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Biphenyl-4-ylcarbamoyl thiophene carboxylic acids as potent DHODH inhibitors

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Abstract—A previously discovered DHODH inhibitor series was further improved by replacing the cyclopentene ring by aromatic heterocycles. Different isomers of these compounds were prepared by the directed *ortho*-metallation procedure. The compounds are more active than the corresponding cyclopentene analogs and show potent effects on PBMC's proliferation.

We have developed a novel series of DHODH inhibitors based on a lead, which came out of a docking procedure using 4Scan® technology and medicinal chemistry exploration. The activity of the initial lead was improved by a QSAR method and yielded low nanomolar inhibitors.[1]

Such compounds promise to have great potential as treatment for autoimmune diseases such as rheumatoid arthritis, graft versus host disease, and multiple sclerosis.[2] To further explore the scope of DHODH inhibitors of types **1**–**3** (Table 1), a replacement of the cyclopentene ring with other small aromatic systems was envisaged. Our X-ray structure indicated that the cyclopentene ring lies virtually planar in a pocket close to the FAD cofactor.[3] Flat small aromatic rings might therefore be of benefit for binding and activity.

Compound **B** (Fig. 1) was synthesized by the directed *ortho*-metallation procedure (DOM)[4] at the stage of the amide using butyl lithium and dry ice as CO_2 source for the introduction of the carboxylic group. Thiophene amides **A** were prepared from commercial 3-thiophenecarboxylic acid by conversion into the acid chloride and reaction with the corresponding 4-phenylaniline, which in turn were obtained by the Suzuki cross-coupling method as described earlier.[1] Compounds with a second carboxylic group, like in **C** or **E**, were either built up by 2-fold DoM/CO_2-quenching or from 3,4-thiophene dicarboxylate upon its conversion into the anhydride with acetic anhydride and nucleophilic anhydride opening with the respective 4-phenylaniline to give compounds **13**–**15**. Regiochemistry for the carboxylate introduction in 3-thiophenamides **A** was in analogy to a literature protocol[4] and was finally deduced from NMR analysis to be in the 2-position.

Introduction of a third carboxylic group into **B** yielded a major and a minor component, which were separated by HPLC. Major product **C** possessed a completely identical NMR spectrum as the major product **E** from carboxylation of 3-thiophene-2-carboxylate **D** by the DoM protocol. Therefore, the structure has to be as depicted in Figure 1.

Enzyme inhibition was measured in an in vitro enzyme assay. For the assay N-terminally truncated recombinant human DHODH was used and the data are presented in Table 1.[5]

Direct comparison of the previously described compounds **1**–**3** with their thiophene analogs **4**, **5**, and **8** showed a clear trend toward an increased inhibitory activity of the thiophene analogs. As with the cyclopentene series, the activity increases with fluoro constituents in the aromatic ring adjacent to the amide bond and with a methoxy group in the terminal aromatic, but the trend is more pronounced in the thiophene series. A X-ray structure investigation of the cyclopentene

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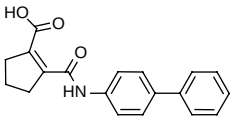
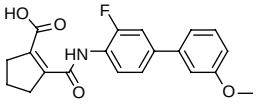
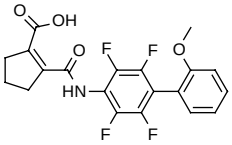
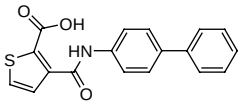
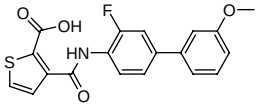
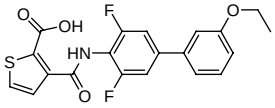
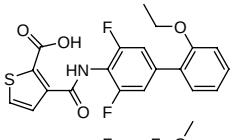
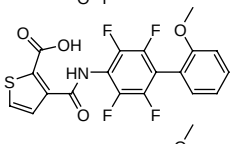
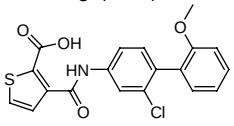
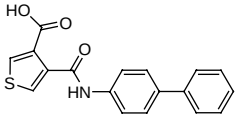
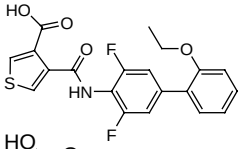
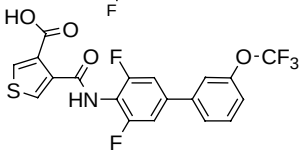
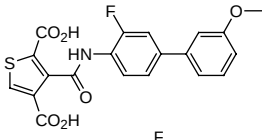
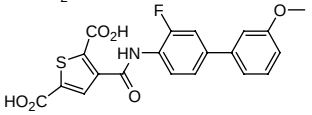
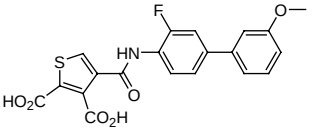
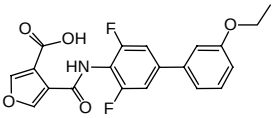
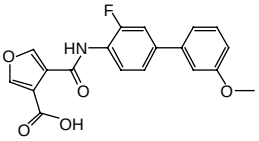
Table 1. Inhibition of human DHODH

Compound	Structure	IC_{50} (nM)
1[1]		410
2[1]		134
3[1]		8
4		303
5		44
6		3
7		9
8		1
9		12
10		1000
11		10
12		10
13		>1000
14		>1000
15		>1000
16		16
17		340
18	Brequinar	8

series revealed a binding mode with the possibility of the carboxylic acid either having an ion bridge toward Arg 136 of the enzyme or pointing into the opposite direction toward Tyr 365 and 147[3] upon turning by 180°. To test the possibility that both binding modes could be obtained within one molecule, compounds with two free carboxylic acids (compounds **13**–**15**) were synthesized. These compounds, however, were inactive, which was explained by the fact that for the alternative binding mode, a conformational change of some residues in the active site of the enzyme was required.

There was not much difference between the regioisomers **7** and **11** in their inhibitory activity, indicating the relatively diminished importance of the sulfur position.

If one compares the X-ray structures of the cyclopentene analog **2** versus those of the thiophene derivative **5**, it is of significance that the cyclopentene analog **2** displayed a single binding mode which we termed 'non-brequinar' like.[3] In contrast, the thiophene analog **5** showed a dual binding mode which was brequinar-like and non-brequinar-like, which can be deduced from the electron density pattern in the crystal structure.

We have previously shown that the brequinar-like binding mode leads to a better inhibitory activity, reflecting a high affinity binding mode. Thus, the higher activity of the compounds of the thiophene series as compared to that of the respective representatives of the cyclopentene series can easily be explained by taking into account their possibility for binding in this high affinity mode. Compound **3** represents an exception within the cyclopentene class, as it likewise binds in both modes, resulting in a comparable excellent activity (cf. compound **3** vs **8**). Furthermore, an increased number of fluoro substituents in the aromatic ring adjacent to the amide bond correlate with an increased tendency toward a 'brequinar-like' binding mode, thus with a better inhibitory activity.

As can be seen from the furan analogs **16** and **17**, a replacement of sulfur by oxygen in the pentacyclic ring is well tolerated. From the few furan analogs prepared,

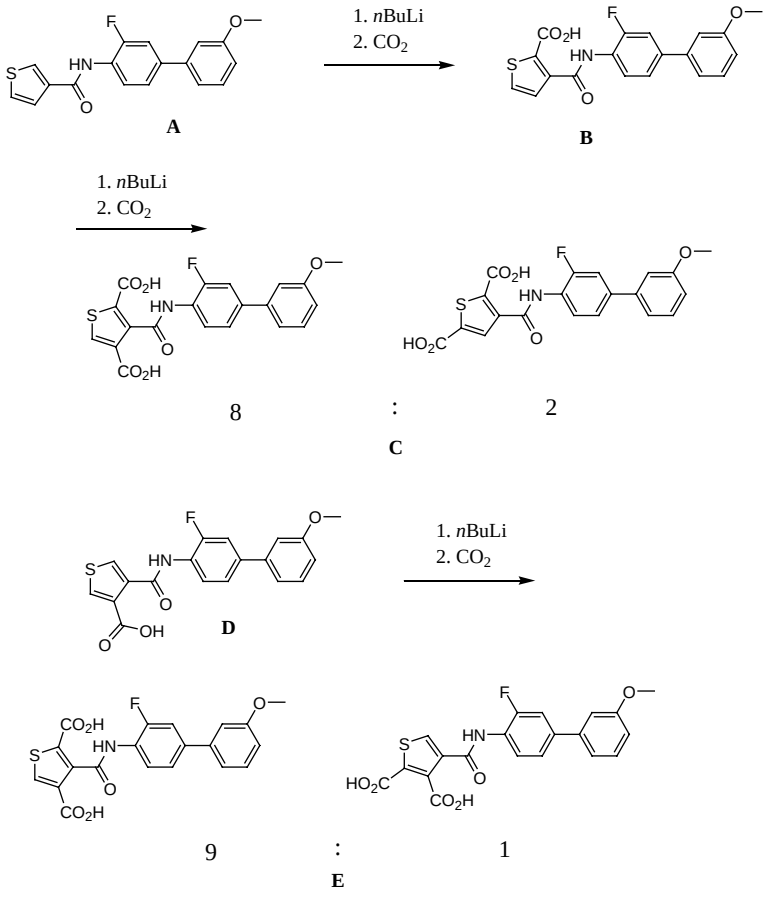


Figure 1.

it can be deduced that this series will have a similar SAR compared with that of the thiophene compounds. A somewhat lower activity can be expected from higher hydrophilicity which results in a hindered diffusion into the hydrophobic environment of the active site, a phenomenon we have encountered with all analogs.

Inhibition of DHODH is reflected by an antiproliferative effect on peripheral blood mononuclear cells (PBMCs), which can be measured by the inhibitory effect on their phytohemagglutinin (PHA) stimulated growth. The test was performed as described in the reference.[6] Data for few selected compounds are presented in Table 2. Compounds **8** and **12** displayed potent antiproliferatory activities on PBMCs and suggest the potential of such compounds to have an effect in animal models for autoimmune diseases.

In conclusion, we have found that pentacyclic aromatic heterocycles can be a potent substitute for a cyclopentene ring for the design of DHODH inhibitors. Such compounds have activities comparable to those of brequinar and promise to be useful to treat autoimmune diseases.

Table 2. Inhibition of human PBMC proliferation

Compound	IC_{50} (μM)
5	20
8	2
12	2
16	15

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References and notes

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6. Cells (mononuclear cells) were isolated from human peripheral blood by Accuspin™ System-Histopaque®-1077 (Sigma,Germany). After washing, the cells were diluted to approximately 1,00,000–2,00,000 cells/well in a sterile 96-well flat-bottomed MP (Corning, Netherlands). T-lymphocytes were stimulated by addition of 20 μg/ml Phytohemagglutinin-L (Roche, Germany). The incubation at 37 °C, 5% CO_2, 90% humidity was made 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 units/ml penicillinG, and 100 μg/ml streptamycin sulfate. After 48 h incubation, the cells were labeled by adding 10 μl BrdU-Solution (Roche, Germany) and reinsulated 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 in a newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction. The reaction product was quantified by measuring the absorbance at the respective wavelengths using an ELISA reader. The EC_{50} values were determined using a fitting function.