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Carbonic anhydrase inhibitors. Inhibition of the transmembrane isozyme XII with sulfonamides—a new target for the design of antitumor and antiglaucoma drugs?

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Abstract—The inhibition of a newly cloned human carbonic anhydrase (CA, EC 4.2.1.1), isozyme XII (hCA XII), has been investigated with a series of sulfonamides, including some clinically used derivatives (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and sulpiride, or indisulam, a compound in clinical development as antitumor drug), as well as the sulfamate antiepileptic drug topiramate. Some simple amino-/hydrazine-/hydroxy-substituted aromatic/heterocyclic sulfonamides have also been included in the study. All types of activity have been detected, with several medium potency inhibitors (K_Is in the range of 34–220 nM), whereas ethoxzolamide and several halogenated sulfanilamides showed stronger potency, with K_Is in the range of 11–22 nM. The antiglaucoma sulfonamides used clinically, except dichlorophenamide, which is a moderate inhibitor (K_I of 50 nM), as well as topiramate, indisulam, and sulpiride behave as very potent hCA XII inhibitors, with K_Is in the range of 3.0–5.7 nM. Several subnanomolar inhibitors (K_Is in the range of 0.30–0.85 nM) have also been detected. Compounds with excellent selectivity against hCA XII over hCA II have been found, showing selectivity ratios in the range of 177.7–566.7. Apparently, hCA XII is a target of the antiglaucoma sulfonamides, and potent hCA XII inhibitors may be developed/used for the management of hypoxic tumors, together with inhibitors of the other tumor-associated isozyme, CA IX.

1. Introduction

The transmembrane human carbonic anhydrase (hCA, EC 4.2.1.1) isozyme XII (CA XII)—the second tumor-associated CA isozyme described, after CA IX[1–3]—has been identified in a human renal cell carcinoma (RCC), cloned and sequenced thereafter from the corresponding cDNA by Sly's group.[4] It was then proved that CA XII is overexpressed in about 10% of RCC patients.[4] Ivanov et al.[5] cloned CA XII almost simultaneously with Sly's group, as a novel von Hippel-Lindau (VHL) target, showing that its expression is strongly inhibited by the wild type VHL in diverse RCC cell lines, also suggesting that it is subject to similar regulation as the gene encoding the other tumor-associated CA, that is, *CA9*.[2] However, suppression of *CA12* requires both the central VHL domain involved in the hypoxia-inducible transcription factor 1α (HIF-1α) binding and the C-terminal elongin-binding domain, whereas only the latter is needed for the negative regulation of *CA9*. Thus, this second tumor-associated CA isozyme is regulated by hypoxia, similarly with CA IX, but by means of diverse biochemical pathways.[2,6]

These first studies also showed that *CA12* mRNA is expressed at very low levels in normal adult kidney, pancreas, colon, prostate, ovary, testis, lung, and brain.[4,5] CA XII protein was then found in normal endometrium,[7] colon,[8] and kidney[9] suggesting an important physiological role for this enzyme in ion transport and fluid concentration. But as mentioned above, it has been observed that many tumors display strong expression of

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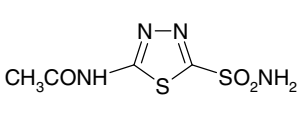
CA XII or co-expression of CA XII and CA IX, mostly in the perinecrotic areas, in accord with the fact that both isozymes are induced by hypoxia.[5,6] Recently, Lerman's group showed that CA XII is highly overexpressed (up to 5 times) in the eyes of glaucoma patients,[10] suggesting that this isozyme may be a target for the treatment of glaucoma (up to now the isozymes thought to be involved in aqueous humor secretion within the eye, and thus considered as targets for the development of antiglaucoma drugs were CA II and CA IV).[1]

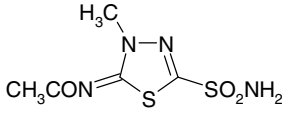
Sly's group[11] then purified and characterized the catalytic activity of hCA XII, whereas Christianson's group resolved the X-ray crystal structure of the native enzyme and of its adduct with the sulfonamide inhibitor acetazolamide.[12] From these studies it appeared that hCA XII has a catalytic activity for the CO_2 hydration reaction similar to that of the cytosolic, slow red blood cell isozyme hCA I or the membrane-bound isozyme hCA IV, also showing a rather weak esterase activity with 4-nitrophenyl acetate as substrate (similarly to CA IV and in contrast to CA I, which is a good esterase with this substrate). Thus, hCA XII is less effective as a catalyst for the CO_2 hydration reaction, as compared to the very rapid isozymes hCA II (cytosolic) or hCA IX (transmembrane),[1–3] but its catalytic activity seems to be relevant to oncogenesis (acidification of the tumor environment),[2] and its potential as a clinically useful tumor marker clearly merit further investigation, together with the design of inhibitors that may show clinical applications as antitumor drugs.

Indeed, in the last period, our and Pastorek's groups[1–3,13–16] developed a large number of potent inhibitors of the best studied tumor-associated CA isozyme, hCA IX, and also demonstrated rigorously its involvement in tumor acidification under hypoxia, as well as the fact that this phenomenon can be reversed by inhibiting CA IX with specific (and potent) sulfonamide or sulfamate CA IX inhibitors.[16] Such studies constitute the proof-of-concept that the inhibition of the tumor-associated CA isozyme(s) (i.e., CA IX and/or CA XII) may represent a novel therapeutic approach for the management of hypoxic tumors overexpressing these proteins.[16]

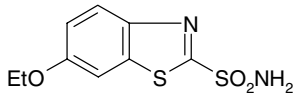
Since no CA XII inhibition studies are available up to now (only the inhibition with ethoxzolamide has been mentioned by Ulmasov et al.[11]), we extend here our previous studies regarding the inhibition of tumor-associated CA isozymes with sulfonamides/sulfamates,[13–16] to this new target. Here we report the first CA XII inhibition study with a series of 34 sulfonamides/sulfamates, among which the clinically used compounds acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, sulpiride **SLP**, and indisulam **IND** (a compound in clinical development as antitumor drug[17–19]), as well



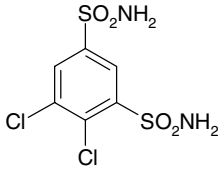
AAZ



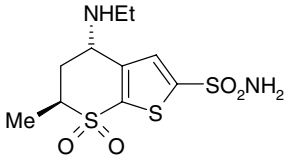
MZA



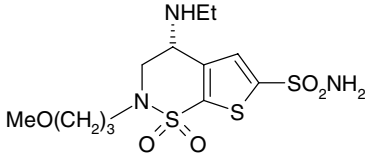
EZA



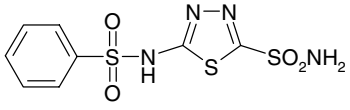
DCP



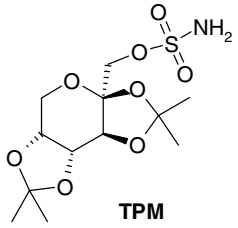
DZA



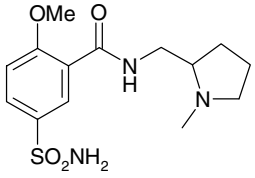
BRZ



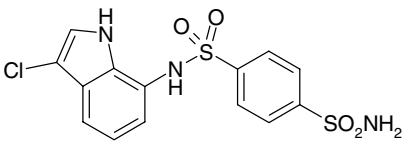
BZA



TPM



SLP

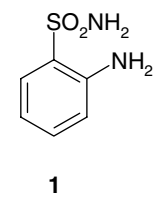


IND

as other simple aromatic/heterocyclic derivatives, which may be used as lead molecules for developing more potent/specific CA inhibitors (CAIs).[20]

2. Chemistry

Sulfonamides investigated for the inhibition of the transmembrane isozyme hCA XII, of types **1**–**24** are shown below. Compounds **1**, **2**, **4**–**6**, **11**–**12**, **18**–**20**, and **23** are commercially available, whereas **3**,[21] **7**–**10**,[14b] **13**–**17**[22] and **21**,[19] **22**,[23] and **24**[21] were prepared as reported earlier by this group. The 10 clinically used compounds **AAZ**–**SLP** (commercially available from Sigma–Aldrich, Merck, Alcon or Johnson & Jonson) were also assayed, since no such data are available in the literature. **IND** was synthesized as described by Owa et al.[17]



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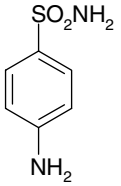

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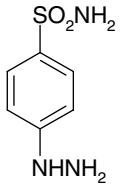

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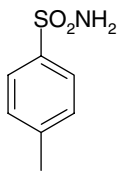

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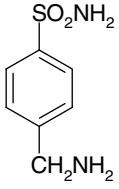

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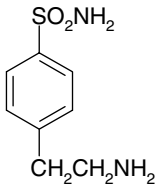

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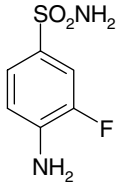

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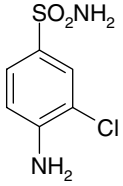

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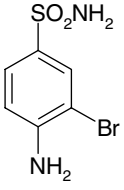

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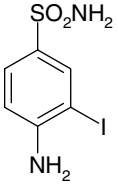

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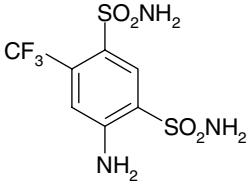

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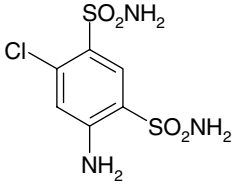

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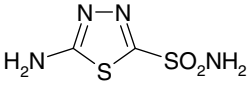

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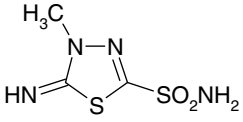

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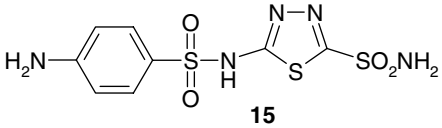

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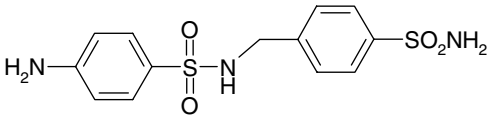

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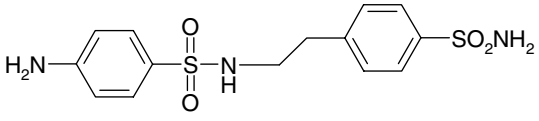

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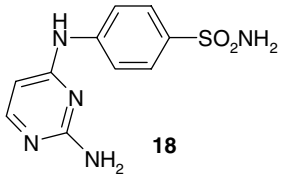

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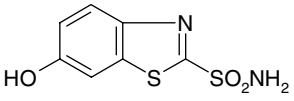

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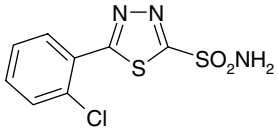

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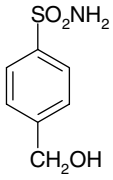

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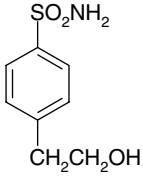

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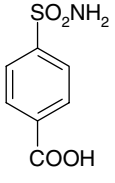

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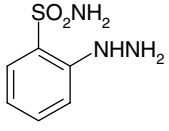

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3. CA inhibition data

We have obtained the cDNA clone encoding the open reading frame of hCA XII by RT-PCR with poly(A)RNA from the human pancreas (Clontech, Palo Alto, CA),[24] and purified large amounts of the CA XII protein produced in a bacterial expression system for screening studies in order to detect potent inhibitors. Thus, in contrast to the procedure reported by Ulmasov et al.[11] who used a mammalian expression vector for obtaining CA XII, we used an *E. coli* expression system from which we purified the catalytic domain of the enzyme, working with the glutathione S-transferase (GST)-fusion protein for simplifying the purification procedure.[24] Similarly to the protein reported earlier,[11] our hCA XII preparation showed a good catalytic activity for the CO_2 hydration reaction, with a k_{cat} of $4.2 \times 10^5\ s^{-1}$ and k_{cat}/K_m of $3.5 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.5 and 20 °C, that is, of the same order of magnitude (within the limits of the experimental errors) as the enzyme reported by Ulmasov et al. from the mammalian expression vector.[11]

Inhibition data of hCA XII with sulfonamides **1**–**24** and the 10 clinically used compounds **AAZ**–**IND** are shown in Table 1, together with hCA I and II inhibition data, since these classical CA isozymes are ubiquitous and highly abundant, potentially novel drugs based on CAIs should address the problem of selectivity toward different isozymes.[1–3] Indeed, selectivity ratios for CA XII versus CA II of the tested inhibitors are also presented in Table 1.

The following should be noted regarding data of Table 1: (i) a first group of compounds, including derivatives **2**, **11**–**13**, **15**, **20**–**24**, and **DCP**, showed medium potency as hCA XII inhibitors, with inhibition constants in the range of 33–220 nM. These compounds mainly include simple benzene-mono- and 1,3-di-sulfonamide derivatives (**2**, **11**, **12**, **DCP**, and **21**–**24**) as well as several 1,3,4-thiadiazole-2-sulfonamide derivatives (**13**, **15**, and **20**). Thus, clearly the benzene-1,3-disulfonamide motif present in compounds **11**, **12**, and **DCP** does not lead to particularly potent CA XII inhibitory properties to these CAIs. It is also amazing that aminobenzolamide **15**, which is a very potent inhibitor of the cytosolic isozymes I and II shows rather weak inhibitory properties against CA XII (Table 1). Also the hydroxy-/carboxy-substituted derivatives **21**–**23** are much weaker CA XII inhibitors as compared to the corresponding methyl/amino-substituted compounds **4**–**6**, which will be discussed shortly; (ii) a small group of derivatives, such as **3**, **9**, **10**, and **EZA** showed stronger hCA XII inhibitory properties than the previously discussed sulfonamides, with inhibition constants in the range of 11–22 nM. These compounds are (except ethoxzolamide) again simple benzenesulfonamide derivatives possessing either the 4-hydrazino- or 3-halogeno-4-amino-substitutions. It should be noted that just the bromo- and iodo-sulfanilamides **9** and **10** showed this behavior, whereas the corresponding fluoro- and chloro-derivatives **7** and **8** are more potent hCA XII inhibitors (see discussion later in the text). It should also be mentioned the rather important difference of activity between ethoxzolamide **EZA** and its de-ethylated derivative **19**, which is 6.5 times a more potent hCA XII inhibitor than **EZA**. Just the reverse is true against the cytosolic isozymes I and II, for which **EZA** is a much more potent CAI as compared to the phenol **19**. It should also be mentioned that Ulmasov et al.[11] reported **EZA** to act as a more potent hCA XII inhibitor, with a K_I of 2 nM. The 11 times difference between their and our data may be due to the fact that the enzyme prepared in the mammalian expression system is glycosylated, whereas the one prepared by us is not; (iii) the large majority of the derivatives investigated here showed a very good CA XII inhibitory activity, with K_Is in the range of 0.3–5.7 nM. Among these compounds, there are simple *ortho*-, *para*-, or 3,4-disubstituted-benzenesulfonamides (such as **1**, **4**–**8**), the heterocyclic imine **14** or the sulfanilyl-derivatives **16**

Table 1. hCA I, II, and XII inhibition data with sulfonamides **1**–**24** and clinically used inhibitors, and the selectivity ratio hCA II over hCA XII (data for hCA I and II are from Ref. 2)

Inhibitor	K_I[a] (nM)			Selectivity ratio
	hCA I[b]	hCA II[b]	hCA XII[c]	hCA II/hCA XII
1	45,400	295	0.85	347
2	25,000	240	37	6.5
3	28,000	300	11	27.2
4	78,500	320	1.8	177.7
5	25,000	170	0.3	566.7
6	21,000	160	3.2	50.0
7	8300	60	1.1	54.5
8	9800	110	3.1	35.5
9	6500	40	20	2.0
10	6000	70	11	6.3
11	5800	63	45	1.4
12	8400	75	44	1.7
13	8600	60	33	1.8
14	9300	19	4.0	4.7
15	6	2	85	0.02
16	164	46	3.5	13.1
17	185	50	4.5	11.1
18	109	33	3.4	9.7
19	95	30	3.7	8.1
20	690	12	36	0.3
21	55	80	220	0.3
22	21,000	125	55	2.2
23	23,000	133	34	3.9
24	24,000	125	58	2.1
AAZ	250	12	5.7	2.1
MZA	50	14	3.4	4.1
EZA	25	8	22	0.4
DCP	1200	38	50	0.7
DZA	50,000	9	3.5	2.6
BRZ	—	3	3.0	1.0
TPM	250[d]	5[d]	3.8	1.3
BZA	15	9	3.5	2.6
SLP	1200[e]	40[e]	3.9	10.2
IND	31[f]	15[f]	3.4	4.4

[a] Errors in the range of 5–10% of the shown data, from three different assays.
[b] Human recombinant isozymes.
[c] Catalytic domain of the human recombinant isozyme, CO_2 hydrase assay method.[25]
[d] From Ref. 26.
[e] From Ref. 27.
[f] From Ref. 18c.

and **17**, the other heterocyclic compounds **18** and **19**, together with all the clinically used compounds except ethoxzolamide and dichlorophenamide, which, as shown above, behave as weaker CA XII inhibitors. Thus, we observe excellent CA XII inhibitory properties for a rather heterogenous class of sulfonamides/sulfamates, including both very simple derivatives (such as **1**, **4**, **5**, and **6** among others) as well as compounds incorporating rather bulky tails/functionalities, such as **16**, **17**, **IND**, **DZA**, **BRZ**, **SLP**, **IND**, or the antiepileptic sulfamate topiramate **TPM**. This is indeed a remarkable result, proving that potent CA XII inhibitors may be still designed belonging to varied chemical classes of sulfonamides and sulfamates among others; (iv) it may be observed that isozyme hCA XII has a completely different inhibition profile as compared to the cytosolic isozymes I and II (Table 1) or the other tumor-associated isozyme, hCA IX (data not shown, see Ref. 13–16 for details). Indeed, most of the investigated compounds show the highest affinity for hCA XII, followed by hCA II; whereas hCA I is an isozyme more resistant to inhibition by these classes of derivatives (Table 1); (v) since hCA II, the rapid blood cell isozyme, ubiquitous in many tissues/cells,[1–3] is known to possess a high affinity for sulfonamides/sulfamates, we determined the selectivity ratios of the tested CAIs against isozyme XII over isozyme II (Table 1). It may be observed that most of the investigated compounds act as more potent hCA XII than hCA II inhibitors, except **15**, **20**, **21**, **EZA**, **DCP**, and **BRZ**, which showed selectivity ratios in the range of 0.02–1. Thus, the most hCA II selective inhibitor was aminobenzolamide **15**. Brinzolamide **BRZ** showed the same potency against both isozymes (selectivity ratio of 1). The most selective hCA XII over hCA II inhibitors, such as derivatives **1**, **4**, and **5**, showed selectivity ratios in the range of 177.7–566.7, which is indeed remarkable. Some other compounds also showed good selectivities, with ratios in the range of 27.2–54.5 (compounds **3**, **6**–**8**). Moderate selectivity has been shown by **2**, **10**, **16**–**19**, and **SLP**, with selectivity ratios in the range of 6.3–13.1, whereas all other derivatives, including the clinically used sulfonamides/sulfamates were only slightly more inhibitory against CA XII than against CA II (selectivity ratios in the range of 1.3–4.7).

These data may be clinically relevant for at least two types of applications. Thus, it was just recently been reported that hCA XII is overexpressed in glaucomatous eyes (from human patients) as compared to the levels of this isozyme in normal human eyes.[10] Furthermore, other investigated isozymes, such as CA IV or CA IX were expressed at similar levels in glaucoma patients and in the normal eyes.[10] These findings, correlated with our present report, suggest that CA XII is clearly a target of the antiglaucoma sulfonamides used clinically, such as **AAZ**, **MZA** (systemically used) or **BRZ**, and **DZA** (topically acting drugs), which all inhibit CA XII with potencies in the low nanomolar range (2.1–4.1 nM). In the second place, as CA XII is present in a rather wide range of tumors (not only RCC but in other types of tumors too, see the recent review of Pastorekova and Pastorek for the tumor-associated isozymes[3]) it appears probable that inhibition of this isozyme with potent and possibly specific inhibitors may have clinical relevance for the development of novel antitumor therapies. We already presented a proof-of-concept study regarding the inhibition of the tumor-associated isozyme CA IX with sulfonamides, and its effect on the tumor pH.[13] Probably compounds inhibiting both CA IX and CA XII have even better chances to lead to such novel approaches for the management of cancer.[28]

4. Conclusions

The first CA XII inhibition study is presented here. A large series of sulfonamides and a sulfamate have been tested for their interaction with the catalytic domain of hCA XII, including clinically used derivatives such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and sulpiride, or indisulam (a compound in clinical development as antitumor drug), as well as the sulfamate antiepileptic drug topiramate. All types of activity have been detected, with several medium potency inhibitors (K_Is in the range of 34–220 nM), whereas ethoxzolamide and several halogenated sulfanilamides showed stronger potency, with K_Is in the range of 11–22 nM. The antiglaucoma sulfonamides used clinically, except dichlorophenamide, which is a moderate inhibitor (K_I of 50 nM), as well as topiramate, indisulam, and sulpiride behave as very potent hCA XII inhibitors, with K_Is in the range of 3.0–5.7 nM. Several subnanomolar inhibitors (K_Is in the range of 0.30–0.85 nM) have also been detected. Compounds with excellent selectivity against hCA XII over hCA II have been found, showing selectivity ratios in the range of 177.7–566.7. Apparently, hCA XII is a target of the antiglaucoma sulfonamides, and potent hCA XII inhibitors may be developed for the management of hypoxic tumors.

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24. The CA XII-GST construct (Amersham): The cDNA fragment encoding the open reading frame of hCA XII was obtained by RT-PCR with poly(A)RNA from the human pancreas (Clontech, Palo Alto, CA). The ploy(A)RNA (0.1 μg) was reverse-transcribed with random hexamers by using a commercial kit (Takara, Kyoto, Japan). The resultant cDNA was amplified by PCR using adopter primers including *Bam*HI and *Sal*I recognition sequences (underlined in the following sequences, respectively): 5′-TTTGGATCCATGCCCCGGCGCAGCCTGCAC-3′ and 5′-TTTGTCGACTCAAGCGTGGGCCTCAGTCTC-3′. The PCR reaction was hot-started with incubation for 2 min at 94 °C and consisted of 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1.5 min at 72 °C. CA XII cDNA was cloned in-frame into the *Bam*HI/*Sal*I site of a pGEX-4T-2 vector to produce a fusion protein with glutathione S-transferase (GST) (Amersham, Piscataway, NJ). The proper cDNA sequence of the CA XII insert included in the vector was reconfirmed by DNA sequencing and then transfected into competent bacteria (*E. coli* JM109). The PCR products were cleaved with the corresponding restriction enzymes, purified and cloned into a modified pGEX-4T2 vector using T4-ligase (Promega). The constructs were then transfected into *E. coli* strain BL21 for production of the CA XII protein, similarly to the procedure already described for hCA IX.[13] The protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside, the cells were harvested when the OD_{600} arrived at 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 spectrophometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[25]

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