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Featured Letter

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N-Acylbenzenesulfonamide Dihydro-1,3,4-oxadiazole Hybrids: Seeking Selectivity toward Carbonic Anhydrase Isoforms

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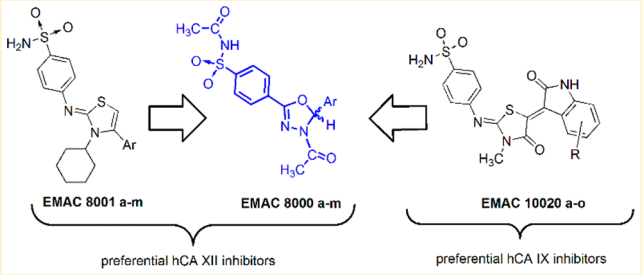
Supporting Information

ABSTRACT: A series of *N*-acylbenzenesulfonamide dihydro-1,3,4-oxadiazole hybrids (**EMAC8000a−m**) was designed and synthesized with the aim to target tumor associated carbonic anhydrase (hCA) isoforms IX and XII. Most of the compounds were selective inhibitors of the tumor associated hCA XII. Moreover, resolution of **EMAC8000d** racemic mixture led to the isolation of the levorotatory eutomer exhibiting an increase of hCA XII inhibition potency and selectivity with respect to hCA II. Computational studies corroborated these data. Overall our data indicate that both substitution pattern and stereochemistry of dihydro-1,3,4-oxadiazole could be considered as key factors to determine activity and selectivity toward hCA isozymes. These results can provide further indication for the design and optimization of selective hCA inhibitors.



KEYWORDS: *human carbonic anhydrase, hCA, N-acylbenzenesulfonamide, 1,3,4-oxadiazole, hybrids, docking*

Carbonic anhydrases (hCA, EC 4.2.1.1) are widespread enzymes that catalyze the hydration reaction of carbon dioxide into bicarbonate.[1] These enzymes are involved in several physiological processes, spacing from pH regulation, ion transport, bone resorption to the secretion of gastric, cerebrospinal fluid, and pancreatic juice.[2] In mammals the hCA family is composed of 16 different isoforms, differing for sequence and, more importantly, for tissue localization, expression, and catalytic activity.[3] Briefly, hCA I−III and hCA VII are cytosolic isoforms, while hCA IV, hCA IX, hCA XII, and hCA XIV are membrane-bound isozymes.[3] Due to their key role in cell metabolism these enzymes have been deeply investigated as drug targets, and as a result, a number of hCA inhibitors have been designed and are currently in clinical use.[4−6] In particular the trans-membrane hCA IX and hCA XII isoforms have been associated with tumor progression and invasion.[7−12] However, most of the clinically available agents inhibit hCA isoforms unselectively, and there is a considerable interest of the scientific community for the development of isozyme-selective agents for the treatment of specific pathologies.[13−16] Moreover, serious drug interactions have been reported for several hCA inhibitors, and therefore, selectivity is mandatory. In order to identify new scaffolds for the selective inhibition of tumor-associated hCAs, we have already synthesized benzenesulfonamide based hybrid molecules[17,18] in which the benzenesulfonamide moiety binds either a *N*-cyclohexyl-thiazoline or to a *N*-methyl-thiazolidine core. With respect to the central core of the hybrid molecule, a different selectivity pathway was observed, being thiazoline derivatives preferential as hCA XII inhibitors, while thiazolidine based compounds exhibit, at least in some cases, a preference toward hCA IX isozyme. Furthermore, saccharin and open derivatives of saccharin, structurally related to compounds **EMAC8000a−m**, have been recently reported as selective inhibitors of the tumor associated hCA isoforms IX and XII,[19,20] suggesting the potential of secondary benzenesulphonamides as isozyme-selective hCA inhibitors. Considering these observations and with the aim to investigate new scaffolds and

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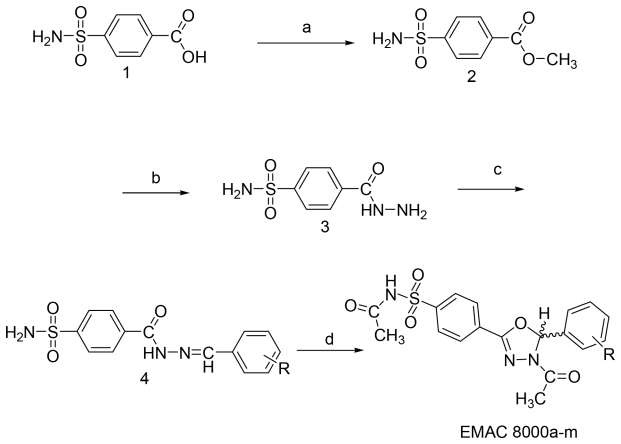
molecular geometries to target hCA IX and hCA XII, we have designed and synthesized a series of *N*-acylbenzenesulfonamide dihydro-1,3,4-oxadiazole hybrids (**EMAC8000a−m**) and evaluated their activity toward hCA isoforms I, II, IX, and XII.

The new compounds were synthesized slightly modifying an already reported multistep synthetic procedure (Scheme 1).[21,22] Briefly, 4-sulfamoylbenzoic acid (**1**) was reacted with methanol in the presence of sulfuric acid as a catalyst.

Scheme 1. Synthetic Pathway to Compounds EMAC 8000a−m[a]

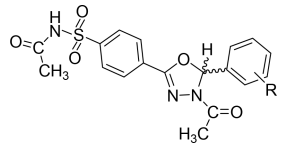


[a]Reagents and conditions: (a) H_2SO_4/MeOH; (b) NH_2NH_2 H_2O, MeOH; (c) substituted benzaldehydes, 2-propanol/CH_3COOH; (d) acetic anhydride/pyridine.

The obtained ester (**2**) was converted into 4-(hydrazinecarbonyl)benzenesulfonamide (**3**) by treatment with hydrazine hydrate in methanol. 4-[*N*′-(Arylmethylidene)-hydrazinecarbonyl]benzenesulfonamide (**4**) was obtained by reaction of **3** with differently substituted benzaldehydes in 2-propanol and acetic acid as a catalyst. The formation of the dihydro-oxadiazole derivatives **EMAC8000a−m**, was accomplished by refluxing **4** in acetic anhydride, as already described, with the addition of pyridine in catalytic amounts. After cooling to room temperature, methanol was added to the reaction mixture. The obtained yellowish solution was then poured into ice water and vigorously stirred. A precipitate was formed that was washed with 10% aqueous $NaHCO_3$ solution and purified by crystallization. Compounds **EMAC8000a−m** were characterized by means of both analytical and spectroscopic methods (see Table S1−S3 and Figure S1−S26) and submitted to biological evaluation toward hCA isoforms I, II, IX, and XII (Table 1).

All **EMAC8000** derivatives displayed a preferential activity toward the isoform hCA XII, except for compounds **EMAC8000 d**, **e**, **f**, and **i** that exhibited a moderate activity on the hCA IX isoform. Surprisingly, although we had previously observed a pseudoconjugative effect between the two aromatic substituents in positions 2 and 5 of the dihydro-oxadiazole,[23] no significant difference in the biological activity of the **EMAC8000** compounds could be related to the electronic nature of the substitution on the phenyl ring in the position 5 of the heterocycle. On the contrary, the steric effects of the substituents seemed to be more relevant for the inhibition potency. In fact, the presence of a bulky substituent in position 4 of the phenyl ring such as −Cl, $-NO_2$, $-OCH_3$, or $-CF_3$ generally lead to a decrease of the inhibitory potency

Table 1. Inhibition Data Towards hCA I, II, IX, and XII of Compounds EMAC8000a−m[a]



		K_i (nM)			
EMAC	R	hCA I	hCA II	hCA IX	hCA XII
8000a	4-Cl	8404	485	502	11.6
8000b	4-CH_3	9410	529	502	5.2
8000c	2-OCH_3	6776	450	378	6.2
8000d	2,4-Cl	3280	83.2	25.0	18.4
8000e	4-F	8245	277	43.6	6.8
8000f	4-NO_2	3872	158	33.1	47.7
8000g	2-NO_2	>10000	293	159	5.0
8000h	4-OCH_3	>10000	239	143	34.0
8000i	3-NO_2	>10000	120	44.3	6.7
8000j	2-CH_3	9376	172	489	6.3
8000k	3-OCH_3	8952	571	430	28.4
8000l	2,4-F	8926	327	373	6.8
8000m	4-CF_3	>10000	892	468	53.8
AAZ		250	12.0	25	5.7

[a]Mean from three different assays, by a stopped flow technique (errors were in the range of ±5−10% of the reported values).

toward hCA XII. According to these observations, we decided to investigate on the role of the stereochemistry at position 5 of the dihydro-oxadiazole ring. **EMAC8000d** was chosen for this preliminary study due to its activity toward both hCA IX and XII.

Thus, the enantioseparation of compound **EMAC8000d** was performed by means of semipreparative HPLC using the amylose-based Chiralpak IA as a chiral stationary phase. Based on the chromatographic results of analytical screening, the ethanol/acetonitrile/H_2O 55/40/5 (v/v/v) mixture was identified as the most suitable eluent for the enantioseparation of **EMAC8000d** on the 1 cm I.D. IA column. An amount of ∼2 mg of racemic samples was resolved for each chromatographic run, and both enantiomers were collected with high enantiomeric purity (>99% ee, Figure S27 and Table S4). The pure enantiomers were then submitted to biological evaluation to investigate the role of stereochemistry on both inhibition potency and isozyme selectivity. Results are reported in Table 2. The activity toward the hCA isoforms was assessed by a previously reported procedure.[24]

Table 2. Inhibition Data toward hCA I, II, IX, and XII and Tumor Associated hCA IX and XII Isoforms Selectivity (SI) versus hCA II of EMAC8000d Pure Enantiomers[a]

	K_i (nM) hCA					
EMAC 8000	I	II	IX	XII	SI hCA XII/II	SI hCA IX/II
(±)-d	3280	83.2	25.0	18.4	4.52	3.33
(+)-d	>10000	59	26.9	38.0	1.55	2.19
(−)-d	1010.02	117.2	24.2	7.5	15.63	4.84
AAZ	250	12.0	25	5.7	2.10	0.48

[a]Mean from three different assays, by a stopped flow technique (errors were in the range of ±5−10% of the reported values).

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Noteworthy, an increase in the inhibitory potency was observed only toward the hCA XII isozyme in the case of the (−)-**EMAC8000d** enantiomer. On the contrary, the (+)-**EMAC8000d** enantiomer resulted as the most active toward the II isozyme, mainly due to the loss of activity of the (−)-**EMAC8000d** enantiomer hCA II. All together this behavior led to a 10-fold increase of the selectivity of the (−)-**EMAC8000d** enantiomer toward hCA XII with respect to the II isozyme. To a lower extent, a similar behavior was observed for the hCA IX isozyme. However, the gain in selectivity toward the hCA IX isoform, with respect to the hCA II isozyme, could only be related to the reduction of the potency of the (−)-**EMAC8000d** enantiomer toward the hCA II isozyme, being the two enantiomers almost equally potent toward the hCA IX. The absolute configuration of (−)-**EMAC8000d** and (+)-**EMAC8000d** enantiomers was not assigned. However, the two enantiomers R and S were considered in the computational investigation. In the light of the biological results exhibited by the pure enantiomers and to rationalize the major activity toward hCA XII of the (−)-**EMAC8000d** enantiomers both **EMAC8000d** enantiomers were subjected to molecular docking applying the Quantum-mechanics polarized Docking (QMPL).[25,26] Prior to perform the docking study on unknown compounds, the protocol has been validated through simulations of self- and cross-docking of known hCA XII inhibitors: the 4-propylthiobenzenesulfonamide (VD9) and acetazolamide (AAZ) into the crystal structure with pdb code 4WW8 (resolution 1.42 Å).[27] Both compounds were successfully docked with the QMPL protocol, displaying root-main-square deviation (RMSD) values lower than 2.0 Å (Figure S28 and Table S5). Both oxadiazolidine derivatives (*S*)-**EMAC8000d** and (*R*)-**EMAC8000d** have been docked into the above-mentioned crystal structure and the best scored poses were subjected to a postdocking protocol based on energy minimization and binding-free energy calculation, due to lack of accuracy of docking score in the overall energy evaluation (Table S6). The total energies of interactions were obtained applying molecular mechanics and continuum solvation models using the molecular mechanics generalized Born/surface area method (MM-GBSA) (Table S7).[28] After these data were collected and analyzed, we were then able to establish which enantiomer was mainly stabilized within the hCA XII active site.

The R enantiomer showed a value of binding free energy of −28.36 kcal/mol, while the S enantiomer a value of −25.75 kcal/mol. This difference could partially explain the better activity of the enantiomer in the biological test. In order to confirm this hypothesis, a detailed analysis of the crucial interactions is required. In fact, the R configuration allows the formation of a hydrogen bond between Asn64 and the amidic oxygen of the dihydro-oxadiazole ring, while the different spatial distribution of the atoms in the S enantiomer does not permit this important interaction. It must be pointed out that the hydrogen bond with Thr198 and His117 and the interaction with the Zn ion is well conserved with both configurations (Figure 1), confirming the same binding mode of this class of compounds.

The putative binding mode confirmed the importance of the substituent in position 5 of the dihydro-oxadiazole ring and the role of stereochemistry in the affinity for this isoform. In addition, the selectivity toward the hCA XII isoform has been demonstrated through the analysis of the binding site of all the tested isoforms. In particular, the substitutions in hCA XII: of

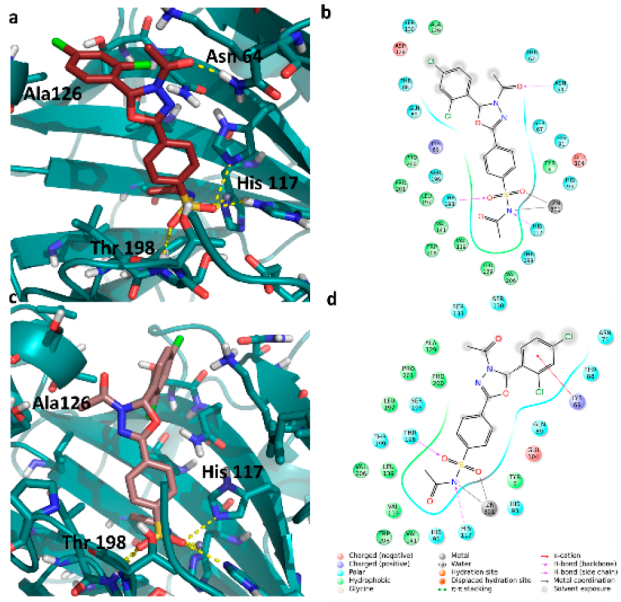

Figure 1. (a,c) Three-dimensional representation of the putative binding mode obtained by docking experiment of (*R*)-**EMAC8000d** (in red) and (*S*)-**EMAC8000d** (in pink) into hCA XII (pdb code 4WW8) and (b,d) 2D representation of the complexes stabilizing interactions with the binding site residues.

Ala126 into Phe131 in hCA II, of Thr88 into Phe 91 in hCA I, and of Asn203 into Tyr204 in hCA I, led to relevant differences between the four isoforms. These residues are located in the entrance of the binding site, which results bigger in the hCA XII compared to the isoform hCA II and hCA I (Figures S29 and S30). Hence this could explain why our compounds are generally more selective toward the hCA XII isoform and could give a rationale for the higher activity of one of the two enantiomers. To corroborate our results, we have docked the compounds **EMAC8000f** and **EMAC8000m** into hCA XII. These compounds are the less active toward the hCA XII isoform and are characterized by the presence of a NO_2 and a CF_3 group in position 4 of the phenyl ring, respectively. The steric hindrance of this group does not allow a fully efficient accommodation in the binding site. This is further confirmed by their lower value of ΔG (Table S7 and Figure S31). In summary, a small library of *N*-acylbenzenesulfonamide dihydro-1,3,4-oxadiazole hybrids has been synthesized and characterized. Our preliminary data indicate that the hybridization of the dihydro-oxadiazole ring with the *N*-acylbenzenesulfonamide moiety leads to preferential inhibition of the hCA XII isoform. In this respect both the substitution pattern and the stereochemistry at position 5 of the oxadiazole ring appear to be key features to modulate the isoform selectivity. With these data in our hands we aim to further develop these derivatives to achieve more effective tumor associated hCA inhibitors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00205.

Experimental procedures, compounds characterization, tables, and schemes (PDF)

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Author Contributions

[∇]These authors contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Biographies

Simona Distinto received her Ph.D. in Pharmaceutical Synthesis from the University of Cagliari (2006). After a postdoctoral fellowship (2006−2011) in Computational Chemistry at the Computational Pharmaceutical Chemistry Laboratory of the University of Catanzaro and at the Computational Computer Aided Molecular Design (CAMD) Group of the University of Innsbruck, she joined the faculty of Biology and Pharmacy of Cagliari as a researcher in 2012. She is currently an Associate Professor in Medicinal Chemistry at the University of Cagliari and heads the Computer Aided Drug Design (CADD) group. Her research focuses on CADD methods and bioinformatic tools applied to several biological relevant targets.

Claudiu T. Supuran is a professor of Medicinal Chemistry at University of Florence, Italy (since 1994). He has published more than 1350 papers and his Hirsch factor is 119. His main research interest is carbonic anhydrase inhibitors, and he discovered several new classes over the years. One of the sulfonamides identified by his group completed Phase I clinical trials in 2014 for the treatment of advanced, metastatic solid tumors and is now in Phase II trials. His laboratory is involved in drug design, synthesis, enzymology, molecular biology, and computational and X-ray crystallographic studies of metalloenzyme inhibitors.

ABBREVIATIONS

hCA, carbonic anhydrase; QMPL, quantum-mechanics polarized docking; VD9, 4-propylthiobenzenesulfonamide; AAZ, acetazolamide; MM-GBSA, molecular mechanics generalized Born/surface area method

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