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SAR, species specificity, and cellular activity of cyclopentene dicarboxylic acid amides as DHODH inhibitors

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Abstract—Novel DHODH inhibitors were developed based on a previously described series by introduction of heteroatoms into the cyclopentene ring and hydroxyl groups attached to it. Also, the hydrophobic biphenyl side chain was replaced with benzyloxy phenyl groups. Activities on human, rat, and mouse enzymes indicate a species specificity of these inhibitors.

Dihydroorotate dehydrogenase (DHODH) is responsible for the conversion of dihydroorotate to orotate, which is the rate-limiting step in pyrimidine biosynthesis. Inhibitors of DHODH show immunosuppressant and antiproliferative activities, which are most pronounced on activated T-cells.[1]

Leflunomide, an inhibitor of DHODH, is currently used to treat rheumatoid arthritis, and analogs are in clinic to treat graft versus host disease and multiple sclerosis.[2]

A novel series of DHODH inhibitors was developed by us based on a lead that was discovered during a docking procedure and medicinal chemistry exploration. The activity of the initial lead was improved by a QSAR method and yielded low nanomolar inhibitors.[3]

To evaluate the compounds further, we introduced heteroatoms into the cyclopentene ring and replaced the biphenyl moiety by the benzyloxy.

Cyclopentene dicarboxylic anhydride was purchased from Aldrich. Thio and oxocyclopentene carboxylic acids were synthesized by the cyanhydrine synthesis, as shown in Figures 1 and 2.

Substituted biarylanilines were obtained by the Suzuki cross-coupling procedure using commercial aromatic boronic acids and halo anilines with Pd and KF in methanol as described.[3] Benzyloxy phenylanilines were prepared, as shown in Figure 3.

The final compounds **1**–**22** were obtained by reacting the appropriate dicarboxylic acid anhydrides with the anilines in an inert solvent.[8]

Enzyme inhibition was measured in an in vitro enzyme assay.[4] For the assay, N-terminally truncated recombinant human DHODH was used.[5] The assay was based on the coupling reaction of the enzyme to the redox dye 2,6-dichlorophenolindophenol (DCIP) as described. The reduction of DCIP can be monitored photometrically by a decreasing absorption at 600 nm.

T-cell (PBMC) proliferation was measured at variance to a published method and is described in reference.[6]

Previously, we have described the discovery of cyclopentene dicarboxylic acid monoamides[3] as potent DHODH inhibitors. Here, we describe further data on the understanding of the SAR of these compounds.

As can be seen on the lead compound **1** (Table 1), introduction of sulfur into the petacyclic ring did not lower the activity significantly in compound **2**. A similar behaviour was seen on all other carbon sulfur replacements (**6**, **10**, **13**, **16**, **17**). Oxidation of the sulfide to the sulfone led to less active compound **3**. Inspection of the binding mode in a X-ray structure of such an

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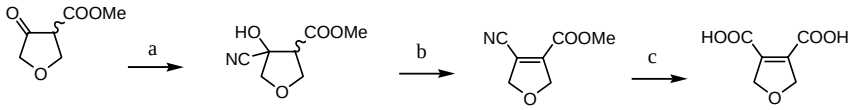


Figure 1. Reagents and conditions: (a) NaCN, water/ether; (b) H_2SO_4, stirring 18 h at room temperature; (c) concd HCl, reflux, 3 h.

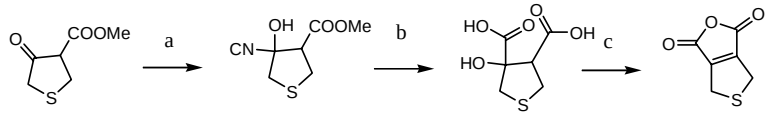


Figure 2. Reagents and conditions: (a) KCN, methanol/water/acetic acid, 0 °C, 8 h; (b) concd HCl, acetic acid, reflux, 20 h; (c) acetic acid anhydride, reflux, 4 h.

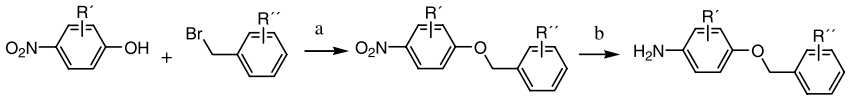


Figure 3. Reagents and conditions: (a) NaOH, water/ethanol, 80 °C, 3 h; (b) Fe, NH_4Cl, water/acetic acid/ethanol, 75 °C, 3 h.

analog indicated a close proximity of the pentacycle to the FMN cofactor in the enzyme, leaving little space for the sulfone without disrupting a good binding mode.[9]

Introduction of oxygen into the pentacyclic ring yielded, in every case, compounds with lower activity (**5**, **9**, **12**, and **19**). A model derived from docking and from the X-ray structure did not indicate any contradiction from a good binding mode. Increased hydrophilicity of the compound may prevent diffusion from into the active side of this enzyme. The enzyme is anchored in a very hydrophobic environment and the inhibitor has to diffuse through a very hydrophobic environment. Such an effect could explain the lower activity of hydrophilic compounds in general. In the biphenyl series, there was a clear trend toward better activity with the number of fluor substitutions in the first aromatic ring (**4**, **7**). We initially attributed the better activity to increased hydrophobicity of the biphenyl ring system, since this pharmacophore binds to a hydrophobic environment in the active site of the enzyme and such compounds diffuse better into the active site.

The X-ray structure of these compounds showed a trend toward a Brequinar-like binding mode with increasing number of fluor substituents in the first aromatic ring. The more Brequinar-like the binding the more active the compounds.[9]

Compounds with hydroxyl groups attached to the pentacyclic ring (**21**, **22**) were synthesized by the Wohl–Ziegler allylbromination with *N*-bromosuccinimide and light from a 300 W lamp, in chloroform under refluxing conditions (Fig. 4). Hydrolysis of the brominated compound, without isolation, yielded two regioisomers that were separated by HPLC (**21**, **22**) in the ratio 7:3. The more active compound **21** was found to be the isomer with the hydroxygroup adjacent to the free carboxylic acid, as shown in the X-ray structure. We did not separate the enantiomers however, the X-ray structure electrodensity of compound **21** accommodates equal binding of both hydroxyl enantiomers. The hydroxylated compound **21** was 20-fold less active than the parent compound **4**.

Another suitable pharmacophore for binding into the hydrophobic pocket of the active site was found to be the benzyloxygroup **14**. Although the parent compound was less active than the unsubstituted biphenyl compound **1**, the introduction of halogen residues in *ortho* and *meta* positions toward the benzylether linkage greatly improved inhibitory activity (**14**–**20**).

In this series, however, the alternative binding mode did not seem to be of importance, but increased activity was considered to be a consequence of lipophilicity, since we did not observe any Brequinar-like binding mode.[9]

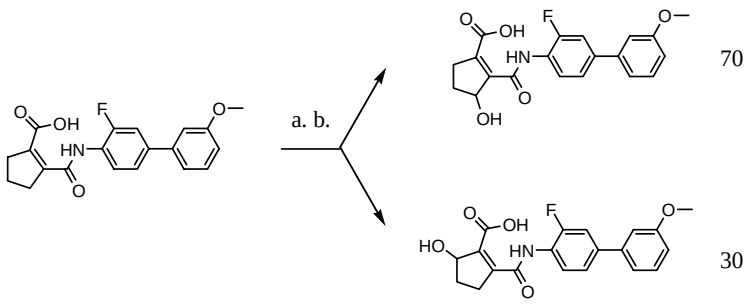


Figure 4. Reagents and conditions: (a) 300 W lamp, NBS, chloroform, reflux; (b) water, RT.

Table 1. Inhibition on human DHODH

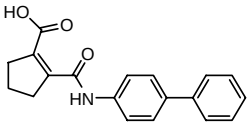
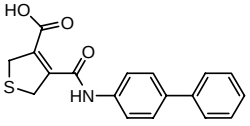
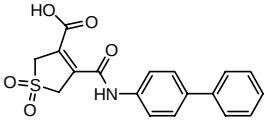
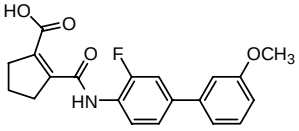
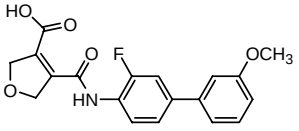
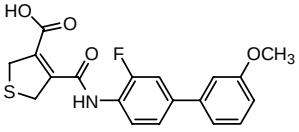
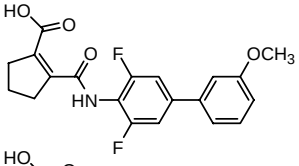
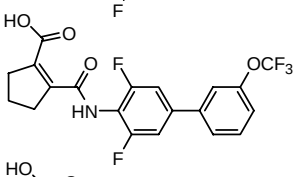
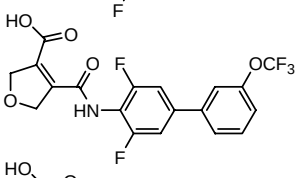
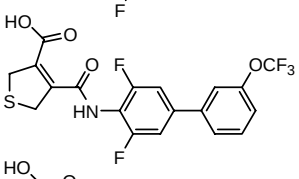
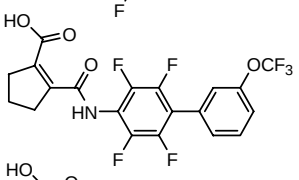
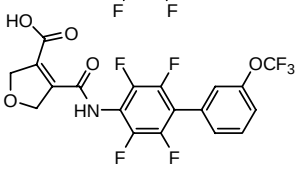
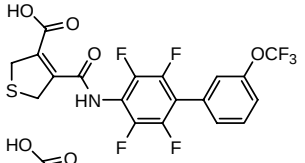
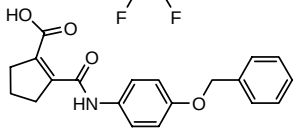
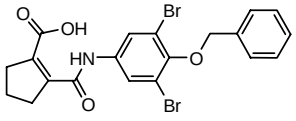
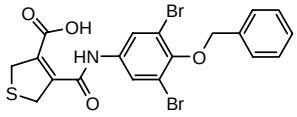
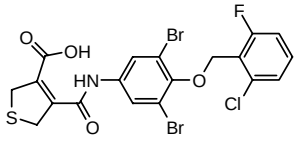
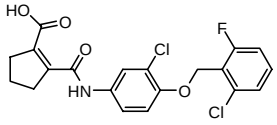
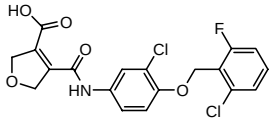
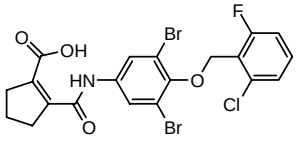
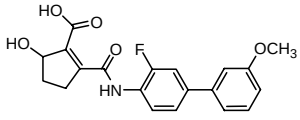
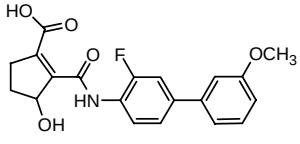
Compound	IC_{50}[a] (μM)	Compound structure
1	0.41	
2	0.667	
3	3.8	
4	0.134	
5	0.360	
6	0.131	
7	0.011	
8	0.033	
9	0.205	
10	0.015	
11	0.007	
12	0.058	

Table 1 (*continued*)

Compound	IC_{50}[a] (μM)	Compound structure
13	0.004	
14	2.0	
15	0.127	
16	0.173	
17	0.105	
18	0.011	
19	0.041	
20	0.110	
21	0.68	
22	1.46	

[a] Values are means of three experiments, errors are usually around 10%.

We also tested some of the most active compounds on the human enzyme, and on rat and mouse DHODH enzymes, and the results were compared. As a reference compound, A771276, the active metabolite of Leflunomide, shows a marked better activity on the mouse versus rat versus human enzyme.

Contrary to this result, all our compounds are better inhibitors on the human versus rat and mouse enzymes.

Table 2. Comparison of selected compounds on human, rat, and mouse DHODH

Compound	IC_{50}[a] (μM)		
	Human	Rat	Murine
1	0.41	1.5	4.0
8	0.033	0.504	2.0
11	0.007	0.078	0.422
19	0.041	1.2	1.8
A771726	0.42	0.009	0.156

[a] Values are means of three experiments, errors being usually around 10%.

Table 3. Inhibition of PBMC proliferation

Compound	IC_{50} (μM)
4	13
8	15
11	0.175–0.327
19	1.0
A771726	108

To show the immunosuppressive effects of compounds, we studied the inhibition of proliferation of our compounds human peripheral blood mononuclear cells (PBMCs) stimulated with Phytohemagglutinin-L.[7]

Data in Table 3 show that this series of DHODH inhibitors was significantly more active than A77127, which is the active metabolite of Leflunomide (Tables 2 and 3).

Further studies on PK and animal models should identify clinical candidates for the development of drugs to treat autoimmune diseases. Such diseases are rheumatoid arthritis, multiple sclerosis, lupus erythematosus, ulcerative colitis, and neurodermitis.

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References and notes

1. Fairbanks, L. D.; Bofill, M.; Ruckemann, K.; Simmonds, H. A. *J. Biol. Chem.* **1995**, *270*, 29682.
2. Allred, A.; Emery, P. *Exp. Opin. Pharmacother.* **2001**, *2*, 125.
3. Leban, J.; Saeb, W.; Garcia, G.; Baumgartner, R.; Kramer, B. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 55.
4. Davis, J. P.; Cain, G. A.; Pitts, W. J.; Magolda, R. L.; Copeland, R. A. *Biochemistry* **1996**, *35*, 1270.
5. Recombinant human, rat and murine DHODH were obtained from Prof. Dr. M. Löffler, University of Marburg, Karl-von-Frisch-Str.1 35043 Marburg.
6. Neidhardt, E. A.; Punreddy, S. R.; McLean, J. E.; Hedstrom, L.; Grossman, T. H. *J. Mol. Microbiol. Biotechnol.* **1999**, *1*, 183.
7. Magaud, J.-P.; Sargent, I.; Mason, D. Y. *J. Immunol. Methods* **1988**, *106*, 95, Cells (Mononuclear cells) were isolated from human peripheral blood by Accuspin™ System-Histopaque®-1077 (Sigma, Germany). After washing, the cells were diluted to approximately 100,000–200,000 cells/well in a sterile 96-well flat-bottomed MP (Corning, Netherlands). T-lymphocytes were stimulated by addition of 20 μg/ml Phytohämagglutinin-L (Roche, Germany). Incubation at 37 °C, 5% CO_2, 90% humidity was carried out in the presence of different concentrations of the compounds. All cells were incubated for 48 h at 37 °C, 5% CO_2, 90% relative humidity over a concentration range of 0.4–50 μM compound solutions with a final volume per well of 100 μl. After the initial incubation period, 10 μM BrdU (Roche, Germany) was added for an additional 4 h incubation. The culture medium employed was RPMI 1640, which contained 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. After 48 h incubation, the cells were labeled by adding 10 μl BrdU-Solution (Roche, Germany) and reincubated for further 4 h. Following incubation, the media plus BrdU and drug were removed, the cells were fixed, and the DNA was denatured in a single step using FixDenat (Roche, Germany) The anti-BrdU-POD (Roche, Germany) binds to the BrdU incorporated into a newly synthesized, cellular DNA. The immune complexes were detected by a subsequent substrate reaction. The reaction product was quantified by measuring the absorbance at the respective wavelength using an ELISA reader. The EC_{50} values were determined using a fitting function.
8. All compounds were characterized by HPLC- MS and NMR (300 MHz), and exhibited satisfactory properties.
9. R. Baumgarter et al., *J. Med. Chem.*, submitted.