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Carbonic anhydrase inhibitors: Inhibition of human, bacterial, and archaeal isozymes with benzene-1,3-disulfonamides—Solution and crystallographic studies☆

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Abstract—Three benzene-1,3-disulfonamide derivatives were investigated for their interaction with 12 mammalian α-carbonic anhydrases (CAs, EC 4.2.1.1), and three bacterial/archaeal CAs belonging to the α-, β-, and γ-CA class, respectively. X-ray crystal structure of the three inhibitors in complex with the dominant human isozyme CA II revealed a particular binding mode within the cavity. The sulfonamide group in *meta*-position to the Zn^{2+}-coordinated SO_2NH_2 moiety was oriented toward the hydrophilic side of the active site cleft, establishing hydrogen bonds with His64, Asn67, Gln92, and Thr200. The plane of the phenyl moiety of the inhibitors was rotated by 45° and tilted by 10° with respect to its most recurrent orientation in other CA II–sulfonamide complexes.

In the last years, all 12 catalytically active human isoforms of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1)[1,2] have been investigated in detail for their inhibition profile against the most important classes of inhibitors, the sulfonamides, sulfamates, and sulfamides.[3–10] Some of these agents have clinical applications for the treatment or prevention of several diseases, such as glaucoma, cancer, obesity, epilepsy, and some other neurological disorders.[1–12] However, up to now few reported compounds show a good selectivity for some CA isoforms with clinical relevance, although important advances have been reported in the design of compounds with some selectivity for inhibiting CA VA over CA II, CA IX over CA II or CA XIII over CA II.[12,13] Recently, many novel representatives of CAs belonging to other gene families were isolated and characterized in organisms such as bacteria (for example *Helicobacter pylori*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Mycobacterium tuberculosis*),[14–16] fungi (*Candida albicans*, *Cryptococcus neoformans*),[17,18] or in the protozoa causing malaria (*Plasmodium falciparum*).[19] Inhibitors of such parasite enzymes may have important therapeutic applications for treating infections caused by these agents (some of which developed high level resistance to the classical antibiotics/antifungal agents),[20–22] in case that potent and selective inhibitors for the pathogenic over the host enzymes could be detected/designed.

There are several clinically used drugs/orphan drugs reported to possess significant CA inhibitory properties (compounds **1**–**10**) and many other such derivatives belonging to the sulfonamide, sulfamate or sulfamide classes are constantly being reported.[1–12] Dichlorophenamide **7a** (daranide) is a disulfonamide, more precisely 4,5-dichloro-benzene-1,3-disulfonamide, which is also a classical CA inhibitor (CAI) used clinically mainly for the management of glaucoma.[23,24] Two structurally related compounds, i.e., **7b** (6-chloro-4-amino-benzene-1,3-disulfonamide) and **7c** (6-trifluoromethyl-4-amino-benzene-1,3-disulfonamide), were also investigated in detail for their inhibition of various human/parasite CA isoforms, and were also used as starting compounds in drug design studies for obtaining antiglaucoma or

Keywords: Carbonic anhydrase; Isozymes; Benzene-1,3-disulfonamide; Dichlorophenamide; X-ray crystallography; Drug design.
☆ Coordinates and structure factors have been deposited with the Protein Data Bank (Accession codes 2POU, 2POV, and 2POW).
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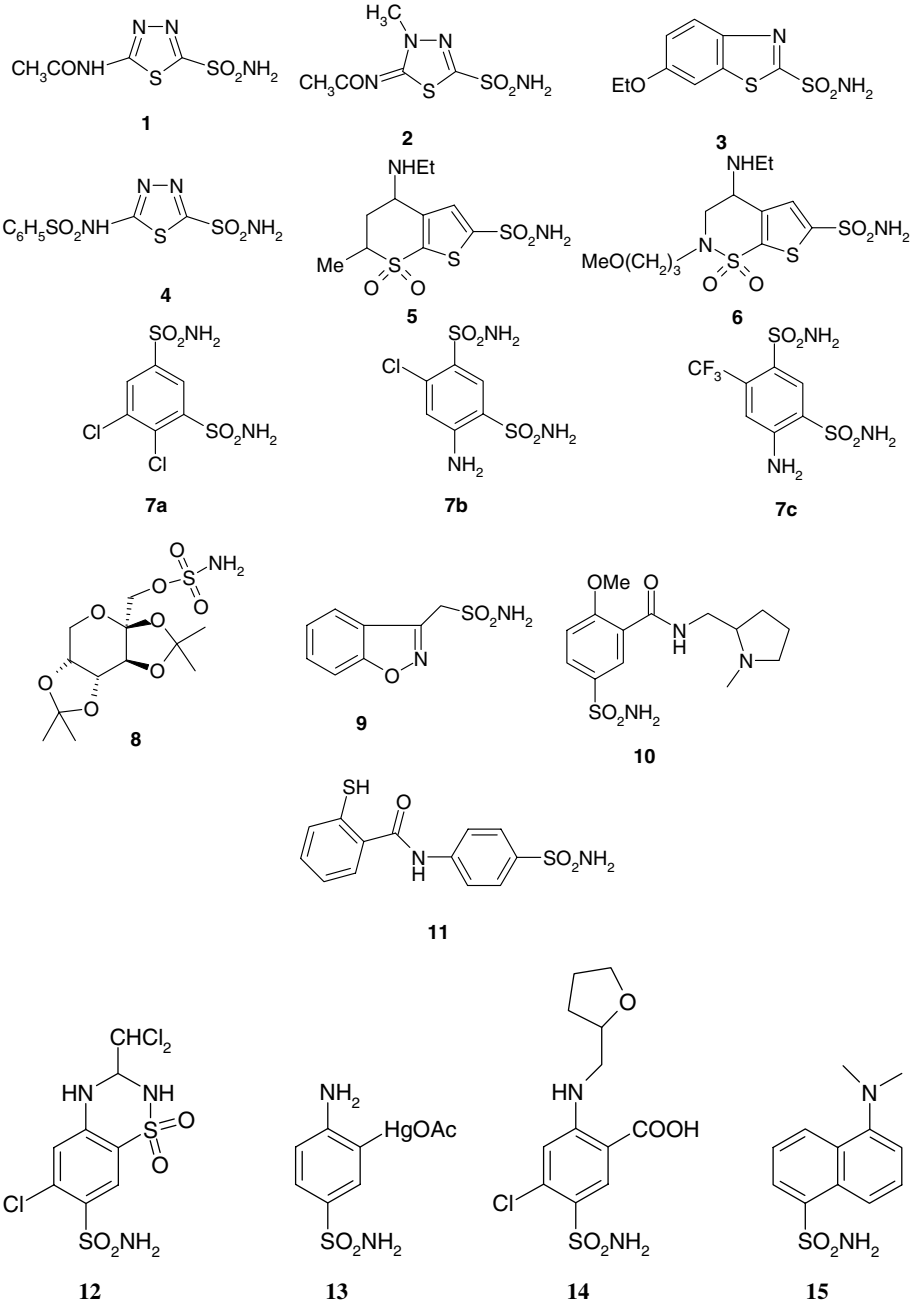
anticancer CAIs.[5–13] It should be observed that in contrast to compounds **7a**–**c**, the other clinically used CAIs, such as the sulfamate antiepileptic topiramate **8**, the sulfonamide antiepileptic zonisamide **9** or the antipsychotic sulpiride **10**, contain only one zinc binding function in their molecule of the sulfamate/sulfonamide type, similarly to the classical inhibitors **1**–**6** mentioned earlier.[25–27]



CAIs of the sulfonamide type, and particularly dichlorophenamide, have also been used in the treatment of several neurological disorders, such as primary periodic paralyses or hypokalemic periodic paralysis, for a long period.[23,28]

Considering the wide clinical use of dichlorophenamide as antiglaucoma and neurological drug,[23,28] it is rather surprising that no structural study is available regarding the binding mode of this sulfonamide and its two structurally related compounds **7b** and **7c** to any CA isozyme. Indeed, all clinically used drugs **1**–**6** and **8**–**10** have been crystallized in adduct with various CA isozymes and their 3D structures investigated by means of X-ray crystallography.[3,25–27,29,30] In this paper we report the high resolution structures of the three compounds **7a**–**c** complexed to the physiologically dominant isoform hCA II. These data are useful for better understanding the mechanism of action of the benzene-1,3-disulfonamide CAIs and also for designing compounds with a better inhibition profile (eventually less side effects) both for human and pathogenic CAs using such derivatives as lead molecules.

Inhibition data of the 12 catalytically active mammalian isozymes, of the bacterial α- and β-CAs from *H. pylori*, and of archaeal γ-CA from *Methanosarcina thermophila*

with clinically used inhibitors of type **1**–**8**, **12**, and **14** are shown in Table 1. In particular, it may be observed that the isoforms which show the highest affinity for these systemically used CAIs are CA II, CA VI, CA VII, CA XII, and CA XIII (affinities in the low nanomolar range, typically 0.8–20 nM), whereas CA VA, CA VB, CA IX, and CA XIV are slightly less sulfonamide avid, but appreciably inhibited anyhow (K_Is typically in the range of 20–65 nM) (Table 1).

Dichlorophenamide **7a** shows inhibitory properties quite different both from the monosulfonamide, classical inhibitors **1**–**3**, as well as the second generation, topically acting compounds **5** and **6** (Table 1). Thus, **7a** is also a weak inhibitor of isozymes hCA I, hCA III, and hCA IV (K_Is in the range of 1.2–15 μM), shows medium potency against hCA VA and hCA XIV (K_Is in the range of 345-630 nm), but acts as an efficient CA II, CA VB, CA VI, CA VII, CA IX, CA XII, and CA XIII inhibitor, with inhibition constants in the range of 21–79 nM (Table 1).[31,32] It is of particular interest the fact that dichlorophenamide is one of the best hCA VB inhibitor detected up to now. It must be also observed that CA VII and CA XIII, two cytosolic isoforms mainly present in the CNS (CA VII)[1,2] and reproductive tract (CA XIII),[1,2] are also well inhibited by this disulfonamide (K_Is in the range of 23–26 nM), whereas the two tumor-associated isoforms CA IX and XII show a slightly reduced affinity (K_Is of 50 nM). A rather similar behaviour to dichlorophenamide is shown by the two structurally related derivatives **7b** and **7c** investigated here (Table 1) against isozymes I, II, III, IX, XII, XIII, and XIV, whereas against the other mammalian isozymes their inhibition profile is rather different from **7a**. It is thus clear that even small structural differences present in the benzene-1,3-disulfonamide scaffold may lead to dramatic differences in the inhibitory properties of such enzyme inhibitors. Thus, derivatives **7b** and **7c** are weak CA I, CA III, CA IV, CA VA, CA VB, CA VI, and CA XIV inhibitors, with affinities in the range of 380–9400 nM, being on the other hand quite effective CA II, CA IX, CA XII, and CA XIII inhibitors (K_Is in the range of 20–75 nM).

Only recently has been investigated the possibility to design potent inhibitors targeting some of the non-vertebrate CAs, such as the enzymes from the gastric pathogen *H. pylori*[20,22] Indeed, many *Bacteria* and *Archaea* contain both CAs belonging to the α-CA family,[1–3] as well as representatives of the β-CA and/or γ-CA class,[14–18] which possess a completely different active site architecture, and susceptibility to inhibition with various classes of compounds. However, only two inhibition studies of such non-classic CA targets are available at this moment: Zimmerman et al.[33] reported the inhibition of the archaeal β-CA (from *Methanobacterium thermoautotrophicum*) and γ-CA (from *M. thermophila*, CAM) with a library of sulfonamides/sulfamates, whereas Data of Table 1 show indeed that among the clinically used derivatives **1**–**8** as well as the two other simple benzene-1,3-disulfonamides **7b** and **7c**, only two compounds are effective inhibitors of hpαCA, i.e., acetazolamide **1** and **7b** (K_Is of 20–49 nM), whereas the β-CA from the same pathogen, hpβCA, was best inhibited by ethoxzolamide **3** and topiramate **8** (K_Is of 32–33 nM), all other investigated compounds being appreciably weaker inhibitors of these bacterial CAs (inhibition constants in the range of 40–4360 nM). CAM also showed a completely different inhibition profile with these compounds as compared to the α- and β-CAs, with the best inhibitors being acetazolamide **1** and again the benzene-1,3-disulfonamide **7b** (K_Is of 63–120 nM). It is thus clear that the investigation of various classes of sulfonamides (as the benzene-1,3-

Table 1. Inhibition data with the clinically used sulfonamides/sulfamate **1**–**8**, **12** (hydrochlorothiazide), and **14** (furosemide) against mammalian isozymes CA I—XIV, bacterial α- and β-CAs from *Helicobacter pylori*, and archaeal γ-CA from *Methanosarcina thermophila*[34]

Isozyme[a]	K_I (nM)										
	1	**2**	**3**	**5**	**6**	**7a**	**7b**	**7c**	**8**	**12**	**14**
hCA I[b]	250	50	25	50,000	45,000	1200	8400	5800	250	328	62
hCA II[b]	12	14	8	9	3	38	75	63	10	290	65
hCA III[b]	3.10^5	1.10^5	5000	8000	nt	6300	6800	7200	nt	nt	nt
hCA IV[b]	74	6200	93	8500	nt	15,000	380	4800	4900	427	564
hCA VA[b]	63	65	25	42	50	630	4150	9400	63	4225	499
hCA VB[b]	54	62	19	33	30	21	6200	6500	30	603	322
hCA VI[b]	11	10	43	10	0.9	79	608	955	45	3655	245
hCA VII[b]	2.5	2.1	0.8	3.5	2.8	26	210	100	0.9	5010	513
hCA IX[c]	25	27	34	52	37	50	39	24	58[d]	367	420
hCA XII[c]	5.7	3.4	22	3.5	3.0	50	44	45	3.8	355	261
mCA XIII[b]	17	19	nt	18	nt	23	21	20	47	3885	550
hCA XIV[b]	41	43	25	27	24	345	570	680	1460	4105	52
hpαCA[b]	21	225	193	4360	210	378	49	412	172	nt	nt
hpβCA[b]	40	176	33	73	128	105	640	973	32	nt	nt
CAM	63	140	200	410	460	190	120	830	1020	nt	nt

nt, not tested (no data available).

[a] h, human; m, murine; hp, *Helicobacter pylori* enzymes; CAM, γ-CA from the archaeon *Methanosarcina thermophila*.

[b] Full length enzyme.

[c] Catalytic domain.

[d] The data against the full length enzyme is of 1590 nM.

disulfonamides investigated in detail here) may provide important insights into the drug design of better CAIs targeting both mammalian (human) as well as bacterial enzymes with medicinal chemistry applications. Thus, we investigated the binding mode of the three benzene-1,3-disulfonamides **7a**–**c** to the physiologically dominant isoform hCA II. All three compounds are effective inhibitors of hCA II (K_Is in the range of 38–75 nM, Table 1).[34]

To evaluate the structural basis responsible for the binding affinity of **7a**–**c** toward hCA II, we determined the crystal structure of hCA II complexed with these three compounds. Three-dimensional structures were analyzed by difference Fourier techniques, the crystals being isomorphous to those of the native enzyme.[30,35,36] Data collection and refinement statistics for each complex structure are shown in Supplementary Table 2.[37,38] Inhibitor binding did not generate major hCA II structural changes in any of the enzyme–inhibitor complexes studied; in fact, the rmsd of Cα atoms between the native enzyme and each enzyme–inhibitor complex ranged from 0.330 to 0.337 Å. In each complex, a clear electron density was visible for the entire inhibitor (Fig. 1). The benzene–disulfonamide moiety of each inhibitor binds identically in the active site (Fig. 2). The ionized sulfonamide NH^- group coordinates to Zn^{2+} and donates a hydrogen bond to Thr199OG, while one sulfonamide oxygen accepts a hydrogen bond from the backbone NH group of Thr199. The substituted phenyl moiety of each inhibitor is stabilized in the active site cleft by various van der Waals contacts with residues delimiting the cavity and several hydrogen bond interactions (see Fig. 2). The sulfonamide group in *meta*-position to the Zn^{2+}-coordinated sulfamoyl moiety, is oriented toward the hydrophilic side of the active site cleft in all three adducts, establishing hydrogen bonds with residues His64, Asn67, Gln92, and Thr200 in hCA II/**7a** and hCA II/**7b** complexes and with residues Gln92 and Thr200 in the hCA II/**7c** adduct (Fig. 2). In the latter complex an additional hydrogen bond is present formed by the inhibitor trifluoromethane moiety and Gln92. This polar interaction could be responsible for the higher binding affinity toward hCA II observed for **7c** as compared to the structurally related compound **7b**, which has an amino group in the corresponding position, which is unable to participate in this hydrogen bond. By using the Marvin software developed by ChemAxon,[37] pK_a calculations have been performed for the two sulfonamide groups present in compounds **7a**–**c** (see Table 2). These calculations show that compounds **7a** and **7b** coordinate to Zn^{2+} ion through the sulfonamide group having the

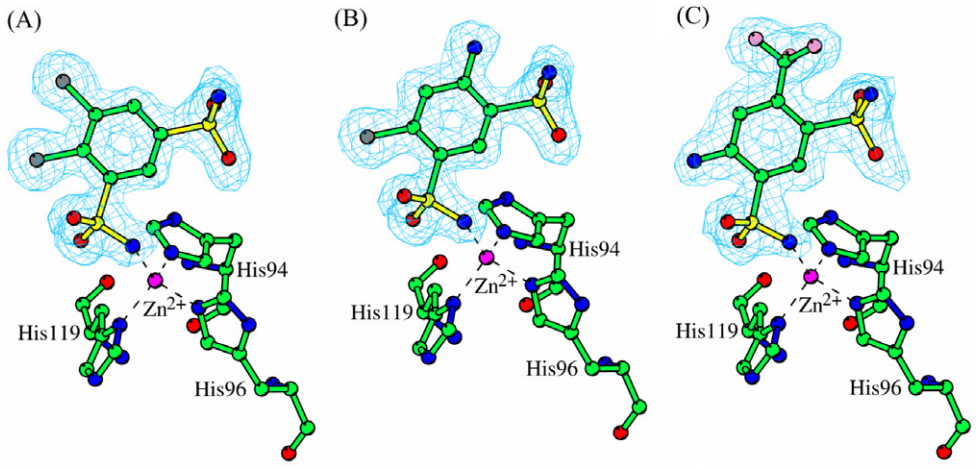


Figure 1. Active site region in the hCAII-**7a** (A), hCAII-**7b** (B), and hCAII-**7c** (C) complexes. The simulated annealing omit $|2F_o - F_c|$ electron density map relative to the inhibitor molecules is shown. Residues coordinating the metal ion are also reported.

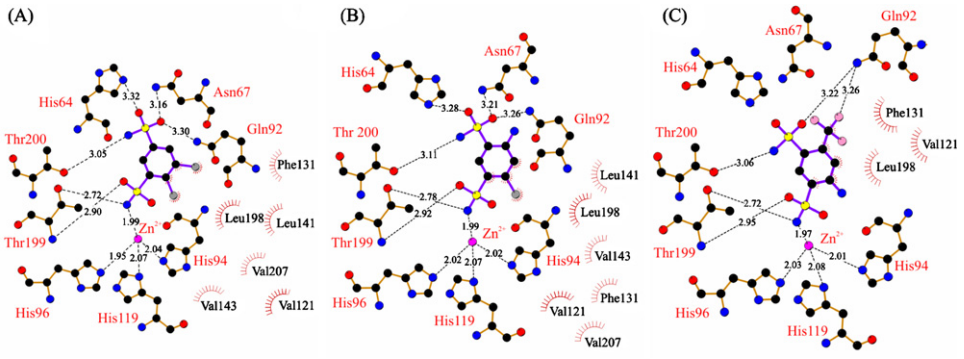


Figure 2. Schematic representation of sulfonamides **7a** (A), **7b** (B) and **7c** (C) bound within the hCA II active site.

Table 2. pK_a data of the sulfonamide moieties of inhibitors **7a–c**

Compound	pK_{a1}[a] Primary sulphonamide	pK_{a2}[a] Secondary sulfonamide
7a	8.64	9.51
7b	9.23	10.05
7c	9.97	9.18

The term 'primary sulfonamide' refers to the group that ionizes and coordinates the active site Zn^{2+} ion. The term 'secondary sulfonamide' refers to the other sulfonamide group present in these CAIs.

[a] pK_a values were calculated at 298 K, by means of the Marvin pK_a prediction plug-in using the MarvinSketch 4.1.3 graphical user interface.[37]

lowest pKa value, while compound **7c** with that having the highest one, which is a rather unusual situation.[23] This unexpected binding mode is described here for the first time, and is probably due to the bulky trifluoromethyl moiety of the inhibitor **7c**, which may render less favorable the interaction of its adjacent more acidic sulfonamide group with the zinc ion, thus causing steric hindrance with the proximal residues in the hCA II active site. In addition, the orientation of compound **7c** within the active site may be associated to the formation of the aforementioned hydrogen bond –CF_3···Gln92NE2. This interaction could be hampered by the eventual coordination of the sulfonamide group having the lowest pKa to the Zn^{2+} ion. Compounds **7a–c** also present a quite peculiar orientation within the enzyme active site, considering other benzene-sulfonamides whose structure in complex with hCA II has been solved so far.[1–5,27–30] Figure 3A shows a structural superposition of inhibitors **7a–c** with one such compound (the antitumor hypoxia-activatable sulfonamide **11**, PDB entry 2HD6) reported earlier.[31] It may be seen that the plane of the phenyl moiety of benzene-1,3-disulfonamides **7a–c** appears clearly rotated by almost 45° and tilted by approximately 10° with respect to its most recurrent orientation, as the one of derivative **11**.[31] This peculiar conformation may be ascribed to the presence of the secondary sulfonamide group in the *meta* position, which also establishes several hydrogen bonds with residues present in the hydrophilic part of the active site cleft, i.e., His64, Asn67, Gln92, and Thr200. These interactions also stabilize His64 in its *in* conformation,[3] whereas this amino acid is usually in equilibrium between the *in* and *out* conformation, in adducts of hCA II with other inhibitors investigated earlier.[3–5,25–27,30,31] It is worth noting that only 3 of the 43 hCA II–benzenesulfonamide complex structures present in the PDB (and one additional naphthalene-sulfonamide derivative) show this peculiar orientation for the substituted-benzene moiety when bound to the enzyme active site. These adducts are those with hydrochlorothiazide **12** (Klebe, G.; Supuran, C.T., manuscript in preparation, PDB entry 1ZGF) (Fig. 3B), 3-acetoxymercuri-4-aminobenzenesulfonamide **13** (PDB entry 3CA2),[35]furosemide **14** (Klebe, G.; Supuran, C.T., manuscript in preparation, PDB entry 1Z9Y), and dansylamide **15** (PDB entry 1OKL).[36] All these compounds, similarly to the benzene-1,3-disulfonamides investigated here, have a rather bulky *meta*-substituted group able to establish polar interactions with the aforementioned active site residues. Thus, these X-ray data allow the possibility to predict structural features regarding the binding of such compounds within the enzyme cavity, which may lead to a rational drug design of tighter/more selective enzyme inhibitors.

In conclusion, three benzene-1,3-disulfonamide derivatives were investigated for their interaction with 12 mammalian α-CAs, and three bacterial/archaeal α-, β-, and γ-CAs. The inhibition profile of the mammalian and bacterial/archaeal isozymes with the three disulfonamides is quite distinct from those of other clinically used derivatives. Dichlorophenamide is one of the best inhibitors of the mitochondrial isoform hCA VB, 6-trifluoromethyl-4-amino-benzene-1,3-disulfonamide is one of the best inhibitors of mCA XIII, whereas 6-chloro-4-amino-benzene-1,3-disulfonamide is one of the best inhibitors detected for hpαCA. The high resolution X-ray crystal structure of the three inhibitors bound to the dominant human isozyme hCA II revealed a particular binding mode within the enzyme cavity, which may explain their inhibition profile. The sulfonamide group in *meta*-position to the coordinated sulfamoyl

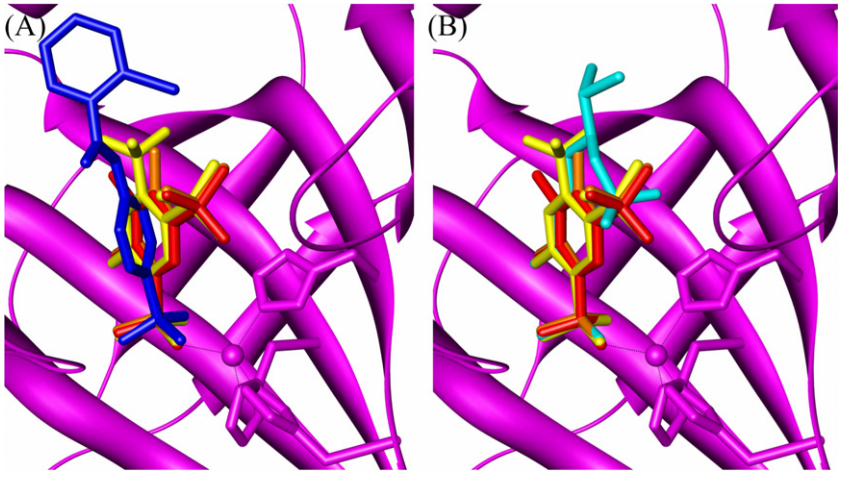


Figure 3. Superposition of hCA II–inhibitor adducts: (A) **7a** is reported in red, **7b** in orange, **7c** in yellow, and **11** in blue.[31] (B), **7a** is reported in red, **7b** in orange, **7c** in yellow, and **12** in cyan. The ribbon diagram of hCA II, the Zn^{2+} ion, and its three protein ligands is also shown.

moiety of the three inhibitors is oriented toward the hydrophilic side of the active site cleft in all three adducts, establishing hydrogen bonds with His64, Asn67, Gln92, and Thr200. As a consequence, the plane of the phenyl moiety of the inhibitors appears rotated by 45° and tilted by 10° with respect to its most recurrent orientation in other CA II–benzene monosulfonamide derivatives. Data here reported suggest that the presence of this additional sulfonamide group in *meta* position of hCA II benzenesulfonamide inhibitors can be used to differently orient the phenyl moiety within the enzyme active site. As a consequence, a putative tail conjugated with the benzene-1,3-disulfonamide group can be opportunely oriented in the enzyme active site, leading to inhibitors with diverse inhibition profiles and selectivity for various mammalian, bacterial or archaeal CAs. These findings constitute the starting basis for the design of selective inhibitors targeting various CA isozymes.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.05.045.

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38. Crystals of the hCA II–inhibitor complexes were obtained using the hanging drop vapor diffusion technique. 5 μL of complex solution and 5 μL of precipitant solution (2.5 M $(NH_4)_2SO_4$, 0.3 M NaCl, 100 mM Tris–HCl (pH 8.2), and 1 mM DTT) were mixed and suspended over a reservoir containing 1 mL of precipitant solution at 4 °C. X-ray diffraction data were collected at 100 K, at the Synchrotron source Elettra in Trieste, Italy, using a Mar CCD detector. Prior to cryogenic freezing, the crystals were transferred to the precipitant solution with the addition of 15 % (v/v) glycerol. Data were integrated and reduced using the HKL crystallographic data reduction package (Denzo/Scalepack).[39] Diffraction data for each crystal were indexed in the *P*21 space group with one molecule in the asymmetric unit. Unit cell parameters and data reduction statistics are recorded in Supplementary Table 2. The atomic coordinates of CA II refined at 2.0 Å resolution (PDB entry 1CA2)[30] were used as a starting model for crystallographic refinement after deletion of non-protein atoms. An initial round of rigid body refinement followed by simulated annealing and individual B factor refinement was performed using the program CNS 1.1.[40] Model visualization and rebuilding was performed using the graphics program O.[41] Inhibitor molecules were identified from peaks in $|F_o| - |F_c|$ maps and were gradually built into the models over several rounds of refinement; restraints on inhibitor bond angles and distances were taken from similar structures in the Cambridge Structural Database and standard restraints were used on protein bond angles and distances throughout refinement. Water molecules were built into peaks $>3\sigma$ in $|F_o| - |F_c|$ maps that demonstrated appropriate hydrogen-bonding geometry. The correctness of stereochemistry was finally checked using PROCHECK.[42] Final refinement statistics for all structures of hCA II–inhibitor complexes are presented in Supplementary Table 2.
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