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Discovery of novel inhibitors for DHODH via virtual screening and X-ray crystallographic structures

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ABSTRACT

Amino-benzoic acid derivatives **1**–**4** were found to be inhibitors for DHODH by virtual screening, biochemical, and X-ray crystallographic studies. X-ray structures showed that **1** and **2** bind to DHODH as predicted by virtual screening, but **3** and **4** were found to be structurally different from the corresponding compounds initially identified by virtual screening.

Dihydroorotate dehydrogenase (DHODH) is an enzyme essential to pyrimidine de novo biosynthesis.[1] It catalyzes oxidative conversion of dihydroorotate to orotate using the co-factors flavin mononucleotide (FMN) and ubiquinone (CoQ) in the redox process. Since blocking pyrimidine biosynthesis has an antiproliferative effect on rapidly dividing cells,[2] inhibitors of human DHODH (*h*DHODH) have been pursued and developed for the treatment of cancer[3,4] and immunological disorders, such as rheumatoid arthritis[5] and multiple sclerosis.[6–8] The DHODH enzymes of parasitic pathogens such as *P. falciparum* (*Pf*DHODH) and *Trypanosoma brucei* are also attractive targets for the development of new therapeutics to combat malaria and sleeping sickness.[9–11]

Some pyrimidine analogs are substrate-based inhibitors that bind to the dihydroorotate binding site,[12] but most reported inhibitors of DHODH bind to the site occupied by the ubiquinone co-factor.[13–30] X-ray crystallographic studies of inhibitor complexes with *h*DHODH and *Pf*DHODH have revealed that known inhibitors, such as brequinar[4] and teriflunomide (the active metabolite of leflunomide),[31] inhibit enzyme activity by displacing the ubiquinone co-factor,[13–16,32–34] either competitively or otherwise.[35] Analogs of brequinar and teriflunomide all contain an acidic head group that interacts with the guanidinyl group of Arg-136 of *h*DHODH, or the corresponding Arg-265 of *Pf*DHODH, at the ubiquinone binding site. An alternative binding mode wherein the acidic group interacts with Tyr-356 has been reported, with some compounds adopting a dual binding mode.[14] This dual binding mode has been observed in a species dependent manner, where the hydroxyl group of teriflunomide interacts with Arg-136 in *h*DHODH but with Tyr-528 in *Pf*DHODH.[33] Compounds with a neutral polar head group have also been reported as DHODH inhibitors,[29,30] including the recently disclosed triazolopyrimidine derivatives[18,20] and S-2678.[21] The X-ray structures showed that the triazolopyrimidine head group acts as a hydrogen-bond acceptor, interacting with the guanidinyl group of Arg-265 in the *Pf*DHODH enzyme.[17]

In a search for novel inhibitors of human DHODH, we carried out virtual screening of the sanofi-aventis compound collection and subsequently tested the selected compounds in a human DHODH enzymatic assay.[36] We also determined the binding mode of selected hits by X-ray crystallography. We report here several amino-benzoic acids as novel inhibitors of *h*DHODH discovered by virtual screening and confirmed by experimental studies.

The software program GLIDE was used for virtual screening with the initial vHTS setting followed by the SP procedure.[37] Virtual screening was carried out by molecular docking (PDB: 1D3G) with constraints of H-bonding interaction to the side-chain of either Arg-136 or Tyr-365 and a hydrophobic pharmacophore feature 6–8 Å away from the hydrogen-bond. Selected compounds were tested in the human DHODH enzymatic assay,[36] which resulted in 200 hits with IC_{50} <10 μM (in comparison to an IC_{50} of 3 nM for brequinar). Four of these hits are shown in Table 1.

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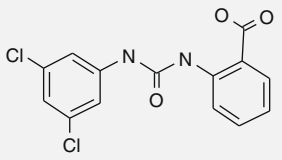
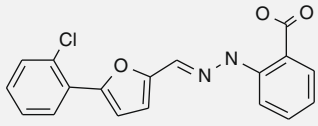
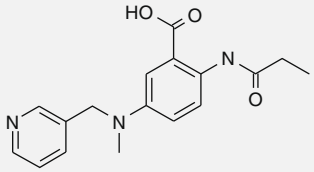
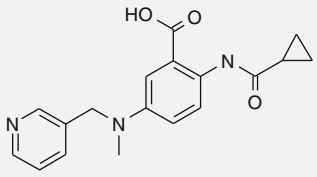
Table 1
Amino-benzoic acid inhibitors for the *h*DHODH

Structure	Compound	IC_{50} (μM)
	**1**	0.7
	**2**	0.5
	**3**	5.5[a]
	**4**	0.1[a]

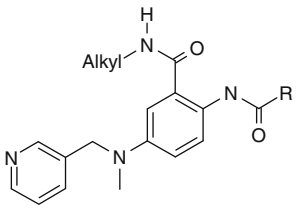
[a] IC_{50} value measured from the initial sample of the presumed amide precursors (see text).

The binding modes of the compounds were revealed by co-crystal structures with *h*DHODH, determined by X-ray crystallography.[38] The compounds are found at the coenzyme Q site, as expected. L-dihydroorotate (DHO), and flavin mononucleotide (FMN) are also observed in the structures which were solved at a resolution of 1.9–2.1 Å. The overall position and the orientation of the acid group toward Arg-136 is similar to that previously observed for brequinar in human DHODH (1D3G).[32]

For compound **1** (shown in Fig. 1), a hydrogen-bond network is observed around the acidic group that makes bifurcated hydrogen-bonds to the guanidinyl group of Arg-136. One carboxylate oxygen atom forms a hydrogen-bond to a crystallographic water molecule, which hydrogen-bonds to Gln-47. The other carboxylate oxygen hydrogen bonds to a second crystallographic water, which in turn hydrogen-bonds to the NHs of the urea linker of the inhibitor and the backbone carbonyl oxygen of Thr-360.

The benzoic acid phenyl ring lies in a hydrophobic pocket formed by the flavin mononucleotide and the side-chains of Val-134 and Tyr-356, limiting potential substitutions on the ring that could increase potency. In contrast, the dichlorophenyl ring points toward the aqueous phase (Fig. 2) and offers several opportunities for structural modification to improve the physical properties of the compounds.

A similar hydrogen-bond network is observed around the acidic group of inhibitor **2** (Fig. 2); the crystallographic water is in a position nearly identical to that observed with compound **1**, being hydrogen-bonded to the benzoic *ortho*-NH. Compared with **1** (in purple), inhibitor **2** (in yellow) extends further toward the solvent, as a result of the interposition of a furan ring. The aromatic chlorophenyl group may be favored in this position, due to its proximity to the side-chains of Leu-42, Tyr-38, Leu-68 (hidden behind inhibitors in Fig. 2), and Phe-62, which form a hydrophobic pocket near the entrance to the ligand binding site. The central region of the inhibitor and the furan ring do not form any close-contact interactions with the protein. The chlorine of the phenyl ring is buried and is 3.0 Å from the side-chain hydroxyl of Thr-63. The carbon of the chlorophenyl ring *para* to the furan is fully exposed to the solvent.



3m (R=ethyl)
4m (R=cyclopropyl)

Compounds **3m** and **4m** were selected by virtual screening. When tested, both compounds showed good activity (IC_{50}s of 5.5 and 0.1 μM, respectively). However, when the amides **3m** and **4m** were re-synthesized, they showed weak inhibition in the *h*DHODH enzymatic assay, suggesting that the activities of the initial samples may be attributed to impurities or hydrolyzed products.

Indeed, the *N*-alkyl group is not seen in the original crystallographic electron density omit map prior to positioning either inhibitor in *h*DHODH, nor can it be found in the final maps. Rather, the electron density maps are consistent with hydrolysis of the amide to the acid. Once the scaffold was clearly defined, compound

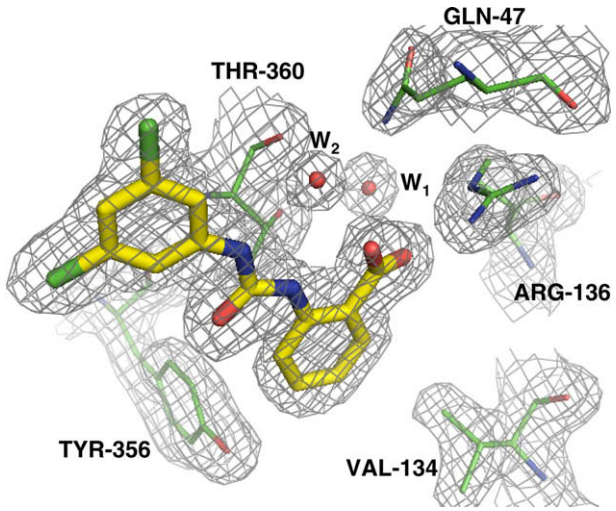


Figure 1. Binding mode of compound **1** (thick stick) with DHODH and water molecules (red balls) revealed by X-ray crystallography. $2F_0–F_c$ electron density contoured at 1σ.

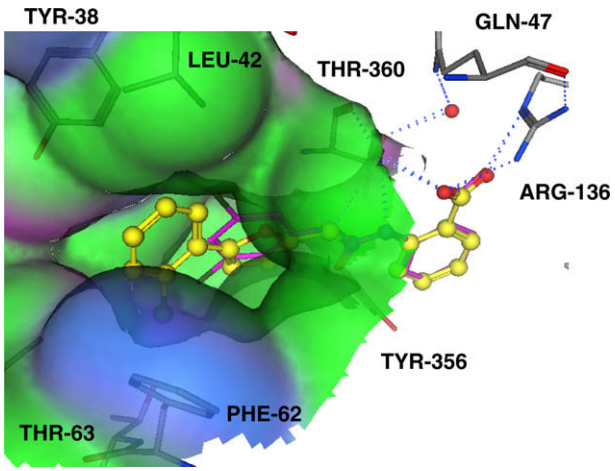


Figure 2. Binding mode of compound **1** (purple) and **2** (yellow) with DHODH determined by X-ray crystallography.

3 was fitted within the electron density, leaving no space for the *N*-alkyl group. The final electron density map is fully consistent with the structure of **3** (shown in Fig. 3a), including the conformations around the ethyl-amide and the N-linked methylpyridine.

In contrast to the initially docked precursor amide **3m**, where the pyridine nitrogen was predicted to interact with Arg-136 (Fig. 3b), the pyridine nitrogen of **3** is hydrogen-bonded to the side-chain hydroxyl of Thr-63. Both carboxylate oxygen atoms of **3** are hydrogen-bonded to the side-chain of Arg-136; one oxygen atom forms a hydrogen-bond to Gln-47 and the second to a crystallographic water molecule. The carbonyl oxygen of the ethyl-amide is hydrogen-bonded to Tyr-356 hydroxyl. The corresponding NH forms an internal hydrogen-bond to the carboxylate oxygen (2.5 Å).

The crystallographic water molecule near the side-chain of Arg-136 in **3** is in the same position (W1 in Fig. 1) as that observed in **1** and **2**. In a comparison with **1**, the acidic group of **3** adopts a different orientation relative to the Arg side-chain (Fig. 4) and the second crystallographic water of **1** (W2 in Fig. 1) is displaced by one of the oxygen atoms of the acidic group in **3**. This altered orientation appears to be the result of steric constraints that prevent the amidoethyl group from burying more deeply into the protein, thus pushing the carboxylate away from the position it adopts in the benzoic acids. The end result is that the acidic group is not as well positioned for interactions with the side-chain of Arg-136 as is the corresponding group in brequinar or other related compounds.

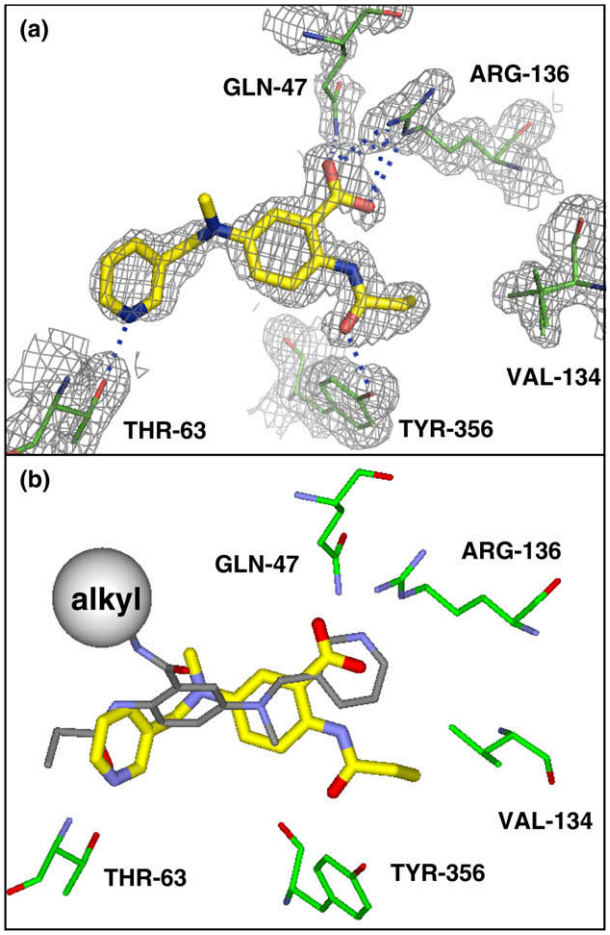


Figure 3. (a) Binding mode of compound **3** (yellow) with DHODH determined by X-ray crystallography $2F_o-F_c$ (electron density countoured at 1σ). (b) Predicted binding mode of corresponding alkyl amide **3m** by GLIDE docking (gray).

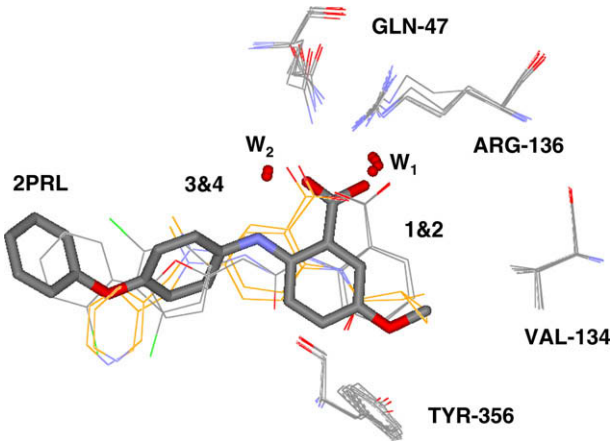


Figure 4. Superposition of compounds **1** and **2** (gray wire-frame), **3** and **4** (orange wire-frame) and inhibitor of 2PRL (gray stick) with water molecules (red balls) and selected DHODH residues.

Similarly, the *N*-alkyl group of the presumed amide derivative **4m** is not seen in the electron density map, which is consistent with the structure of the acid **4**. The resulting structure of **4** is the same as that of **3** (including the lengths of the hydrogen-bonds), except for the amidocyclopropyl group in place of amidoethyl. The cyclopropyl ring lies in a hydrophobic pocket and points toward Val-134. It appears that the suboptimal orientation of the carboxylate is partly compensated by hydrogen-bonding interactions of the pyridine with Thr-63 and the amide oxygen with Tyr-356. The near perfect fit of the cyclopropyl ring in a hydrophobic pocket may explain the improved activity of compound **4** relative to compound **3**.

Figure 4 shows the superposition of compounds 1–4 with a recently reported amino-benzoic acid inhibitor in complex with *h*DHODH (PDB: 2PRL, with a reported IC_{50} of 81 nM).[34] The acidic group is seen to span a range of orientations, all of which maintain hydrogen-bonds to the guanidinyl group of Arg-136. The specific orientation appears to be related to the size of the group that buries in the pocket. The acidic group of compounds **1** and **2** is most deeply buried, as no steric hindrance from a substitution on the benzoic acid is present. In compounds **3** and **4**, the acidic group is pushed away from the buried position by the relatively large substitution in the *ortho* position. 2PRL has a substitution of intermediate size, which results in an orientation intermediate between those reported for the current sanofi-aventis compounds.

Although all of the amino-benzoic acid scaffolds occupy the same binding pocket, subtle changes (such as ethyl to cyclopropyl) have dramatic effects on inhibitory activities. The potency of the cyclopropyl analogue may be attributed to an accessible bisected conformation[39] or, simply result from a snug hydrophobic packing of the cyclopropyl group.

In summary, the repertoire of carboxylic acid inhibitors for DHODH was expanded by the discovery of *meta*- and *meta*–*ortho*-substituted benzoic acids by means of structure-based virtual screening and X-ray crystallographic structures of the bound ligand complexes. The structure–activity information gained from these inhibitors is expected to aid the discovery of novel DHODH inhibitors that may be effective therapeutics for cancer and immunological disorders.

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35. McLean, J. E.; Neidhardt, E. A.; Grossman, T. H.; Hedstrom, L. *Biochemistry* **2001**, *40*, 2194.
36. The assay method used for screening was based on that published by the group of Monika Loeffler et al. (Bader, B. et. al., *Protein Expr. Purif.* **1998**, *13*, 414 and Ref. 16), from whom the human DHODH was initially purchased. In this method, a blue indicator dye (2,3-dichlorophenolindo-phenol, DCIP) is substituted for the co-factor flavin normally found in the biological system. The DCIP turns from dark blue to clear during the course of the enzyme reaction and this can be monitored by absorbance at 600 nm. Since the reduced DCIP is eventually re-oxidized, a kinetic assay run over the first few minutes of the reaction was used for compound screening.
37. Schrodinger, Inc., New York, NY, USA (www.schrodinger.com).
38. Recombinant *h*DHODH was prepared essentially as described by Liu et al. (Ref. 32), with the inclusion of NV-10 to minimize aggregation. The protein was concentrated to 21 mg/mL in 50 mM Hepes, pH 7.7, 300 mM NaCl, 10% glycerol, and 10 mM *N*,*N*-dimethylundecylamine-*N*-oxide. Apo-crystals and co-crystals were obtained by hanging drop vapor diffusion by mixing equal volumes of protein and reservoir solution (2.4–2.5 M ammonium sulfate, 15% glycerol, 0.1 M sodium acetate, pH 5.0–5.2, 40 mM *N*,*N*-dimethylundecylamine-*N*-oxide, 20.8 mM *N*,*N*-dimethyldecylamine-*N*-oxide) at 25 °C. Data were collected either with a Rigaku R-Axis IV++ image plate and RU-H3R X-ray generator equipped with Xenocs Fox-2D optics or at the Advanced Photon Source (APS). Data were processed with HKL2000 and CNX. Molecular replacement (MOLREP) used 1D3G as starting structure. Coordinates have been deposited and are available from the PDB using accession code 3KVK (**1**), 3KVM (**2**), 3KVL (**3**), and 3KVJ (**4**).
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