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Identification of novel inhibitors of UDP-Glc 4′-epimerase, a validated drug target for african sleeping sickness

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Abstract—Novel inhibitors of *Trypanosoma brucei* and mammalian UDP-Glc 4′-epimerase were identified by screening a small library of natural products and commercially available drug-like molecules. The inhibitors possess low micromolar potency against the *T. brucei* and human enzymes in vitro, display a degree of selectivity between the two enzymes, and are cytotoxic to cultured *T. brucei* and mammalian cells.

Trypanosoma brucei, a protozoan parasite transmitted by the bite of the Tsetse fly, is the causative agent of African sleeping sickness in humans and the related disease Nagana in cattle. *T. brucei* cells are able to survive in the blood of the host by virtue of a dense surface coat of variant surface glycoprotein that protects the parasite from the complement pathway and undergoes antigenic variation to evade specific immune responses, making the production of a vaccine unfeasible.[1] Sleeping sickness is invariably fatal if not treated and is responsible for upwards of 30,000 deaths per year in Sub-Saharan Africa.[2] Current treatments are expensive, toxic, and difficult to administer, leaving an urgent need for new therapeutic agents.

Bloodstream form *T. brucei* contains many galactose-containing glycoproteins,[3–5] yet unlike mammalian cells, the parasite hexose transporters are unable to uptake galactose directly.[6,7] Instead, *T. brucei* must obtain its galactose via the action of the enzyme UDP-glucose 4′-epimerase (GalE) that interconverts UDP-glucose (UDP-Glc) and UDP-galactose (UDP-Gal).[8] The generation of a *T. brucei* GalE (TbGalE) conditional null genetic mutant has demonstrated that this enzyme is essential for survival of the parasite in both the disease-relevant bloodstream form and the Tsetse-fly dwelling procyclic form.[8,9] The high-resolution crystal structures of human GalE (hGalE) and TbGalE show that the enzymes share a common topology, and despite the low level of sequence identity (33%), many protein–ligand interactions are conserved.[10,11] Selective inhibition of the parasite enzyme may be possible due to a non-conservative substitution in the UDP-binding pocket from Gly237 in hGalE to Cys266 in TbGalE.[11] However, such selectivity may not be essential, as deficiency in the human enzyme is either asymptomatic or leads to a rare form of galactosaemia,[12] a chronic condition that can be partially controlled by diet and that may be tolerable over a short period. Therefore, we believe that TbGalE is a suitable target for the development of drugs to treat African sleeping sickness.

We are not aware of any precedent for developing inhibitors of UDP-Glc 4′-epimerase as drug leads; indeed, there are few reported inhibitors of GalE. The substrate analogue UDP-4-deoxy-4-fluorogalactose is reported to bind *Escherichia coli* GalE (K_d = 1.1 mM),[13] and a competitive inhibitor of hGalE (1–143) has been identified from a combinatorial chemistry library of 1338 uridine-based Schiff bases (IC_{50} = 10 μM),[14] but neither are sufficiently potent or drug-like to be pursued as lead compounds. In order to identify novel and potent inhibitors that are not substrate analogues, we decided to

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screen a small library of natural product consisting of 44 compounds isolated from marine invertebrates, and a small commercial library of 880 structurally diverse drug-like molecules (Prestwick) against TbGalE.

The activity of GalE can be conveniently measured by using an excess of UDP-glucose dehydrogenase (UGD) in a coupled enzyme assay (Fig. 1), enabling the reaction to be followed by monitoring the production of NADH at 340 nm.[15] Although *E. coli* UGD has previously been cloned and over-expressed in *E. coli*,[16] and the enzyme is commercially available, we decided to clone and express the *E. coli* UGD to provide a cheap and readily available source of the enzyme for our screening efforts. The *E. coli* K12 UGD sequence (GenBank Accession No. U00096) was PCR amplified from genomic DNA and cloned into the expression vector pET15b (Novagen), appending an N-terminal hexahistidine tag. Over-expression in *E. coli* and single step purification using an immobilized nickel column produced $\geqslant$100 mg of purified UGD per litre of cell culture.

Prior to compound screening, the kinetics of the TbGalE–UGD coupled assay were examined to ensure that the activity of UGD was in excess; the specific activity of 6.5 U/mg and K_m for UDP-Gal of 95 ± 10 μM obtained were in good agreement with literature values for TbGalE of 5.9 U/mg and 77 μM, respectively.[8] The natural products were screened in triplicate using the coupled assay with an inhibitor concentration of 50 μM,[17] revealing three compounds that showed >75% inhibition. The commercial compounds were screened once with an inhibitor concentration of 50 μM,[17] identifying thirteen compounds that showed >75 % inhibition (Z' 0.85).

To confirm that these initial hits were inhibiting TbGalE rather than the coupling enzyme UGD, their inhibition was measured directly using high pH anion exchange chromatography (HPAEC) to follow the conversion of UDP-Gal to UDP-Glc by TbGalE.[18] Whilst the majority of compounds displayed significant inhibition of TbGalE at 50 μM, five of the initial hits (all commercial compounds) showed no significant inhibition. The most potent seven inhibitors (Fig. 2) that gave greater than 75% inhibition of TbGalE at 50 μM were selected for further study.

The potencies of the selected inhibitors against *T. brucei*- and human-GalE were determined using the HPAEC assay,[18] confirming the low micromolar IC_{50} values expected (Table 1). All the inhibitors showed

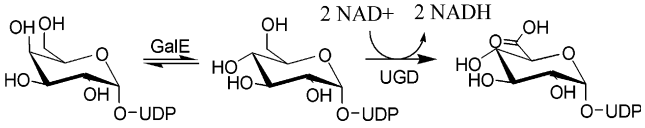

Figure 1. The GalE–UGD coupled enzyme assay. Provided UGD is in excess, the kinetics of GalE can be measured by following the production of NADH at 340 nm.

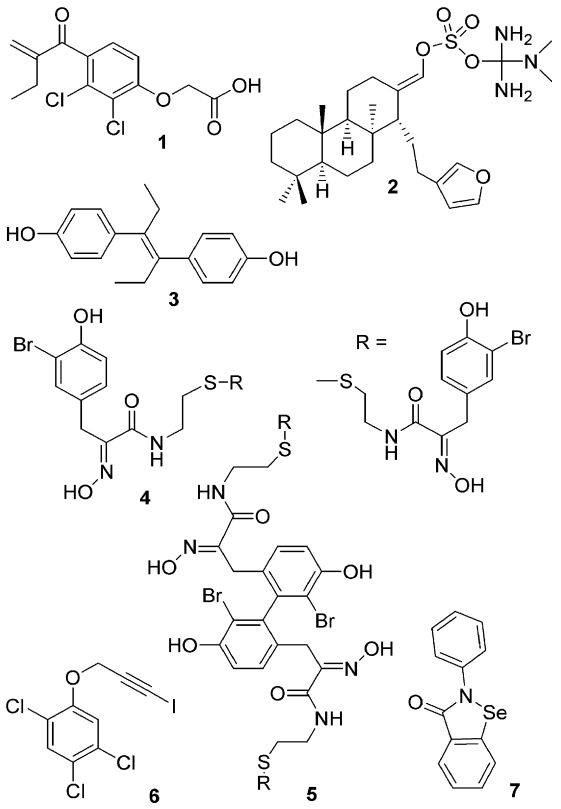

Figure 2. Structure of the GalE inhibitors selected for further study.

significantly different potencies against the two enzymes, which may indicate that the compounds have distinct interactions with each enzyme. However, only three of the compounds (**1**–**3**) were found to be more potent against the *T. brucei* enzyme than the human enzyme.

To further investigate the binding of the new inhibitors we docked the three compounds that showed selectivity for TbGalE (**1**–**3**) into the substrate binding site of the TbGalE crystal structure.[19,20] Compound **2** was too large to be accommodated without structural rearrangements of the binding site. Compounds **3** and **1** show a good shape complementarity to the pocket and occupy roughly the same space as the UDP part of the natural substrate. In addition, in the predicted binding mode, compound **1** forms a hydrogen bond to Phe255 and a salt bridge to Arg268 (Fig. 3). Both interactions are also found in the crystal structure complex with the substrate. These docking results suggest that at least compounds **1** and **3** can act as competitive inhibitors of TbGalE.

In order to determine if any of the compounds were covalently modifying TbGalE, compounds were co-incubated with the enzyme prior to mass spectrometric analysis.[21] Whereas compounds **3**, **6** and **7** did not form any covalent adducts, compounds **1**, **2**, **4** and **5** were found to form multiple adducts with TbGalE at high compound concentrations (50 μM), indicating that non-selective covalent modifications were occurring, probably due to the intrinsic reactivity of the molecules.

Table 1. Potency of the selected GalE inhibitors in vitro

Compound	In vitro IC_{50}[a] (μM)		Cytotoxicity EC_{50}[a] (μM)	
	TbGalE	hGalE	*T. brucei*	CHO-K1
1	3.1 ± 0.1	14 ± 7.7	3.3 ± 0.27	56 ± 38
2	5.6 ± 1.5	12 ± 7.2	>100	>100
3	17 ± 8.1	75 ± 22	2.8 ± 1.6	8.5 ± 4.2
4	15 ± 5.1	3.6 ± 0.5	2.6 ± 2.4	0.9 ± 0.6
5	14 ± 5.6	ND[b]	5.2 ± 2.6	6.0 ± 2.4
6	13 ± 3.2[c]	0.5 ± 0.6	0.3 ± 0.25	0.6 ± 0.35
7	0.62 ± 0.2	0.014 ± 0.003	4.1 ± 2.7	6.1 ± 1.8

[a] Values are means of three experiments, standard deviation is given.
[b] Insufficient material for determination.
[c] Pre-incubation with TbGalE for 1 h, otherwise IC_{50} > 100 μM.

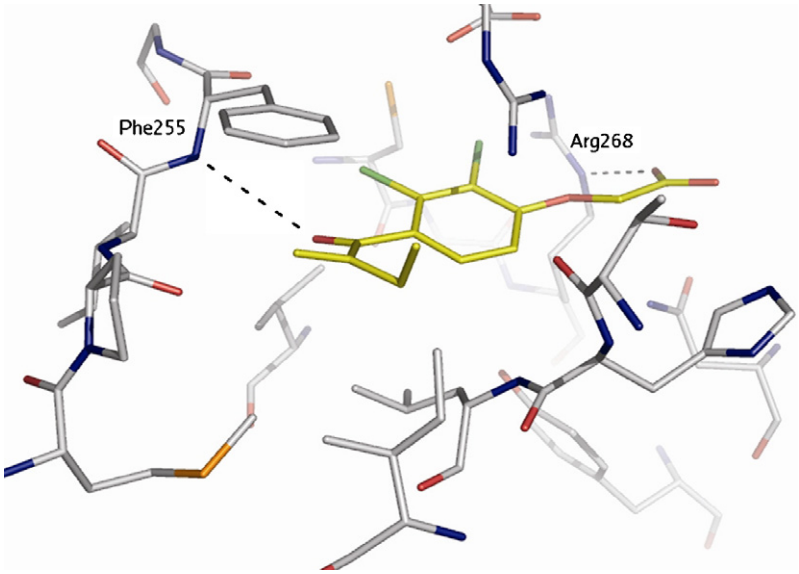

Figure 3. Molecular model of compound **1** bound to TbGalE. Atoms are coloured as follows: carbon grey (protein)/yellow (compound **1**) nitrogen—blue, oxygen—red and chlorine—green.

Compound **1** contains an enone moiety that is susceptible to Michael addition, compound **2** can tautomerise to an enol sulfate and *N*,*N*-dimethylguanidine and compounds **4** and **5** are able to undergo disulfide bond exchange.

The cytotoxicity of the inhibitors against cultured bloodstream-form *T. brucei* and mammalian CHO-K1 cells was also determined (Table 1).[22] All the inhibitors were found to display significant cytotoxicity against both cell types except for the natural product **2**, which may reflect poor cell permeability or inherent reactivity of this compound. Unfortunately, only **1** displayed the desired selective cytotoxicity against *T. brucei* cells, and the most potent in vitro inhibitor **7** gave only micromolar cytotoxicity. However, for the structurally unrelated compounds **1**, **3** and **4** the trends in IC_{50} values against trypanosomal and mammalian GalE correlate well with the trends in the EC_{50} cytotoxicity values against the respective cultured cells, suggesting that the two properties are linked.

The cytotoxicity of the inhibitors against CHO-K1 cells is somewhat unexpected since it is not clear if GalE is essential to the survival of these cells, and is instead likely to result from separate off-target effects. Indeed, many of these compounds are known to possess other biological activity; ethacrynic acid (**1**) is a clinically used loop diuretic, diethylstilbestrol (**3**) is a carcinogenic oestrogen mimic, psammaplin A (**4**) and the related bisaprasin (**5**) inhibit topoisomerase and histone deactylase activity, haloprogin (**6**) is a clinically used topical fungicide and ebselen (**7**) has entered stage III clinical trials for ischaemic stroke treatment. The formation of multiple covalent adducts observed for compounds **1**, **4** and **5** may account in part for their cytotoxicity, but does not adequately explain the observed differences in potency between cell types.

In conclusion, we have identified novel inhibitors of UDP-glucose 4′-epimerase by screening a small library of natural products and commercially available compounds. The inhibitors possess low micromolar potency against the *T. brucei* and human enzymes in vitro, display a degree of selectivity between the two enzymes and are cytotoxic to cultured *T. brucei* and CHO-K1 cells. However, none of the compounds are promising drug leads, and the number of intrinsically chemically

reactive compounds obtained as hits was somewhat disappointing.

Nevertheless, *T. brucei* GalE is a validated drug target for African sleeping sickness, and these results demonstrate that the *T. brucei* enzyme can be selectively inhibited by small molecules that are not substrate analogues in vitro, although the mechanism of cytotoxicity of these inhibitors is unconfirmed. Of the compounds screened, ethacrynic acid displays the best therapeutic index, but is clinically used as a loop diuretic and was found to form multiple covalent adducts with TbGalE in vitro, limiting its potential as a lead compound. However, these results show that novel inhibitors of TbGalE may be readily identified by screening small compound libraries, demonstrating that high-throughput screening of larger compound libraries of drug-like and lead-like molecules against TbGalE using the methodology described here is a feasible approach to the discovery of drug leads for the treatment of African sleeping sickness.

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17. GalE–UGD coupled assay: The reaction mixture (100 mM glycine, pH 8.7, 0.5 mM β-NAD^+, 0.2 mM UDPGal, 0.2 mg/ml UGD and 80 ng/ml TbGalE) was aliquoted into 96-well plates (198 μl/well) with or without inhibitor (in 2 μl DMSO), and the reaction monitored at 340 nm for 15 min at 30 °C on a SpectraMax 380 (Molecular Devices).
18. GalE HPAEC assay: The reaction mixture (1 mM Tris, pH 7.6, 0.02% BSA, 250 μM UDP-Gal, 250 μM β-NAD^+ and 2.5 g/mL TbGalE) was incubated at 30 °C for 30 min with or without inhibitor, quenched with 10-fold excess of 1 mM NaOH and then subjected to HPAEC chromatography on a CarboPac PA-1 column (Dionex) using conditions adapted from Tomiya et al. (Tomiya, N., Ailor, E., Lawerence, S. M., Bettenbaugh, M. J., Lee, Y. C. *Anal. Biochem.* **2002,** *293*, 129). The eluant was monitored at 260 nm, and peaks assigned by comparison to commercial standards.
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21. The reaction mixture (1 mM Tris, pH 7.6, 0.02% BSA and 100 μg/mL TbGalE) was incubated for 30 min with or without inhibitor (50 μM) prior to analysis by MALDI-TOF mass spectrometry.
22. Cytotoxicity assay: 200 microlitres of cells (1×10^4 cells/ml) was aliquoted into 96-well plate and incubated with or without inhibitor (100 mM to 30 nM) for a further three days before the number of viable cells was counted using the Alamar blue assay. For a description of the Alamar blue assay, see: Raz, B.; Iten, M.; Grether-Buhler, Y.; Kaminski, R.; Brun, R. *Acta Trop.* **1997**, *68*.