

High-throughput screening affords novel and selective trypanothione reductase inhibitors with anti-trypanosomal activity

Derek C. Martyn,^{a,†} Deuan C. Jones,^b Alan H. Fairlamb^b and Jon Clardy^{a,*}

^aDepartment of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA

^bDivision of Biological Chemistry and Molecular Biochemistry, University of Dundee, Dundee, UK

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Abstract—Trypanothione reductase (TR), an enzyme that buffers oxidative stress in trypanosomatid parasites, was screened against commercial libraries containing approximately 134,500 compounds. After secondary screening, four chemotypes were identified as screening positives with selectivity for TR over human glutathione reductase. Thirteen compounds from these four chemotypes were purchased, and their *in vitro* activity against TR and *Trypanosoma brucei* is described.

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Trypanosomatid protozoan parasites cause several devastating diseases in large parts of the developing world. The three most important are human African trypanosomiasis (sleeping sickness) caused by *Trypanosoma brucei*, Chagas disease caused by *Trypanosoma cruzi*, and leishmaniasis caused by members of the genus *Leishmania*. These three diseases are estimated to cause 113,000 deaths annually and more than four million disability adjusted life years—the healthy life years lost due to disability and death.¹ Currently available therapies are inadequate due to issues involving safety, efficacy, resistance, toxicity, ease of administration in poor conditions, and cost. Despite the urgent need for new drugs, these diseases have been neglected, largely due to market forces, by most pharmaceutical companies.

As part of a concerted campaign to discover new treatments organized by DNDi (Drugs for Neglected Diseases Initiative), we undertook a high-throughput screen for inhibitors of trypanothione reductase (TR).² Trypanosomatids use a unique biosynthetic pathway that conjugates glutathione and spermidine to form trypanothione (1, Fig. 1) to perform the same antioxidant role as glutathione (2) in mammalian cells.³ TR, the parasite analog of glutathione reductase (GR), plays an essential

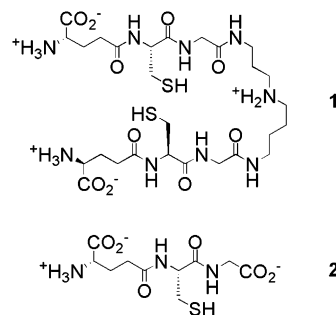


Figure 1. Structures of trypanothione (1) and glutathione (2).

role in maintaining the intracellular redox balance by returning trypanothione disulfide to its reduced state. Genetic knockouts have illustrated the importance of TR for parasite viability,⁴ and therefore as a target for drug development for all three diseases. Comparison of TR and human GR (hGR) crystal structures reveals significant differences between their active sites, and these differences are consistent with the selectivity shown by GR and TR for their respective substrates.⁵ TR possesses a more open active site with an overall negative charge to accommodate the positively charged spermidine, while the more compact GR active site has a positive charge to compensate the glycine carboxylate.⁶ These active site differences could be exploited in the development of TR-selective inhibitors.

The HTS program utilized an assay developed by Fairlamb et al.⁷ that measures TR activity by coupling the

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* Corresponding author. Tel.: +1 617 432 2845; fax: +1 617 738 3702; e-mail: jon_clardy@hms.harvard.edu

† Present address: Infectious Diseases Initiative, Broad Institute of Harvard University and MIT, Cambridge, MA 02141, USA.

formation of **1** to the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), regenerating trypanothione disulfide, and forming the yellow thionitrobenzoate ion (TNB), which absorbs strongly at 405 nm. Minor alterations to the assay procedure made it amenable to the 384-well format (see Ref. 8 for details). The primary screening of approximately 134,500 compounds from commercial libraries was performed. A Z factor of 0.71 was calculated for the entire data set. At the nominal compound concentration of 11.1 $\mu\text{g/mL}$ present in the assay, $\geq 90\%$ inhibition was used as an arbitrary cut-off point to identify 89 compounds (0.07% 'hit rate') from 24 chemotypes (based on a shared central scaffold) as having a level of activity sufficient for further analysis. The distribution of compound activities by percent inhibition is summarized in Figure 2.

Within the 24 chemotypes, we identified several classes of compounds, including the maleimides represented by **3** in Figure 3. These chemotypes all had an explicit enone functionality that could react with the free thiols present in the coupled assay (TNB, reduced **1**, or TR active site cysteines). We adapted the classical assay⁹ for measuring TR activity to the 384-well format (see Ref. 10 for details), resulting in the elimination of 12 chemotypes that were either inactive (i.e., reacted with TNB in the coupled assay) or weak inhibitors of TR. Maleimides, while active in the classical assay, were eliminated due to the high proportion of this chemotype displaying $>75\%$ inhibition during primary screening (65% of tested compounds). Additionally, succinimide-based screening positives were represented at a relatively high rate (20% of tested compounds gave $>75\%$ inhibition), and this chemotype was eliminated from further consideration due to concerns regarding selectivity.

ChemBank¹¹ was used to perform substructure similarity searches on the screening positives from the remaining 10 chemotypes (represented by the general structures **4–13** in Fig. 4) that displayed TR inhibition in the classical assay. Between 6 and 19 compounds from each

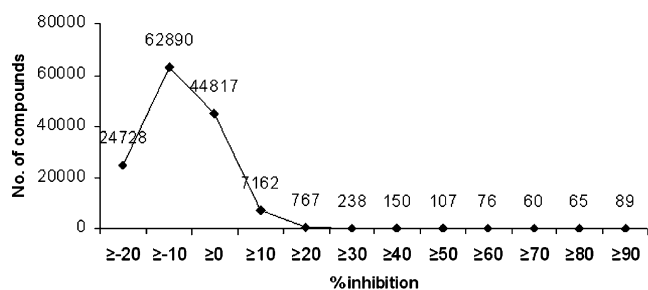


Figure 2. Distribution of compounds from primary screen by percent inhibition.

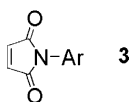


Figure 3. Representative structure of maleimide Michael acceptors.

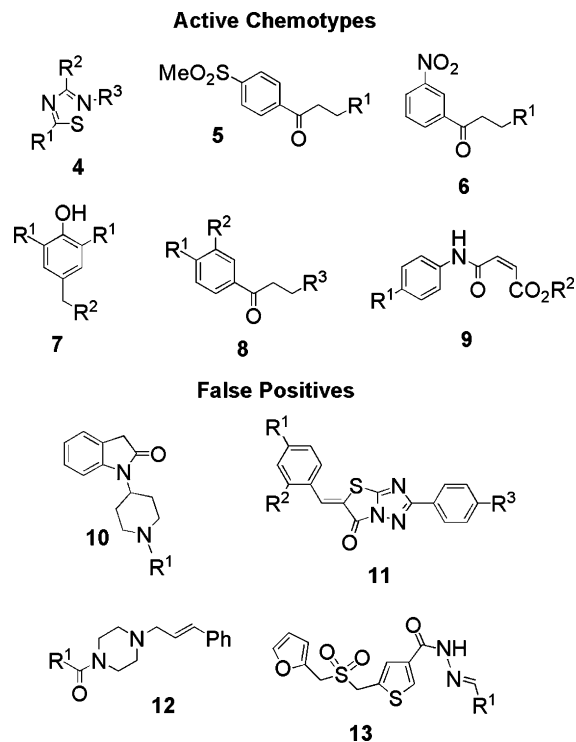


Figure 4. Representative structures **4–13** of cherry-picked chemotypes, split into active chemotypes and false positives.

chemotype were selected on the basis of structural similarity[†] and percent TR inhibition generated during primary screening, and then selected from the compound library stock plates for additional assays. These 115 compounds were tested in the DTNB-coupled assay in a concentration range of 1.91 ng/mL to 31.2 $\mu\text{g/mL}$, and IC_{50} values were determined.

Data analysis revealed that chemotypes **10–13**, which had few representatives, were false positives, and were therefore eliminated from future consideration (data not shown). The remaining six chemotypes had selected examples reselected, which were tested for activity against hGR. Compound classes **4** and **9** did not display any selectivity for TR over hGR, and were not investigated further (data not shown).

Solid samples of selected compounds from the remaining chemotypes (**5–8**), which displayed selective inhibition of TR over hGR, were purchased to confirm their activity (Table 1). Purity was determined by LCMS and ^1H NMR, and by these methods all compounds were $\geq 95\%$ pure. In general, the nitrobenzenes **6a–e** gave the greatest selectivity for TR over hGR, and they afforded the most potent inhibitor tested (**6a**, $\text{IC}_{50} = 0.34 \mu\text{M}$). Replacing the dimethylamino group of **6a** with an arylamino group lowered activity, although **6b–e** still gave an $\text{IC}_{50} < 10 \mu\text{M}$. The trisubstituted phenols **7a** and **7b** displayed moderate selectivity for TR, and activity decreased with the incorporation

[†] The screening positive itself gives a similarity score of 1.0, and only compounds with a score ≥ 0.7 were considered.

Table 1. Assay results for chemotypes **5–8**

| Compound ^a | Structure ^b | | | TR IC ₅₀ (μM) ^c | GR IC ₅₀ (μM) ^c | Selectivity for TR ^e | <i>T. brucei</i> EC ₅₀ (μM) ^f |
|-----------------------|---|---|--|--|--|------------------------------------|--|
| | R ¹ | R ² | R ³ | | | | |
| 5a | NMe ₂ ·HCl | — | — | 2.9(±1.4) | 20.4(±6.3) | 7.0 | 0.55(±0.04) |
| 5b | <i>N</i> -piperidyl | — | — | 7.8(±3.7) | 105(±36) | 13 | 0.95(±0.09) |
| 6a | NMe ₂ ·HCl | — | — | 0.34(±0.13) | 20.3(±8.4) | 59 | 0.68(±0.10) |
| 6b | NH-4-(CO ₂ - <i>n</i> -Bu)Ph | — | — | 2.4(±0.92) | >135 ^d | >56 | 0.96(±0.11) |
| 6c | NHPh | — | — | 1.7(±0.78) | 58.0(±16) | 35 | 1.01(±0.07) |
| 6d | NH-2,3-diMePh | — | — | 1.5(±0.71) | 86.3(±41) | 58 | 5.87, 0.68, 0.15 ^g |
| 6e | NH-3-FPh | — | — | 3.3(±1.1) | >173 | >52 | 0.82(±0.16) |
| 7a | ^t Bu | N((CH ₂) ₂ OH)·HCl | — | 2.0(±0.21) | 24.9(±6.0) | 13 | 3.01(±0.30) |
| 7b | ⁱ Pr | NMe ₂ | — | 11.8(±3.2) | 79.3(±25) | 6.7 | >50 |
| 7c | ^t Bu | <i>N</i> -homopiperidyl | — | 38.8(±5.0) | >155 | >4 | 3.30(±0.30) |
| 8a | Cl | Cl | R ³ = NMe ₂ ·HCl | 3.3(±2.2) | 64.8(±18) | 20 | 1.00(±0.21) |
| 8b | Cl | H | R ³ = <i>N</i> -morpholinyl | 148(±31) | >212 | >1.4 | 0.98(±0.07) |
| 8c | Cl | Cl | R ³ = NH-4-EtPh | 107(±0.71) | >157 | >1.5 | 1.65(±0.18) |

^a All compounds were purchased from ChemBridge, except for **5a** and **5b** (Peakdale).^b Dash signifies that this chemotype did not possess variable groups at this position.^c Values are means of two experiments, standard deviation is given in parentheses.^d IC₅₀ value greater than the highest tested inhibitor concentration.^e GR IC₅₀/TR IC₅₀.^f Values are weighted means of three independent experiments, standard errors are given in parentheses. IC₅₀ values for pentamidine are 4.6(±0.2) nM.^g Compound **6d** gave highly variable EC₅₀ results, all three are listed.

of an *N*-homopiperidyl group into **7c**. Replacement of the dimethylamino moiety possessed by the phenylketone **8a** with a bulkier group led to a substantial loss of activity. For all four chemotypes, the most potent compound in each class was the hydrochloride salt. Three of these compounds possess the dimethylamino functionality found in previously described TR inhibitors, such as clomipramine.²

All compounds in Table 1 were then tested for their ability to restrict *T. brucei* proliferation using an in vitro assay that determines a compound's activity via the conversion of a redox-sensitive dye to a fluorescent product by viable cells.^{12,13} Six compounds (**5a–b**, **6a–b**, and **6e**, **8b**) gave submicromolar EC₅₀ values, and with the exception of **6a**, these values were moderately lower than their IC₅₀ against TR. Compounds **7c** and **8b–c** were noticeably more active against the parasite than TR, perhaps indicating some off-target effects. The presence of a dimethylamino group at R² of **7b**, and conversion of the R¹ group from *tert*-butyl to *iso*-propyl, resulted in a substantial loss of activity compared to **7a** and **7c**.

It should be noted that all of the compounds that emerged from this set of screens have the potential to eliminate an amine to generate a compound that could undergo a conjugate addition. In the case of **5**, **6**, and **8**, an α,β-unsaturated ketone could be formed, and in the case of **7**, a *para*-quinone methide could be formed.

Conversely, amino groups could be selected because they mimic the positively charged central amine in trypanothione's spermidine moiety, increasing an inhibitors' affinity for the negatively charged binding site of TR. In any case, the compounds all show selectivity for TR over hGR and activity in a whole organism assay. A ChemBank search for compounds that possessed the 3-(dimethylamino)-1-arylpropan-1-one motif shared by **5a**, **6a**, and **8a** found ten compounds, of which five showed negligible (<15%) TR inhibition.

In conclusion, we have performed a HTS program against TR to identify novel inhibitors. Secondary screening revealed four chemotypes that displayed selectivity for TR over hGR, and selected representatives all displayed activity against *T. brucei* in a whole organism assay. Focused library synthesis of some of these compound classes will be undertaken.

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8. An assay solution containing potassium HEPES (40 mM, pH 7.4), EDTA (1 mM), trypanothione disulfide (4.22 μ M), DTNB (70.3 μ M), and TR (2.81 mU/mL) was prepared. The positive control contained the above reagents plus the TR inhibitor clomipramine hydrochloride (200 μ M). Eighty microliter per well of assay solution or positive control is added to the appropriate columns of clear Nunc 384-well Polysorb polystyrene plates using a BioTek μ Fill liquid handler. A CyBio liquid handler was used to transfer 200 nL of library compound, dissolved in DMSO at a concentration of 5 mg/mL, to each well. Enzymatic activity is initiated by the addition of 10 μ L of an NADPH solution (135 μ M in potassium HEPES, 40 mM, pH 7.4, and EDTA, 1 mM). The NADPH solution is added to four plates at a time, with 4 min 20 s delay between each set of plates. Plates are incubated for 60 min at 30 °C, before being removed and read in batches of four on a Perkin-Elmer Envision plate reader at 405 nm. Raw data are processed using Microsoft Excel.
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10. An assay solution containing potassium HEPES (40 mM, pH 7.4), EDTA (1 mM), NADPH (130 μ M), and TR (10 mU/mL) was prepared. The positive control does not use enzyme. Twenty microliter per well of reagent A or positive control is added to the appropriate columns of clear Nunc 384-well Polysorb polystyrene plates. One-hundred nanoliter of library compound, dissolved in DMSO at a concentration of 5 mg/mL, was added to each well. Enzymatic activity is initiated by the addition of 20 μ L of a trypanothione disulfide solution (110 μ M in potassium HEPES, 40 mM, pH 7.4, and EDTA, 1 mM). The trypanothione disulfide is added to four plates at a time, with 4 min 20 s delay between each set of plates. Incubation, plate reading, and data analysis are performed as in Ref. 9, except that the plates are read at 355 nm.
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13. EC₅₀ determinations were performed as in Ref. 12 with the following modifications. Test compounds (10 mM in DMSO) were serially diluted in HMI9 culture medium in 96-well plates (Greiner). Bloodstream *T. brucei* (S427) were added to give a final concentration of 10³ ml⁻¹ cells in 0.5% DMSO with inhibitor ranging from 50 to 0.2 μ M in a total volume of 200 μ L. Wells containing media only and media plus cells were used as controls. Pentamidine (250–1 nM) was included as a standard drug control on each test plate. Plates were incubated at 37 °C in a 5% CO₂ humidified atmosphere for 72 h, then 45 μ M resazurin (Sigma) added. After a further 4-h incubation, fluorescence due to formation of resorufin was measured at λ excitation 528 nm, λ emission 590 nm. Each inhibitor set was tested in triplicate and the experiments repeated on three separate occasions. Data are presented as weighted means and standard errors of the mean.