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Carbonic anhydrase inhibitors. Interaction of 2-*N*,*N*-dimethylamino-1,3,4-thiadiazole-5-methanesulfonamide with 12 mammalian isoforms: Kinetic and X-ray crystallographic studies☆

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Abstract—2-*N*,*N*-Dimethylamino-1,3,4-thiadiazole-5-methanesulfonamide was tested for its interaction with the 12 catalytically active mammalian carbonic anhydrase (CA, EC 4.2.1.1) isozymes, CA I–XIV. The compound is a potent inhibitor of CA IV, VII, IX, XII, and XIII (K_Is of 0.61–39 nM), a medium potency inhibitor of CA II and VA (K_Is of 121–438 nM), and a weak inhibitor against the other isoforms (CA III, VB, VI, and XIV), making it a very interesting candidate for situations in which a strong/selective inhibition of certain isozymes is needed. The crystal structure of the hCA II adduct of this sulfonamide revealed interesting interactions between the inhibitor and the enzyme which are quite different from those observed in the adducts of CA II with the structurally related aliphatic derivatives zonisamide, 2-amino-1,3,4-thiadiazolyl-5-difluoromethanesulfonamide, and 2-dimethylamino-5-[sulfonamido-(aminomethyl)]-1,3,4-thiadiazole reported earlier.

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in bacteria, archaea, and eukaryotes, catalyzing a critically important physiologic reaction, hydration of carbon dioxide to bicarbonate and protons.[1–4] These enzymes are inhibited by several classes of compounds, such as sulfonamides,[1,5–9] sulfamates,[1,2] and sulfamides,[1,2] some of which have pharmacologic applications for the treatment of glaucoma,[5] obesity,[6] cancer,[8–12] epilepsy,[7] and other neurological disorders,[1,2] or as diuretics.[5] Bacterial, fungal, and protozoan CAs belonging to the α-, β-, γ-, and/or δ-CA gene families, which are present in many pathogens, started also to be considered recently as potential targets for the development of inhibitors with therapeutic applications.[13–18] Inhibitors belonging to the chemical classes mentioned above bind to the catalytic zinc ion within the enzyme cavity, as shown by means of X-ray crystallographic studies for many representatives, mainly in complex with the ubiquitous human isoform II (hCA II).[5a,19–24] A number of such derivatives are clinically used drugs, such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, topiramate, zonisamide, sulpiride, sulthiame, celecoxib, and valdecoxib among others.[1,25] Other compounds are in clinical development, such as indisulam and COUMATE-667.[1]

As mentioned above, CA inhibitors (CAIs) are mainly used in therapy as diuretics and antiglaucoma agents but some of them also show marked anticonvulsant, antiobesity, and antitumor effects.[1,2,5–11] This is due to the fact that such inhibitors target different isozymes

Keywords: Carbonic anhydrase; Isozyme selective inhibitor; X-ray crystallography; Aliphatic sulfonamide.
☆ The coordinates of the hCA II–sulfonamide adduct have been deposited in PDB, ID code 3BL0.
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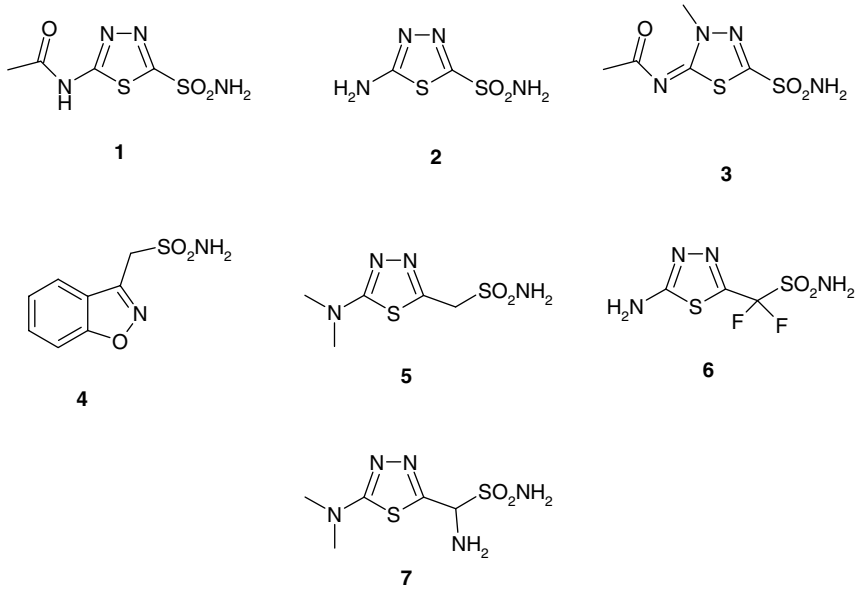
among the 16 presently known in vertebrates.[1,2] However, most of the presently available CAIs show undesired side effects due to indiscriminate inhibition of CA isoforms other than the target one.[1,2,5–14] Thus, many new CAI classes are being developed in the search of isozyme selective compounds as potential drugs with less side effects.[1,5,25]

The most investigated CAIs belong to the sulfonamide class.[1,2,24–27] Aromatic, heterocyclic, and aliphatic such derivatives were investigated, such as for example acetazolamide **1**, its deacetylated precursor **2**, methazolamide **3** and zonisamide **4**.[1,2,24–27] Compounds**1**, **3**, and **4** have clinical applications as diuretics, antiglaucoma, and antiepileptic drugs.[1,2,26,27] An interesting class of novel CAIs was recently reported by Blackburn's group,[24,26] consisting of aliphatic derivatives possessing $CF_2SO_2NH_2$ and $CH(R)SO_2NH_2$ (where R = H or amino) zinc binding groups (ZBGs), attached to the classical 2(5)-substituted-1,3,4-thiadiazole ring present in two of the classical CAIs, acetazolamide and methazolamide. Compounds **5**–**7** are representatives of this new class of CAIs recently described in the literature, but they were not assayed in detail for the inhibition of different CA isoforms.[26] The X-ray crystal structure of the adducts of hCA II with **6** and **7** was reported recently by Fisher et al., but no CA inhibitory data were available for these two derivatives.[24] Compounds structurally similar to **6** and **7** were however reported by Boyle et al.[26] to act as good to moderate CA II inhibitors (K_Is in the range of 15–208 nM). On the other hand, Cecchi et al.[27c] investigated similar aliphatic sulfonamides possessing $CF_2SO_2NH_2$ and $CH_2SO_2NH_2$ ZBGs attached to benzene, coumarin or steroid scaffolds, which showed selective inhibition of the mitochondrial isoform CA VA over the ubiquitous, cytosolic isozymes CA I and II or the transmembrane, tumor-associated one CA IX. Thus, this type of relatively little

Table 1. Inhibition data with the sulfonamide derivatives **1**–**5** against 12 mammalian α-CA isoforms



Isozyme[a]	K_I[b] (nM)					
	1	**2**	**3**	**4**	**5**	**6**
hCA I[d]	250	8600	50	56	1710	nt
hCA II[d]	12	60	14	35	121	15.1[c]
hCA III[d]	2×10^5	1.5×10^5	7×10^5	2.2×10^6	3.6×10^5	nt
hCA IV[d]	74	940	6200	8590	23.5	nt
hCA VA[d]	63	2300	65	20	438	nt
hCA VB[d]	54	2150	62	6033	1422	nt
hCA VI[d]	11	798	10	89	2150	nt
hCA VII[d]	2.5	5.2	2.1	117	0.61	nt
hCA IX[e]	25	41	27	5.1	39	nt
hCA XII[e]	5.7	33	3.4	11,000	7.7	nt
mCA XIII[d]	17	nt	19	430	20	nt
hCA XIV[d]	41	215	43	5250	2900	nt

Data for the inhibition of these CAs with compounds **1**–**4** are from Refs. 1,28. No inhibition data of compounds **6** and **7** are available in the literature.[26]

[a] h, human; m, murine isozyme.

[b] Errors in the range of ±5% of the reported data from three different assays ($n = 3$), by a stopped flow CO_2 hydration method.[29]

[c] Boyle N. A. Ph.D., Thesis, Sheffield University, 2001, nt = not tested.

[d] Recombinant full length enzyme.

[e] Recombinant enzyme, catalytic domain.

investigated CAIs shows great interest in the search of compounds with different and possibly improved selectivity/inhibition profiles, as well as enhanced solubility, as compared to those of the classical, clinically used sulfonamide CAIs.

In this work, we report the first detailed inhibition study of all 12 catalytically active mammalian CA isoforms with an aliphatic sulfonamide of this type, that is, **5**,[26,28] as well as a high resolution X-ray crystal structure for its adduct with the ubiquitous and physiologically relevant hCA II.

Sulfonamide **5** has been investigated for the inhibition of the 12 catalytically active mammalian CA isoforms CA I–XIV (h, human; m, mouse isozyme) (Table 1). Data for the structurally related, clinically used derivatives **1**–**4** are also included for comparison, as they are available in the literature.[1,27,29] It may be observed that compound **5** acts as a potent inhibitor of isoforms CA IV, VII, IX, XII, and XIII, with K_Is in the range of 0.61–39 nM. On the other hand, **5** is a medium potency inhibitor of CA II and VA (K_Is of 121–438 nM), and a weak inhibitor against the other isoforms (CA III, VB, VI, and XIV), with K_Is in the range of 1422–3.6×10^5 nM (Table 1). It is thus clear that the inhibition profile of **5** is completely different from those of the clinically used sulfonamides **1**, **3**, and **4** or the acetazolamide precursor **2** with which **5** is structurally related. Indeed, **5** is one of the most potent CA IV and CA VII inhibitors ever reported, while acting as a much weaker inhibitor of the ubiquitous isoform CA II, which is often considered as a house-keeping enzyme, whose inhibition is not desired.[1,2,6] This is clearly a very important result, and one which makes **5** a quite unique CAI in the armamentarium of compounds showing isoform selective inhibitory properties.[1,5–7] Furthermore, in addition to the isozymes mentioned above, **5** shows potent inhibitory activity only against CA XII and IX, which are tumor-associated and thus not present in normal tissues,[7–10] and against CA XIII, an isoform present in restricted compartments of the genito-urinary tract in normal tissues.[6c] All these facts make **5** a selective inhibitor of isoforms IV and VII, a profile which is completely new for any known CAI.[1–6] This distinct inhibition profile is in fact unsimilar both to those of the thiadiazole/thiadiazoline clinically used inhibitors (**1** and **3**), as well as to that of the related aliphatic antiepileptic sulfonamide zonisamide, **4**. Unlike **5**, compounds **1** and **3** possess a quite promiscuous inhibitory activity, acting as potent inhibitors against many isozymes (e.g., acetazolamide **1** and methazolamide **2** are potent inhibitors of all isoforms except CA I and III), whereas zonisamide **4** already shows a more modulated activity, being a potent inhibitor of only isoforms I, II, VA, VI, and IX.

In order to better understand the interesting inhibitory activity of **5** and also to learn some lessons for the drug design of new CAIs based on this ring system/spacer, we report the X-ray crystal structure of the hCA II–**5** adduct (Table 2).[30–34] The three-dimensional structure of the enzyme is very similar to that of hCA II without any ligand bound,[19,21,23–25] as judged by an rms deviation for Cα atoms of only 0.30 Å. Examination of the initially calculated electron density maps in the active-site region showed clear evidence for the binding of a single inhibitor molecule within the active site cavity. The electron density of all moieties of the inhibitor is in fact very well defined (Fig. 1).

Table 2. Crystallographic parameters and refinement statistics for the hCA II–**5** adduct

Parameter	Value
Crystal parameter	
Space group	$P2_I$
Cell parameters (Å)	a = 41.5
	b = 42.1
	c = 72.4
Data collection statistics (*20.0–1.9* Å)	
No. of total reflections	99,060
No. of unique reflections	21,015
Completeness (%)[a]	98.0 (96.7)
F2/sig (*F2*)	6.5 (1.3)
R-sym (%)	17.0 (33.0)
Refinement statistics (*20.0–1.9* Å)	
R-factor (%)	19.8
R-free[b] (%)	24.8
Rmsd of bonds from ideality (Å)	0.009
Rmsd of angles from ideality (°)	1.36

[a] Values in parentheses relate to the highest resolution shell (2.0–1.9 Å).
[b] Calculated using 5% of data.

The tetrahedral geometry of the Zn^{2+} binding site and the key hydrogen bonds between the SO_2NH_2 moiety of **5** and enzyme active site are all retained with respect to other hCA II-sulfonamide/sulfamate/sulfamide complexes structurally characterized so far (Figs. 1

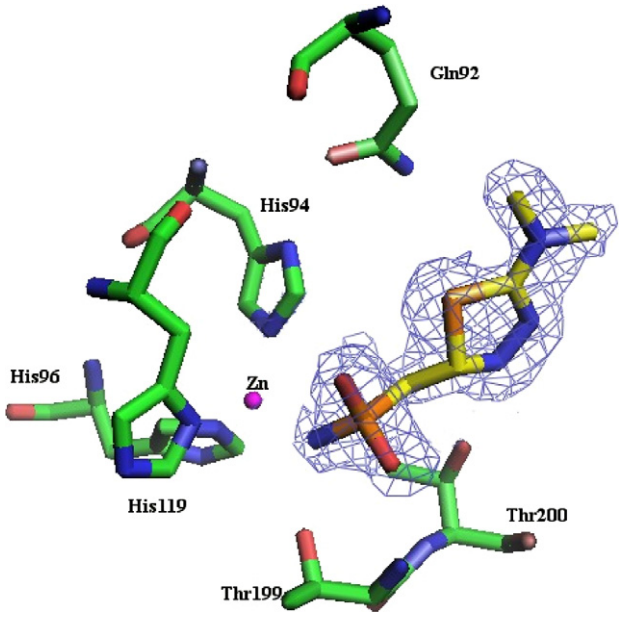


Figure 1. Electron density map of **5** (in yellow) bound within the hCA II active site. The Zn(II) ion of the enzyme, its three histidine ligands (His94, 96, and 119), and residues involved in the binding of the inhibitor (Thr199, Thr200, and Gln92) are also shown.

and 2).[5,19,21,23–25,35] In particular, the ionized nitrogen atom of the sulfonamide group of **5** is coordinated to the zinc ion at a distance of 1.90 Å. This nitrogen atom is also hydrogen bonded to the hydroxyl group of Thr199 (N–Thr199OG = 2.63 Å), which in turn interacts with the Glu106OE1 atom (2.50 Å, data not shown). One oxygen atom of the coordinated sulfamoyl moiety is hydrogen bonded to the backbone amide of Thr199 (ThrN–O2 = 2.70 Å), whereas the second oxygen atom of this moiety is 3.20 Å away from the catalytic Zn^{2+} ion, being considered as weakly coordinated to the metal ion.[1,19,21,23–25,35] All these interactions have also been observed in the adducts of hCA II with other sulfonamide inhibitors, such as acetazolamide **1**, 5-amino-1,3,4-thiadiazole-2-sulfonamide **2** or zonisamide **4**, but the corresponding distances are of course different.[19,21–25,35] Interestingly, one of the endocyclic nitrogen atoms of the thiadiazole ring makes a strong hydrogen bond with the OH moiety of Thr200, of 3.14 Å, an interaction not observed earlier in any other adduct of hCA II with 1,3,4-thiadiazole-sulfonamide derivatives.[35] The scaffold of **5** is accommodated perfectly within the active site channel, being oriented toward the hydrophobic half of it, and participating in several favorable interactions with various amino acid residues (Figs. 1 and 2). Thus, the 1,3,4-thiadiazole-methyl scaffold of the inhibitor makes favorable van der Waals contacts (around 4 Å) with amino acids lining the hydrophobic half of the hCA II cavity (such as Gln92, Phe131, Val121, Val143, and Leu198). For example, the distance between the endocyclic sulfur atom of **5** and the nitrogen atom of the carboxamido side chain of Gln92 is of 4 Å (Fig. 2). It is also interesting to note that the dimethylamino moiety of **5** does not participate in any interaction with the enzyme nor with water molecules from the active site, which may in fact explain the medium potency inhibitory activity of this compound. The same is true for the CH_2 spacer between the ZBG and the substituted-1,3,4-thiadiazole ring (Figs. 1 and 2). From this point of view (i.e., scarcity of interactions between the enzyme and the moiety in position 2 of the 1,3,4-thiadiazole ring), **5** is rather similar to **2** (also a medium potency CA II inhibitor, K_I of 60 nM) for which the crystal structure in adduct with hCA II was recently reported by our group.[35]

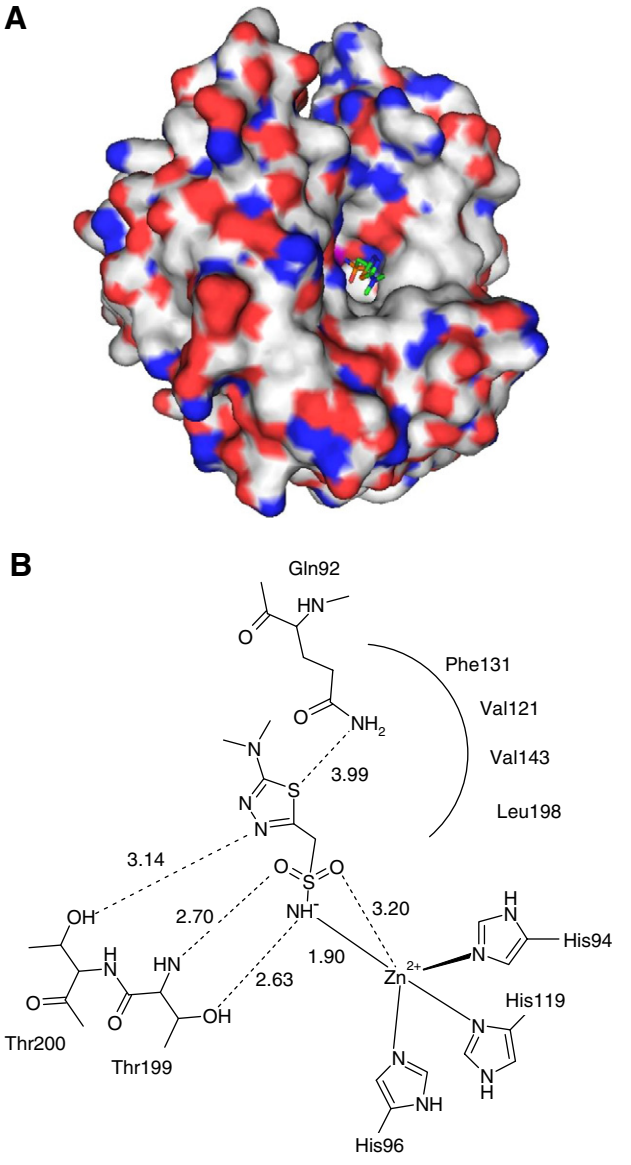


Figure 2. (A) 3D overview of the hCA II–**5** complex. The surface of the enzyme is colored according to the hydropathy (blue, hydrophilic; red, hydrophobic residues). Zinc is violet and the inhibitor **5** is green. (B) Detailed schematic representation for interactions in which sulfonamide **5** participates when bound to the hCA II active site. Figures represent distances (in Å).

In order to try to understand the different inhibition profiles of compound **5** and the structurally related derivatives **4**, **6**, and **7** investigated earlier,[19,24] we have also superposed the 3D structures of these four sulfonamides complexed within the hCA II active site (Fig. 3). Figure 3A and B shows that although the last three compounds have a common 1,3,4-thiadiazolyl-methanesulfonamide scaffold, their binding to the enzyme is very different. Indeed, only the SO_2NH_2 moieties of the three inhibitors are superposable, whereas the orientation of the 1,3,4-thiadiazole rings and of the spacers between the ring and the ZBG is completely different. For example, as shown by Fisher et al.[24] in the adduct of **6** with hCA II the 1,3,4-thiadiazole ring participates in a π-stacking interaction with the imidazole ring of His94, an interaction not seen in the adducts of hCA II with **5** or **7** (Fig. 3A). On the other hand, in the adduct of hCA II with **7**, the 1,3,4-thiadiazole ring adopted a puckered conformation, which is again typical only for that adduct. On the contrary, the derivative **5** investigated by us here shows an extended conformation of the thiadiazole-methyl scaffold, which enables it to participate in an increased number of hydrophobic interactions (in addition to the polar ones mentioned above) (Fig. 3B). These data also indicate that as the 2-dimethylamino-moiety of **5** and **7** or the 2-amino- one in **6** does not participate in any particular interactions with amino acid residues in the active site, substituting this moiety with functionalities which can interact with amino acid residues situated at the entrance of the cavity, such as,

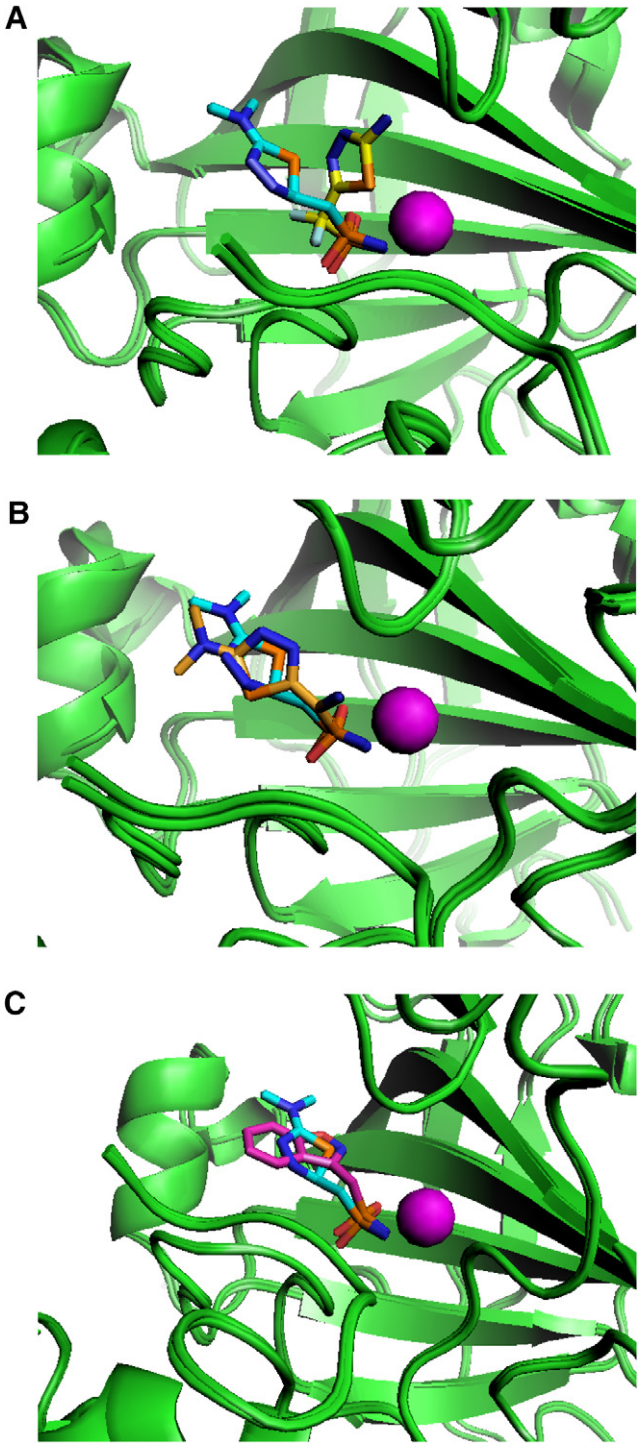


Figure 3. Superposition of the: (A) hCA II–**5** adduct (in blue) with hCA II–**6** adduct (PDB file 2EU3),[24] in yellow; (B) hCA II–**5** adduct (in blue) with hCA II–**7** adduct (PDB file 2EU2),[24] in gold, and (C) hCA II **5** adduct (in blue) with hCA II–zonisamide **4** adduct (magenta).[19]

for example, dimethylaminoethyl-carboxamido,[36] benzenesulfonamido,[37] etc., may lead to analogues with increased affinity for hCA II and probably also with a diverse inhibition profile versus other isoforms of interest. On the other hand, as shown in Figure 3C, the inhibitors **4** and **5** possessing both a methanesulfonamide ZBG are again not very much superposable, except for the sulfamoyl moiety. In fact, the orientation of the CH_2 spacer is rather different in the two adducts, but the two ring systems (1,2-benzoxazole in **4**, and 1,3,4-thiadiazole in **5**, respectively) lie approximately in the same region of the CA II active site. In fact, among derivatives **1**–**5**, the inhibition profile of **5** is the most similar just to that of zonisamide **4**, which can be understood also from these X-ray crystallographic data.

In conclusion, we investigated the interaction of 2-*N*,*N*-dimethylamino-1,3,4-thiadiazole-5-methanesulfonamide with the 12 catalytically active mammalian CA isozymes, CA I–XIV. The compound is a potent inhibitor of CA IV, VII, IX, XII, and XIII (K_Is of 0.61–39 nM), a medium potency inhibitor of CA II and VA (K_Is of 121–438 nM), and a weak inhibitor against the other isoforms (CA III, VB, VI, and XIV), making it a very interesting candidate for situations in which a strong/selective inhibition of certain isozymes is needed. The crystal structure of the hCA II adduct of this sulfonamide revealed interesting interactions between the inhibitor and the enzyme which are quite different from those observed in the adducts of CA II with the structurally related 2-amino-1,3,4-thiadiazolyl-5-difluoromethanesulfonamide or 2-dimethylamino-5-[sulfonamido-(aminomethyl)]-1,3,4-thiadiazole reported earlier.

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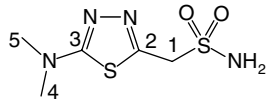
28. *2-N,N-Dimethylamino-5-methyl-1,3,4-thiadiazole*. $POCl_3$ (0.82 ml, 8.8 mmol) was added dropwise to an ice-cooled mixture of glacial acetic acid (0.5 ml, 8.8 mmol) and 4,4-dimethylthiosemicarbazide (0.50 g, 4.2 mmol), under argon. The resulting suspension was heated at 70 °C for 3 h, ice-cold water (100 ml) was added, and the residue dissolved with gentle heating and stirring. The resulting suspension was filtered and the filtrate was adjusted to pH 8 with saturated $NaHCO_3$. The mixture was extracted with EtOAc (3× 50 ml) and the combined organic extracts were washed with brine and dried over $MgSO_4$. Evaporation in vacuo gave the product as a white solid (0.49 mg, 82%); TLC (100% EtOAc): $R_f = 0.2$; δ_H (250 MHz; $CDCl_3$) 3.08 (6H, s, 2× NCH_3), 2.53 (3H, s, CCH_3); δ_C (250 MHz; $CDCl_3$) 172.1 (C3), 154.2 (C2), 41.4 (C4 and 5), 15.8 (C1); MS (EI^+) *m/z* 143 (M^+, 100%); HRMS calcd for $C_5H_9N_3S$: 143.0517. Found: 143.0518; IR (KBr, cm^{-1}) 3414, 1554, 1420, 1337.

2-N,N-Dimethylamino-1,3,4-thiadiazole-5-methanesulfonamide ***5***. BuLi (0.84 ml, 2.1 mmol) was added to 2-*N*,*N*-dimethylamino-5-methyl-1,3,4-thiadiazole (0.3 g, 2.1 mmol) in anhydrous THF (10 ml), under argon, at −78 °C. Sulfur dioxide was introduced into the solution at −78 °C for 1 h, causing a precipitate and the solution to turn purple. The mixture was concentrated in vacuo and the residue dissolved in aq sodium acetate (20 ml, 1.1 M). Hydroxylamine-*O*-sulfonic acid (1.13 g, 10 mmol) was added and the solution stirred at rt overnight. The solution was adjusted to pH 7 with saturated $NaHCO_3$, extracted with EtOAc (3× 35 ml), and the combined organic extracts washed with brine, dried ($MgSO_4$), and evaporated. The resulting pale yellow solid was suspended in warm $CHCl_3$, collected by filtration, and washed with $CHCl_3$ to give the *product* as an off-white solid (0.12 g, 25%); TLC (100% EtOAc): $R_f = 0.2$ (stained yellow with ninhydrin); mp 191–193 °C (decomposition); δ_H (250 MHz; DMSO-d_6) 7.16 (2H, s, NH_2), 4.62 (2H, s, CH_2), 3.09 (6H, s, 2× NC**H**$_3$); δ_C (250 MHz; DMSO)-d_6 172.8 (C3), 147.4 (C2), 55.3 (C1), 41.0 (C4 and 5); MS (EI^+) *m/z* 222 (M^+, 20%); HRMS calcd for $C_5H_{10}N_4O_2S_2$: 222.0245. Found: 222.0246; IR (KBr, cm^{-1}) 3332, 1568, 1330, 1164, 1124.



29. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, 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 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following 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 (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done 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 non-linear least-squares methods using PRISM 3, as reported earlier,[12–14] and represent the mean from at least three different determinations. Enzyme concentrations in the assay system were in the range of 7.1– 13 nM.[12–14].

30. The hCA II–**5** complex was crystallized as previously described. Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using CuKα radiation (1.5418 Å). The unit cell dimensions were: $a = 41.5$ Å, $b = 42.1$ Å, $c = 72.41$ Å and $\alpha = \gamma = 90°$, $\beta = 104.25°$ in the space group $P2_1$. Data were processed with CrysAlis RED (Oxford Diffraction 2006).[31] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2 as starting model. The refinement was carried out with the program REFMAC5[32]; model building and map inspections were performed using the COOT program.[33] The final model of the complex hCA II–**5** had an *R*-factor of 19.8% and *R*-free 24.8% in the resolution range 20.0–1.9 Å, with a rms deviation from standard geometry of 0.009 Å in bond lengths and 1.36° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[34] Coordinates and structure factors have been deposited with the Protein Data Bank (accession code 3BL0). Crystallographic parameters and refinement statistics are summarized in Table 2.
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