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Carbonic anhydrase inhibitors. Inhibition of the prokariotic beta and gamma-class enzymes from *Archaea* with sulfonamides

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Abstract—A detailed inhibition study of carbonic anhydrases (CAs, EC 4.2.1.1) belonging to the β- and γ-families from *Archaea* with sulfonamides has been performed. Compounds included in this study were the clinically used sulfonamide CA inhibitors, such as acetazolamide, methazolamide, ethoxzolamide, topiramate, valdecoxib, celecoxib, dorzolamide, sulfanilamide, dichlorophanamide, as well as sulfanilamide analogs, halogenated sulfanilamides, and some 1,3-benzenedisulfonamide derivatives. The two γ-CAs from *Methanosarcina thermophila* (Zn-Cam and Co-Cam) showed very different inhibitory properties with these compounds, as compared to the α-CA isozymes hCA I, II, and IX, and the β-CA from *Methanobacterium thermoautotrophicum* (Cab). The best Zn-Cam inhibitors were sulfamic acid and acetazolamide, with inhibition constants in the range of 63–96 nM, whereas other investigated aromatic/heterocylic sulfonamides showed a rather levelled behavior, with K_Is in the range of 0.12–1.70 μM. The best Co-Cam inhibitors were topiramate and *p*-aminoethyl-benzenesulfonamide, with K_Is in the range of 0.12–0.13 μM, whereas the worst one was homosulfanilamide (K_I of 8.50 μM). In the case of Cab, the inhibitory power of these compounds varied to a much larger extent, with sulfamic acid and sulfamide showing millimolar affinities (K_Is in the range of 44–103 mM), whereas the best inhibitor was ethoxzolamide, with a K_I of 5.35 μM. Most of these sulfonamides showed inhibition constants in the range of 12–100 μM against Cab. Thus, the three CA families investigated up to now possess a very diverse affinity for sulfonamides, the inhibitors with important medicinal, and environmental applications.

1. Introduction

Ever since the report of Domagk in 1935[1] that prontosil and its metabolite, sulfanilamide, showed potent biological activity as antibacterials, sulfonamides have been widely investigated by medicinal chemists in the search of compounds with putative applications as drugs.[2–10] Indeed, considering this simple molecule, sulfanilamide as lead compound, nowadays a very large number of different pharmacological agents incorporating the sulfonamide moiety have been developed, such as among others the antibacterial sulfadrugs (exemplified by sulfathiazole, Fig. 1),[2] the carbonic anhydrase (CA) inhibitors (CAIs) among which acetazolamide (Fig. 1) is the best known representative,[3,4] different types of diuretics, among which furosemide, a high ceiling diuretic is widely used clinically,[5] a large number of matrix metalloprotease inhibitors,[6] the cycloxygenase 2 (COX-2) 'specific' inhibitors, such as valdecoxib,[7] the HIV-1 protease inhibitor amprenavir,[8] the antitumor sulfonamide in clinical development indisulam,[9] as well as the hypoglycemic agent glibenclamide (Fig. 1).[10] Interestingly, among the small number of privileged building blocks recurrently used in designing potent drug molecules, the sulfonamide one confers to the derivatives incorporating such very different biological activities, correlated with a low toxicity or a lack of toxicity.[2–10]

The CAIs of the sulfonamide type are widely used pharmacological agents for the treatment or prevention of a variety of diseases, such as glaucoma, cystoid macular edema, diabetic retinopathy, epilepsy, neurological/neuromuscular disorders (hemiplegic migraine, ataxia, periodic paralysis, neuropathic pain, essential tremor),

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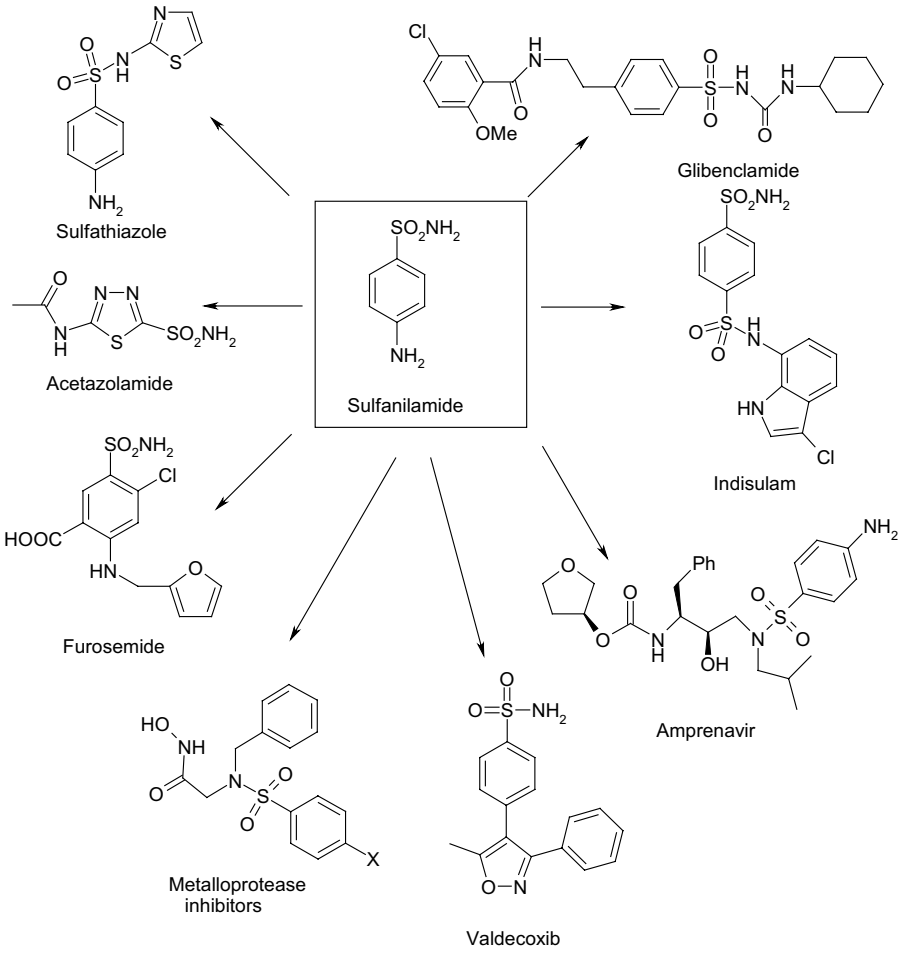


Figure 1. Drugs developed from sulfanilamide as lead molecule: antibacterials such as sulfathiazole; carbonic anhydrase inhibitors (CAIs) such as acetazolamide; diuretics, such as furosemide; matrix metalloprotease inhibitors of the sulfonylated amino acid hydroxamate type; cycloxygenase 2 (COX-2) 'specific' inhibitors, such as valdecoxib; HIV-1 protease inhibitors, such as amprenavir; antitumor drugs such as indisulam or hypoglycemic agents, such as glibenclamide.

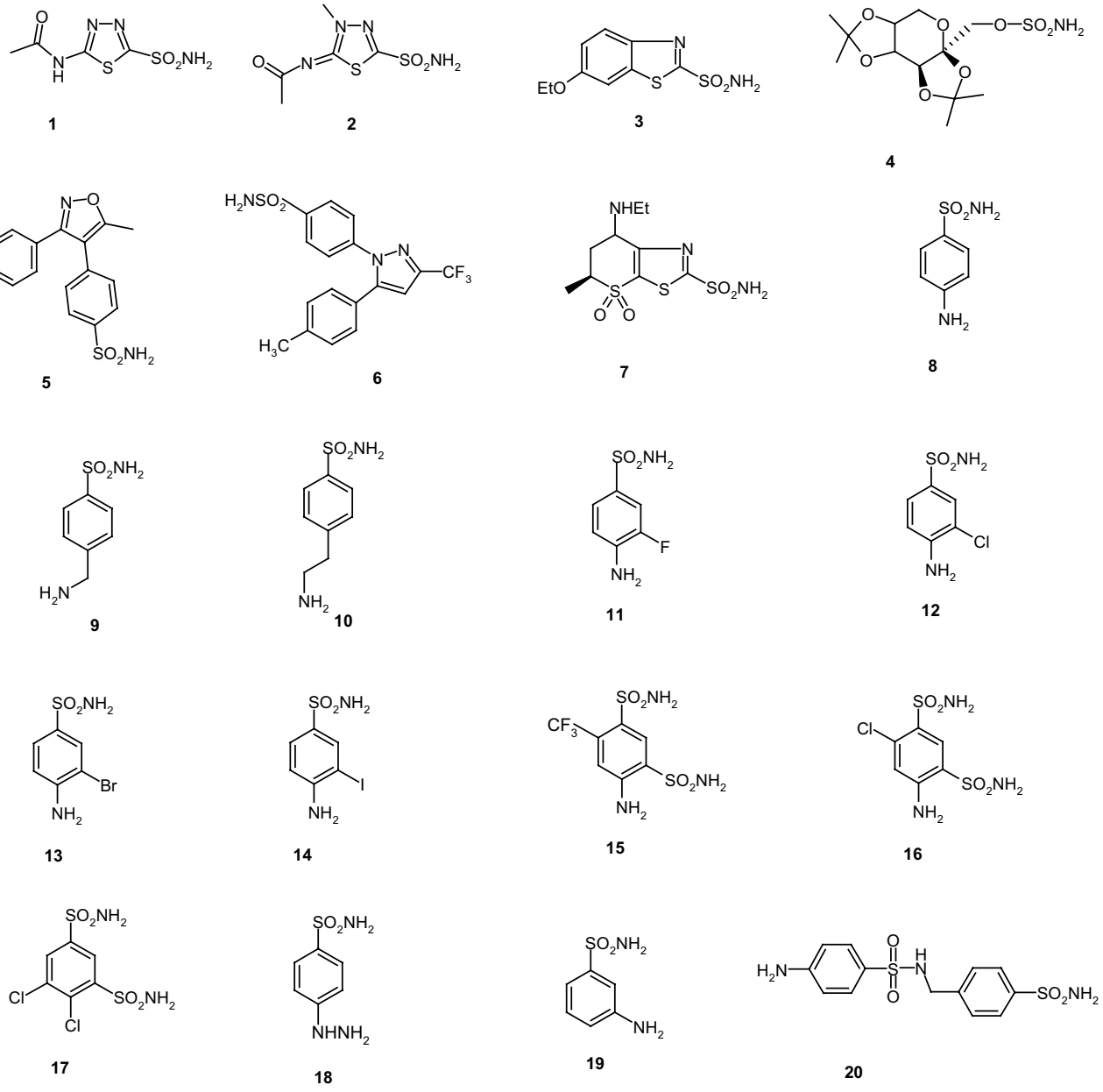
edema (due to congestive heart failure, renal/hepatic disease, diabetes, drugs), obstructive pulmonary disease, sleep apnea, osteoporosis, and cancer.[3–5,11] These multiple applications of the sulfonamide CAIs are mainly due to the fact that at least 14 different α-CA isozymes are present in diverse tissues/organs of higher vertebrates, including humans, and in fact these are the best studied CAs up to now. Still, in recent years, four other gene families encoding CAs have been discovered in different organisms all over the phylogenetic tree, the β- to ε-CA classes of enzymes.[3,12–16] These CAs are far less investigated as compared to the different α-CA isozymes mentioned earlier. Two of them were isolated from *Archaea* by one of our groups: a β-CA, denominated Cab, in the methanoarchaeon *Methanobacterium thermoautotrophicum*,[16] and a γ-CA, denominated Cam, in the methanogenic archaeon *Methanosarcina thermophila*.[13] Recently, this group investigated the inhibition of both Cam (the zinc(II)- and cobalt(II)-containing enzymes)[17] as well as Cab with anions,[18] finding important differences between these enzymes and α-CAs in their behavior toward anions, which represent the second class of CAIs, in addition to the sulfonamides.[3] Since the interaction between β-CAs or γ-CAs with sulfonamides received little attention up to now, we present here a detailed inhibition study of three such enzymes, that is, Cab (a zinc containing β-CA), Zn-Cam, and Co-Cam with a large series of aromatic and heterocyclic sulfonamides, among which are also the clinically used CAIs.

2. Chemistry

Buffers, sulfamide, sulfamic acid, and sulfonamides **1**–**17** were commercially available, of highest purity available, and were used without further purification. Sulfonamides **18**–**20** were prepared as reported earlier by this group.[19,20] Recombinant Cab and Cam isozymes were obtained as previously reported.[13,16] The recombinant α-CA isozymes used for comparison in this study were obtained as previously reported.[21]

3. CA inhibition

There are very few literature data on the inhibition of non-α-CAs with sulfonamides. Forsman[29] reviewed these data recently, showing that only acetazolamide **1**



and ethoxzolamide **3** inhibition data of several plant β-CAs (such as e.g., the enzymes isolated from the dicotyledons *Petroselinus crispus*, *Solanum tuberosum*, *Spinacia oleracea*, *Pisum sativum*, and *Phaseolus vulgaris*, the monocotyledons *Tradescantia albiflora* and *Hordeum vulgare*, and algae belonging to the *Coccomyxa* genera) are available, these sulfonamides inhibiting such β-CAs with inhibition constants in the range of 2–50 μM, being thus much more resistant to these inhibitors as compared to the higher vertebrates CAs (such as e.g., the human red cell isozyme, hCA II, for which these sulfonamides show low nanomolar inhibition constants).[3,4] Thus, this is one of the first systematic inhibition studies of non-α-CAs with sulfonamides. Compounds included in this study were the clinically used sulfonamide CAIs,[3,4] such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, topiramate **4**,[30] valdecoxib **5**,[7b] celecoxib **6**,[7b] dorzolamide **7**, sulfanilamide **8**, dichlorophanamide **17**, as well as sulfanilamide analogs (compounds **9**, **10**, **18**–**20**), halogenated sulfanilamides recently developed by this group,[31] as well as some 1,3-benzenedisulfonamide derivatives (**15** and **16**), structurally related to dichlorophenamide.

Inhibition data against three α-class CA isozymes (the cytosolic hCA I and hCA II, and the membrane-associated hCA IX), Cab and Zn(II)- and Co(II)-Cam with sulfamic acid, sulfamide (the most simple compounds incorporating a sulfonamide moiety), and sulfonamides **1**–**20** are shown in Table 1. Two Cam isozymes were included in this study, one with Zn(II), the other with Co(II) at the active site, since it is not known up to now which is the metal ion present in this enzyme in nature, as purification of Cam in anaerobic conditions led to a very active Fe(II)-containing enzyme, which is unstable in the presence of oxygen.[32]

Table 1. Inhibition constants of sulfonamide/sulfamate inhibitors against α-isozymes hCA I, II, IX, γ isozymes Zn-Cam and Co-Cam, and β-class enzyme Cab, for the CO_2 hydration reaction, at 20 °C[20]

Inhibitor	K_I[a]					
	hCA I[c]	hCA II[c] [μM]	hCA IX[c] [μM]	Zn-Cam [μM]	Co-Cam [μM]	Cab [μM]
$HOSO_2NH_2$[b]	21	390,000	nt	0.096	3.36	103,000
$H_2NSO_2NH_2$	310	1,130,000	nt	69.86	5.96	44,000
1 (Acetazolamide)	0.25	12	25	0.063	1.43	12.1
2 (Methazolamide)	0.05	14	27	0.14	0.17	32.1
3 (Ethoxzolamide)	0.025	8	34	0.20	0.74	5.35
4 (Topiramate)	0.25	5	58	1.02	0.12	23.5
5 (Valdecoxib)	54	43	27	0.13	0.24	61
6 (Celecoxib)	50	21	16	0.14	1.01	38.5
7 (Dorzolamide)	50,000	9	52	0.41	1.71	30.7
8 (Sulfanilamide)	28	300	294	0.25	3.93	57.8
9	25	170	103	0.35	8.50	28.5
10	21	160	33	0.27	0.13	25.7
11	8.3	60	245	0.97	0.42	53.4
12	9.8	110	264	0.14	0.74	111
13	6.5	40	269	1.72	0.28	77
14	6.0	70	285	0.28	0.66	98
15	5.8	63	24	0.83	0.95	50.0
16	8.4	75	39	0.12	0.61	62.1
17	1.2	38	50	0.19	0.15	105
18	78.5	320	305	2.20	0.22	127
19	25	240	238	0.17	1.24	14.3
20	0.10	33	31	0.18	6.36	52.4

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] As sodium salt.
[c] Data for hCA I, II, and IX inhibition from Refs. 7b,9c,27,28.

The following SAR can be drawn from data of Table 1, regarding Zn-Cam inhibition with the investigated derivatives: (i) very potent Zn-Cam inhibitors were sulfamic acid (as sulfamate, since the experiments have been done at pH 7.4 when this compound is ionized) and acetazolamide **1**, which showed inhibition constants in the range of 63–96 nM. The data of sulfamate are really surprising, since this compound whose X-ray crystal structure in the adduct with hCA II has recently been reported,[33] is a much weaker inhibitor of the α-CA isozymes hCA I and II, as well as for Co-Cam and Cab. Sulfamic acid may be thus considered a true Zn-Cam-specific inhibitor. At this point it is also important to note the very weak Zn-Cam inhibitory properties of sulfamide, which has an inhibition constant of around 70 μM for this enzyme, being the worst inhibitor in the entire series of investigated derivatives. We cannot explain these important differences between the two simple and structurally related compounds (sulfamic acid and sulfamide), nor the differences between these diverse CAs in their behavior toward these two inhibitors; (ii) a rather large number of the investigated sulfonamides, such as **2**, **3**, **5**–**10**, **12**, **14**, **16**, **17**, **19**, and **20**, showed a rather efficient Zn-Cam inhibition, with K_Is in the range of 0.12–0.41 μM. The first feature hitting our attention in this case was the rather similar inhibitory properties of such very diverse sulfonamides, which in the case of the different α-CA isozymes showed a much more variable behavior, with affinities between the low nanomolar to the micromolar range. It is thus probable that the active site of Zn-Cam is somehow less differentiated for the interaction with these relatively large organic molecules, as compared to the active site of the α-CAs, but this speculation must be checked experimentally by resolving the X-ray crystal structure of adducts of Zn-Cam with diverse sulfonamides; (iii) the sulfamate **4** (topiramate) as well as several other sulfonamides, such as **11**, **13**, **15**, and **18**, showed a decreased affinity for Zn-Cam as compared to the previously mentioned derivatives, with K_Is in the range of 0.83–1.72 μM.

Co-Cam showed a completely different behavior toward these sulfonamide/sulfamate inhibitors, as compared to Zn-Cam. Thus, (i) several of these derivatives, such as the clinically used derivatives methazolamide **2**, topiramate **4**, valdecoxib **5**, dichlorophenamide **17**, or the simple sulfanilamide analogs **12**, **13**, and **18**, showed good Co-Cam inhibitory properties, with K_Is in the range of 0.12–0.28 μM. It is obvious that these compounds showed completely different inhibitory properties toward the α-CA isozymes and Cab investigated here (Table 1); (ii) another group of derivatives, including acetazolamide **1**, ethoxzolamide **3**, celecoxib **6**, dorzolamide **7**, as well as derivatives **11**, **12**, **14**–**16**, and **19**, showed less effective Co-Cam inhibitory properties, with K_Is in the range of 0.42–1.71 μM. Again these compounds belong to rather disparate classes of sulfonamides, and no clear-cut SAR can be drawn; (iii) some other derivatives such as sulfamic acid, sulfamide, sulfanilamide **8**, compounds **9**, and **20**, were the least effective Co-Cam inhibitors, with K_Is in the range of 3.36–8.50 μM. The difference of activity between homosulfanilamide **9** (the worst Co-Cam inhibitor) and its close derivative **10** (the second best Co-Cam inhibitor discovered here) is rather impressive, considering that the two compounds differ by only a CH_2 moiety, and show again

that much is to be understood regarding inhibition of non-α-CAs by sulfonamides.

Inhibition data of the β-class enzyme, Cab, are again very different from those of the γ-CAs (Zn-Cam and Co-Cam) as well as those of the three α-CA isozymes considered here (hCA I, II, and IX) (Table 1). Thus, (i) sulfamic acid and sulfamide are very weak Cab inhibitors, with affinities in the range of 44–103 mM; (ii) a rather large group of sulfonamides, such as **5**, **8**, **11**–**18**, and **20**, showed moderate Cab inhibitory activity, with K_Is in the range of 50–127 μM. It is thus apparent that Cab is less prone to be inhibited by sulfonamides as compared to both the α- and γ-CAs, being thus more similar to the plant-type β-class enzymes investigated by Forsman,[29] who reported comparable inhibition data of acetazolamide and ethoxzolamide for some of these enzymes; (iii) the best Cab inhibitors detected here were acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, topiramate **4**, celecoxib **6**, dorzolamide **7**, as well as derivatives **9**, **10**, and **19**, with K_Is in the range of 5.35–38.5 μM. It appears that heterocyclic sulfonamides (and especially the bicyclic derivative ethoxzolamide) lead to the strongest inhibitors, but clear-cut SAR is again difficult to draw from these data.

In conclusion, a detailed inhibition study of CAs belonging to the β- and γ-CA families from *Archaea* with sulfonamides is presented for the first time here. The two γ-CAs from *Methanosarcina thermophila* (Zn-Cam and Co-Cam) showed very different inhibitory properties with these compounds, as compared to the α-CA isozymes hCA I, II, and IX, and the β-CA from *Methanobacterium thermoautotrophicum* (Cab). The best Zn-Cam inhibitors were sulfamic acid and acetazolamide, with inhibition constants in the range of 63–96 nM, whereas other investigated aromatic/heterocylic sulfonamides showed a rather levelled behavior, with K_Is in the range of 0.12–1.70 μM. The best Co-Cam inhibitors were topiramate and *p*-aminoethyl-benzenesulfonamide, with K_Is in the range of 0.12–0.13 μM, whereas the worst one was homosulfanilamide (K_I of 8.50 μM). In the case of Cab, the inhibitory power of these compounds varied to a much larger extent, with sulfamic acid and sulfamide showing millimolar affinities (K_Is in the range of 44–103 mM), whereas the best inhibitor was ethoxzolamide, with a K_I of 5.35 μM. Most of these sulfonamides showed inhibition constants in the range of 12–100 μM against this enzyme. Thus, the three CA families investigated up to now possess a very diverse affinity for sulfonamides, the inhibitors with important medicinal, and environmental applications.

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21. Human CA I, and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the corresponding plasmids described by Lindskog's group (Lindskog, S.; Behravan, G.; Engstrand, C.; Forsman, C.; Jonsson, B. H.; Liang, Z.; Ren, X.; Xue, Y. In *Carbonic Anhydrase—From Biochemistry and Genetics to Physiology and Clinical Medicine*; Botrè, F., Gros, G., Storey, B. T., Eds.; VCH: Weinheim, 1991; pp 1–13). Cell growth conditions were those described in Ref. 15 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[26] 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.[22–24] The cDNA of the catalytic domain of hCA IX (isolated as described in Pastorek, J.; Pastorekova, S.; Callebaut, I.; Mornon, J. P.; Zelnik, V.; Opavsky, R.; Zatovicova, M.; Liao, S.; Portetelle, D.; Stanbridge, E. J.; Zavada, J.; Burny, A.; Kettmann, R. *Oncogene* **1994**, *9*, 2877–2888) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100. The homogenate thus obtained was extensively centrifuged in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione, 1 mM oxidized glutathione. Active hCA IX was extensively dialyzed into a solution of 10 mM Hepes (pH 7.4), 10 mM Tris HCl, 100 mM Na_2SO_4, and 1 mM $ZnCl_2$, passed on a sulfonamide affinity column from which it was eluted with sodium azide, as described earlier.[25] The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate.[26]
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26. An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA hydration activity. 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—this anion is not inhibitory anyhow, see Table 1), 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. Stock solutions of inhibitors were prepared at a concentration of 10–200 mM (in the assay buffer) and dilutions up to 0.01 μM done with the assay buffer mentioned above. Enzyme concentrations were 0.09 μM for hCA I and hCA IX, 0.1 μM for Cab, and 0.01 μM for hCA II, Zn-Cam and Co-Cam. Inhibition constants were calculated as described by Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573, and are the mean form three different determinations.
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