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Carbonic anhydrase inhibitors: Inhibition of the transmembrane isozyme XIV with sulfonamides

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Abstract—The inhibition of the last human carbonic anhydrase (CA, EC 4.2.1.1) isozyme (hCA XIV) discovered has been investigated with a series of sulfonamides, including some clinically used derivatives (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and zonisamide), as well as the sulfamate antiepileptic drug topiramate. The full-length hCA XIV is an enzyme showing a medium-low catalytic activity, quite similar to that of hCA XII, with the following kinetic parameters at 20 °C and pH 7.5, for the CO_2 hydration reaction: $k_{cat} = 3.12 \times 10^5\ s^{-1}$ and $k_{cat}/K_M = 3.9 \times 10^7\ M^{-1}\ s^{-1}$. All types of activities have been detected for the investigated compounds, with several micromolar inhibitors, including zonisamide, topiramate, and simple sulfanilamide derivatives (K_I-s in the range of 1.46–6.50 μM). In addition, topiramate and zonisamide were observed to behave as weak hCA XII inhibitors, while zonisamide was an effective hCA IX inhibitor (K_I of 5.1 nM). Some benzene-1,3-disulfonamide derivatives or simple five-membered heteroaromatic sulfonamides showed K_I-s in the range of 180–680 nM against hCA XIV, whereas the most effective of such inhibitors, including 3-chloro-/bromo-sulfanilamide, benzolamide-like, ethoxzolamide-like, and acetazolamide/methazolamide-like derivatives, showed inhibition constant in the range of 13–48 nM. The best hCA XIV inhibitor was aminobenzolamide (K_I of 13 nM), but no CA XIV-selective derivatives were evidenced. There are important differences of affinity of these sulfonamides/sulfamates for the three transmembrane CA isozymes, with CA XII showing the highest affinity, followed by CA IX, whereas CA XIV usually showed the lowest affinity for these inhibitors.
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At least 15 isoforms of the widely spread metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) were discovered up to now in humans.[1–4] These are hCA I–hCA XIV, but there are two CA V-like enzymes, i.e., hCA VA and hCA VB,[5,6] which lead to the final number of 15 isozymes (although they are numbered from I to XIV). Isozyme CA XIV was the last to be discovered by Nishimori's group,[7] who sequenced the gene encoding this protein, mapped it on chromosome 1, and expressed/purified the enzyme. The catalytic domain of the corresponding murine enzyme (mCA XIV) was then crystallized and the X-ray structure reported by Christianson's group,[8] whereas research from Nishimori's,[7,9] Parkkila's and Sly's groups[10–12] revealed its distribution in the human body as well as potential physiological/pathological roles. Indeed, it has been observed that hCA XIV is highly abundant in the brain, kidney, colon, small intestine, urinary bladder, liver, and spinal cord.[7,9–12] It has been then shown that in kidneys, the luminal CA XIV is involved in bicarbonate reabsorption (a function previously considered to be played by CA IV),[10] whereas the same group also showed that in hepatocytes plasma membranes CA XIV is involved in the control of pH and ion transport processes among the hepatocytes, sinusoids, and bile canaliculi.[11] Finally, this extracellular CA isoform is highly abundant in neurons and axons in the murine and human brains, where it seems to play an important role in modulating excitatory synaptic transmission.[12]

Similar to isozymes CA IX[1,13] and CA XII,[14] CA XIV is a transmembrane isozyme with the active site oriented extracellularly,[7,8] but unlike these two CAs, it seems to be not associated predominantly with tumors.[1] Further-

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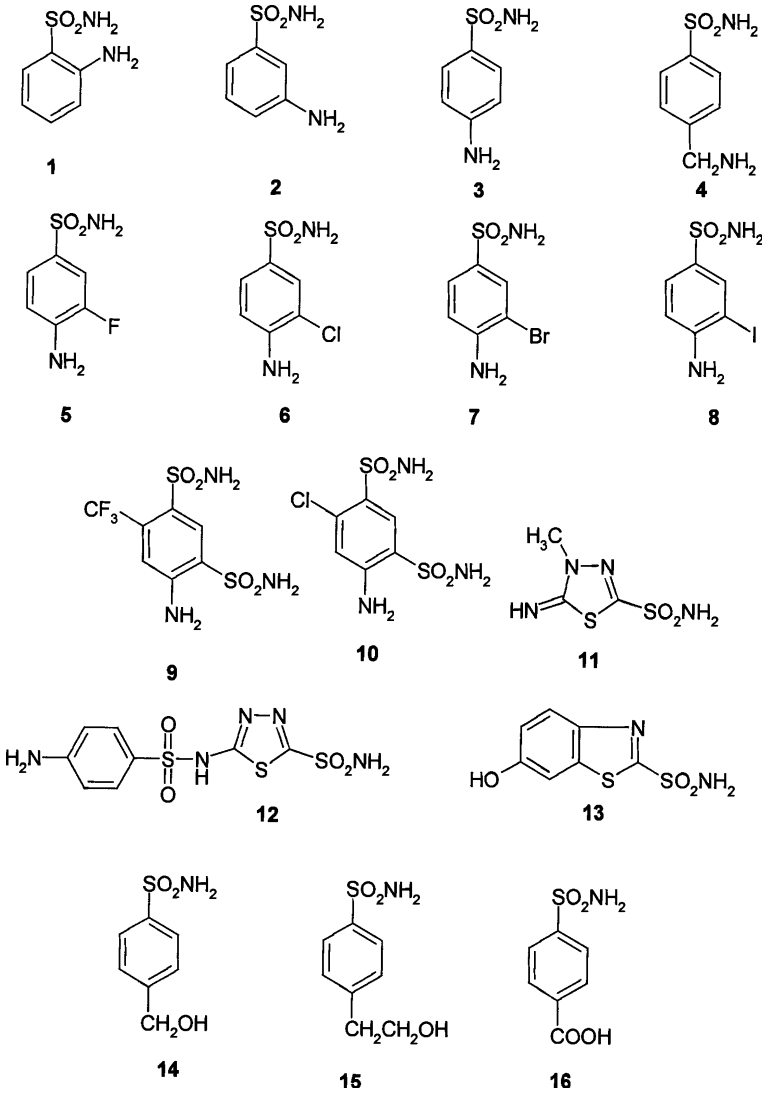
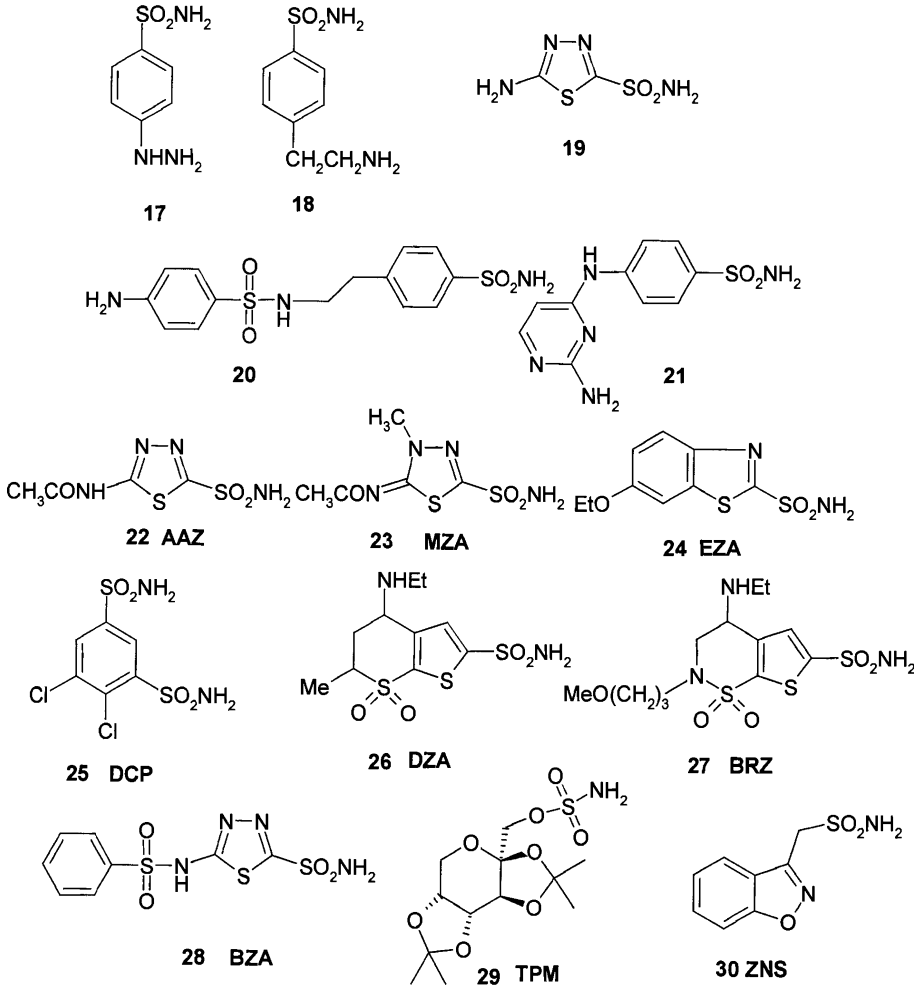
more, similar to the above-mentioned two isozymes, CA XIV possesses a highly conserved catalytic domain rather similar even with that of the cytosolic isozymes CA I and CA II, in addition to a short intracellular polypeptide segment and a single transmembrane helix (CA IX also possesses in addition to these domains an extracellular quite large proteoglycan-like segment).[1,7,8,13,14] As the X-ray crystal structures of both the catalytic domains of hCA XII[15] and mCA XIV[8] were reported, a high degree of similarity between these two proteins, has been noted as they both contain: (i) a Zn(II) ion coordinated by three histidine residues (His94, His96, and His119, CA II numbering of the amino acid residues)[8] and a water molecule (similar to all known α-CAs investigated up to now),[1] (ii) identical door-keeping residues in the neighborhood of the catalytic zinc (i.e., Thr199 hydrogen bonded to the zinc-bound water molecule/hydroxide ion and to the carboxylate moiety of Glu106), and (iii) the same proton shuttle residue, His64.[16] All these active site amino acid residues play important roles in the α-CAs catalytic cycle or to their inhibition by sulfonamides and other types of inhibitors.[16]

We have previously investigated the inhibition of most of the known human CAs (except CA Vb and CA XIV) with sulfonamides and anions, the most investigated classes of CA inhibitors (CAIs).[16–25] It has thus been shown that many of these CA isozymes are druggable targets, and that their inhibitors may have applications in the design of antiglaucoma, antitumor, anticonvulsant or antiobesity therapies among others.[16–25] Here, we report the first hCA XIV inhibition study with a large series of sulfonamides/sulfamates, showing that this widely distributed isoform may also be a target for the design of therapeutic agents. We also report a method for producing high amounts of the full-length (337-amino acid polypeptide) isozyme hCA XIV.

Sulfonamides/sulfamates investigated for the inhibition of the transmembrane isozyme hCA XIV, of types **1**–**30** are shown below. Derivatives **22**–**30** are clinically used drugs, such as acetazolamide **22**, methazolamide **23**, ethoxzolamide **23**, and dichlorophenamide **24**—the classical CAIs. Dorzolamide **26** and brinzolamide **27** are topically acting antiglaucoma agents, benzolamide **28** is an orphan drug belonging to this class of pharma-





cological agents,[16] whereas topiramate **29** and zonisamide 30 are antiepileptic drugs.[26,27] Compounds **1**–**4**, **9**, **10**, **16**, **21**–**27**, **29**, and **3** are commercially available from Sigma–Aldrich, Merck, Alcon, Johnson & Jonson or DaiNippon, whereas **5**–**8**,[28] **11**–**15**,[29,30] **20**,[31] and **28**,[31] were prepared as reported earlier by this group.

There are conflicting literature data regarding the catalytic activity of CA XIV: Nishimori's group[7] reported full-length hCA XIV (obtained in *Escherichia coli*) to possess a medium-low catalytic activity (of around 10% that of hCA II), whereas Whittington et al.[8] reported that their preparations of mCA XIV (obtained in Chinese hamster ovary cells as expressing system) show very high catalytic activity for the CO_2 hydration reaction (of around 130% that of hCA II), but in both these valuable studies no kinetic parameters have been obtained. These discrepancies may be due to the fact that the murine and human isozymes may show indeed quite different catalytic properties (a recent such example is furnished by the human and bovine CA IV which show very different catalytic activities and affinities for sulfonamides),[17] or to the fact that the enzymes produced in prokaryotic expressing systems are not glycosylated, in contrast to those obtained in eukaryotes. However, usually the glycosylation sites are far from the active site, as seems to be the case for CA XIV too,[7] and probably the above-mentioned discrepancy is due to the different mammalian species from which the two enzymes have been obtained. Here, we confirm the original finding of Fujikawa-Adachi et al.[7] that hCA XIV is an enzyme showing a medium-low catalytic activity, quite similar to that of hCA XII, to which this isozyme is the most similar both from the point of view of the sequence[7] as well as from that of the tridimensional structure[8] (Table 1). Furthermore, the originally reported[7] GST–hCA XIV construct and expressing system in *E. coli* affords the enzyme in good yields and with a rather simple purification procedure, involving two affinity column purification steps: one on a glutathione–Sepharose column and the other one on a sulfonamide affinity column.[32]

As seen from data of Table 1, hCA XIV has a catalytic activity similar to that of the slow cytosolic red blood cell isozyme hCA I or that of the related transmembrane isoform hCA XII (catalytic domain).[2,19] The kinetic parameters for the CO_2 hydration reaction catalyzed by full-length hCA XIV at 20 °C and pH 7.5, determined by a stopped-flow technique,[34] were $k_{cat} = 3.12 \times 10^5\ s^{-1}$, and $k_{cat}/K_M = 3.9 \times 10^7\ M^{-1}\ s^{-1}$. These parameters are at least one order of magnitude lower than those of the very efficient catalyst which is

Table 1. Kinetic parameters for CO_2 hydration reaction catalyzed by the cytosolic α-CA isozymes I–III, and the transmembrane isozymes hCA XII (catalytic domain) and hCA XIV (full length), at 20 °C and pH 7.5, and their inhibition data with acetazolamide **AAZ** (5-acetamido-l,3,4-thiadiazole-2-sulfonamide), a clinically used compound

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I[a]	Low	2.0×10^5	5×10^7	250
hCA II[a]	Very high	1.4×10^6	1.5×10^8	12
hCA III[b]	Very low	1.0×10^4	3×10^5	300,000
hCA XII[c]	Low	4.20×10^5	3.5×10^7	5.7
hCA XIV	Low	3.12×10^5	3.9×10^7	41

[a] From Ref. 2.
[b] From Ref. 16.
[c] From Ref. 19.

hCA II,[1,16] and quite comparable with the corresponding kinetic parameters of hCA I and hCA XII (catalytic domain). On the other hand, hCA XIV is a much better catalyst as compared to the catalytically very inefficient isozyme hCA III (Table 1). It may be thus speculated that the extracellular CA isoforms (CA IX, CA XII, and CA XIV) may have evolved quite early during the evolution of life on earth, as catalysts needed to convert carbon dioxide to bicarbonate which may subsequently be used in biosynthetic reactions involving C1 units. Indeed, many of these isoforms are overexpressed in hypoxic tumors[33] in which the metabolic pathways are completely different from those found in normal cells, due to the lack of oxygen and the acidic environment. Data in Table 1 also show that similar to hCA II and hCA XII, the new isozyme investigated here, hCA XIV, is inhibited by acetazolamide, the sulfonamide CAI par excellence, whereas hCA I has a lower affinity for this inhibitor. hCA III has very low affinity for sulfonamide CAIs,[1,16] as also seen from data in Table 1.

Detailed inhibition data of hCA XIV with sulfonamides/sulfamates **1**–**30** are shown in Table 2, together with hCA IX and hCA XII inhibition data, since these transmembrane CA isozymes are most similar to the target isoform investigated here.

The following should be noted regarding data in Table 2: (i) a group of compounds, including the clinically used sulfamate topiramate **29** and the antiepileptic sulfonamide zonisamide **30**, as well as derivatives **1**–**4**, **14**, **15**, **17**, and **18**, showed weak hCA XIV inhibitory activity, with inhibition constant in the range of 1.45–6.50 μM. It may be observed that most of these compounds (except the two clinically used drugs **29** and **30)** are simple benzenesulfonamide derivatives, monosubstituted in *ortho*-, *meta*- or *para* with compact moieties such as amino, hydroxy, aminomethyl/ethyl, hydroxymethyl/ethyl or hydrazino. Furthermore, it should be noted that many of these simple benzenesulfonamides act as much more effective inhibitors of the other two transmembrane CA isozymes, CA IX and CA XII, with efficiencies in the low nanomolar range in some cases. Thus, there are important differences between the inhibitory properties of these sulfonamides/sulfamates against the three transmembrane CA isozymes, with CA XIV being the most resistant to inhibition, followed by CA IX, whereas CA XII is the most prone to be inhibited by most of these compounds. Since

Table 2. hCA IX, hCA XII, and hCA XIV inhibition data with compounds **1–30**

Inhibitor	K_I[a] (nM)		
	hCA IX[b]	hCA XII[b]	hCA XIV[c]
1	33	085	6500
2	238	125	4800
3	294	37	5400
4	103	0.3	3200
5	245	1.1	180
6	264	3.1	21
7	269	20	15
8	285	11	79
9	24	45	680
10	39	44	570
11	30	4	245
12	38	85	13
13	14	3.7	48
14	21	220	3840
15	22	55	3255
16	26	34	780
17	305	11	1450
18	33	3.2	2900
19	41	33	280
20	24	4.5	76
21	16	3.4	89
AAZ 22	25	5.7	41
MZA 23	27	3.4	43
EZA 24	34	22	25
DCP 25	50	50	345
DZA 26	52	3.5	27
BRZ 27	37	3.0	24
BZA 28	47	3.5	33
TPM 29	1590	3800	1460
ZNS 30	5.1	11,000	5250

Data for hCA IX and hCA XII are from Refs. 19 and 25 except the hCA IX topiramate and benzolamide data, and the zonisamide hCA IX and hCA XII data which are new.
[a] Errors in the range of 5–10% of the shown data, from three different assays.
[b] Catalytic domain of the human recombinant isozymes.
[c] Full length, human recombinant isozyme, CO_2 hydrase assay method.[34]

both topiramate and zonisamide were not investigated in detail up to now against the other two cancer-associated transmembrane isozymes, CA IX and CA XII, it must be stressed that these data show topiramate to be a weak hCA IX inhibitor, whereas zonisamide to be a very effective one (K_I of 5.1 nM). Both compounds act on the other hand as extremely weak hCA XII inhibitors

(inhibition constant in the range of 3.8–11.0 μM). Again we confirm the early data of Fujikawa-Adachi et al.[7] that sulfanilamide **3** is a weak CAI; (ii) another group of derivatives, such as **5**, **9**–**11**, **16**, **19**, and **DCP 25**, behaved as medium potency hCA XIV inhibitors, with K_I-s in the range of 180–680 nM, being usually much more potent hCA IX and hCA XII inhibitors (except **5**, which is a rather weak hCA IX inhibitor). These compounds are either benzene-1,3-disulfonamide derivatives (**9**, **10**, and **25**), simple five-membered heteroaromatic sulfonamides (**11** and **19**) or substituted benzenesulfonamides incorporating fluorine, amino, and carboxyl moieties (**5** and **16**); (iii) a small number of derivatives, including iodosulfanilamide **8**, and the derivatives **20** and **21** incorporating two cyclic moieties in their molecule, behaved as effective hCA XIV inhibitors, with inhibition constant in the range of 76–89 nM; (iv) the most effective hCA XIV inhibitors detected in the library of derivatives investigated here were **6**, **7**, **12**, **13**, **22**–**24**, and **26**–**28**, which showed K_I values in the range of 13–48 nM. Several structural classes of potent hCA XIV inhibitors are thus so far evidenced. They include among others some halogenated sulfanilamides, such as the 3-chloro- and 3-bromo-sulfanilamides **6** and **7** (it should be noted that the corresponding fluoro- and iodo-derivatives are weaker inhibitors, as discussed above, and that all these compounds are generally ineffective hCA IX inhibitors but very effective hCA XII inhibitors). Other structural classes among the best hCA XIV inhibitors detected here, include the benzolamide-like derivatives **12** and **28**, the ethoxzolamide-like compounds **13** and **24**, together with the other bicyclic ring systems incorporating the thienothiopyran/thiazine rings present in dorzolamide and brinzolamide, respectively, as well as the 1,3,4-thiadiazole/thiadiazoline-sulfonamide derivatives used clinically, acetazolamide **22**, and methazolamide **23**. It may be thus observed that acetylation of the imine **11** or the amine **19** leads to a drastic increase in hCA XIV inhibitory properties of the corresponding derivatives, whereas the corresponding differences for the inhibition of hCA IX or hCA XII with such pairs of compounds are not at all so important. All these data suggest that hCA XIV is a druggable enzyme and that SAR of sulfonamide/sulfamate inhibitors may be quite different from those corresponding to the related transmembrane isozymes hCA IX and hCA XII investigated earlier. This may also lead to the possible design of hCA XIV-selective inhibitors, since the compounds investigated here do not show selectivity for any of the three transmembrane isozymes CA IX, CA XII or CA XIV.

In summary, the first CA XIV inhibition study is presented here. The full-length hCA XIV is an enzyme showing a medium-low catalytic activity, quite similar to that of hCA XII, with the following kinetic parameters at 20 °C and pH 7.5, for the CO_2 hydration reaction: $k_{cat} = 3.12 \times 10^5\ s^{-1}$ and $k_{cat}/K_M = 3.9 \times 10^7\ M^{-1}\ s^{-1}$. A large series of sulfonamides and a sulfamate have been tested for their interaction with this isoform, including clinically used derivatives such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and zonisamide, as well as the sulfamate antiepileptic drug topiramate. All types of activities have been detected, with several micromolar inhibitors, including zonisamide, topiramate, and simple sulfanilamide derivatives (K_I in the range of 1.46–6.50 μM). It has also been observed that topiramate and zonisamide are very weak hCA XII inhibitors, whereas zonisamide is an effective hCA IX inhibitor (K_I of 5.1 nM). Some benzene-1,3-disulfonamide derivatives or simple five-membered heteroaromatic sulfonamides showed K_I-s in the range of 180–680 nM against hCA XIV, whereas the most effective of such inhibitors, including 3-chloro-/bromo-sulfanilamide, benzolamide-like, ethoxzolamide-like, and acetazolamide/methazolamide-like derivatives, showed inhibition constant in the range of 15–48 nM. The best hCA XIV inhibitor was aminobenzolamide (K_I of 13 nM), but no CA XIV-selective derivatives were evidenced. There are important differences of affinity of these sulfonamides/sulfamates for the three transmembrane CA isozymes, with CA XII showing highest affinity, followed by CA IX, whereas CA XIV usually showed the lowest affinity.

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32. The CA XIV-GST construct: A putative full-length cDNA of hCA XIV (Accession No. AB025904) was obtained by RT-PCR with poly(A) RNA from the human spinal cord (Clontech, Palo Alto, CA) and the 5′ and 3′ rapid amplification of cDNA ends (RACE) has been performed by the previously described method.[7] The cDNA fragment encoding the open reading frame of hCA XIV was amplified by PCR using adopter primers including *Eco*RI and *Sal*I recognition sequences (underlined in the following sequences, respectively): 5′-CCGAATTCGGACTGC ATGTTGTTCTCCGCCCTCCT-3′ and 5′-TTTGTCG ACTTATGCCTCAGTCGTGGCTT-3′. The PCR was hot-started with incubation for 5 min at 94 °C and consisted of 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The PCR products were cleaved with the corresponding restriction enzymes, purified, and cloned in-frame into a modified pGEX-4T2 vector using T4-ligase (Promega). The proper cDNA sequence of the hCA XIV insert included in the vector was reconfirmed by DNA sequencing. The constructs were then transfected into *E. coli* strain BL21 for production of the hCA XIV protein, similar to the procedure already described for hCA IX.[33] The protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside, 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) 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 (Amersham). The column was extensively washed with buffer and then the fusion (GST–hCA XIV) 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 hCA XIV is purified quite easily and the procedure is quite simple. The obtained hCA XIV was further purified by sulfonamide affinity chromatography, the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[34]
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34. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 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), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of 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, to allow for the formation of the E–I complex. The inhibition constant were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier,[16,26] and represent the mean from at least three different determinations. The other cloned enzymes (hCA IX and hCA XII) were obtained as reported earlier by this group.[18–23]