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Carbonic anhydrase inhibitors: Novel sulfonamides incorporating 1,3,5-triazine moieties as inhibitors of the cytosolic and tumour-associated carbonic anhydrase isozymes I, II and IX

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This paper is dedicated to the memory of Mircea D. Banciu (1942–2005)

Abstract—A new series of aromatic benzenesulfonamides incorporating 1,3,5-triazine moieties in their molecules is reported. This series was obtained by reaction of cyanuric chloride with sulfanilamide, homosulfanilamide or 4-aminoethylbenzenesulfonamide. The prepared dichlorotriazinyl-benzenesulfonamides were subsequently derivatized by reacting them with various nucleophiles, such as ammonia, hydrazine, primary and secondary amines, amino acid derivatives or phenol. 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, tumour-associated hCA IX. The new compounds inhibited hCA I with inhibition constants in the range of 31–8500 nM, hCA II with inhibition constants in the range of 14–765 nM and hCA IX with inhibition constants in the range of 1.0–640 nM. Structure–activity relationship was straightforward and rather simple in this class of CA inhibitors, with the compounds incorporating compact moieties at the triazine ring (such as amino, hydrazino, ethylamino, dimethylamino or amino acyl) being the most active ones, and the derivatives incorporating such bulky moieties (*n*-propyl, *n*-butyl, diethylaminoethyl, piperazinylethyl, pyridoxal amine or phenoxy) being less effective hCA I, II and IX inhibitors. Some of the new derivatives also showed selectivity for inhibition of hCA IX over hCA II (selectivity ratios of 23.33–32.00), thus constituting excellent leads for the development of novel approaches for the management of hypoxic tumours.

1. Introduction

In a previous contribution from this laboratory[1] it was reported that triazinyl-containing sulfonamides obtained from cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) and amino-benzenesulfonamides, also incorporating hydroxy, alkoxy or amino moieties, act as highly effective inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1).[2–5] The designed inhibitors[1] have been tested for the inhibition of three physiologically relevant CA isozymes (of the 15 currently known in higher vertebrates),[2–5] that is, the cytosolic CA I and II (of human origin, hCA I and hCA II) and the tumour-associated transmembrane isozyme hCA IX.[2–5] Indeed, the levels of hCA IX—the best studied tumour-associated CA at this moment—dramatically increase in response to hypoxia, a characteristic of many tumours, via a direct transcriptional activation of the *CA9* gene by the hypoxia inducible factor HIF-1,[4] being also proven that the expression of this protein in tumours is generally a sign of poor prognosis.[4] Recently, we and Pastorekova and co-workers[6] showed that hCA IX is involved in the tumour acidification processes, providing H^+ ions to the extracellular milieu by means of the CO_2 hydration reaction to bicarbonate and protons. The pH of tumours is in fact more acidic by 0.5–1.0 pH unit than that

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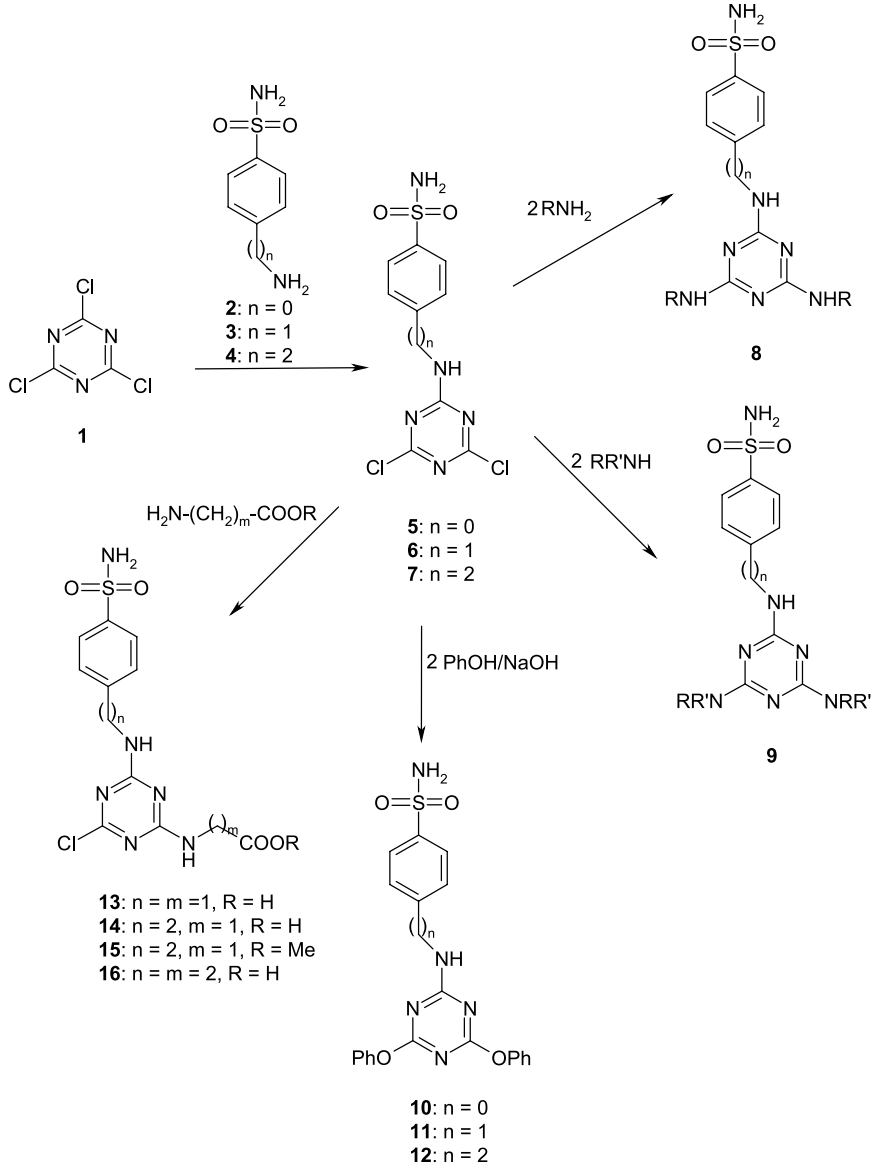
of the surrounding normal tissue,[4] and this acidic environment seems to play a very important role both in the growth, dissemination and propagation of tumour cells and in their nonresponsiveness to chemo- and radiotherapy.[4,6,7] We have also proved[6] that inhibition of hCA IX in transfected cells or in cultured tumour cells by means of potent CA IX inhibitors developed in our laboratory leads to a diminution of the acidifying effects in these cells, with restoration of a more physiologic pH. This constitutes the proof-of-concept that inhibition of the tumour-associated CAs (two such isozymes are known at this moment, hCA IX and hCA XII)[4] may lead to novel therapeutic approaches in the fight against hypoxic tumours, which are generally less responsive or nonresponsive to all the classical chemotherapeutic drugs or to radiotherapy.[6]

Since the sulfonamides incorporating triazinyl moieties previously reported[1] were among the most potent and selective hCA IX inhibitors obtained up to now, in this paper we continue the investigation of this class of derivatives, reporting the synthesis and hCA I, II and IX inhibitory properties of a large series of such novel derivatives.

2. Chemistry

Considering the versatile chemistry of cyanuric chloride **1** (2,4,6-trichloro-1,3,5-triazine),[1,8] and its reactions with various nucleophiles such as amines, amino-sulfon amides, alcohols, phenols, etc., we extend here our previous investigations[1] in the design of novel CA inhibitors[9] containing triazinyl moieties (Scheme 1).

Reaction of cyanuric chloride **1** with sulfanilamide **2**, homosulfanilamide **3** or 4-aminoethyl-benzenesulfon amide **4**, in a 1:1 molar ratio, afforded the dichlorotriazine-substituted key intermediates **5**–**7** reported previously (Scheme 1).[1] Reaction of **5**–**7** with primary



Scheme 1.

amines in molar ratios of 1:2 afforded the bis-amino-triazinyl derivatives **8**. A large series of such compounds have been obtained, since in the previous report only three amino-derivatives were synthesized—the methyl-amino ones—which were among the best hCA IX inhibitors in the series of investigated triazinyl-sulfonamides. Diverse alkyl amines possessing both normal- and branched C_2–C_4 chains (ethyl, *n*-propyl, *iso*-propyl, *n*-butyl) have been employed in the reaction, in order to prepare a large library of such derivatives and detect the best substitution pattern for the CA inhibitory properties of these derivatives. Some heterocyclic amines have also been included in our study, such as pyrid oxal-amine or *N*-aminoethyl-piperazine among others (leading to derivatives **8s**–**x**); but a limited number of such compounds have been prepared, since due to steric impairment of these rather bulky derivatives it is probable that they should not fit easily within the enzyme active site (see discussion later in the text). However, it is interesting to note that although these two compounds possess different nucleophilic moieties in their molecules in addition to the NH_2 one, unexpectedly only the primary amine reacted with **5**–**7**, leading to only one reaction product in each case (**8s**–**x**) that could be separated and purified without problems. Similarly, reaction of **5**–**7** with secondary amines containing 2–4 carbon atoms in their molecules led to a smaller series of tertiary bis-amines of type **9**, incorporating derivatives with both identical substituents (R = R′ in structure **9**) as well as compounds with such different moieties (Scheme 1 and Table 1). The key intermediates **5**–**7** have also been reacted with sodium phenoxide, leading to the bis-ethers **10**–**12** (in the previous report only the reaction with alkoxides has been investigated).[1] Finally, the reaction of the intermediates **5**–**7** with amino acids and some of their derivatives has also been investigated. However, only four mono-substituted derivatives **13**–**16** could be obtained at this moment, when working at 1:1 or 1:2 molar ratios between the halogeno-substituted intermediates **5**–**7** and the amino acid derivative. Thus, the second chlorine atom from **5**–**7** is probably deactivated to the nucleophilic substitution with amino acid derivatives; however, this reaction deserves further investigation, as only a limited number of amino acid derivatives was obtained in this study (Scheme 1).[10]

3. CA inhibition

Among the physiologically most relevant CA isozymes in humans are the cytosolic ubiquitous hCA I and II,[2,3] as well as the tumour-associated transmembrane isozyme IX, which plays a relevant role in tumourigenesis, as mainly investigated by Pastorek and co-workers.[4,6] Data of Table 1 show hCA I, II and IX inhibition with the new compounds reported here of types **8**–**16**, as well as clinically used CA inhibitors, such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA** and brinzolamide **BRZ**.[9] Indisulam (E7070) **IND**, an antitumour sulfonamide in phase II clinical trials for which we recently demonstrated potent CA inhibitory properties, has also been included for comparison in this study.[11,12] Furthermore, the X-ray crystal structure of **IND** in adduct with isozyme hCA II has recently been reported by our group.[11]

The following SAR should be noted from data of Table 1:[13] (i) against the cytosolic, slow isozyme hCA I, the new derivatives incorporating triazine moieties of types **8**–**16** showed all types of inhibitory properties, with K_Is in the range of 31–8500 nM. Thus, a first group of derivatives, such as **8s**–**x**, behaved as quite weak hCA I inhibitors, with K_Is in the range of 1560–8500 nM. It may be observed that all these compounds incorporate two bulky piperazinyl-ethyl- or pyridoxal amine moieties in their molecules, which are obviously too bulky

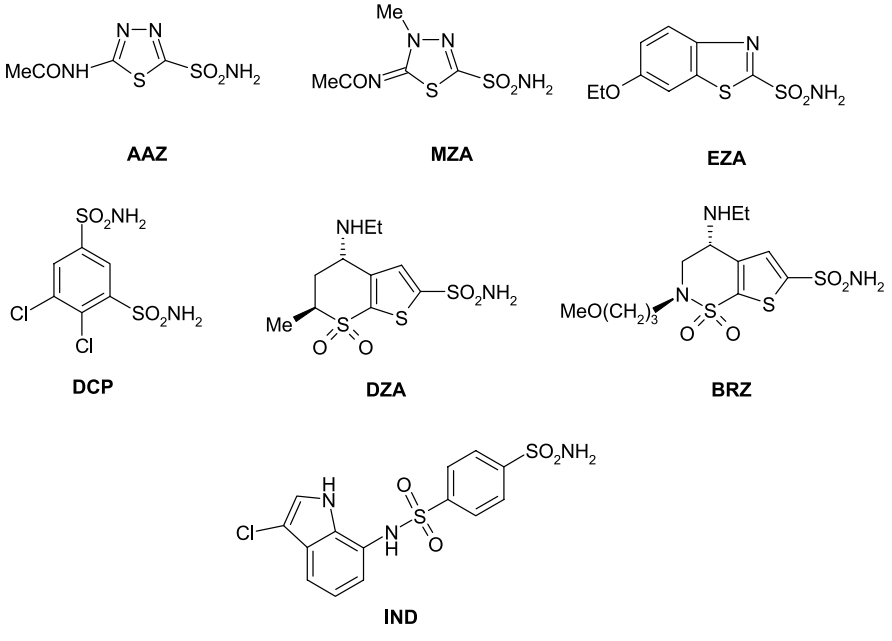
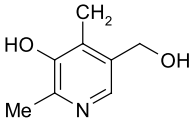
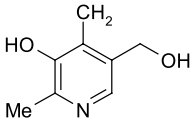
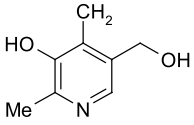


Table 1. Inhibition data for derivatives **8**–**16** investigated in the present paper and standard sulfonamide CA inhibitors, against isozymes hCA I, II and IX, and their selectivity ratios for isozyme IX over isozyme II[13]

Compound	R,R′	*n*	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
8a	H	1	87	16	1.2	13.33
8b	H	2	95	21	1.0	21.00
8c	NH_2	0	93	18	1.5	12.00
8d	NH_2	1	104	27	1.4	19.28
8e	NH_2	2	113	31	1.3	23.84
8f	Et	1	105	14	2.5	5.60
8g	Et	2	112	23	2.9	7.93
8h	*i*-Pr	0	235	38	13	2.92
8i	*i*-Pr	1	247	46	16	2.87
8j	*i*-Pr	2	241	50	18	2.77
8k	*n*-Pr	0	368	49	56	0.87
8m	*n*-Pr	1	425	63	78	0.80
8n	*n*-Pr	2	540	83	125	0.66
8o	*n*-Bu	0	561	146	185	0.79
8p	*n*-Bu	1	622	175	210	0.83
8q	*n*-Bu	2	613	163	204	0.80
8r	$Et_2NCH_2CH_2$	0	558	216	174	1.24
8s	$[HN(CH_2CH_2)_2N]CH_2CH_2$	0	1560	326	287	1.13
8t	$[HN(CH_2CH_2)_2N]CH_2CH_2$	1	1635	338	274	1.23
8u		0	3500	450	386	1.16
8v		1	4200	596	423	1.40
8x		2	8500	765	640	1.19
9a	Me, Me	0	63	39	1.5	26.00
9b	Me, Me	1	75	33	1.3	25.38
9c	Me, Me	2	76	35	1.5	23.33
9d	Et, Et	0	128	65	5.9	11.01
9e	Et, Et	1	130	62	7.5	8.26
9f	Et, Et	2	156	77	6.3	12.22
9g	Me, *n*-Pr	0	176	39	10.5	3.71
9h	Me, *n*-Pr	1	180	33	12.6	2.61
9i	Me, *n*-Pr	2	198	36	13.7	2.62
10	—	—	875	138	254	0.54
11	—	—	631	124	197	0.63
12	—	—	549	83	113	0.73
13	—	—	35	29	1.7	17.05
14	—	—	33	32	1.0	32.00
15	—	—	39	33	1.4	23.57
16	—	—	31	28	1.2	23.33

NT = not tested.

* 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.

for their favourable binding within the active site of the enzyme. Several other compounds, such as the bis-amines **8k**–**r** and the bis-ethers **10**–**12**, were more effective hCA I inhibitors, showing K_Is in the range of 368–875 nM. Again the bulky moieties (phenoxy in **10**–**12** or *n*-propyl/*n*-butyl in **8k**–**r**) are detrimental to the good inhibitory activity of these compounds, which is again probably due to steric hindrance effects. Good hCA I inhibitory activity, with K_Is in the range of 104–247 nM, has been detected for derivatives **8d**–**j** and **9d**–**i**. These compounds incorporate less bulky moieties than the previously discussed ones, such as hydrazino, ethylamino, isopropylamino, *N*,*N*-diethylamino- or *N*-methyl-*N*-propylamino. Finally, the best hCA I inhibitors in this series were **8a**–**c**, **9a**–**c** and **13**–**16**, which showed K_Is in the range of 31–95 nM, in the same range as the potent, clinically used inhibitors ethoxzolamide, methazolamide and indisulam (Table 1). It is easy to observe that the best inhibitors in the series incorporate the most compact substituents at the triazine ring, such as amino, hydrazine (in **8a**–**c**), dimethylamino (in **9a**–**c**) as well as the monosubstituted amino acid derivatives (**13**–**16**), which contain a chlorine atom and an amino acyl moiety, being thus among the less bulky ones in the entire series. Generally (although there are many exceptions) the sulfanilamide derivatives ($n = 0$ in structures **8**–**16**) were more effective hCA I inhibitors than the corresponding homosulfanilamides ($n = 2$), which in turn were more active inhibitors than the corresponding derivatives of 4-aminoethylbenzenesulfonamide ($n = 2$); (ii) against isozyme hCA II, one of the physiologically most relevant CAs, the new derivatives **8**–**16** also showed interesting inhibitory power, with K_Is in the range of 14–765 nM. SAR is also in this case similar to what was discussed for hCA I above, rather straightforward and simple, with the derivatives incorporating the compact moieties at the triazine ring being the most active ones, and the derivatives incorporating bulky moieties the most ineffective inhibitors. As for hCA I, generally activity diminished with increasing *n* from 0 (sulfanilamide derivatives) to 2 (4-aminoethylbenzenesulfonamide derivatives) for all compounds **8**–**16** investigated here (but more exception as for hCA I can be easily observed from data of Table 1). Thus, the most ineffective hCA II inhibitors were the bulky derivatives **8n**–**x** and **10**–**12** (K_Is in the range of 83–765 nM), followed by the less bulky derivatives **8h**–**m**, **9** and **13**–**16**, which are medium potency—effective hCA II inhibitors (K_Is in the range of 28–77 nM). The most effective hCA II inhibitors (with potencies comparable to those of the clinically used sulfonamides shown in Table 1) were **8a**–**g**, with K_Is in the range of 14–27 nM; (iii) against the tumour-associated isozyme hCA IX, the derivatives **8**–**16** investigated here showed inhibition constants in the range of 1.0–640 nM. Considering the triazine ring substituents, SAR is similar to what disclosed above for the inhibition of hCA I and II, with the bulky compounds being the most ineffective inhibitors, and those incorporating compact, smaller moieties being the most effective hCA IX inhibitors. This is clearly due to the fact that the bulky derivatives difficultly may bind within the enzyme active site. On the other hand, unlike as with hCA I and II, generally the sulfanilamide derivatives were less effective hCA IX inhibitors than the corresponding homosulfanilamides, which in turn were less inhibitory than the corresponding derivatives with $n = 2$ (of course, also in this case several exceptions may be observed from data of Table 1, but the general trend was different for the transmembrane isozyme than for the cytosolic ones discussed above). The less effective hCA IX inhibitors were again the amines/ethers incorporating the bulky *n*-propyl, *n*-butyl, diethylaminoethyl, piperazinyl-ethyl, pyridoxalamine or phenoxy moieties (**8n**–**x** and **10**–**12**), which showed K_Is in the range of 113–640 nM. Medium potency hCA IX inhibitors were two *n*-propyl derivatives (**8k** and **8m**) with inhibition constants of 56–78 nM, whereas the remaining compounds (incorporating compact substituents at the triazine ring) showed very effective hCA IX inhibitory properties, with K_Is in the range of 1.0–18 nM, similar to the previously reported derivatives belonging to this class.[1] Thus, the drug design of triazinyl sulfonamides may indeed lead to very effective hCA IX inhibitors, both belonging to the amine, amino acid or alkoxy[1] derivatives. The best hCA IX inhibitors were the amino, hydrazine, ethyl amino, dimethylamino and amino acid derivatives, which showed inhibition constants in the range of 1–3 nM, being among the most potent hCA IX inhibitors reported up to now;[1,16,17] (iv) since hCA II is a ubiquitous enzyme showing high affinity for sulfonamide inhibitors, a critical issue in the design of inhibitors of other CA isozymes which show potential as medicinal chemistry targets regards the selectivity of an inhibitor for the target isozyme over hCA II. In this case, the selectivity ratios for hCA IX over hCA II of the new inhibitors are shown in Table 1. It may be observed that all the clinically used sulfonamides and a small number of the new inhibitors reported here (e.g., **8k**–**q** and **10**–**12**) behave better as hCA II than hCA IX inhibitors, with selectivity ratios <1. Another group of new derivatives, such as **8h**–**j**, **8r**–**x** and **9g**–**i**, were better hCA IX than hCA II inhibitors, but with a modest selectivity, in the range of 1.13–3.71. Finally, the remaining new derivatives showed good or excellent selectivity ratios, being 5.60–32.00 times more effective hCA IX than hCA II inhibitors. Among the most selective hCA IX inhibitors were some of the amino acid derivatives (**14**–**16**) or the secondary amines **9a**–**c** (selectivity ratios in the range of 23.33–32.00). As some of these compounds are also very effective hCA IX inhibitors (in the low nanomolar range), it is obvious that this class of derivatives affords potent and quite selective hCA IX inhibitors, with potential to be developed for the management of hypoxic tumours.

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 sub sequently derivatized by reacting them with various nucleophiles, such as ammonia, hydrazine, primary and secondary amines, amino acid derivatives or phenol.

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, tumour-associated hCA IX. The new compounds reported here inhibited hCA I with K_Is in the range of 31–8500 nM, hCA II with K_Is in the range of 14–765 nM and hCA IX with inhibition constants in the range of 1.0–640 nM. SAR was straightforward and rather simple in this class of CA inhibitors, with the derivatives incorporating compact moieties at the triazine ring (such as amino, hydrazino, ethylamino, dimethylamino or amino acyl) being the most active ones, and the derivatives incorporating such bulky moieties (*n*-propyl, *n*-butyl, diethylaminoethyl, piperazinylethyl, pyridoxal amine or phenoxy) being the most ineffective hCA I, II and IX inhibitors. Some of the new derivatives also showed selectivity for inhibition of hCA IX over hCA II, thus constituting excellent leads for the development of novel approaches in the management of hypoxic tumours.

Acknowledgments

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10. Synthesis of the key intermediate **5**–**7**:[1] An acetone solution containing 0.1 mol of either sulfanilamide **2**, homosulfanilamide (HCl salt) **3** or 4-(2-aminoethyl)benzenesulfonamide **4** (17.2, 22.3 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.[1,8] 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 was 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.[8] Reaction of intermediates **5**–**7** with ammonia, amines or phenol: 0.005 mol of the appropriate intermediate **5**–**7** was added to a suspension/solution of 0.01 mol of nucleophile in water. The mixture was refluxed for 4–6 h and the corresponding amount of a NaOH solution dissolved in a small volume of water was added dropwise. The desired derivatives, **8**–**12** precipitated after cooling the reaction mixture at 4 °C, were filtered and recrystallized from MeOH–water (1:1) or EtOH–water (1:1 or 1:2). Yields were generally in the range of 70–95%. Reaction of intermediates **5**–**7** with amino acid derivatives: 0.005 mol of the halogeno intermediates **5**–**7** was added to a solution of 0.005 mol (or 0.01 mol) of the appropriate amino acid derivative in water. The mixture was heated to reflux and 0.005 or 0.01 mol of aqueous NaOH was slowly added to the mixture. Refluxing was continued for 4–5 h. If necessary, the pH of the mixture was changed to neutral using diluted (5%) HCl water solution, and the insoluble products were easily isolated by filtration and recrystallized from MeOH or EtOH. Yields were in the range of 50–65%. The purity of all products was checked by TLC using methanol as mobile phase and confirmed by ^{1}H NMR and MS. Some representatives of this class could not be obtained in pure enough state and were not included in Table 1 (e.g., the reaction products of **5** with ammonia or ethylamine among others).
4-(4,6-Bis-isopropylamino-[1,3,5]triazin-2-yl-amino)-benzenesulfonamide **8h**: mp 129–131 °C, ^{1}H NMR (DMSO-d_6, 250 MHz): δ 9.2 (s, 1H), 8 (d, 2H, J = 8 Hz), 7.7 (d, 2H, J = 8 Hz), 7.2 (s, 2H), 6.7 (m, 2H), 4.1 (m, 2H), 1.15 (d, 12H, J = 6 Hz); MS ESI$^+$ m/z 366 $(M+H)^+$, 731 $(2M+H)^+$. ESI$^-$ m/z 364 $(M-H)^-$, 729 $(2M-H)^-$.
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13. hCA I and hCA 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.[14] Cell growth conditions were those described in Ref.14 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[15] 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 for CA I, and 29.3 for CA II, respectively. A variant of the previously published[16,17] 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.[18]) 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™ 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[16,17] 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 chromato graphy,[15] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[19] The specific activity of the obtained enzyme was the same as the one previously reported,[16,17] but the yields in active protein were 5–6 times higher per liter of culture medium.

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19. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An SX.18MV-R Applied Photophysics stopped-flow instrument was 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) was 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 were used to determine the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. The kinetic constants k_{cat} and k_{cat}/K_m were obtained by nonlinear least-squares methods using PRISM 3. 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. K_Is of the inhibitors were determined by using Lineweaver–Burk plots, as reported earlier,[16,17] and represent the means from at least three different determinations.