete protease inhibitor mixture tablet (Roche Applied Science) per 50 ml of buffer), and cell lysis was performed using a continuous flow cell disrupter (Constant Systems). Cell debris was separated and discarded after centrifugation (30,000 × *g*, 30 min, 4 °C). The supernatant containing soluble protein was passed through a 0.2-μm filter unit and loaded onto a 5-ml nickel-chelated HisTrap column (GE Healthcare) and then cleaved using thrombin to remove the hexahistidine tag. The final purification step was achieved using a HiLoad Q Sepharose 16/10 column (GE Healthcare), and the buffers for chromatography and thrombin cleavage steps were essentially as described previously (12).

TryS Assay Development and Kinetic Parameter Determinations—The enzymatic activity of TryS was determined by monitoring release of phosphate using BIOMOL Green reagent (BIOMOL International UK), which gives an absorbance readout at 650 nm (21). Following optimization of the assay buffer and determination of the enzyme linearity, assays were carried out at room temperature in a 50-μl reaction volume containing 100 mM Hepes, pH 8.0, 0.5 mM EDTA, 2 mM dithiothreitol, 0.01% Brij-35, 10 mM magnesium acetate, 10 nM recombinant TryS, and varying concentrations of substrates. Michaelis constants for each of the three substrates (Spd, GSH, and ATP) were determined in an end point assay, using these buffer and enzyme conditions.

TryS Hit Identification—The TryS high throughput screen was performed using our in-house diverse compound collection, containing 63,362 diverse structures based around ~4000 chemical scaffolds. All library compounds were solubilized in 100% DMSO to a concentration of 3 mM.

Single point inhibition assays were carried out at room temperature in clear, flat bottom, polystyrene, 384-well plates (Matrix). Each assay was performed in a 50-μl reaction volume containing 100 mM Hepes, pH 8.0, 0.5 mM EDTA, 2 mM dithiothreitol, 0.01% Brij-35, 10 mM magnesium acetate, 10 nM TryS, 25 μM Spd, 20 μM GSH, 35 μM ATP, and 30 μM test compound.

Test compound (0.5 μl in DMSO) was transferred to all assay plates using a Cartesian Hummingbird (Genomics Solutions) before 25 μl of a reaction mix, containing all assay components except GSH, was added to assay plates using a Thermo Scientific PlateMate Plus (Matrix). The reaction was initiated and stopped with the additions of 25 μl of GSH and 50 μl of BIOMOL Green, respectively, on a Thermo Scientific WellMate (Matrix). The TryS assay was run at room temperature for 60 min, and the BIOMOL Green signal was allowed to develop for 30 min before the absorbance of each well was read at 650 nm using an EnVision multilabel plate reader (PerkinElmer Life Sciences).

ActivityBase from IDBS was used for data processing and analysis. Compounds were designated hits if the A_{650} was greater than three S.D. values from the mean DMSO-only control.

TryS Inhibitor Studies—Following medicinal chemistry analysis of the data set, hit compounds of interest were cherry picked (from original library plates using a JANUS work station) based upon structural similarity, chemical tractability, and defined physicochemical properties. To generate IC_{50} data for these putative TryS inhibitors, 10-point inhibitor curves (consisting of half-log serial dilutions of compound in DMSO) were prepared in 384-well plates using a JANUS work station (PerkinElmer Life Sciences). Each compound plate created contained 10-point inhibitor curves for 30 test compounds and two curves of prochlorperazine (dimaleate salt) (Sigma), which was used as the standard compound in this screen. Following preparation of the inhibitor curves, assays were carried out using the BIOMOL Green reagent as described above.

ActivityBase from IDBS was again used for data processing and analysis. All IC_{50} curve fitting was undertaken using XLFit version 4.2 from IDBS. A four-parameter logistic dose-response curve was utilized using XLFit 4.2 model 205. All test compound curves had floating top and bottom, and prefit was used for all four parameters.

To establish modes of inhibition of representative inhibitors, data sets were collected at six inhibitor concentrations with six varied concentrations of each substrate. Each data set was individually fitted to the Michaelis-Menten equation, and the resulting Lineweaver-Burk plots were examined for diagnostic patterns for competitive, mixed, or uncompetitive inhibition. Simple linear inhibition was tested by plotting K_m/V_{max} *versus* inhibitor concentration. Data sets were then globally fitted to the appropriate model. If more than one model appeared possible, then data were fitted to both and examined for significance using the F-test function in GraFit. For GSH as varied substrate, data were fitted to equations for competitive high substrate inhibition (Equation 1), uncompetitive high substrate

inhibition (Equation 2), and allosteric high substrate inhibition (Equation 3).

$$v = \frac{V_{max}}{1 + \frac{K_m}{S}\left(1 + \frac{i}{K_i}\right) + \frac{S}{K_i^s}\left(1 + \frac{i}{K_i}\right)} \quad \text{(Eq. 1)}$$

$$v = \frac{V_{max}}{1 + \frac{K_m}{S} + \frac{i}{K_i} + \frac{S}{K_i^s}} \quad \text{(Eq. 2)}$$

$$v = \frac{V_{max}}{1 + \frac{K_m}{S} + \frac{S}{K_i^s}\left(1 + \frac{i}{K_i}\right)} \quad \text{(Eq. 3)}$$

Compound Synthesis—Detailed syntheses of the compounds used in this study (28), and the analytical methods used to confirm the molecular identity of these novel compounds can be found in the supplemental material.

Coupled TryS Enzyme Assay—Several hit compounds identified from the high throughput screen were also tested using an orthogonal assay platform, namely the coupled assay described previously (20), which is a continuous spectrophotometric assay at 340 nm. These assays were run at 25 °C in polystyrene cuvettes because it was found that certain inhibitors in DMSO bound to acrylic cuvettes. Each 1-ml assay was prepared in 100 mM (K^+) HEPPS, pH 8, and contained 0.2 mM NADH, 1 mM phosphoenolpyruvate, 5 mM dithiothreitol, 0.5 mM EDTA, 10 mM $MgSO_4$, 2 units/ml L-lactate dehydrogenase, 2 units/ml pyruvate kinase, 0.5 μM TryS, 300 μM ATP, 100 μM GSH, 1.2 mM Spd, and varying concentrations of test compound.

Compound Efficacy in Cultured T. brucei Parasites—Bloodstream *Trypanosoma brucei* S427 were cultured at 37 °C in modified HMI9 medium (56 μM 1-thioglycerol was substituted for 200 μM 2-mercaptoethanol). Routine screening of test compounds against parasites was performed in 96-well plates using a modification of the Alamar Blue cell viability assay (22). Cell culture plates were stamped with 1 μl of an appropriate concentration of test compound in DMSO (to give final assay concentrations between 50 μM and 2 nM), followed by the addition of 200 μl of trypanosome culture (10^4 cells/ml) to each well, except for one column, which received medium only. MRC-5 cells were cultured in Dulbecco's modified Eagle's medium, seeded at 2000 cells/well, and allowed to adhere overnight. One microliter of test compound (10 point dilutions to give final assay concentrations between 50 μM and 2 nM) was added to each well at the start of the assay. The maximum tolerability of the cell assays was 0.5% DMSO, precluding testing at higher concentrations of inhibitor. Culture plates of *T. brucei* and MRC-5 cells were incubated at 37 °C in an atmosphere of 5% CO_2 for 69 h, prior to the addition of 20 μl of resazurin (final concentration 50 μM). After a further 4 h of incubation, fluorescence was measured (excitation 528 nm; emission 590 nm) using a BioTek FLX800 plate reader (23).

The EC_{50} values for test compounds used in thiol analysis were also determined using triplicate flasks of *T. brucei* containing 1×10^5 trypanosomes/ml and various concentrations of inhibitor. Growth of the parasites after culture for 72 h was assessed by measuring cell densities using the CASY model TT cell counter (Schärfe). EC_{50} values were determined using the four-parameter IC_{50} equation provided by GraFit. The data were background-corrected. In this equation, *s* was the slope factor.

$$y = \frac{\text{range}}{1 + \left(\frac{x}{IC_{50}}\right)^s} + \text{background} \quad \text{(Eq. 4)}$$

EC_{50} values were also determined in TryS single knock-out (SKO) and overexpression *T. brucei* cell lines (16) as previously described; however, TryS SKO cultures were maintained in the presence of puromycin (0.1 μg/ml), whereas TryS overexpression cell lines were maintained in the presence of phleomycin (2.5 μg/ml) and tetracycline (1 μg/ml).

Analysis of Intracellular Thiols—Cultures containing 1×10^5 bloodstream trypanosomes/ml were incubated with varying concentrations of compound DDD86243 equivalent to 0.5, 1.0, 1.5, and 2 times the previously determined EC_{50}. Following incubation for 72 h, cells (1×10^8) were collected by centrifugation (900 × *g*, 10 min, 4 °C), washed once in ice-cold PSG buffer (phosphate-buffered saline, pH 8.0, 1.5% (w/v) glucose, and 0.5 mg/ml bovine serum albumin), and derivatized with monobromobimane, as described previously (24). Acid-soluble thiols were separated by ion-paired, reverse phase high pressure liquid chromatography on an ion-paired Ultrasphere C_{18} column using a Dionex Ultimate 3000 instrument fitted with a Dionex RF-2000 fluorometer.

Analysis of TryS Expression—Western blotting for TryS in *T. brucei* cell lines (wild type, SKO, and overexpression equivalents) was performed as described previously (16).

RESULTS

Assay Development and Kinetic Characterization of TryS Activity—A novel assay for measuring *T. brucei* TryS activity was developed using the BIOMOL Green reagent, which provides a colorimetric quantification of phosphate levels via measurement at A_{650}. Using an optimized assay buffer and BIOMOL Green, linear TryS activity could be detected in a 60-min assay up to an enzyme concentration of 12.5 nM (Fig. 1*A*).

Michaelis constants for each of the three TryS substrates (ATP, Spd, and GSH) were determined (Fig. 1, *B–D*). When fixed substrate concentrations were required, Spd and ATP were used at saturating concentrations of 1.2 mM and 100 μM, respectively. When the GSH concentration was fixed, 100 μM was used because this concentration gave a maximal signal (note that GSH displays high substrate inhibition with an apparent K_i of 55 ± 6 μM). The substrate K_m values calculated were 45.4 ± 2.0 μM for Spd, 8.6 ± 0.6 μM for ATP, and 23.8 ± 2.3 μM for GSH.

For the compound screen, Spd and GSH concentrations were fixed at sub-K_m concentrations (25 and 20 μM, respectively), whereas the ATP concentration was fixed at a saturating level (35 μM). Under these conditions, linearity of the assay with respect to time was monitored. Fig. 1*E* reveals that the assay is

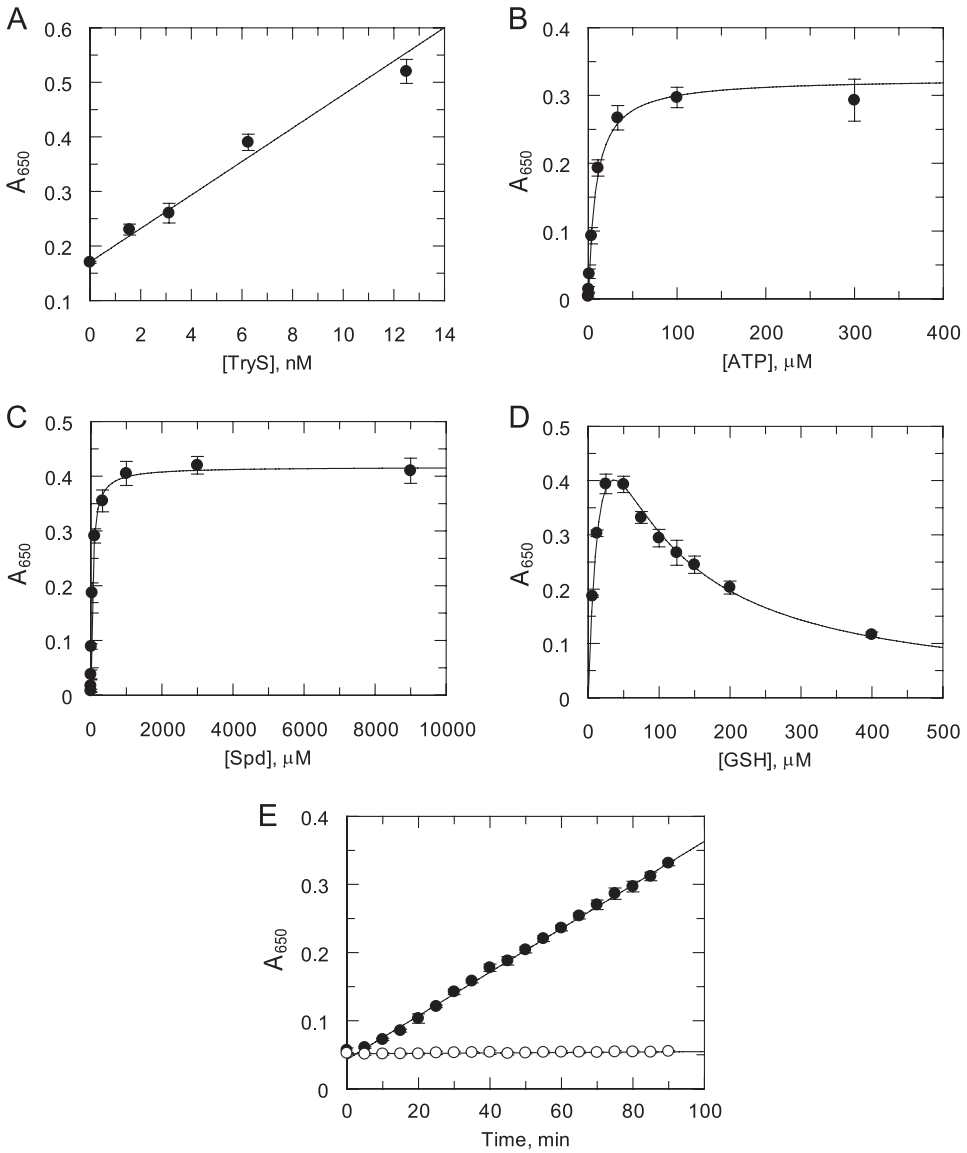


FIGURE 1. **Summary of TryS assay development.** *A*, linearity of the TryS assay with respect to enzyme concentration. *B*, ATP K_m determination in presence of 1.2 mM Spd and 100 μM GSH. *C*, Spd K_m determination in the presence of 100 μM ATP and 100 μM GSH. *D*, GSH K_m determination in the presence of 100 μM ATP and 1.2 mM Spd. *E*, linearity with respect to time under the following conditions: 10 nM TryS, 35 μM ATP, 25 μM Spd, and 20 μM GSH (●) or 35 μM ATP, 25 μM Spd, and 20 μM GSH without TryS enzyme (○). All data are presented as mean ± S.D. ($n = 4$).

linear for at least 90 min under these conditions. A 60 min time point was selected for screening.

Identification of TryS Inhibitors—Because there was no known TryS inhibitor available to us, an attempt was made to identify a compound for use as a standard during the screening campaign. A selection of inhibitors from an in-house trypanothione reductase program was assessed in the TryS assay, and a number of these compounds were shown to also inhibit TryS activity. One of the most potent of these (DDD66604 (prochlorperazine) (Fig. 2*A*)) returned an IC_{50} of ~19 μM (Fig. 2*B*) and was selected for use as a standard on the basis of its commercial availability.

TryS was subsequently screened against our in-house, diverse, leadlike library of 63,362 compounds (30 μM). To assess the robustness and reproducibility of the assay and the quality of the hit discovery campaign, various criteria were assessed following completion of the primary screen. These data reveal a high quality screening campaign, with a mean Z' factor (25) of 0.91 ± 0.04 and a mean DDD66604 pIC_{50} of 4.61 ± 0.12. In addition, the mean signal to background was 3.54 ± 0.23, and the mean percent coefficient of variation was 1.26 ± 0.46%.

Compounds from the primary screen displaying >33% inhibition were designated hits (a percentage inhibition of 33% corresponded to an absorbance read-out three S.D. values from the mean of DMSO-only controls). Based on this threshold, 776 putative TryS inhibitors were identified (Fig. 2*C*). These active compounds were retested in duplicate yielding 725 confirmed hits, giving a confirmation rate of 93% and a final hit rate of 1.14%.

After chemistry assessment and primary clustering of the hit compounds, 174 compounds were selected for progression to potency testing. For these compounds, 10-point dose-response curves were generated and assayed in duplicate against TryS. Fig. 2*D* shows the excellent correlation between the two replicate IC_{50} determinations, with the active compounds identified displaying potencies ranging from 90 nM to 30 μM. The identities of these 174 TryS inhibitors were confirmed by liquid chromatography-mass spectrometry analysis with a pass rate of >95% (correct MH^+ and ≥90% purity). Compounds of particular interest were resynthesized or resupplied, which, upon reassay, confirmed these compounds as inhibitors of TryS (data not shown). Based on their core structure, many hit compounds were assigned to one of eight hit series. Three of these hit series (Table 1) displayed excellent structure-activity relationships and also provided potent lead compounds (Table 2); therefore, future work proceeded with representative compounds from these hit series.

As a further confirmation of TryS hits, representative compounds from hit series 1–3 (compounds DDD60632, DDD73385, and DDD85811; Table 2) were tested against TryS using the traditional coupled assay platform. For direct comparison, the BIOMOL Green assay was modified to correlate directly with enzyme and substrate conditions in the coupled assay. Under these conditions, the key compounds

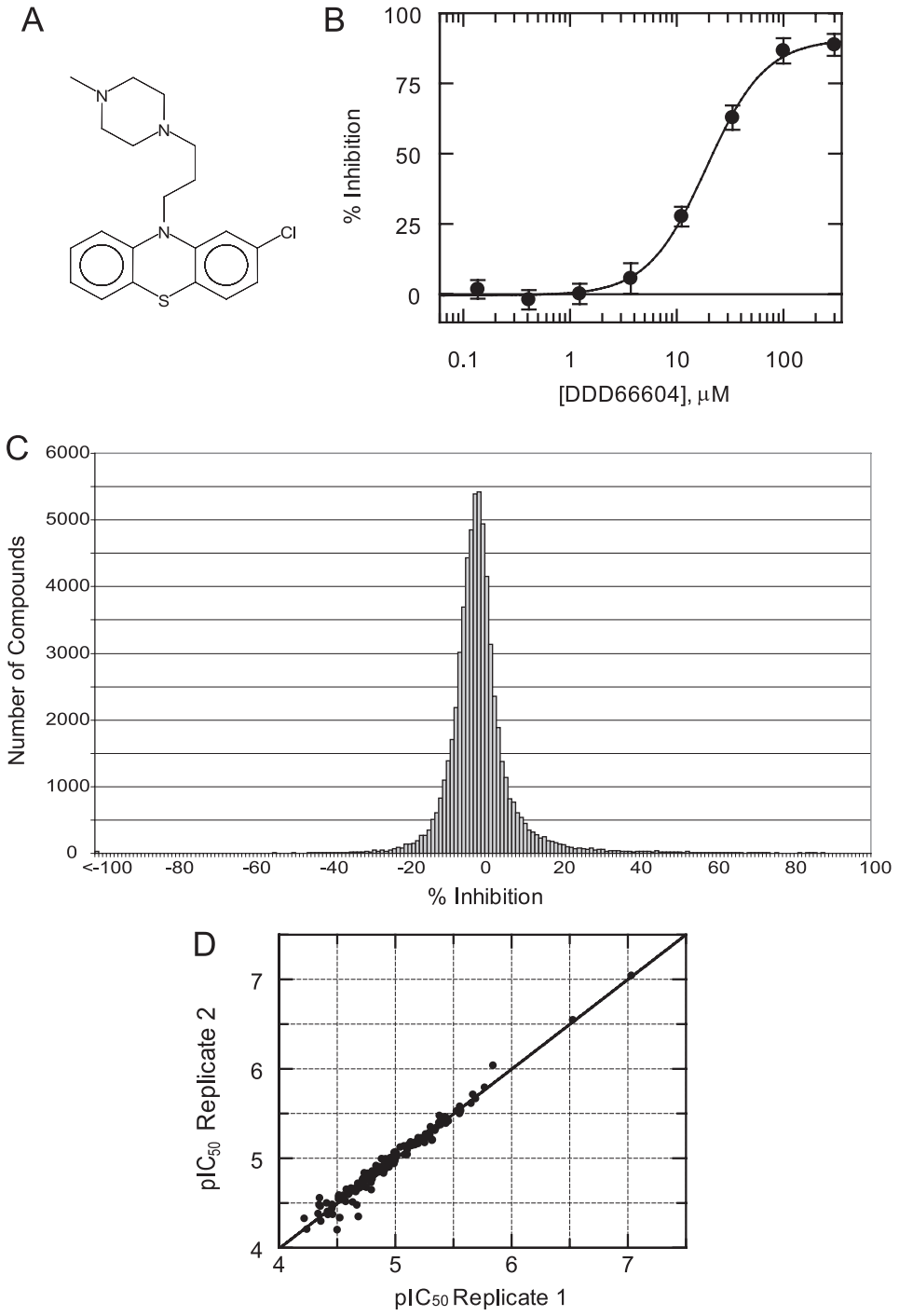


FIGURE 2. **Summary of TryS hit discovery campaign.** *A*, structure of DDD66604, a compound identified for use as a standard inhibitor in the TryS screen. *B*, representative IC_{50} determination for DDD66604. Data points are mean ± S.D. ($n = 16$). This representative example returns an IC_{50} for DDD66604 of 19.02 ± 1.26 μM. *C*, frequency histogram of hits identified during the primary screen. In total, 776 active compounds were identified. *D*, correlation between replicate pIC_{50} ($-\log IC_{50}$) values for each of the 174 compounds advanced to potency testing. Linear regression of these data returned a correlation coefficient of 0.987.

tested returned highly equivalent potency values in the two platforms, with DDD60632 returning IC_{50} values of 383 ± 64 and 575 ± 64 nM in the BIOMOL Green and coupled assays, respectively. DDD73385 returned IC_{50} values of 326 ± 39 and 365 ± 23 nM, and DDD85811 returned IC_{50} values of 181 ± 22 and 274 ± 30 nM, again in the BIOMOL Green and coupled assays, respectively. Notably, the shifts in potency of these compounds were minimal when increasing the substrate concentration toward physiological levels, providing an initial indication that these compounds were not acting through a competitive mechanism.

Mode of Inhibition—To establish the exact mode of inhibition of the identified hit series, the effect of varying each substrate concentration at various inhibitor concentrations was examined. Each data set was individually fitted to the Michaelis-Menten equation, and the resulting Lineweaver-Burk plots were examined for diagnostic patterns for competitive, mixed, or uncompetitive inhibition.

Mode of inhibition studies were performed using representative compounds from hit series 1–3. All of the compounds tested displayed the same mode of inhibition and were not competitive for any of the three substrates. These compounds displayed mixed inhibition with respect to Spd (Fig. 3*A*) and uncompetitive inhibition with respect to ATP (Fig. 3*B*). The GSH data proved more challenging to analyze due to the high substrate inhibition displayed by GSH (Fig. 3*C*). This data set was therefore analyzed using the high substrate inhibition equations described under "Experimental Procedures" (Equations 1–3) and found to fit the allosteric model best.

Mode of inhibition studies were also performed using DDD66604, which was the standard compound used during the hit discovery campaign. This compound displayed characteristic competitive inhibition with respect to Spd (Fig. 3*D*) and uncompetitive inhibition with respect to both ATP and GSH (Fig. 3, *E* and *F*, respectively). These data are included as a reference for compounds from hit series 1–3, none of which display competitive inhibition. In all cases, the K_i values generated by the above mode of inhibition analyses were within 2-fold of the IC_{50} value determined.

The Effects of TryS Inhibitors on T. brucei—The efficacy of several of the lead compounds were assessed in a cell-based assay using bloodstream *T. brucei* parasites. In addition, hit compounds were tested against the human cell line, MRC-5, to

TABLE 1
Core structures of TryS hit series

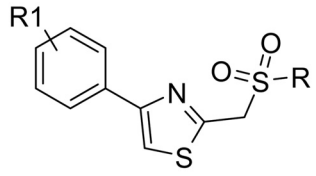
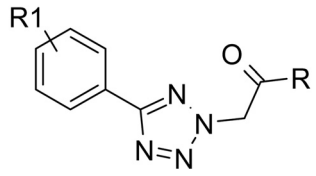
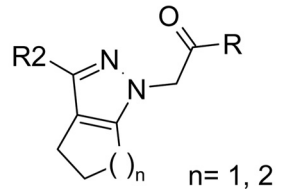
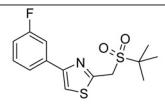
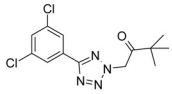
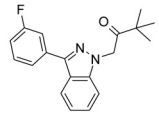
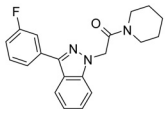
Hit Series	Core Structure
1	
2	
3	

TABLE 2
Structure-activity relationships from hit series 1–3
IC_{50} and EC_{50} values were determined as described under "Experimental Procedures."

Compound ID	Hit Series	Structure	IC_{50} TryS, μM	EC_{50} *T. brucei*, μM	EC_{50} MRC-5, μM (% Inhibition at 50 μM[a])
DDD60632	1		0.273	27.1	>50 (7.6%)
DDD73385	2		0.317	21.2	>50 (0%)
DDD85811	3		0.095	8.8	>50 (2.4%)
DDD86243	3		0.140	5.1	>50 (0%)

[a] Top concentration of cell-based assay limited to 50 μM to keep assay within DMSO tolerance.

ascertain whether these compounds showed selectivity in inhibiting parasite growth over human cell growth. These data are shown in Table 2 and indicate that none of the compounds tested were active against this human cell line at concentrations up to 50 μM, whereas a range of measurable EC_{50} values between 5 and 30 μM were determined for all compounds against the parasite. These data provide an initial indication of these TryS inhibitors selectively inhibiting parasite growth over the prototypical mammalian MRC-5 cells.

In order to better understand the relationship between IC_{50} values against TryS and the relatively weak (5–30 μM) EC_{50} values in the *T. brucei* proliferation assay, studies were conducted to confirm that compounds were indeed acting on TryS in the parasite. Intracellular thiol levels were determined in bloodstream *T. brucei* parasites following incubation with concentrations of the lead compound, DDD86243 (Table 2), corresponding to 0.5, 1.0, 1.5, and 2.0 times the established EC_{50} value (20 μM) (Fig. 4*A*). (This EC_{50} value was established by growing parasites in flasks rather than in a 96-well format). Treatment with DDD86243 resulted in a significant, dose-dependent decrease in the levels of intracellular trypanothione. Indeed, parasites incubated with the highest levels of DDD86243 (40 μM) were found to retain less than 10% of their original trypanothione levels after 72 h. In contrast, glutathione, the substrate for TryS, accumulated within treated parasites, reaching levels 5.2-fold higher than those seen in untreated cells.

Altering the abundance of a specific drug target within a cell is likely to lead to changes in drug potency. With this mind, TryS SKO and TryS-overexpressing *T. brucei* cell lines were generated (16). Thiol analysis confirmed that TryS function had been modulated such that SKO and overexpressing cell lines showed significantly lower and higher levels of trypanothione compared with wild-type cells, respectively (Table 3).

These transgenic cell lines were then compared with wild type cells for their relative sensitivity to DDD86243. Changes in the level of TryS in these cells directly correlated with their relative sensitivity to DDD86243, with EC_{50} values of 20.4 ± 0.4, 6.9 ± 0.2, and 44.5 ± 0.5 μM for wild-type, SKO, and TryS-overexpressing cell lines, respectively (Fig. 4*B*). These observations are entirely consistent with inhibition of TryS activity in DDD86243-treated bloodstream trypanosomes and strongly suggest that this compound specifically targets this enzyme.

DISCUSSION

Many potential drug targets from *T. brucei*, the causative agent of human African trypanosomiasis, have been identified, with enzymes involved in the trypanothione pathway providing particularly attractive targets. In this study, a hit discovery campaign was initiated to identify novel inhibitors of the *T. brucei* TryS enzyme.

Initially, a novel assay for measuring TryS activity was developed because the traditional continuous, spectrophotometric assay was not amenable to high throughput screening. The BIOMOL Green reagent was successfully used to develop a highly robust screening assay. Kinetic analysis using this new TryS assay platform revealed substrate K_m values of 45.4 μM for Spd, 8.6 μM for ATP, and 23.8 μM for GSH. These were highly comparable with the previously published substrate K_m values (37.8, 7.1, and 56.2 μM for Spd, ATP, and GSH, respectively) using the traditional coupled assay (12). In addition, the high substrate inhibition previously observed with GSH in the coupled assay (12) was also replicated using the BIOMOL Green assay platform. This excellent degree of correlation between the

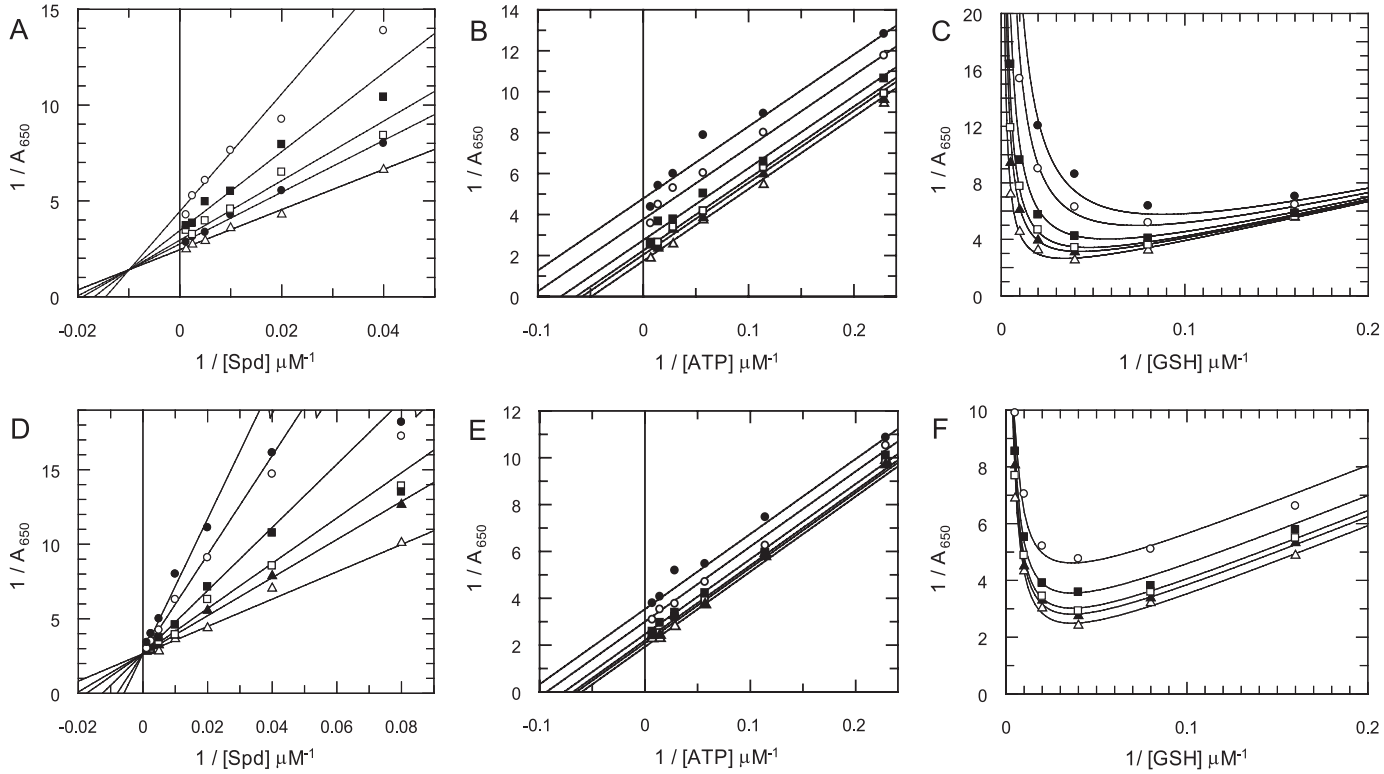


FIGURE 3. **Mode of inhibition of TryS inhibitors.** *A–C*, reciprocal plots of A_{650} *versus* substrate concentration reveal DDD60632, a representative example of all hit compounds tested, displays characteristic mixed inhibition with respect to Spd, uncompetitive inhibition with respect to ATP, and allosteric inhibition with respect to GSH. *D–F*, reciprocal plots of A_{650} *versus* substrate concentration reveal DDD66604 displays characteristic competitive inhibition with respect to Spd and uncompetitive inhibition with respect to both ATP and GSH. In all *panels*, inhibitor concentrations used were as follows: 3 × IC_{50} (●), 2 × IC_{50} (○), 1 × IC_{50} (■), 0.5 × IC_{50} (□), 0.3 × IC_{50} (▲), 0 × IC_{50} (△).

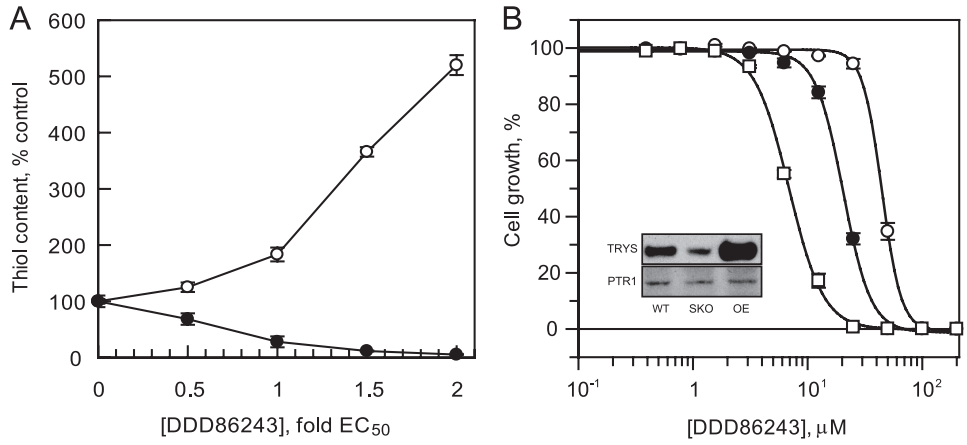


FIGURE 4. **Assessment of *in vivo* on target effects of TryS inhibitors.** *A*, thiol analysis of DDD86243-treated *T. brucei* bloodstream parasites. Data are represented as a percentage of trypanothione (●) and GSH (○) levels of untreated cells ± S.D. Each data point is the mean of at least four individual measurements. *B*, EC_{50} values for compound DDD86243 against wild-type and transgenic *T. brucei* cell lines. The EC_{50} values for wild-type (●), TryS single knock-out (□), and TryS-overexpressing (○) *T. brucei* cell lines were determined using Equation 4. The *inset* shows TryS expression levels in each cell line; PTR-1 levels were determined for loading control purposes. Data are presented as mean ± S.D. (n = 3). EC_{50} values of 20.4 ± 0.4, 6.9 ± 0.2, and 44.5 ± 0.5 μM were determined for wild-type, single knock-out, and TryS-overexpressing cell lines, respectively.

two assay platforms validated BIOMOL Green as a suitable alternative for use in the TryS screening campaign.

A number of hit series were identified from the hit discovery campaign, with three in particular displaying excellent SAR. Chemistry exploration around these hit series resulted in the identification of compounds with potencies less than 100 nM. Several of these hit compounds were also shown to be TryS inhibitors using the orthogonal coupled assay platform, thus confirming the enzymatic target of the compound and further validating the use of the BIOMOL Green assay platform, with highly comparable potency values returned in both assays.

One potential challenge associated with targeting TryS is that the physiological concentrations of the Spd, ATP, and GSH substrates in *T. brucei* are very high (the intracellular concentrations of these substrates in the mammalian stage of the parasite are 2 mM Spd, 2 mM ATP, and 0.2 mM GSH). This could clearly present a problem if the inhibitors identified were found to act competitively; therefore, mode of inhibition studies were conducted. These studies revealed that representative compounds from the three hit series of interest were not competitive for any of the substrate binding sites and instead acted through an allosteric mechanism. This, of course, is hugely advantageous, since the high physiological concentrations of substrates should not mask the potent effects of these

TABLE 3

Intracellular thiols of *T. brucei* transgenic cell lines

Thiols were determined as described under "Experimental Procedures." Each value represents the mean of triplicate determinations.

Cell line	Thiols	
	GSH	Trypanothione
	nmol/10^8 cells	
Wild type	0.47 ± 0.05	0.24 ± 0.02
TryS SKO	0.37 ± 0.02[a]	0.16 ± 0.01[b]
TryS overexpressor	0.56 ± 0.06[c]	0.66 ± 0.05[d]

[a] $p < 0.05$, Student's t test.
[b] $p < 0.005$, Student's t test.
[c] Not significant, Student's t test.
[d] $p < 0.0001$, Student's t test.

TryS inhibitors in the trypanosome. Because the crystal structure of TryS from *L. major* has recently been solved (19), this provides an opportunity to model these hit compounds to determine their actual binding site and could provide important information to assist in the future development of more potent compounds.

DDD66604, which was used as a standard during the screening campaign, showed a different mode of inhibition, competing with Spd for binding. Although in the current study, compounds of this class were not advanced further, it is interesting to note that similar tricyclic compounds have long been known to bind to the equally essential trypanothione reductase enzyme (26). Indeed, DDD66604 was a hit originally identified in an in-house trypanothione reductase program. Although the competitive nature of this compound presents problems in terms of overcoming high intracellular Spd concentrations, it does present an exciting opportunity to utilize network pharmacology (27). Screening for potent compounds that inhibit two or more essential enzyme targets in the trypanosome could present an attractive and feasible alternative approach in the search for new drugs to treat HAT.

Despite the fact that potent, non-competitive inhibitors of recombinant TryS were identified, a large potency shift was observed when they were tested against *T. brucei* with cell EC_{50} values ~100-fold greater than enzyme IC_{50} values. This result was unexpected, because the physicochemical properties of these compounds indicate that they should be taken up into the trypanosome (*e.g.* these compounds have acceptable solubility, lipophilicity, and molecular weight, as predicted by StarDrop). To confirm these compounds were entering the cell and acting on target to inhibit TryS, intracellular thiol levels were measured in the presence and absence of inhibitor (DDD86243). These experiments showed a clear dose-dependent reduction in trypanothione levels with increasing inhibitor concentration. In addition, there was a corresponding increase in GSH levels, which may also be advantageous because GSH shows high substrate inhibition *in vitro*; therefore, this increase in intracellular GSH may potentially feedback to inhibit the TryS enzyme further. These data provide strong evidence that DDD86243 is acting on target to inhibit TryS activity, and this is further supported by data that show that TryS SKO and TryS-overexpressing cell lines display changes in potency to DDD86243 compared with wild type *T. brucei*.

Although we have shown that DDD86243 is acting on target, further explanations are required to account for the observed high EC_{50} values. After a 72-h exposure to twice the EC_{50} inhibitor concentration, trypanothione drops to <10% of its original level (Fig. 4*A*). Since this causes a 90% inhibition of cell growth (Fig. 4*B*), it can be estimated that treated cells have undergone approximately four cell divisions over 72 h in culture. In the absence of *de novo* synthesis or turnover of trypanothione, it can be calculated that trypanothione would drop to 6% of its original level. This is consistent with what is observed experimentally and implies that the parasites can survive with very low levels of trypanothione, as observed in conditional gene knock-out studies, where complete loss of viability was only seen after 6 days (16). In order to see improved cell efficacy, it may be that our standard 72-h cell-based assay must be extended to see the potent effects of these drugs. In addition, improving the potency of the lead compounds to ensure that trypanothione levels are reduced to concentrations low enough to be detrimental to the parasite should increase cell efficacy. The medicinal chemistry carried out to date has achieved significant improvements in potency with each round of chemistry exploration; thus, it is entirely feasible that further rounds of chemistry exploration can drive this potency down further. Finally, it should also be noted that the medium used to culture *T. brucei* is supplemented with many factors, including reducing agents (*e.g.* 1.5 mM cysteine), which may protect these parasites under low trypanothione conditions. It is therefore possible that these compounds will appear more efficacious in an *in vivo* model, which provides a better model of conditions in the human host.

In conclusion, this study has reported the discovery and characterization of novel *T. brucei* TryS inhibitors. Furthermore, these inhibitors have been shown to act on target in *T. brucei* to inhibit TryS, thus chemically validating TryS as a drug target.

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