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Carbonic anhydrase inhibitors: Inhibition of the tumor-associated isozymes IX and XII with a library of aromatic and heteroaromatic sulfonamides

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Abstract—The inhibition of the two transmembrane, tumor-associated isozymes of carbonic anhydrase (CA, EC 4.2.1.1) of human origin, hCA IX and XII, with a library of aromatic and heteroaromatic sulfonamides has been investigated. Most of them were sulfanilamide, homosulfanilamide, and 4-aminoethyl-benzenesulfonamide derivatives, to which tails that should induce diverse physico-chemical properties have been attached at the amino moiety, whereas several of these compounds were derived from metanilamide, benzene-1,3-disulfonamide or the 1,3,4-thiadiazole/thiadiazoline-2-sulfonamides. The tails were of the alkyl/aryl-carboxamido/sulfonamido-, ureido or thioureido type. Against hCA IX the investigated compounds showed inhibition constants in the range of 3–294 nM, whereas against hCA XII in the range of 1.9–348 nM, respectively. The best hCA IX inhibitors were ureas/thioureas incorporating 4-aminoethyl-benzenesulfonamide and metanilamide moieties. The best hCA XII inhibitors were 1,3,4-thiadiazole/thiadiazoline-2-sulfonamides incorporating 5-acylamido or 5-arylsulfonylamido moieties. These compounds also inhibited appreciably the cytosolic isozymes hCA I and II, but some selectivity for the transmembrane, tumor-associated isozymes was observed for some of them, which is an encouraging result for the design of novel therapies targeting hypoxic tumors, in which these carbonic anhydrases are highly overexpressed.

The carbonic anhydrases (CAs, EC 4.2.1.1)[1–4] constitute interesting targets for the design of pharmacological agents useful in the treatment or prevention of a variety of disorders such as glaucoma, acid–base disequilibria, epilepsy and other neuromuscular diseases, altitude sickness, edema, and obesity.[5,6] A quite new and unexpected application of the CA inhibitors (CAIs) regards their potential use in the management (imaging and treatment) of hypoxic tumors,[7–14] since at least two CA isozymes of the 15 presently known in humans, that is, CA IX and XII, are predominantly found in tumor cells and lack (or are present in very limited amount) in normal tissues.[15–18]

These enzymes catalyze the simplest physiological reaction, CO_2 hydration to bicarbonate and a proton.[1–6] Their involvement in many physiological/pathological processes in which CO_2, H^+ ions, and bicarbonate anions participate, as well as the fact that generally different isozymes of the 15 mentioned above are involved in particular such processes, allows the development of diverse medicinal chemistry applications of their inhibitors.[1,2] Thus, as mentioned above, the human isozymes hCA IX and hCA XII are the targets for the development of novel antitumor therapies,[5,7–10] hCA II and XII for the development of antiglaucoma drugs,[19–22] hCA Va and hCA Vb for the design of new antiobesity agents,[6,23,24] and hCA VII for the development of anticonvulsant/antiepileptic drugs,[25] whereas non-vertebrate CAs, such as the α-CA present in *Plasmodium falciparum* (pfCA), may lead to novel types of antimalaria drugs.[26] The enzyme from the ulcer-producing bacteria *Helicobacter pylori* was, on the other hand, recently shown to be involved in the acclimation of the pathogen in the highly acidic medium within the stomach,[27] being

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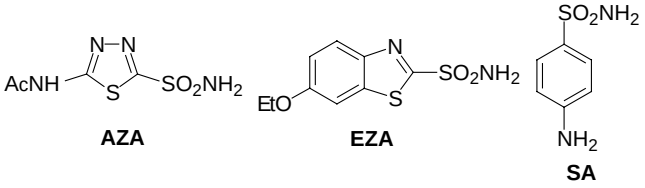
essential for the growth of this widespread pathogen, leading thus to the possibility to develop novel types of antiulcer therapies.

Hypoxia constitutes a challenging clinical problem, being common in many cancer types which are inaccessible to radio- and chemotherapy.[7,15,18] Acidic extracellular pH is also a typical attribute of the hypoxic tumor microenvironment, with a strong impact on cancer progression and treatment outcome.[7,15,18] CA IX and to a smaller extent also CA XII are highly overexpressed in hypoxic tumors.[7,15,18] We have recently shown that both in transfected and cells in tumor cells (HeLa and SiHa) endogenously expressing these enzymes, inhibition of CA IX by potent sulfonamide inhibitors leads to a significant increase of the tumor microenvironment pH of 0.5–1.0 unit. Correlated with the fact that many such sulfonamides were shown earlier to possess strong in vitro and in vivo anticancer activity,[28–31] these findings constitute the proof-of-concept that such enzyme inhibitors may lead to the development of novel antitumor therapies.

In previous reports from this laboratory, we have investigated various classes of sulfonamides[11,12,16] or sulfamates[13,14] mainly as CA IX inhibitors, whereas very recently we reported the first CA XII inhibition study with a series of simple aromatic and heterocyclic compounds.[22] Here we report an inhibition study of both these cancer-associated isozymes with a library of sulfonamides synthesized earlier during our work for the design of topically acting antiglaucoma agents.[24,32–37] Since some tumors overexpress both CA IX and CA XII, it may be important to detect compounds with strong inhibitory activities against both of them to possess candidates for in vivo studies and also derivatives showing appropriate physico-chemical properties (water solubility, balanced hydro-/liposolubility, etc.) for a potential drug.

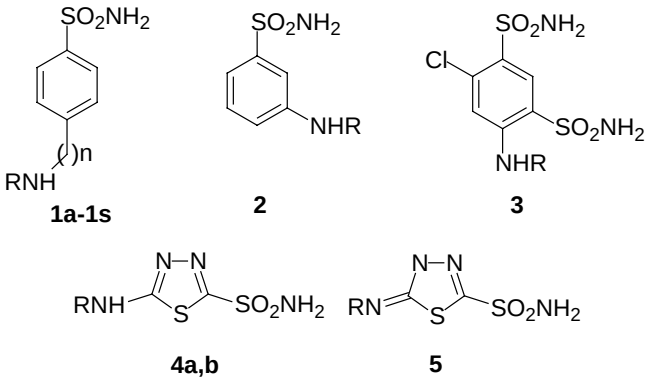
Acetazolamide (AZA), ethoxzolamide (EZA), and sulfanilamide (SA) were from Sigma–Aldrich (Milan, Italy). Compounds **1**–**5** used in the assay were previously reported by one of our groups.[24,32–37] Most of them are sulfanilamide, homosulfanilamide, and 4-aminoethyl-benzenesulfonamide derivatives (compounds **1a**–**1s**), to which tails that should induce diverse physico-chemical properties have been attached at the amino moiety. However, several of these compounds are derived from metanilamide (**2**), benzene-1,3-disulfonamide (**3**) or the thiadiazole/thiadiazoline-2-sulfonamides (**4a**, **4b**, and **5**). They were chosen in such a way as to include a rather wide range of different classes of compounds to which acyl-, alkyl-/arylsulfonyl-, ureido, and thioureido tails have been attached to modulate some of their physico-chemical properties important for a potential drug, such as hydro- and liposolubility among others.



Inhibition data of four physiologically relevant CA isozymes, that is, hCA I, II, IX, and XII with sulfonamides **1**–**5** and standard, clinically used inhibitors are shown in Table 1.[38] The inhibition data of the human red blood cell isozymes hCA I and II are also included in Table 1, for comparative reasons, although these data were reported earlier.[24,32–37,39]

The following should be noted regarding inhibition of hCA IX and XII with this series of sulfonamides: (i) Against hCA IX, a group of sulfonamides among the derivatives investigated here, including **1a**–**1c**, **1e**, **1g**, **1i**, **3**, and SA showed moderate inhibitory activity, with inhibition constants in the range of 106–294 nM. It may be observed that SA is the weakest such inhibitor and that its derivatives of type **1** already showed an enhanced efficiency as inhibitors of this isozyme. The only 1,3-benzene-bissulfonamide derivative investigated here, **3**, also showed moderate activity. For the sulfanilamide derivatives **1** mentioned above, it may be seen that activity increases with the length of the acyl moiety of the RCONH tail, but branched chains such as *iso*-propyl- or *tert*-butyl generally lead to a decreased activity as compared to the corresponding unbranched derivatives (incorporating, for example, *n*-Pr or *n*-Bu chains) (Table 1). Another group of derivatives, including **1d**, **1f**, **1h**, **1j**, and EZA, were more effective hCA IX inhibitors as compared to those discussed above, with K_I values in the range of 50–83 nM. It may be observed that these derivatives incorporate again the acylamido substitution in position 4 of the sulfanilamide lead molecule, but with longer acyl chains as the previously mentioned derivatives or with the benzoylamido- (**1h**) or benzenesulfonamido- (**1j**) substitutions. It should also be noted that the RSO_2NH– type of derivative is slightly more efficient than the RCONH one bearing the same R, in both the aliphatic and aromatic series (compare **1a** and **1i**, or **1h** and **1j**, respectively). A last subgroup of derivatives, such as **1k**–**1s**, **2**, **4**, and **5**, showed very good hCA IX inhibitory properties, with K_I values in the range of 3–40 nM, of the same order of magnitude as the clinically used derivatives among which AZA is shown in Table 1. Except for the sulfanilamide derivative **1k**, bearing the substituted phenylsulfonamide moiety that was shown above to lead to an increased activity, the other compounds in this group include either homosulfanilamide/4-aminoethylbenzenesulfonamide derivatives (**1m**–**1s**), metanilamide (**2**) or heteroaromatic derivatives (**4** and **5**), as well as aryl-thioureido, arylureido, aryl-carboxamido/sulfonamido tails. Indeed, it has previously been discovered that this type of tails induces strong potency against isozymes I and II,[24,28–33] as can be seen also from data of Table 1. Thus, against hCA IX best activity is observed for homosulfanilamide, 4-aminoethylbenzenesulfonamide or thiadiazole/thiadiazoline-2-sulfonamide derivatives bearing this type of tails, that is, aryl-thioureido, arylureido, aryl-carboxamido/sulfonamido. (ii) Against hCA XII very few inhibition studies are available up to now.[22] As seen from data of Table 1, a first group of derivatives, including **1e**–**1g** and **3**, show moderate hCA XII inhibitory properties, with K_I values in the range of 147–348 nM. These compounds incorporate sulfanilamide and benzene-1,3-disulfonamide heads

Table 1. hCA I, II, IX, and XII inhibition data with sulfonamides **1**–**5**, acetazolamide (AZA), ethoxzolamide (EZA), and sulfanilamide (SA)



Compound	*n*	R	K_I (nM)[c]			
			hCA I[a]	hCA II[a]	hCA IX[b]	hCA XII[b]
1a	0	CH_3CO	21,400	246	135	49
1b	0	CF_3CO	14,600	133	112	31
1c	0	EtCO	19,700	232	106	56
1d	0	*n*-PrCO	19,300	227	83	85
1e	0	*i*-PrCO	23,500	258	139	138
1f	0	*n*-BuCO	17,650	214	79	147
1g	0	*t*-BuCO	20,600	230	136	249
1h	0	PhCO	3300	37	73	21
1i	0	$MeSO_2$	125	64	113	33
1j	0	$PhSO_2$	103	49	52	68
1k	0	4-$AcNHC_6H_4$	245	82	37	76
1m	1	$PhSO_2$	81	40	40	83
1n	1	PhNH–C(=S)	266	12	26	18
1p	2	PhNH–C(=S)	50	53	21	24
1q	2	PhNH–C(=O)	430	75	18	13
1r	2	4-$H_2NO_2SC_6H_4NH$–C(=S)	37	4	3	79
1s	2	4-$H_2NO_2SC_6H_4CO$	40	5	12	13
2	—	PhNH–C(=O)	1500	150	14	10
3	—	PhNH–C(=O)	900	100	146	348
4a	—	4-$BrC_6H_4SO_2$	3	2	21	3.3
4b	—	4-$O_2NC_6H_4SO$	3	1	16	1.9
5	—	Furan-2-yl-CO	290	6	13	2.4
AZA	—	—	250	12	25	5.7
EZA	—	—	25	8	50	22
SA	—	—	28,000	300	294	37

Data of hCA I and II are from Refs. 24,32–37.
[a] Human, recombinant enzyme.
[b] Catalytic domain of the human cloned isozyme.
[c] Mean from three assays (errors in the range of 5–10% of the reported value).

and the rather bulky *iso*-propyl-, *n*-*tert*-butyl tails (for derivatives **1**). Another subgroup of the investigated compounds, such as **1c**, **1d**, **1j**–**1m**, and **1r**, showed an enhanced hCA XII inhibition, with K_I values in the range of 56–85 nM. All these compounds are benzenesulfonamide derivatives incorporating acylamido/arylsulfonamido/thioureido trails in the sulfanilamide homosulfanilamide or 4-aminoethyl-benzenesulfonamide series. A last subgroup of derivatives, among which **1a**, **1b**, **1h**, **1i**, **1n**–**1q**, **1s**, **2**, **4**, **5**, and the clinically used compounds AZA, EZA, and SA, showed much more effective hCA XII inhibitory activity, with K_I values in the range of 1.9–49 nM. Several important SAR observations should be made: unlike for other investigated CA isozymes (such as hCA I, II, and IX among others), derivatization of sulfanilamide at the N-4 amino group does not always lead to an enhanced hCA XII inhibition. In fact, most of the substituted sulfanilamides investigated here (except for **1b**, **1h**, and **1i**) were less effective hCA XII inhibitors as compared to SA. On the other hand, good hCA XII inhibitory properties were observed for the ureido/thioureido derivatives of metanilamide, homosulfanilamide, and 4-aminoethyl-benzenesulfonamide as well as for the heteroaromatic derivatives **4** and **5**, which were more effective inhibitors than acetazolamide AZA, the lead compound, which is already a very potent inhibitor. It should also be noted that ethoxzolamide EZA is a much less effective inhibitor as compared to acetazolamide (the same situation as for hCA IX; see Table 1), which seems to be thus a general feature of the transmembrane, tumor-associated isozymes. These isozymes seem to be less inhibited by

bicyclic, ethoxzolamide-like compounds, which, on the other hand, are the best hCA I and II inhibitors. (iii) The compounds investigated here inhibit indiscriminately all CA isozymes, both the cytosolic ones (hCA I and II) and the transmembrane ones (hCA IX and XII), but important differences of activity between the various classes are observed, as already mentioned above for ethoxzolamide. Thus, the best selectivity for the tumor-associated over the cytosolic isozymes is observed for the metanilamide derivative 2 which is a very potent hCA IX/XII inhibitor, being at the same type a medium potency inhibitor of hCA II and a very weak hCA I inhibitor (K_I values in the range of 150–1500 nM). Such compounds may thus lead to isozyme-specific or more selective inhibitors with potential applications in the development of novel antitumor therapies.

The inhibition of the two transmembrane, tumor-associated isozymes hCA IX and XII with a library of aromatic and heteroaromatic sulfonamides has been investigated. Most of them were sulfanilamide, homosulfanilamide, and 4-aminoethyl-benzenesulfonamide derivatives, to which tails that should induce diverse physico-chemical properties have been attached at the amino moiety, whereas several of these compounds were derived from metanilamide, benzene-1,3-disulfonamide or the 1,3,4-thiadiazole/thiadiazoline-2-sulfonamides. The tails were of the alkyl/aryl-carboxamido/sulfonamido-, ureido or thioureido type. Against hCA IX the investigated compounds showed inhibition constants in the range of 3–294 nM, whereas against hCA XII in the range of 1.9–348 nM. The best hCA IX inhibitors were ureas/thioureas incorporating 4-aminoethyl-benzenesulfonamide and metanilamide moieties. The best hCA XII inhibitors were 1,3,4-thiadiazole/thiadiazoline-2-sulfonamides incorporating 5-acylamido or 5-arylsulfonylamido moieties. These compounds also inhibited appreciably the cytosolic isozymes hCA I and II, but some selectivity for the transmembrane, tumor-associated isozymes was observed for some of them, which is an encouraging result for the design of novel therapies targeting hypoxic tumors, in which these carbonic anhydrases are highly overexpressed.

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38. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for measuring the initial velocities for the CO_2 hydration reaction catalyzed by different CA isozymes, by following the change in absorbance of a pH indicator. 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. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the 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 inhibitors were prepared at a concentration of 1–3 mM (in DMSO/water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier,[16,26] and represent the mean from at least three different determinations.
39. A variant of the previously published[12] CA IX purification protocol has been used for obtaining high amounts of hCA IX needed in these experiments. The catalytic domain of hCA IX cloned into pGEX-4T-1 vector (details described previously in Svastova et al.[7]) was expressed in *Escherichia coli* BL21 Codon Plus bacterial strain (Stratagene). The bacterial cells were resuspended in the lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8, 150 mM NaCl, and 0.2% Triton X-100) and incubated for 20 min on ice with lysozyme (Sigma) in a final concentration of 1 mg/ml. To suspension was added COMPLETE cocktail of protease inhibitors (Roche) and bacterial cells were sonicated (5×30 s). The obtained lysate was centrifuged for 30 min at 10,000 rpm, at +4 °C and the supernatant was then applied to a prepacked glutathione Sepharose 4B column (Amersham), extensively washed with the lysis buffer followed by phosphate-buffered saline, pH 7.4, and the fusion 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 (Sigma). The advantage of this method over the previous one[12] 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,[19] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[38] The specific activity of the obtained enzyme was the same as the one previously reported,[12] but the yields in active protein were 5–6 times higher per liter of culture medium. The GST-hCA XII construct previously reported[22] was transfected into *E. coli* strain BL21 for production of the CA XII protein, similar to the procedure already described for hCA IX above.[12] The protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside, the cells were harvested when the OD_{600} reached a value of 1.00 and lysed by sonication in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized twice with a Polytron (Brinkmann) twice for 30 s each at 4 °C. Centrifugation at 30,000*g* for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatant was then applied to a prepacked glutathione Sepharose 4B column, extensively washed with buffer, and the fusion (GST-CA XII) 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 is that CA XII is purified quite easily and the procedure is quite simple. The obtained CA XII was further purified by sulfonamide affinity chromatography, the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[38]