A novel series of human dihydroorotate dehydrogenase inhibitors discovered by *in vitro* screening: inhibition activity and crystallographic binding mode

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**Keywords**
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Human dihydroorotate dehydrogenase (DHODH), the enzyme that catalyzes the rate-limiting step in *de novo* pyrimidine biosynthesis, is considered to be an attractive target for potential treatment of autoimmune disease and cancer. Here, we present a novel class of human DHODH inhibitors with high inhibitory potency. The high-resolution crystal structures of human DHODH complexed with various agents reveal the details of their interactions. Comparisons with the binding modes of teriflunomide and brequinar provide insights that may facilitate the development of new inhibitors targeting human DHODH.

The dihydroorotate dehydrogenase (DHODH) is a key enzyme involved in the *de novo* pyrimidine biosynthetic pathway, which is conserved among all living organisms [1]. DHODH uses flavin mononucleotide (FMN) as its redox cofactor and catalyzes the fourth step in this biosynthesis pathway, the conversion of D-hydroorotate to orotate. DHODHs have been shown to substantially be diverse in their structural, kinetic, and functional properties [1]. Via phylogenetic analysis, DHODH could be clustered into two major classes: class 1 and class 2 [1]. The different classes differ also in their cell location: DHODHs from class 1 are found in the cytosol, whereas those from class 2 are membrane-associated. Human DHODH belongs to class 2 and is anchored at the inner mitochondrial leaflet [2].

Inhibition of pyrimidine metabolism by selectively targeting DHODHs has been exploited in the development of new therapies against cancer, immunological disorders, bacterial and viral infections, and parasitic diseases [3,4]. Although a variety of inhibitors targeting human DHODH has been studied over the years, only leflunomide and its *in vivo* metabolite teriflunomide have been approved as human DHODH-targeting drugs [4–6]. The severe side effects, narrow therapeutic window, and inconsistent pharmacokinetics of the available DHODH inhibitors raise the need of new and more efficient human DHODH inhibitors [7,8].

Here, we present a novel class of human DHODH inhibitors, which are based on 6-isopropyl-1,5,6,7-

**Abbreviations**
C11DAO, N,N-dimethylundecylamine N-oxide; DDAO, N,N-dimethyldecylamine N-oxide; DHODH, dihydroorotate dehydrogenase; DHO, dihydroorotic acid; FMN, flavin mononucleotide.
tetrahydro-4H-benzo[d][1,2,3]triazol-4-one scaffold. In vitro inhibitory assay revealed that the compounds 1289 and 1291 have high potency against human DHODH. High-resolution crystal structures of human DHODH and inhibitor complex elucidated their interactions. Our studies provide a solid structural basis for the design and development of new chemo-diverse inhibitors targeting human DHODH.

**Materials and methods**

**Chemical synthesis of compounds**

*6-Isopropyl-1-(2,2',6-trifluoro-5'-(hydroxymethyl)-1,1'-biphenyl)-4-yl)-1,5,6,7-tetrahydro-4H-benzo[d][1,2,3]triazol-4-one (1289)*

Under N₂ atmosphere, a mixture of 4-bromo-3,5-difluoroaniline (0.720 g, 3.49 mmol), (2-fluoro-5-(hydroxymethyl)phenyl) boronic acid (0.889 g, 5.23 mmol), Pd (dppf) CCl₂.DCM (0.120 mg, 0.147 mmol), and potassium carbonate (0.720 g, 3.49 mmol) was added to a mixture of 5-isopropylcyclohexane-1,3-dione (0.720 g, 3.49 mmol) in THF (10 mL). After stirring for 15°C for 1 h, the temperature was raised to 88°C for overnight and then was allowed to cool to room temperature. The mixture was heated to 100°C over night and then was allowed to cool to room temperature. The mixture was stirred at 75°C for 7 h, the temperature was raised to 90°C for overnight and then was allowed to cool to room temperature. The reaction was quenched with water and extracted with dichloromethane (3 × 50 mL). The combined organic layer was washed with brine and then evaporated in vacuo. The crude product was purified using silica gel chromatography with a petroleum ether/dichloromethane gradient to afford the desired product as white powder (96 mg, 34.5% yield).

**1H NMR (400 MHz, CDCl₃)**

δ 7.52–7.47 (m, 1H), 7.44 (d, J = 6.2 Hz, 1H), 7.33 (s, 2H), 7.22 (d, J = 8.8 Hz, 1H), 4.76 (s, 2H), 3.10 (dd, J = 12.0, 4.4 Hz, 1H), 2.89 (dd, J = 10.8, 5.6 Hz, 1H), 2.76 (dd, J = 13.6, 2.8 Hz, 1H), 2.49 (dd, J = 13.2, 3.2 Hz, 1H), 2.22 (m, 1H), 1.83 (dd, J = 6.4, 6.4 Hz, 2H), 1.04 (d, J = 6.4 Hz, 6H); **13C NMR (101 MHz, CDCl₃)** δ 189.75, 160.59, 144.26, 142.87, 137.08 (d, J = 3.03 Hz), 136.49, 130.46, 129.99 (d, J = 8.08 Hz), 116.23 (d, J = 22. 22 Hz), 115.47 (d, J = 20. 2 Hz), 107.15, 64.27, 58.48, 50.87, 42.65, 42.09, 31.74, 29.70, 25.34, 19.65 (d, J = 8.08 Hz), 18.43.

**1-(3'-(Dimethylamino)-2,6-difluoro-[1,1'-biphenyl]-4-yl)-6-isopropyl-1,5,6,7-tetrahydro-4H-benzo[d][1,2,3]triazol-4-one (1291)**

Compound 1291 was synthesized by a similar procedure as 1289. **1H NMR (400 MHz, CDCl₃)** δ 7.36 (t, J = 8.0 Hz, 1H), 7.29 (d, J = 8.0 Hz, 2H), 6.83 (dd, J = 12.0, 6.0 Hz, 3H), 3.08 (dd, J = 16.6, 4.0 Hz, 1H), 3.00 (s, 6H), 2.91 (s, 6H). The crude product was purified using silica gel chromatography with a petroleum ether/dichloromethane gradient to afford the desired product as white powder (388 mg, 29.1% yield).

**Table 1. Chemical structures and in vitro inhibitory activities of human DHODH inhibitors.**

| Compound | Chemical formula | IC₅₀ (nM) |
|----------|-----------------|-----------|
| 1289     | ![Structure](image1.png) | 171       |
| 1291     | ![Structure](image2.png) | 39        |

IC₅₀ value of teriflunomide is from the reference [17].
1H), 2.76 (dd, J = 13.2, 3.2 Hz, 1H), 2.50 (s, 1H), 2.27–2.18 (m, 1H), 1.83 (dd, J = 13.0, 6.6 Hz, 1H), 1.03 (d, J = 6.4 Hz, 6H). 13C NMR (101 MHz, CDCl3) δ 189.81, 161.76 (d, J = 8.08 Hz), 161.68 (d, J = 9.09 Hz), 159.27, 159.18, 150.54, 144.29, 142.76, 129.19, 128.13, 120.98, 118.12, 113.98, 113.09, 107.36 (d, J = 31.31 Hz), 107.26 (d, J = 11.1 Hz), 53.43, 42.64, 42.11, 40.52, 31.74, 25.27, 19.64 (d, J = 6.06 Hz).

**In vitro inhibitory activity of compounds**

Human DHODH inhibition profiles are obtained from human DHODH-inhibitor profiler services provided by ChemPartner (Shanghai, China).

**Protein preparation**

Human DHODH was cloned into a vector derived from pET-28a (+) (Novagen, Madison, WI, USA), which contains an N-terminal SUMO tag and an N-terminal His6 tag, and overexpressed in *Escherichia coli* strain Rosetta (DE3) (Novagen) at 18 °C for 18 h. The cells were harvested by centrifugation, and the cell pellet was resuspended in binding buffer (50 mM Tris/HCl pH 7.5, 500 mM NaCl, 0.33% Thesit, 10% glycerol, 1 mM PMSF). The cells were lysed by an ultrahigh-pressure homogenizer (JNBIO) and centrifuged. The resultant supernatant was collected and loaded onto a Ni-NTA column pre-equilibrated with binding buffer. After washed with binding buffer supplemented with 20 mM imidazole to remove nonspecifically binding proteins, the target protein was eluted using binding buffer supplemented with 250 mM imidazole. The eluted target protein was collected and dialyzed against binding buffer with ULP1 protease (1 : 100) for 16 h at 8 °C to remove the SUMO tag. The digested protein was then passed through a Ni-NTA column (GE Healthcare, Marlborough, MA, USA) to remove free SUMO tag, uncleaved protein, and ULP1 protease. The flow-through was collected and was further purified via gel filtration (Superdex 200 10/300 GL; GE Healthcare) in a buffer consisting of 50 mM HEPES, pH 7.5, 400 mM NaCl, 10% glycerol, 1 mM EDTA, and 0.05% Thesit, on an AKTA system (GE Healthcare).
The purified proteins were concentrated to 20 mg mL\(^{-1}\) and stored at \(-80^\circ\text{C}\) until use.

**Cocrystallization of human DHODH and inhibitors**

Purified DHODH was incubated with 2 mM dihydroorotic acid (DHO), 40 mM \(N,N\)-dimethylundecylamine N-oxide (C11DAO), 20.8 mM \(N,N\)-dimethyldecylamine N-oxide (DDAO), and fivefold small molecule inhibitor for 2 h at 4 \(^\circ\text{C}\). Crystals of DHODH and inhibitor were grown using the hanging-drop vapor diffusion method at 20 \(^\circ\text{C}\), in a buffer consisting of 0.1M acetate pH 4.6, 1.8–2.0 m ammonium sulfate, and 30–35% glycerol. Cubic crystals appeared after a week.

**Data collection and structure determination**

Crystals were fished directly from the growing drop and flash-frozen in liquid nitrogen. Diffraction data were collected on beamline BL19U1 of National Facility for Protein Science Shanghai (NFPS) at Shanghai Synchrotron Radiation Facility. The data collected were processed by the HKL-3000 program suite [9]. Details of the data collection and processing statistics are summarized in Table 1. Structures were determined by molecular replacement using the human DHODH structure (PDB ID 1D3G) as search model.
A novel class of human DHODH inhibitors

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A novel class of potent inhibitors of human DHODH

Biphenyl moiety was observed in several DHODH inhibitors, such as leflunomide, brequinar, and vidofludimus [13–15], and plays a key role in ligand–DHODH interactions. Therefore, the compounds with a biphenyl moiety in our in-house library were screened for their DHODH inhibitory effects. Finally, the screens led to the discovery of a series of compounds which have a 6-isopropyl-1,5,6,7-tetrahydro-4H-benzo[d][1,2,3]triazol-4-one scaffold, which are significantly different from available DHODH inhibitors.

Recently, a series of hDHODH inhibitors were discovered by scaffold-hopping strategy or structural modification based on previous reported lead compounds [16,17]. In our instance, the active compounds were selected based on in vitro screening. Our screening discovered a novel class of human DHODH inhibitors, which have a 6-isopropyl-1,5,6,7-tetrahydro-4H-benzo[d][1,2,3]triazol-4-one scaffold. Among these compounds, 1289 and 1291 have shown high in vitro inhibitory activity against human DHODH with nM range IC_{50} values (Table 1). The IC_{50} values of other two compounds, the approved and market-established drug teriflunomide and one of the strongest known DHODH inhibitors brequinar, are presented here for comparison. The synthesis of 1289 and 1291 has been shown in Scheme 1.

Crystal structures of DHODH-inhibitor complex

To elucidate the binding modes of these two inhibitors, we determined the crystal structures of human DHODH complexed with 1289 or 1291 to 1.6 and 1.85 Å resolutions, respectively (Fig. 1A). Details of the data collection and refinement statistics are summarized in Table 2. The high-resolution and clear density maps enable us to determine the position and orientation of these inhibitors unambiguously (Fig. 1B). Superposition of these two structures shows an identical binding mode for the two inhibitors (Fig. 1A).

Human DHODH has an α/β-barrel fold (Fig. 1A), and the inhibitors and FMN are located at a tunnel within DHODH (Fig. 1C,D). Our structures reveal the detailed interactions between the inhibitors and human DHODH (Fig. 2). Both compounds are stabilized by a substantial number of hydrophobic interactions involving M43, L46, L58, F62, F98, M111, and L359. Compound 1289 forms hydrogen bonds with the side chains of R136 and Y38 (Fig. 2A). Compound 1291 forms hydrogen bonds with R136 as same as compound 1289, but loses the hydrogen bond with Y38 due to the substitution of the hydroxymethyl group for the N,N-dimethyl group (Fig. 2C). Meanwhile, such substitution reinforces the hydrophobic interactions between 1291 and the local hydrophobic environment, which may contribute to the lower IC_{50} value of this inhibitor (Table 1). The full binding environments of inhibitors 1289 and 1291 have been shown in Fig. 2B and D, respectively.
Comparison of the binding modes of 1289, 1291, teriflunomide, and brequinar

Comparison with the previously solved DHODH–teriflunomide and DHODH–brequinar complex structures has been carried out by superposition (Fig. 3). The FMN molecules from different structures are completely overlapped, suggesting the superposition is well performed. The two inhibitors we present here adopt a brequinar-like binding mode, which is different from that of teriflunomide (Fig. 3). The bifurcated moiety of 1289 and 1291 occupied a very similar position as that of brequinar (Fig. 3B). However, the isopropyl group of 1289 and 1291 is mimicking the isopropyl alcohol group of teriflunomide, which is approaching to the FMN cofactor (Fig. 3A).

It has been shown that the DHODH-inhibitor binding site has intrinsic plasticity [18], so human DHODH may accommodate a diverse range of inhibitors, rationalizing the strategy of designing inhibitors with diverse scaffolds. The high-resolution structures of human DHODH-inhibitor complex we report here elucidate the interactions between these new inhibitors and their target, and thus facilitate the design and development of novel, efficient, and chemo-diverse inhibitors for human DHODH. Further in vivo efficacy studies and compound optimization are ongoing to evaluate this novel class of human DHODH inhibitors.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

QC and YY conceived and designed the project. TZ, ZZ, and YY acquired the data. YL, YZ, and QC analyzed and interpreted the data. QC wrote the paper.

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