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Carbonic anhydrase inhibitors: synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, and IX with sulfonamides incorporating 1,2,4-triazine moieties

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Abstract—A series of benzenesulfonamide derivatives incorporating triazine moieties in their molecules was obtained by reaction of cyanuric chloride with sulfanilamide, homosulfanilamide, or 4-aminoethylbenzenesulfonamide. The dichlorotriazinyl-benzenesulfonamides intermediates were subsequently derivatized by reaction with various nucleophiles, such as water, methylamine, or aliphatic alcohols (methanol and ethanol). The library of sulfonamides incorporating triazinyl moieties was tested for the inhibition of three physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes, the cytosolic hCA I and II, and the transmembrane, tumor-associated hCA IX. The new compounds reported here inhibited hCA I with K_Is in the range of 75–136 nM, hCA II with K_Is in the range of 13–278 nM, and hCA IX with K_Is in the range of 0.12–549 nM. The first hCA IX-selective inhibitors were thus detected, as the chlorotriazinyl-sulfanilamide and the bis-ethoxytriazinyl derivatives of sulfanilamide/homosulfanilamide showed selectivity ratios for CA IX over CA II inhibition in the range of 166–706. Furthermore, some of these compounds have subnanomolar affinity for hCA IX, with K_Is in the range 0.12–0.34 nM. These derivatives are interesting candidates for the development of novel unconventional anticancer strategies targeting the hypoxic areas of tumors. Clear renal cell carcinoma, which is the most lethal urologic malignancy and is both characterized by very high CA IX expression and chemotherapy unresponsiveness, could be the leading candidate of such novel therapies.

1. Introduction

It has only recently been discovered that invasive growth and metastatic spread of many tumors types are closely associated with hypoxia.[1] Tumor hypoxia is the result of the imbalance between neoplastic cells growth and the recruitment of new blood supply for the maintenance of oxygen and nutrients, termed tumor angiogenesis.[1] Thus, changes in tumor metabolism and microenvironment connected with adaptation of cells to hypoxia are important components of tumor progression.[1,2] Hypoxic conditions elicit cellular responses designed to improve cell oxygenation and survival by means of several mechanisms such as neoangiogenesis, improved glycolysis, and enhanced energy production, as well as upregulation of molecules related to cell survival/apoptosis.[1] Nonterminally differentiated proliferating hypoxic cells are significant for the disease outcome because of their resistance to radiotherapy and possibly other cytotoxic treatments.[1c] The most important molecule regulating the mammalian response to hypoxia is the heterodimeric protein hypoxia-inducible factor 1 (HIF-1), which in turn up-regulates genes involved in adaptation responses to hypoxic conditions.[1] Two such genes encode for the transmembrane carbonic anhydrase (CA, EC 4.2.1.1) isozymes CA IX and CA XII, containing extracellular enzyme active sites. These CAs appear to participate in tumorigenetic processes via their

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ability to catalyze hydration of CO_2 to bicarbonate and protons, regulating in this way the intratumoral pH.[2] In addition, CA IX, possessing a unique N-terminal domain, has a capacity to perturb E-cadherin mediated cell–cell adhesion via interaction with β-catenin and may potentially contribute to tumor invasion.[2] CA IX shows restricted expression in normal tissues but is tightly associated with different types of tumors, mostly due to its strong induction by tumor hypoxia that involves HIF-1 binding to a hypoxia response element in the *CA9* gene promoter.[1,2] CA IX was proposed to serve as a marker of tumor hypoxia and its predictive and prognostic potential has been demonstrated in clinical studies (reviewed in Ref. 2) CA XII is expressed in many normal tissues and overexpressed in some tumors.[2] It is also induced by hypoxia, but the underlying molecular mechanism remains undetermined. Both CA IX and CA XII are negatively regulated by von Hippel Lindau (VHL) tumor suppressor protein and their expression in clear renal cell carcinomas (RCC) is related to inactivating mutation of *VHL* gene.[2] Necrosis and oxidative stress also regulate CA IX expression in RCC.[2c] The high catalytic activity of these two CA isoforms supports their role in acidification of tumor microenvironment, which enhances tumor growth and progression with earlier acquisition of metastatic phenotypes.[2–4] Therefore, modulation of extracellular tumor pH via inhibition of CA activity represents a promising novel approach to anticancer therapy.[2–4] Sulfonamide CA inhibitors were shown to compromise in vitro tumor cells proliferation and invasiveness, such as renal cell carcinoma cell lines and improve the effect of conventional chemotherapy in vivo.[2–5] However, their precise targets are not known in detail at this moment, but it is presumed that these two tumor-associated CA isozymes, that is, CA IX and XII, may represent important molecules for targeting cancer cells, by an unconventional therapeutic approach.[2–5]

In previous work from this laboratory, we showed that CA IX is a druggable target.[6–9] In such papers we have explored the design of potent and preferably selective sulfamate/sulfonamide CA IX inhibitors belonging to various chemical classes.[6–9] It was thus observed among others that unlike for other CA isozymes (such as for example CA I, II, or V among others)[10–13] aromatic sulfonamides are generally better CA IX inhibitors, as compared to the heterocyclic derivatives. Thus, it appeared of interest to explore other chemical scaffolds incorporating aromatic (benzene) sulfonamide derivatives that led to the best CAIs targeting CA IX reported up to now.[13] In this work we consider a new such approach, taking advantage of the facile and versatile chemistry of cyanuric chloride (2,4,6-trichloro-1,3,5-triazine),[14] which was used to generate a library of aromatic sulfonamides possessing various substituents at the triazine moiety.

2. Chemistry

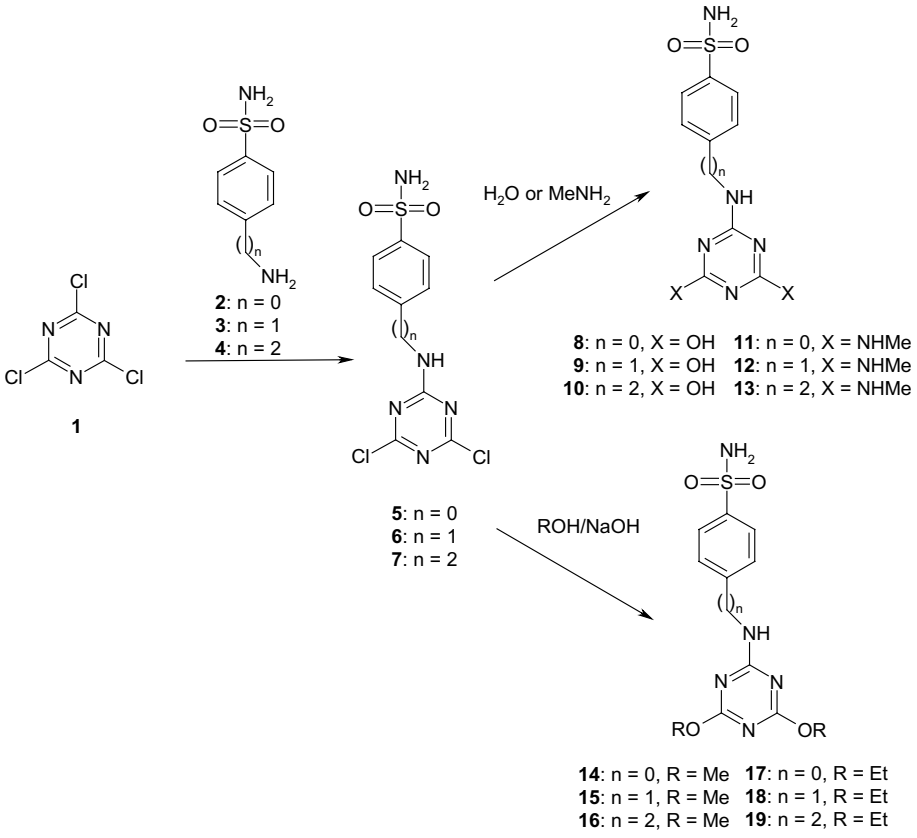
Benzenesulfonamide derivatives show well-known CA inhibitory properties, and a wide range of such compounds have been used in the design of inhibitors with various medicinal chemistry applications.[15] Here we decided to investigate another approach for obtaining such derivatives, taking advantage of the versatile (and facile) chemistry involving cyanuric chloride **1** (2,4,6-trichloro-1,3,5-triazine),[14] and its reactions with various nucleophiles. Indeed, D'Alelio and White[14] reported already in 1959 the reaction of **1** with sulfanilamide, as well as the subsequent substitution of the remaining chlorine atoms with alcohols and amines (ammonia), but the small number of prepared derivatives has never been investigated for their CA inhibitory properties. Here we re-explored these derivatives reported in the previous study,[14] and extend the series of investigated compounds, including some sulfanilamide analogues in our work (Scheme 1).

Reaction of cyanuric chloride **1** with sulfanilamide **2**, homosulfanilamide **3** or 4-aminoethyl-benzenesulfonamide **4**, in a 1:1 molar ratio, afforded the dichlorotriazine-substituted sulfonamides **5**–**7** (of which only **5** has been reported previously).[14] Reaction of these key intermediates **5**–**7** with water afforded derivatives **8**–**10**, whereas with methylamine, they were converted to the bis-dimethylamino-triazino-substituted compounds **11**–**13** (Scheme 1). Alternatively, the chlorine atoms of **5**–**7** may be substituted by alkoxy moieties, by reaction with alcohols in the presence of sodium hydroxide, when derivatives **14**–**19**, incorporating methoxy and ethoxy moieties have been obtained, of which **14** and **17** have been reported earlier[14] (Scheme 1).[16]

3. CA inhibition

Data of Table 1 show CA I, II, and IX inhibition with the compounds reported here of types **5**–**19**, the parent sulfonamides used in the synthesis of types **2**–**4**, as well as clinically used CAIs, such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, and brinzolamide **BRZ**.[17] Indisulam (E7070) **IND**, an antitumor sulfonamide in phase II clinical trials for which we recently demonstrated potent CA inhibitory properties has also been included for comparison in this study.[5d,10,25] Furthermore, the X-ray crystal structure of **IND** in adduct with isozyme hCA II has recently been reported by our group.[10]

The following SAR should be noted from data of Table 1: (i) against the cytosolic, slow isozyme hCA I, the three parent sulfonamides **2**–**4** act as weak inhibitors, with K_Is in the range of 21–28 μM, whereas the new derivatives incorporating triazine moieties, of types **5**–**19** show decisively better inhibitory properties, with K_Is in the range of 75–136 nM. Basically, all these derivatives show a rather similar inhibitory activity against this isozyme, with rather small variations when different substitution patterns at the triazine ring or the length of the spacer between this and the benzenesulfonamide moieties are considered. As a whole, the best hCA I inhibitors were the amines **11**–**13**, the chloroderivative **7**, and the ethoxyderivative **19**, but as mentioned above, differences of



Scheme 1.

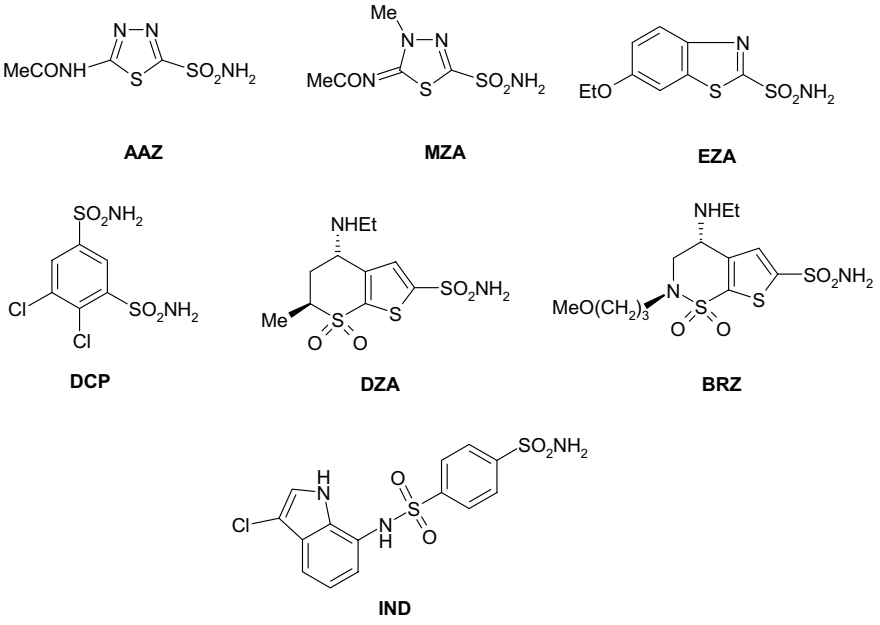
Table 1. Inhibition data for derivatives **2**–**19** investigated in the present paper and standard sulfonamide CAIs, against isozymes I, II, and IX[17]

Compound	K_I* (nM)			Selectivity ratio
	hCA I[a]	hCA II[a]	hCA IX[b]	K_I (hCA II)/K_I (hCA IX)
AAZ	250	12	25	0.48
MZA	50	14	27	0.52
EZA	25	8	34	0.23
DCP	1200	38	50	0.76
DZA	50,000	9	52	0.17
BRZ	nt	3	37	0.08
IND	31	15	24	0.62
2	28,000	300	294	1.02
3	25,000	170	103	1.65
4	21,000	160	33	4.84
5	120	106	0.15	706.67
6	136	13	124	0.10
7	75	21	138	0.15
8	103	278	142	1.95
9	94	130	360	0.36
10	96	98	549	0.17
11	83	23	153	0.15
12	90	15	1.3	11.53
13	88	27	1.1	24.54
14	85	36	150	0.24
15	116	112	155	0.72
16	105	21	162	0.13
17	78	20	0.12	166.67
18	110	84	0.34	247.05
19	82	23	12	1.91

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

[a] Human cloned isozyme, by the CO_2 hydration method.

[b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method; nt = not tested.



activity are unexpectedly small. Thus, these compounds are weaker hCA I inhibitors as compared to clinically used derivatives methazolamide, ethoxzolamide, and indisulam, but more effective as compared to acetazolamide, dichlorophenamide, or dorzolamide (Table 1); (ii) against isozyme hCA II, one of the physiologically most relevant CAs, the parent sulfonamides **2**–**4** show medium potency activity, with K_Is in the range of 160–300 nM, whereas the new derivatives **5**–**19** show a better inhibitory power, with K_Is in the range of 13–278 nM. It may be observed that the increase of inhibitory power was not very significant in some cases, comparing the parent sulfonamides and the triazinyl-substituted derivatives, such as, for example, in the case of the hydroxy-derivatives **8**–**10**, which show only slightly better inhibitory activity as compared to the corresponding parent sulfonamides. The corresponding chloroderivatives **5**–**7** already show a better inhibitory power than derivatives **8**–**10**, whereas for the methylamino-, methoxy-, and ethoxy-substituted derivatives **11**–**19**, a clear-cut SAR is somehow more difficult to draw. All these derivatives are better hCA II inhibitors as compared to the parent sulfonamides **2**–**4** or the hydroxy-triazines **8**–**10** discussed above. Still, for the alkoxy-derivatives, the homosulfanilamides **15** and **18** were much less active as compared to the corresponding sulfanilamides (**14** and **17**) or 4-aminoethylbenzenesulfonamides (**16** and **19**). On the contrary, for the amines **11**–**13**, the best inhibitory activity was shown just by the homosulfanilamide **12**. It is quite difficult to rationalize these results, without knowing the X-ray crystal structure of these adducts, but such a situation has not been encountered up to now in other libraries of aromatic sulfonamides derived from these three derivatives (**2**–**4**), containing the same substitution patterns.[15] It may also be observed that the compounds investigated here, of the type **5**–**19** are generally weaker hCA II inhibitors as compared to the clinically used sulfonamides, with several exceptions, such as **6** or **12** among others (Table 1); (iii) against the tumor-associated isozyme hCA IX, the activity of the new sulfonamides **5**–**19** reported here was even more intriguing. Thus, several subnanomolar hCA IX inhibitors were detected, such as the chloro-derivative **5** and the ethoxy-derivatives **17** and **18**, whereas two other compounds, the amines **12** and **13** were also very effective inhibitors, with K_Is in the range of 1.1–1.3 nM. Except for the other ethoxy-derivative, **19**, which is a potent inhibitor (K_I of 12 nM—being more potent than the clinically used sulfonamides, including indisulam), the other triazine-substituted derivatives investigated here showed modest or weak CA IX inhibitory properties, with K_Is in the range of 124–549 nM, similarly with two of the parent sulfonamides, sulfanilamide **2**, and homosulfanilamide **3** (compound **4** is a rather good hCA IX inhibitor). On the other hand, it should be noted the dramatic difference of hCA IX inhibitory power of the amines **11**–**13**, with one such derivative (**11**) being rather inactive, whereas the other two behaving as very potent inhibitors. The differences between the methoxy-derivatives **14**–**16** and the corresponding ethoxy-derivatives **17**–**19** are again very important, and cannot be explained at this moment; (iv) the three CA isozymes investigated here showed a very different behavior toward these inhibitors, with isozyme IX being generally the most prone to be inhibited by some of them, followed by isozyme II and isozyme I; (v) the selectivity of some of these inhibitors against hCA IX over hCA II is quite interesting, and favorable for considering the design of isozyme-specific CA IX inhibitors. Thus, from data of Table 1 it may be observed that the clinically used derivatives are all more inhibitory against hCA II than against hCA IX, having selectivity ratios <1, in the range of 0.08–0.76 (the most hCA II selective inhibitor is brinzolamide, whereas the most hCA IX 'selective' is dichlorophenamide). On the contrary, the three aromatic sulfonamides used for the preparation of the new derivatives reported here, of type **2**–**4**,

are all better hCA IX than hCA II inhibitors, but their selectivity ratios are not very high, being in the range of 1.02–4.84. For the compounds **5**–**19** investigated here, the selectivity ratio for the two isozymes discussed in detail vary over a very large range, that is, between 0.10 and 706.67. This is the highest such variation seen up to now for all the investigated CA IX inhibitors,[6–10] and we consider this as a very significant result in the search of CA IX-specific CA inhibitors. Thus, three of the compounds investigated here, namely **4**, **17**, and **18**, may really be considered as CA IX-selective inhibitors, as they inhibit this isozyme 166–706 times better than hCA II (whereas their selectivity ratios over hCA I are even higher, but hCA I is known to be an isozyme with lower affinity for sulfonamide inhibitors).[15] Other two compounds, the amines **12** and **13**, are also 11.5–24.5 times better hCA IX than hCA II inhibitors, which is also an important result, since selectivity ratios of this amplitude were rarely seen up to now for other investigated CA IX inhibitors.[6–10] The other investigated compounds were on the other hand better hCA II than hCA IX inhibitors, showing selectivity ratios <1 (Table 1). We are unable to explain these very high differences of selectivity of our compounds for hCA IX, an isozyme for which the X-ray crystal structure is not available at this moment.

4. Conclusions

We report here a series of aromatic, benzenesulfonamide derivatives incorporating triazine moieties in their molecules. They were obtained by reaction of cyanuric chloride with sulfanilamide, homosulfanilamide, or 4-aminoethylbenzenesulfonamide. The dichlorotriazinylbenzenesulfonamides obtained in this way were subsequently derivatized by reacting them with various nucleo- philes, such as water, methylamine, or aliphatic alcohols (methanol and ethanol). The library of sulfonamides incorporating triazinyl moieties was tested for the inhibition of three physiologically relevant CA isozymes, the cytosolic hCA I and II, and the transmembrane, tumor-associated hCA IX. The new compounds reported here inhibited hCA I with K_Is in the range of 75–136 nM, hCA II with K_Is in the range of 13–278 nM, and hCA IX with K_Is in the range of 0.12–549 nM. The first hCA IX-selective inhibitors were detected, as the chlorotriazinyl-sulfanilamide as well as the bis-ethoxytriazinyl derivatives of sulfanilamide and homosulfanilamide showed selectivity ratios for CA IX over CA II inhibition in the range of 166–706, having thus a much higher affinity for the tumor-associated isozyme. Furthermore, some of these compounds have subnanomolar affinity for hCA IX, with K_Is in the range 0.12–0.34 nM. These derivatives are interesting candidates for the development of novel therapies targeting hypoxic tumors such as RCC, which is both characterized by very high CAs IX/XII expression and chemotherapy unresponsiveness, with an exceptionally elevated mortality rate (40%). Synthesis of such derivatives represents an example of alternative investigation advocated by the urologist community, which needs effective therapeutic strategies for the management of metastatic RCC.

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16. Synthesis of the key intermediate **5**–**7**:[14] An acetone solution containing 0.1 mol of either sulfanilamide **2**, homosulfanilamide (HCl salt) **3**, or 4-(2-aminoethyl)-benzenesulfonamide **4** (17.2 g, 22.3 g, and 20.0 g, respectively), was dropped into a solution of 0.1 mol (18.5 g) of cyanuric chloride **1** in 100 mL of acetone.[14] The temperature was maintained at 0–5 °C. The mixture was stirred for 0.5 h and then a solution of 0.1 mol (4.0 g) of sodium hydroxide in 60 mL of water was added dropwise. In the case of the reaction with homosulfanilamide hydrochloride, 0.2 mol (8.0 g) of NaOH were used. Stirring was continued for an additional 0.5 h. Ice-water (200 mL) was added to the reaction mixture and the solid was filtered. The product was washed with cold water until free of chloride ions. The product was purified by dissolving in hot acetone and precipitated with ice-water, as described by D'Alelio and White.[14]
Reaction of intermediates **5**–**7** with alcohols or water: 0.005 mol of the appropriate intermediate **5**–**7** was added to a suspension/solution of 0.01 mol of NaOH in the corresponding alcohol (methanol or ethanol) or water. The mixture was refluxed for 4–6 h. The alcoxide derivatives **14**–**19** were isolated by precipitation with water and if necessary, purified by dissolving in hot acetone and re-precipitation with water. Reaction of intermediates **5**–**7** with methylamine: 0.005 mol of the appropriate intermediate **5**–**7** was added to a solution of 0.01 mol of methylamine hydrochloride in water. The mixture was heated to reflux and 0.02 mol of aqueous sodium hydroxide was slowly added to the mixture. Refluxing was continued for 3 h. The insoluble products **11**–**13** were easily isolated by filtration and crystallized from ethanol–water 1:1.
2,4-Dimethylamino-6-(4-sulfamoylanilino)-1,3,5-triazine **11**: mp 232–234 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 9.3 (s, 1H), 8 (d, 2H, J = 8 Hz), 7.7 (d, 2H, J = 8 Hz), 7.2 (s, 2H), 6.8 (m, 2H), 2.75 (m, 6H); MS ESI$^+$ *m*/*z* 310 (M+H)$^+$, 332 (M+Na)$^+$, 619 (2M+H)$^+$, 641 (2M+Na)$^+$. ESI$^-$ *m*/*z* 308 (M−H)$^-$, 617 (2M−H)$^-$.
2,4-Diethoxy-6-(4-sulfamoylanilino)-1,3,5-triazine **17**: mp 208–210 °C (lit.[14] mp 210–211 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 10.3 (s, 1H), 7.9 (d, 2H, J = 7.2 Hz), 7.8 (d, 2H, J = 7.2 Hz), 7.2 (s, 2H), 4.4 (q, 4H, J = 6.8 Hz), 1.35 (t, 6H, J = 5 Hz); MS ESI$^+$ *m*/*z* 362 (M+Na)$^+$, 701 (2M+Na)$^+$. ESI$^-$ *m*/*z* 338 (M−H)$^-$, 677 (2M−H)$^-$.
17. 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.[18] Cell growth conditions were those described in Ref. 19 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[20] 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.[21,22] A variant of the previously published[6,7] 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.[23]) 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 *E. 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^{TM} 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,[6,7] 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,[20] the amount of enzyme being determined by spectrophometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[25] The specific activity of the obtained enzyme was the same as the one previously reported,[6,7] but the yields in active protein were 5–6 times higher per liter of culture medium. AnSX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[25] 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.[24] 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.
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