MedChemComm

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

CONCISE ARTICLE

Synthesis and Evaluation of Phenoxymethylbenzamide Analogues as Anti-Trypanosomal Agents

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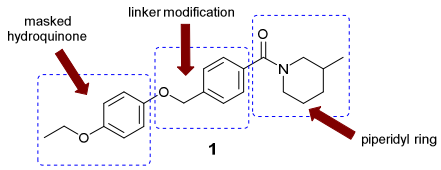
Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

The synthesis and anti-trypanosomal activity of a compound library based on a phenoxymethylbenzamide hit discovered in a high throughput screen is described. Several of the analogues exhibited potent activity against *Trypanosoma brucei rhodesiense,* a human infective strain of the trypanosome parasite, that serve as lead compounds for further optimisation.

Human African trypanosomiasis (HAT), also known as African sleeping sickness, is a neglected tropical disease that largely affects the population of sub-Saharan Africa. Currently the World Health Organisation (WHO) estimates that there are approximately 20,000 cases of HAT, with a further 70 million people predicted to be at risk of infection.[1, 2] HAT is caused by infection with one of two subspecies of parasitic trypanosomatid protozoa of the *Trypanosoma brucei* family, *Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense.* They are transmitted to humans through the bite of a tsetse fly (*Glossina* genus). There are two stages of infection. Stage 1 involves the invasion of the haemolymphatic system by the parasite and is often accompanied by bouts of fever, headaches and joint pain.[3] Stage 2 occurs once protozoa in the blood stream cross the blood brain barrier (BBB) and enter the central nervous system (CNS) and the cerebrospinal fluid (CSF).[2] This latter stage of infection is more aggressive, resulting in higher levels of patient presentation due to the onset of identifiable neurological symptoms,[4] and are usually fatal if left untreated.[5-7]

Despite the decline in the number of cases of HAT in recent years,[1] the widespread nature of latent infections,[8, 9] together with diagnoses often only in later stages of infection, means that the potential for a HAT epidemic is still present.[10, 11] Currently, the only drugs available for second stage treatment are melarsoprol, eflornithine and nifurtimox. All three drugs are associated with severe side effects and melarsoprol and eflornithine must be delivered intravenously.[1, 6, 10, 12-14] Eflornithine alone or in combination with nifurtimox is the WHO-preferred HAT treatment, however this regime is not effective against *T.b. rhodesiense*[15-17] and is also not suitable for use in individuals co-infected with HIV as it requires a regularly functioning immune system.[18, 19] Consequently there is a significant need for the development of new drugs for the more effective treatment of HAT.

High throughput screening (HTS) of compound libraries against whole cell *T. brucei* has recently gained momentum as a rapid and cost effective mechanism for elucidating new inhibitors of the protozoa that may provide new HAT drug leads.[20, 21] Avery and co-workers recently reported a whole organism HTS of 87,926 compounds against *T.b. brucei*, the non-human transmissible *T. brucei* subspecies.[22] This screen led to a number of novel "hit" compounds that exhibited low micromolar inhibitory activity against *T.b. brucei* and limited mammalian cell toxicity, including phenoxymethylbenzamide hit compound **1** (Figure 1).[23, 24] This compound possessed low micromolar inhibition of *T.b. brucei* and a calculated polar surface area indicative of a molecule that may be able to cross the BBB, an important consideration for the treatment of *T. brucei* infections that have progressed into the CNS (Figure 1).[25] Herein, we describe the first detailed examination of the structure-activity relationships for **1**, to allow for optimization of anti-trypanosomal activity.



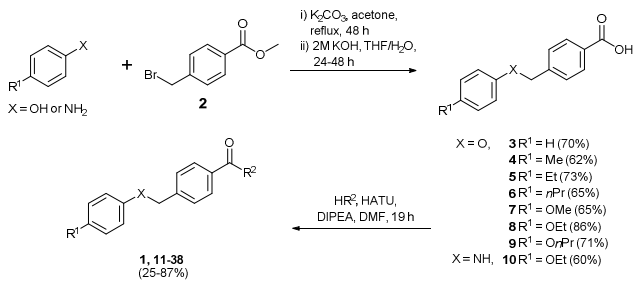
IC_{50} (*T.b. brucei*) = 1.15 μM
Molecular weight = 353 gmol^{-1},
Calculated aqueous solubility = 25 mM,
Polar surface area = 39 Å^2, **cLogP** = 4.8

Fig. 1 Activity and physicochemical parameters of the phenoxymethylbenzamide lead **1** revealing regions identified for SAR manipulation.

At the outset, investigation of the SAR of **1** was proposed by division of the structure into three key regions: the masked hydroquinone moiety, the central aromatic core and the carboxamide piperidyl ring (Figure 1). Initial studies then centred on modification of the two outer regions, namely the masked hydroquinone and the 3-methylpiperidyl moiety.

Synthesis of a 50 analogue compound library began from methyl (4-bromomethyl)benzoate **2**,[26] which was reacted with a range of 4-substituted phenols, or 4-ethoxyaniline, which after base hydrolysis afforded free acids **3-10** in good yields (see Scheme 1 for the general synthesis of a selection of the analogue library). Reaction of **3-10** with a range of amines, using HATU as the coupling reagent and *N,N*-diisopropylethylamine as the base

in DMF, then generated the benzamide library members in moderate to good yields.



Scheme 1 Synthesis of phenoxymethylbenzamide **1** and analogues **11-38**

Table 1 Inhibitory activity of **1**, **8** and **11-38** against *T. b. brucei* together with selectivity indices (SI)

compound	R^1	X	R^2	*T.b. brucei* IC_{50} [μM]	Sel. Index (SI) [a]
8	OEt	O		>50	-
1	OEt	O		1.15	36
11	OMe	O		4.60	18
12	*n*Pr	O		>50	-
13	OEt	NH		3.13	27
14	Me	O		9.20	9
15	*n*Pr	O		10.0	8
16	OMe	O		3.75	22
17	OEt	O		0.49	84
18	OEt	NH		1.93	43
19	Me	O		>50	-
20	Et	O		>50	-
21	OMe	O		>50	-
22	OEt	O		>50	-
23	OEt	O		2.70	31
24	OEt	NH		2.90	29
25	Et	O		14.8	6
26	*n*Pr	O		4.17	20
27	OEt	O		4.20	20
28	OEt	NH		20.6	4
29	Et	O		>50	-
30	OMe	O		>50	-
31	OEt	O		>50	-
32	Et	O		4.84	17
33	OMe	O		18.0	5
34	OEt	O		2.88	29
35	OEt	NH		4.64	18
36	OEt	O		>50	-
37	OEt	O		1.73	48
38	OEt	NH		3.51	24

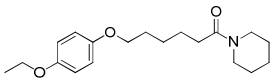
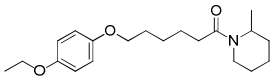
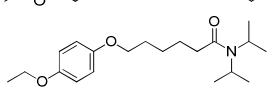
[a] SI = (IC_{50} against HEK293)/(IC_{50} against *T.b. brucei*).

The target compound library was then subjected to a preliminary screen against non-pathogenic *T.b. brucei* to assess the anti-protozoa activity as well as against the human cell line HEK293 as a measure of selectivity (Table 1). These assays were performed using the HTS assay previously described by Avery and co-workers.[27] Unfortunately, a number of the compounds (not shown in Table 1) proved to be insoluble under the assay conditions and thus IC_{50} values could not be obtained in these cases (for full results table, see Supplementary Information).

A number of interesting structure-activity trends were elucidated from these preliminary screening results. Notably compound **8**, a fragment of the original lead **1**, was found to be inactive (IC_{50} > 50 μM), thus confirming the importance of the piperidyl ring for anti-trypanosomal activity. In general, compounds possessing a 4-alkoxy substituted masked hydroquinone moiety possessed more potent activity when compared with 4-alkyl substitution. Of all the compounds bearing a piperidyl functionality (**11-24**), those also containing an ethoxy substituted masked hydroquinone possessed the most potent anti-trypanosomal activity. This included analogues **13**, **17**, **18**, **23** and **24** (IC_{50} = 0.49 – 3.13 μM), with compound **17** exhibiting slightly increased potency and selectivity [IC_{50} = 0.49 μM, selectivity index (SI) = 84] relative to initial hit **1**. Interestingly, incorporation of a 4-methyl piperidyl substituent in **19-22** led to a complete loss in activity (IC_{50} > 50 μM). This observation was mirrored in analogues **29-31**, containing a 4-methylpiperazine moiety. Other substitutions of the methylpiperidyl ring appeared to be tolerated with the morpholino series **25-28** and aniline-based analogues **32-35** possessing significant activity (IC_{50} = 4.17 – 20.6 μM), albeit less potent than the homologues bearing a piperidyl moiety. Inclusion of a smaller *N*-isopropylamide substituent in **36** in place of the 3-piperidyl substituent in **1** led to loss of activity. However, inclusion of a *N,N*-diisopropylamide substituent led to recovery of activity with **37** and **38** exhibiting IC_{50} values of 1.73 and 3.51 μM, respectively.

Having identified the importance of both the ethoxy-derived phenol moiety and either a piperidyl or *N,N*-diisopropylamide functionality, we were next interested in investigating the requirements of the central aryl ring for anti-trypanosomal activity. To this end, analogues **39-41** were synthesised in which the central aryl ring was replaced with an alkyl chain (Table 2, for synthetic details please see Supplementary Information). Compounds **40** and **41** exhibited only modestly reduced activity when compared with the parent compound possessing a rigid aryl linker. These results suggest that the central aromatic ring is not essential for inhibitory activity but rather serves as a spacer unit to appropriately position the two terminal functional groups with respect to each other.

Table 2 Active alkyl-linked analogues **39-41** against *T.b. brucei*

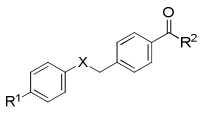
compound	*T.b. brucei* IC_{50} [μM]	Sel. Index (SI) [a]
39 	7.35	5
40 	1.66	50
41 	2.03	41

[a] SI = (IC_{50} against HEK293)/(IC_{50} against *T.b. brucei*).

Having determined the inhibitory activity of **11-41** against *T.b. brucei* in a preliminary screen, we were next interested in selecting compounds for screening against a human infective

trypanosomal strain. To this end, 13 compounds (all with IC_{50} values < 5 μM in the preliminary screen) were screened against *T.b. rhodesiense* (strain STIB 900) (Table 3). In addition, these compounds were assessed against three other parasitic protozoa, namely, *Trypanosoma cruzi* (the causative agent of American trypanosomiasis or Chagas disease), *Leishmania donovani* (the cause of visceral leishmaniasis) and *Plasmodium falciparum* (the most common causative agent of human malaria) to gauge for the selectivity of the anti-trypanosomal activity. The compounds were also screened against rat skeletal myoblasts (L6 cells), as an additional measure of the selectivity of the anti-trypanosomal activity over mammalian cells. Gratifyingly, all of the compounds proved to be more potent inhibitors of *T.b. rhodesiense* compared with the other species of protozoa investigated (Table 2). Indeed, while compounds exhibited high nanomolar-low micromolar inhibition of *T.b. rhodesiense* (IC_{50} = 0.37 – 9.7 μM), most compounds displayed moderate activity against *T. cruzi, L. donovani* and *P. falciparum* (Table 3). For the most part, the compounds also showed significant selectivity for *T.b. rhodesiense* over the rat skeletal myoblast (L6) cell line. The most potent inhibitors of *T.b. rhodesiense* were the 4-ethoxy substituted compounds **17** and **37** (IC_{50} values of 0.37 μM and 0.46 μM) containing 2-methylpiperidyl and *N,N*-diisopropylamide functionalities, respectively. Notably, both **17** and **37** were more potent inhibitors of *T.b. rhodesiense* growth than against the non-human infective protozoa *T.b. brucei*. Substituting the 2-piperidyl substituent in **17** with a piperidyl (in **23**), 3-piperidyl (in **1**) or aniline (in **34**) moiety led to a significant drop in activity against *T.b. rhodesiense.* Compounds **39-41**, where the central aryl moiety was replaced with a flexible alkyl linker, proved to be equipotent against *T.b. rhodesiense* (IC_{50} = 4.7−7.9 μM) and *T.b. brucei* (see Supplementary Information for data). Of all the compounds screened, **37** showed the most significant selectivity for *T.b. rhodesiense* over human cell lines and therefore serves as a promising lead for further development.

Table 3. Results from *T.b. rhodesiense* screening (and other parasitic protozoa) of 13 active first generation compounds from preliminary *T.b. brucei* testing.



compound	R^1	X	R^2	*T.b. rhodesiense* IC_{50} [μM]	*T. cruzi* IC_{50} [μM]	*L. donovani* IC_{50} [μM]	*P. falciparum* IC_{50} [μM]	Cytotoxicity L6 IC_{50} [μM] (SI)[a]
11	OMe	O		2.64 ± 0.243	65.4 ± 22.7	35.1 ± 4.27	35.9 ± 2.27	79.3 ± 44.6 (30)
1	OEt	O		0.985 ± 0.076	107 ± 34.5	35.7 ± 6.22	22.3 ± 1.06	186 ± 94.2 (190)
13	OEt	NH		4.94 ± 1.96	15.3 ± 6.73	37.8 ± 2.70	24.9 ± 1.04	26.9 ± 1.24 (5.4)
16	OMe	O		1.82 ± 0.022	59.8 ± 21.1	39.2 ± 3.98	25.7 ± 5.45	85.7 ± 35.8 (47)
17	OEt	O		0.365 ± 0.059	50.1 ± 23.5	28.9 ± 1.02	17.6 ± 3.43	116 ± 6.93 (320)
18	OEt	NH		1.88 ± 0.122	60.8 ± 19.6	45.4 ± 1.42	20.3 ± 1.72	71.6 ± 36.8 (38)
23	OEt	O		1.74 ± 0.230	172 ± 49.5	94.0 ± 40.1	48.3 ± 11.3	263 ± 31.71 (150)
26	nPr	O		9.73 ± 1.28	32.1 ± 11.5	40.4 ± 11.6	19.1 ± 5.13	106 ± 14.2 (11)
32	Et	O		7.16 ± 3.17	168 ± 37.1	64.0 ± 23.2	113 ± 31.4	>275 (>38)
34	OEt	O		3.38 ± 1.58	135 ± 71.2	136.5 ± 87.5	117 ± 38.3	>275 (>81)
35	OEt	NH		5.28 ± 0.472	16.2 ± 5.38	38.6 ± 4.30	15.4± 0.222	75.0 ± 23.3 (14)
37	OEt	O		0.464 ± 0.162	229 ± 26.3	>275	88.3 ± 41.4	>275 (>590)
38	OEt	NH		3.61 ± 1.12	50.5 ± 15.1	44.9 ± 0.423	12.3 ± 1.21	83.6 ± 24.4 (23)

Control IC_{50} values as follows: IC_{50} (Melarsoprol) = 0.010 μM (*T.b. rhodesiense*); IC_{50} (Benznidazole) = 2.325 μM (*T. cruzi*); IC_{50} (Miltefosine) = 0.572 μM (*L. donovani*); IC_{50} (Chloroquine) = 0.013 μM (*P. falciparum*); IC_{50} (Podophylotoxin) = 0.022 μM (L6 cells). [a]SI = (IC_{50} against L6)/(IC_{50} against *T.b. rhodesiense*). Errors are standard errors of the mean of three independent experiments provided to three significant figures.

In summary, we have reported the synthesis of a library of analogues based around the structure of a phenoxymethylbenzamide hit elucidated through an HTS campaign. Several members of the compound library exhibited potent activity against the non-human infective trypanosome strain *T.b. brucei*, as well as good selectivity over mammalian cell lines. Several compounds also demonstrated potent inhibitory activity against *T.b. rhodesiense* with the most active compound **17** possessing an IC_{50} value of 365 nM. The novel class of anti-trypanosomal agents described here provides an opportunity for further medicinal chemistry efforts, both to optimise the activity against pathogenic *T. brucei* species and to assess the ADME and PK properties of this class of compounds. Furthermore, chemical biology approaches will also be pursued in order to elucidate the molecular target of these compounds in the parasite, to aid the second generation of compounds.

Notes and references

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† Electronic Supplementary Information (ESI) available: detailed experimental procedures, characterisation data and original NMR spectra. See DOI: 10.1039/b000000x/

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