CHEMICAL VALIDATION OF TRYPANOTHIONE SYNTHETASE: A POTENTIAL DRUG TARGET FOR HUMAN TRYPANOSOMIASIS
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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 x EC50 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 knockout and TryS over-expressing 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) is endemic in sub-Saharan Africa and is estimated to be responsible for more than 30,000 deaths per 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 which 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 underway 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 (N1,N8-bis (glutathionyl)spermidine) as their principal thiol, in contrast to most other organisms (including their mammalian hosts) which utilise 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 knockout and RNA interference (RNAi), thus highlighting the essentiality of the trypanothione pathway in T. brucei function.

In T. brucei trypanothione is synthesised from glutathione (GSH) and spermidine (Spd) by an ATP-dependent C-N ligase, trypanothione synthetase (TryS; EC 6.3.1.9), with N1- and N8-glutathionylspermidine as intermediates (12, 13). Despite a lack of chemical validation, TryS has been genetically validated as a drug target, with RNAi and gene knockout studies confirming
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 as 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 crystallised (19) providing an opportunity to co-crystallise 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 NADH (20). Although this cuvette based assay has been successfully used to characterise 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 characterisation 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 and purification of recombinant *Tb*TryS utilized the construct pET15b-*TbTRY* (12) however expression conditions were adapted to ensure elevated levels of protein sufficient for the HTS. 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 media, containing 50 µg ml⁻¹ carbenicillin and 20 µg ml⁻¹ gentamycin, with agitation (250 rpm). Transformed cells were centrifuged briefly (4000 x g, 10 min, 4°C) and used to inoculate 500 ml LB media and grown at 37°C until OD600 > 0.6. Cells were harvested by centrifugation (4000 x g, 30 min, 4°C) and subsequently used to equally inoculate 8 x 1L of autoinduction growth media (no antibiotic selection) to bulk up cell mass (30 °C, 250 rpm) until OD600 ≈ 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 MgCl₂, DNase, 1 mM tris(2-carboxyethyl)phosphine plus one complete protease inhibitor cocktail tablet (Roche) 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) then cleaved using thrombin to remove the hexa-histidine tag. The final purification step was achieved using a HiLoad Q Sepharose 16/10 column (GE Healthcare), 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 the BIOMOL Green reagent (BIOMOL International UK) which gives an absorbance read-out at 650 nm (21). Following optimisation 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 DTT, 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 ~ 4,000 chemical scaffolds. All library compounds were solubilised in 100% dimethyl sulphoxide (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 and 2 mM DTT, 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 Solution) 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 GSH and 50 µl BIOMOL Green respectively on a Thermo Scientific WellMate (Matrix). The reaction 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 (Perkin Elmer).

ActivityBase from IDBS was used for data processing and analysis. Compounds were designated hits if the A 650 was greater than three standard deviations 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 workstation) based upon structural similarity, chemical tractability and defined physicochemical properties. To generate IC₅₀ 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 workstation (Perkin Elmer). 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 IC₅₀ curve fitting was undertaken using XLFit version 4.2 from IDBS. A 4 Parameter Logistic dose response curve was utilised using XLFit 4.2 Model 205. All test compound curves had floating top and bottom and pre-fit was used for all 4 parameters.

To establish modes of inhibition of representative inhibitors, data sets were collected at 6 inhibitor concentrations with 6 varied concentrations of each substrate. Each data set was individually fitted to the Michaelis-Menten equation and the resulting Lineweaver-Burke plots 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 was fitted to equations for competitive high substrate inhibition (equation 1), uncompetitive high substrate inhibition (equation 2) and allosteric high substrate inhibition (equation 3), shown below:

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

$$v = \frac{V_{max}}{1 + \frac{K_m}{S} \left( 1 + \frac{i}{K_i} \right) \left( 1 + \frac{i}{K_i} \right)}$$ (Eq. 2)

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

Compound Synthesis - Detailed syntheses of the compounds used in this study (UK Patent application 815947.7) and the analytical methods used to confirm the molecular identity of these novel compounds can be found in the Supplementary Experimental Procedures.

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 in (20), which is a continuous spectrophotometric assay at 340 nm. These assays were run at 25°C in polystyrene cuvettes since it was found that certain inhibitors in DMSO bound to acrylic cuvettes. Each 1ml assay was prepared in 100 mM (K⁺) HEPPS, pH8 and contained 0.2 mM NADH, 1mM phosphoenolpyruvate, 5 mM DTT, 0.5 mM EDTA, 10 mM MgSO₄, 2 U ml⁻¹ L-lactate dehydrogenase, 2 U ml⁻¹ 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 trypanosome culture (10⁴ cells ml⁻¹) to each well, except for one column which received media only. MRC-5 cells were cultured in Dulbecco’s Modified Eagle’s Medium, seeded at 2,000 cells per well and allowed to adhere overnight. One microlitre of test compound (10 point dilutions to give final assay concentrations between 50 μM to 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% CO₂ for 69 h, prior to the addition of 20 μl resazurin (final concentration 50 μM). After a further 4 h incubation, fluorescence was measured (excitation 528 nm; emission 590 nm) using a BioTek FLX800 plate reader (23).

The EC₅₀ for test compounds used in thiol analysis were also determined using triplicate flasks of T. brucei containing 1 x 10⁵ bloodstream trypanosomes ml⁻¹ 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). EC₅₀ values were determined using the four-parameter IC₅₀ equation provided by GraFit. The data was background corrected. In this equation, s was the slope factor.

\[ y = \frac{\text{Range}}{1 + \left( \frac{x}{\text{IC}_{50}} \right)} + \text{Background} \]  

(Eq. 4)

EC₅₀ values were also determined in TryS single knockout (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⁻¹) while TryS overexpression cell lines were maintained in the presence of phleomycin (2.5 μg ml⁻¹) and tetracycline (1 μg ml⁻¹).

**Analysis of intracellular thiols** - Cultures containing 1 x 10⁵ bloodstream trypanosomes ml⁻¹ were incubated with varying concentrations of compound DDD86243 equivalent to 0.5, 1.0, 1.5 and 2x the previously determined EC₂₀. Following incubation for 72h, cells (1 x 10⁵) were collected by centrifugation (900 x 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⁻¹ BSA) and derivatised with monobromobimane, as described previously (24). Acid-soluble thiols were separated by ion-paired, reverse phase HPLC on an ion-paired Ultrasphere C₁₈ 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 over-expression equivalents) was performed as previously described (16).

**RESULTS**

**Assay Development and Kinetic Characterisation of TryS Activity** - A novel assay for measuring T. brucei TryS activity was developed using the BIOMOL Green reagent, which provides a colourimetric quantification of phosphate levels via measurement at A₆₅₀. Using an optimised 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. 1a).

Michaelis constants for each of the three TryS substrates (Spd, GSH and ATP) were determined (Fig. 1b-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 as this concentration gave a maximal signal (note that GSH displays high substrate inhibition with an apparent Kᵢ of 55 ± 6 µM). The substrate Kₘ 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-Kₘ concentrations (25 μM 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. 1e reveals that the assay is linear
for at least 90 min under these conditions. A 60-
min time point was selected for screening.

**Identification of TryS Inhibitors** - As 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. 2a)) returned an IC_{50} of ~19 µM (Fig. 2b) and was selected for use as a standard on the basis of its commercial availability.

TryS was subsequently screened against our in-house, diverse, lead-like library of 63,362 compounds (30µM). 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 S:B was 3.54 ± 0.23 and the mean %CV 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 standard deviations from the mean of DMSO-only controls). Based on this threshold, 776 putative TryS inhibitors were identified (Fig. 2c). These active compounds were progressed to a duplicate point retest with 725 found to be 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. Figure 2d 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 identity of these 174 TryS inhibitors were confirmed by LC-MS analysis with a pass rate >95% (correct MH’ and ≥90% purity). Compounds of particular interest were re-synthesised 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 progressed 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 tested returned highly equivalent potency values in the two platforms, with DDD60632 returning IC_{50} values of 383 ± 64 nM and 575 ± 64 nM in the BIOMOL Green and coupled assays, respectively. DDD73385 returned IC_{50} values of 326 ± 39 nM and 365 ± 23 nM and DDD85811 returned IC_{50} values of 181 ± 22 nM 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 towards 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-Burke plots 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 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. 3a) and uncompetitive inhibition with respect to ATP (Fig. 3b). The GSH data proved more challenging to analyse due to the high substrate inhibition displayed by GSH (Fig. 3c). This data set was therefore analysed using the high substrate inhibition equations described in the **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. 3d) and uncompetitive inhibition with respect to both ATP and GSH (Fig. 3e and 3f respectively). This data is 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 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 5-30 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) (Figure 4a). (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 over-expressing $T. brucei$ cell lines were generated (16). Thiol analysis confirmed that TryS function had been modulated such that SKO and over-expressing cell lines showed significantly higher and lower levels of trypanothione compared to 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 over-expressing cell lines, respectively (Figure 4b). 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 as 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 to the previously published substrate $K_m$ values (37.8 µM, 7.1 µM 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 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 TryS inhibitors in the trypanosome. As the crystal structure of TryS from *Leishmania 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 progressed 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 utilise 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 tested against *T. brucei* with cell EC<sub>50</sub> values ~100-fold greater than enzyme IC<sub>50</sub> values. This result was unexpected as the physicochemical properties of these compounds indicate 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 since 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 TryS SKO and TryS over-expressing 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<sub>50</sub> values. After 72 hours exposure to twice the EC<sub>50</sub> inhibitor concentration, trypanothione drops to <10% of its original level (Figure 4a). Since this causes a 90% inhibition of cell growth (Figure 4b) 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 knockout 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 hour 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 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 media 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 characterisation 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.
REFERENCES


Abbreviations: GSH, Glutathione; HAT, Human African Trypanosomiasis; Spd, spermidine; SKO, single knock out; TryS, trypanothione synthetase

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FIGURE LEGENDS

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 presence of 100 µM ATP and 100 µM GSH.  D, GSH $K_m$ determination in 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 ± standard deviation (n=4).

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 ± standard deviation (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 progressed to potency testing. Linear regression of these data returned a correlation coefficient of 0.987.

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 x IC$_{50}$ (●); 2 x IC$_{50}$ (○); 1 x IC$_{50}$ (■); 0.5 x IC$_{50}$ (□); 0.3 x IC$_{50}$ (▲); 0 x 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 is represented as a percentage of trypanothione (●) and GSH (○) levels of untreated cells ± standard deviation. 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 knockout (□) and TryS over-expressing (○) T. brucei cell lines were determined using Eq. 4. Inset shows TryS expression levels in each cell line, PTR-1 levels were determined for loading control purposes. Data are presented as mean ± standard deviation (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 knockout and TryS over-expressing cell lines, respectively.
### TABLE 1
Core structures of TryS hit series

<table>
<thead>
<tr>
<th>Hit Series</th>
<th>Core Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Structure 1" /></td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure 2" /></td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure 3" /></td>
</tr>
</tbody>
</table>

For hit series 3, \( n = 1, 2 \)
**TABLE 2**
Structure-activity relationships from hit series 1-3.
IC$_{50}$ and EC$_{50}$ values were determined as described in *Experimental Procedures*

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Hit Series</th>
<th>Structure</th>
<th>IC$_{50}$ TryS, µM</th>
<th>EC$_{50}$ T. brucei, µM</th>
<th>EC$_{50}$ MRC-5, µM (% Inhibition at 50 µM$^a$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDD60632</td>
<td>1</td>
<td><img src="image1" alt="Structure" /></td>
<td>0.273</td>
<td>27.1</td>
<td>&gt;50 (7.6%)</td>
</tr>
<tr>
<td>DDD73385</td>
<td>2</td>
<td><img src="image2" alt="Structure" /></td>
<td>0.317</td>
<td>21.2</td>
<td>&gt;50 (0%)</td>
</tr>
<tr>
<td>DDD85811</td>
<td>3</td>
<td><img src="image3" alt="Structure" /></td>
<td>0.095</td>
<td>8.8</td>
<td>&gt;50 (2.4%)</td>
</tr>
<tr>
<td>DDD86243</td>
<td>3</td>
<td><img src="image4" alt="Structure" /></td>
<td>0.140</td>
<td>5.1</td>
<td>&gt;50 (0%)</td>
</tr>
</tbody>
</table>

*a* Top concentration of cell-based assay limited to 50µM to keep assay within DMSO tolerance.
TABLE 3
Intracellular thiols of *T. brucei* transgenic cell lines.
Thiols were determined as described in *Experimental Procedures*. Each value represents the mean of triplicate determinations.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Thiols nmol ((10^8 \text{ cells})^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH</td>
</tr>
<tr>
<td>WT</td>
<td>0.47 ± 0.05</td>
</tr>
<tr>
<td>TryS -SKO</td>
<td>0.37 ± 0.02 (^a)</td>
</tr>
<tr>
<td>TryS-over-expressor</td>
<td>0.56 ± 0.06 (^b)</td>
</tr>
</tbody>
</table>

\(^a\) P < 0.05; \(^b\) not significant; \(^c\) P < 0.005; \(^d\) P < 0.0001 Students \(t\) test
Figure 2

A

B

C

D

\[ \text{DDD66604}, \mu \text{M} \]

\[ \% \text{Inhibition} \]

\[ \text{pIC}_{50} \text{ Replicate 1} \]

\[ \text{pIC}_{50} \text{ Replicate 2} \]
Figure 3

A, B, C, D, E, F: Diagrams showing the reciprocal of absorbance at 650 nm (1/A_{650}) plotted against the reciprocal of Spd concentration, ATP concentration, and GSH concentration, respectively. Each graph includes multiple data points represented by different symbols and lines, indicating various conditions or treatments.

- **A**: Graph showing 1/A_{650} vs. 1/[Spd] µM⁻¹.
- **B**: Graph showing 1/A_{650} vs. 1/[ATP] µM⁻¹.
- **C**: Graph showing 1/A_{650} vs. 1/[GSH] µM⁻¹.

The graphs illustrate concentration-response relationships for the indicated substances, with data points suggesting non-linear behavior and potential saturation effects.

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Figure 4

A

[DDD86243], fold EC$_{50}$

Thiol content, % control

B

[DDD86243], µM

Cell growth, %

TRYS
PTR1

WT SKO OE
Chemical validation of trypanothione synthetase: a potential drug target for human trypanosomiasis
Leah S. Torrie, Susan Wyllie, Daniel Spinks, Sandra L. Oza, Stephen Thompson, Justin R. Harrison, Ian H. Gilbert, Paul G. Wyatt, Alan H. Fairlamb and Julie A. Frearson

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