UDP-galactose 4′-epimerase from the liver fluke, *Fasciola hepatica*: biochemical characterization of the enzyme and identification of inhibitors

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SUMMARY

The Leloir pathway enzyme uridine diphosphate (UDP)-galactose 4′-epimerase from the common liver fluke *Fasciola hepatica* (FhGALE) was identified and characterized. The enzyme can be expressed in, and purified from, *Escherichia coli*. The recombinant enzyme is active: the K_m (470 μM) is higher than the corresponding human enzyme (HsGALE), whereas the k_{cat} (2·3 s^{-1}) is substantially lower. FhGALE binds NAD^+ and has shown to be dimeric by analytical gel filtration. Like the human and yeast GALEs, FhGALE is stabilized by the substrate UDP-galactose. Molecular modelling predicted that FhGALE adopts a similar overall fold to HsGALE and that tyrosine 155 is likely to be the catalytically critical residue in the active site. *In silico* screening of the National Cancer Institute *Developmental Therapeutics Program* library identified 40 potential inhibitors of FhGALE which were tested *in vitro*. Of these, 6 showed concentration-dependent inhibition of FhGALE, some with nanomolar IC_{50} values. Two inhibitors (5-fluoroorotate and *N*-[(benzyloxy)carbonyl]leucyltryptophan) demonstrated selectivity for FhGALE over HsGALE. These compounds also thermally destabilized FhGALE in a concentration-dependent manner. Interestingly, the selectivity of 5-fluoroorotate was not shown by orotic acid, which differs in structure by 1 fluorine atom. These results demonstrate that, despite the structural and biochemical similarities of FhGALE and HsGALE, it is possible to discover compounds which preferentially inhibit FhGALE.

Key words: neglected tropical disease, Leloir pathway, GALE, UDP-glucose 4′epimerase, 5-fluoroorotate, *N*-[(benzyloxy) carbonyl]leucyltryptophan.

INTRODUCTION

Fascioliasis, or infection with the liver fluke species *Fasciola hepatica* and *Fasciola gigantica*, is recognized by the World Health Organisation as a neglected tropical disease. It is estimated that 7 million humans are infected and many more millions are at risk of infection (Robinson and Dalton, 2009). The majority of these people live in the developing world. The parasites also infect farm animals causing billions of pounds of agricultural losses worldwide and providing a reservoir for the human disease (Boray, 1994). The treatment of choice for both animals and humans is the benzimidazole derivative triclabendazole. This drug is effective against both adult and juvenile flukes. However, in the past decade, resistance to triclabendazole has been reported in liver flukes infecting farm animals (Brennan *et al*. 2007; Brockwell *et al*. 2014). It is highly probable that resistance will soon emerge in liver flukes which infect humans. Consequently, there is considerable interest in identifying and characterizing liver fluke proteins as potential drug targets. There has been recent revival of interest in targeting metabolic enzymes in the chemotherapeutic control of infectious diseases (Srinivasan and Morowitz, 2006). This has resulted in a number of selective inhibitors of metabolic enzymes from unicellular parasites (for example Aronov *et al*. 1999; Rodriguez-Romero *et al*. 2002; Enriquez-Flores *et al*. 2008; Durrant *et al*. 2010; Friedman *et al*. 2012; Soares *et al*. 2013).

Uridine diphosphate (UDP)-galactose 4′-epimerase (UDP-glucose 4′-epimerase, galactowaldenase, GALE, EC 5.1.3.2) catalyses the conversion of UDP-galactose to UDP-glucose (McCorvie and Timson, 2014). This reaction, which reverses the stereochemistry at position 4 of the sugar moiety, is required for the Leloir pathway of galactose catabolism (Leloir, 1951). In addition to this catabolic role, the enzyme also has biosynthetic roles. GALE helps maintain the cellular pools of UDP-galactose and UDP-glucose along with the structurally related sugars UDP-*N*-acetylgalactosamine and UDP-*N*-acetylglucosamine (Daenzer *et al*. 2012).

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These compounds are precursors for the synthesis of the oligosaccharide units of glycoproteins and glycolipids.

GALE enzymes are typically homodimeric with 2 active sites and 2 tightly bound NAD^+ cofactors. The reaction mechanism requires the NAD^+-dependent oxidation of the C_4-OH of the sugar to a ketone, rotation of the sugar moiety and re-reduction of the ketone to a hydroxyl in the opposite configuration (Maitra and Ankel, 1971). The oxidation/reduction cycle is assisted by a tyrosine residue (Tyr-157 in human GALE, HsGALE) in the active site (Thoden *et al*. 2000).

In humans, lack of GALE activity resulting from mutations in the gene coding for the enzyme causes type III galactosaemia (Holton *et al*. 1981; Timson, 2006). Type III galactosaemia varies considerably in severity, depending on the mutations present in the individual. The most severe forms result in progressive damage to the kidneys, liver and brain with consequent physical and cognitive disabilities (Holton *et al*. 1981; Fridovich-Keil *et al*. 1993; Walter *et al*. 1999; Fridovich-Keil and Walter, 2008). These severe consequences for the organism resulting from reduced GALE activity suggest that the enzyme may be a potential target for novel therapeutics in pathogens such as *F. hepatica*. GALE has been shown to be essential for normal development in *Drosophila melanogaster* (Sanders *et al*. 2010; Daenzer *et al*. 2012). Although the molecular pathology of type III galactosaemia is not completely elucidated, the fundamental molecular cause has been shown to be protein misfolding which results in reduced enzymatic activity, reduced cofactor binding or an increased propensity to aggregate (Wohlers and Fridovich-Keil, 2000; Timson, 2005; Chhay *et al*. 2008; Bang *et al*. 2009; McCorvie *et al*. 2011, 2012). A reduction in GALE activity results in a build-up of galactose 1-phosphate, which is believed to be toxic in high concentrations (Mayes *et al*. 1970; Tsakiris *et al*. 2005). Cellular galactose concentrations also increase, most likely leading to increased free radical production; the biochemical mechanism of this is uncertain (Lai *et al*. 2009; Jumbo-Lucioni *et al*. 2013). In addition, altered protein and lipid glycosylation may occur in type III galactosaemia (Fridovich-Keil *et al*. 1993).

Therefore, it is a reasonable assumption that selective inhibition of GALE from the liver fluke would be detrimental to the organism. In *Trypanosoma* spp., which cannot take up galactose and have an absolute requirement for GALE for the production of UDP-galactose, loss of GALE activity leads to defects in cell growth and division accompanied by reduced amounts of galactose moieties in cell surface mucins (Roper *et al*. 2002; MacRae *et al*. 2006; Urbaniak *et al*. 2006*b*). Therefore, considerable efforts have been made to identify specific inhibitors of this organism's GALE (Shaw *et al*. 2003; Alphey *et al*. 2006; Urbaniak *et al*. 2006*a*; Durrant *et al*. 2010; Friedman *et al*. 2012). Here we describe, for the first time, the biochemical characterization of *F. hepatica* GALE (FhGALE), the *in silico* identification of potential inhibitors and the *in vitro* testing of these compounds against FhGALE and HsGALE.

MATERIALS AND METHODS

Cloning, expression and purification of FhGALE

The *FhGALE* coding sequence was amplified by PCR using primers based on sequences in the *F. hepatica* expressed sequenced tag (EST) and transcriptome libraries (Ryan *et al*. 2008; Young *et al*. 2010). The amplicon was inserted into the *Escherichia coli* expression vector, pET46 Ek-LIC (Merck, Nottingham, UK) by ligation-independent cloning according to the manufacturer's instructions. This vector inserts nucleotides encoding a hexahistidine tag at the 5′-end of the coding sequence. The complete coding sequence was obtained by DNA sequencing (GATC Biotech, London, UK). FhGALE protein was expressed in, and purified from, *E. coli* Rosetta (DE3) (Merck) using the same method as previously reported for *F. hepatica* triose phosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase (Zinsser *et al*. 2013*b*).

Bioinformatics

Selected GALE protein sequences were aligned using ClustalW and a neighbour-joining tree constructed using MEGA5.0 (Larkin *et al*. 2007; Kumar *et al*. 2008; Tamura *et al*. 2011). The molecular mass and isoelectric point of the protein were estimated using the ProtParam application in the ExPASy suite of programs (Gasteiger *et al*. 2005).

Molecular modelling and computational identification of potential inhibitors

The predicted protein sequence of FhGALE was submitted to Phyre2 in the intensive mode to generate an initial molecular monomeric model of the protein (Kelley and Sternberg, 2009). Two copies of this model were aligned using PyMol (www.pymol.org) to the subunits of *Burkholderia pseudomallei* GALE structure (PDB ID: 3ENK) and NAD^+ molecules from this structure inserted in order to generate an initial dimeric model. This was energy minimized using YASARA to generate the final substrate free dimeric model (Krieger *et al*. 2009). The minimized model was realigned to 3ENK and UDP-glucose molecules from this structure inserted. This initial ligand bound structure was then minimized using YASARA. The final, minimized models with and without UDP-glucose bound are provided as Supplementary material to this paper.

For docking studies the final, minimized model containing UDP-glucose and NAD^+ molecules was prepared. Docking was performed with 2 different tools – AutoDock Vina (Trott and Olson, 2010) and Schroedinger's Glide (Friesner *et al*. 2004, 2006; Halgren *et al*. 2004). Ligands and cofactors were removed from the AutoDock Vina receptor files. The AutoDock script (Morris *et al*. 2009) prepare_receptor4.py was used to prepare the final receptor pdbqt files after the UDP-glucose coordinates had been removed. The docking box centre was chosen based on the PA phosphate coordinates of the UDP-glucose molecule. For the Glide screens, the receptors were prepared using the tools provided in the Maestro Protein Preparation Wizard and the Glide Receptor Grid Generation. Only the UDP-glucose ligand was removed from the Glide receptor grid files, while the cofactor was left in the binding site.

The virtual screen was performed using the National Cancer Institute (NCI) diversity set III, a subset of the full NCI compound database. Ligands were prepared using LigPrep, adding missing hydrogen atoms, generating all possible ionization states, as well as tautomers. The final set used for virtual screening contained 1013 compounds. Docking simulations were performed with both AutoDock Vina (Trott and Olson, 2010) as well as Glide (Friesner *et al*. 2004, 2006; Halgren *et al*. 2004). The AutoDock script (Morris *et al*. 2009) prepare_ligand4.py was used to prepare the ligand pdbqt files for the AutoDock Vina screens. A docking grid of size $28{\cdot}0 \times 28{\cdot}0 \times 28{\cdot}0$ Å, centred on the position of the ligand in the active site, was used for docking. For Glide docking, all compounds were scored with the Glide XP scoring function. The individual AutoDock Vina and Glide rankings were combined to form a consensus list of compounds that scored well with both methods, a method that has been reported previously to be successful in virtual screening (Lindert *et al*. 2013).

Developmental Therapeutics Program (DTP) compounds

DTP compounds were obtained from the NCI/DTP Open Chemical Repository, USA (http://dtp.cancer.gov) in 3 mg aliquots. Initial stocks were created by dissolving these solids to 10 mM in 100% dimethyl sulfoxide (DMSO). Compounds that do not dissolve immediately were shaken overnight at 37 °C. Initial stocks were diluted 10-fold in 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid (HEPES)-OH, pH 7·5 to give working stocks. Initial and working stocks were stored at −20 °C.

Analytical methods

Protein–protein cross-linking of FhGALE was carried out with bis(sulphosuccinimidyl)suberate (BS^3) (Partis *et al*. 1983). Protein (50 μM) was incubated at 37 °C for 5 min before the addition of BS^3 (1·6 mM); where required NAD^+ or UDP-galactose was included at a final concentration of 1 mM. Reactions were allowed to proceed for 30 min at 37 °C and were stopped by the addition of an equal volume of SDS-PAGE loading buffer (120 mM Tris–HCl pH 6·8, 4% (w/v) SDS, 20% (v/v) glycerol, 5% (w/v) bromophenol blue and 1% (w/v) dithiothreitol (DTT)). Reactions were analysed by 10% SDS-PAGE.

Analytical gel filtration and thermal scanning fluorimetry were carried out as previously described (Banford *et al*. 2013; McCorvie *et al*. 2013; Zinsser *et al*. 2013*a*, *b*, 2014). Protein concentrations were estimated by the method of Bradford using bovine serum albumin as a standard (Bradford, 1976). Intrinsic fluorescence measurements were made using a Spectra Max Gemini XS fluorescence plate reader controlled by SOFTmax PRO software. FhGALE (15 μM) samples (200 μL) were made up in triplicate in FluoroNunc black 96 well plates and excited at 280 nm. Fluorescence emission spectra between 310 and 500 nm were collected. Samples containing buffer only were also measured as used a background measurements and were subtracted from the protein spectra to generate the final spectra.

Enzyme kinetic analysis

The activity of FhGALE was measured by coupling the reaction to the NAD^+-dependent oxidation of UDP-glucose catalysed by UDP-glucose dehydrogenase (Ng *et al*. 1967). The coupling enzyme was prepared as previously described (McCorvie *et al*. 2011). Reactions (150 μL) were set up in triplicate in 96 well plates and contained 10 mM HEPES-OH, pH 8·8, 10 mM NAD^+, 2·4 μM UDP-glucose dehydrogenase and a variable concentration of UDP-galactose (0–1 mM). Reactions were incubated at 37 °C for 10 min and then initiated by the addition of FhGALE (16 nM). The absorbance was monitored at 340 nm in a Multiskan Spectrum platereader (Thermo Scientific) at 37 °C for 30 min. The linear part of the progress curve was used to estimate the initial rate and these initial rates plotted against the concentration of UDP-galactose. These data were fitted to the Michaelis–Menten equation by non-linear curve fitting as implemented in GraphPad Prism 6.0 (GraphPad software, CA, USA) (Michaelis and Menten, 1913; Marquardt, 1963). All points were weighted equally.

DTP compounds were initially screened, in triplicate, at a concentration of 100 μM in the presence of 16 nM FhGALE and 1 mM UDP-galactose using the coupled enzyme assay described above. Those compounds which caused a significant reduction ($P < 0{\cdot}05$ in Student's *t*-test (Student, 1908)) in the rate were then tested for their ability to inhibit the

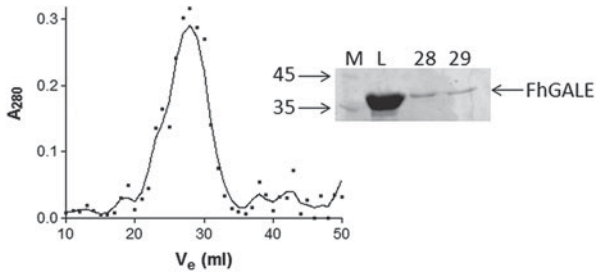

Fig. 1. Dimerization of FhGALE. FhGALE (90 μM) was analysed by gel filtration using a Sephacryl S-300 (Pharmacia) column of total volume (V_t) 46·0 mL and void volume (V_0, estimated using blue dextran) 14·6 mL. The protein eluted in 1 major peak (left) with an elution volume (V_e) of 28·5 mL corresponding to a K_{av} of 0·428 and a molecular mass of 71 kDa. That this peak contained FhGALE was confirmed by SDS-PAGE (right). L, FhGALE as loaded onto the column; 28 and 29, material from the fractions corresponding to 28 and 29 mL. The sizes of molecular mass markers (M) are shown to the left of the gel in kDa.

coupling enzyme UDP-glucose dehydrogenase (reactions contained 0·17 mM UDP-glucose, 1·3 mM NAD^+, 100 μM DTP compound in 1% (v/v) DMSO/50 mM HEPES-OH, 1·2 μM human UDP-galactose dehydrogenase in a total volume of 150 μL and absorbance measured at 37 °C for 15 min at 340 nm). Any compounds which caused significant inhibition ($P<0·05$ in Student's t-test) of this enzyme were eliminated from the screen. The remaining compounds were tested for their ability to cause dose-dependent inhibition of FhGALE and human GALE (HsGALE; prepared as described previously (Timson, 2005)). Reactions (150 μL) contained 1% DMSO/50 mM HEPES-OH, pH 7·5 buffer, 2·4 μM UGDH, 10 mM NAD^+, 0·2 mM UDP-galactose and 1 nM–100 μM DTP compound. Reaction mixes were pre-incubated at 37 °C for 10 min and were then initiated by addition of FhGALE (16 nM) or HsGALE (24 nM). The absorbance at 340 nm was monitored in triplicate in 96 well plates at 37 °C for 30 min with measurements taken every 8 s. Initial rates were calculated from absorbance *vs* time plots and the relative rate (compared to the uninhibited rate) derived. This relative rate was plotted against the common logarithm of the inhibitor concentration and the IC_{50} for each compound determined by non-linear curve fitting (GraphPad Prism 6.0).

RESULTS AND DISCUSSION

FhGALE is a dimeric protein

The coding sequence for *FhGALE* (GenBank: KF700240) encodes a polypeptide of 343 amino acid residues, an estimated, monomeric molecular mass of 38 kDa and a predicted isoelectric point of 6·75. The most similar protein sequence is that of *Clonorchis sinensis* GALE (72% identity) and protein sequence analysis shows that FhGALE clusters with other trematode GALEs (Supplementary Figure S1).

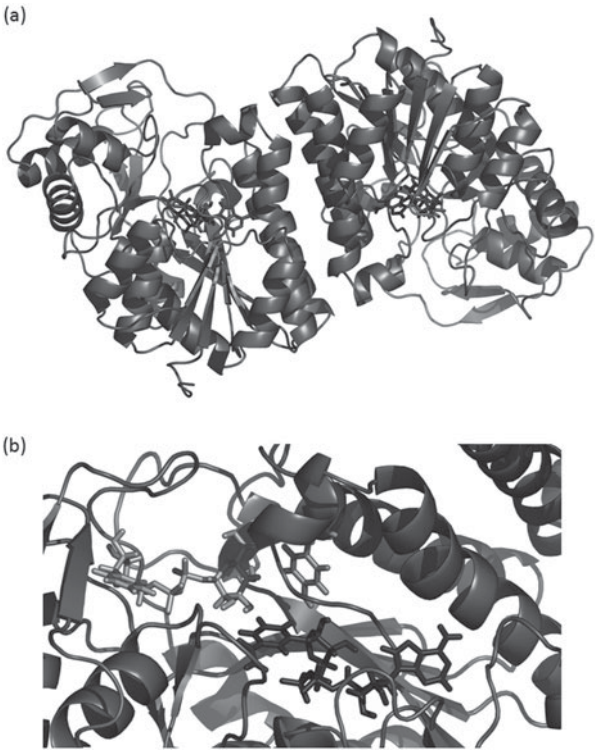

Fig. 2. Predicted 3-dimensional structure of FhGALE. (a) The overall predicted fold of the FhGALE dimer with the NAD^+ cofactor shown in red. (b) A close up of the active site of FhGALE showing the NAD^+ cofactor in red, the product, UDP-glucose, in pink and the catalytically important Tyr-155 residue in orange.

The protein could be expressed in, and purified from, *E. coli* (Supplementary Figure S2a). Cross-linking with BS^3 showed that the protein is likely to be a homodimer in solution (Supplementary Figure S2b). In common with human and *Saccharomyces cerevisiae* GALE proteins, higher order oligomers were also observed in this cross-linking experiment (Supplementary Figure S2b (Timson, 2005; Scott and Timson, 2007; McCorvie *et al*. 2012). This was further demonstrated by analytical gel filtration analysis which estimated a solution molecular mass for the protein of 71 kDa, consistent with a globular homodimer (Fig. 1).

A molecular model of the dimeric protein was generated (Fig. 2). FhGALE is structurally similar to HsGALE (PDB: 1EK6 (Thoden *et al*. 2000)) with a root mean squared deviation (rmsd) of 1·378 Å over 4421 equivalent atoms. It is slightly less similar to *Trypanosoma brucei* GALE (PDB: 1GY8 (Shaw *et al*. 2003)) with an rmsd of 2·067 Å over 3671 equivalent atoms. By comparison with HsGALE, it was predicted that Tyr-155 is the catalytically important tyrosine in FhGALE.

FhGALE binds NAD$^+$, is stabilized by substrate binding and has Michaelis–Menten kinetics

It has been demonstrated previously that the presence of NAD$^+$ in human GALE causes a small, but detectable Förster resonance energy transfer (FRET). Irradiation of the protein at 280 nm results in a large tryptophan emission peak at ∼330 nm. However, some of the emission transfers to the NAD$^+$ which emits at ∼420 nm (McCorvie *et al*. 2012). A similar effect was observed with FhGALE (Fig. 3a).

In the absence of added ligands, the melting temperature (T_m) of recombinant FhGALE (3·0 μM) was 49·5 ± 0·8 °C. UDP-galactose increased the T_m in a concentration-dependent manner and the apparent dissociation constant ($K_{D,app}$) was estimated to be 0·13 ± 0·01 mM (Fig. 3b). NAD$^+$ had no measurable effect on the T_m (data not shown).

The activity of FhGALE shows a saturatable, hyperbolic dependence on UDP-galactose concentration (Fig. 3c). The K_m for UDP-galactose is 470 ± 90 μM which is higher than the human enzyme (69 μM (Timson, 2005)). The turnover number was estimated to be 2·3 ± 0·2 s^{-1}, which is substantially lower than that for the human enzyme (36 s^{-1} (Timson, 2005)).

FhGALE is inhibited by a range of different types of compound

The molecular model of FhGALE was used to identify possible binding molecules from the NCI DTP repository. A virtual screen of the NCI diversity set III against the model was conducted using both AutoDock Vina (Trott and Olson, 2010) and Schroedinger's Glide (Friesner *et al*. 2004, 2006; Halgren *et al*. 2004). The individual docking results were combined into a consensus list of compounds that scored well with both methods. The top 40 hits from that list were obtained and used in *in vitro* testing (Supplementary Table 1). Of these compounds, 31 were soluble in 1% (v/v) DMSO/50 mM HEPES-OH. Twelve compounds reduced the rate of NADH production in an initial screen and 1 increased it; of these 1 (NSC91378) has been shown to affect the rate of the coupling enzyme UDP-glucose dehydrogenase and was not studied further. Six compounds (NSC13575, 16722, 50690, 91355, 116702 and 139105) gave unreliable or inconsistent results in concentration-dependent assays and so were eliminated. The ability of the remaining 6 compounds to inhibit FhGALE and HsGALE was then tested. All 6 compounds inhibited both enzymes (Fig. 4, Table 1). One, NSC27305, gave a sub-nanomolar IC_{50} under the conditions tested. Four more (NSC14303, NSC31712, NSC50690 and NSC335979) had submicromolar IC_{50} values and only 1 (NSC36525) had an IC_{50} in the millimolar range. This identification of high-affinity binders in

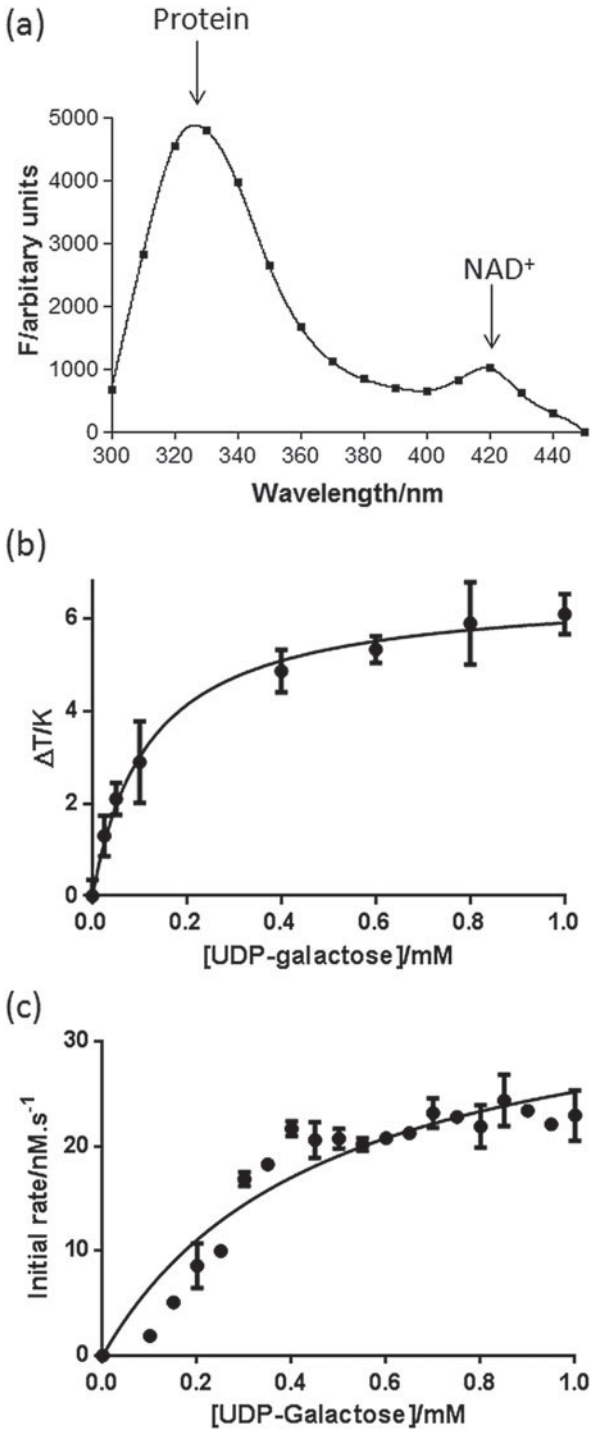

Fig. 3. FhGALE ligand binding and kinetics. (a) The intrinsic fluorescence spectrum of FhGALE (excitation 280 nm) shows 2 peaks, a major one at ∼330 nm corresponding to the aromatic residues in the protein and a smaller one at ∼420 nm corresponding to FRET from the NAD$^+$ cofactor. (b) The change in melting temperature (ΔT_m) of FhGALE (3·0 μM) depends on the UDP-galactose concentration. Each point represents the mean of 3 independent determinations and the error bar represents the S.E.S of these means. (c) The substrate dependence of the initial rate was determined and plotted as a function of substrate concentration (see Materials and methods). Each point represents the mean of 3 independent determinations and the error bar represents the S.E.S. of these means.

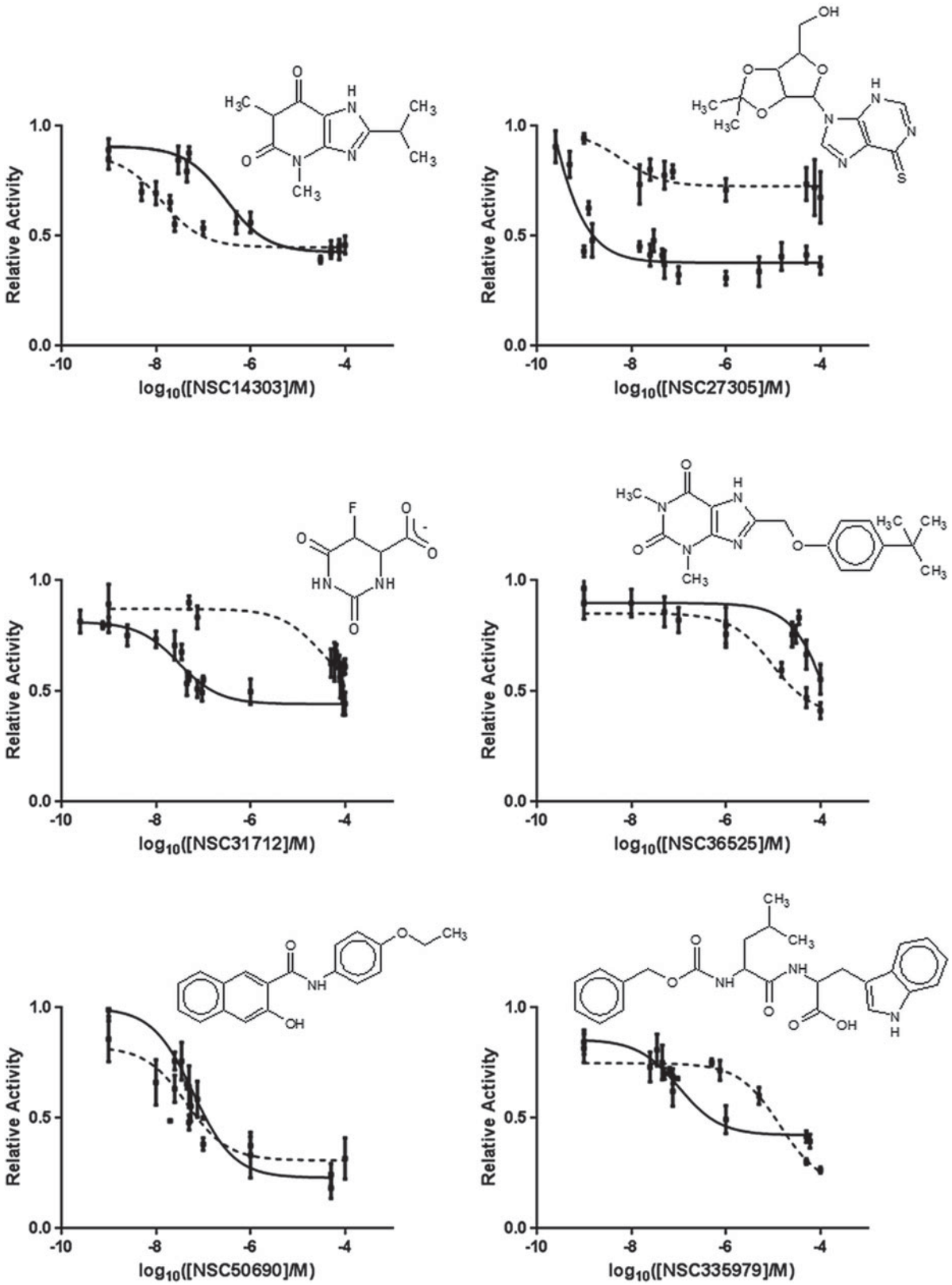

Fig. 4. Six novel inhibitors of FhGALE. In each case, the solid line represents the fit to the data for FhGALE and the dashed line represents the fit for HsGALE. Each point represents the mean of 3 independent determinations and the error bar represents the S.E.S. of these means.

the first round of screening gives us confidence that additional inhibitors in the nanomolar range can be discovered in future.

NSC27305 and NSC50690 inhibited both enzymes to approximately the same extent (as judged by their respective IC_{50} values). NSC14303 and

Table 1. IC_{50} values for FhGALE inhibitors

Systematic name	CAS #	NSC code	FhGALE IC_{50} (nM)	HsGALE IC_{50} (nM)
4,6-Dimethyl-2-(propan-2-yl)-1*H*-imidazo[4,5-*b*] pyridine-5,7(4*H*,6*H*)-dione	2850-40-0	NSC14303	300 ± 110	13 ± 6
9-[6-(Hydroxymethyl)-2,2-dimethyltetrahydrofuro [3,4-*d*][1,3]dioxol-4-yl]-3,9-dihydro-6*H*-purine-6-thione[a]	5856-48-4	NSC27305	0·11 ± 0·12	6·1 ± 9·2
5-Fluoro-2,6-dioxohexahydropyrimidine-4-carboxylate[b]	703-95-7	NSC31712	30 ± 11	33 000 ± 54 000
8-[(4-*Tert*-butylphenoxy)methyl]-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione	na	NSC36525	260 000 ± 550 000	9500 ± 8200
N-(4-ethoxyphenyl)-3-hydroxynaphthalene-2-carboxamide	4711-68-6	NSC50690	61 ± 20	45 ± 23
(3-(1H-indol-3-yl)-2-[[4-methyl-2-(phenylmethoxycarbonylamino)pentanoyl] amino]propionate)[c]	53262-00-3	NSC335979	100 ± 46	14 000 ± 5400
2,6-Dioxohexahydropyrimidine-4-carboxylate[d]	65-86-1	na	600 ± 810	630 ± 650

[a] 2′,3′-O-isopropylidene-6-mercaptopurine riboside.
[b] 5-Fluoroorotate.
[c] (*N*-[(benzyloxy)carbonyl]leucyltryptophan).
[d] Orotate.

NSC36525 inhibited the human enzyme to a greater extent than the liver fluke one. However, 2 compounds, NSC31712 and NSC335979, inhibited FhGALE several orders of magnitude more strongly than HsGALE (Fig. 4, Table 1). Both these compounds also affected the thermal stability of FhGALE, reducing the T_m in a concentration-dependent manner (Supplementary Figure S3). Destabilization can result from the compound preferentially binding to partially unfolded forms of the protein (Cooper *et al*. 2001). The $K_{D,app}$ values for NSC31712 and NSC335979 were 1·9 ± 0·9 and 0·21 ± 0·02 mM, respectively. It was not possible to get meaningful fits to the T_m data with HsGALE and these compounds.

NSC31712 is 5-fluoroorotate (5-fluoro-2,6-dioxo-hexahydropyrimidine-4-carboxylate, 5FOA; CAS# 703-95-7). This compound is widely used in yeast molecular biology to select for transformants (Boeke *et al*. 1984). It has also been tested as a potential anti-cancer and anti-malarial agent (Heidelberger *et al*. 1958; Heath *et al*. 1985; Rathod *et al*. 1989; Muregi *et al*. 2009; Riviere *et al*. 2011). In these applications, the target is not GALE; 5FOA can be converted to 5-fluorouracil which then inhibits thymidylate synthase – an enzyme required for the synthesis of thymidine monophosphate (dTMP) (Chaudhuri *et al*. 1958; Heidelberger *et al*. 1958; Boeke *et al*. 1984). In malarial parasites, 5FOA also directly targets and inhibits thymidylate synthase (Rathod *et al*. 1992).

NSC335979 is the peptide derivative, *N*-[(benzyloxy)carbonyl]leucyl tryptophan (3-(1H-indol-3-yl)-2-[[4-methyl-2-(phenylmethoxy carbonylamino) pentanoyl]amino]propionate; CAS# 5326-00-3). Only limited pharmacological characterization is available for this compound: of the 114 bioassays currently recorded in PubChem (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?cid=333588), 113 showed no activity and 1 was considered inconclusive.

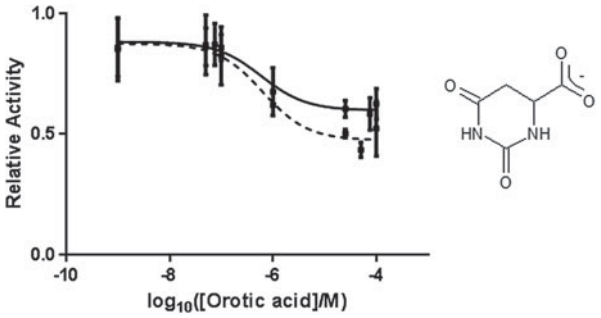

Fig. 5. Unlike 5FOA, orotic acid does not distinguish between FhGALE and HsGALE. The solid line represents the fit to the data for FhGALE and the dashed line represents the fit for HsGALE. Each point represents the mean of 3 independent determinations and the error bar represents the S.E.S. of these means.

The fluorine atom in 5FOA is critical for selectivity over HsGALE

Docking of 5FOA into the FhGALE model structure, followed by alignment of the human structure revealed no structural differences in the predicted binding site which would be likely to account for the selectivity (data not shown). Interestingly, when orotic acid (which lacks the fluorine atom of 5FOA but is otherwise structurally identical) was tested as an inhibitor of both FhGALE and HsGALE, no selectively was observed (Fig. 5, Table 1).

Concluding remarks

Overall, FhGALE has similar properties to human GALE and the majority of other GALE enzymes characterized to date. It is a homodimer in solution and is stabilized by substrate binding. Despite these similarities, selective sub-micromolar inhibition could be obtained with 2 structurally different compounds. Further *in silico* and biochemical work will be required to elucidate the molecular basis of this specificity. This will facilitate the further development of selective FhGALE inhibitors. FhGALE may also have potential as a vaccine target: recent work has shown that *Schistosoma mansoni* GALE is located on the worm's tegument and that vaccination with the recombinant protein provides some protection against infection (Liu *et al*. 2012). Furthermore, this tegumental location may also indicate that the protein is involved in the production of the complex oligosaccharides found in the glycocalyx. If so, inhibition of FhGALE would have at least 2 negative consequences for the parasite: disruption of intermediary metabolism similar to that observed in type III galactosaemia and reduced ability to produce tegumental glycoproteins and glycolipids. These glycosylated molecules have been shown to be important in infection and host immune system evasion (Hanna, 1980; Duffus and Franks, 1981; Wilson *et al*. 2011; Vukman *et al*. 2013). In this context, it is interesting to note that galactose moieties have been observed in tegumental glycoproteins (Hanna, 1976). These observations and our identification of selective inhibitors of FhGALE suggest that attempts to disrupt galactose metabolism in *F. hepatica* may be a useful line of enquiry in the search for novel drugs to control fascioliasis.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S003118201400136X.

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