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

Veronika L. Zinsser¹, Steffen Lindert², Samantha Banford¹, Elizabeth M. Hoey¹, Alan Trudgett^{1,3}, and David J. Timson^{1,3,*}

¹School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL. UK.

²Department of Pharmacology and Center for Theoretical Biological Physics, University of California San Diego, La Jolla, CA, 92093. USA.

³Institute for Global Food Security, Queen's University Belfast, 18–30 Malone Road, Belfast, BT9 5BN. UK.

Abstract

The Leloir pathway enzyme UDP-galactose 4'-epimerase from the common liver fluke *Fasciola hepatica* (FhGALE) was identified and characterised. 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 was shown to be dimeric by analytical gel filtration. Like the human and yeast GALEs, FhGALE is stabilised 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 NCI DTP library identified 40 potential inhibitors of FhGALE which were tested *in vitro*. Of these, six 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 destabilised FhGALE in a concentration-dependent manner. Interestingly the selectivity of 5-fluoroorotate was not shown by orotic acid, which differs in structure by one 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.

Keywords

Neglected tropical disease; Leloir pathway; GALE; UDP-glucose 4'-epimerase; 5-fluoroorotate; N-[(benzyloxy)carbonyl]leucyltryptophan

* Author to whom correspondence should be addressed at: School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL. UK., Telephone: +44(0)28 9097 5875, Fax: +44(0)28 9097 5877, d.timson@qub.ac.uk.

Introduction

Fascioliasis, or infection with the liver fluke species *Fasciola hepatica* and *Fasciola gigantica*, is recognised by the World Health Organisation as a neglected tropical disease. It is estimated that seven million humans are infected and many millions more 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 characterising 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; Durrant et al. 2010; Enriquez-Flores et al. 2008; Friedman et al. 2012; Rodriguez-Romero et al. 2002; Soares et al. 2013).

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

GALE enzymes are typically homodimeric with two active sites and two tightly bound NAD⁺ cofactors. The reaction mechanism requires the NAD⁺-dependent oxidation of the C₄-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 galactosemia (Holton et al. 1981; Timson 2006). Type III galactosemia 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 (Fridovich-Keil et al. 1993; Fridovich-Keil and Walter 2008; Holton et al. 1981; Walter et al. 1999). 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*

(Daenzer et al. 2012; Sanders et al. 2010). Although the molecular pathology of type III galactosemia 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 (Bang et al. 2009; Chhay et al. 2008; McCorvie et al. 2011; McCorvie et al. 2012; Timson 2005; Wohlers and Fridovich-Keil 2000). 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 (Jumbo-Lucioni et al. 2013; Lai et al. 2009). In addition, altered protein and lipid glycosylation may occur in type III galactosemia (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 (MacRae et al. 2006; Roper et al. 2002; Urbaniak et al. 2006b). Therefore, considerable efforts have been made to identify specific inhibitors of this organism's GALE (Alphey et al. 2006; Durrant et al. 2010; Friedman et al. 2012; Shaw et al. 2003; Urbaniak et al. 2006a). Here we describe, for the first time, the biochemical characterisation of *Fasciola 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* 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; Zinsser et al. 2014).

Bioinformatics

Selected GALE protein sequences were aligned using ClustalW and a neighbour-joining tree constructed using MEGA5.0 (Kumar et al. 2008; Larkin et al. 2007; 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 was 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 minimised using YASARA to generate the final substrate free dimeric model (Krieger et al. 2009). The minimised model was realigned to 3ENK and UDP-glucose molecules from this structure inserted. This initial ligand bound structure was then minimised using YASARA. The final, minimised models with and without UDP-glucose bound are provided as supplementary information to this paper.

For docking studies the final, minimised model containing UDP-glucose and NAD⁺ molecules was prepared. Docking was performed with two different tools – AutoDock Vina (Trott and Olson 2010) and Schroedinger's Glide (Friesner et al. 2004; Friesner et al. 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 center 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; Friesner et al. 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 28.0 Å × 28.0 Å × 28.0 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% DMSO. Compounds which did not dissolve immediately were shaken overnight at 37 °C. Initial stocks were diluted ten-fold in 50 mM Hepes-OH, pH 7.5 to give working stocks. Initial and working stocks were stored at –20 °C.

Analytical methods

Protein-protein crosslinking of FhGALE was carried out with bis(sulfosuccinimidyl)suberate (BS³) (Partis et al. 1983). Protein (50 μ M) was incubated at 37 °C for 5 min before the addition of BS³ (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, 1 % (w/v) 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; Zinsser et al. 2013; Zinsser et al. 2014). Protein concentrations were estimated by the method of Bradford using BSA as a standard (Bradford 1976). Intrinsic fluorescence measurements were made using a Spectra Max Gemini XS fluorescence platereader controlled by SOFTmax PRO software. FhGALE (15 μ M) samples (200 μ l) 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 μ l) were set up in triplicate in 96 well plates and contained 10 mM HEPES-OH, pH 8.8, 10 mM NAD⁺, 2.4 μ M 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 in at 340 nm 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) (Marquardt 1963; Michaelis and Menten 1913). All points were weighted equally.

DTP compounds were initially screened, in triplicate, at a concentration of 100 μ M 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 ($p < 0.05$ in Student's t-test (Student 1908)) in the rate were then tested for their ability to inhibit the coupling enzyme UDP-glucose dehydrogenase (reactions contained 0.17 mM UDP-glucose, 1.3 mM NAD⁺, 100 μ M DTP compound in 1% (v/v) DMSO/50 mM Hepes-OH, 1.2 μ M human UDP-galactose dehydrogenase in a total volume of 150 μ l 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 μ l) contained 1% DMSO/50 mM Hepes-OH, pH 7.5 buffer, 2.4 μ M UGDH, 10 mM NAD⁺, 0.2 mM UDP-galactose, 1 nM to 100 μ M 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 versus 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 IC₅₀ 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).

The protein could be expressed in, and purified from, *E. coli* (Supplementary Figure S2a). Crosslinking with BS³ 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 crosslinking experiment (Figure 1b) (McCorvie et al. 2012; Scott and Timson 2007; Timson 2005). 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 (Figure 1).

A molecular model of the dimeric protein was generated (Figure 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 *T. 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 stabilised 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 (Figure 3a).

In the absence of added ligands, the melting temperature (T_m) of recombinant FhGALE (3.0 μ M) was 49.5 $\pm$ 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 (Figure 3b). NAD^+ had no measurable effect on the T_m (data not shown).

The activity of FhGALE shows a saturable, hyperbolic dependence on UDP-galactose concentration (Figure 3c). The K_m for UDP-galactose is 470 ± 90 μ M which is higher than the human enzyme (69 μ M (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 Schrodinger's Glide (Friesner et al. 2004; Friesner et al. 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 one increased it; of these one (NSC91378) was shown to affect the rate of the coupling enzyme UDP-glucose dehydrogenase and was not studied further. Six compounds (NSC13575, 16722, 50690, 91355, 116702, 139105) gave unreliable or inconsistent results in concentration-dependence assays and so were eliminated. The ability of the remaining six compounds to inhibit FhGALE and HsGALE was then tested. All six compounds inhibited both enzymes (Figure 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 one (NSC36525) had an IC_{50} in the millimolar range. This identification of high affinity binders in 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 NSC36525 inhibited the human enzyme to a greater extent than the liver fluke one. However, two compounds, NSC31712 and NSC335979, inhibited FhGALE several orders of magnitude more strongly than HsGALE (Figure 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). Destabilisation 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 mM 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-dioxohexahydropyrimidine-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 (Heath et al. 1985; Heidelberger et al. 1958; Muregi et al. 2009; Rathod et al. 1989; 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) (Boeke et al. 1984; Chaudhuri et al. 1958; Heidelberger et al. 1958). In malarial parasites, 5FOA also directly targets and inhibits thymidylate synthase (Rathod et al. 1992).

NSC335979 is the peptide derivative, N-[(benzyloxy)carbonyl]leucyltryptophan (3-(1H-indol-3-yl)-2-[[4-methyl-2-(phenylmethoxycarbonylamino)pentanoyl]amino]propionate; CAS# 5326-00-3). Only limited pharmacological characterisation 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 one was considered inconclusive.

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 selectivity was observed (Figure 5, Table 1).

Conclusions

Overall, FhGALE has similar properties to human GALE and the majority of other GALE enzymes characterised to date. It is a homodimer in solution and is stabilised by substrate binding. Despite these similarities, selective sub-micromolar inhibition could be obtained with two 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 *S. 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 two negative consequences for the parasite: disruption of intermediary metabolism similar to that observed in type III galactosemia 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 (Duffus and Franks 1981; Hanna 1980; Vukman et al. 2013; Wilson et al. 2011). 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

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Dimerisation of FhGALE

FhGALE (90 μ M) 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 one 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.

Figure 2. Predicted three-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.

Figure 3. FhGALE ligand binding and kinetics

(a) The intrinsic fluorescence spectrum of FhGALE (excitation 280 nm) shows two 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 UDP-galactose concentration dependence of the change in melting temperature (T_m) of FhGALE (3.0 μ M). Each point represents the mean of three independent determinations and the error bar the standard errors 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 three independent determinations and the error bar the standard errors of these means.

Figure 4. Six novel inhibitors of FhGALE

In each case, the solid line represents the fit to the data for FhGALE and the dashed line the fit for HsGALE. Each point represents the mean of three independent determinations and the error bars the standard errors of these means.

Figure 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 the fit for HsGALE. Each point represents the mean of three independent determinations and the error bars the standard errors of these means.

Table 1IC₅₀ 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 <i>H</i> -imidazo[4,5- <i>b</i>]pyridine-5,7(4 <i>H</i> ,6 <i>H</i>)-dione	2850-40-0	NSC14303	300±110	13±6
9-[6-(hydroxymethyl)-2,2-dimethyltetrahydrofuro[3,4- <i>d</i>][1,3]dioxol-4-yl]-3,9-dihydro-6 <i>H</i> -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- <i>tert</i> -butylphenoxy)methyl]-1,3-dimethyl-3,7-dihydro-1 <i>H</i> -purine-2,6-dione	na	NSC36525	260,000±550,000	9500±8200
<i>N</i> -(4-ethoxyphenyl)-3-hydroxynaphthalene-2-carboxamide	4711-68-6	NSC50690	61±20	45±23
(3-(1 <i>H</i> -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