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Carbonic anhydrase inhibitors. Inhibition studies of the human secretory isoform VI with anions

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Abstract—The unique secretory isozyme of human carbonic anhydrase (hCA, EC 4.2.1.1), hCA VI, has been cloned, expressed, and purified. The kinetic parameters for the CO_2 hydration reaction proved hCA VI to possess a k_{cat} of 3.4×10^5 s^{-1} and k_{cat}/K_M of 4.9×10^7 M^{-1} s^{-1} (at pH 7.5 and 20 °C). hCA VI has a significant catalytic activity for the physiological reaction, of the same order of magnitude as isoforms CA I or CA IX. A series of anions (such as bicarbonate, chloride, nitrate, etc.) were shown to inhibit the activity of the enzyme, with inhibition constants typically in the range of 0.60–0.90 mM. The best hCA VI inhibitors were cyanide, azide, sulfamide, and sulfamate, with inhibition constants in the range of 70–90 μM.

Isozyme VI is the only secreted form among the 16 carbonic anhydrases (CAs, EC 4.2.1.1) described up to now in mammals.[1–3] Indeed, this family of metalloenzymes is widespread all over the phylogenetic tree,[4] being fundamental to a variety of physiological processes. By catalyzing the interconversion between carbon dioxide and bicarbonate, with generation of a proton, CAs operate on three very simple molecules/ions involved in a variety of critical life processes.[1–4] Among them, the most important ones are pH regulation, respiration, secretion of electrolytes, biosynthesis of some important biomolecules such as urea, glucose, lipids, and pyrimidines, excretion of acid and salts, carcinogenesis, signaling, etc.[1–7] Different isozymes among the 16 presently known participate in such processes.[1–7] Indeed, these isoforms show a very diverse cellular localization, catalytic activity, and susceptibility to be inhibited/activated by various endogenous or exogenous regulators of activity.[1–7] In humans only 15 of the 16 mammalian isoforms are present, due to the fact that CA XV is encoded by a pseudogene in all primates investigated so far.[8] Among these remaining 15 isoforms, 12 possess catalytic activity for the CO_2 hydration reaction, whereas CA VIII, X, and XI are devoid of this activity, as these proteins lack one or more histidine residues coordinating the catalytically critical Zn(II) ion within the enzyme active site.[9] CA I–III, VII, and XIII are soluble, cytosolic isozymes, CA IV and XV are extracellular, membrane-anchored enzymes by means of glycosylphosphatidylinositol (GPI) tails, CA VA and VB are mitochondrial enzymes, whereas CA IX, XII, and XIV are transmembrane proteins with the active site situated outside the cell.[1–8] CA VI, the only secreted CA isoform, has been initially identified in sheep saliva and parotid glands by Fernley's group in 1979.[10,11] CA VI was thereafter shown to be secreted in saliva, tears, and milk of all mammals,[10–14] where it may play important physiological roles, some of which are little understood at this moment. More recently, the enzyme was also evidenced in enamel organs in the rodent teeth,[15] but it is not well understood whether the enzyme is secreted into the enamel layers or onto the intercellular spaces of the enamel organ.

There are quite conflicting literature data[11,12] regarding the catalytic activity of CA VI, and virtually no inhibition study of this enzyme. Here, we present the first study regarding the DNA cloning, purification, enzymatic characterization, and inhibition data with a large

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number of simple anions of the human secreted isoform hCA VI.

hCA VI cloning, purification, and catalytic activity. The secretory isoform hCA VI contains an N-terminal 17 amino acid signal sequence typical for secreted proteins.[11a] We obtained a cDNA clone encoding an open-reading frame of hCA VI and successfully produced the full-length enzyme in soluble form by the GST-fusion protein method.[16–19] To date, complete amino acid sequences in the open-reading frame of three hCA VI clones have been deposited in the GenBank (Accession Nos. NM_001215, M57892, and AF128411). The amino acid sequence of our clone was identical to that of clone NM_001215. The other two clones showed different amino acid usage at three sites, that is, residues Ser:Gly at position 70 (numbering is based on CA I sequence, see Fig. 1), Ser:Thr at position 130, and Asn:Lys at position 253. Considering the significance of these amino acids in the CA active site (mainly those in position 70 and 130 which are adjacent to two critical residues involved in the binding of inhibitors/activators,[20–23] that is, amino acids 69 and 131), these substitutions probably represent normal polymorphisms.

The amino acid sequence of the CA domain which was deduced from the cDNA sequence of our hCA VI clone was aligned with that of three CA isozymes, hCA I, hCA II (cytosolic isozymes) and hCA IX (transmembrane, cancer-associated isoform), as shown in Figure 1. hCA VI showed an overall similarity of 31–40% with these isozymes. The highest similarity was observed with hCA IX, probably because both hCA VI and hCA IX are extracellular isozymes. In Figure 1, the 36 residues that were previously shown to form the active site cavity in all α-CAs[23–25] are indicated by a mixture of asterisk, plus sign, and 'z' above the hCA II sequence. Among these active site residues, 25 amino acids are conserved between hCA VI and hCA II, the isozyme showing the strongest CA activity in the α-CA family (Table 1). Interestingly, hCA I and hCA IX also showed comparable sequence homology of 24 and 26 amino acid residues with hCA II, respectively, whereas the overall hCA VI versus hCA I homology was 30.8%, that of hCA VI versus hCA II 34.1%, and that of hCA VI versus hCA IX 39.8%. It is important to note that all the amino acid residues which are critical in the CA catalytic cycle are conserved in all these isozymes: (i) the three zinc ligands, His94, 96, and 119; (ii) the 'gate-keeping' residues Thr199 and Glu106, which orient the substrate in the right position to be attacked by the zinc-bound hydroxide ion; and (iii) His64, the proton shuttle residue, which transfers protons from the zinc bound water molecule toward the external medium, leading to the generation of the active form of the enzyme with hydroxide as the fourth zinc ligand.[1–4] Thus, hCA VI has all the requisites to show a catalytic activity comparable to that of

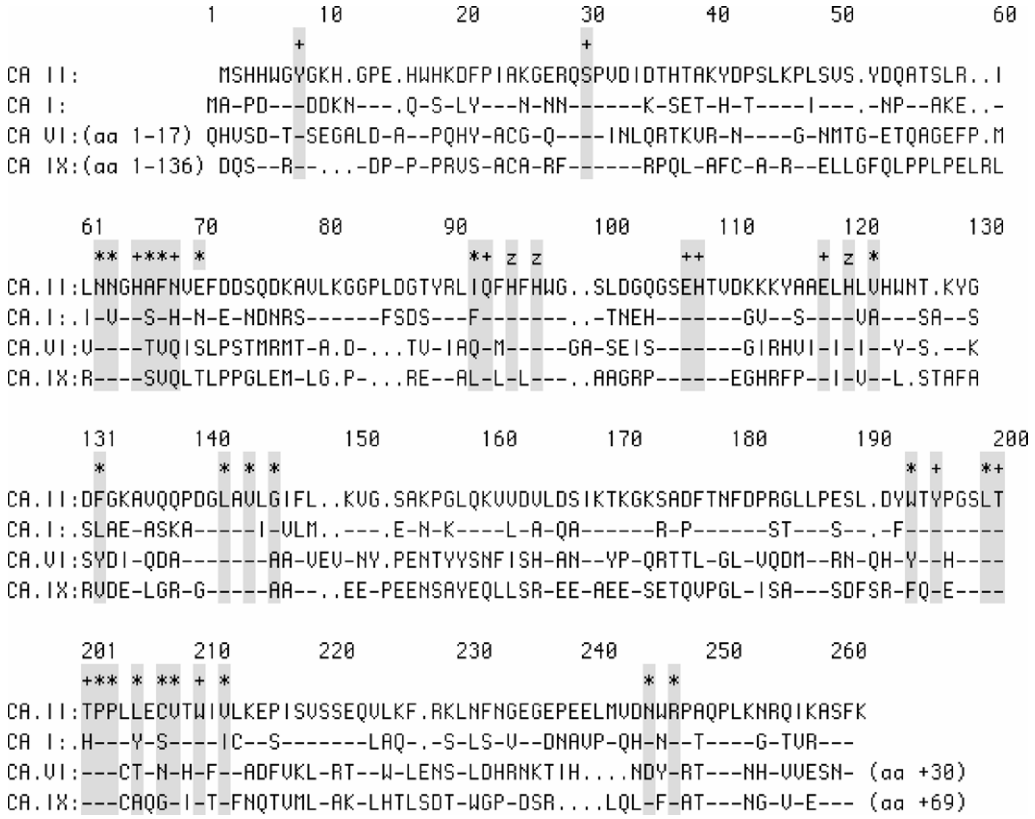

Figure 1. Sequence alignment of the CA domain of human isozymes hCA I, II, VI, and IX. The residues indicated by a mixture of asterisk, plus sign, and 'z' above the hCA II sequence are those forming the active site. Seventeen residues known to participate in a network of hydrogen bonds crucial for the binding of inhibitors/activators are indicated by plus; 'z' indicates the three zinc liganded histidines. Conserved amino acids in comparison to hCA II are indicated by a bar. A dot indicates no amino acid residue comparable to some other isozyme in the sequence.

Table 1. Kinetic parameters for the CO_2 hydration reaction catalyzed by the cytosolic α-hCA isozymes I and II, the mitochondrial isozyme VA, the secreted isoform hCA VI (full-length), and the transmembrane isozymes hCA IX (catalytic domain) and hCA XII (catalytic domain), at 20 °C and pH 7.5, and their inhibition data with acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used drug[1]

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I	Moderate	2.0×10^5	5.0×10^7	250
hCA II	Very high	1.4×10^6	1.5×10^8	12
hCA VA	Low	2.9×10^5	2.9×10^7	63
hCA VI	Moderate	3.4×10^5	4.9×10^7	11
hCA IX	High	3.8×10^5	5.5×10^7	25
hCA XII	Moderate	4.2×10^5	3.5×10^7	5.7

isozymes I, II or IX investigated in great detail earlier.[1] There are however several amino acid residues which are characteristic only to CA VI among the α-CA isozymes, such as among others those in position 20, 31, 40, 50, 60, 65, 69, 91, 93, 131, 170, 171, 193, 205, 207, and 209 (Fig. 1). In some cases these amino acids are burried within the protein structure, but some of them are known to be important for the active site architecture, being involved in the binding of inhibitors and/or activators.[5,7,19,23–26] Among these amino acid substitutions typical for the secreted form CA VI, we should mention Thr65 which is nearby the conserved proton shuttle residue of all α-CAs, His64. This amino acid is a Ser in CA I and IX, and an Ala in CA II. It is tempting to hypothesize that the decreased catalytic efficiency of CA I, VI (see discussion later in the text), and IX as compared to CA II (possessing Ala in this position) may be due to the fact that the bulkier Ser/Thr residues (as compared to Ala) are interfering with the flip movement of His64. In fact it is well known that this proton shuttle residue has a great mobility in order to assist the proton transfer processes between the enzyme active site and the environment, being observed in many X-ray crystal structures with at least two conformation, the 'in' and 'out' ones.[26] Residues 69 and 131, which are an Ile and Tyr in CA VI, respectively, were shown to be involved in the binding of inhibitors/activators in several other isozymes.[23–26] Furthermore, residues close to the zinc ligands, for example, those in positions 91 and 93, are also characteristic only to CA VI (Fig. 1). All these particular amino acids explain in fact the unique activity and inhibition profile of this isozyme, as compared to all other characterized α-CAs investigated up to now (see the kinetics and inhibitor binding discussion later in the text).

The kinetic parameters for the CO_2 hydration reaction catalyzed by this newly purified recombinant isozyme, hCA VI, as well as for other isoforms which are targets for the drug design (such as hCA I, II, VA, IX, and XII), are shown in Table 1. As shown above, literature data regarding the catalytic activity of hCA VI are rather conflicting,[11,12] with Murakami and Sly[12] reporting hCA VI to have about 1% of the catalytic activity of hCA II, whereas Fernley[11] showed the secreted isoform to be much more active, with a catalytic activity of around 25–30% that of the perfectly evolved catalyst which is hCA II.[1] It should be mentioned that both groups used hCA VI purified from saliva by sulfonamide column chromatography. The catalytic activity of our recombinant isoform, which is practically free of any contaminant protein, showed hCA VI to have indeed a lower catalytic activity as compared to hCA II, but of the same order of magnitude as that of the highly abundant, cytosolic isoform hCA I (Table 1). Indeed, both isoforms have very similar k_{cat} and almost an identical k_{cat}/K_m, close to 5.0×10^7 M^{-1} s^{-1} (at pH 7.5 and 20 °C, see Table 1). This is indeed around 33% of the catalytic activity (if one compares k_{cat}/K_M of various isoforms, Table 1) of hCA II. Thus, hCA VI is less effective than hCA II as a catalyst for CO_2 hydration to bicarbonate, but it has a comparable activity with that of two highly investigated and important isozymes, such as CA I (cytosolic isoform) and IX (transmembrane, tumor-associated isozyme). Furthermore, hCA VI is also more active than isozymes VA or XII, some of which were previously shown to be important drug targets.[1–4] It may be also observed from data of Table 1 that all these isozymes are susceptible to inhibition by acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), **AAZ**, the clinically used sulfonamide CA inhibitor par excellence.[1]

hCA VI inhibition by anions. Apart from the sulfonamides, anions constitute the second class of CA inhibitors.[1] Being zinc-containing enzymes, CAs bind both metal-complexing as well as metal non-complexing anions with different affinities.[27–30] Indeed, many human CA isoforms have been investigated for their interaction with anions, such as the typical metal poisons cyanide, (thio)cyanate, hydrogen sulfide, azide, etc., but also by some anions which show less propensity to complex metal ions in solution (such as sulfate, nitrate, perchlorate, etc.).[27–30] Thus, anion inhibition studies of the human isozymes I, II, IV, VA, VII, IX, and XIII have been reported by our group in recent years.[27–30] However, no anion inhibition data of hCA VI are available in the literature. Such data might be important from the physiological point of view, since it has been shown that in metabolically active tissues, and especially in situations requiring efficient ion transport, many CA isoforms interact with bicarbonate transporters or biosynthetic enzymes to form spatially and functionally orchestrated protein complexes called metabolons.[31] CAs can improve the transmembrane movement of bicarbonate or other membrane-impermeable anions which are transported by integral membrane proteins including the Cl^-/HCO_3^- anion exchangers (AEs), Na^+-coupled HCO_3^- co-transporters (NBCs), and SLC26A transporters.[31] The role of CAs (which physically interact with these anion transporters) in these metabolons is to increase the local availability of bicarbonate (or other

anions, such as sulfate or carboxylates) and thereby accelerate their transmembrane flux.[31] Via this mechanism, various CA isoforms contribute to modulation of pH at both sides of the plasma membranes.[31] It is thus clear that in addition to their catalytic activity, most CAs are also involved in much more complex processes in which they interact with anions present in rather high concentrations in various tissues, as well as with their transporters. Continuing our investigations on the interactions of CAs with various classes of modulators of their activity, we report here the first anion inhibition study of the secreted isozyme hCA VI.

Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, and sulfate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid were also commercially available compounds.

Data of Table 2 show the following regarding hCA VI inhibition with this set of physiological and non-physiological anions (literature inhibition data[27,28] of isozymes I, II, and IX are also included in the table for the sake of comparison, as they are useful for better understanding the significance of CA VI inhibition data): (i) the least effective hCA VI anion inhibitors were bisulfite, sulfate, and phenylarsonic acid, which showed K_Is in the range of 9.9–14.2 mM; (ii) a large number of physiologically relevant, as well as non-physiological anions, such as the halides and pseudohalides (cyanate, thiocyanate), bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, and phenylboronic acid, showed a very similar inhibition behavior, all these derivatives possessing K_Is in the range of 0.60–0.89 mM. It should be noted that our data for chloride and iodide inhibition are very much different from the corresponding data reported by Murakami and Sly.[12] This may be due to the different assay systems used in the two studies, or most probably, to the level of purification/contamination of CA VI from saliva in the earlier study[12] with other proteins/CAs; (iii) the most effective CA VI inhibitors were cyanide, azide, sulfamic acid (as sulfamate anion), and sulfamide, these compounds possessing inhibition constants in the range of 70–90 μM (Table 2). Thus, hCA VI has a very characteristic behavior toward anion inhibitors, which is completely different from that of the cytosolic (hCA I and hCA II) or extracellular (CA IX) isozymes. In fact, data of Table 2 show that hCA I generally has a much higher affinity to many of these anions (as compared to hCA VI, II, and IX), for example, cyanate, cyanide, azide, and hydrogen sulfide are submicromolar inhibitors of this ioform, but hCA I is resistant to inhibition by fluoride, sulfate, and phenylboronic and phenylarsonic acids (Table 2). On the other hand, both CA II and IX show a rather similar behavior toward many anions, which is again different from the corresponding behavior of CA VI. Thus, halides, bicarbonate/carbonate, and sulfate are generally weak inhibitors of hCA II and IX, because both these enzymes are probably forming metabolons with AEs involved in the transport of bicarbonate/chloride/sulfate out (or inside) the cell.[31] However, similarly to CA VI, the best anion inhibitor of CA II and IX is cyanide, known to possess a high affin-

Table 2. Inhibition constants of anionic inhibitors against the cytosolic human isozymes hCA I, II, the secreted human isoform hCA VI, and the transmembrane, human cancer-associated isoform hCA IX, for the CO_2 hydration reaction, at 20 °C and pH 7.5[19]

Inhibitor	K_I[e] (mM)			
	hCA I[a]	hCA II[a]	hCAVI[b]	hCA IX[c]
F^-	>300	>300	0.60	48
Cl^-	6	200	0.72 (14)[d]	33
Br^-	4	63	0.73	16
I^-	0.3	26	0.81 (56)[d]	7
CNO^-	0.0007	0.03	0.69	0.043
SCN^-	0.2	1.6	0.89	0.13
CN^-	0.0005	0.02	0.07	0.004
N_3^-	0.0012	1.5	0.07	0.005
HCO_3^-	12	85	0.80	13
CO_3^{2-}	15	73	0.69	29
NO_3^-	7	35	0.76	46
NO_2^-	8.4	63	0.82	42
HS^-	0.0006	0.04	0.71	0.007
HSO_3^-	18	89	14.2	75
SO_4^{2-}	63	>200	9.9	>200
H_2NSO_3H[f]	0.021	0.39	0.09	0.092[d]
$H_2NSO_2NH_2$	0.31	1.13	0.07	0.096[d]
$PhB(OH)_2$	58.6	23.1	0.82	0.12
$PhAsO_3H_2$[f]	31.7	49.2	13.9	0.055

[a] Human recombinant isozyme, data from Ref. 27,28.
[b] Human recombinant isozyme, this work.
[c] Catalytic domain, human recombinant isoform, data from Ref. 27a.
[d] Values reported in Ref. 12 (in a different assay system).
[e] Errors were in the range of 3–5% of the reported values, from three different assays.
[f] As sodium salt.

ity for many metal ions in solution or bound within metalloenzyme active sites.[1–3] What is really particular to hCA VI is the fact that sulfamide, which is a rather weak hCA I and II inhibitor, shows micromolar affinity to the secreted isoform (as well as to the other extracellular CA investigated here, hCA IX). Azide is equipotent as a hCA VI inhibitor to cyanide and sulfamide, being at the same time a very potent hCA I and IX inhibitor, and a quite effective hCA II inhibitor too.

But what is the significance of our results? As mentioned in the introduction, the saliva/milk secreted isozyme hCA VI is the least understood isozyme of this large family of metalloproteins. However, several important physiological studies showed the involvement of this enzyme in olfaction, taste, and pH regulation in the oral cavity.[10–15] Thus, our results show CA VI to possess an enzymatic activity for the CO_2 hydration reaction much higher than previously reported. In fact CA VI has around 33% of the activity of hCA II (if one compares k_{cat}/K_M values of Table 1), a very efficient catalyst for this crucial physiological reaction. Furthermore, CA VI has the same catalytic power as CA I, another very abundant and ubiquitous CA isoform.[1–4] CA VI is on the other hand inhibited well only by few anions, such as cyanide, azide, sulfamate, and sulfamide, most of the physiological anions (chloride, bicarbonate, carbonate) showing inhibition constants in the range of 0.60–0.80 mM. Thus, among all investigated isozymes, hCA VI is in fact the most sensitive to both bicarbonate and chloride inhibition. Although we are unable to explain these results at this moment, clearly this behavior represents an evolutionary adaptation of this enzyme to the harsh environment in which it must work, that is, in secretions (saliva or milk) which may contain variable amounts of other proteins and anions.

In conclusion, the first anion hCA VI inhibition study is presented here. The full-length enzyme has a significant catalytic activity for the physiological reaction, of the same order of magnitude as the ubiquitous isoform CA I or the transmembrane, tumor-associated isozyme CA IX. A series of anions were also shown to inhibit the activity of the enzyme, with inhibition constants typically in the range of 0.60–0.90 mM. The best hCA VI inhibitors were cyanide, azide, sulfamide, and sulfamate, with inhibition constants in the range of 70–90 μM.

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References and notes

1. Supuran, C. T.; Scozzafava, A.; Conway, J., Eds.; *Carbonic Anhydrase—Its Inhibitors and Activators*. CRC Press: Boca Raton, New York, London, 2004; p 1–363.
2. (a) Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199; (b) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Pat.* **2004**, *14*, 667.
3. (a) Supuran, C. T. Carbonic anhydrases: catalytic and inhibition mechanisms, distribution and physiological roles. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton, 2004; pp 1–23; (b) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146; (c) Supuran, C. T.; Scozzafava, A.; Casini, A. Development of Sulfonamide Carbonic Anhydrase Inhibitors. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton, 2004; pp 67–147; (d) Winum, J. Y.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. *Mini-Rev. Med. Chem.* **2006**, *6*, 921.
4. (a) Smith, K. S.; Jakubzick, C.; Whittam, T. S.; Ferry, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 15184; (b) Sly, W. S.; Hu, P. Y. *Annu. Rev. Biochem.* **1995**, *64*, 375.
5. Lehtonen, J.; Shen, B.; Vihinen, M.; Casini, A.; Scozzafava, A.; Supuran, C. T.; Parkkila, A. K.; Saarnio, J.; Kivela, A. J.; Waheed, A.; Sly, W. S.; Parkkila, S. *J. Biol. Chem.* **2004**, *279*, 2719.
6. (a) Wykoff, C. C.; Beasley, N. J.; Watson, P. H.; Turner, K. J.; Pastorek, J.; Sibtain, A.; Wilson, G. D.; Turley, H.; Talks, K. L.; Maxwell, P. H.; Pugh, C. W.; Ratcliffe, P. J.; Harris, A. L. *Cancer Res.* **2000**, *60*, 7075; (b) Potter, C. P. S.; Harris, A. L. *Brit. J. Cancer* **2003**, *89*, 2.
7. (a) Supuran, C. T.; Scozzafava, A. Activation of Carbonic Anhydrase Isozymes. In *The Carbonic Anhydrases—New Horizons*; Chegwidden, W. R., Carter, N., Edwards, Y., Eds.; Birkhauser: Verlag, Basel, Switzerland, 2000; pp 197–219; (b) Ilies, M.; Scozzafava, A.; Supuran, C. T. Carbonic Anhydrase Activators. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton (FL), USA, 2004; pp 317–352; (c) Bahn, Y. S.; Muhlschlegel, F. A. *Curr. Opin. Microbiol.* **2006**, *9*, 1.
8. Hilvo, M.; Tolvanen, M.; Clark, A.; Shen, B.; Shah, G. N.; Waheed, A.; Halmi, P.; Hanninen, M.; Hamalainen, J. M.; Vihinen, M.; Sly, W. S.; Parkkila, S. *Biochem. J.* **2005**, *392*, 83.
9. Nishimori, I. Acatalytic CAs: Carbonic Anhydrase-Related Proteins. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton (FL), USA, 2004; pp 25–43.
10. Fernley, R. T.; Wright, R. D.; Coghlan, J. P. *FEBS Lett.* **1979**, *105*, 299.
11. (a) Aldred, P.; Fu, P.; Barrett, G.; Penschow, J. D.; Wright, R. D.; Coghlan, J. P.; Fernley, R. T. *Biochemistry* **1981**, *30*, 569; (b) Fernley, R. T. Secreted Carbonic Anhydrases. In *Carbonic Anhydrase—From Biochemistry and Genetics to Physiology and Clinical Medicine*; Botrè, F., Gros, G., Storey, B. T., Eds.; VCH: Weinheim, 1991; pp 178–185; (c) Kivela, J.; Parkkila, S.; Parkkila, A. K.; Leinonen, J.; Rajaniemi, H. *J. Physiol.* **1999**, *520*, 315.
12. Murakami, H.; Sly, W. S. *J. Biol. Chem.* **1987**, *262*, 1382.
13. Karhumaa, P.; Leinonen, J.; Parkkila, S.; Kaunisto, K.; Tapanainen, J.; Rajaniemi, H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 11604.
14. Ogawa, Y.; Matsumoto, K.; Maeda, T.; Tamai, R.; Suzuki, T.; Sasano, H.; Fernley, R. T. *J. Histochem. Cytochem.* **2002**, *50*, 821.
15. Smith, C. E.; Nanci, A.; Moffatt P. *Eur. J. Oral Sci.* **2006**, *114*, Suppl 1, 147.
16. Nishimori, I.; Vullo, D.; Innocenti, A.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *J. Med. Chem.* **2005**, *48*, 7860.

17. The cDNA fragment encoding the open-reading frame of hCA VI was amplified from polyA(+) RNA obtained from human salivary gland (Clontech, Palo Alto, CA, USA) by using a commercial RT-PCR kit (Takara, Kyoto, Japan) with adopter primers including *Eco*RI and *Sal*I recognition sequences (underlined in the following sequences, respectively): 5′-CGGAATTCCCAT GAGGGCCCTGGTGCTTCT-3′ and 5′-GCGTC GACTCAGTTCAATGCTCTTCT-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 57 °C, and 90 s at 72 °C. The PCR products were cleaved with *Eco*RI and *Sal*I, purified, and cloned in-frame into the pGEX-4T2 vector (Amersham). The cDNA sequence of the hCA VI insert included in the vector was reconfirmed by DNA sequencing. The constructs were then transfected into *Escherichia coli* strain BL21 for production of the GST-hCA VI fusion protein, similarly to the procedure already described for hCA VB, IX, and XII.[16] Following induction of the protein expression by addition of 1 mM isopropyl-β-D-thiogalactopyranoside, the bacteria were harvested and sonicated 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 GST-hCA VI fusion 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 VI is purified easily and the procedure is quite simple. The obtained hCA VI was further purified by sulfonamide affinity chromatography,[18] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[19]
18. Khalifah, R. G.; Strader, D. J.; Bryant, S. H.; Gibson, S. M. *Biochemistry* **1977**, *16*, 2241.
19. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An 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 or $NaClO_4$ (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 (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 μM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier, and represent the mean from at least three different determinations.
20. Casini, A.; Antel, J.; Abbate, F.; Scozzafava, A.; David, S.; Waldeck, H.; Schafer, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 841.
21. Abbate, F.; Coetzee, A.; Casini, A.; Ciattini, S.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 337.
22. Temperini, C.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 3019.
23. Tashian, R. E.; Venta, P. J.; Nicewander, P. H.; Hewett-Emmett, D. *Prog. Clin. Biol. Res.* **1990**, *344*, 159.
24. Vullo, D.; Franchi, M.; Gallori, E.; Antel, J.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2004**, *47*, 1272.
25. Vullo, D.; Innocenti, A.; Nishimori, I.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 963.
26. (a) Nair, S. K.; Christianson, D. W. *J. Am. Chem. Soc.* **1991**, *113*, 9455; (b) Christianson, D. W.; Fierke, C. A. *Acc. Chem. Res.* **1996**, *29*, 331; (c) Whittington, D. A.; Grubb, J. H.; Waheed, A.; Shah, G. N.; Sly, W. S.; Christianson, D. W. *J. Biol. Chem.* **2004**, *279*, 7223.
27. (a) Vullo, D.; Franchi, M.; Gallori, E.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 403; (b) Winum, J. Y.; Innocenti, A.; Nasr, J.; Montero, J. L.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2535.
28. Innocenti, A.; Firnges, M.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5769.
29. (a) Lehtonen, J.; Parkkila, S.; Vullo, D.; Casini, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3757; (b) Innocenti, A.; Vullo, D.; Scozzafava, A.; Casey, J. R.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 573; (c) Franchi, M.; Vullo, D.; Gallori, E.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2857.
30. (a) Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Biorg. Med. Chem. Lett.* **2005**, *15*, 567; (b) Innocenti, A.; Antel, J.; Wurl, M.; Vullo, D.; Firnges, M. A.; Scozzafava, A.; Supuran, C. T. *Biorg. Med. Chem. Lett.* **2005**, *15*, 1909; (c) Vullo, D.; Ruusuvuori, E.; Kaila, K.; Scozzafava, A.; Supuran, C. T. *Biorg. Med. Chem. Lett.* **2006**, *16*, 3139.
31. (a) Sterling, D.; Reithmeier, R. A.; Casey, J. R. *J. Biol. Chem.* **2001**, *276*, 47886; (b) McMurtrie, H. L.; Alvarez, B. V.; Loiselle, F. B.; Sterling, D.; Morgan, P. E.; Cleary, H. J.; Johnson, D. E.; Casey, J. R. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 231.