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Research paper

Design, synthesis, biological evaluation and X-ray structural studies of potent human dihydroorotate dehydrogenase inhibitors based on hydroxylated azole scaffolds

Stefano Sainas [a, 1], Agnese C. Pippione [a, 1], Marta Giorgis [a], Elisa Lupino [b], Parveen Goyal [c], Cristina Ramondetti [b], Barbara Buccinnà [b], Marco Piccinini [b], Rodolpho C. Braga [d], Carolina H. Andrade [d], Mikael Andersson [c], Ann-Christin Moritzer [c, e], Rosmarie Friemann [c, f], Stefano Mensa [g], Salam Al-Karadaghi [h], Donatella Boschi [a], Marco L. Lolli [a, *]

[a] Department of Science and Drug Technology, University of Torino, via Pietro Giuria 9, 10125 Torino, Italy
[b] Department of Oncology, University of Torino, via Michelangelo 27/B, 10126 Torino, Italy
[c] Department of Chemistry and Molecular Biology, University of Gothenburg, Box 462, S-40530 Gothenburg Sweden
[d] LabMol, Faculty of Pharmacy, Federal University of Goias, 74605-170 Goiania, Brazil
[e] Department of Chemistry, Biochemistry, University of Bielefeld, Universitätsstraße 25, 33615 Bielefeld, Germany
[f] Department of Structural Biology, Stanford University School of Medicine, 299 Campus Drive West Stanford, CA 94305-5126, USA
[g] Stephenson Institute for Renewable Energy, Department of Chemistry, University of Liverpool, L69 7ZF Liverpool, UK
[h] Department of Biochemistry and Structural Biology, Lund University, Sweden

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ABSTRACT

A new generation of potent *h*DHODH inhibitors designed by a scaffold-hopping replacement of the quinolinecarboxylate moiety of brequinar, one of the most potent known *h*DHODH inhibitors, is presented here. Their general structure is characterized by a biphenyl moiety joined through an amide bridge with an acidic hydroxyazole scaffold (hydroxylated thiadiazole, pyrazole and triazole). Molecular modelling suggested that these structures should adopt a brequinar-like binding mode involving interactions with subsites 1, 2 and 4 of the *h*DHODH binding site. Initially, the inhibitory activity of the compounds was studied on recombinant *h*DHODH. The most potent compound of the series in the enzymatic assays was the thiadiazole analogue **4** (IC_{50} 16 nM). The activity was found to be dependent on the fluoro substitution pattern at the biphenyl moiety as well as on the choice/substitution of the heterocyclic ring. Structure determination of *h*DHODH co-crystallized with one representative compound from each series (**4**, **5** and **6**) confirmed the brequinar-like binding mode as suggested by modelling. The specificity of the observed effects of the compound series was tested in cell-based assays for antiproliferation activity using Jurkat cells and PHA-stimulated PBMC. These tests were also verified by addition of exogenous uridine to the culture medium. In particular, the triazole analogue **6** (IC_{50} against *h*DHODH: 45 nM) exerted potent *in vitro* antiproliferative and immunosuppressive activity without affecting cell survival.

1. Introduction

Human dihydroorotate dehydrogenase (*h*DHODH), a flavin-dependent mitochondrial enzyme involved in *de novo* pyrimidine biosynthesis, is a validated therapeutic target for the treatment of autoimmune diseases such as rheumatoid arthritis and cancer [1–3]. Leflunomide (Fig. 1) is a disease-modifying anti-rheumatic drug that was approved more than 15 years ago for the treatment of

Abbreviations used: human dihydroorotate dehydrogenase, (*h*DHODH); L-dihydroorotate, (DHO); orotate, (ORO); reduced flavin mononucleotide, ($FMNH_2$); Quantum mechanics/molecular mechanics, (QM/MM); QM-Polarized Ligand Docking, (QPLD); Palladium on carbon, (Pd/C); phytohaemagglutinin, (PHA); peripheral blood mononuclear cell, (PBMC); isopropyl β-D-1-thiogalactopyranosidel, (IPTG); n-Undecyl-*N*,*N*-Dimethylamine-*N*-Oxide, (UDAO); potassium thiocyanate, (KSCN); poly-γ-glutamic acid low molecular weight, (PGA-LM).

* Corresponding author.
E-mail address: marco.lolli@unito.it (M.L. Lolli).

[1] Both authors contributed equally to this work.

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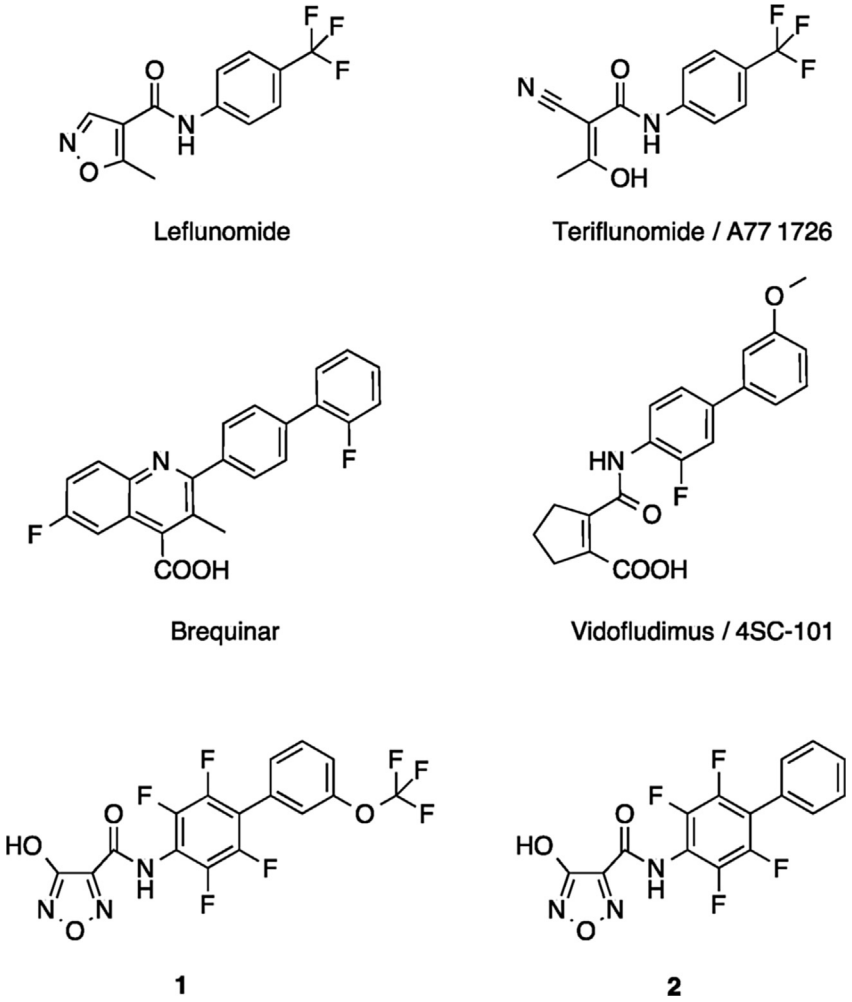

Fig. 1. Presentation of scaffolds of leflunomide, its active metabolite teriflunomide (A77 1726), brequinar, vidofludimus (4SC-101) and the hydroxyfurazan analogues **1** [5] and **2** [5].

rheumatoid arthritis and other autoimmune diseases [4].

Although associated with severe side effects such as diarrhea, abnormal liver tests, nausea, and hair loss [6], leflunomide acts as a prodrug and is rapidly converted into its active metabolite teriflunomide (also called A77 1726, Fig. 1), which is able to inhibit *h*DHODH in the low μM range [7,8]. Since the introduction of leflunomide, the search for new potent *h*DHODH inhibitors that would display similar clinical benefits as leflunomide but without associated side effects, has been on going. One of the promising compounds was brequinar [9], which was discarded as a therapeutic agent due to a narrow therapeutic window and inconsistent pharmacokinetics [2]. Another compound, 4SC-101 (vidofludimus, Fig. 1) [10], is currently undergoing phase II clinical trials for inflammatory bowel disease [6,11]. However, despite recent efforts [1,6,12–15], the quest to add new *h*DHODH inhibitors to the human pharmacopoeia remains an urgent area of research.

Earlier, we reported a series of innovative *h*DHODH inhibitors [5] designed by merging some structural features of leflunomide and brequinar and based on the acidic 4-hydroxy-1,2,5-oxadiazol-3-yl (hydroxyfurazan) moiety (Fig. 1, compounds **1** and **2**). The acidic hydroxyfurazan, connected through an amide bridge to a substituted biphenyl lipophilic moiety, was suggested to play the role of brequinar's carboxylic group by interacting with Arg136 in the *h*DHODH subsite 2 [16]. Compounds **1** and **2** were able to potently inhibit DHODH on murine liver mitochondrial membranes (50 and 66 nM respectively) [5]. The degree of fluorine substitution at the phenyl ring adjacent to the oxadiazole moiety was strongly correlated with activity. In addition, the correlation between activity and stabilization of the compounds' bioactive conformations was extensively studied [17].

Using a similar approach, in this work we describe new potent *h*DHODH inhibitors designed by selecting other acidic hydroxylated azoles, ideally substituting hydroxyfurazan in **1** and **2**. The selection of hydroxylated azole systems (specifically hydroxythiadiazole, pyrazole and triazole) was run by the possibility of establishing interactions with the small lipophilic pocket created by Val143 and Val134 (subsite 4) and by their different acidic properties [18]. Supported by promising docking scores, nine candidate structures based on three acidic heterocycles were designed and synthetized (compounds **3**–**9**, Fig. 2, Table S1).

Synthetic strategies and detailed enzymatic and cell-based studies of the designed series are presented and discussed. The suggested binding modes of the most representative molecules were confirmed by high-resolution crystal structures of *h*DHODH in complex with the compounds **4**–**6**.

2. Result and discussion

2.1. Molecular modelling

A molecular simulation of compounds **1**–**9** docked inside the *h*DHODH binding site was initially performed. Docking results,