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Inhibition of Human DHODH by 4-Hydroxycoumarins, Fenamic Acids, and *N*-(Alkylcarbonyl)anthranilic Acids Identified by Structure-Guided Fragment Selection

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A strategy that combines virtual screening and structure-guided selection of fragments was used to identify three unexplored classes of human DHODH inhibitor compounds: 4-hydroxycoumarins, fenamic acids, and *N*-(alkylcarbonyl)anthranilic acids. Structure-guided selection of fragments targeting the inner subsite of the DHODH ubiquinone binding site made these findings possible with screening of fewer than 300 fragments in a DHODH assay. Fragments from the three inhibitor classes identified were subsequently chemically expanded to target an additional subsite of hydrophobic character. All three classes were found to exhibit distinct structure–activity relationships upon expansion. The novel *N*-(alkylcarbonyl)anthranilic acid class shows the most promising potency against human DHODH, with IC_{50} values in the low nanomolar range. The structure of human DHODH in complex with an inhibitor of this class is presented.

Introduction

Dihydroorotate dehydrogenase (DHODH, EC 1.3.5.2) is a mitochondrial enzyme involved in the rate-limiting step of the de novo biosynthesis of pyrimidine bases.[1] In humans, it has been demonstrated that resting T-lymphocytes meet their metabolic requirement of pyrimidines through a salvage pathway, but for proliferating T-lymphocytes, de novo synthesis of pyrimidines is crucial.[2] Stimulated T-cells switch on their de novo synthetic route to produce additional precursors necessary for RNA and DNA synthesis and other metabolic activities needed for clonal expansion. Inhibition of DHODH decreases cellular levels of rUMP and arrests proliferating T-cells in the G_1 or early S phase of the cell cycle, which results in suppression of the immune system.[3] Inhibitors of human DHODH have potential as drugs against autoimmune diseases and cancer as well as in the treatment of transplant rejection. The most widely known human DHODH inhibitors brequinar (**1**) and A771726 (**2**, the active metabolite of leflunomide) have been developed for the treatment of cancer and rheumatoid arthritis (RA), respectively (Figure 1).[4] However, both compounds have suboptimal properties. Brequinar failed in clinical trails due to a limited therapeutic window.[5] A771726 has a disadvantageous plasma half-life of approximately two weeks in humans and is associated with liver toxicity.[6,7] Furthermore, it cannot be ruled out that tyrosine kinase inhibition also contributes to the in vivo activity of A771726.[8] Thus, it is still of

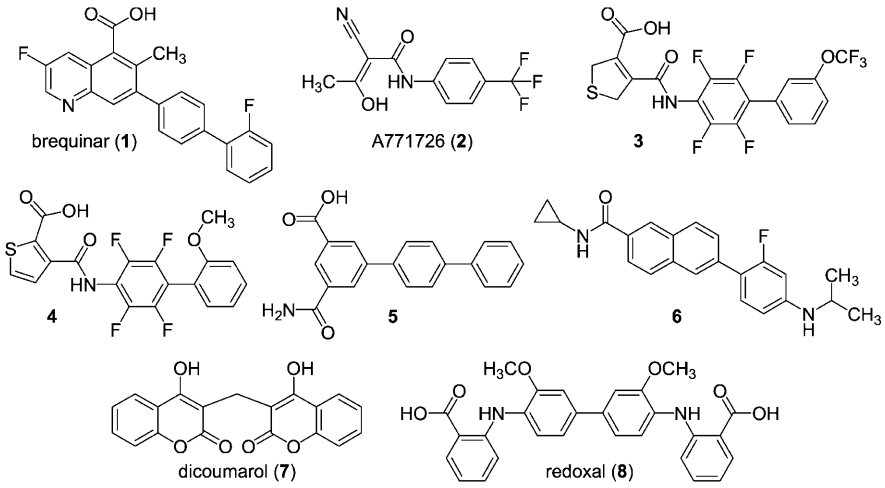

Figure 1. Structures of known inhibitors of human DHODH.

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interest to find potent and selective inhibitors of human DHODH for use as immunosuppressants. The first crystallographic structures of human DHODH in complex with a brequinar analogue and in complex with the active metabolite of A771726 were published in August 2000.[9] In these experiments brequinar and A771726 were found to bind to the site at which the cofactor ubiquinone is believed to bind, and not to the substrate binding site. After publication of the three-dimensional structures, additional compounds that inhibit the human enzyme in the nanomolar or low micromolar range were reported (examples **3**–**6** in Figure 1).[10] Most compounds share one or more common motifs with brequinar: 1) a biphenyl moiety; 2) a quinoline portion, sometimes exchanged for naphthalene; or 3) a carboxylic acid group on quinoline or other aromatic rings. Considering their dissimilarity to the substrate orotate and their structural resemblance to brequinar, all these compounds probably target the ubiquinone binding site.

The aim of our work was to identify, with limited screening effort, novel human DHODH inhibitors that could serve as lead structures for subsequent optimisation as drug candidates. The suggested ubiquinone binding site of DHODH was chosen for the design of the new compounds (Figure 2). This channel-like binding site, as described by Liu et al.[9] and others, is located in a region of the protein that is expected to be involved in membrane association.[11] The membrane association creates a highly hydrophobic environment around the entrance to the binding site. This is also reflected by the hydrophobic character of the beginning (entrance) of the channel, which is surrounded mainly by hydrophobic amino acid side chains (Figure 2). From inspection of the crystal complexes, it can be concluded that this part of the binding site offers hydrophobic interactions with nonpolar groups such as biphenyl in brequinar or *para*-(trifluoromethyl)phenyl in A771726.[9] The channel ends in a narrow cavity that contains the protein redox site next to the redox cofactor flavin mononucleotide (FMN). Structural information available from the DHODH–A771726 or DHODH–brequinar crystal complexes allow the identification of several electron donor/acceptor and ionisable interactions between potential ligands and the inner enzyme region.[9] Virtual screening along with visual inspection of the inner subsite structure was used to guide us in a selection procedure whereby fragments were selected for the DHODH screen. This procedure allowed us to substantially decrease the number of fragments to be tested relative to the commonly used fragment screening, in which libraries with several thousand fragments may be screened against a given target.[12]

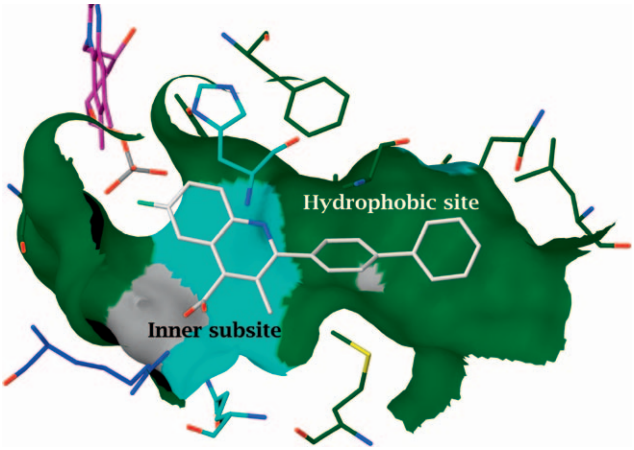


Figure 2. The ubiquinone binding site: The binding mode of brequinar (shown in grey) from crystallographic structure data.[9] Hydrophobic residues and surfaces are coloured green, and water molecules give grey surfaces. The inner subsite (i.e., proximal redox site) that was the target for fragment screening has a high proportion of polar residues and surfaces (coloured cyan). The Arg 136 residue is coloured blue. The redox cofactor flavin mononucleotide (FMN) is shown in magenta.

Results and Discussion

The general procedure for selection of a small fragment library targeting the inner DHODH subsite was divided into two parts (Figure 3): virtual screening and inner subsite-guided extraction of a scaffold. The fragments identified in this procedure were then screened at high concentration in the DHODH enzyme assay.

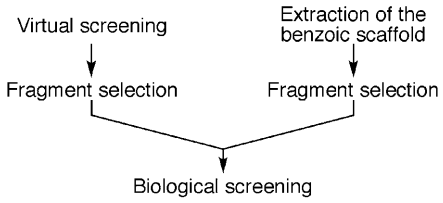


Figure 3. Schematic summary of the procedure used for the selection of fragments for the DHODH screen.

Virtual screening

Virtual screening was based on the crystallographic coordinates of human DHODH in complex with brequinar and A771726 (PDB accession numbers 1D3G and 1D3H, respectively).[9] A notable difference between the inhibitor binding sites in the two structures is the presence of one buried water molecule in the case of brequinar and two buried water molecules in the case of A771726.

Pharmacophores were created by using the structure-based focusing method.[13] A LUDI interaction map was created for the binding site in the absence of inhibitor but in the presence of the buried water molecules.[14] This allowed the identification of hydrophobic, acceptor, donor, and ionisable groups available for interaction with inhibitors. The interaction site vectors and points were subjected to clustering, and the remaining pharmacophore features were selected and combined into twelve different feature combinations. All pharmacophores created contained a total of four or five features, that is, hydrophobic groups, hydrogen bond acceptors, hydrogen bond donors, and a negative ionisable group. The default definitions of the sizes of the pharmacophore features were used. The query also included around 200 excluded volume spheres of 1.2 Å radius. These were positioned on the protein heavy atoms in the vicinity of the binding site.

The Catalyst-formatted databases NCI2000 and Maybridge2001 from Accelrys were screened using the pharmacophore models created. The conformers that superimposed favourably with the pharmacophores and that did not overlap with the excluded volumes spheres were retrieved as hits. The hits were subjected to consensus scoring based on the scoring functions LigScore, LUDI, PLP, and PMF using LigandFit software.[13,15–18] Visual inspection of the proposed interaction with the protein was used together with the consensus score to select fragments for screening and use as scaffolds.

Extraction of the benzoic acid scaffold from the brequinar structure

Brequinar is one of the most potent inhibitors of human DHODH currently known (IC_{50} = 6 nM).[4] It is about 100-fold more potent than A771726 in vitro,[11b,19] and this justified the choice of brequinar as the starting point for scaffold extraction. Benzoic acid, a substructure of brequinar, formed the base for the selection of the major part of the inner subsite-guided fragments (Figure 4). Extraction of benzoic acid was based on analysis of the interactions of the inhibitor with the protein. The salt bridge between the carboxylic acid of brequinar and the guanidine group of Arg 136 was considered to be an important feature for inhibition of DHODH by brequinar; thus, the carboxylic acid was preserved in the scaffold extraction. On the other hand, the biphenyl portion of brequinar was omitted because only the inner subsite was the target for the fragment screen, while the quinoline ring was simplified to a benzene ring. This extraction procedure resulted in the choice of benzoic acid as a scaffold for fragment selection. This scaffold offered many diverse and commercially available derivatives and was expected to generate hits that would, for the most part, be reasonably easy to synthesise and modify. Two target interactions and the geometry relative to the benzoic acid scaffold can be observed from superposition of the DHODH crystal structures with bound A771726 and brequinar (Figure 4).

Selection of fragments

The inner subsite has a high proportion of polar residues (Figure 2), and it is expected that interactions in this part of the enzyme are crucial for the determination of target specificity and inhibitor affinity. Accordingly, it was decided that one criterion for a fragment structure should be the presence of a minimum of two polar functionalities: hydrogen bond donors, hydrogen bond acceptors, and/or negatively ionisable groups. The fragment should also fit the inner subsite, having a suitable size and complementary volume. Starting from benzoic acid, a fragment library was selected by combining up to four types of structural modification (Figure 4). The first modification was the addition of either a functional group or functionality included in the aromatic ring of the benzoic acid scaffold that could create a second polar interaction site. For several reasons, Tyr 356 was identified as the best target for the second polar interaction. Firstly, it seems likely that ubiquinone uses this residue to bind to the inner subsite because, as with Arg 136, Tyr 356 is invariant in family 2 DHODHs.[9] Secondly, the short distance (2.8 Å) between one of the oxygen atoms of bound A771726 and Tyr 356 in the crystallographic structure clearly indicates that the two groups form a hydrogen bond (see Figure 4). Thirdly, the three-dimensional structure of the inhibitor-free enzyme shows that Tyr 356 is not involved in internal hydrogen bond interactions.[20] Based on these data and from visual inspection of the enzyme structure, we could conclude that substituents at position 3 of the benzoic acid moiety should have the best geometry for interaction with Tyr 356. Small groups such as methoxy, hydroxy, nitro, and carbonyl were mainly selected to probe these interactions. Other possible interaction targets were Glu 47, Tyr 147, and His 56.[21]

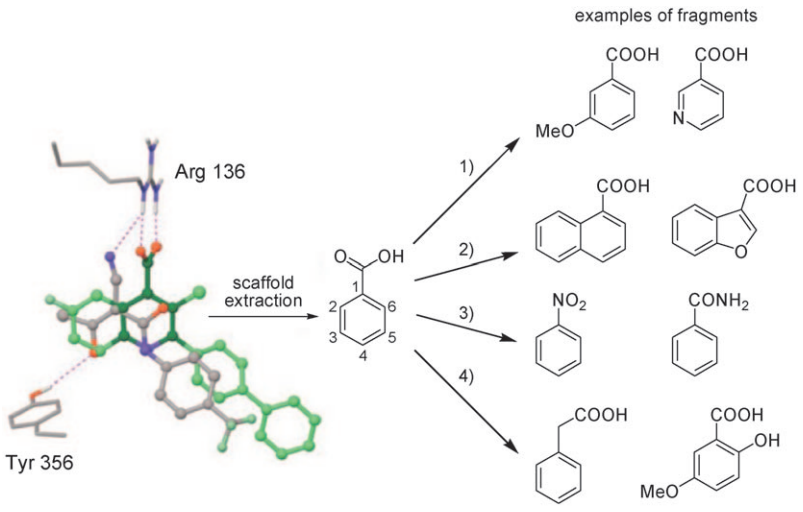


Figure 4. Extraction of the benzoic acid scaffold from the brequinar structure and example of modification to select new fragments. At left is the overlay of bound A771726 (grey) with brequinar (green, with the benzoic acid portion in dark green) from the superimposed crystallographic structure of DHODH together with the relative positions of Arg 136 and Tyr 356.[9] Starting from the benzoic acid scaffold, one of four types of modification is made in each example to form new fragments. Several of the modifications 1)–4) are then combined to produce a diverse set of fragments shown at right.

The second modification involved exchange of the benzoic acid aromatic ring for naphthalene or a heteroaromatic mono- or bicyclic system. About 35% of the compounds selected were based on a bicyclic ring. To fill the same volume in the enzyme as the quinoline ring of brequinar, the second ring was attached mainly at positions 2 and 3 of the benzoic acid.

The third modification involved exchange of the carboxylic acid group. A nitro or hydroxy group was frequently used to model the interaction with Arg 136. Also, cyano groups (as in A771726), carboxamides,

or methyl esters were examined for their ability to form hydrogen bonds with Arg 136.

The fourth modification included random addition of one or several small chemical entities such as methyl, methoxy, hydroxy, amine, halogen, and phenyl, including the insertion of methylene groups between the phenyl and the carboxylic acid functionality in the benzoic acid scaffold (see Figure 4).

In parallel with the target-orientated selection from benzoic acid, virtual screening hits and derivatives thereof were selected. The proposed inner subsite binding parts of some of the hits from the virtual screen were used as scaffolds and treated according to the scheme used for the benzoic acid scaffold above. In both cases, the choice of derivatives was restricted by commercial availability. The final result of the total selection process was a small, target-directed but diverse set of fragments.

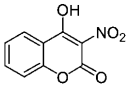
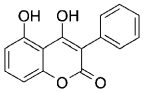
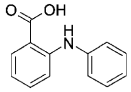
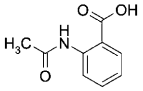
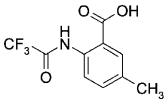
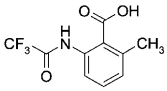
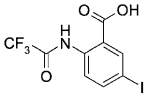
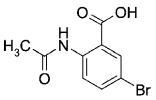
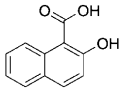
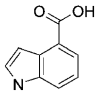
Evaluation in a human DHODH enzyme assay

A DHODH enzyme assay was set up and validated using mitochondrial membrane preparations from the human lymphoma cell line U937 for the initial screening of fragments.[22] To allow detection of low-affinity binders, inhibition of DHODH was measured at concentrations of up to 1 mM, which, in many cases, were close to the given compound's solubility limit. In total, 265 fragments were tested, half of which were selected from databases with commercially available compounds and the rest from an in-house collection of compounds. The molecular weight varied between 123 and 384 Da, and the average of the predicted log*P* values was 2.2.[23] A list of the most potent hits is presented in Table 1.

Three interesting fragment types were identified from the low-affinity screen: 4-hydroxycoumarin derivatives (compounds **9** and **10**), fenamic acid (compound **11**), and derivatives of *N*-(alkylcarbonyl)anthranilic acids (compounds **12**–**16**, Table 1), where the *N*-(alkylcarbonyl)anthranilic acids originate from the virtual screen, and the others originate from the selection based on the benzoic acid scaffold. Figure 5 shows the core fragment structure for each of these three unexplored DHODH inhibitor classes. The known compound dicoumarol (**7**, Figure 1) shares the 4-hydroxycoumarin motif with compounds **9** and **10**; dicoumarol was recently found to impair pyrimidine biosynthesis at the DHODH-catalysed step.[24] The fenamic acid derivative redoxal (**8**), originally found in a tumour cell screen, is the only substance of these classes with a documented value for DHODH inhibition (IC_{50} = 45 nM in recombinant human DHODH), although no SAR has been reported for it.[11b,25,26] Recently, compounds related to fenamic acid have been patented as DHODH inhibitors.[27]

Besides the three found classes, two additional fragments from the benzoic acid scaffold selection, **17** and **18**, were identified as hits. The potency of compounds **17** and **18** could be explained by their close resemblance to the quinoline–carboxylic acid moiety of brequinar. Indeed, a hydroxy derivative of brequinar analogous to fragment **17** has been reported as an anti-arthritic and immunosuppressant in vivo.[28]

Table 1. Selected hits from screening of chemical fragments in a DHODH membrane assay.

	Compound	DHODH Inhibition [%][a] 1 mM	0.1 mM
9		46	18
10		71	38
11		n.d.[b]	85
12		101	40
13		n.d.[b]	100
14		91	68
15		n.d.[b]	96
16		n.d.[b]	79
17		106	78
18		41	14

[a] Assays were run as single samples on two different occasions, and values represent the mean of the duplicate measurements carried out at the indicated compound concentrations. [b] Not determined.

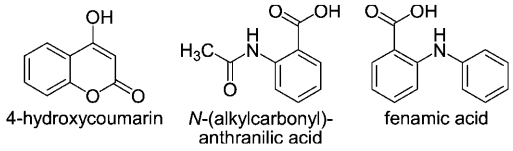


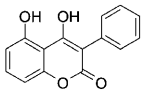
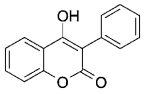
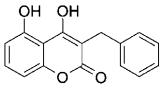
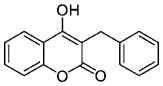
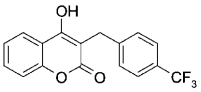
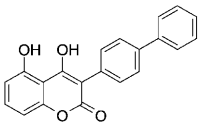
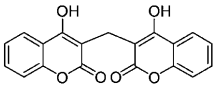
Figure 5. Fragment structures of three compound classes found to inhibit human DHODH activity.

Chemical expansion of fragment hits

The objective of the chemical expansion was to determine the potency of the three inhibitor classes identified (Figure 5) before initiation of a structure optimisation phase that would include extensive efforts involving synthesis. The strategy was to expand the fragments of these compound classes with non-polar moieties pointing towards the hydrophobic part of the ubiquinone binding site. An analysis of possible geometries of

bound fragments using the crystallographic structure of the enzyme was conducted to decide on anchor points for chemical expansion. A few fragments of each class were then merged with a phenyl- or biphenyl-containing moiety at the anchor point, and the derivatives were screened to determine the IC_{50} value for DHODH inhibition (compounds **19**–**32**, Tables 2–4). As expected, successive filling of the hydrophobic pocket gives higher calculated log*P* values. Another general observation was that just expanding the fragment hits with a biphenyl moiety, as in brequinar, gave markedly lower IC_{50} values for all three classes (Table 2–4; compounds **23**, **26**, **27**, and **30**).

Table 2. Inhibition of recombinant DHODH by 4-hydroxycoumarin derivatives.

	Compound	IC_{50} [μM]	LE[a]	Qlog *P*[b]
10		120	0.21	1.96
19		>2000	0.15	2.42
20		11	0.25	2.17
21		860	0.16	2.81
22		190	0.16	3.81
23		0.23	0.27	3.50
7		6.6	0.21	1.79

[a] Ligand efficiencies are calculated as pIC_{50} divided by the number of heavy atoms. [b] Calculated log*P* in octanol/H_2O.[23]

4-Hydroxycoumarin derivatives

4-Hydroxycoumarin derivatives originate from the benzoic acid scaffold. They contain a bicyclic system that can be regarded as a substitute for the quinoline ring of brequinar. In these compounds, the carboxylic group of benzoic acid is exchanged with a hydroxy group. The 4-hydroxycoumarins were expanded at position 3 of coumarin (compounds **19**, **20**, **21**, **22**, and **23**, Table 2) and evaluated for inhibition of DHODH together with the known compound dicoumarol (**7**) as a reference. One interesting feature is the acidity of 4-hydroxycoumarin, with a pK_a value of 5.1.[29] The acidity indicates that the coumarin compounds are negatively charged at neutral pH. The negative charge is delocalised to the enolic hydroxy group as well as the lactone oxygen atoms, and either part could interact with the positively charged Arg 136 (possibly mediated by water). It seems likely that the hydrophobic groups at position 3 of the coumarins bind in the outer hydrophobic subsite of the enzyme, and that this restricts the possible binding conformations of the coumarin portion; this suggests a binding mode that places the lactone part closest to Arg 136 as illustrated for the particularly potent derivative **23** (IC_{50} = 230 nM; Figure 6a). If the coumarin part was rotated 180°, placing the hydroxy groups closest to Arg 136, overlap between the biphenyl groups from brequinar and coumarin **23** would not be possible. It was observed that 4,5-dihydroxycoumarins **10** and **20** are more potent and have higher ligand efficiencies (see Table 2) than the corresponding 4-hydroxycoumarins **19** and **21**, perhaps due to the possibility that the 5-hydroxy group may be involved in additional polar interactions, for example with Tyr 356.

HL-60 leukaemia cells treated with the reference compound dicoumarol (**7**) were recently found to accumulate in the S phase of the cell cycle, due to impairment of pyrimidine biosynthesis at the DHODH step.[24] We determined the IC_{50} to be 6.6 μM, which confirms dicoumarol as a DHODH inhibitor.

Fenamic acid derivatives

Fenamic acid derivatives also originate from the fragment selection based on benzoic acid as scaffold. As mentioned above, the carboxylic acid may form a salt bridge with Arg 136. With such a salt bridge, compound **11** has two possible orientations in the binding site. Either the anilinic phenyl group points inwards in the binding site or it points in the opposite direction, towards the outer and more lipophilic subsite. In compounds **24**, **26**, **27**, and **28** (Table 3), possible hydrophobic interactions were explored in greater detail. Because the anilinic phenyl group in compound **11** has no polar interaction points, the phenyl ring was expected to be directed towards the outer part of the channel. In this binding mode, there is no interaction with Tyr 356; however, it is possible to accomplish this with a small substituent. A close look at Figure 4 shows that substituents at position 5 of fenamic acid should have a good fit for interaction with Tyr 356. To investigate this, two methoxy analogues (compounds **25** and **27**, Table 3) were synthesised. The SAR of these compounds supports the hypothesis that the phenyl ring of compound **11** is directed towards the outer part of the channel. In compounds **27** and **28**, parent fragment **11** was modified with an additional phenyl group to increase the hydrophobic interaction and with a methoxy group to introduce interaction with Tyr 356. This markedly improved the inhibition of DHODH and resulted in IC_{50} values of 39 and 81 nM for compounds **27** and **28**, respectively, as compared with 38 μM for fragment **11**. Subsequently, as part of this work, human DHODH was crystallised in complex with compound **28**, and the structure was published.[20] The binding mode of compound **28**, shown in Figure 6b, is consistent with the assumed interactions.

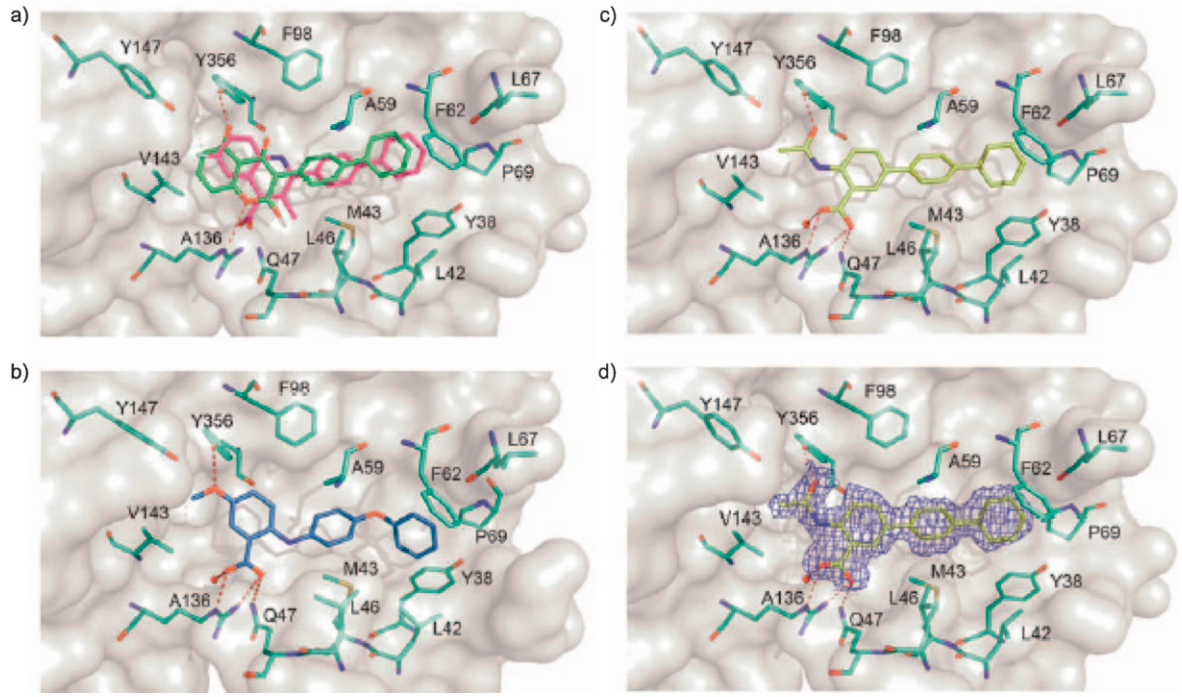
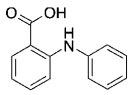
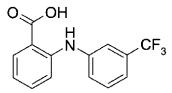
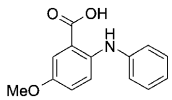
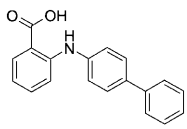
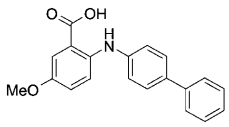
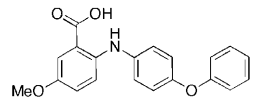


Figure 6. a) Proposed binding mode for coumarin derivative **23**. The lactone part is placed close to Arg 136 to facilitate polar interactions. The biphenyl groups from brequinar (pink) and coumarin derivative **23** (green) occupy almost the same positions in the suggested binding mode; van der Waals surfaces and residues are from the crystallographic structure of DHODH in complex with brequinar.[9] b) Binding site for fenamic acid **28** (blue) in the crystal complex with human DHODH.[20] c) Binding site for anthranilic acid **30** (yellow) in the crystal complex with human DHODH. d) Binding site for anthranilic acid **30** (yellow) in the crystal complex with human DHODH, shown with electron density. The amino acid residues are shown in cyan, and a $3F_o-2F_c$ electron density map contoured at 1.0 σ is displayed in blue mesh. Selected residues within 5 Å of the bound inhibitor are shown in all four panels a)–d); images were generated with the PyMOL software package (The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA (USA), 2002).

Table 3. Inhibition of recombinant DHODH by fenamic acid derivatives.

	Compound	IC_{50} [μM]	LE[a]	Qlog *P*[b]
11		38	0.28	3.68
24		19	0.24	5.24
25		25	0.26	3.57
26		0.19	0.31	4.89
27		0.039	0.31	4.99
28		0.081[20]	0.28	4.94

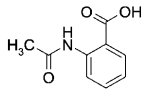
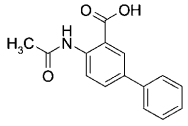
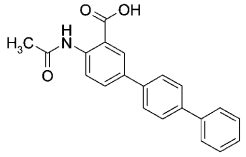
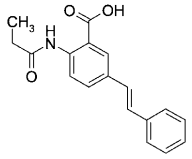
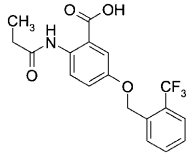
[a] Ligand efficiencies are calculated as pIC_{50} divided by the number of heavy atoms. [b] Calculated log *P* in octanol/H_2O.[23]

N-(Alkylcarbonyl)anthranilic acid derivatives

The *N*-(alkylcarbonyl)anthranilic acids (compounds **12**–**16**, Table 1) were derived from the virtual screen. The results indicate a binding mode in which the negatively charged carboxylate group of the anthranilic acid interacts with Arg 136, and the carbonyl oxygen of the amide group serves as a hydrogen bond acceptor in an interaction with Tyr 356. Comparison of the IC_{50} values for each of the compounds **12**–**16** reveals that position 5 of the anthranilic acid should be a good anchor point for substitution with a lipophilic group directed towards the outer hydrophobic subsite. Thus, compounds **29** and **30** (Table 4) were synthesised to fill the hydrophobic subsite with a considerable gain in potency. Optimisation of the novel *N*-(alkylcarbonyl)anthranilic acid class, in which additional nonpolar moieties were probed in combination with various alkyl substituents in the alkylcarbonyl portion, resulted in compounds with further improved potency. Here, an ethylcarbonyl substituent on the anthranilic nitrogen atom proved beneficial for potency. The optimised anthranilic acids **31** and **32** (with IC_{50} values of 33 and 15 nM, respectively, Table 4) have IC_{50} values that are four orders of magnitude lower than that of parent fragment **12** (190 μM), and compound **31** has the highest ligand efficiency (0.34, Table 4) found in this study.

DHODH was crystallised in complex with compound **30**, and a 1.9 Å resolution data set was collected (coordinates deposited in the Protein Data Bank; PDB ID: 2WV8). The binding

Table 4. Inhibition of recombinant DHODH by *N*-(alkylcarbonyl)anthranilic acid derivatives.

	Compound	IC_{50} [μM]	LE[a]	Qlog *P*[b]
12		190	0.29	1.56
29		8.4	0.27	2.69
30		0.21	0.27	4.01
31		0.033	0.34	3.98
32		0.015	0.30	4.30

[a] Ligand efficiencies are calculated as pIC_{50} divided by the number of heavy atoms. [b] Calculated log *P* in octanol/H_2O.[23]

mode for compound **30** (Figure 6c) can be referred to as brequinar-like in that it occupies basically the same position and involves interaction with Arg 136 with a carboxylic acid group. Thus, the structure also confirms our fragment-based design strategy. The carboxylic acid of compound **30** is rotated by ~30° relative to the position of the previously published complex between brequinar and human DHODH; this is possible because, unlike brequinar, its rotation is not restricted by a methyl substituent at the *ortho* position of the aromatic ring.[9] This facilitates more optimal interactions with Arg 136 and Gln 47, which gets closer to the bound ligand by 0.5–1.0 Å. The distance between the carboxylic oxygen atoms and Arg 136 and Gln 47 is 2.8 and 2.9 Å, respectively. Additional polar interactions are formed between the carboxylic acid of the ligand and the bound water molecule, and also, to a lesser extent, with the carbonyl group of Thr 360 (not shown in Figure 6). One of the key interactions of the anthranilic acids is the hydrogen bond that is formed between the carbonyl oxygen of the amide group and the side chain of Tyr 356 (2.6 Å), which probably contributes to the high potency of the anthranilic acid class. The interactions between both the carboxylic acid and the alkylcarbonyl functionalities on compound **30** with the enzyme give rise to a shift in the position of the biphenyl portion, ~1 Å further out towards the entrance of the binding site relative to brequinar, but no conformational differences in that part of the protein are observed.

Conclusions

A fragment screen was performed with a diverse selection of fragments that target the inner subsite of human DHODH. The selection of fragments was guided by structural analysis of crystallised human DHODH and the binding modes of two inhibitors, A771726 and brequinar. The selection process gave a library of fragments based on either virtual screening hits or a benzoic acid scaffold. From fragment screening of only 265 selected entities, three unexplored DHODH inhibitor classes were identified: 4-hydroxycoumarins, fenamic acids, and *N*-(alkylcarbonyl)anthranilic acids.

The knowledge-based selection of fragments not only decreased the number of compounds to screen in the DHODH assay, but also guided the chemical expansion of the fragment hits. Upon expansion of the fragments with hydrophobic elements targeting the outer hydrophobic subsite of the enzyme, the compound classes identified exhibited distinct SAR with up to 10000-fold difference in potency between expanded fragments and the parent fragment. With gradual filling of the hydrophobic subsite, higher calculated log *P* values were observed, along with a gain in potency. Of the three DHODH inhibitor classes identified, only dicoumarol (**7**) and the fenamic acid redoxal (**8**) are known to affect pyrimidine biosynthesis or inhibit DHODH (Figure 1). Redoxal (**8**) is a known DHODH inhibitor (IC_{50} = 45 nM toward recombinant human DHODH), although no SAR has been reported.[11b,26] Recently dicoumarol (**7**) was found to cause accumulation of treated HL-60 leukaemia cells in the S phase due to impairment of pyrimidine biosynthesis at the DHODH step.[24] An IC_{50} value of 6.6 μM for dicoumarol in our recombinant DHODH assay confirms these findings. In addition, a ~30-fold more potent coumarin, compound **23**, was found in the structure–activity evaluation. The coumarin derivates, however, were not as potent as the other two inhibitor classes found; that is, the best coumarin **23** was about tenfold less potent than the fenamic acids **27** and **28** and the anthranilic acids **31** and **32**. A ligand efficiency of 0.3 (calculated as pIC_{50} divided by the number of heavy atoms) is sometimes considered as a cutoff value for an efficient binder, and four compounds (**26**, **27**, **31**, and **32**) of both the fenamic acid class and the *N*-(alkylcarbonyl)anthranilic acid class fulfill this criterion. The *N*-(alkylcarbonyl)anthranilic acids represent a new class of DHODH inhibitors, and within this class the lowest IC_{50} value was measured: 15 nM for compound **32**.

In this study, where a wealth of structural information could be found on both target protein and different ligands, it was most likely a good idea to complement virtual screening with knowledge-based selection of compounds to increase the number of screening hits. Because the *N*-(alkylcarbonyl)anthranilic acids derive from the virtual screen and the fenamic acids originate from the benzoic acid scaffold, and the classes are almost equally potent, both selection procedures can be regarded as valuable.

Although the best compounds found have chemical features that are similar to those of brequinar, we found a second hydrogen bond, to Tyr 356, which could be important for target specificity. The calculated log *P* value is about one unit lower

for the best anthranilic acid (Clog $P = 4.30$ for compound **32**) relative to brequinar (Clog $P = 5.58$), which indicates that polar interactions contribute more to the potency of this class. Both the second hydrogen bond and the decreased Clog P value are features that could improve the pharmaceutical properties and contribute to a wider therapeutic window of our compound relative to brequinar.

The most potent expanded compounds in each class are promising lead structures for further optimisation as drug candidates. The hydrophobic part of the lead compounds were not subjected to fragment screening, and this part has a particularly high potential to be modified further, to increase potency and optimise the drug properties.

Experimental Section

Chemistry

The following commercially available compounds were included in DHODH screening (the bold numbers in parentheses correspond to compound numbers in Tables 1–4): dicoumarol (**7**), *N*-acetylanthranilic acid (**12**), 3-methyl-6-[(2,2,2-trifluoroacetyl)amino]benzoic acid (**13**), 2-methyl-6-[(2,2,2-trifluoroacetyl)amino]benzoic acid (**14**), 3-iodo-6-[(2,2,2-trifluoroacetyl)amino]benzoic acid (**15**), 4-hydroxy-3-phenylcoumarin (**19**), and 3-benzyl-4-hydroxy-2*H*-1-benzopyran-2-one (**21**) were from Maybridge; 2-acetamido-5-bromobenzoic acid (**16**) was from Lancaster; 4-hydroxy-3-nitrocoumarin (**9**), 2-hydroxynaphthoic acid (**17**), and indole-4-carboxylic acid (**18**) were from Aldrich; *N*-(3-*tri*fluoromethylphenyl)anthranilic acid (**24**) (flufenamic acid) was from Acros; and *N*-phenylanthranilic acid (**11**) was from Fluka. 3-Benzyl-4,5-dihydroxycoumarin (**20**) was prepared as described previously.[30] The synthesis of compound **28** is described in reference [20].

NMR spectra were recorded on a Bruker 500 MHz spectrometer. Chemical shifts (δ), determined from residual solvent peaks, are reported in parts per million relative to $(CH_3)_4Si$. Combustion analysis was performed using a Fisons EA 1108 CHNS-O instrument for all compounds synthesised. Nominal molecular weight ($M+H^+$) for all synthesised compounds was confirmed by electrospray ionisation LC–MS.

4,5-Dihydroxy-3-phenylcoumarin (10): Compound **10** was prepared by heating resorcinol (1.1 g, 10 mmol) and 2-phenylmalonic acid diethyl ester (2.4 g, 10.2 mmol) for 2 h at 250°C. The reaction mixture was allowed to cool to room temperature, and then EtOH (10 mL) was added. The mixture was stirred for 2 h before the crude product was filtered off and washed with a small volume of cold EtOH. Recrystallisation from acetic acid gave the product as ivory-white crystals (140 mg, 5%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 8$–10 (bs, 2H), 7.45 (dd, $J = 8.1$, 1.0 Hz, 2H), 7.39 (t, $J = 8.2$ Hz, 1H), 7.33 (t, $J = 7.7$ Hz, 2H), 7.23 (t, $J = 7.4$ Hz, 1H), 6.80 (d, $J = 8.2$ Hz, 1H), 6.69 (d, $J = 8.2$ Hz, 1H); ^{13}C NMR ([D$_6$]DMSO): $\delta = 165.8$, 162.8, 157.2, 154.2, 133.7, 133.2, 131.6, 128.2, 127.3, 110.8, 108.0, 105.4, 103.1; Anal. calcd for $C_{15}H_{10}O_4$: C 70.86, H 3.96, found: C 70.13, H 3.95.

4-Hydroxy-3-(4-trifluoromethylbenzyl)coumarin (22): A mixture of 4-hydroxycoumarin (240 mg, 1.5 mmol) and 4-(trifluoromethyl)-benzaldehyde (260 mg, 1.5 mmol) was heated for 3.5 h at 150°C in triethylammonium formate (2 mL).[31] The reaction mixture was poured into ice water (20 mL), acidified with dilute HCl (1 M, 5 mL), and the crude product was filtered off. Recrystallisation from EtOH gave the desired product as a white solid (150 mg, 31%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 11.8$ (bs, 1H), 8.01 (dd, $J = 8.0$, 1.1 Hz, 1H), 7.62–7.66 (m, 3H), 7.47 (d, $J = 8.0$ Hz, 2H), 7.39 (m, 2H), 3.98 (s, 2H); ^{13}C NMR ([D$_6$]DMSO): $\delta = 163.7$, 161.9, 153.0, 145.7, 132.9, 129.8, 127.5 (q, $J = 32$ Hz), 126.0 (q, $J = 3.8$ Hz) 125.3 (q, $J = 272$ Hz), 124.8, 124.2, 117.2, 117.1, 104.1; Anal. calcd for $C_{17}H_{11}F_3O_3$: C 63.76, H 3.46, found: C 63.53, H 3.57.

4,5-Dihydroxy-3-(4-biphenyl)coumarin (23): The starting material 2-biphenyl-4-ylmalonic acid diethyl ester was prepared according to a method already described.[32] Compound **23** was synthesised from resorcinol and 2-biphenyl-4-ylmalonic acid diethyl ester according to the procedure for compound **10**, to provide the product as an ivory-white powder (170 mg, 5%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 7.68$ (d, $J = 7.2$ Hz, 2H), 7.57–7.65 (m, 4H), 7.46 (t, $J = 7.7$ Hz, 2H), 7.33–7.41 (m, 2H), 6.80 (d, $J = 8.2$ Hz, 1H), 6.68 (d, $J = 8.2$ Hz, 1H); ^{13}C NMR ([D$_6$]DMSO): $\delta = 166.7$, 162.9, 157.6, 154.2, 141.1, 138.8, 133.24, 133.20, 132.1, 129.8, 128.1, 127.4, 126.4, 110.8, 107.8, 105.6, 102.2; Anal. calcd for $C_{21}H_{14}O_4$: C 76.36, H 4.27, found: C 76.02, H 4.22.

5-Methoxy-2-(phenylamino)benzoic acid (25): 2-Bromo-5-methoxybenzoic acid (690 mg, 3.0 mmol) was reacted with aniline (560 mg, 6.0 mmol) in the presence of copper powder (20 mg) and K_2CO_3 (210 mg, 1.5 mmol) in *N*,*N*-dimethylformamide (DMF; 5 mL) at 150°C for 2 h. The cooled mixture was added to 5 M HCl (5 mL) and ice. After filtration, the crude product was recrystallised from EtOH/H_2O to give a yellow powder (360 mg, 49%). ^{1}H NMR ($CDCl_3$/TFA): $\delta = 10.9$ (bs, 1H), 7.60 (d, $J = 2.9$ Hz, 1H), 7.40 (t, $J = 7.6$ Hz, 2H), 7.32–7.37 (m, 1H), 7.25–7.28 (m, 2H), 7.17–7.25 (m, 1H), 7.15 (dd, $J = 2.9$, 9.1 Hz, 1H), 3.87 (s, 3H); ^{13}C NMR ($CDCl_3$/TFA): $\delta =$ 173.3, 153.9, 140.9, 140.3, 130.2, 125.7, 124.7, 122.7, 120.1, 115.3, 114.0, 56.4; Anal. calcd for $C_{14}H_{13}NO_3$: C 69.12, H 5.39, N 5.76, found: C 68.84, H 5.31, N 5.79.

2-[(4-Biphenyl)amino]benzoic acid (26): 2-Bromobenzoic acid was reacted with 4-bromoaniline according to the procedure for compound **25**. The resulting 2-[(4-bromophenyl)amino]benzoic acid (230 mg, 0.8 mmol) was subsequently coupled using the Suzuki reaction by stirring it at room temperature for 48 h with phenylboronic acid (100 mg, 0.82 mmol), $Pd(OAc)_2$ (2 mg), and Na_2CO_3 (250 mg, 2.4 mmol) in H_2O/1,2-dimethoxyethane (50 mL:12 mL). The solution was filtered and then acidified with dilute HCl (1 M, 5 mL). The product was collected by filtration as an ivory-white powder (150 mg, 65%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 13.1$ (bs, 1H), 9.73 (s, 1H), (dd, $J = 8.0$, 1.5 Hz, 1H), 7.66–7.68 (m, 4H), 7.42–7.48 (m, 3H), 7.32–7.36 (m, 4H), 6.83 (t, $J = 7.5$ Hz, 1H); ^{13}C NMR ([D$_6$]DMSO): $\delta = 170.8$, 147.4, 140.9, 140.5, 135.4, 135.1, 132.8, 129.8, 128.5, 127.8, 127.0, 122.1, 118.6, 115.1, 113.8; Anal. calcd for $C_{19}H_{15}NO_2$: C 78.87, H 5.23, N 4.84, found: C 76.28, H 5.08, N 4.80.

2-(Biphenyl-4-ylamino)-5-methoxybenzoic acid (27): 2-(Biphenyl-4-ylamino)-5-methoxybenzoic acid was synthesised from 2-bromo-5-methoxybenzoic acid according to the procedure used for compound **26**. Recrystallisation from EtOH/H_2O yielded 45% of the title product as light-green crystals. ^{1}H NMR ([D$_6$]DMSO): $\delta = 13.3$ (bs, 1H), 9.3 (bs, 1H), 7.59–7.76 (m, 4H), 7.42–7.47 (m, 3H), 7.35 (d, $J =$ 9.1 Hz, 1H), 7.31 (t, $J = 7.3$ Hz, 1H), 7.24 (d, $J = 8.5$ Hz, 2H), 7.13 (dd, $J = 3.1$, 9.1 Hz, 1H), 3.75 (s, 1H); ^{13}C NMR ([D$_6$]DMSO): $\delta = 170.2$, 152.3, 142.4, 140.8, 140.7, 134.0, 129.8, 128.4, 127.6, 126.9, 122.4, 120.2, 118.5, 116.0, 115.5, 56.3; Anal. calcd for $C_{20}H_{17}NO_3$: C 75.22, H 5.37, N 4.39, found: C 74.85, H 5.30, N 4.45.

2-Acetylamino-5-phenylbenzoic acid (29): A mixture of 2-acetylamino-5-bromobenzoic acid methyl ester (540 mg, 2.0 mmol), phenylboronic acid (240 mg, 2.0 mmol), and $Pd(PPh_3)_4$ (23 mg, 0.02 mmol) in 1,2-dimethoxyethane (20 mL) and 1 M $NaHCO_3$

(7 mL) was heated at 100°C for 10 min. After cooling to room temperature, 5 M NaOH (2.5 mL) was added and stirred overnight for ester hydrolysis, and then H_2O (10 mL) was added before the solution was acidified with 5 M HCl (4 mL). After 1 h the resulting mixture was filtered, washed with H_2O, and recrystallised from EtOH/H_2O to provide the product (380 mg, 74%) as a white solid. ^{1}H NMR ([D$_6$]DMSO): δ=13.8 (bs, 1H), 11.1 (s, 1H), 8.57 (d, J=8.7 Hz, 1H), 8.22 (d, J=3.6 Hz, 1H), 7.90 (dd, J=8.7, 2.2 Hz, 1H), 7.66 (d, J=7.6 Hz, 2H), 7.47 (t, J=7.5 Hz, 2H), 7.37 (t, J=7.4 Hz, 1H), 2.17 (s, 3H); ^{13}C NMR ([D$_6$]DMSO): δ=170.2, 169.4, 141.0, 139.6, 135.0, 132.9, 129.9, 129.6, 128.4, 127.2, 121.4, 117.9, 25.9; Anal. calcd for $C_{15}H_{13}NO_3$: C 70.58, H 5.13, N 5.49, found: C 70.00, H 5.03, N 5.51.

2-Acetylamino-5-(4-biphenyl)benzoic acid (30): A mixture of 2-acetylamino-5-bromobenzoic acid (520 mg, 2.0 mmol), 4-biphenylboronic acid (400 mg, 2.0 mmol), and $Pd(PPh_3)_4$ (72 mg, 0.062 mmol) in 1,2-dimethoxyethane (10 mL) and 1 M $NaHCO_3$ (7 mL) was heated at 100°C for 2 h. The solution was cooled and then acidified with 1 M H_2SO_4 (10 mL). The resulting precipitate was filtered off and washed with a small amount of H_2O. The crude product was then dissolved in EtOAc, filtered through a short column of silica and then concentrated to ~10 mL. Upon standing at room temperature, crystallisation occurred. The resulting ivory-white crystals were filtered off and dried to yield the desired product (230 mg, 35%). ^{1}H NMR ([D$_6$]DMSO): δ=13.8 (bs, 1H), 11.2 (bs, 1H), 8.56 (d, J=8.7 Hz, 1H), 8.27 (d, J=2.4 Hz, 1H), 7.95 (dd, J=2.4, 8.7 Hz, 1H), 7.76 (s, 4H), 7.71 (d, J=7.3 Hz, 2H), 7.48 (t, J=7.7 Hz, 2H), 7.37 (t, J=7.4 Hz, 1H), 2.15 (s, 3H); ^{13}C NMR ([D$_6$]DMSO): δ=170.3, 169.4, 141.0, 140.4, 140.0, 138.6, 134.4, 132.7, 129.9, 129.5, 128.4, 128.2, 127.7, 127.4, 121.5, 118.1, 25.9; Anal. calcd for $C_{21}H_{17}NO_3$: C 76.12, H 5.17, N 4.23, found: C 74.82, H 5.10, N 4.26.

2-Propionylamino-5-[(*E*)-styryl]benzoic acid (31): A mixture of methyl-2-amino-5-bromobenzoic acid methyl ester (6.8 mmol, 1.4 g) and propionyl chloride (12 mmol, 1.1 g) in 1,2-dichloroethane (30 mL) was stirred for 5 h. $CHCl_3$ (30 mL) was added, and the organic phase was washed with 1 M $NaHCO_3$ and H_2O. It was then dried over anhydrous Na_2SO_4, filtered, and concentrated to give the intermediate 2-propionylamino-5-bromobenzoic acid methyl ester (1.6 g, 82%). A mixture of 2-propionylamino-5-bromobenzoic acid methyl ester (3.5 mmol, 1.0 g), K_2CO_3 (3.8 mmol, 0.53 g), tributylamine (3.8 mmol, 0.71 g), $Pd(PPh_3)_2Cl_2$ (0.05 mmol, 35 mg), and styrene (4.2 mmol, 0.44 g) in dry DMF (20 mL) was stirred and heated overnight at 150°C. H_2O (10 mL) and 5 M NaOH (2 mL) were added, and heating at 100°C was continued for 1 h. The mixture was allowed to cool to room temperature and was diluted with H_2O (50 mL), filtered through Celite, and acidified with 5 M HCl (4 mL). The precipitate was filtered off, washed with H_2O and recrystallised from EtOH to provide the title compound (0.35 g, 34%) as an ivory-white powder. ^{1}H NMR ([D$_6$]DMSO): δ=13.8 (bs, 1H), 11.2 (bs, 1H), 8.55 (d, J=8.7 Hz, 1H), 8.17 (d, J=2.1 Hz, 1H), 7.87 (dd, J=8.7, 2.1 Hz, 1H), 7.62 (d, J=7.3 Hz, 2H), 7.38 (t, J=7.7 Hz, 2H), 7.51–7.64 (m, 2H), 7.23 (d, J=16.5 Hz, 1H), 2.43 (q, J=7.5 Hz, 2H), 1.14 (t, J=7.5 Hz, 3H); ^{13}C NMR ([D$_6$]DMSO): δ=172.8, 170.4, 141.1, 137.8, 132.2, 130.2, 129.6, 128.9, 128.5, 128.0, 127.3, 121.0, 117.4, 31.5, 10.2; Anal. calcd for $C_{18}H_{17}NO_3$: C 73.20, H 5.80, N 4.74, found: C 72.59, H 5.70, N 4.76.

2-Propionylamino-5-(2-trifluoromethylbenzyloxy)benzoic acid (32): 2-Amino-5-hydroxybenzoic acid (20 mmol, 3.0 g) dissolved in 0.5 M NaOH (150 mL) was cooled to 0–5 °C. Propionic anhydride (50 mmol, 6.5 g) was added, and the resulting solution was stirred for 10 min and then acidified with 5 M HCl (16 mL). The precipitated material was filtered off and hydrolysed in EtOH (120 mL) and 5 M NaOH (25 mL) at 70°C for 7 h. Most of the EtOH was evaporated, H_2O (100 mL) was then added, and the solution was filtered. After acidification with 5 M HCl (25 mL), the precipitate was filtered off and dried to provide the intermediate 2-propionylamino-5-hydroxybenzoic acid (2.3 g, 55%). A mixture of 2-propionylamino-5-hydroxybenzoic acid (0.24 mmol, 50 mg) and 2-(trifluoromethyl)-benzyl bromide (0.38 mmol, 90 mg) in acetone (1 mL) and 0.5 M NaOH (1 mL) was heated for 4 h at 70°C. MeOH (0.5 mL) and 5 M NaOH (0.5 mL) were added and heated at 70°C for an additional 50 min to hydrolyse the intermediate ester; 5 M HCl (0.7 mL) was added to the warm solution, and the slurry was stirred and allowed to cool to room temperature. The crude product was filtered off, dried, and re-crystallised from MeOH/H_2O (9:1 *v/v*) to provide the title compound (40 mg, 45%) as an ivory-white powder. ^{1}H NMR ([D$_6$]DMSO): δ=13.7 (bs, 1H), 10.8 (bs, 1H), 8.38 (d, J=9.1 Hz, 1H), 7.70–7.76 (m, 2H), 7.73 (t, J=7.5 Hz, 1H), 7.60 (t, J=7.5 Hz, 1H), 7.51 (d, J=3.1 Hz, 1H), 7.29 (dd, J=9.1, 3.1 Hz, 1H), 5.26 (s, 2H), 2.38 (q, J=7.5 Hz, 2H), 1.12 (t, J=7.5 Hz, 3H); ^{13}C NMR ([D$_6$]DMSO): δ=172.4, 169.8, 153.6, 135.6 (d, J=2.5 Hz), 133.7, 131.3, 129.7, 127.7 (q, J=30 Hz), 127.0 (q, J=5.7 Hz), 125.2 (q, J=274 Hz), 122.9, 121.7, 119.1, 116.6, 67.4 (d, J=2.4 Hz), 31.3, 10.3; Anal. calcd for $C_{18}H_{16}F_3NO_4$: C 58.86, H 4.39, N 3.81, found: C 57.87, H 4.34, N 3.84.

Biochemistry

The DHODH enzyme assay was established and validated using mitochondrial membrane preparations from human lymphoma U937 cells for the initial DHODH screening of fragments (Table 1).[22] The results presented are average values of duplicate samples run on two different occasions. Relative standard deviations of mean inhibition were $<$40%. IC_{50} values were determined for expanded derivatives and the most interesting fragment compounds from the low-affinity assay (Tables 2–4). For determination of IC_{50} values, the recombinant DHODH enzyme was purified, and an in vitro enzyme assay was established using N-terminally truncated recombinant human DHODH.[11b] The assay was based on coupling of the ubiquinone reduction to the redox dye 2,6-dichloroindophenol (DCIP), and the reduction of DCIP was monitored photometrically by decreasing absorption at λ 630 nm according to reference [33]. A771726 was used as control, and IC_{50} values varied between 0.7 and 1.1 μM (reported IC_{50}: 0.77±0.08).[11b] The assay was run with duplicate samples on two to five different occasions. Standard errors of mean IC_{50} were usually $<$20% for tested compounds (see the Supporting Information).

Crystallisation of human DHODH in complex with compound 30

Cloning, expression, and purification of human DHODH and co-crystallisation with compound **30** as well as data collection and structure determination were done according to the methods described by Walse et al.[20] (see the Supporting Information). Auto-Dep EBI-41387 has been assigned wwPDB ID 2wv8 for the coordinate entry; we assigned the code r2wv8sf for the structure factors.

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Keywords: dihydroorotate dehydrogenase · drug discovery · fragment screening · inhibitors · structure–activity relationships

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