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ORIGINAL PAPER

The role of fluorine in stabilizing the bioactive conformation of dihydroorotate dehydrogenase inhibitors

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Abstract Dihydroorotate dehydrogenase (DHODH) is an important drug target due to its prominent role in pyrimidine biosynthesis. Leflunomide and brequinar are two well-known DHODH inhibitors, which bind to the enzyme in the same pocket with different binding modes. We have recently realized a series of new inhibitors based on the 4-hydroxy-1,2,5-oxadiazole ring, whose activity profile was found to be closely dependent on the degree of fluorine substitution at the phenyl ring adjacent to the oxadiazole moiety; a positive influence of fluorine on the DHODH inhibitory potency was observed previously [Baumgartner et al. (2006) J Med Chem 49:1239–1247]. Potential energy surface scans showed that fluorine plays an important role in stabilizing the bioactive conformations; additionally, fluorine influences the balance between leflunomide-like and brequinar-like binding modes. These findings may serve as a guide to design more potent DHODH inhibitors.

Keywords DHODH inhibitor · Bioactive conformation · PES scan · Fluorine · Strain energy

Introduction

Dihydroorotate dehydrogenase (DHODH) is a flavine-containing enzyme that catalyzes the stereospecific conversion of (*S*)-dihydroorotate (DHO) to orotate (ORO). Electrons resulting from this oxidation are transferred to ubiquinone (CoQ) and finally to the cytochrome *c* oxidase of the respiratory chain [1]. Since this transformation is the rate-limiting step of the de novo pyrimidine biosynthesis, DHODH has become an appealing pharmaceutical target; its inhibition leads to antiproliferative and immunomodulatory effects, which can be exploited for the treatment of autoimmune diseases [2, 3]. The best known DHODH inhibitors are leflunomide (**1**) and brequinar (**2**) (Fig. 1). The former is a prodrug used widely in the treatment of rheumatoid arthritis; upon absorption, it undergoes ring opening to its active metabolite A771726 (Fig. 1, **3**) [3]. The latter was developed for cancer therapy and to combat the rejection of organ transplants, but failed in clinical trials due to its narrow therapeutic window [4].

Previous crystallographic studies showed that DHODH has two distinct binding sites: one for DHO/ORO and one for ubiquinone. Both A771726 and brequinar bind the protein at the narrow end of the pocket used by ubiquinone to interact with the reduced coenzyme: this channel contains lipophilic amino acids, especially leucines and valines, and several polar residues such as Gln47, Arg136, His56, Tyr356 and Thr360. The deprotonated enolic group of A771726 interacts via hydrogen bonding with the phenolic moiety of Tyr356, while the amide carbonyl forms a water-mediated hydrogen bond with Gln47 and Arg136 (Fig. 2a). The binding mode of brequinar is quite different: the carboxylate group forms a salt bridge with Arg136 and a hydrogen bond with Gln47, while the biphenyl moiety establishes a number of hydrophobic interactions with the lipophilic residues of the channel (Fig. 2b) [5].

Our research group has recently explored the possibility of using the 1,2,5-oxadiazole ring (furazan) as a bioisoster of the isoxazole moiety present in **1**. Although, like leflunomide, these compounds undergo ring opening under physiological conditions, the resulting products proved to

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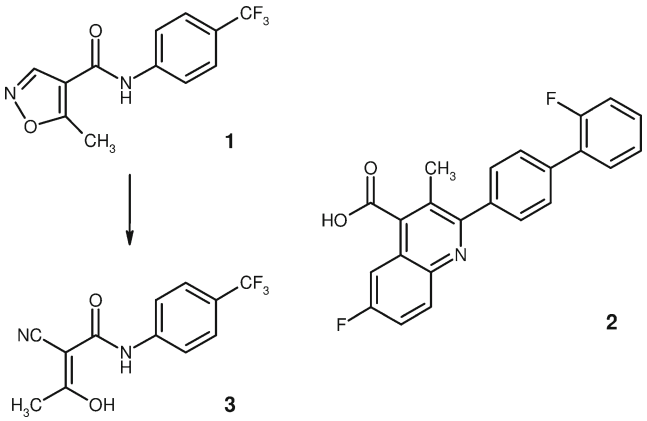


Fig. 1 Structures of leflunomide (**1**), its active metabolite A771726 (**3**), and brequinar (**2**)

be very poor DHODH inhibitors [6]. In order to improve their activity, the unsubstituted furazan moiety was functionalized with a hydroxyl group. The hydroxyfurazan system, which is stable under physiological conditions, should potentially maintain the correct orientation of the deprotonated hydroxyl group, mimicking the enolic moiety of A771726 [7]. In the attempt to validate this hypothesis, a docking simulation was carried out using both rat and human enzymes. All these inhibitors appeared to bind the protein in a brequinar-like fashion, with the deprotonated hydroxyl group facing Arg136. However, using a different X-ray structure of human DHODH as docking target, a 180° flip of the hydroxyfurazan moiety was observed, such that the enolate group interacted with Tyr356 in a leflunomide-like fashion [7]. Marked variations of the ligand binding mode upon minor structural modifications were observed also by Baumgartner and co-workers [8] on another series of inhibitors based on a fluorinated biphenyl scaffold. In order to shed light on the relationship between the structure of these inhibitors and the binding mode they adopt in the DHODH pocket, we analyzed their complexes with the human enzyme crystallized by Baumgartner (Table 1). These molecules bind the protein in a leflunomide-like or brequinar-like fashion, and some of them show both binding modes at once. The authors linked the in vitro activity data with the prevailing mode of binding that the molecule adopts inside the DHODH pocket: the more brequinar-like it is, the more active the inhibitor [8]. However, we noticed that the inhibitory activity of these compounds is also related to their substitution pattern, especially in the ortho–ortho' positions of the central phenyl ring. It is well known that flexible molecules do not bind the protein in their lowest energy conformation [9]. The energy difference between the bioactive conformation and the global minimum in solution configures a strain energy penalty; its magnitude is inversely related to the activity of the molecule [9]. In an attempt to investigate if these considerations hold true for DHODH, a systematic conformational study was carried out on Baumgartner's series of inhibitors, enhanced by two virtual models (**9** and **10**, Fig. 3) lacking fluorine atoms on the central aromatic ring.

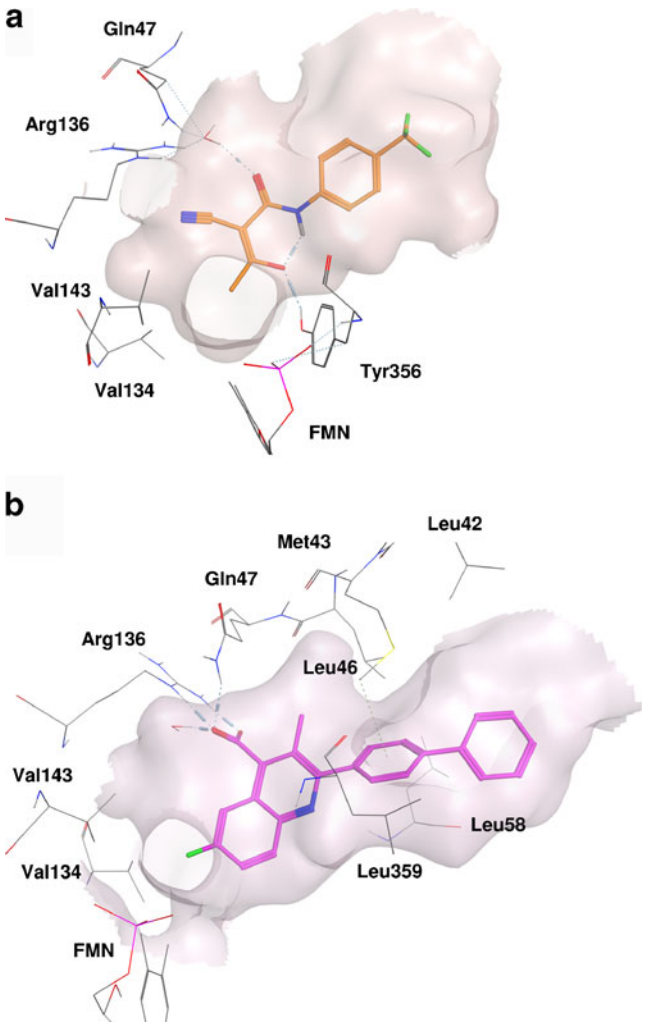


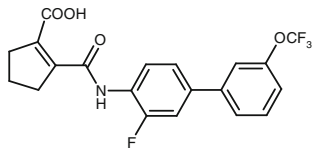
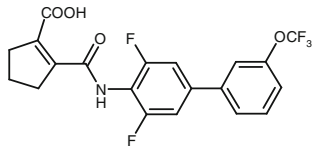
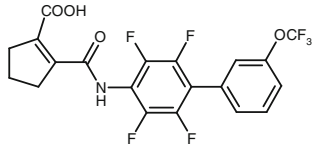
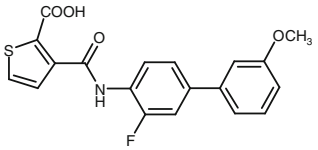
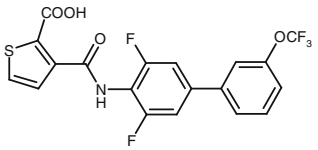
Fig. 2 Binding mode of A771726 (**a**) and a close analogue of brequinar (**b**) inside dihydroorotate dehydrogenase (DHODH) (PDB IDs 1D3H and 1D3G, respectively)

Methods

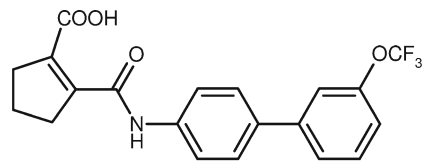
Conformational search

All molecules were modeled in their dissociated form, in accordance with their pK_a values [6]. For compounds **4**–**8** (Table 1) crystallographic coordinates were available [8], while 3D models **9** and **10** were built with the MOE modeling suite [10], removing fluorine from compounds **4** and **7**, respectively. A gas phase optimization of all structures was carried out using the Newton-Raphson method (MMFF94s force field, dielectric constant 4.0, no non-bonded cut-off) until the gradient was lower than 0.05 kcalmol^{-1} Å^{-1}. In order to identify the most stable geometries, a systematic

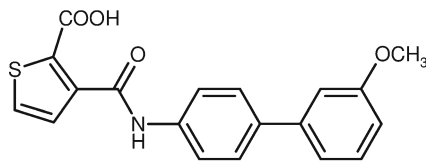
Table 1 Baumgartner's series of dihydroorotate dehydrogenase (DHODH) inhibitor discussed throughout this work (adapted from ref. [8])

	Compound	PDB entry code	Resolution (Å)	IC_{50} (nM)	Binding mode
4		2BXV	2.15	280	leflunomide-like
5		2FPT	2.40	33	leflunomide-like + brequinar-like
6		2FQI	1.95	7	leflunomide-like + brequinar-like
7		2FPV	1.80	44	leflunomide-like + brequinar-like
8		2FPY	2.00	2	brequinar-like

conformational search was carried out by means of a two-step procedure. In the first step the two torsional angles C3-N4-C5-C6 (ϕ) and C7-C8-C11-C12 (ψ) (see Fig. 4) were varied over 10° increments, obtaining 1,296 conformers. These structures underwent a constrained geometry optimization blocking the two dihedrals at their initial values, while the rest of the molecules was allowed to relax. A quantum-mechanical (QM) single-point DFT calculation at the RB3LYP/6-31G(d) level of theory was run on the MMFF94s minimum energy geometries, thus obtaining two potential energy surfaces (PES), one purely molecular mechanical (MM) and the other MM/QM. Once the local minima were identified from the MM/QM PES, they were fully relaxed through a second unconstrained DFT optimization carried out at the same level of theory. Once the stationary points were characterized as true minima through a Hessian matrix calculation, potential energies were refined through single-point calculations at the RB3LYP/6-311G(2d, 2p) level. All QM calculations were performed using FIREFLY [11]. Energy values for each structure were reported relative to the global minimum.



9



10

Fig. 3 Virtual models lacking aromatic fluorine atoms added to Baumgartner's series

Docking simulation

The starting conformations of **9** and **10** used for docking simulation were obtained refining the MM local minima by an ab initio QM optimization at the RHF/6-31G(d) level of theory using FIREFLY [11]. Atom-centred charges were fit to the ab initio electrostatic potential through the RESP method [12].

The experimental crystallographic structures of DHODH complexes used as docking targets were retrieved from the Protein Data Bank (PDB IDs 1D3G and 2BXV; resolutions

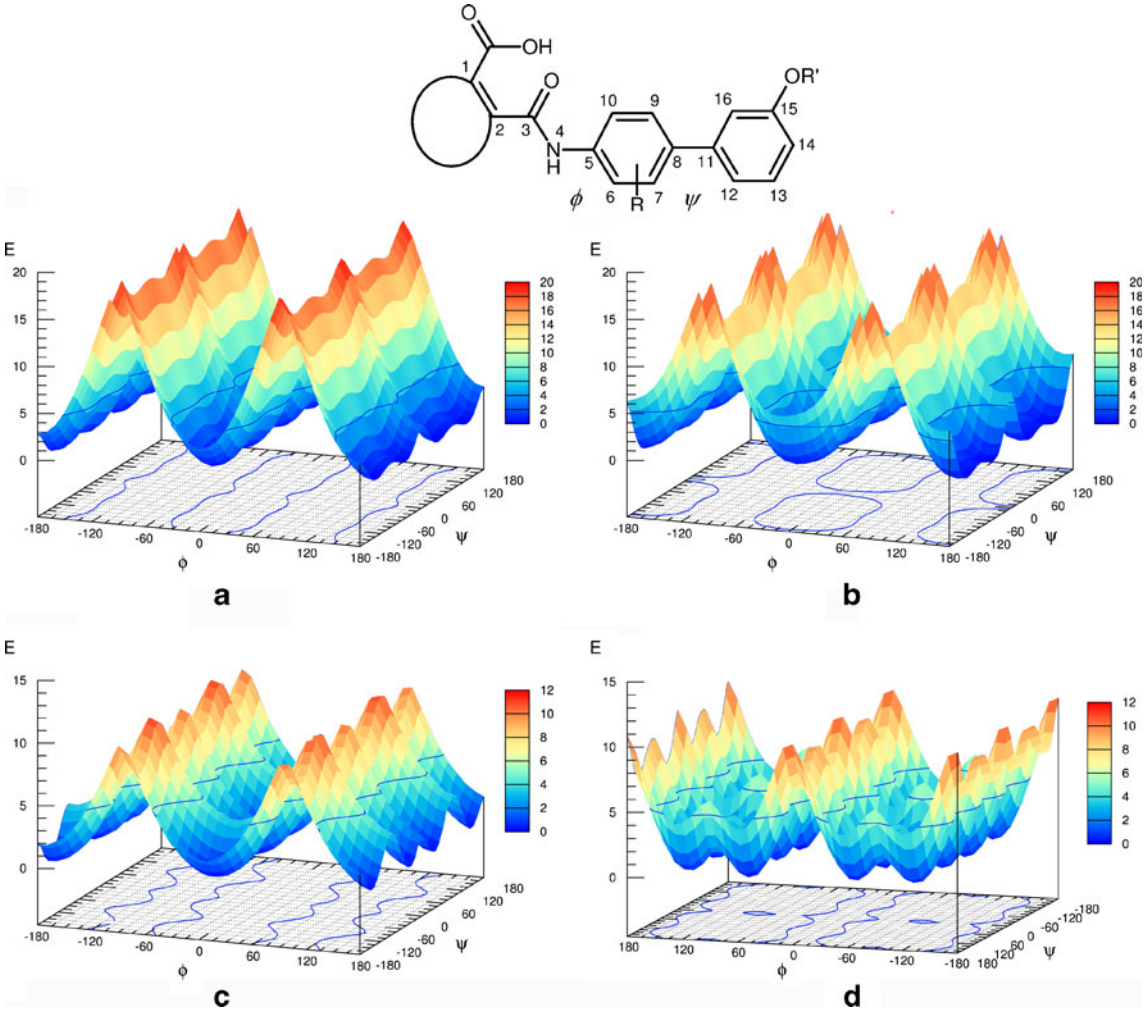


Fig. 4 Molecular mechanical (MM) potential energy surfaces (PES) for compounds **9** (**a**) and **6** (**b**) and their respective MM/QM (quantum mechanical) curves (**c** and **d**). The potential energy values relative to the global minimum (kcal mol^{-1}) are reported on the z axis vs the torsional angles ϕ and ψ (values expressed in degrees)

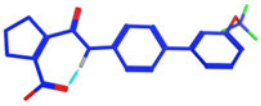
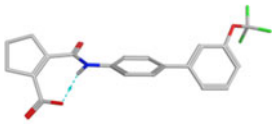
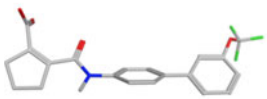
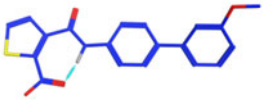
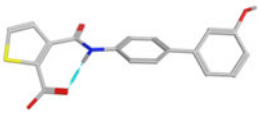
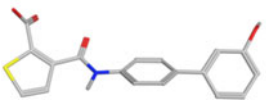
1.60 Å and 2.15 Å, respectively) [13]. Missing hydrogen atoms were added in standard positions, then optimized using the SANDER module of the AMBER 10 software package [14], while keeping heavy atoms harmonically constrained to initial crystallographic coordinates with a force constant of 32 kcal mol^{-1} $Å^{-2}$. AMBER FF99 parameters and charges were assigned to protein atoms, GAFF parameters coupled with QM-fitted RESP charges [12] were used for the co-crystallized inhibitor and ORO, while values for the FMN cofactor were taken from literature [15]. After removing the co-crystallized inhibitor, docking of **9** and **10** was carried out using AutoDock 4.2 [16]. A 40×40×40 grid with 0.375 Å step size was centered on the inhibitors' binding site and energy grid maps were pre-computed with AutoGrid, then flexible docking was accomplished with AutoDock. The target proteins were kept rigid, while ligands were left free to explore the conformational space inside the DHODH cavity; 100 separate docking simulations were run on each protein using the Lamarckian genetic algorithm with default parameters. This docking protocol was able to closely reproduce the poses of the co-crystalllized ligands present in 1D3G and 2BXV (RMSD 0.60 Å and 0.42 Å, respectively; Fig. S1, Electronic supplementary material).

Results and discussion

MM PESs generated using the MMFF94s force field looked very similar to each other; in particular, the fluorine atoms seemed not to exert any significant effects on the conformational preferences of the molecules (Fig. 4a,b). These results are in contrast with the well-known effect of fluorine atoms on aromatic rings, especially when they occupy the ortho–ortho' positions [17].

In contrast, MM/QM PESs were dramatically different from the purely MM ones (Fig. 4c,d); most importantly, the

Table 2 Stable conformers of compounds **9** and **10** as obtained by density functional theory (DFT) optimization (**a**; *blue*) and docking simulation (**b**, **c**; *light grey*)

Conformer		Description	ϕ [a]	ψ [a]	ΔE [b]
9a		DFT-optimized structure	+179.00	-32.03	0.00
9b		leflunomide-like docked pose	+170.55	+52.78	+0.96
9c		brequinar-like docked pose	+134.80	+42.12	+24.95
10a		DFT-optimized structure	+178.50	+33.61	0.00
10b		leflunomide-like docked pose	+173.09	+49.00	+0.62
10c		brequinar-like docked pose	+154.50	+48.46	+18.88

[a] Torsional angles are expressed in degrees; see Fig. 4 for the definition of ϕ and ψ

[b] Potential energies for each cluster of conformers are expressed in kcal mol^{-1} relative to the global minimum; see Methods for details

QM method was able to put into evidence the effect of fluorine substituents on the central phenyl ring, as expected. This effect is indeed impressive, since the torsional angles that yielded minima on PESs of non-fluorinated compounds correspond to maxima when fluorines are introduced in the structures. In light of these considerations, only the MM/QM PESs will be discussed further.

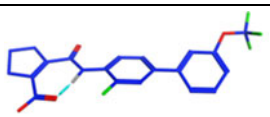
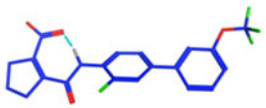
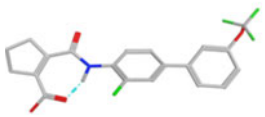
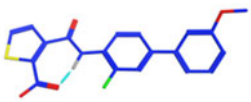
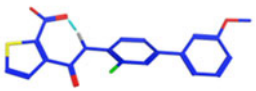
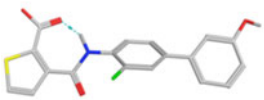
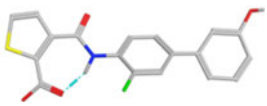
Non-fluorinated models

PESs of models lacking aromatic fluorine atoms (Fig. 4c) showed two symmetrical minima at 0 and 180° along the ϕ dihedral, while the ψ vs E curve is characterized by two symmetric pairs of minima due to the presence of the meta substituent in the distal phenyl ring; the same trend can be observed in all other PESs. In global minimum conformations, the amide group lies in the same plane as the phenyl moiety (**9a** and **10a**, Table 2), allowing the formation of a charge-enhanced hydrogen bond between the deprotonated carboxylic group and the amide hydrogen.

To avoid biasing the outcome of our simulations towards either brequinar-like or leflunomide-like poses, we decided to carry out docking of compounds **9** and **10** on 1D3G and 2BXV protein templates whose co-crystallized ligands show both binding modes. As expected, the binding mode thus obtained was dependent on the protein used as target, just as described above for the hydroxyfurazanyl inhibitors. Both brequinar-like and leflunomide-like putative bioactive conformations are tilted around ϕ, since the constraints imposed by the enzyme cavity do not allow the amide group and the central phenyl ring to lie in the same plane; however, the extent to which coplanarity is lost is quite different. In leflunomide-like poses (**9b** and **10b**, Table 2) the amide portion is tilted by less than 10°, making docked poses fairly superimposable to the global minima in gas phase (Fig. S2a, b, Electronic supplementary material; RMSD 0.71 Å and 0.50 Å, respectively).

Moving to brequinar-like docked poses **9c** and **10c**, marked differences from global minima are observed. Firstly, the charge-enhanced hydrogen bond found in the leflunomide-like docked conformations is missing, probably due to an underestimation of hydrogen bonding interactions in AutoDock's force field; as a consequence, these structures are extremely unstable in gas phase (+25 kcal mol^{-1} for **9c** and +19 kcal mol^{-1} for **10c**). Moreover, the degree to which the amide group and phenyl ring are tilted compared to the QM global minima is much higher (ϕ=−44° and −24°, respectively), resulting in large root mean square deviations (RMSDs) from the gas phase conformations: 1.18 Å and 0.99 Å, respectively (Fig.

Table 3 Stable conformers (**a**, **b**; *blue*) of compounds **4** and **7** and their co-crystallized poses (**c**, **d**; *light grey*)

	Conformer	Description	ϕ [a]	ψ [a]	ΔE [b]
4a		DFT-optimized structure	+175.80	+31.70	0.00
4b		DFT-optimized structure	-45.61	+32.83	+2.62
4c		leflunomide-like co-crystallized pose	+142.48	+45.93	+1.24
7a		DFT-optimized structure	+177.70	+32.94	0.00
7b		DFT-optimized structure	-48.00	+34.26	+1.88
7c		brequinar-like co-crystallized pose	-60.64	+43.86	+0.72
7d		leflunomide-like co-crystallized pose	+154.50	+42.48	+0.08

[a] Torsional angles are expressed in degrees; see Fig. 4 for the definition of ϕ and ψ

[b] Potential energies for each cluster of conformers are expressed in kcal mol^{-1} relative to the global minimum; see Methods for details

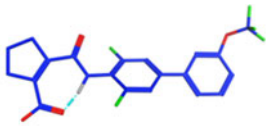
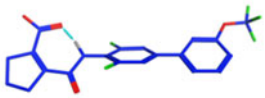
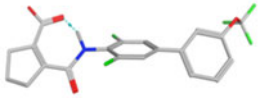
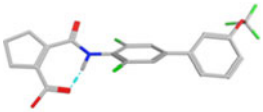
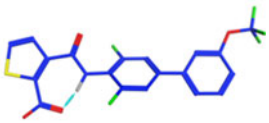
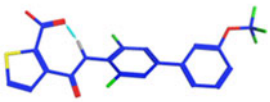
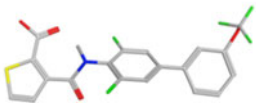
S2c,d, Electronic supplementary material). This indicates that brequinar-like bioactive poses are very unlikely for these compounds, suggesting that, in the absence of fluorine atoms, the leflunomide-like binding poses are largely favored.

Since the same considerations apply to the non-fluorinated inhibitors we published recently [7], the low inhibitory activity of the latter may be reasonably attributed to the prevalence of leflunomide-like poses that, according to Baumgartner, have a lower affinity for the DHODH pocket.

Monofluorinated compounds

The presence of a fluorine atom in the ortho position of the central phenyl ring gives rise to three different minima depending on the ϕ torsional value (Fig. S3a, Electronic supplementary material). For both the cyclopentene and the thiophene derivatives, the most stable structures (**4a** and **7a**, Table 3) are characterized by coplanarity of the amide group and the adjacent benzene ring, allowing for an electrostatic interaction between the amide hydrogen and the aromatic fluorine. The other, less stable local minima (**4b** and **7b**, Table 3) have quite different geometries, in which the coplanarity between the amide group and the ortho-fluorophenyl ring is lost together with the H···F interaction, which is replaced by the less favorable C=O···F contact. Experimental bioactive poses obtained via X-ray crystallography by Baumgartner and co-workers are also reported in Table 3 for comparison. For both **4** and **7** the leflunomide-like conformations are more stable than the brequinar-like ones, which again accounts for their relatively low activity. However, the energy gap between brequinar-like and leflunomide-like poses is much higher for **4** (> 2 kcal mol^{-1}) than for **7** (0.72 kcal mol^{-1}); this explains why a fraction of the experimentally determined complexes shows a brequinar-like binding mode only in the case of **7**. Additionally, the conformational strain penalty to assume the leflunomide-like binding mode is almost negligible for the thiophene derivative **7** (0.08 kcal mol^{-1}), but not for the cyclopentene derivative **4** (1.24 kcal mol^{-1}). This finding justifies the IC_{50} value for compound **7** (44 nM) being one order of magnitude lower than for **4** (280 nM).

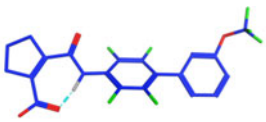
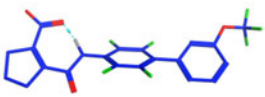
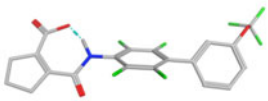
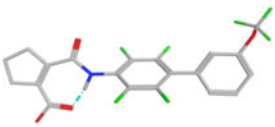
Table 4 Stable conformers (**a**, **b**; *blue*) of compounds **5** and **8** and their co-crystallized poses (**c**, **d**; *light grey*)

Conformer		Description	ϕ [a]	ψ [a]	ΔE [b]
5a		DFT-optimized structure	+135.90	+31.47	0.00
5b		DFT-optimized structure	-47.68	+31.32	+0.11
5c		brequinar-like co-crystallized pose	-65.00	+48.69	+0.32
5d		leflunomide-like co-crystallized pose	+134.10	+50.52	+0.54
8a		DFT-optimized structure	+134.90	+32.67	0.00
8b		DFT-optimized structure	-48.93	+33.07	+0.04
8c		brequinar-like co-crystallized pose	-65.80	+48.50	+0.31

[a] Torsional angles are expressed in degrees; see Fig. 4 for the definition of ϕ and ψ

[b] Potential energies for each cluster of conformers are expressed in kcal mol^{-1} relative to the global minimum; see Methods for details

Table 5 Stable conformers (**a**, **b**; *blue*) of compound **6** and its co-crystallized poses (**c**, **d**; *light grey*)

Conformer		Description	ϕ [a]	ψ [a]	ΔE [b]
6a		DFT-optimized structure	+137.70	+36.38	0.00
6b		DFT-optimized structure	-45.97	+38.21	+0.14
6c		brequinar-like co-crystallized pose	-64.07	+56.68	+0.29
6d		leflunomide-like co-crystallized pose	+131.40	+57.02	+0.54

[a] Torsional angles are expressed in degrees; see Fig. 4 for the definition of ϕ and ψ

[b] Potential energies for each cluster of conformers are expressed in kcal mol^{-1} relative to the global minimum; see Methods for details

Difluorinated compounds

Derivatives **5** and **8** are characterized by a fluorine atom in both the ortho and ortho' positions of the central benzene ring. PESs contain 16 almost equivalent minima; minor energetic differences are due only to the long-range interactions between the arylcarbamoyl moiety and the meta substituent on the distal benzene ring (Fig. S3b, Electronic supplementary material). In contrast with non-fluorinated and monofluorinated compounds, ortho–ortho' substituents force the amide group to lie in a different plane with respect to the benzene ring, in order to avoid steric and electrostatic clashes between the carbonyl oxygen and the halogen. Potential energies of calculated and experimental conformations are almost equivalent (Table 4): this suggests that both poses likely have similar affinity for the DHODH pocket, the brequinar-like pose being slightly favored (0.30 kcal mol^{-1} above the global minimum for **5c** compared to 0.54 for the leflunomide-like pose **5d**). All co-crystallized conformers are more closely superimposable to the gas phase conformations than the monofluorinated analogues (Fig. S2e,g, Electronic supplementary material, RMSD 0.30 Å, 0.44 Å and 0.41 Å, respectively); again, the lower conformational energy strain required to assume the bioactive pose would account for their higher activity.

Tetrafluorinated compound

The only compound bearing four fluorine atoms published by Baumgartner et al. is the cyclopentene derivative **6** (Table 1); its potential energy surface is similar to that of difluorinated inhibitors. The only remarkable difference is in the E vs ψ profile, because the double ortho–ortho' substitution exerts its effect also on the distal benzene ring, tilting it out of plane as observed for the amide group (Fig. 4d). Also in this case brequinar-like and leflunomide-like gas phase conformations **6a** and **6b** are almost isoenergetic (Table 5). Similarly to the difluorinated analogue, the crystallographic brequinar-like pose suffers a moderately lower strain energy penalty than the leflunomide-like pose, confirming that fluorine has a beneficial effect in stabilizing the higher-affinity brequinar-like binding mode.

In addition to the potential energy considerations discussed so far, it is reasonable to expect that the higher rigidity imposed by the double ortho–ortho substitution pattern may favor binding also from an entropic point of view, since the loss of conformational freedom upon binding will be incrementally lower moving from tetra- to di-, mono-, and non-fluorinated analogues.

While the increasing degree of fluorination of the central benzene ring may contribute to improving interactions between the molecule and the hydrophobic amino acids lining the DHODH cavity, especially leucines 46, 58 and 359, it would be difficult to justify only on these bases the 100-fold increase in activity observed in Baumgartner's series of inhibitors, particularly in the absence of specific electrostatic or hydrogen bonding interactions.

Summary

Conformational preferences of a series of DHODH inhibitors were analyzed in order to determine whether a correlation between their experimentally determined binding mode and their affinity could be found. The MMFF94s force-field failed to properly address ortho-ortho' effects; therefore, a systematic conformational scan was carried out with a DFT method, in order to obtain MM/QM PESs of higher quality. Analysis of the latter allowed a clear link to be established between the degree of fluorine substitution, the preferred binding mode and the inhibitory activity. Translating these observations to the non-fluorinated models **9** and **10**, we were able to find a sound justification for the low activity of a series of inhibitors we realized in the recent past, which shared a scaffold largely reminiscent of Baumgartner's compounds but lacked fluorine substituents. Our conformational analysis also underlined the role of incremental fluorine substitution in stabilizing the brequinar-like binding mode, which has been found previously to be connected with higher inhibitory potency. Our work sheds light on the molecular determinants that lead to effective DHODH inhibition, and may serve as a guide to design more potent analogues by molecular modeling techniques.

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