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Carbonic anhydrase inhibitors: Inhibition of human cytosolic isozymes I and II and tumor-associated isozymes IX and XII with S-substituted 4-chloro-2-mercapto-5-methyl-benzenesulfonamides

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Abstract—A series of S-substituted 4-chloro-2-mercapto-5-methyl-benzenesulfonamides has been investigated as inhibitors of four isoforms of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), that is, the cytosolic, ubiquitous isozymes CA I and II, as well as the transmembrane, tumor-associated isozymes CA IX and XII. The new derivatives were inefficient inhibitors of isoform I (K_Is in the range of 2.7–18.7 μM) but generally had low nanomolar affinity for the inhibition of the other three isoforms (K_Is in the range of 2.4–214 nM against hCA II; 1.4–47.5 nM against hCA IX, and 1.7–569 nM against hCA XII, respectively). Some selectivity for the inhibition of the tumor-associated versus the cyctosolic isoform II with some of these compounds has also been evidenced. As CA IX is an important marker of tumor hypoxia and its predictive, prognostic, and druggability potentials for designing antitumor therapies were recently validated, detection of selective, potent CA IX inhibitors may be relevant in the fight against cancers overexpressing CA isozymes.

1. Introduction

It is well known that aryl/heteroaryl sulfonamides may act as antitumor agents through a variety of mechanisms such as cell cycle perturbation in the G1 phase, distribution of microtubule assembly, angiogenesis inhibition, and functional suppression of the transcriptional activator NF-Y. Moreover, following an extensive evaluation, numerous sulfonamides were found to act as carbonic anhydrase (CA, EC 4.2.1.1) inhibitors.[1–4] In brief, the α-CAs are a family of metalloenzymes involved in the catalysis of an important physiological reaction: the hydration of CO_2 to bicarbonate and a proton ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). At least 13 enzymatically active isoforms have been discovered in higher vertebrates.[1–4] CAs are involved in pH regulation, secretion of electrolytes, respiration,[5–7] biosynthetic reactions which require CO_2/bicarbonate as substrate such as gluconeogenesis, lipogenesis, ureagenesis, and pyrimidines synthesis among others.[8] Other roles for these enzymes were highlighted, such as calcification and bone resorption.[9] The discovery that CA IX, a transmembrane tumor-associated protein,[10] was prevalently expressed in several human cancer cells and not in their normal counterparts[11–19] suggests a role for some CA isoforms in oncogenesis.[8] Several studies showed a clear-cut relationship between high CA IX levels in tumors and a poor prognosis.[12–22] CA IX also acts on cells adhesion and differentiation by its N-terminal proteoglycan related-region which is absent in other transmembrane CA isozymes, such as CA XII (which is present in some tumors[8]) and CA XIV (which is not associated with tumors).[14] The level of CA IX, which efficiently catalyzes CO_2 hydration to bicarbonate with release of a

Keywords: Carbonic anhydrase; Sulfonamide; Tumor-associated isoform IX and XII; Selective inhibitor.
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proton,[1,3] is strongly increased by tumor hypoxia via a direct transcriptional activation of *CA9* gene by the hypoxia inducible factor type 1 (HIF-1).[8] Furthermore, CA IX is negatively regulated by von Hippel Lindau (VHL) tumor suppressor protein, and its expression in renal cell carcinomas is related to inactivating mutation of the VHL gene.[21,22] CA IX was also proposed to serve as a marker of tumor hypoxia and its predictive and prognostic potential has been demonstrated in a number of clinical studies.[8,12,13,22]

The high catalytic activity of CA IX and CA XII isozymes leading to formation of protons by the hydration of CO_2, was demonstrated to participate to the tumor microenvironment acidification by maintaining the extracellular acidic pHe, and thus contributes to tumor propagation and malignant progression.[23] Indeed, Svastova et al.[23] showed that the acidic extracellular pHe of the tumor microenvironment, is generated by the activity of the tumor-associated isozymes, that is, CA IX and probably also CA XII, and that this acidification can be perturbed by deletion of the enzyme active site and inhibited by CA-selective inhibitors of the sulfonamide type which bind only to hypoxic cells containing the active enzyme.[23] As a consequence, targeting the tumor microenvironment via CA IX inhibition constitutes an attractive new approach for the management of hypoxic tumors.[24,25] The potential use of carbonic anhydrase inhibitors as antitumor agents opens thus a new important research direction.[24,25]

Recently, we have reported on the strong inhibition of human cytosolic isozymes I and II and tumor-associated isozymes IX and XII with some S-substituted 4-chloro-2-mercapto-6-methyl-benzenesulfonamides of type I.[5] Some of those compounds also showed a certain degree of selectivity for the inhibition of the tumor-associated over the cytosolic CA isoforms.[5] These findings prompted us to investigate the inhibitory activity of the related derivatives of type II with a methyl group placed at position 5 of the phenyl ring and various substituents attached to the sulfur atom.

2. Results and discussion

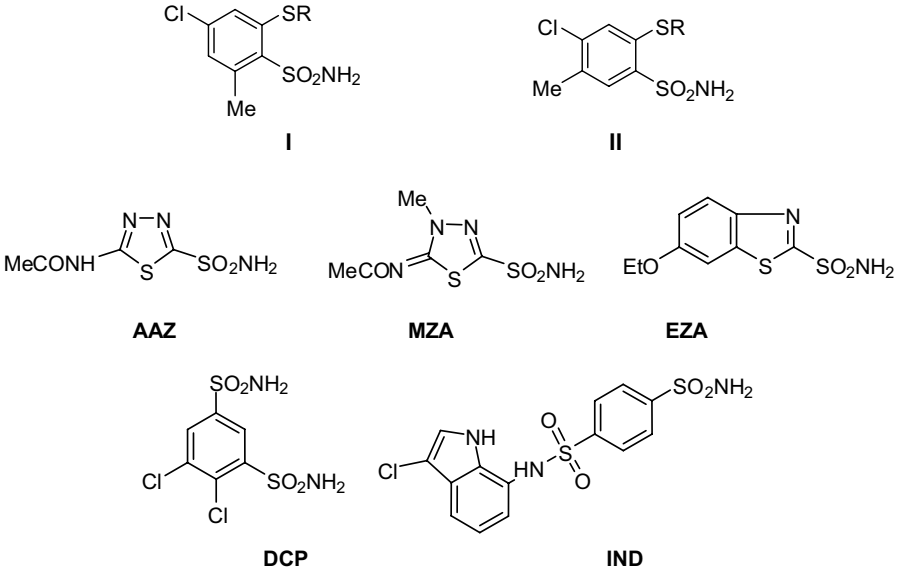
2.1. Chemistry

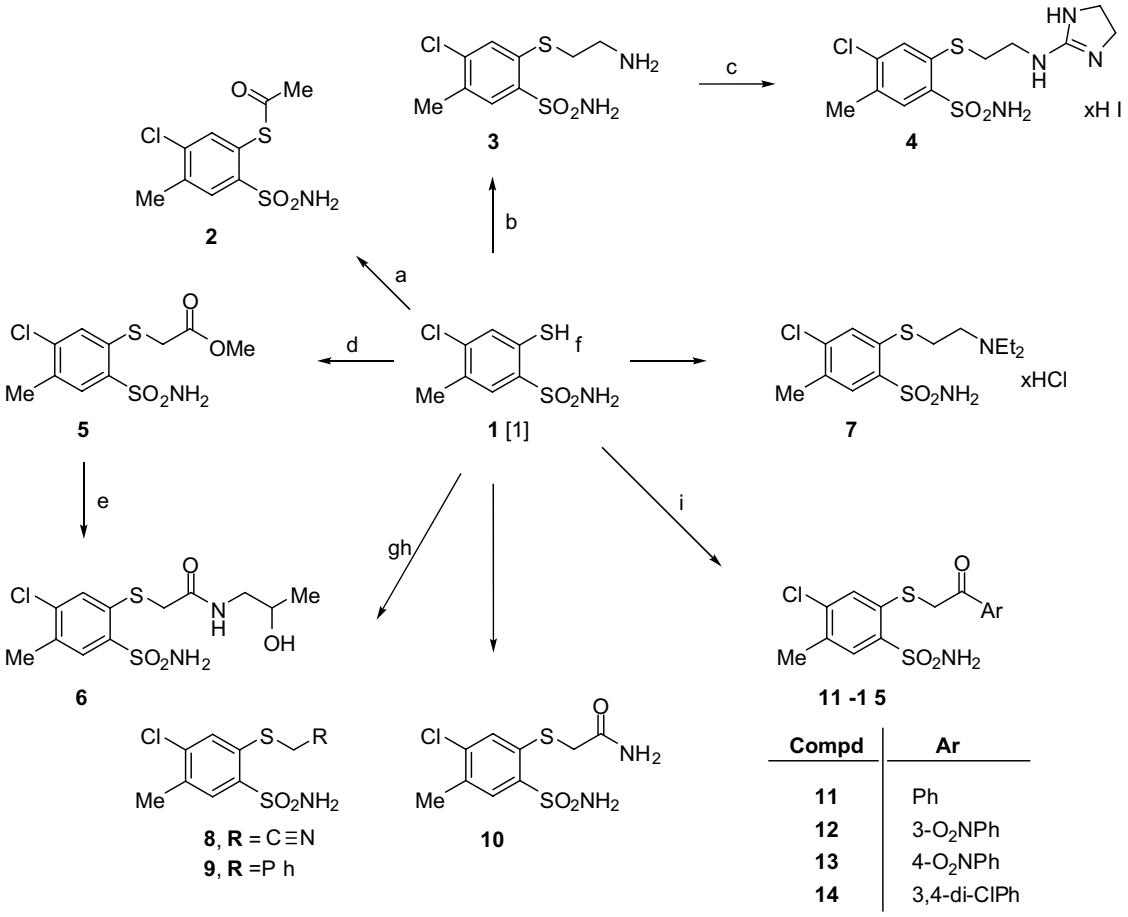
A series of S-substituted 4-chloro-2-mercapto-5-methyl-benzenesulfonamides of type **2**–**26** has been obtained starting from 2-mercapto-4-chloro-5-methyl-benzenesulfonamide **1** by the procedures shown in Schemes 1 and 2.[32] The synthesis of these compounds has been reported earlier[32–36] but their interaction with CAs has never been investigated. It should be emphasized, that esters **5**, **18**–**20** and the acetic acid derivative **15** proved to be relatively unstable in aqueous solution, and therefore, have not been tested for their biological activities.

2.2. CA inhibition studies

The compounds **2**–**4**, **6**–**14**, **19**, and **21**–**26** as well as standard, clinically used CAIs, such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND**, have been tested for the inhibition of two cytosolic, ubiquitous isozymes of human origin, that is, hCA I and hCA II,[1,3] as well as the two human, tumor-associated isoforms hCA IX and XII (Table 1).

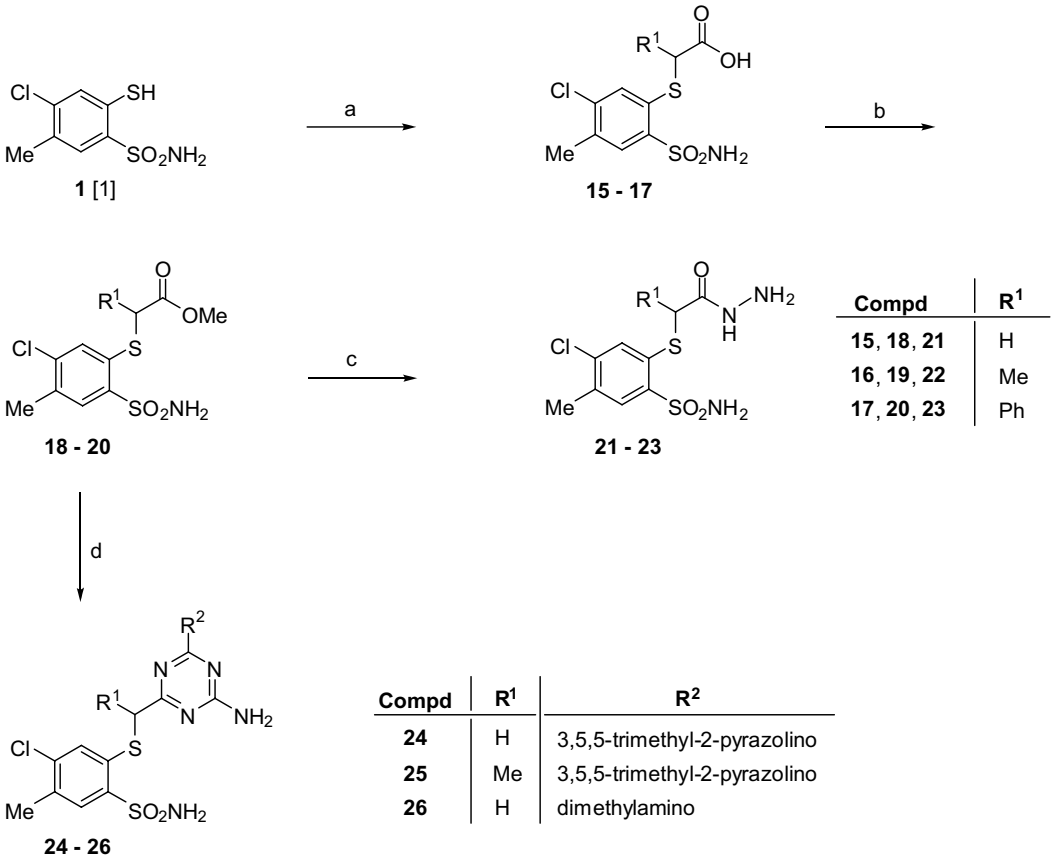
The following should be noted regarding CA inhibitory data of Table 1: (i) against the slow cytosolic isoform hCA I, the sulfonamides **2**–**26** investigated here showed moderate to weak inhibitory properties. Thus, derivatives **4**, **6**, **8**, **10**, **12**, and **21** showed weak inhibition of this isoform, with K_Is in the range of 10.7–18.7 μM, being thus much weaker inhibitors as compared to the clinically used compounds **AAZ**–**IND** (Table 1). The other investigated derivatives were slightly more inhibitory against hCA I, with K_Is in the range of 2.7–8.1 μM. No clear-cut SAR is obvious from these data since compounds with both bulky *ortho*-substituents to the sulfamoyl moiety (such as for example **24**–**26**) as well as some with more compact such functionalities (e.g., **2** and **3**) showed comparable activity; (ii) against the ubiquitous and dominant rapid cytosolic isozyme hCA II, com-





Scheme 1. Synthesis of S-substituted 4-chloro-2-mercapto-benzenesulfonamides **2**–**14**. Reagents and conditions: (a) acetic anhydride (5.0 M equiv), acetic acid, room temperature, 24 h; (b) aziridine (1.08 M equiv), MeOH, 0–5 °C, 3 h; (c) 2-methylthioimidazoline hydroiodide (1.1 M equiv), MeOH, reflux, 17 h; (d) $ClCH_2COOMe$ (1.0 M equiv), MeONa (1.07 M equiv), MeOH, room temperature, 8 h, reflux, 5 h; (e) 3-amino-2-propanol (2.04 M equiv), toluene, 20–100 °C, 10 h, reflux, 6 h; (f) 2-(diethylamino)ethyl chloride (1.1 M equiv), 2-propanol, reflux, 6 h; (g) $BrCH_2R$ (1.1 M equiv), TEA (1.1 M equiv), CH_2Cl_2, 0–20 °C, 7 h, reflux, 1 h; (h) chloroacetamide (1.1 M equiv), TEA (1.1 M equiv), CH_2Cl_2, room temperature, 3 h, reflux, 7 h; (i) $ArCOCH_2Br$ (1.1 M equiv), TEA (1.1 M equiv), CH_2Cl_2, 18–24 °C, 2 h, reflux, 6 h.

pounds **2**–**26** showed a much better inhibitory activity (Table 1). Thus, except for **24**–**26**, possessing the very bulky triazinyl-methylthio groups in *ortho* to the zinc-binding sulfamoyl moiety, and acting as weaker hCA II inhibitors (K_Is in the range of 173–214 nM), the other compounds are much stronger CA II inhibitors. Thus, a quite compact behavior of potent inhibitor was observed for all of them, with K_Is in the range of 3.6–12.6 nM, of the same order of magnitude as most of the clinically used compounds (Table 1). Indeed, this class of derivatives shows a typical hCA II inhibitory profile of tight-binding sulfonamide, irrespective of the nature of the *ortho*-S-substituted moiety near the sulfamoyl group. It is thus rather difficult to rationalize this behavior but work is in progress in our laboratories for resolving the X-ray crystal structure of adducts of hCA II with some of the most active hCA II sulfonamides inhibitors investigated here; (iii) a quite good inhibition profile of the tumor-associated isoform hCA IX has also been observed with all the investigated sulfonamides of type **2**–**26**. Thus, compounds **3**, **7**, and **24**–**26** were the weakest inhibitors (K_Is in the range of 18.1–47.5 nM), whereas the remaining derivatives were more effective inhibitors, with K_Is in the range of 1.4–10.3 nM. As a class, these S-substituted 4-chloro-2-mercapto-5-methyl-benzenesulfonamides are among the most effective hCA IX inhibitors detected up to now, with some of them showing low nanomolar inhibition, which makes them extremely attractive derivatives for detailed pharmacological evaluation in cell cultures[23,29] or animal models of hypoxic tumors overexpressing CA IX.[8,24,25] Again no clear-cut SA regarding the role of the *ortho*-substituent to the sulfamoyl group in the CA IX inhibitory activity of these compounds can be drawn from these data, which is rather frustrating, but we have recently demonstrated by means of X-ray crystallography and kinetic measurements that even one atom difference between otherwise isostructural/isosteric compounds (e.g., replacing of the sulfamate oxygen from the zinc-binding function by an NH moiety in the sulfamide one, maintaining the same organic scaffold of the inhibitor) may lead to dramatic differences of CA inhibitory activity (of several hundreds times) for the various isoforms.[37] Thus, X-ray crystallography is probably the only way of better understanding the intricate interactions between these enzymes and their inhibitors, even for a congeneric series as the one



Scheme 2. Synthesis of S-substituted 4-chloro-2-mercapto-benzenesulfonamides **16**–**27**. Reagents and conditions: (a) ClCH(R^1)COOH (1.6 M equiv), NaOH (2.6 M equiv), water, room temperature, 24 h; (b) MeOH (large excess), H_2SO_4, reflux, 20 h; (c) $H_2NNH_2{\cdot}H_2O$ (2.0 M equiv or 6.0 M equiv in the case of **24**), MeOH, reflux, 5–7 h; (d) H_2N–C(=NH)–NH–C(=NH)–$R^2{\cdot}$HCl (2.0 M equiv), MeONa (4.0 M equiv), MeOH, reflux, 30 h.

investigated here possessing a large variety of substituents in *ortho* to the sulfamoyl moiety critical for the binding to the Zn(II) ion within the enzyme active site; (iv) a quite larger variation of inhibitory activity was on the other hand observed for the inhibition of the second tumor-associated isoform, hCA XII (Table 1). Thus, the compounds with bulky *ortho*-substituents **23**–**26** showed weak CA XII inhibitory activity, with K_Is in the range of 420–569 nM. Another groups of compounds, such as **9** and **11**–**14**, showed moderate CA XII inhibition, with K_Is in the range of 24.1–76.3 nM, whereas the remaining derivatives were very effective hCA XII inhibitors (K_Is in the range of 1.7–14.7 nM, Table 1). As mentioned above, again the SAR is difficult to understand, except for the bulky compounds **23**–**26** mentioned earlier in this paragraph; (v) one of the important issues regarding the design of CAIs regards the selectivity of such compounds for the inhibition of the target isoform over that of the ubiquitous ones CA I and II.[1,3] As seen from data of Table 2, clinically used sulfonamides such as **AAZ**, **MZA**, **EZA** do indiscriminately inhibit both the cytosolic isoforms CA I and II as well as the tumor-associated ones CA IX and XII with rather similar potency. Furthermore, as observed from Table 2, many times the selectivity ratio for inhibiting the tumor-associated isoform CA IX over the cytosolic ubiquitous one CA II are in the range of 0.23–0.51 for these compounds, meaning that all of them are better CA II than CA IX inhibitors. However, data of Table 2 also show that some of the compounds investigated here, such as **2**, **8**, **22**, and **24** among others, show good selectivity ratios for the inhibition of the transmembrane over the cytosolic isozymes, in the range of 100–4675 for the inhibition of CA IX over CA I, of 1.48–6.77 for the inhibition of CA IX over CA II, in the range of 7.62–8904 for the inhibition of CA XII over CA I, and of 0.51–4.23, for the inhibition of CA XII over CA II, respectively.

In order to rationalize some of these results, we shall consider the 3D structure and amino acid sequence of the four CA isozymes investigated here for their inhibition with this class of sulfonamides. X-ray crystallographic studies are available for the following isozymes and their adducts with inhibitors (sulfonamides, sulfamates, and sulfamides), among those considered here: CA II (the best studied isozyme in this family),[37–40] CA I,[41,42] and CA XII.[43] The X-ray crystal structure of CA IX has not been reported yet. Figure 1 shows that the 3-D fold of isozymes CA I, II and XII is very similar: the shape of these three proteins is practically the same, the active sites are very similar with each other, the only important difference being the fact that CA XII is a bitopic, dimer protein, possessing two identical active sites,[43] whereas CA I and II are monomers, with only one active site.[37–42] The active site of these

Table 1. Inhibition data of sulfonamides **2–4**, **6–14**, **19**, and **21–26** reported in the present paper and standard CA inhibitors, against isozymes I, II, IX, and XII, by a stopped-flow, CO_2 hydration assay[31]

Inhibitor	K_I[c]			
	hCA I[a] (μM)	hCA II[a] (nM)	hCA IX[b] (nM)	hCA XII[b] (nM)
AAZ	0.31 ± 0.03	12 ± 0.6	25 ± 1.1	5.7 ± 0.4
MZA	0.78 ± 0.08	14 ± 0.8	27 ± 2.0	3.4 ± 0.2
EZA	0.025 ± 0.002	8 ± 0.4	34 ± 2.1	22 ± 1.1
DCP	1.20 ± 0.09	38 ± 2.5	50 ± 3.7	50 ± 4.1
IND	0.031 ± 0.003	15 ± 0.9	24 ± 1.6	3.4 ± 0.2
2	4.8 ± 0.25	3.6 ± 0.2	1.4 ± 0.1	1.7 ± 0.1
3	5.6 ± 0.31	9.2 ± 0.8	18.4 ± 1.4	13.6 ± 1.2
4	14.8 ± 1.3	9.0 ± 0.7	9.7 ± 0.9	12.9 ± 0.5
6	14.9 ± 1.1	5.4 ± 0.6	10.3 ± 0.8	8.6 ± 0.4
7	8.1 ± 0.9	8.0 ± 0.4	18.1 ± 1.5	14.7 ± 0.9
8	18.7 ± 1.3	8.9 ± 0.6	4.0 ± 0.3	2.1 ± 0.1
9	2.7 ± 0.3	9.1 ± 1.0	9.2 ± 0.8	34.0 ± 2.7
10	14.9 ± 1.3	5.4 ± 0.2	10.3 ± 1.0	8.6 ± 0.6
11	5.3 ± 0.2	8.6 ± 0.6	4.5 ± 0.3	24.1 ± 1.9
12	15.1 ± 1.2	4.9 ± 0.4	6.3 ± 0.5	48.6 ± 3.7
13	3.5 ± 0.3	5.2 ± 0.4	4.4 ± 0.2	35.1 ± 2.8
14	6.1 ± 0.4	4.1 ± 0.1	8.0 ± 0.7	76.3 ± 5.3
19	5.0 ± 0.4	7.3 ± 0.6	2.8 ± 0.2	13.8 ± 1.1
21	10.7 ± 1.1	2.4 ± 0.3	3.7 ± 0.2	2.5 ± 0.1
22	3.4 ± 0.2	7.7 ± 0.5	5.2 ± 0.3	5.0 ± 0.1
23	7.2 ± 0.4	12.6 ± 1.1	14.1 ± 1.0	420 ± 23
24	2.8 ± 0.3	189 ± 15	27.9 ± 2.4	367 ± 33
25	3.2 ± 0.2	214 ± 18	35.2 ± 3.0	438 ± 29
26	5.1 ± 0.3	173 ± 15	47.5 ± 4.6	569 ±38

[a] Human (cloned) isozymes, by the CO_2 hydration method.
[b] Catalytic domain of human, cloned isozymes,[29,30] by the CO_2 hydration method.[31]
[c] Mean ± standard error (from three different assays).

Table 2. Selectivity ratios for the inhibition of the tumor-associated (CA IX and XII) over the cytosolic (CA I and II) isozymes with selected CAIs reported here

Compound	Selectivity ratio			
	hCA I/hCA IX	hCA II/hCA IX	hCA I/hCA XII	hCA II/hCA XII
AAZ	10	0.48	43.8	2.10
MZA	1.85	0.51	14.7	4.11
EZA	0.73	0.23	1.13	0.36
2	3428	2.57	2823	2.11
8	4675	2.22	8904	4.23
22	653	1.48	680	1.54
24	100.3	6.77	7.62	0.51

three CAs (and presumably also of CA IX, based on the amino acid sequence shown in Fig. 2)[1,25] is formed by a cavity at the bottom of which is placed the catalytically essential Zn(II) ion (violet sphere in Fig. 1), coordinated in all isoforms by three histidine residues, His94, 96, and 119 (CA II numbering system).[37–40] Other residues involved in the binding of inhibitors are also identical in these four isozymes. These are: (i) the gate-keepers, that is, residues Thr199 and Glu106 which form a network of hydrogen bonds with the non-protein zinc ligand (the water molecule in the un-inhibited enzyme and the inhibitor coordinated to zinc in E–I adducts), which represents one of the factors explaining the potent inhibition of CAs by sulfonamides and their isosteres[1–3,37–43];

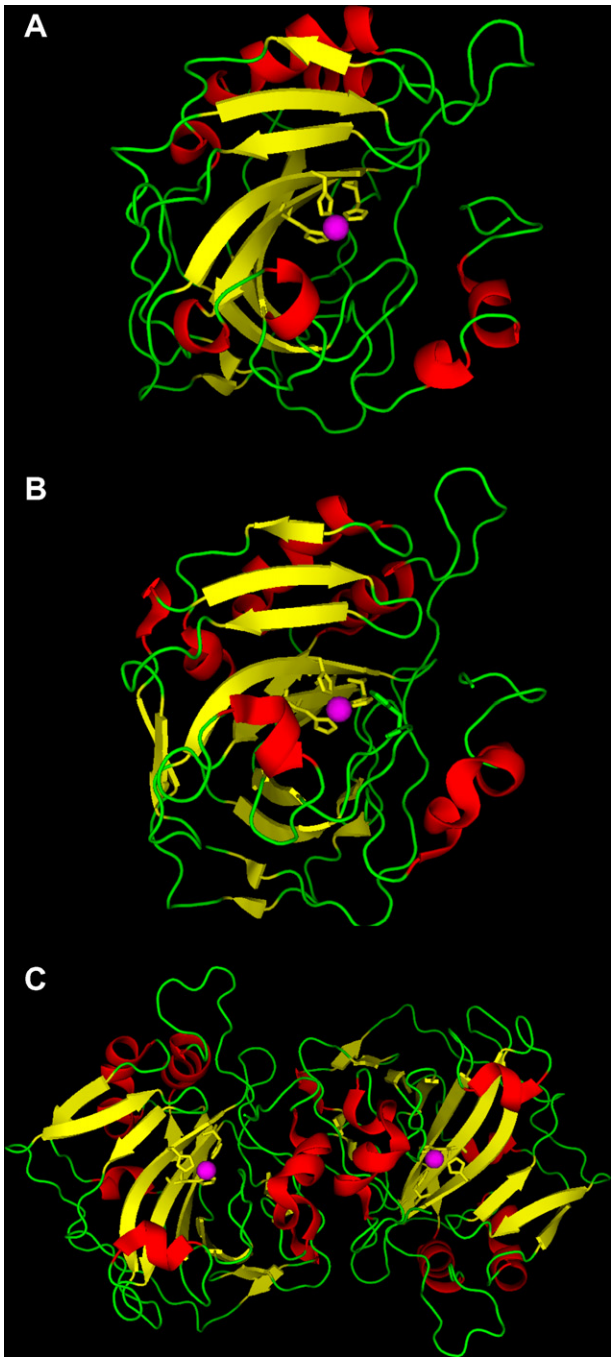


Figure 1. Folding of CA isozymes hCA II[37–40] (A), hCA I[41] (B) and hCA XII[43] (C) as determined by X-ray crystallography. The Zn(II) ion (violet sphere), its three histidine ligands (in yellow, His94, 96, and 119, CA II numbering) and the ribbon diagram of the polypeptide chains are also shown (α-helices in red; β-sheets in yellow).

(ii) the proton shuttle, which is His64 in all these isozymes,[1–3,37–43] although for CA I additional shuttling may be also provided by His67 (Fig. 2).[44] However, as seen from Figure 2, where the amino acid sequences of the four isozymes are aligned, many of the other residues involved in the active site architecture of these enzymes differ substantially between the four isoforms. This clearly explains on one hand the different catalytic activities of these CAs (CA I and XII are slow catalysts for CO_2 hydration, whereas CA II and IX are much more

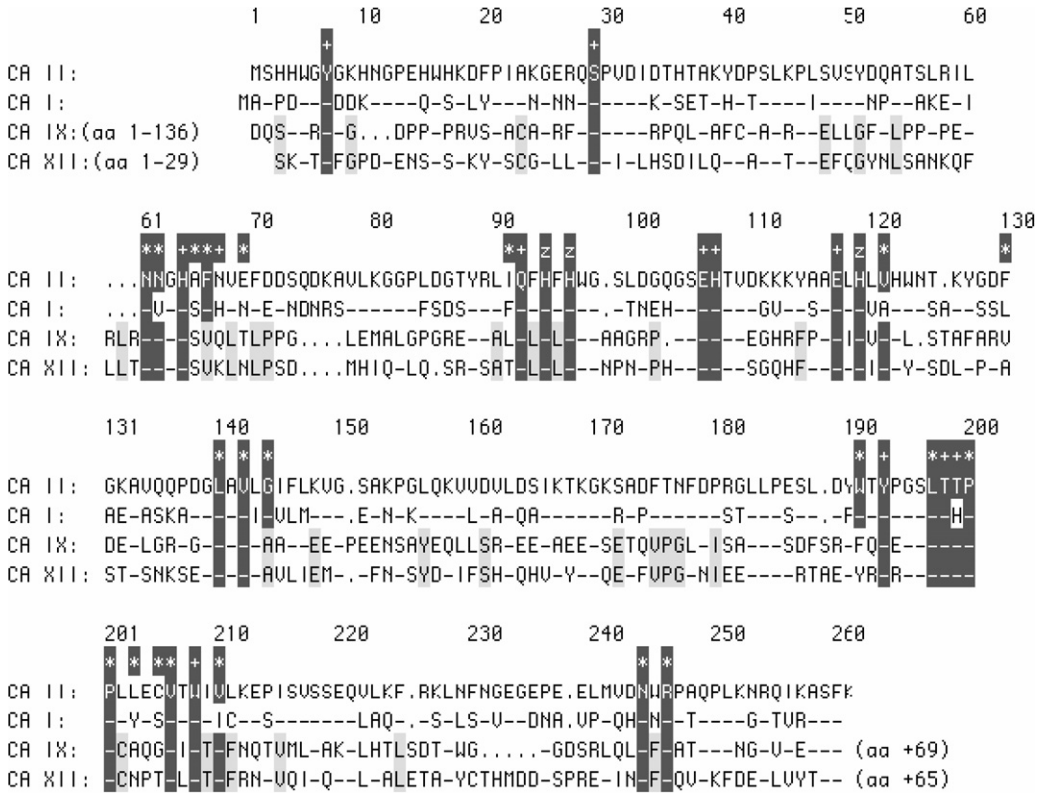


Figure 2. Alignment of amino acid sequences of hCA I, II, IX and XII. Residues involved in the active site architecture are represented by a combination of z, + and ∗ signs (hCA I numbering). Gray bars represent conserved amino acid residues in all these four isozymes. In the discussion the hCA II numbering system was employed (in which amino acids from position 125 on, become 125 +1, e.g., Phe130 becomes Phe131, etc.).

effective catalysts for the physiological reaction),[1–4,25] and probably also the different inhibition profiles with various classes of inhibitors.[1–4,7,8,25,26]

For example, an important difference between various CA isozymes is constituted by the amino acid in position 131, which is Phe for hCA II, Leu for CA I, Val for hCA IX, and Ala for CA XII (Fig. 2). Phe131 is known to be very important for the binding of sulfonamide inhibitors to CAs[45]: in many cases this bulky side chain limits the space available for the inhibitor aromatic moieties, or it may participate in stacking interactions with groups present in it (for recent examples see Refs. 45,46). Thus, the presence of a less bulky residue in hCA IX (i.e., a valine), which is also unavailable for participation to stacking interactions, has as a consequence the fact that the hCA IX active site is larger than the hCA II active site. A second residue that drew our attention is 132, which is Gly in hCA II, Ala in hCA I, Asp in hCA IX, and Ser in hCA XII. This residue is situated on the rim of the hydrophilic half of the entrance to the active site of hCA II (and presumably also of hCA IX) and it is critical for the interaction with inhibitors possessing elongated molecules, as recently shown by us.[47] Strong hydrogen bonds involving the CONH moiety of Gly132 were shown to stabilize the complex of this isozyme with a *p*-aminoethylbenzenesulfonamide derived inhibitor.[47] In the case of hCA IX, the presence of aspartic acid in this position at the entrance of the active site may signify that: (i) stronger interactions with polar moieties of the inhibitor bound within the active site should be possible, since the COOH moiety possesses more donor atoms; (ii) this residue may have flexible conformations, fine-tuning in this way the interaction with inhibitors. Thus, the stronger hCA IX inhibition with some of these inhibitors (as compared to their affinity for isozyme II), such as for example **2**, **8**, **22**, and **24**, might be explained just by the different interactions with the two active site residues mentioned above. Anyhow, the final answers may arrive only after the report of the X-ray crystal structure of this isozyme and its complexes with inhibitors. pK_a of the sulfonamide moiety may also be an important factor for the binding of the inhibitors to the enzyme (as the sulfonamidate anions is coordinated to zinc),[1–4] but no exact values of this parameter for the newly investigated derivatives is available yet. However, considering the very complex interactions between the inhibitor and the enzyme, pK_a is just one of the many factors influencing the binding of these compounds within the enzyme cavity, being probably less important than the steric interactions (favorable and clashing ones) in which the inhibitor participates when bound to the enzyme.[37]

3. Conclusions

A rather large series of S-substituted 4-chloro-2-mercapto-5-methyl-benzenesulfonamides, incorporating various *ortho*-substituents to the sulfamoyl moiety has been investigated as inhibitors of four CA isoforms, the cytosolic, ubiquitous isozymes CA I and II, as well

as the transmembrane, tumor-associated isozymes CA IX and XII. The new derivatives were inefficient inhibitors of isoform I (K_Is in the range of 2.7–18.7 μM) but generally had low nanomolar affinity for the inhibition of the other three isoforms (K_Is in the range of 2.4–214 nM against hCA II; 1.4–47.5 nM against hCA IX, and 1.7–569 nM against hCA XII, respectively). Some selectivity for the inhibition of the tumor-associated versus the cyctosolic isoform II with some of these compounds has also been evidenced. As CA IX is an important marker of tumor hypoxia and its predictive, prognostic and druggability potentials for designing antitumor therapies were recently validated, detection of selective, potent CA IX inhibitors may be relevant in the fight against cancers overexpressing CA isozymes.

4. Experimental

4.1. Chemistry

The clinically used sulfonamide CA inhibitors (CAIs) acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND**, employed as standard inhibitors in the enzyme assays, are commercially available from Sigma–Aldrich or have been prepared as previously described.[26] Recombinant human CA isoforms I, II, and IX have been prepared as reported earlier by our group,[27–30] and their activity assayed by the stopped-flow method of Khalifah.[31]

Compounds **2**–**26** investigated in the present study belong to the substituted-2-mercapto-benzenesulfonamide class. Starting from 4-chloro-2-mercapto-5-methylbenzenesulfonamide (**1**)[32] compounds **2**–**26** were synthesized according to the previously described procedures as shown in Schemes 1 and 2.[33–36]

4.2. CA inhibition assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[31] 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 (1 mM) were prepared in distilled–deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) 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,[27–30] and represent the mean from at least three different determinations. Enzyme concentrations in the assay system were: 9.2 nM for hCA I, 7.6 nM for hCA II, 12 nM for hCA IX, and 13 nM for hCA XII.

Acknowledgments

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