Carbonic Anhydrase Inhibitors. DNA Cloning, Characterization, and Inhibition Studies of the Human Secretory Isoform VI, a New Target for Sulfonamide and Sulfamate Inhibitors

Isao Nishimori,[†] Tomoko Minakuchi,[†] Saburo Onishi,[†] Daniela Vullo,[‡] Andrea Scozzafava,[‡] and Claudiu T. Supuran*[,‡]

Department of Gastroenterology and Hepatology, Kochi Medical School, Nankoku, Kochi 783-8505, Japan, and Laboratorio di Chimica Bioinorganica, Rm. 188, Università degli Studi di Firenze, Via della Lastruccia 3, I-50019 Sesto Fiorentino, Firenze, Italy

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The secretory isozyme of human carbonic anhydrase (hCA, EC 4.2.1.1), hCA VI, has been cloned, expressed, and purified in a bacterial expression system. 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 on the same order of magnitude as the ubiquitous isoform CA I or the transmembrane, tumor-associated isozyme CA IX. A series of sulfonamides and one sulfamate have been tested for their interaction with this isozyme. Simple benzenesulfonamides were rather ineffective hCA VI inhibitors, with inhibition constants in the range of 1090−6680 nM. Better inhibitors were detected among such derivatives bearing 2- or 4-amino-, 4-aminomethyl-, or 4-hydroxymethyl moieties or among halogenated sulfanilamides (K_I values of 608−955 nM). Some clinically used compounds, such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, topiramate, sulpiride, and indisulam, or the orphan drug benzolamide, showed effective hCA VI inhibitory activity, with inhibition constants of 0.8−79 nM. The best inhibitors were brinzolamide and sulpiride (K_I values of 0.8−0.9 nM), the latter compound being also a CA VI-selective inhibitor. The metallic taste reported as a side effect after the treatment with systemic sulfonamides may be due to the inhibition of the salivary CA VI. Some of the compounds investigated in this study might be used as additives in toothpastes for reducing the acidification produced by the relevant CO_2 hydrase activity of enamel CA VI, which leads to the formation of protons and bicarbonate and may have a role in cariogenesis.

Introduction

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. In fact, 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. 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; signalling; etc.[1−7] Different isozymes among the 16 presently known participate in such processes.[1−7] Indeed, these isoforms present 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] Indeed, CA I−III, VII, and XIII are soluble, cytosolic isozymes, CA IV and XV are extracellular, membrane-anchored enzymes by means of glycosylphosphatidyl-inositol (GPI) tails, and 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, most of which are little understood at this moment. More recently, the enzyme was also evidenced in enamel organs in 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 number of aromatic/heterocyclic sulfonamides and one sulfamate of the human secreted isoform hCA VI. This is in fact the only human CA that was not investigated up to now for its inhibition by the main class of CA inhibitors (CAIs), i.e., the sulfonamides and their related derivatives (sulfamates, sulfamides). As many pharmacological agents possess such functional groups (of the $X-SO_2NH_2$ type, where X is carbon, oxygen, or nitrogen), this study may reveal on one hand possible side effects of clinically used drugs, but may be also useful for the drug design of CAIs with novel applications that would specifically target this poorly understood isozyme.

Results

hCA VI Cloning, Purification, and Catalytic Activity. The alignment of amino acid sequences of the human isoforms CA I, II (cytosolic), VI (secreted), and IX (transmembrane) is shown in Figure 1. The catalytic activity of several α-CA isozymes of human origin, among which also hCA I, II, III, VA, VB, VI,

* Correspondence author. Tel: +39-055-457 3005. Fax: +39-055-4573385. E-mail: claudiu.supuran@unifi.it.
[†] Kochi Medical School.
[‡] Università degli Studi di Firenze.

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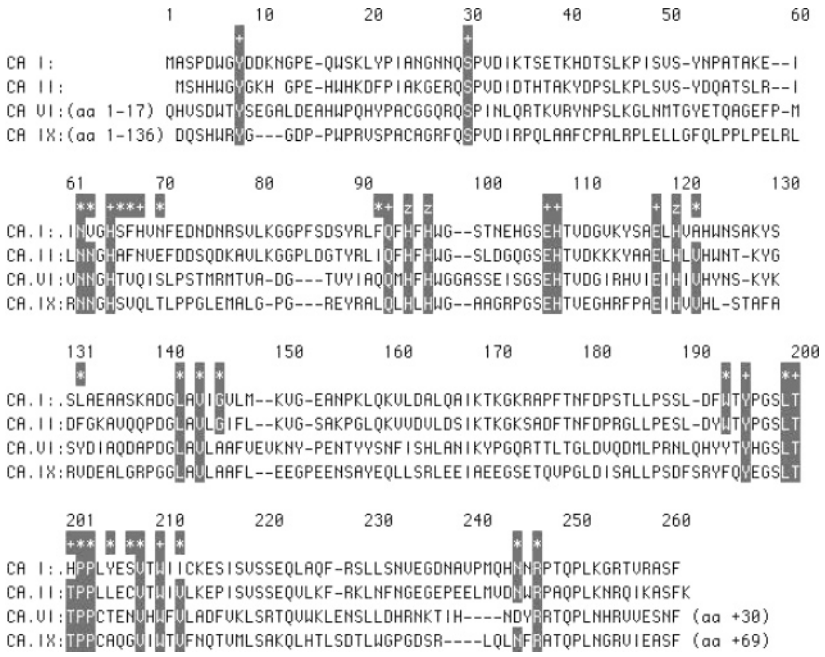

Figure 1. Sequence alignment of the CA domain of human isozymes hCA I, II, VI, and IX. The residues indicated by a mixture of an asterisk, plus sign, and "z" above the hCA I sequence are those forming the active site. Seventeen residues known to participate in a network of hydrogen bonds crucial for the binding of inhibitors/activators are indicated by plus; "z" indicates the three zinc-liganded histidines. Among these residues, conserved amino acids of hCA VI in comparison to other isoforms are indicated by a closed box.

Table 1. Kinetic Parameters for the CO_2 Hydration Reaction Catalyzed by the Cytosolic α-hCA Isozymes I−III, the Mitochondrial Isozymes VA and VB, 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 (5-Acetamido-1,3,4-thiadiazole-2-sulfonamide), a Clinically Used Drug

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 III	very low	1.0×10^4	3.0×10^5	300 000
hCA VA	low	2.9×10^5	2.9×10^7	63
hCA VB	high	9.5×10^5	9.8×10^7	54
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

IX, and XII, for the physiologic reaction catalyzed by them (CO_2 hydration), is shown in Table 1.

Chemistry and CA Inhibition. Sulfonamides investigated for the inhibition of the newly purified, secreted CA isoform hCA VI of types **1**−**24** (Figure 2) are shown below. Derivatives AAZ through IND (Figure 3) are clinically used drugs: acetazolamide (AAZ), methazolamide (MZA), ethoxzolamide (EZA), and dichlorophenamide (DCP) are the classical, systemically acting CAIs.[3b] Dorzolamide (DZA) and brinzolamide (BRZ) are topically acting antiglaucoma agents,[1−3] and benzolamide (BZA) is an orphan drug belonging to this class of pharmacological agents,[3b] whereas topiramate (TPM) is a widely used antiepileptic drug.[16] Sulpiride (SLP)[17] and indisulam (IND)[18] were recently shown by this group to belong to this class of pharmacological agents. Compounds **1**, **2**, **4**−**6**, **11**, **12**, **18**−**20**, **23** and AAZ through SLP are commercially available or were a gift from the company producing them (see Experimental Section for details), whereas **3**,[19] **7**−**10**,[19] **13**−**17**,[20] **21**,[21] **22**,[22] and **24**,[23] were prepared as reported earlier by this group. Inhibition data of these sulfonamides and the sulfamate TPM against isozymes hCA I, II, VI, and IX are shown in Table 2.

Discussion

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] In this work, we initially tried to obtain the truncated enzyme lacking the N-terminal 17 amino acid residues for a GST−hCA VI construct. However, we failed to obtain any bacterial colony expressing the truncated hCA VI protein. The full-length enzyme was on the other hand obtained in soluble form without complications by the GST-fusion protein method.[24] A similar phenomenon was previously experienced during the construction of the GST−hCA VB fusion protein: no truncated enzyme could be obtained, but the full length enzyme was successfully cloned and purified.[24] Although the exact reasons for such phenomena are uncertain, it is likely that ectopically overexpressed hCAs in the cytoplasm of bacteria (normally CA VB is expressed in the mitochondria and CA VI is secreted out of the cell) are toxic to the prokaryotic cell (*Escherichia coli*) used for their production.

To date, complete amino acid sequences in the open reading frame of three hCA VI clones have been deposited in the GenBank, with the accession numbers 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, i.e., residues Ser:Gly at position 70 (numbering is based on CA I sequence), 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 positions 70 and 130, which are adjacent to two critical residues involved in the binding of inhibitors/activators,[16,17,25,26] i.e., 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, and hCA IX (Figure 1). hCA VI showed an overall similarity of 31−40% with these isozymes. The highest similarity was

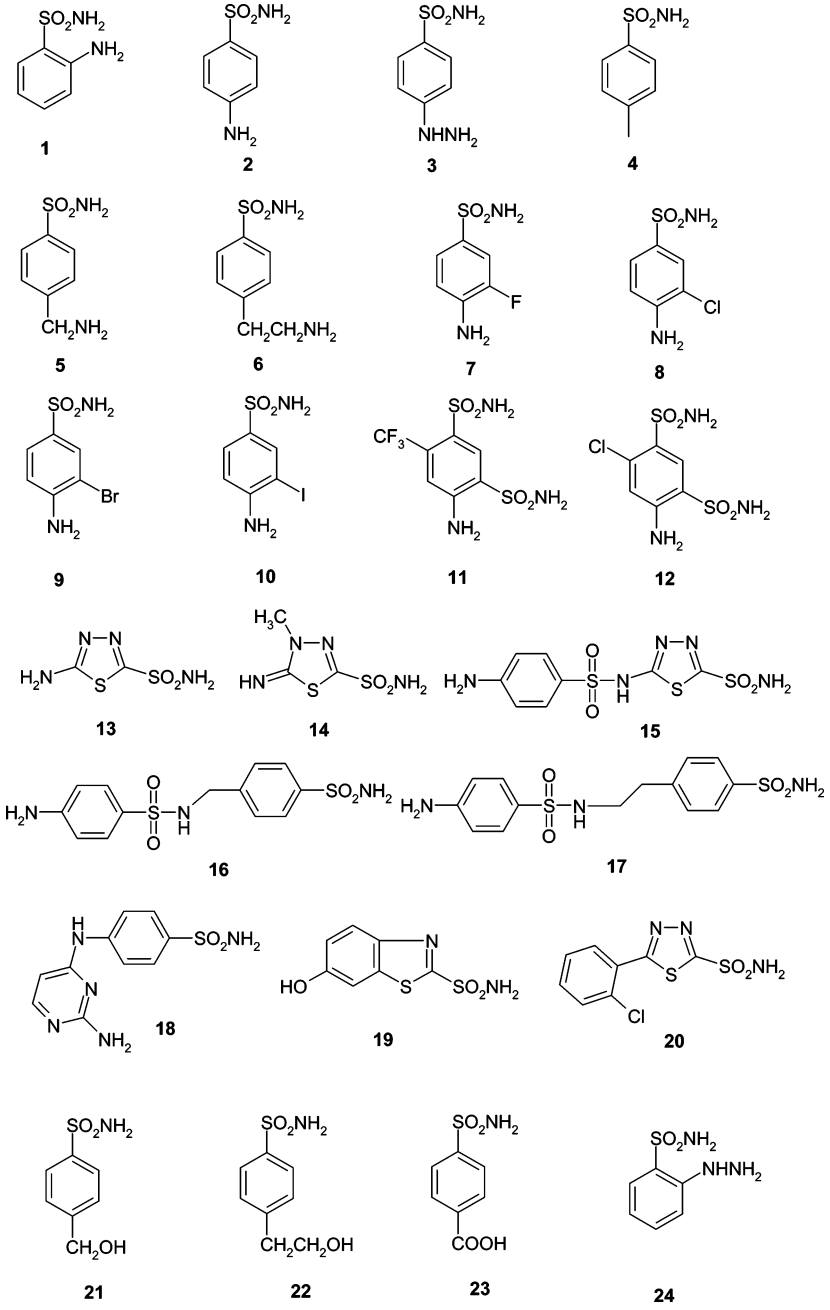

Figure 2.

observed with hCA IX, probably because both hCA VI and CA IX are extracellular isozymes. In Figure 1, the 36 residues that were previously shown to form the active site cavity in all α-CAs[27−29] are indicated by a mixture of an asterisk, plus sign, and "z" above the hCA I 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 vs hCA I homology was 30.8%, that of hCA VI vs hCA II was 34.1%, and that of hCA VI vs hCA IX was 39.8%. However, it is important to note that all the amino acid residues that 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 isozymes I, II, or IX investigated in great detail earlier.[1] There are, however, several amino acid residues that are characteristic only to CA VI among the α-CA isozymes, such as among those in positions 20, 31, 40, 50, 60, 65, 69, 91, 93, 131, 170, 171, 193, 205, 207, and 209 (Figure 1). In some cases, these amino acids are buried 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.[5,7,16−26,30] Among these amino acid substutions 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, and IX as

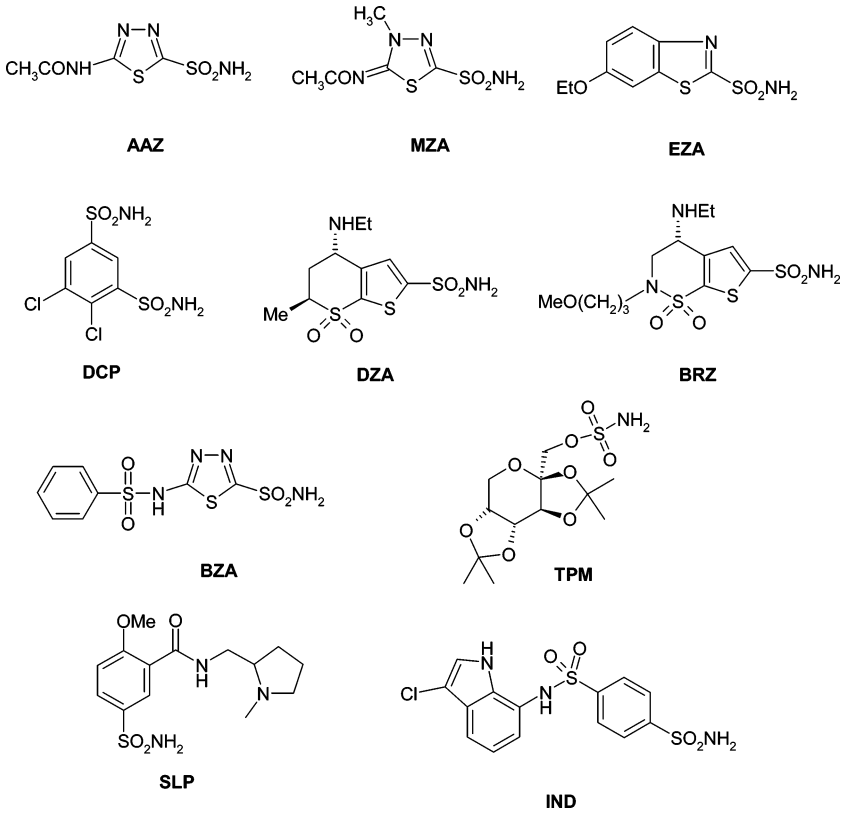

Figure 3.

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.[30] 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.[16−30] Furthermore, residues close to the zinc ligands, e.g., those in positions 91 and 93, are also characteristic only of CA VI (Figure 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, VB, IX, and XII), are shown in Table 1. As stressed in the introduction, 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. 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 on 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% the catalytic activity (as turnover number) of hCA II. Thus, hCA VI is less effective than hCA II or hCA VB, 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 III, VA, or XII, some of which were previously shown to be important drug targets.[28,29] It may be also observed from the data of Table 1 that all these isozymes except CA III are susceptible to inhibition by acetazolamide (AAZ), the most investigated CAI (see extensive discussion later in the text).[1]

Chemistry and hCA VI Inhibition. Sulfonamides and sulfamates are among the clinically used CAIs.[1−4,16] A library of such derivatives, including the simple aromatic/heteroaromatic derivatives **1**−**24** and the clinically used compounds AAZ through IND [benzolamide (BZA) is an orphan drug][1] were included in this study, basically for two reasons: (i) most of the compounds **1**−**24** were used as lead molecules for designing potent inhibitors targeting other CA isozymes, such as CA I, II, IV, VA, VII, IX, and XII among others, by the tail approach (i.e., derivatization of the free amino/hydrazine/hydroxy moieties contained in the molecule).[1,3,4,16−24] Thus, it in not improbable that detecting good hCA VI inhibitors among such simple sulfonamides may thereafter lead to the development of more effective inhibitors; (ii) the inhibition profile against hCA VI of the clinically used compounds AAZ through IND may be important both for explaining possible side effects of some of these drugs as well as for the design of novel therapeutic applications, for them or some of their derivatives. Table 2 shows CA VI inhibition data with the panel of sulfonamides **1**−**24** and AAZ through IND and the sulfamate TPM. hCA I, II, and IX inhibition data of the same compounds have been included for the sake of comparison, in order to better understand

Table 2. hCA I, II, VI, and IX Inhibition Data with Sulfonamides **1**−**24** and the Clinically Used Derivatives AAZ through IND

	K_I[a] (nM)			
inhibitor	hCA I[c]	hCA II[c]	hCA VI[d]	hCA IX[e]
1	45 400	295	772	33
2	25 000	240	941	238
3	28 000	300	1275	294
4	78 500	320	1582	460
5	25 000	170	4800	103
6	21 000	160	813	33
7	8 300	60	96	245
8	9 800	110	1097	264
9	6 500	40	4680	269
10	6 000	70	1024	285
11	5 800	63	955	24
12	8 400	75	608	39
13	8 600	60	798	41
14	9 300	19	740	30
15	6	2	73	38
16	164	46	55	31
17	185	50	24	24
18	109	33	86	16
19	95	30	103	14
20	690	12	114	30
21	55	80	6680	21
22	21 000	125	4150	22
23	23 000	133	887	26
24	24 000	125	1090	176
AAZ	250	12	11	25
MZA	50	14	10	27
EZA	25	8	43	34
DCP	1 200	38	79	50
DZA	50 000	9	10	52
BRZ	45 000	3	0.9	37
BZA	15	9	93	47
TPM	250	10	45	58[b]
SLP	12 000	40	0.8	46
IND	31	15	47	24

[a] Errors are in the range of 5−10% of the shown data, from three different assays. Data of isoforms I, II, and IX are from refs 24b,c. [b] K_I against the full length hCA IX is 1590 nM.[31] [c] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[32] [d] Full length, human recombinant enzyme. [e] Catalytic domain of human, recombinant enzyme.

the significance of the new data presented here. In fact, both CA II and CA IX are well-established drug targets in this family of enzymes, and their inhibitors are antiglaucoma or anticancer drugs/diagnostic tools.[1−4] hCA I is on the other hand a widely spread isoform in many tissues/organs, but its role is presently less well understood.[1−4]

The following SAR may be drawn from the inhibition data of Table 2: (i) A first group of sulfonamides, including **3**−**5**, **8**−**10**, **21**, **22**, and **24**, showed weak CA VI inhibitory activity, with K_I values in the range of 1090−6680 nM. It may be observed that all these compounds are simple benzenesulfonamide derivatives, possibly 2- or 4-substituted with compact groups of the hydrazino, methyl, aminomethyl, or hydroxymethyl/ethyl type, whereas three of them (**8**−**10**) are halogenosulfanilamides. It must be also noted that most of these derivatives act as much better inhibitors of isoforms CA II and IX and are generally quite weak CA I inhibitors (Table 2). (ii) Medium-potency hCA VI inhibitors were compounds **1**, **2**, **6**, **11**−**14**, and **23**, which showed K_I values in the range of 608−955 nM. These sulfonamides also belong to rather heterogeneous classes, some of them being structurally related to the compounds mentioned above (such as the simple orthanilamide **1** and sulfanilamide **2** or some of their derivatives, of type **6** and **23**). Compounds **11** and **12** are 1,3-benzenedisulfonamide derivatives, whereas **13** and **14** are heterocyclic sulfonamides, being in fact the precursors of two widely clinically used drugs,[1−4] acetazolamide (AAZ) and methazolamide (MZA), respectively. As for the derivatives mentioned earlier, these sulfonamides also generally act as much better CA II/CA IX inhibitors and weak CA I inhibitors. (iii) Strong CA VI inhibitors were detected among the following derivatives: **7**, **15**−**20**, AAZ, MZA, EZA, DCP, DZA, BZA, TPM, and IND, which showed excellent inhibition profiles, with K_I values in the range of 10−114 nM. It may be observed that the clinically used CAIs act as very good CA VI inhibitors. Other interesting compounds were fluorosulfanilamide **7**, BZA and its 4-amino derivative **15**, the structurally related sulfanilated-sulfonamides **16** and **17**, the pyrimidine derivative **18**, the ethoxzolamide precursor **19**, and chlorazolamide **20**. It is obvious that potent CA VI inhibitors can be obtained both from the class of the substituted benzensulfonamides, as well as those belonging to the heterocyclic sulfonamide classes, e.g., 1,3,4-thiadiazole-2-sulfonamide, 1,3,4-thiadiazoline-2-sulfonamide, benzothiazole-2-sulfonamide, and thienothiopyran-*S*,*S*-dioxide-2-sulfonamide derivatives among others. However, the most interesting fact that emerged in this study was that two clinically used compounds, brinzolamide (BRZ) and sulpiride (SLP), act as subnanomolar inhibitors of CA VI, with inhibition constants of 0.9 and 0.8 nM, respectively (Table 2). Among the compounds in this group of potent CA VI inhibitors, some of them, such as **15**−**20**, EZA, BZA, DCP, TPM, and IND, generally act as better CA II and CA IX than CA VI inhibitors, whereas their affinity for CA I is generally low. However, other derivatives (such as AAZ, MZA, BRZ, and SLP) inhibited better CA VI than any other known/investigated CA isozyme, and this is indeed a very important finding. Thus, SLP can be considered as an isozyme VI-selective inhibitor, since its selectivity ratio for inhibiting CA VI over CA II is 50, the selectivity ratio for the inhibition of CA VI over CA IX is 120, and that of inhibiting CA VI over CA I is 15 000 (this compound is a very weak CA I inhibitor).[17]

The inhibition data obtained in this study are quite different from the data reported by Murakami and Sly,[12] who reported an inhibition constant of 1.1 μM for AAZ, 0.56 μM for MZA, and 9.9 μM for sulfanilamide **2**. A possible explanation for the discrepancy with our data is confered by the different glycosylation of hCA VI protein. Distinct glycoforms of bovine CA VI, between the parotid and submaxillary gland, were reported to be caused by different expression of certain types of glycosyltrasnferases by Hooper et al.[34] Murakami and Sly[12] purified their hCA VI from human saliva, whereas we produced hCA VI by using a bacterial recombinant protein system. It is likely that the post-traslational glycosylation reactions are different between human and *E. coli*, which may thus explain the very low enzymatic activity and insensitivity to acetazolamide inhibition observed in the study of the cited authors.[12] It should be also mentioned that the enzyme from the Murakami and Sly[12] report showed an unexplainably low catalytic activity (as we mentioned above), which is reflected thereafter also in its low affinity for these sulfonamide inhibitors.

But what is the significance of our results? As mentioned in the Introduction, the saliva secreted isozyme hCA VI is the least understood isoform of this large family of metalloproteins. However, several important physiological studies demonstrated its involvement in olfaction, taste, and pH regulation in the oral cavity.[10−15,36,37] Our results show CA VI to possess a much higher enzymatic activity for the CO_2 hydration reaction than previously reported.[12] In fact, CA VI has around 33% of the enzymatic activity of hCA II, a very efficient catalyst for this crucial physiologic reaction. Furthermore, CA VI has the same catalytic power as CA I, another very abundant and ubiquitous CA isoform, but in contrast to CA I, which is generally less

susceptible to inhibition by sulfonamides (Table 2),[1−4] CA VI has a good affinity for many such compounds, some of which are widely used clinical drugs. Thus, the metallic taste[35] observed as a side effect after the treatment with systemic sulfonamide CAIs may in fact be due to the inhibition of the salivary CA VI. A possible solution to this unpleasant (but non-life-threatening) side effect would be that of designing compounds without CA VI inhibitory properties that at the same time inhibit the other targeted isoform(s), such as for example CA II and CA IX.

There is strong evidence from Parkkila's and Kivela's laboratories[36,37] showing that CA VI is present in the enamel pellicle of the teeth. However, the same authors showed that CA VI concentrations in the saliva did not correlate with salivary pH or the buffer capacity of this fluid.[36,37] Thus, the prevalent hypothesis up to now regarding the role of CA VI found in the enamel/saliva was that it provides bicarbonate for the neutralization of acid produced by microbe-delivered H^+ ions, in this way protecting teeth from caries.[36,37] However, tooth enamel, similarly to bone, is formed of the same mineral matrix, hydroxyapatite, and it is well-established that another CA isoform, CA II, is involved in bone homeostasis in osteoclasts.[38] By catalyzing CO_2 hydration to bicarbonate and protons in osteoclasts, CA II provides H^+ ions needed in the processes of bone formation, resorption, and remodeling.[38] Furthermore, a long-term treatment with acetazolamide, a potent CA II inhibitor, was shown to be one of the few effective treatments for the prevention of osteoporosis.[39] Thus, our hypothesis regarding the physiological role of CA VI is the alternative one to the Parkkila-Kivela[36,37] hypothesis mentioned above, i.e., that this enzyme may be involved in the acidification of the enamel pellicle, by catalyzing CO_2 hydration (resulted from the bacterial metabolism) to bicarbonate and protons and that its inhibition may be thus useful in prevention of caries. The acid formed due to the enzymatic activity of this isoform might be used for mobilization of calcium from the mineral matrix, thus leading to the formation of caries. Thus, we suggest that CA VI may have the same physiological role (in teeth) as CA II in the bone resorption processes operating in osteoclasts. In fact, we recently showed that CA VI has a rather high sensitivity to inhibition by bicarbonate, with a K_I of 0.80 mM (whereas the K_I of hCA I is 12 mM, of hCA II is 85 mM, and of hCA IX is 13 mM against this anion).[40] Such data reveal that CA VI would not be particularly active enzymatically in the presence of high amounts of bicarbonate, and especially not so for the dehydration reaction (bicarbonate conversion to carbon dioxide in the presence of H^+, a reaction that is much more rapid—and needs no catalyst—as compared to the reverse one, CO_2 hydration to bicarbonate).[1] Thus, similarly to other CA isozymes,[1−4] we consider CA VI to be more active as a catalyst for the hydration of carbon dioxide to bicarbonate and a proton, eventually in the highly acidic conditions present in some tissues where the enzyme is found, such as the enamel or some epithelial surfaces of the upper alimentary tract,[36,37] and not as a catalyst for converting bicarbonate to CO_2, since this reaction is spontaneous, very fast, and needs no catalyst.

Some of the compounds investigated in this study, such as the clinically used derivatives AAZ, MZA, DZA, BRZ, and SLP (which are low nanomolar CA VI inhibitors), might be used to verify our hypothesis, i.e., whether inhibition of CA VI is detrimental or useful for caries formation. If the second situation is the true one, CA VI inhibitors might be used as additives in some toothpastes for reducing the acidification produced by the relevant CO_2 hydrase activity of salivary CA VI and reducing in this way cariogenesis.

4. Conclusions

The first hCA VI inhibition study is presented here. The full length enzyme has a high catalytic activity with the following kinetic parameters at 20 °C and pH 7.5, for the CO_2 hydration reaction: $k_{cat} = 9.5 \times 10^5\ s^{-1}$, and $k_{cat}/K_M = 9.8.\ 10^7\ M^{-1}\ s^{-1}$. Thus, hCA VI has a significant catalytic activity for the physiological reaction, on the same order of magnitude as the ubiquitous isoform CA I or the transmembrane, tumor-associated isozyme CA IX. A series of sulfonamides and a sulfamate have been tested for their interaction with this isozyme. Simple benzenesulfonamides were rather ineffective hCA VI inhibitors, with inhibition constants in the range of 1090−6680 nM, whereas better inhibitors were detected among such derivatives bearing 2- or 4-amino-, 4-aminomethyl-, or 4-hydroxymethyl moieties or among halogenated sulfanilamides (K_I values in the range of 608−955 nM). The clinically used compounds acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, topiramate, sulpiride, and indisulam showed effective hCA VI inhibitory activity, with inhibition constants in the range of 0.8−79 nM. The best inhibitors were brinzolamide and sulpiride (K_I values of 0.8−0.9 nM), the last compound being also a CA VI-selective inhibitor. The metallic taste reported as a side effect after the treatment with systemic sulfonamide CAIs may in fact be due to the inhibition of the salivary CA VI. Some of the compounds investigated in this study, such as the clinically used derivatives showing low nanomolar CA VI inhibitory activity, might be used as additives in toothpastes for reducing the acidification produced by the relevant CO_2 hydrase activity of salivary CA VI, which leads to the formation of protons and bicarbonate in the enamel pellicle.

Experimental Section

Chemistry. Compounds **1**, **2**, **4**−**6**, **11**, **12**, **18**−**20**, **23**, and AAZ through SLP are commercially available from Sigma-Aldrich, except DZA (a gift from Merck), BRZ (a gift from Alcon), and TPM (which was extracted from Topamax pills, from Johnson & Johnson). Derivatives **3**,[19] **7**−**10**,[19] **13**−**17**,[20] **21**,[21] **22**,[22] and **24**[23] were prepared as reported earlier by this group.

The GST−hCA VI Fusion Protein. 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) by using a commercial RT-PCR kit (Takara, Kyoto, Japan) with adopter primers including *Eco* RI and *Sal* I recognition sequences (italic in the following sequences, respectively): 5′-CG*GAATTC*CCATGAGGGCCCTGGTGCTTCT-3′ and 5′-GC*GTCGAC*TCAGTTCAATGCTCTTCT-3′. The PCR reaction 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 *E. coli* strain BL21 for production of the GST−hCA VI fusion protein, similarly to the procedure already described for hCA VB, IX, and XII. 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,[33] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[32]

CA Inhibition Assay. An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[32] 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 and 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, in order to allow for the formation of the E−I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, from Lineweaver−Burk plots, as reported earlier, and represent the mean from at least three different determinations.

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