Journal of Medicinal Chemistry

Article

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Synthesis, Structure–Activity Relationship Studies, and X-ray Crystallographic Analysis of Arylsulfonamides as Potent Carbonic Anhydrase Inhibitors†

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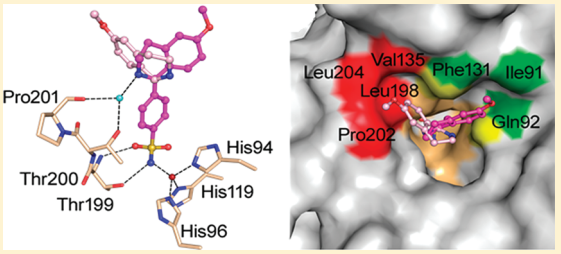
Supporting Information

ABSTRACT: A series of arylsulfonamides has been synthesized and investigated for the inhibition of some selected human carbonic anhydrase isoforms. The studied compounds showed significant inhibitory effects in the nanomolar range toward druggable isoforms (hCA VII, hCA IX, and hCA XIV) (K_i values from 4.8 to 61.7 nM), whereas they generally exhibited significant selectivity over hCA I and hCA II, that are ubiquitous and considered off-target isoforms. On the basis of biochemical data, we herein discussed structure–affinity relationships for this series of arylsulfonamides, suggesting a key role for alkoxy substituents in CA inhibition. Furthermore, X-ray crystal structures of complexes of two active inhibitors (**I** and **2a**) with hCA II allowed us to elucidate the main interactions between the inhibitor and specific amino acid residues within the catalytic site.

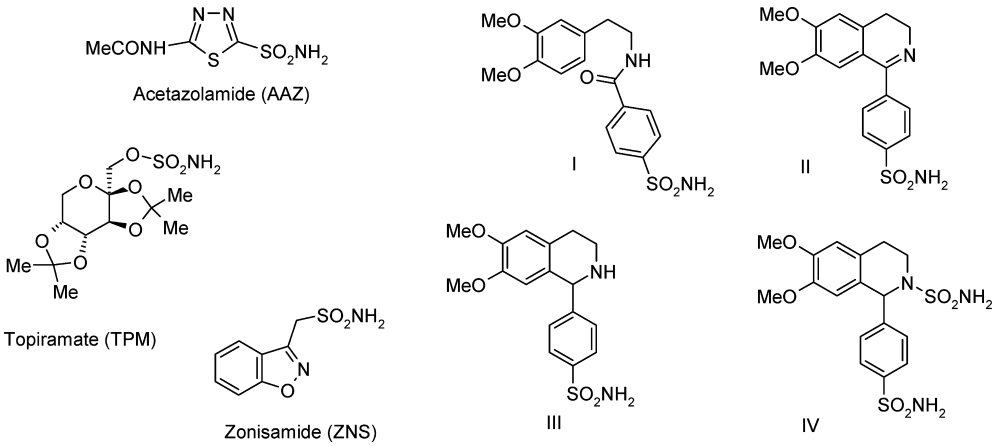


INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc metalloenzymes that catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion. To date, 16 human CA (hCA) isoforms have been identified that display significant differences in catalytic activity, subcellular localization, and tissues expression.[1] They play relevant roles in a large series of physiological processes such as intracellular and extracellular pH homeostasis, cell proliferation and differentiation, modulation of neuronal transmission, and several biochemical pathways where either CO_2 or bicarbonate is required. Furthermore, this family of enzymes is involved in some pathological pathways and thus are interesting targets for the treatment of glaucoma, cancer, obesity, and epilepsy. On this basis, the development of CA inhibitors (CAIs) possessing high potency and selectivity against specific isoforms represents an attractive strategy to obtain new active compounds able to avoid side effects, thus improving therapeutic safety.[1−8] Among the different mammalian isoforms, the hCA VII, hCA IX, and hCA XIV have recently been shown to be druggable targets. hCA VII is one of the least investigated cytosolic CA isoforms; it presents a limited distribution, being mainly expressed in the cortex, hippocampus, and thalamus regions within the mammalian brain where it is considered involved in generating neuronal excitation and seizures.[9,10] Recently, the hCA VII involvement in neuropathic pain control was proposed.[11] This could represent an interesting pharmacological mechanism for the design of new pain killers useful for therapeutic applications. hCA IX is expressed in a limited number of normal tissues whereas it is overexpressed in many solid tumors and considered involved in critical processes connected with cancer progression. The expression of hCA IX is strongly up-regulated by hypoxia via the hypoxia inducible factor-1 (HIF-1) transcription factor. The overexpression of hCA IX induces the pH imbalance of tumor tissue contributing significantly to the extracellular acidification of solid tumors; thus, hCA IX inhibitors could specifically bind hypoxic tumor cells expressing this isoform. Consequently, they have been proposed as antitumor agents.[12−19] hCA XIV is a transmembrane isozyme with the active site oriented extracellularly; it is highly abundant in neurons and axons in the murine and human brain, where it seems to play an important role in modulating excitatory synaptic transmission.[20]

Received: January 26, 2012
Published: March 26, 2012

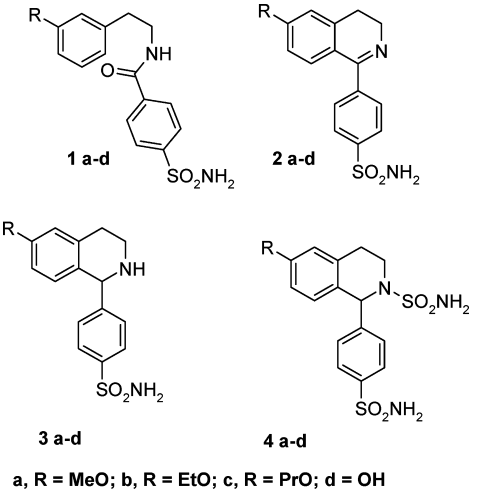
Chart 1. Well-Known CAIs and Previously Reported Arylsulfonamides[24]



The catalytic domain of CA is generally composed of three histidine residues (His94, His96, and His119) coordinating the zinc(II) ion. So the active zinc ion can bind hydroxide ion that reacts with carbon dioxide to give bicarbonate.[21] This implies that the CAIs are generally molecules containing specific chemical groups able to coordinate the zinc ion of the catalytic binding site thus blocking the CA enzymatic activity. The most well-known CAIs contain a sulfamide/sulfonamide/sulfamate moiety (Chart 1, acetazolamide, topiramate, and zonisamide) that in deprotonated form binds the active zinc ion in place of physiological substrates in the catalytic cycle of CAs.[22] We recently reported that some sulfamide-based heterocyclic compounds such as *N*-sulfonamide-1,2,3,4-tetrahydroisoquinolines can bind the CA catalytic site, displaying significant inhibitory effects as well as very high selectivity toward hCA VII, hCA IX, and hCA XIV.[23−26] The CA interaction has been demonstrated by crystallographic data of hCA II in complexes with some active *N*-sulfonamide-1,2,3,4-tetrahydroisoquinolines (PDB codes 3IGP and 3PO6);[25,27] from this study, their sulfonamide moiety has been demonstrated to be bound within the catalytic site through zinc ion and specific amino acid residues. Moreover, by means of docking studies we explored the involvement of other amino acid residues driving the isoform selectivity against hCA IX and hCA XIV.[25] We also found that the arylsulfonamide derivatives **I**−**IV** (Chart 1)[24] were active and selective CAIs, showing significant inhibitory effects at nanomolar concentration and a good selectivity ratio over hCA I and hCA II isozymes that are abundant in many tissues and are considered responsible for some unwanted side effects of CAIs currently used in therapy. Particularly, it was highlighted that 4-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)benzenesulfonamide (**III**, Chart 1) displayed very interesting inhibitory effects against hCA VII isoform (K_i value of 4.6 nM) and low hCA II activity (K_i value of 3640 nM).[24]

In this context we herein report the synthesis of 16 arylsulfonamides **1a**−**d**, **2a**−**d**, **3a**−**d**, and **4a**−**d** (Chart 2) strictly related to our previously studied compounds **I**−**IV**. Our idea was first and foremost to understand the role of methoxy substituents. So we planned the synthesis of monomethoxy analogues (R= MeO) as well as of other monalkoxy derivatives (R = EtO or PrO) and of the corresponding hydroxyl ones (R = OH). In this way we hoped to explore how these modifications could influence the interaction with hydrophilic/hydrophobic areas of the catalytic pocket, thus controlling the activity/selectivity against some CA isoforms (hCA I, hCA II, hCA VII, hCA IX, and hCA XIV). Therefore, in the current paper both biochemical screening and X-ray crystal structures of some active derivatives in complex with hCA II have been carried out to explore the CA inhibitory effects and the different protein interactions of this new series of arylsulfonamides (**1**−**4**, Chart 2).

Chart 2. Designed Compounds

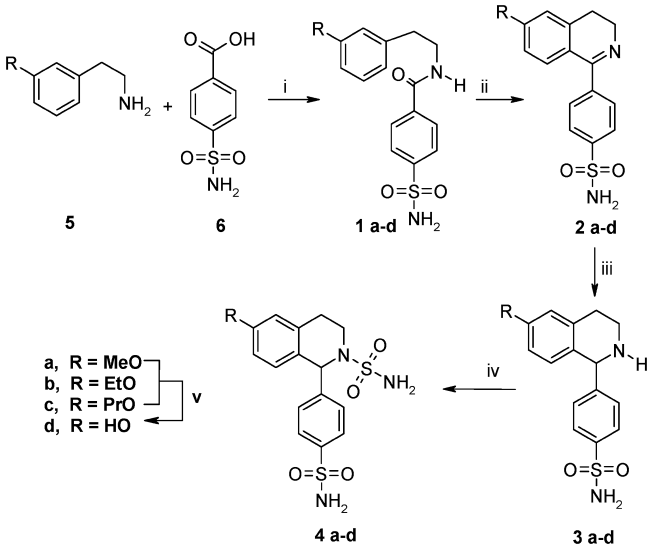


CHEMISTRY

As depicted in Scheme 1, a series of arylsulfonamides derivatives (**1**−**4**) was prepared by following a previously reported procedure[24] with slight modifications. By coupling of 4-(aminosulfonyl)benzoic acid (**6**) with suitable 2-(3′-alkoxyphenyl)ethylamine derivatives **5a**−**c**, we obtained the corresponding 4-(aminosulfonyl)-*N*-[2-(3′-phenylethyl]-benzamides **1a**−**c** that were cyclized by treatment with phosphorus oxychloride, giving the 3,4-dihydroisoquinoline derivatives **2a**−**c**; then by reduction of the intermediates **2a**−**c** with sodium borohydride we prepared the 1,2,3,4-tetrahydroisoquinolines **3a**−**c** as racemic mixture. Successively, the treatment of compounds **3a**−**c** with an excess of sulfamide yielded 1-[4-(aminosulfonyl)phenyl]-6-alkoxy-1,2,3,4-tetrahydroisoquinolinesulfonamides **4a**−**c**. Finally, by dealkylation of the suitable alkoxy parent compounds, we obtained in good yields the corresponding hydroxyl analogues (**1d**, **2d**, **3d**, and **4d**).

dx.doi.org/10.1021/jm300112w | J. Med. Chem. 2012, 55, 3891−3899

Scheme 1[a]



[a]Reagents and conditions: (i) HBTU, DMF, NEt_3, rt, overnight; (ii) $POCl_3$, toluene, Δ, 6 h; (iii) $NaBH_4$, CH_3OH, rt, 1 h; (iv) $CH_3CH(OCH_3)_2$, $NH_2SO_2NH_2$, MW, two steps of 20 min at 100 °C, 150 W; (v) BBr_3 (1 M in DCM), DCM, rt, overnight.

RESULTS AND DISCUSSION

All new synthesized compounds were assayed for their ability to inhibit some human carbonic anhydrases selected among ubiquitous and druggable isoforms (hCA I, hCA II, hCA VII, hCA IX, and hCA XIV). The K_i values for the 16 arylsulfonamides (**1a−d**, **2a−d**, **3a−d**, and **4a−d**) have been determined using a stopped-flow assay[28] monitoring CA-catalyzed hydration of carbon dioxide and are reported in Table 1. These results were compared with data previously obtained for the corresponding dimethoxy analogues (**I−IV**) and the reference compounds acetazolamide (AAZ), topiramate (TPM), and zonisamide (ZNS). From the data of Table 1 some relevant structure−activity relationships (SARs) can be highlighted for the four classes of synthesized compounds. In the benzamide series (i.e., compounds **1a−d**) the removal of one methoxy substituent generally gave an improvement in selectivity with respect to dimethoxy derivative **I** that resulted in a very potent inhibitor (K_i 2.7−8.7 nM) against all studied isoforms. In particular the 3-methoxy derivative **1a** was a more selective inhibitor than **1b−d** toward the druggable isoform hCA IX; the 3-ethoxy-substituted **1b** was optimal to improve hCA VII activity whereas the 3-propoxy derivative **1c** behaved like the dimethoxy analogue **I**, thus showing high inhibitory efficacy against hCA VII and hCA IX and no selectivity over hCA II. The substitution with a 3-hydroxyl group provided an increase in hCA II activity (**1d**). Among dihydroisoquinoline derivatives **2a−d**, the 6-methoxy derivative **2a** demonstrated an impressive improvement of hCA II affinity (250-fold) when compared with the corresponding 6,7-dimethoxy analogue **II**, thus furnishing some suggestions about the mode of interaction within the catalytic site (vide infra, X-ray studies). The most interesting inhibitor was the 6-ethoxy-substituted compound **2b** that showed high affinity toward hCA VII and hCA IX (K_i 6.5 and 7.2 nM, respectively) and very low efficacy against off-target isoforms hCA I and **II**. Compound **2c** displayed low selectivity toward hCA I unlike **2d** (R = OH) for which very

Table 1. K_i Values (nM) against hCA I, hCA II, hCA VII, hCA IX, and hCA XIV Isoforms Shown by Arylsulfonamide Derivatives Acetazolamide (AAZ), Topiramate (TPM), and Zonisamide (ZNS)

	K_i (nM)[a]				
	hCA I	hCA II	hCA VII	hCA IX	hCA XIV
I[b]	8.7 ± 0.4	2.7 ± 0.1	5.8 ± 0.3	3.8 ± 0.2	4.3 ± 0.4
II[b]	3000 ± 76	2150 ± 16	73.3 ± 6.2	210 ± 14	103 ± 9
III[b]	920 ± 43	3640 ± 127	4.6 ± 0.3	130 ± 11	112 ± 8
IV[b]	1540 ± 39	3130 ± 168	7.0 ± 0.5	76.0 ± 5	87.0 ± 7
1a	474 ± 21	86.7 ± 7	61.7 ± 4	6.5 ± 0.3	50.2 ± 4
1b	514 ± 28	33.0 ± 2	4.8 ± 0.5	8.1 ± 0.3	8.6 ± 0.7
1c	50.4 ± 5	5.3 ± 0.3	5.9 ± 0.4	8.3 ± 0.2	51.0 ± 5
1d	24.6 ± 2	4.9 ± 0.5	13.9 ± 1.1	41.7 ± 3	22.4 ± 2
2a	681 ± 51	8.6 ± 0.6	10.2 ± 1.0	18.3 ± 1.2	8.9 ± 0.5
2b	1050 ± 54	87.5 ± 3.7	6.5 ± 0.4	7.2 ± 0.1	17.9 ± 1.5
2c	83.1 ± 7.1	8.8 ± 0.8	8.0 ± 0.3	16.1 ± 1.2	8.7 ± 0.6
2d	5.1 ± 0.3	31.1 ± 2.5	53.6 ± 5	51.5 ± 1.0	46.9 ± 3.7
3a	9.2 ± 0.6	8.8 ± 0.2	5.7 ± 0.5	15.4 ± 1.0	8.6 ± 0.6
3b	667 ± 25	43.5 ± 3.1	5.8 ± 0.4	9.2 ± 0.7	8.7 ± 0.1
3c	72.3 ± 5.9	8.9 ± 0.8	8.2 ± 0.7	9.2 ± 0.8	15.3 ± 1.3
3d	24.3 ± 2.1	84.5 ± 6.4	34.7 ± 1.5	68.3 ± 3.9	55.9 ± 1.8
4a	1210 ± 35	48.5 ± 4.0	23.4 ± 1.4	6.5 ± 0.5	5.2 ± 0.3
4b	4165 ± 93	33.2 ± 1.9	17.8 ± 0.8	52.1 ± 2.8	6.7 ± 0.4
4c	6.7 ± 0.1	6.3 ± 0.3	7.9 ± 0.7	7.8 ± 0.5	9.2 ± 0.3
4d	6.7 ± 0.4	6.7 ± 0.2	50.8 ± 2.5	7.9 ± 0.6	24.8 ± 1.9
AAZ[b]	250 ± 12	12 ± 0.6	2.5 ± 0.1	25 ± 1.4	41 ± 1.7
TPM[b]	250 ± 10	10 ± 1.0	0.9 ± 0.1	58 ± 3.7	1460 ± 62
ZNS[b]	56 ± 1.6	35 ± 1.8	117 ± 9.1	5.1 ± 0.3	5250 ± 174

[a]Mean ± standard error, from three different assays. Recombinant full length hCA I, II VII, and XIV and catalytic domain of hCA IX were used. [b]Data are taken from ref 24.

dx.doi.org/10.1021/jm300112w | J. Med. Chem. 2012, 55, 3891−3899

high hCA I activity (K_i value of 5.1 nM) was observed (Table 1).

Among the 1,2,3,4-tetrahydroisoquinolines (**3**), the monoalkoxy derivatives (**3a−c**) were potent inhibitors of hCA VII, hCA IX, and hCA XIV isoforms (K_i 5.7−15.4 nM); in particular, the 6-ethoxy-substituted compound **3b** was the best inhibitor in terms of activity/selectivity. This compound had inhibition constants ranging from 5.8 to 9.2 nM against the selected druggable isoforms and showed a good selectivity ratio exclusively over hCA I when compared with 6,7-dimethoxy analogue **III** (see Table 1). Compound **3d** (R = OH) exhibited good inhibitory effects but no significant selectivity. Finally, the *N*-sulfonamide-1,2,3,4-tetrahydroisoquinolines **4a−d** were active against all tested isoforms but displayed very low selectivity with to respect compound **IV**.

Overall, the newly synthesized compounds show K_i values ranging from 4.8 to 68.3 nM toward hCA VII, hCA IX, and hCA XIV isozymes, displaying in some cases (**2−4**) a CA inhibition profile better than that of their parent (**2−4** vs **II−IV**) and standard compounds. The most effective and selective inhibitors are ethoxy-substituted derivatives **1b−4b** whereas the presence of a hydroxyl function (e.g., compounds **1d−4d**) generally gives a reduction in activity. Therefore, this study shows that these new arylsulfonamide derivatives generally maintain high affinity whereas they behave as weak selective inhibitors toward only one CA isoform.

To investigate the inhibitor−protein interactions, we cocrystallized two of the most active inhibitors (compound **I** and compound **2a**) with the isoform hCA II, and the crystal structures were determined at atomic resolution (PDB codes 3V7X and 3VBD). The data collection and refinement statistics are summarized in Table 2. The crystal structures of hCA II in complex with compounds **I** and **2a** were determined by the difference Fourier method and refined using data to 1.03 Å and 1.05 Å, respectively. Both complexes crystallized in the monoclinic $P2_1$ space group with one hCA II molecule in the asymmetric unit and solvent content of 41.2% and 40.5% for compounds **I** and **2a**, respectively. All hCA II amino acid residues could be traced into a well-defined electron density map with the exception of side chains of some terminal and surface amino acid residues (Ser2, Lys9, and Lys261 in hCA II in complex with compound **I**, and Ser2, Lys9, Lys45, and Lys261 in hCA II in complex with compound **2a**), which were thus not included in the final model.

Compounds **I** and **2a** could be modeled into a well-defined electron density present within the enzyme active site (see Figure 1A,B). The high quality of the experimental data allowed the interpretation of the electron maps by two alternative conformations of inhibitor. The sulfonamide aryl group of **I** and **2a** is bound in one unambiguous conformation, while both the 2-(3′,4′-dimethoxyphenyl)ethylamine fragment (**I**) and 6-methoxy-3,4-dihydroisoquinoline nucleus (**2a**), that are distal from sulfonamide group, acquire two different conformations with equivalent partial occupancies (Figure S1, Supporting Information).

Interactions of compounds **I** and **2a** with the active site of hCA II are depicted in panels C and E of Figure 1, and the complete lists of interactions between the two inhibitors and hCA II are reported in Table S1, Supporting Information. Major interactions of the inhibitors with the enzyme active site are mediated through the deeply buried sulfonamide group, which makes polar interactions with the zinc ion and residues located at the bottom of the active site cavity (His94, His96, and His119), similar to those observed for other arylsulfonamides previously reported in the literature.[5,29−31] The ionized nitrogen atom of the sulfonamide moiety is coordinated to the zinc ion at a distance of about 2.0 Å. The sulfonamide nitrogen also forms a hydrogen bond with the hydroxyl group of the Thr199 side chain, and one oxygen atom from the sulfonamide moiety forms a hydrogen bond with the backbone amine group of Thr199. The position of the sulfonamide group as well as the position of the aryl moiety within the enzyme active site is identical for **I** and **2a** (see Figure 1C,D).

An additional polar interaction of compounds **I** and **2a** with the enzyme is added through water-mediated interaction of the amine group present in both inhibitors with the side chain of Thr200 and main chain of Pro201. In compound **I**, the carbonyl group is involved in an additional water-mediated interaction with the side chain of Gln92 (Figure 1C). Figure 1 shows that the substituents distal from sulfonamide group interact with residues at the opening of the enzyme active site. These groups apparently have some degree of freedom in their binding to the enzyme active site because they are able to acquire two alternative conformations (see Figure 1C,D). Compound **I** binds in an extended conformation in which the positions of the two alternative conformations are quite similar.

Table 2. Data Collection Statistics and Refinement Statistics

	hCA II in complex with **I**	hCA II in complex with **2a**
space group	$P2_1$	$P2_1$
unit cell parameters		
a, *b*, *c* (Å)	42.14, 41.51, 71.84	42.01, 41.18, 71.76
α, β, γ (deg)	90, 104.23, 90	90, 104.33, 90
wavelength (Å)	0.918	0.915
resolution range (Å)	15.47−1.03 (1.06−1.03)	16.76−1.05 (1.08−1.05)
no. of unique reflections	117 997 (8733)	109 951 (7248)
multiplicity	3.7 (3.4)	3.5 (2.6)
completeness (%)	99.8 (100.0)	99.1 (89.0)
R_{merge}[a] (%)	5.8 (58.4)	5.3 (41.3)
average $I/\sigma(I)$	9.8 (2.3)	9.6 (2.0)
Wilson B (Å^2)	11.3	6.0
refinement statistics		
resolution range	10.0−1.03	10.0−1.05
no. of reflections in working set	112 061	104 286
no. of reflections in test set	5916	5556
R[b] (%)	11.65	12.71
R_{free}[c] (%)	14.81	16.03
rmsd bond length (Å)	0.020	0.015
mean ADP protein−inhibitor (Å^2)	13.9	15.1
PDB code	3V7X	3VBD

[a] $R_{merge} = \sum_{hkl}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where the $I_i(hkl)$ is an individual intensity of the *i*th observation of reflection *hkl*, and $\langle I(hkl)\rangle$ is the average intensity of reflection *hkl* with summation over all data. [b] $R = ||F_o| - |F_c||/|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. [c] R_{free} is equivalent to *R* but is calculated for 5% of the reflections chosen at random and omitted from the refinement process.[49]

dx.doi.org/10.1021/jm300112w | J. Med. Chem. 2012, 55, 3891−3899

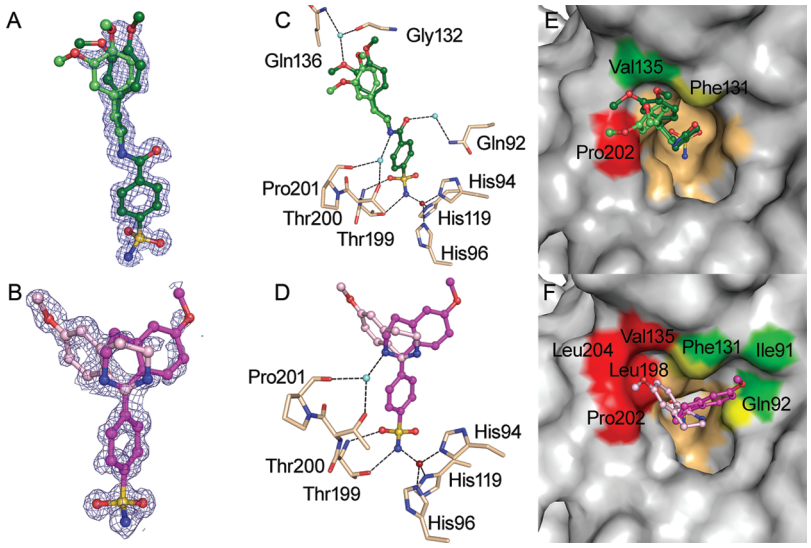


Figure 1. Panels A and B show electron density maps for the inhibitory compounds **I** (labeled A) and **2a** (labeled B). The $2F_o - F_c$ electron density map for compounds **I** and **2a** are contoured at 0.9σ and 0.8σ, respectively. The inhibitors are in stick representation with carbon atoms of compound **I** colored green and compound **2a** magenta (heteroatoms are colored according to standard color coding: oxygen, red; nitrogen, blue; sulfur, yellow). Carbon atoms with alternative conformations are shown in lighter shades of original colors. Panels C and D show the compounds and active site residues involved in either direct or water-mediated polar interactions in the active site (same color coding as was used in left-hand panels is used for inhibitor atoms and amino acid residues). Panels E and F show inhibitors in the active site cavity represented by solvent-accessible surface with residues involved in van der Waals interactions color coded as follows: residues involved in van der Waals interactions with arylsulfonamide moiety are colored light brown, residues forming interactions with both alternative conformations are colored yellow, residues forming interactions with alternative conformation 1 only are colored green, and residues forming interactions with alternative conformation 2 only are colored red.

The dimethoxyphenyl substituent is mostly solvent-exposed, having only minor van der Waals interactions with specific residues such as Phe131, Val135, and Pro202. The oxygen of the 3-methoxy group of conformation 1 makes a water-mediated hydrogen bond with residues Gly132 and Gln136. On the contrary, in compound **2a** the binding of two alternative conformations differ substantially and each conformation occupies a different surface pocket located on the opposite side of the enzyme active site entrance (see panel D of Figure 1). We found that conformation 1 (magenta) interacts with residues Ile91, Gln92, and Phe131. On the contrary, conformation 2 (pink) interacts with the hydrophobic pocket formed by residues Phe131, Val135, Leu198, Pro202, and Leu204. Thus, the hCA II/binding was useful to reveal that two different atropisomers of compound **2a** cocrystallized within the enzymatic catalytic pocket. On the contrary, the existence of these two atropisomers remained undetected in the ^{1}H NMR spectra of compound **2a**. Because each conformer adopted an orientation which ensured the formation of a good interaction with the hydrophobic pocket, the observed atropisomerism might not have significant impact on inhibitory effects.

The crystal structure of inhibitor **2a** in complex with hCA II was employed to decipher the CA inhibitory effects toward all studied isoforms (see Table 1). Analyzing the differences in the amino acid composition of the active site of hCA I, hCA II, hCA VII, hCA IX, and hCA XIV isoforms, we tried to explain the CA selectivity and the different degree of inhibitory effects displayed by the most active compounds among this new series of CAIs.

We superimposed the coordinates of hCA I (PDB code 3LXE, orange),[32] hCA VII (PDB code 3MDZ, magenta), hCA IX (PDB code 3IAI, cyan),[33] and hCA XIV (PDB code 1RJ6, green)[34] onto the coordinates of hCA II (gray) in complex with both conformers of compound **2a**, and the results are reported in Figure 2A–D. Although the five studied isoforms are similar and some crucial residues are conserved (Gln92, Leu198, Pro202), there are several differences in the amino acid composition within the catalytic site. The weaker K_i value of compound **2a** toward hCA I compared to hCA II could be mainly attributed to the different hydrophobicity of the area where the two crucial residues Leu/Phe131 and Ala/Val135 (see Figure 2A) are located. In particular, these residues are involved in relevant interactions with the hydrophobic portion of the methoxy substituent on the benzene fused ring of both **2a** conformers. So we can speculate that the reduction of hydrophobicity of hCA I might reduce the affinity of compound **2a**.

The superposition between hCA II in complex with **2a** and hCA VII (Figure 2B) confirms that the hydrophobic contact with Phe131 (Phe133 in hCA VII numbering) seems to play an important role in the binding mode of compound **2a** via the methoxy group within the catalytic site of hCA VII.

Of particular note are the results of the superposition of the hCA IX catalytic site and hCA II in complex with compound **2a**. In fact, in this case the three hydrophobic residues Leu91, Val131, and Leu135 create a hydrophobic region (see Figure 2C) that could compensate for the absence of the previously mentioned crucial Phe residue.

In a similar way the Leu131 and Ala135 residues model the hydrophobic area in hCA XIV, creating the surface for inhibitor **2a** interaction, thus justifying its high K_i value against this isoform. Considering the high similarity between hCA I and hCA XIV, this result appears to be a contradiction; however, it is possible to hypothesize that the presence of the bulky phenyl ring of the Phe91 residue in hCA I could lead to a steric clash, thus precluding the interaction within the catalytic pocket.

dx.doi.org/10.1021/jm300112w | J. Med. Chem. 2012, 55, 3891–3899

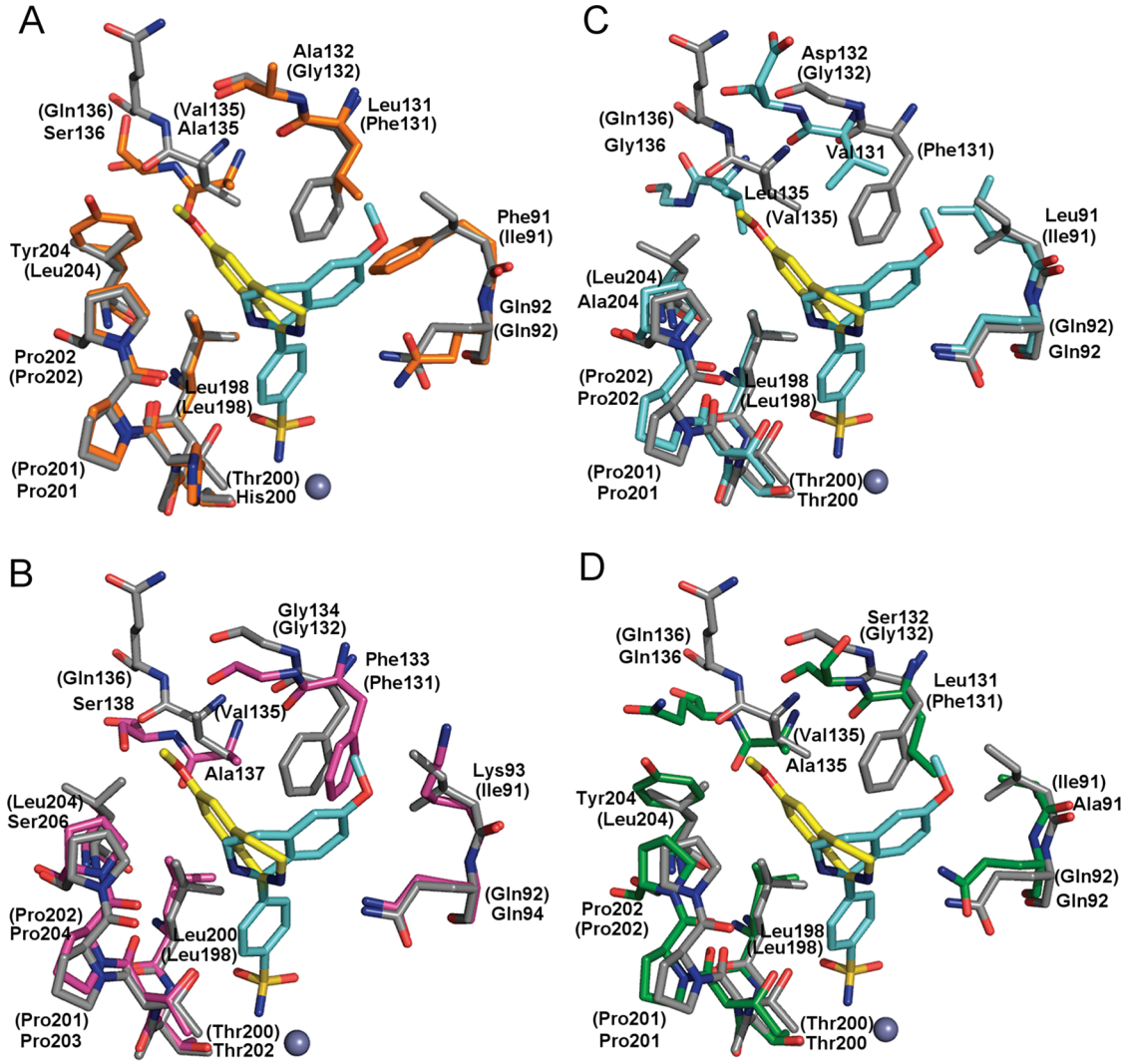


Figure 2. Representation of (A) hCA I (orange), (B) hCA VII (magenta), (C) hCA IX (cyan), and (D) hCA XIV (green) active sites superimposed onto hCA II (gray) complexed with compound **2a** (both conformers: cyan and yellow, respectively). The zinc is depicted as a black sphere. hCA II residues are labeled in parentheses.

CONCLUSIONS

The new synthesized arylsulfonamides **1**−**4** are structurally related to compounds **I**−**IV** that displayed significant CA inhibitory effects and generally high selectivity toward druggable isozymes hCA VII, hCA IX, and hCA XIV. We kept the arylsulfonamide moiety but modified the alkoxy substituents with the aim to explore their role in the interactions within the hydrophobic pocket of the enzyme catalytic site. Overall, the new synthesized compounds **1**−**4** generally showed lower selectivity when compared to previously reported **I**−**IV** analogues. We interpreted this finding as evidence that the dimethoxy substitution plays a key role in controlling the interaction within the catalytic pocket. The cocrystal structure of compound **2a** with hCA II confirmed that the benzylsulfonamide portion binds the zinc ion within the catalytic site, thus generating a blockade of enzymatic activity. Interestingly, the bottom of the hydrophobic pocket accepts the two alternative conformations generated by ring flipping. In these two completely different orientations, each **2a** conformer is involved in a number of interactions with the crucial residues of hCA II, thus suggesting that compound **2a** could be an effective inhibitor in both conformations.

EXPERIMENTAL SECTION

Chemistry. All starting materials and reagents were purchased from Sigma-Aldrich Milan, Italy, and used without further purification. Microwave-assisted reactions were carried out in a CEM focused microwave synthesis system. Melting points were determined on a Buchi B-545 apparatus and are uncorrected. By combustion analysis (C, H, N) carried out on a Carlo Erba Model 1106 Elemental Analyzer, we determined the purity of synthesized compounds; the results confirmed a ≥95% purity. Merck silica gel 60 F254 plates were used for analytical TLC. R_f values were determined on TLC plates using a mixture of CH_2Cl_2/CH_3OH (94:6) as eluent. Flash chromatography (FC) was performed on a Biotage SP_1 EXP. ^1H NMR spectra were measured in DMSO-d_6 and CD_3OD with a Varian Gemini 300 spectrometer; chemical shifts are expressed in δ (ppm) and coupling constants (J) in hertz. All exchangeable protons were confirmed by addition of deuterium oxide (D_2O).

*General Procedure for the Synthesis of 4-(Aminosulfonyl)-N-[2-(3′-alkoxy)phenethyl]benzamides (**1a**−**c**).* A mixture of 4-(aminosulfonyl)benzoic acid (**6**) (1 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (1 mmol) in dimethylformamide (1.5 mL) was stirred for 1 h at room temperature. Successively, the suitable 2-(3′-alkoxyphenyl)ethylamine (**5a**−**c**) (1 mmol) in triethylamine (1 mmol) was added. The reaction mixture was left overnight and then quenched with water (10 mL) and

dx.doi.org/10.1021/jm300112w | J. Med. Chem. 2012, 55, 3891−3899

extracted with ethyl acetate (3 × 5 mL). The combined extract was dried with Na_2SO_4, and solvent was removed in vacuo. The residue was powdered with diethyl ether and crystallized by treatment with ethanol to give the desired final compounds **1a−c** as white crystals.

*4-(Aminosulfonyl)-N-[2-(3′-methoxy)phenyl)ethyl]benzamide (**1a**).* Yield 85%; mp 172−174 °C. R_f = 0.58. ^{1}H NMR (DMSO-d_6) δ 2.83 (t, J = 7.25, 2H, CH_2), 3.49 (m, 2H, CH_2), 3.73 (s, 3H, OCH_3), 6.76−6.83 (m, 3H, ArH), 7.21 (m, 1H, ArH), 7.47 (bs, 2H, NH_2), 7.90 (d, 2H, J = 8.6, ArH), 7.96 (d, 2H, J = 8.6, ArH), 8.75 (bs, 1H, NH). Anal. ($C_{16}H_{18}N_2O_4S$) C, H, N.

*4-(Aminosulfonyl)-N-[2-(3′-ethoxy)phenyl)ethyl]benzamide (**1b**).* Yield 65%; mp 138−140 °C. R_f = 0.58.

*4-(Aminosulfonyl)-N-[2-(3′-propoxy)phenyl)ethyl]benzamide (**1c**).* Yield 56%; mp 146−148 °C. R_f = 0.56.

*General Procedure for the Synthesis of 4-(6-Alkoxy-3,4-dihydroisoquinolin-1-yl)benzenesulfonamides (**2a−c**).* Phosphorus oxychloride ($POCl_3$, 10 mmol) was added dropwise to a solution of 4-(aminosulfonyl)-*N*-[2-(3′-alkoxyphenyl)ethyl]benzamide (**1a−c**) (1 mmol) in dry toluene (1 mL), and the mixture was refluxed for 6 h at 110 °C. After cooling, ammonia aqueous solution (10 mL, 10%) was added to quench the reaction, and the mixture was extracted with ethyl acetate (3 × 5 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure. The residue was powdered by treatment with diethyl ether and crystallized from ethanol to give derivatives **2a−c** as yellow crystals.

*4-(6-Methoxy-3,4-dihydroisoquinolin-1-yl)benzenesulfonamide (**2a**).* Yield 86%; mp 234−236 °C. R_f = 0.56. ^{1}H NMR (DMSO-d_6) δ 2.73 (t, J = 7.25, 2H, CH_2), 3.71 (t, J = 7.25, 2H, CH_2), 3.81 (s, 3H, OCH_3), 6.84 (dd, J = 2.8, J = 8.5, 1H, ArH), 6.95 (d, J = 2.8, 1H, ArH), 7.05 (d, J = 8.5, 1H, ArH), 7.44 (bs, 2H, NH_2), 7.69 (d, J = 8.2, 2H, ArH), 7.89 (d, J = 8.2, 2H, ArH); Anal. ($C_{16}H_{16}N_2O_3S$) C, H, N.

*4-(6-Ethoxy-3,4-dihydroisoquinolin-1-yl)benzenesulfonamide (**2b**).* Yield 46%; mp 200−202 °C. R_f = 0.55.

*4-(6-Propoxy-3,4-dihydroisoquinolin-1-yl)benzenesulfonamide (**2c**).* Yield 81%; mp 232−234 °C. R_f = 0.55.

*General Procedure for the Synthesis of 4-(6-alkoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)benzenesulfonamides (**3a−c**).* A solution of compounds **2a−c** (1 mmol) and $NaBH_4$ (10 mmol) in methanol (18 mL) was stirred for 1 h at room temperature. The reaction mixture was quenched by adding water (10 mL), extracted with ethyl acetate (3 × 5 mL), dried over Na_2SO_4, and then concentrated under reduced pressure. The crude products were crystallized by treatment with a small amount of diethyl ether to afford the final products **3a−c** as white powders.

*4-(6-Methoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)-benzenesulfonamide (**3a**).* Yield 60%; mp 182−184 °C. R_f = 0.30. ^{1}H NMR (DMSO-d_6)δ 2.68−3.07 (m, 4H, CH_2−CH_2), 3.70 (s, 3H, OCH_3), 4.99 (s, 1H, CH), 6.50 (d, J = 8.5, 1H, ArH), 6.61 (dd, J = 2.8, J = 8.5, 1H, ArH), 6.70 (d, J = 2.8, 1H, ArH), 7.30 (bs, 2H, NH_2), 7.42 (d, J = 8.24, 2H, ArH), 7.75 (d, J = 8.24, 2H, ArH); Anal. ($C_{16}H_{18}N_2O_3S$) C, H, N.

*4-(6-Ethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)-benzenesulfonamide (**3b**).* Yield 89%; mp 212−214 °C. R_f = 0.36.

*4-(6-Propoxy-1,2,3,4-tetrahydroisoquinolin-1-yl) benzenesulfonamide (**3c**).* Yield 30%; mp 187−189 °C. R_f = 0.33.

*Synthesis of 1-[4-(Aminosulfonyl)phenyl]-6-alkoxy-1,2,3,4-tetrahydroisoquinoline-2-sulfonamides (**4a−c**).* A mixture of compounds **3a−c** (0.5 mmol) and sulfamide (6 mmol) in dimethoxyethane (2 mL) was placed in a cylindrical quartz tube (Ø 2 cm) and then stirred and irradiated in a microwave oven at 150 W for two steps of 20 min at 100 °C. The reaction was quenched by addition of water (5 mL) and extracted with ethyl acetate (3 × 5 mL). The organic layer was washed with an aqueous saturated solution of $NaHCO_3$ (2 × 5 mL), dried over Na_2SO_4, and concentrated until dryness under reduced pressure. The residue crystallized from ethanol to give final compounds **4a−c** as white powders.

*1-[4-(Aminosulfonyl)phenyl]-6-methoxy-1,2,3,4-tetrahydroisoquinoline-2-sulfonamide (**4a**).* Yield 74%; mp 102−104 °C. R_f = 0.55. ^{1}H NMR (DMSO-d_6) δ 2.68−2.73 (m, 1H, CH_2CH_2), 3.03−3.29 (m, 2H, CH_2CH_2), 3.33−3.52 (m, 1H, CH_2CH_2), 3.73 (s, 3H, OCH_3), 5.88 (s, 1H, CH), 6.73−6.79 (m, 2H, ArH), 6.95 (m, 1H, ArH), 6.99 (bs, 2H, NH_2), 7.29 (bs, 2H, NH_2), 7.36 (d, J = 8.2, 2H, ArH), 7.72 (d, J = 8.2, 2H, ArH). Anal. ($C_{16}H_{19}N_3O_5S_2$) C, H, N.

*1-[4-(Aminosulfonyl)phenyl]-6-ethoxy-1,2,3,4-tetrahydroisoquinoline-2-sulfonamide (**4b**).* Yield 61%; mp 119−120 °C. R_f = 0.55.

*1-[4-(Aminosulfonyl)phenyl]-6-propoxy-1,2,3,4-tetrahydroisoquinoline-2-sulfonamide (**4c**).* Yield 89%; mp 79−84 °C. R_f = 0.55.

*General Procedure for the Synthesis of Hydroxyl Derivatives **1d, 2d, 3d,** and **4d**.* The suitable alkoxy derivative (1 mmol) was dissolved in methylene chloride (DCM) (5 mL) and then treated with BBr_3 (1 M in DCM) (6 mmol) and stirred overnight. Successively, methanol (7 mL) was carefully added at 0 °C and the solvents were removed under reduced pressure. The residue was dissolved in ethyl acetate (10 mL) and washed with water (10 mL × 3). The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The crude product was crystallized from ethanol to give the desired hydroxyl derivative.

*4-(Aminosulfonyl)-N-[2-(3′-hydroxy)phenyl)ethyl]benzamide (**1d**).* Yield 78%; mp 194−196 °C. R_f = 0.27. ^{1}H NMR (DMSO-d_6) δ 2.74 (t, J = 7.5, 2H, CH_2), 3.44 (m, 2H, CH_2), 6.57−7.07 (m, 4H, ArH), 7.45 (s, 2H, NH_2), 7.87 (d, J = 6.7, 2H, ArH), 7.95 (d, J = 6.7, 2H, ArH), 8.72 (bs, 1H, NH), 9.26 (s, 1H, OH). Anal. ($C_{15}H_{16}N_2O_4S$) C, H, N.

*4-(6-Hydroxy-3,4-dihydroisoquinolin-1-yl)benzenesulfonamide (**2d**).* Yield 42%; mp 244−246 °C. R_f = 0.11. ^{1}H NMR (DMSO-d_6) δ 2.67 (t, J = 7.2, 2H, CH_2), 3.69 (t, J = 7.2, 2H, CH_2), 6.65−6.97 (m, 3H, ArH), 7.45 (s, 2H, NH_2), 7.68 (d, J = 8.0, 2H, ArH), 7.88 (d, J = 8.0, 2H, ArH), 10.08 (s, 1H, OH). Anal. ($C_{15}H_{14}N_2O_3S$) C, H, N.

*4-(6-Hydroxy-1,2,3,4-tetrahydroisoquinolin-1-yl)-benzenesulfonamide (**3d**).* Yield 70%; mp 137−139 °C. R_f = 0.05. ^{1}H NMR (DMSO-d_6) δ 2.65− 3.06 (m, 4H, CH_2CH_2), 4.96 (s, 1H, CH), 6.38−6.52 (m, 3H, ArH), 7.31 (s, 2H, NH_2), 7.42 (d, J = 8.3, 2H, ArH), 7.75 (d, J = 8.3, 2H, ArH), 9.15 (s, 1H, OH). Anal. ($C_{15}H_{16}N_2O_3S$) C, H, N.

*1-[4-(Aminosulfonyl)phenyl]-6-hydroxy-1,2,3,4-tetrahydroisoquinoline-2-sulfonamide (**4d**).* Yield 37%; mp 183−185 °C. R_f = 0.21.^{1}H NMR (DMSO-d_6) δ 2.62− 3.55 (m, 4H, CH_2−CH_2), 5.84 (s, 1H, CH), 6.59−6.88 (m, 3H, ArH), 6.96 (bs, 2H, NH_2), 7.28 (bs, 2H, NH_2), 7.37 (d, J = 8.3, 2H, ArH), 7.73 (d, J = 8.3, 2H, ArH), 9.35 (bs, 1H, OH). Anal. ($C_{15}H_{17}N_3O_5S_2$) C, H, N.

CA Inhibition Assay. An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10−20 mM HEPES (pH 7.5) or Tris (pH 8.3) as buffers, and 20 mM Na_2SO_4 or 20 mM $NaClO_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10−100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5−10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled−deionized water, and dilutions up to 0.01 nM were performed thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E−I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier, and represent the mean from at least three different determinations. CA isoforms were recombinant ones obtained as reported earlier by this group.[35−38]

Protein Crystallography. *Protein Crystallization and X-ray Data Collection.* Complexes of human carbonic anhydrase II (purchased from Sigma, catalog no. C6165) with compounds **I** and **2a** were prepared by adding a 4-fold molar excess of inhibitor (dissolved in pure DMSO) to 10 mg mL^{-1} protein solution in water without pH adjustment. The best crystals of the complexes were prepared by the vapor-diffusion hanging drop method using crystallization conditions recently published by Behnke et al.[39] A 2 μL amount of complex solution was mixed with 2 μL of precipitant solution containing 3 M ammonium sulfate, 50 mM Tris-HCl, and 2 mM 4-

(hydroxymercuri)benzoate (Sigma, catalog no. 55540) and equilibrated over a 1 mL reservoir of precipitant solution at 18 °C. The final DMSO concentration in the drop was 2% (v/v). Crystals with dimensions of 0.5 mm × 0.3 mm × 0.2 mm grew within 5 days. Before data collection, the crystals were soaked for 5−10 s in a reservoir solution supplemented with 25% (v/v) glycerol and stored in liquid N_2. Diffraction data were collected at 100 K at beamlines BL14.1 and BL14.2 of BESSY, Berlin, equipped with an MX-225 CCD detector from Rayonics (Evanston, IN). A crystal of hCA II in complex with compound **I** diffracted to 1.03 Å, and the diffraction data were processed using the HKL-3000 suite of programs.[40] A crystal of hCA II in complex with compound **2a** diffracted to 1.05 Å, and the diffraction data were integrated and reduced using MOSFLM[41] and scaled using SCALA[42] from the CCP4 suite of programs.[43] Crystal parameters and data collection statistics for both complexes are summarized in Table 2.

Structure Determination, Refinement, and Analysis. The structures of hCA II complexes with compounds **I** and **2a** were solved using the difference Fourier technique, using the hCA II structure (Protein Data Bank entry 3PO6) as the initial model. Initial rigid-body refinement and subsequent restrained refinement for both hCA II complexes with **I** and **2a** were performed using the program REFMAC 5[43] with isotropic ADPs. The structures were refined with one inhibitor molecule in the enzyme active site with occupancy 1 and 0.84 for compounds **I** and **2a**, respectively. Atomic coordinates and the geometric library for the inhibitor were generated using the PRODRG server.[44] The Coot program[45] was used for inhibitor fitting, model rebuilding, and addition of water molecules. Final refinement at resolution was carried out with SHELXL-97[46] in conjugate-gradient least-squares mode, with 5% of the reflections reserved for cross-validation. Both structures were first refined with isotropic ADPs. After addition of solvent atoms and metal ions (zinc and mercury), building inhibitor molecules in the active site, and several alternate conformations for a number of residues, anisotropic ADPs were refined for nearly all atoms (with the exception of spatially overlapping atoms in segments with alternate conformations; also oxygen atoms of water molecules with an unrealistic ratio of ellipsoid axes were refined with isotropic ADPs) including the inhibitor molecules. The quality of the crystallographic model was assessed with MolProbity.[47] The final refinement statistics are summarized in Table 2. All figures showing structural representations were prepared using PyMOL.[48]

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR assignment for compounds **1b,c**, **2b,c**, **3b,c**, and **4b,c**, additional X-ray data, and a complete list of interactions between hCA II and the two inhibitors **I** and **2a**. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

†Atomic coordinates for the crystal structure of hCA II A with compounds **I** and **2a** can be accessed using PDB codes 3V7X and 3VBD, respectively.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support for this research awarded by MiUR (Prin2008, grant number No 20085HR5JK_002), Academy of Sciences of the Czech Republic (Projects AV0Z50520514 and AV0Z40550506), and Grant Agency of the Czech Republic (Grant GA203/09/0820) is gratefully acknowledged. Research from CTS lab was financed by a seventh FP grant (METOXIA project). We thank MX 14.1 and 14.2 beamline staff at the BESSY synchrotron for expert assistance during data collection.

■ ABBREVIATIONS USED

AAZ, acetazolamide; CA, carbonic anhydrase; HIF-1, hypoxia inducible factor-1; PDB, protein data bank; TPM, topiramate; ZNS, zonisamide

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