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Carbonic anhydrase inhibitors: synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, and IX with sulfonamides derived from 4-isothiocyanato-benzolamide

Alessandro Cecchi,[a] Jean-Yves Winum,[a,b] Alessio Innocenti,[a] Daniela Vullo,[a] Jean-Louis Montero,[b] Andrea Scozzafava[a] and Claudiu T. Supuran[a,*]

[a]*Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy*
[b]*Université Montpellier II, Laboratoire de Chimie Biomoléculaire, UMR 5032, Ecole Nationale Supérieure de Chimie de Montpellier, 8 rue de l'Ecole Normale, 34296 Montpellier Cedex, France*

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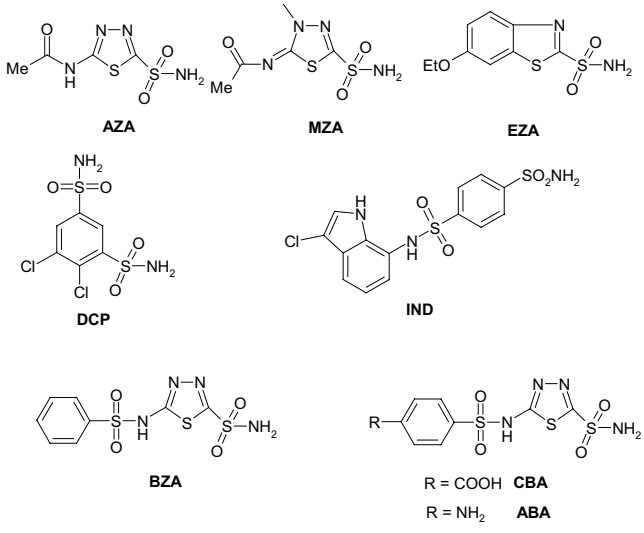
Abstract—A series of sulfonamides incorporating 4-thioureido-benzolamide moieties have been prepared from aminobenzolamide and thiophosgene followed by the reaction of the thiocyanato intermediate with aliphatic/aromatic amines or hydrazines. The new derivatives have been investigated as inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), and more precisely of the cytosolic isozymes hCA I and II, as well as the tumor-associated isozyme hCA IX (all of human origin). The new compounds showed excellent inhibitory properties against all three isozymes with inhibition constants in the range of 0.6–62 nM against hCA I, 0.5–1.7 nM against hCA II and 3.2–23 nM against hCA IX, respectively. These derivatives are interesting candidates for the development of novel therapies targeting hypoxic tumors.

1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) are widely used therapeutic agents in the management or prevention of many diseases.[1–4] This is mainly due to the wide distribution of the 14 presently described human CA isozymes in many cells, tissues, and organs, where they play crucial physiological functions.[1–4] Still, the available pharmacological agents are far from being perfect, as they possess many undesired side effects, mainly due to their lack of selectivity for the different isozymes. Thus, development of isozyme-specific or at least organ-selective inhibitors would be highly beneficial both for obtaining novel types of drugs, devoid of major side effects, as well as for physiological studies in which specific/selective inhibitors may constitute valuable tools for understanding the physiology/physiopathology of these enzymes.[1–4]



Sulfonamide CAIs such as acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA**, or dichlorophenamide **DCP** among others, played a crucial role in the understanding of renal physiology and pharmacology,

* Corresponding author. Tel.: +39 055 4573005; fax: +39 055 4573385; e-mail: claudiu.supuran@unifi.it

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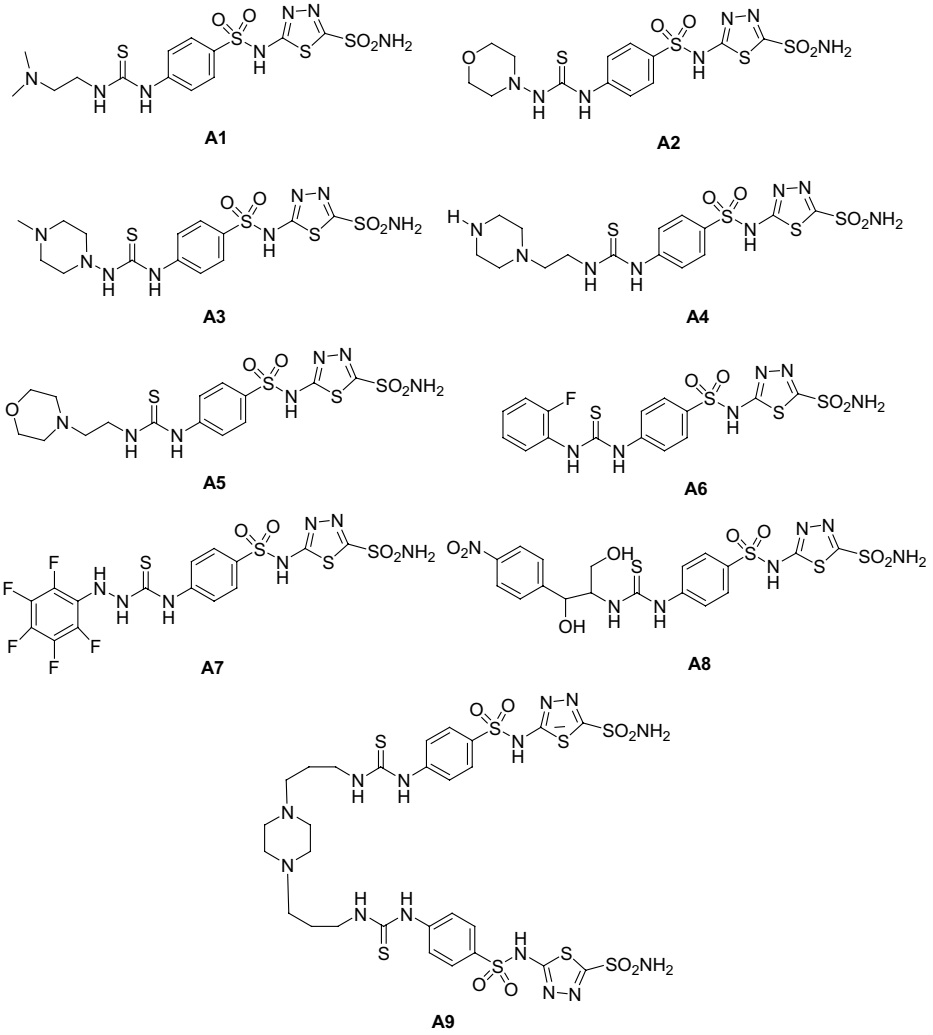
and led to the development of widely used diuretic drugs such as the benzothiadiazine and high ceiling diuretics,[1–5] but more recently, also to other types of pharmacological agents, as it will be shown here shortly. Thus, a recent and new field in CAI research has been opened by the report of the potent antitumor properties of a rather large number of sulfonamide CAIs, as well as by the isolation of some CA isozymes predominantly present in tumor cells, such as CA IX and CA XII.[6–10] The mechanisms by which such compounds inhibit tumor cell growth only now begin to be understood, and important advances in this direction have recently been achieved, since several laboratories are involved in the synthesis, evaluation and in vitro/in vivo antitumor testing of novel types of CAIs with potential application as anticancer therapeutic agents.[6–10] Indeed, a compound of this type—indisulam **IND**—has progressed to Phase II clinical trials for the treatment of solid tumors.[11,12]

In previous work from this laboratory,[13] we have developed thioureido-containing sulfonamide CAIs derived from simple aromatic sulfonamides, such as sulfanilamide and homosulfanilamide, which showed excellent CA I, II, and IV inhibitory properties and were effective topically acting antiglaucoma agents in an animal model of this disease (the above-mentioned isozymes are the main ones involved in aqueous humor secretion within the eye and are the targets of antiglaucoma sulfonamides).[1–4] Continuing this work and the same type of chemistry,[13] but using as lead molecule benzolamide **BZA**, an orphan drug belonging to the family of CAIs,[14] we report here novel thioureido-containing benzolamide-like compounds, which were designed mainly as inhibitors of the transmembrane, tumor-associated isozyme CA IX. These compounds were also tested for their inhibitory properties against the major cytosolic isoforms CA I and II.

2. Chemistry

Benzolamide **BZA** has already been used by our group as lead compound for developing potent topically acting antiglaucoma CAIs.[15] Indeed, a 4-carboxybenzolamide (**CBA**) derivative in which the carboxylic acid moiety has been converted to the diethylaminoethyl-carboxamide was shown not only to act as an excellent antiglaucoma compound in an animal model of the disease (due to its low nanomolar affinity for isozyme hCA II—K_I of 1.4 nM)[15] but also to bind within the active site of hCA II



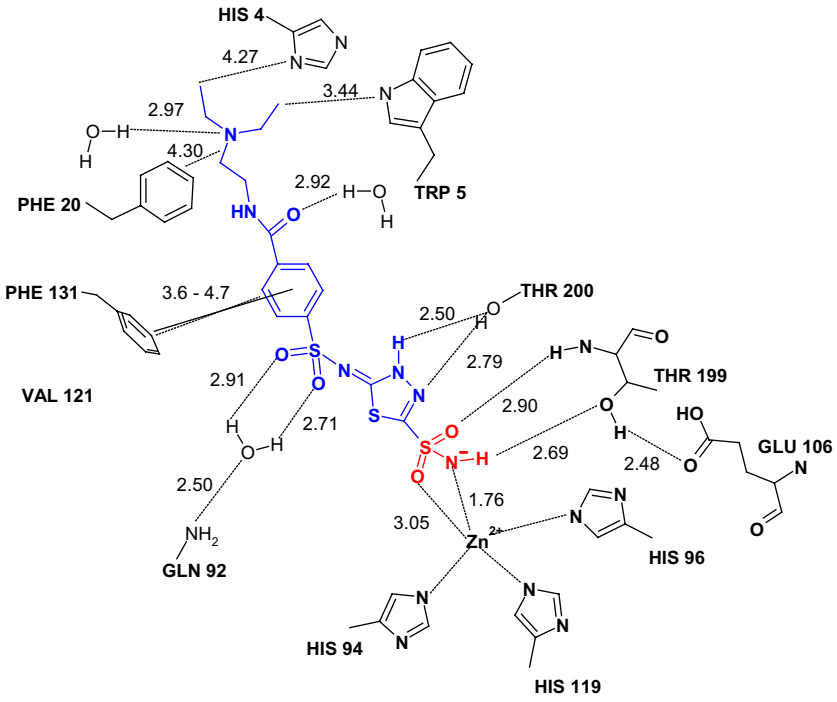


Figure 1. Detailed schematic representation for the binding within the hCA II active site of a 4-carboxybenzolamide (**CBA**)-derived inhibitor—the 2-*N*,*N*-diethylaminoethylamide of 5-(4-carboxybenzenesulfonamido-1,3,4-thiadiazole-2-sulfonamide (figures represent distances in Å). Reproduced with permission from Elsevier from Ref. 16.

in a completely different manner as compared to other such derivatives for which the X-ray crystal structures in complexes with hCA II have been reported.[16] Thus, it has been observed that the inhibitor bound within the enzyme active site was in the sulfonylimido-4*H*-δ^2-1,3,4-thiadiazoline tautomeric form (Fig. 1), with its deprotonated primary sulfonamide moiety being coordinated to the Zn(II) ion of the enzyme, also participating to the classical hydrogen bond network involving amino acid residues Thr 199 and Glu 106.[16] The 1,3,4-thiadiazoline fragment of the inhibitor was shown to make two hydrogen bonds with the active site residue Thr 200, the secondary sulfonamide moiety participated in two hydrogen bonds involving a water molecule and the residue Gln 92, whereas the phenyl ring of the inhibitor participated to an edge-to-face interaction with the phenyl ring of Phe 131, the two cycles being almost perfectly perpendicular to each other. The tertiary amine fragment of the carboxamido tail and the carboxamido moiety itself made hydrogen bonds with water molecules present at the rim of the active site entrance and van der Waals contacts with His 4, Trp 5, and Phe 20 (Fig. 1).[16] All these multiple interactions never evidenced previously in CA–sulfonamide complexes, explained the very high affinity of this inhibitor for the hCA II active site and prompted us to use such benzolamide-like derivatives for the drug design of the compounds reported here.

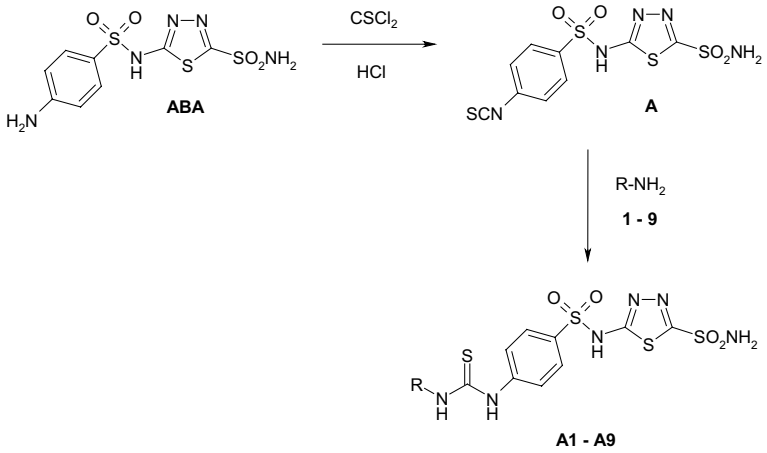
The new compounds reported here, of type **A1**–**A9** have been prepared from aminobenzolamide **ABA**,[17] as shown in Scheme 1.

Reaction of aminobenzolamide **ABA**[17] with thiophosgene in the presence of hydrochloric acid afforded the key intermediate 4-isothiocyanato-benzolamide **A**, by the procedure previously described for the preparation of the isothiocyanato derivatives of sulfanilamide or homosulfanilamide.[13] This compound was then reacted with different aliphatic, heterocyclic, or aromatic amines/hydrazines/diamines **1**–**9** (commercially available derivatives, chosen in such a way as to possess heteroatoms able to participate in hydrogen bonds or other interactions with amino acid residues situated at the rim of the active site entrance of CAs, as those shown in Fig. 1), affording thioureas **A1**–**A9**.[13,18]

3. Carbonic anhydrase inhibition

Inhibition data against three physiologically relevant isozymes, that is, the cytosolic isozymes hCA I and II and the membrane-bound, tumor-associated isozyme hCA IX (of human origin all of them) with the new compounds **A1**–**A9** as well as the standard, clinically used CAIs acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND** are shown in Table 1.[19]

The following SAR should be noted from data of Table 1: (i) sulfonamides **A1**–**A9** behave as effective inhibitors of all the investigated isozymes, typically showing low nanomolar affinity for all of them, similarly with the lead molecules benzolamide **BZA** and aminobenzolamide **ABA** from which they were obtained. This may be explained taking into account the X-ray crystal data of the adduct of a structurally related such sulfonamide in complex with hCA II, mentioned above (Fig. 1), which evidenced a large number of favorable interactions



Scheme 1.

Table 1. Inhibition data for sulfonamides **A1**–**A9** investigated in the present paper and standard sulfonamide CAIs, against isozymes hCA I, II, and IX

Inhibitor	K_I* (nM)			Selectivity ratio
	hCA I[a]	hCA II[a]	hCA IX[b]	K_I (hCA II)/K_I (hCA IX)
AZA	900	12	25	0.48
MZA	780	14	27	0.52
EZA	25	8	34	0.23
DCP	1200	38	50	0.76
IND	31	15	24	0.62
BZA	15	9	42	0.21
ABA	6	2.0	38	0.05
A	15	1.7	23	0.07
A1	0.6	0.9	13	0.06
A2	58	0.9	10	0.09
A3	0.6	0.7	3.2	0.21
A4	60	0.9	3.0	0.30
A5	24	0.8	19	0.04
A6	0.7	0.5	18	0.02
A7	0.7	0.6	20	0.03
A8	0.6	0.6	18	0.03
A9	62	0.9	4.6	0.19

* Errors in the range of 5–10% of the reported value (from three different assays).

[a] Human (cloned) isozymes, by the CO_2 hydration method.

[b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.[26]

between the inhibitor and key amino acid residues present within the active site of the enzyme; (ii) against hCA I, the sulfonamides **A1**–**A9** reported here showed inhibition constants in the range of 0.6–62 nM. Thus, four such derivatives, that is, **A2**, **A4**, **A5**, and **A9** were medium potency inhibitors, with K_I values in the range of 24–62 nM. These compounds incorporate moieties rather similar to that of other derivatives described here, which showed K_I values in the range of 0.6–0.7 nM. For example, **A2** and **A3** differ only by the heteroatom present in the six-membered ring (with an N-Me group in **A3** substituting the oxygen atom of **A2**) and the difference of activity between these two compounds is almost 10-fold. It is rather difficult to explain these results without X-ray crystal structures, which only prove that a very small modification in the molecule of a CAI leads to dramatic changes in the biological activity. The most active compounds against this isozyme, that is, **A1**, **A3**, and **A6**–**A8** were generally 10 times more efficient than **ABA**, already a potent hCA I inhibitor (K_I of 6 nM), whereas the ABA–isothiocyanate **A** was a less effective inhibitor (K_I of 15 nM). It should also be mentioned that these compounds are much more effective hCA I inhibitors than the clinically used derivatives **AZA**–**IND** (Table 1), being in fact among the best CA I inhibitors ever reported; (iii) against hCA II, all the new derivatives **A1**–**A9** showed excellent inhibitory properties, with K_I values in the range of 0.5–0.9 nM. It is practically almost impossible to discuss these results, as all these derivatives showed a very potent and homogenous behavior as CA II inhibitors, being almost twice as effective as compared to **ABA** or ABA–isothiocyanate **A** (K_Is of 1.7–2 nM). So potent hCA II inhibitors were so far reported only in the preceding paper in which we designed benzolamide-like derivatives with potent antiglaucoma properties.[15,16] The new compounds **A1**–**A9** are 10–20 times more effective CA II inhibitors as compared to the clinically used drugs (Table 1); (iv) very good inhibitory properties were also shown by compounds **A1**–**A9** against the tumor-associated isozyme hCA IX, with K_I in the range of 3.0–20 nM. Thus, the lead compound **BZA** is not a very effective CA IX inhibitor, similarly to **ABA** (K_I of 38–42 nM). The isothiocyanate **A** is already a better inhibitor (K_I of 23 nM), whereas these properties are further augmented for the thioureas **A1**–**A9**. Among these derivatives, compounds **A5**–**A8** show K_I values in the range of 18–20 nM, being more potent than the clinically used compounds acetazolamide or indisulam (Table 1). Two other derivatives, **A1** and **A2** show a further enhancement of activity (K_I of 10–13 nM), whereas the most potent inhibitors were **A3**, **A4**, and **A9**, with inhibition constants in the range of 3.0–4.6 nM. It is thus clear that all the substitution patterns investigated here may lead to potent hCA IX inhibitors; (v) the compounds investigated here are much better hCA II than hCA IX inhibitors. Indeed, the selectivity ratios shown in Table 1 against the two sulfonamide-avid isozymes, hCA II and IX, are $\ll 1$,

proving that these compounds cannot be considered as CA IX-selective inhibitors.

4. Conclusions

A small library of sulfonamides has been obtained using benzolamide as lead compound. Aminobenzolamide was treated with thiophosgene followed by reaction of the thiocyanato intermediate with aliphatic/aromatic amines, or hydrazines, leading to thioureas incorporating aliphatic, aromatic, or heterocyclic moieties. The new derivatives have been investigated as inhibitors of the cytosolic isozymes hCA I and II, as well as the tumor-associated isozyme hCA IX. The new compounds showed excellent inhibitory properties against all three isozymes with inhibition constants in the range of 0.6–62 nM against hCA I, 0.5–1.7 nM against hCA II and 3.2–23 nM against hCA IX, respectively. These derivatives are interesting candidates for the development of novel therapies targeting hypoxic tumors.

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18. Reaction of the isothiocyanate **A** with amines **1**–**9** has been done in water or *N*,*N*-diethylacetamide as solvents, as previously described.[13]
5-(4-[3-(2-Dimethylamino-ethyl)-thioureido]-benzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide **A1**: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.1 (s, 1H), 8.1 (s, 1H), 7.8 (s, 2H), 7.7 (d, 2H, J = 8.6 Hz), 7.5 (d, 2H, J = 8.6 Hz), 3.8 (m, 2H), 3.2 (m, 2H), 2.8 (s, 6H), 2.2 (s, 1H); MS ESI$^+$ *m*/*z* 466 $(M+H)^+$. ESI$^-$ *m*/*z* 464 $(M-H)^-$.
5-[4-(3-Morpholin-4-yl-thioureido)-benzenesulfonylamino]-1,3,4-thiadiazole-2-sulfonamide **A2**: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10 (s, 1H), 9.6 (s, 1H), 8.4 (s, 2H), 7.9 (d, 2H, J = 8.6 Hz), 7.8 (d, 2H, J = 8.6 Hz), 3.7 (m, 5H), 2.8 (m, 4H), 2.8 (s, 6H), 2.2 (s, 1H); MS ESI$^+$ *m*/*z* 480 $(M+H)^+$, 502 $(M+Na)^+$. ESI$^-$ *m*/*z* 478 $(M-H)^-$.
5-(4-[3-(4-Methyl-piperazin-1-yl)-thioureido]-benzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide **A3**: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.1 (s, 1H), 9.6 (s, 1H), 7.8 (s, 1H), 7.7 (m, 5H), 3.2–3 (m, 8H), 2.8 (s, 3H). MS ESI$^-$ *m*/*z* 491 $(M-H)^-$.
5-(4-[3-(2-Morpholin-4-yl-ethyl)-thioureido]-benzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide **A5**: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.1 (s, 1H), 8.1 (s, 1H), 7.9

(s, 2H), 7.7 (d, 2H, $J = 7.8$ Hz), 7.5 (d, 2H, $J = 7.8$ Hz), 3.9 (m, 4H), 3.8 (m, 2H), 3.2 (m, 6H), 2.2 (s, 1H); MS ESI$^+$ *m/z* 508 $(M+H)^+$, 530 $(M+Na)^+$. ESI$^-$ *m/z* 506 $(M-H)^-$.

5-(4-[3-(2-Fluorophenyl)-thioureido]-benzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide **A6**: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.3 (s, 1H), 9.8 (s, 1H), 8.5 (s, 2H), 7.8 (m, 4H), 7.6 (t, 1H, $J = 7.7$ Hz), 7.3–7.2 (m, 3H); MS ESI$^+$ *m/z* 489 $(M+H)^+$, 511 $(M+Na)^+$. ESI$^-$ *m/z* 487 $(M-H)^-$.

5-[4-(3-pentafluoroanilyl)-thioureido)-benzenesulfonylamino]-1,3,4-thiadiazole-2-sulfonamide **A7**: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.4 (s, 1H), 10 (s, 1H), 8.5 (s, 1H), 8.45 (s, 2H), 7.85 (d, 2H, $J = 8.4$ Hz), 7.8 (d, 2H, $J = 8.4$ Hz). MS ESI$^+$ *m/z* 576 $(M+H)^+$, 598 $(M+Na)^+$. ESI$^-$ *m/z* 574$(M-H)^-$.

19. Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group.[20] Cell growth conditions were those described in Ref. 21 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[22] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1}cm^{-1}$ for CA I and 54 $mM^{-1}cm^{-1}$ for CA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.3 kDa for CA II, respectively.[23,24] A variant of the previously published[9,10] CA IX purification protocol has been used for obtaining high amounts of hCA IX needed in these experiments. The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[25]) was amplified by using PCR and specific primers for the glutathione S-transferase (GST)-Gene Fusion Vector pGEX-3X. The obtained fusion construct was inserted in the pGEX-3X vector and then expressed in *Escherichia coli* BL21 Codon Plus bacterial strain (from Stratagene). The bacterial cells were sonicated, then suspended in the lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8, 150 mM NaCl, and 0.2% Triton X-100). After incubation with lysozime (approx. 0.01 g/L) the protease inhibitors Complete™ were added to a final concentration of 0.2 mM. The obtained supernatant was then applied to a prepacked Glutathione Sepharose 4B column, extensively washed with buffer and the fusion (GST-CA IX) protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin. The advantage of this method over the previous one,[9,10] is that CA IX is not precipitated in inclusion bodies from which it has to be isolated by denaturing–renaturing in the presence of high concentrations of urea, when the yields in active protein were rather low, and the procedure much longer. The obtained CA IX was further purified by sulfonamide affinity chromatography,[22] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[26] The specific activity of the obtained enzyme was the same as the one previously reported,[9,10] but the yields in active protein were 5–6 times higher per liter of culture medium). An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[26] 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. Saturated CO_2 solutions in water at 20 °C were used as substrate.[26] 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 0.01 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. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The activity of these enzyme preparations seem to be rather similar with the in vivo activity of CA IX in tumors as recently proved by CA inhibitor binding studies and disturbance of tumor pH following CA IX inhibition as compared to normal tissue pH (Švastová, E.; Hulíková, A.; Rafajová, M.; Zat'ovičová, M.; Gibadulinová, A.; Casini, A.; Cecchi, A.; Scozzafava, A.; Supuran, C. T.; Pastorek, J.; Pastoreková, S. *FEBS Lett.*, in press).
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