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Discovery of 2-iminobenzimidazoles as a new class of trypanothione reductase inhibitor by high-throughput screening

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Abstract—A high-throughput screening campaign of a library of 100,000 lead-like compounds identified 2-iminobenzimidazoles as a novel class of trypanothione reductase inhibitors. These 2-iminobenzimidazoles display potent trypanocidal activity against *Trypanosoma brucei rhodesiense*, do not inhibit closely related human glutathione reductase and have low cytotoxicity against mammalian cells.

Parasitic protozoa of the family Trypanosomatidae are the causative agent of many significant tropical diseases including African trypanosomiasis, Chagas disease and Leishmaniasis. In the 2004 world health report[1] African trypanosomiasis was reported to cause 48 thousand deaths and a disease burden of 1525 thousand DALYs (disability adjusted life years) annually, Chagas disease 14 thousand deaths and a disease burden of 667 thousand DALYs and Leishmaniasis 51 thousand deaths and a disease burden of 2090 DALYs. There are currently nine key drugs in use for the treatment of these disease states (Fig. 1): suramin and pentamidine against early stage African trypanosomiasis, and eflornithine and melarsoprol against late stage disease; nifurtimox and benznidazole against early stage Chagas disease; meglumine antimoniate and sodium stibogluconate against Leishmaniasis, and amphotericin B against antimony-resistant strains. All of these drugs have severe limitations including administration difficulties, long treatment regimes, life-threatening side effects, varied parasitological cure rates for different strains, lack of efficacy against late stage diseases, and increasing incidence of drug resistance.[2–5]

The intracellular reducing environment of trypanosomatids is maintained by a unique thiol redox system where the glutathione/glutathione reductase (GR) couple found in mammalian cells is replaced by the (bis-glutathionyl)spermidine trypanothione/trypanothione reductase (TR) couple.[6] TR is a key enzyme of the parasite antioxidant defence,[7,8] does not occur in the mammalian host and has been found to be essential for all trypanosomatids currently studied.[9,10]

TR and human GR have similar catalytic mechanisms with 14 of the 19 amino acid residues close to the substrate binding site being conserved. However, they are specific to their respective disulfide substrates (Fig. 2).[11] GR has a hydrophilic, positively charged region in its active site that interacts with the glycine carboxylates of glutathione disulfide, while TR has a larger binding site with a negatively charged region with which the spermidine moiety of trypanothione disulfide binds.[12] The absence of TR from the mammalian host and the sensitivity of trypanosomatids to oxidative stress makes TR an attractive target for trypanosomiasis therapeutics.[13] The objective of this work, therefore,

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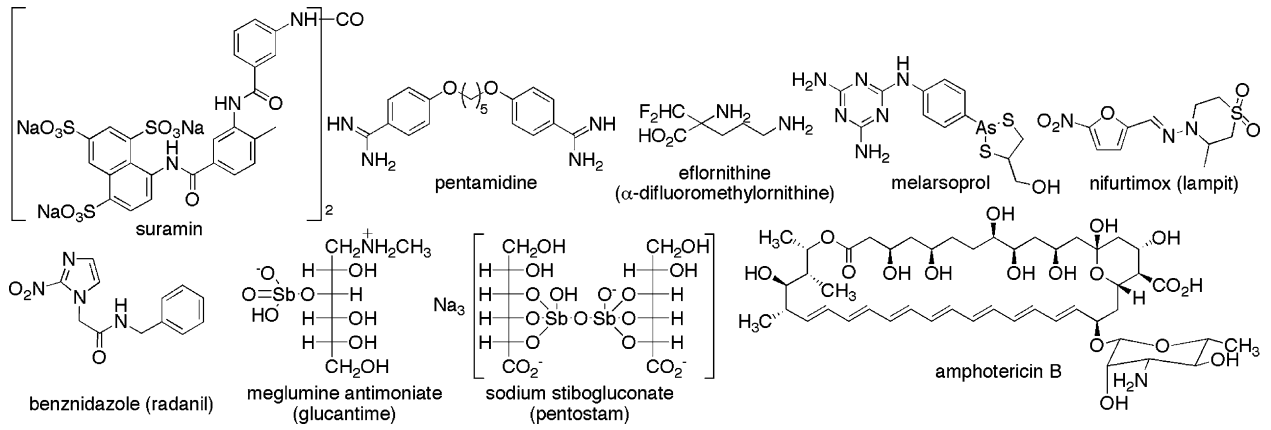


Figure 1. Current trypanosomiasis drugs on the market.

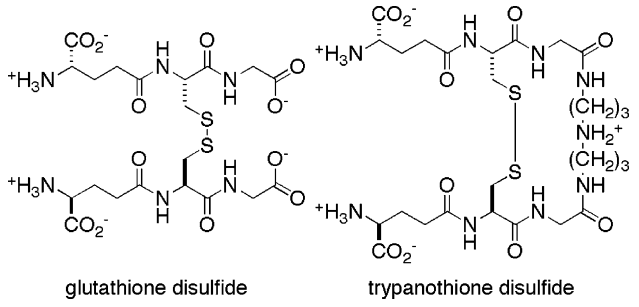


Figure 2. Trypanothione disulfide and glutathione disulfide.

was to identify novel classes of TR inhibitors by high-throughput screening (HTS) of a diverse chemical library.

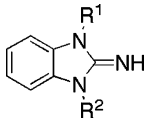
Our chemical compound library is a collection of approximately 100,000 compounds purchased from commercial vendors. The compounds in this chemical library were selected to provide 'lead-like' chemical structures with a guiding philosophy that most successful drug development projects have started from leads that are smaller and more polar than the drug itself. 'Lead-like' chemical structures were defined as having simple molecular structures that are chemically unreactive, synthetically accessible and have 'drug-like' properties. The 100,000 compounds in our 'lead discovery library' represent a diverse set of molecules as judged by Tanimoto dissimilarity analysis ($T \leqslant 0.85$), and although simple filters based on the Lipinski criteria were not used in the selection process, 89% of the compounds in the library are Lipinski compliant[14] and 81% conform with Oprea's criteria for 'lead-likeness'.[15]

An automated screening protocol for TR was developed using the photometric assay described by Hamilton et al.[16,17] The assay was adapted for operation in 384-well microtitre plates and was found to be exceptionally robust and reproducible. The average Z' and Z values[18] achieved over the entire primary screen were 0.72 and 0.54, respectively. The primary screen of 100,000 compounds identified 120 compounds that inhibited TR activity by more than 50% at a concentration of 25 μM. The potencies of the hits were confirmed by assaying compounds as 11 point titrations. In summary, the hit set contained compounds from 13 distinct structural classes, and the IC_{50} values of the hits ranged from 1 to 67 μM. A focus set of 43 compounds was selected from the population of primary screen hits based on inhibitory potency, synthetic accessibility and compound novelty. These compounds were re-ordered from the original chemical vendors and the TR inhibitory potency, structural identity and purity of the re-supplied material confirmed. The focus set contained compounds from nine distinct structural classes and the 2-iminobenzimidazoles were prominent having four close analogues in the focus set. The development of this 2-iminobenzimidazole structural class will be discussed in this communication.

The 2-iminobenzimidazoles are a novel class TR inhibitors that are chemically suitable for optimization and scored well in a drug-likeness analysis. A search of the patent literature revealed few 2-iminobenzimidazoles, none of which were reported to have anti-trypanosomal activity. A number of other 2-iminobenzimidazoles were contained within the lead discovery library; eight were selected and their potency determined to investigate structure–activity relationships (SAR) (Table 1).

Defined SAR were observed with the highest inhibitory activity obtained when one side chain contained a basic moiety (R^2) and the other (R^1) an aromatic ring (**3**, **5**, **7**). Replacement of the basic group in the R^2 side chain with a hydrophobic group (**1**, **2**, **4**), or removal of the phenyl ring in the R^1 side chain (**6**, **8**) resulted in a significant loss of inhibitory activity. None of the 2-iminobenzimidazoles in the patent literature possessed the general structure **I** (Table 2). A search of chemical vendors revealed 26 analogues with the general structure **I** that were purchased and tested, revealing further SAR (selected compounds: Table 2). The most potent compounds ($IC_{50} \leqslant 10$ μM) possessed a piperidine (**9**, **11**, **14**, **16**, **20**) or diethylamine (**13**, **15**, **7**, **22**) basic amine moiety. The inhibitory activity was significantly reduced by extension of the R^2 side chain (**18**) and completely lost when the basic amine group was a morpholine moiety (**10**, **12**). A variety of alcohol substituents (**9**, **11**, **15**,

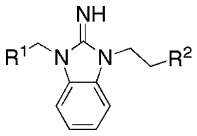
Table 1. Inhibition of TR by selected 2-iminobenzimidazoles in the lead discovery library



Compound	R^1	R^2	% inhibition[a]	IC_{50}[b] (μM)
1	4-BrPhCH(OH)CH_2	$CH_2CH_2CH_2CH_3$	42	>100
2	4-ClPhCH(OH)CH_2	CH_2Ph	32	>100
3	3,4-DiClPhCH(OH)CH_2	$CH_2CH_2NMe_2$	92	10
4	4-(EtO)PhCH(OH)CH_2	$CH_2CH{=}CH_2$	6	>100
5	4-(MeO)PhCH(OH)CH_2	CH_2CH_2Npiperidine	85	24
6	$CH_2{=}CHCH_2$	$CH_2CH_2NEt_2$	31	>100
7	4-MePhC(O)CH_2	$CH_2CH_2NEt_2$	91	7
8	$Me_3CC(OH)CH_2$	CH_2CH_2Npiperidine	66	>100

[a] At 25μM.
[b] Number of assay replicates performed = 3.

Table 2. Inhibition of TR by selected commercially available 2-iminobenzimidazoles



(I)
R^1 = aryl containing group
R^2 = basic amine group

Compound	R^1	R^2	IC_{50}[a] (μM)
9	$PhOCH_2CH(OH)$	Npiperidine	5
10	$PhOCH_2CH(OH)$	Nmorpholine	>100
11	4-$MePhOCH_2CH(OH)$	Npiperidine	9
12	4-$MePhOCH_2CH(OH)$	Nmorpholine	>100
13	$PhOCH_2$	NEt_2	8
14	$PhOCH_2$	Npiperidine	4
15	3,4-DiClPhCH(OH)	NEt_2	9
16	3,4-DiClPhCH(OH)	Npiperidine	4
17	4-MePhCH(OH)	NMe_2	29
18	4-MePhCH(OH)	CH_2NMe_2	35
7	4-MePhC(O)	NEt_2	7
19	4-PhPhC(O)	NEt_2	25
20	2,4-di$ClPhOCH_2CH(OH)$	Npiperidine	5
21	4-(MeO)$PhOCH_2CH(OH)$	Npiperidine	16
5	4-(MeO)PhCH(OH)	Npiperidine	24
22	3,4-DiClPhC(O)	NEt_2	10
23	4-MePhCH(OH)	NEt_2	19

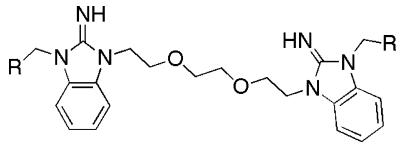
[a] Number of assay replicates performed = 3.

16, **20**), carbonyl groups (**7**, **22**) or ether linkages (**9**, **11**, **13**, **14**, **20**) were accommodated in the R^1 side chain without significant loss of inhibitory activity. Furthermore, variation of the number of methylene groups (n = 2–4) linking the endocyclic benzimidazole nitrogen atom and the R^1 aryl group also did not result in a significant loss of inhibitory activity (e.g., **7** **15**, **16** and **22** (n = 2) cf **13** and **14** (n = 3) cf. **9**, **11** and **20** (n = 4)). With n = 2, the 3,4-dichloro aryl group was tolerated regardless of linker composition (**15**, **16**, **22**). The 4-methoxyphenyl substituent was distinctly less favoured (**5**). A 4-methyl group was well tolerated in **7** where the linker contained a carbonyl but less so in **23** where the linker contained an alcohol. Replacement of the 4-methyl group (**7**) with a 4-phenyl group (**19**) resulted in a significant drop in inhibitory activity. The results obtained on this limited number of analogues indicate that the TR binding site will tolerate a relatively wide range of different linker structures and substitution patterns on the R^1 aromatic group. This is consistent with previous studies in which inhibitors have been reported to have multiple binding modes.[19–21]

The 2-iminobenzimidazole dimer, **25**, is one of the most potent compounds identified from the screen (Table 3). It does not fit the general pharmacophore pattern of one basic amine and one bulky hydrophobic side chain, but it is possible that one of the 2-iminobenzimidazoles is acting as the basic amine moiety. This hypothesis is supported by the observation that when the benzyl side chain was replaced with an ethyl group (**24**), there is a significant loss of inhibitory activity.

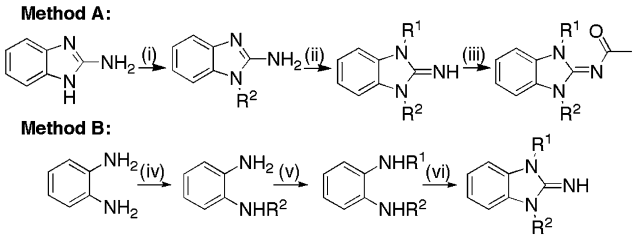
Selected analogues were synthesized to further explore structure–inhibitory activity relationships in the

Table 3. Inhibition of TR by commercially available dimer 2-iminobenzimidazoles



Compound	R	IC_{50}[a] (μM)
24	Me	>100
25	Ph	4

[a] Number of assay replicates performed = 3.



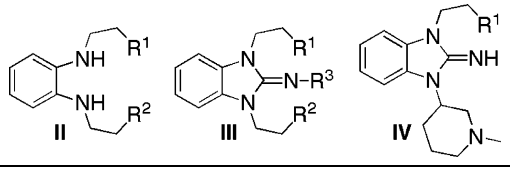
Scheme 1. Synthesis of 2-iminobenzimidazoles. Yields displayed are for the synthesis of **27**, **29** via Method A and **30** via Method B. (i) $R^2Cl{\cdot}HCl$, KOH, EtOH, Δ, 16 h (42%); (ii) R^1Br, 2-butanone, Δ, 48 h (64%); (iii) AcCl, *N*,*N*-diisopropylamine, DMAP, CH_3CN, 60 °C, 6 h (59%); (iv) R^2Br, MeOH, Δ, 24 h (77%); (v) R^1Br, NaI, DMF, Δ, 2 h or R^1OTs, Na_2CO_3, 120 °C, 24 h; (vi) CNBr, EtOH, rt, 48 h (74%).

2-iminobenzimidazole series that were not accessible through the commercially available analogues.[22]

Two methods were utilized to synthesize the desired 2-iminobenzimidazole analogues. In Method A 2-aminobenzimidazole was substituted with a alkylamine hydrochloride[23] and then the monosubstituted product was reacted with a series of phenoxyalkylbromides[24] to give the desired 2-iminobenzimidazoles (Scheme 1, Method A). Despite the good stability of the compounds once isolated, a number of challenges were encountered during the purification of the 2-iminobenzimidazoles and their intermediates. In addition, the second substitution in Method A was slow and accompanied by significant decomposition. Therefore, an alternative method was explored to synthesize further 2-iminobenzimidazole analogues (Scheme 1, Method B). 1,2-Phenylenediamine was sequentially substituted[25–27] and then the ring closed upon treatment with cyanogen bromide.[28] Finally, a 2-acyliminobenzimidazole analogue was synthesized by treatment of the 2-iminobenzimidazole with acetyl chloride in the presence of four equivalents of *N*,*N*-diisopropylethylamine and catalytic 4-dimethylaminopyridine in acetonitrile at 60 °C for 6 h (Scheme 1, Method A).

The compounds in Table 4 both confirm earlier observations and reveal more information about the SAR within this chemical series. First, the general structure **I** was confirmed as the essential pharmacophore since compound **30**, which contains two basic amine side chains, also has poor inhibitory activity (Table 4). Second, in agreement with earlier observations, the results obtained with **14**, **28** and **29** indicate that the enzyme binding site will tolerate a range of R^1 linker lengths. Third, the loss of inhibitory activity of the acyclic compound **26** suggests that the 2-iminobenzimidazole moiety is an important part of the pharmacophore and does not merely serve as a scaffold to appropriately present the R^1 and R^2 side chains for binding. Finally, we postulate that the loss of inhibitory activity of the acylimino analogue, **27**, is likely to be due to the reduced basicity of its 2-imino group, and that the guanidyl moiety of inhibitory 2-iminobenzimidazoles must be protonated for high affinity binding.[29] This hypothesis is supported by the observation that **3**, **16**, and **25** are competitive inhibitors of trypanothione binding[30] (Table 5) and that the trypanothione binding site has a negatively charged region which binds the spermidine moiety of trypanothione disulfide.[12]

Table 4. Inhibition of TR by 2-iminobenzimidazole analogues



Compound	Gen Struct	R^1	R^2	R^3	IC_{50}[c] (μM)
14[a]	III	PhO	Npiperidine	H	4[d]
26[b]	II	PhO	Npiperidine	—	>100
27[a]	III	PhO	Npiperidine	Ac	>100
28[a]	III	$PhOCH_2$	Npiperidine	H	7
29[a]	III	$PhOCH_2CH_2CH_2$	Npiperidine	H	8
30[b]	III	NEt_2	NEt_2	H	59
31[b]	IV	PhO	—	—	>100

[a] Synthesized using Method A.
[b] Synthesized using Method B.
[c] Number of assay replicates performed = 2.
[d] IC_{50} of batch synthesized in-house.

Table 5. Biological activity of selected 2-iminobenzimidazoles

Compound	TR enzyme K_i for competitive binding (μM)	*T. brucei rhodesiense* IC_{50} (μM)	Cytotoxicity IC_{50} (μM)	Human GR enzyme IC_{50} (μM)
3	5.4	0.6	46	>100
16	3.6	0.2	9	>100
25	1.9	1.3	144	>100

The biological inhibitory activity of a small selection of 2-iminobenzimidazoles (**3**, **16**, **25**) was further explored in whole parasite and cytotoxicity assays (Table 5).[31] The 2-iminobenzimidazoles tested displayed potent trypanocidal activity against *Trypanosoma brucei rhodesiense* (STB 900) and relatively low cytotoxicity against a human bladder carcinoma cell line (HT-29). It is possible that the cytotoxicity observed, particularly with compound **16**, may be due to inhibition of human GR. However this is unlikely due to their lack of activity in an in vitro GR assay (Table 5).[32] Considering the low micromolar K_i values obtained for these compounds against the TR enzyme, the trypanocidal activity particularly for **3** and **16** is unexpectedly potent. This could be due to the active uptake of these compounds by the parasite which is known to occur via a variety of mechanisms for other basic trypanocidal compounds.[33,34]

In summary, the application of high-throughput screening of a lead discovery library of 100,000 compounds identified nine novel chemical classes of TR inhibitors. In particular the 2-iminobenzimidazole class was found to have good development potential. The essential pharmacophore for TR inhibitory activity was identified by investigation of a series of analogues and further biological testing revealed that members of this new class of TR inhibitor have potent trypanocidal activity against *T. brucei rhodesiense*, and low cytotoxicity against human cells. This chemical series has significant potential for further development as a new class of therapeutics for trypanosome-mediated diseases.

Acknowledgments

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