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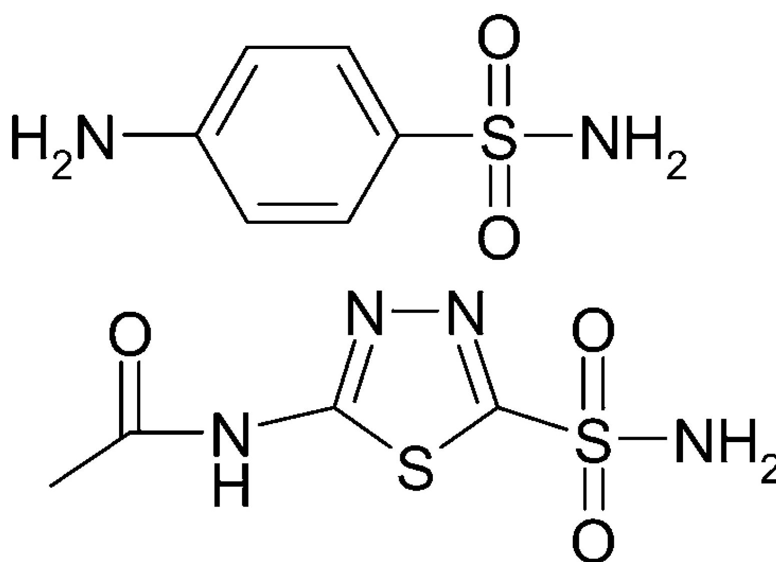

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Carbonic Anhydrase Inhibitors. The Mitochondrial Isozyme VB as a New Target for Sulfonamide and Sulfamate Inhibitors

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A lately discovered carbonic anhydrase (hCA, EC 4.2.1.1), the mitochondrial hCA VB, was cloned, expressed, and purified. Kinetic parameters proved it to be 3.37 times more effective than hCA VA as a catalyst for the physiological reaction, with $k_{cat} = 9.5 \times 10^5$ s^{-1} and $k_{cat}/K_M = 9.8 \times 10^7$ M^{-1} s^{-1}, being second only to hCA II among the 16 isoforms presently known in humans. We investigated the inhibition of hCA VB with a library of sulfonamides/sulfamates, some of which are clinically used compounds. Benzenesulfonamides were ineffective inhibitors, whereas derivatives bearing 4-amino, 4-hydrazino, 4-methyl, 4-carboxy moieties or halogenated sulfanilamides were more effective (K_i's of 1.56−4.3 μM). Among the 10 clinically used compounds, acetazolamide, benzolamide, topiramate, and indisulam showed effective inhibitory activity (K_i's of 18−62 nM). Three compounds showed better activity against hCA VB over hCA II, among which were sulpiride and ethoxzolamide, which were 2 times more effective inhibitors of the mitochondrial over the cytosolic isozyme. hCA VB is a druggable target and some of its inhibitors may lead to the development of novel antiobesity therapies.

1. Introduction

At least 15 isoforms of the widely spread metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) were discovered up to now in humans, all belonging to the α-CA gene family.[1−5] These are hCA I through hCA XIV, but there are two mitochondrial enzymes, denoted hCA VA and hCA VB,[6,7] which leads to the final number of 15 isozymes (although they are numbered from I to XIV). Isozymes CA VB and CA XIV were the last to be discovered by Nishimori's group,[6,8a] who sequenced the genes encoding these proteins, mapped them on different chromosomes (isozyme VB is situated on chromosome X whereas CA XIV on chromosome 1) expressed the enzymes, and studied their tissue distribution.[6,8a] Isoform CA XV has very recently been reported, but it is not expressed in humans or other primates (where it is encoded by a pseudogene), being however quite abundant in rodents and other vertebrates.[8b]

The first mitochondrial CA discovered, isoform CA VA, which is present only in hepatocytes,[6,9−12] was shown mainly by Forster's group to be involved in several important biosynthetic reactions, such as lipogenesis, gluconeogenesis, and ureagenesis, among others.[9−13] The catalytic mechanism of CA VA is not so well understood,[14] although the murine enzyme (mCA VA) has been crystallized and its X-ray structure reported by Christianson's group.[15] Thus, the coordination of the catalytically critical Zn(II) ion in the active site of all α-CA isozymes, including CA VA, is identical: three histidine residues (His94, 96, and 119, CA I numbering) and a water molecule/hydroxide ion, which acts as a nucleophile in the hydration of carbon dioxide to bicarbonate and a proton (the physiological reaction catalyzed by these metalloenzymes).[1,15,16] However, in contrast to isozyme CA II, the most investigated and physiologically relevant isoform,[1,16] which possesses a histidine residue in position 64, acting as a proton shuttle between the active site and the environment in the rate-determining step of the catalytic cycle,[1] CA VA has a Tyr residue in that position, which probably acts as a rather inefficient proton-transfer group at the physiologic pH. Indeed, the catalytic activity of CA VA at pH values of 7.0−7.5 is lower compared to that of the very efficient isozyme II,[14] but it should be also stressed that the pH of mitochondria where CA VA is present is around 8.5, so in vivo its activity may be higher.[1] Recently, our group showed[17−20] that CA VA is a druggable target and that sulfonamide/sulfamate inhibitors directed against this isoform may have applications for the development of antiobesity drugs,[21,22] due to the impaired provision of substrate (i.e., bicarbonate) for carboxylating enzymes involved in fatty acid biosynthesis, such as pyruvate carboxylase and acetyl-coenzyme A carboxylase, ensued by inhibition of CA VA.[21−23] Indeed, CA inhibitors (CAIs) targeting diverse isozymes are clinically used as antiglaucoma drugs,[1,16] diuretics,[16] and in the treatment of epilepsy and some minor neurological disorders,[24,25] whereas some of them show promising applications as antitumor agents.[26,27] Different isozymes of the 15 mentioned above are being

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CA VB -33:MVVMNSLRVILQASPGKLLWRKFQIPRFMPARPCSLYTCTYKTRNRALHPLWESVDLVPG
CA VA -38:-LGR-TWKTSAFSFLVEQM-APLWSRSMR-G-W--QRS-AWQ-S-NT-----TVPVS---

CA VB  28:GDRQSPINIRWRDSVYDPGLKPLTISYDPATCLHVWNNGYSFLVEFEDSTDKSVIKGGPL
CA VA  23:-T-------Q--------Q----RV--EA-S--YI--T--L-Q---D-A-EA-G-S----

                                      ▼
CA VB  88:EHNYRLKQFHFHWGAIDAWGSEHTVDSKCFPAELHLVHWNAVRFENFEDAALEENGLAVI
CA VA  83:-NH------------VNEG-------GHAY----------S-KYQ-YKE-VVG-------

                                                ▼
CA VB 148:GVFLKLGKHHKELQKLVDTLPSIKHKDALVEFGSFDPSC-MPTCPDYWTYSGSLTTPPLS
CA VA 143:-------A--QT--R---I--E------RAAMRP----T-L---W-----A--------T

CA VB 208:ESVTWIIKKQPVEVDHDQLEQFRTLLFTSEGEKEKRMVDNFRPLQPLMNRTVRSSFRHDY
CA VA 203:-------Q-E----APS--SA------SAL--E--M--N-Y---------K-WA--QATN

CA VB 268:VLNVQAKPKPATSQATP
CA VA 263:EGTRS
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Figure 1. Alignment of amino acid sequences of CAVA and VB. The N-terminal underlined segments indicate a putative signal sequence for mitochondrial localization. Note CA VB has two additional cysteine residues (bold face) indicated by triangles.

	+	+			+			+			+	Z	Z	+	+	+	Z							+		+	+						+		+	+
Residue number	7	29	61	62	64	65	66	67	69	91	92	94	96	106	107	117	119	121	131	141	143	145	192	194	198	199	200	201	202	204	206	207	209	211	244	246
CA II	Y	S	N	N	H	A	F	N	E	I	Q	H	H	E	H	E	H	V	F	L	V	G	W	Y	L	T	T	P	P	L	C	V	W	V	N	R
CA VB	T	-	-	-	Y	S	-	L	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	-	-	I	-	-
CA VA	T	-	-	T	Y	L	-	Q	-	K	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	T	S	-	-	I	-	-

Figure 2. An alignment of active-site residues of CAs VA and VB with CA II. Thirty-six active site residues that were previously[44] defined as forming the active site are aligned; +, active-site hydrogen bond network; Z, zinc-liganded histidine. Three residues of CA II that participate in the hydrogen bond network are replaced with different residues in the mitochondrial isozymes (shown in boxes). In comparison with CA II, eight residues are substituted in CA VB and two additional substitutions are observed in CA VA (bold boxes). Residue numbers are based on the CA I sequence.[16]

targeted for such applications, mainly by sulfonamide, sulfamate, or sulfamide compounds, some of which act as low nanomolar inhibitors.[28,29]

CA VB has a much wider tissue distribution[6,7,30] as compared to CA VA, suggesting different physiological roles for the two mitochondrial isozymes. Indeed, Nishimori's and Sly's groups showed CA VB to be present in pancreas, kidney, salivary glands, spinal cord, heart, and skeletal muscle, but not in the liver, where CA VA is present.[6,7,30] On the other hand, the precise catalytic activity, as well as susceptibility to inhibition, of this widely distributed isoform has never been investigated up to now. Here we report the first such study showing that CA VB is catalytically more efficient as compared to CA VA and that this isoform is also a druggable target. An inhibition study with a library of 34 sulfonamides/sulfamates has been performed by using this newly characterized isozyme cloned/purified in a new expression system. Many potent inhibitors were detected that may be useful both in the design of novel applications of CAIs as well as in explaining possible side effects of some of the clinically used drugs from this family of pharmacological agents.

Results

hCA VB Cloning, Purification, and Catalytic Activity. The alignment of amino acid sequences of the two mitochondrial isoforms CA VA and VB is shown in Figure 1, whereas the 36 active site amino acid residues of isozymes hCA II, VA, and VB are shown in Figure 2.

The catalytic activity of several α-CA isozymes of human origin, among which are also hCA II, VA, and VB, is shown in Table 1.

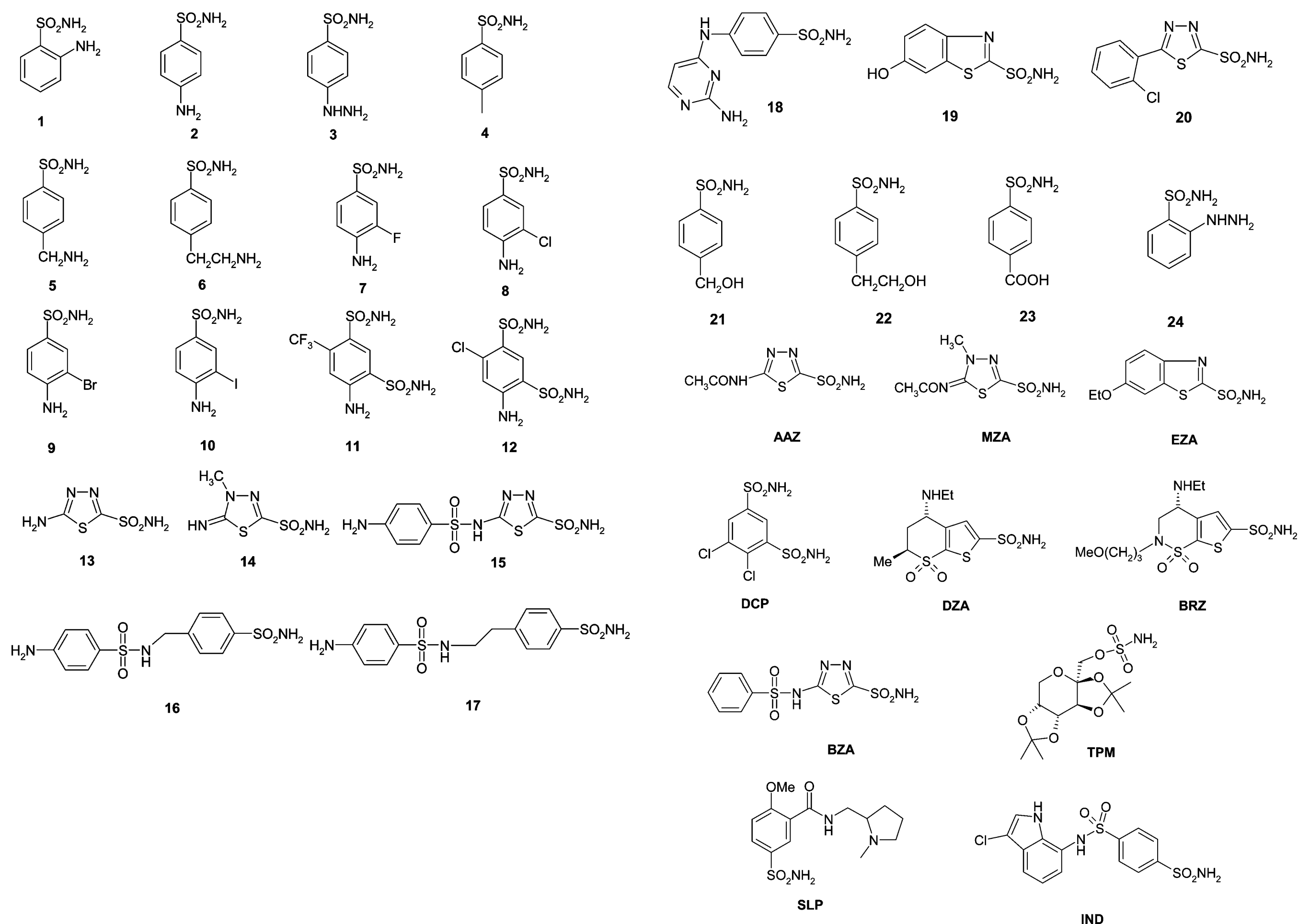
Table 1. Kinetic Parameters for CO_2 Hydration Reaction Catalyzed by the Cytosolic α-CA Isozymes I−III, the Mitochondrial Isoforms hCA VA and hCA VB, and the Transmembrane Isozymes hCA XII (catalytic domain) and hCA XIV (full length), at 20 °C and pH 7.5, and Their Inhibition Data with Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a Clinically Used Sulfonamide

isozyme[a]	activity level	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_i (acetazolamide) (nM)
hCA I	low	2.0×10^5	5×10^7	250
hCA II	very high	1.4×10^6	1.5×10^8	12
hCA III	very low	1.0×10^4	3×10^5	300000
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 XII	low	4.20×10^5	3.5×10^7	5.7
hCA XIV	low	3.12×10^5	3.9×10^7	41

[a] h = human.

Chemistry and CA Inhibition. Sulfonamides investigated for the inhibition of the mitochondrial enzymes hCA VA and hCA VB, of types **1**−**24** are shown below. Derivatives AAZ through IND are clinically used

Chart 1



drugs: acetazolamide (AAZ), methazolamide (MZA), ethoxzolamide (EZA), and dichlorophenamide (DCP) are the classical, systemically acting CAIs.[16] Dorzolamide (DZA) and brinzolamide (BRZ) are topically acting antiglaucoma agents,[1,4] and benzolamide (BZA) is an orphan drug belonging to this class of pharmacological agents,[31] whereas topiramate (TPM) is a widely used antiepileptic drug.[32] Sulpiride (SLP)[33] and indisulam (IND)[34] 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, whereas **3**, **7**−**10**,[35] **13**−**17**,[36,37] **21**, **22**,[38] and **24**[39] were prepared as reported earlier by this group (Chart 1). Inhibition data of these sulfonamides/sulfamates against isozymes hCA II, VA, and VB are shown in Table 2.

Discussion

hCA VB Cloning, Purification, and Catalytic Activity. The mitochondrial isoform hCA VB has been cloned from the cDNA previously described[6] in a bacterial expression system in order to produce large amounts of protein needed for medicinal chemistry purposes (see Experimental Section for details). In the previous work,[6] an eukaryotic expression system (COS-7 cells) has been employed for obtaining CA VB. In this work, a GST-hCA VB construct was obtained both for the full length enzyme (of 36.4 kDa)[6] as well as for the truncated enzyme lacking the first N-terminal 33 amino acid residues (which represents a putative mitochondrial signal sequence).[6] However, this last construct (but not the first one) was toxic to the prokaryotic cells in which we intended to express the protein (*Escherichia coli* strain BL21), and thus, no truncated hCA VB could be obtained by this approach. The full length enzyme was on the other hand obtained in soluble form without complications by the GST-fusion protein method, leading to a simplified procedure for obtaining large amounts of protein needed for inhibitor screening purposes. The GST part of the fusion protein was cleaved with thrombin and hCA VB further purified by affinity chromatography, as for other CA isozymes ultimately purified by the same approach (such as CA VII, IX, and XII, among others).[40−42]

The kinetic parameters for the CO_2 hydration reaction catalyzed by this new mitochondrial isozyme, as well as other isoforms that are targets for the drug design (such as hCA I, II, VA, XII, and XIV), are shown in Table 1. It may be observed that hCA VB is about 3.37 times more effective as a catalyst for CO_2 hydration to bicarbonate as compared to hCA VA, with a k_{cat} of 9.5 $\times$ 10^5 s^{-1} and k_{cat}/K_M of 9.8 $\times$ 10^7 M^{-1} s^{-1} (at pH 7.5 and 20 °C). Thus, hCA VB is second only to hCA II as a catalyst for the physiological reaction (hCA II is the most effective human isoform investigated to date and also one of the best biocatalysts known in nature).[16] Indeed, hCA VB is almost 2 times more effective than the slow red blood cell isozyme hCA I, being also more

Table 2. hCA II, VA, and VB Inhibition Data with Compounds **1**−**24** and the Ten Clinically Used Derivatives AAZ through IND

inhibitor	$K_i{}^a$ (nM)		
	hCA II[b]	hCA VA[b]	hCA VB[b]
1	295	15300	9560
2	240	32000	3650
3	300	7700	3500
4	320	4700	2430
5	170	27600	12300
6	160	42400	8950
7	60	9300	4220
8	110	7400	4300
9	40	7600	3540
10	70	9400	3300
11	63	9400	6560
12	75	4170	6210
13	60	2300	2150
14	19	2160	2075
15	2	34	30
16	46	176	24
17	50	121	42
18	33	17000	10200
19	30	54	41
20	12	43	30
21	80	9300	7450
22	125	8700	7000
23	133	2650	1560
24	125	9600	3400
AAZ	12	63	54
MZA	14	65	62
EZA	8	25	19
DCP	38	630	21
DZA	9	42	33
BRZ	3	50	30
BZA	9	37	34
TPM	10	63	30
SLP	40	174	18
IND	15	79	23

[a] Errors in the range of 5−10% of the shown data, from 3 different assays. [b] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[48]

active than isozymes XII or XIV for the physiological reaction catalyzed by CAs. The least effective among the active CA isozymes is CA III, which has a very low catalytic activity (Table 1).[16] It may also be observed from the data of Table 1 that all these isozymes except CA III are susceptible to inhibition by acetazolamide, the CAI par excellence (see extensive discussion later in the text).[16]

Among the α-CA gene family, hCA VB shows the highest degree of similarity in amino acid sequence, 64.2%, with the other mitochondrial isozyme, hCA VA[6] (Figure 1). However, there is a different number of cysteine residues in the two proteins, which are possibly involved in intramolecular disulfide bond formation:[6,43] CA VA contains four cysteine residues, whereas CA VB has six. The additional two cysteines in CA VB (at positions 149 and 219; see Figure 1) probably further stabilize its molecular structure as compared to that of CA VA and thus may explain the higher activity of isoform VB. The 36 amino acid residues forming the active site in the human α-CAs[44] were aligned for the two mitochondrial CAs (VA and VB) with those corresponding to CA II (Figure 2). Three residues of CA II that take part in the active-site hydrogen bond network critical for the catalytic/inhibition mechanism (indicated by boxes in Figure 2) are replaced with different residues in the mitochondrial CAs. These residues are Tyr7 (replaced with Thr7), His64 (replaced with Tyr64), and Asn67 (replaced with Leu67 in CA VB and with Gln67 in CA VA). These amino acid substitutions may explain the lower catalytic activity of the two mitochondrial CAs as compared to CA II, since His64 plays an important role in catalysis. However, the proton shuttle residue of CA VA (and VB) is not known at this moment. Among these active site residues, interestingly, CA VB has eight substitutions as compared to CA II, whereas CA VA has two other additional substitutions. An important amino acid for the binding of inhibitors[27,45] to the active site among those discussed here is 131, which is Phe in CA II and VB and Tyr in CA VA. Computer analysis of the total amino acid sequences showed higher homology of CA II with CA VB (54.9%) as compared to that of CA VA (51.0%). These findings might explain the higher catalytic activity of CA VB as compared to CA VA and the different affinity for inhibitors of the three isozymes (see later in the text), furnishing a basis for the rational drug design of CA VB-targeted inhibitors. Indeed, some of the amino acid residues mentioned above (i.e., those in positions 67 and 131) were shown to be involved in the binding of sulfonamide/sulfamate inhibitors by means of X-ray crystallography of enzyme−inhibitor adducts.[27,45]

Chemistry and hCA VB 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 10 clinically used compounds AAZ through IND 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 their molecule).[1,3,4,16,29,37] Thus, it in not improbable that detecting good hCA VB inhibitors among such simple sulfonamides may thereafter lead to the development of more effective inhibitors; (ii) the inhibition profile against hCA VB of the clinically used compounds AAZ through IND may be important both for explaining possible side effects of some of these derivatives as well as for the design of novel therapeutic applications, for them or some of their derivatives.

The following SAR may be drawn from the inhibition data of Table 2. (i) Several compounds investigated here, such as **1**, **5**, **6**, **11**, **12**, **18**, **21**, and **22**, showed weak hCA VB inhibitory properties, with inhibition constants in the range of 6.21−12.3 μM. It may be observed that all these compounds are either simple 2- or 4-substituted-benzenesulfonamide or benzene-1,3-disulfonamide derivatives, bearing moieties such as amino, aminomethyl/ethyl or hydroxymethyl/ethyl. (ii) The following derivatives were medium potency hCA VB inhibitors (K_i's in the range of 1.56−4.3 μM): **2**−**4**, **7**−**10**, **13**, **14**, **23**, and **24**. These compounds either belong to the benzensulfonamide group of derivatives bearing 4-amino, 4-hydrazino, 4-methyl, or 4-carboxy moieties or are halogenated sulfanilamides (derivatives **7**−**10**). Two such derivatives are heteroaromatic sulfonamides (**13** and **14**), being in fact the deacetylated precursors of two clinically used drugs, AAZ and MZA, respectively. (iii) A rather large number of derivatives, such as **15**−**17**, **19**, **20**, and the 10 clinically used drugs AAZ through

IND showed strong inhibitory activity against the mitochondrial isozyme hCA VB, with inhibition constants in the range of 18−62 nM. It may be observed that from the chemical point of view these derivatives are quite heterogeneous, with both aromatic sulfonamides/bis-sulfonamides (such as the sulfanilylsulfonamides **16** and **17**, the 1,3-benzenedisulfonamide DCP, IND, or SLP), heteroaromatic compounds (**15**, **19**, **20**, AAZ, MZA, EZA, DZA, BRZ, and BZA), and sulfamate TPM showing this compact behavior of potent hCA VB inhibitors. It is interesting to note that the most efficient hCA VB inhibitors detected up to now were EZA (K_i of 19 nM) and SLP (K_i of 18 nM). (iv) The mitochondrial isozyme hCA VB has an inhibition profile with these derivatives quite different from those of the other mitochondrial isoform, hCA VA, or the ubiquitous cytosolic isozyme hCA II (Table 2). Thus, generally, isozyme hCA II showed the highest affinity for these inhibitors, followed by the mitochondrial isozyme hCA VB, whereas the other mitochondrial isozyme, hCA VA, was the least inhibitable by this class of sulfonamides/sulfamates. However, several important exceptions to this rule have been evidenced. For example, three of the investigated compounds, **16**, DCP, and SLP, showed the highest affinity just for this new isozyme, hCA VB, over the other two investigated here (hCA VA and hCA II). Thus, the selectivity ratio of these compounds (for hCA VB over hCA II) was 1.91 for **16** and SLP and 1.80 for DCP, meaning that these three derivatives were on average almost 2 times more effective inhibitors of the mitochondrial isozyme hCA VB than of the cytosolic isoform hCA II. Comparing the selectivity ratio of these derivatives for the inhibition of isozyme hCA VB over isozyme hCA VA, the selectivity ratios are much better, in the range of 7.33 for **16**, 30.00 for DCP, and 9.66 for SLP, respectively. Thus, it is obvious that, even from this small library of derivatives investigated, it is possible to evidence compounds with selectivity for this new mitochondrial isozyme over the related isoform hCA VA or the ubiquitous cytosolic isozyme hCA II. However, many of the clinically used sulfonamides, such as AAZ, MZA, and BZA, showed almost the same potency against the two mitochondrial isozymes hCA VA and hCA VB. It is interesting to note that TPM, a compound showing important antiobesity effects that are probably due to the inhibition of the mitochondrial isozyme hCA VA,[17,21] is a 2 times more effective hCA VB inhibitor (K_i of 30 nM) as comparted to hCA VA inhibitor (K_i of 63 nM). At this point it may be important to investigate in detail the role of this new isoform (hCA VB) in lipogenesis, provided that potent and rather selective inhibitors have been evidenced in this study.

4. Conclusions

The first hCA VB inhibition study is presented here. The full length 36.4 kDa 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}. A large series of sulfonamides and a sulfamate have been tested for their interaction with this isozyme as well as the other mitochondrial enzyme (hCA VA) and the cytosolic, ubiquitous isoform hCA II. Simple benzenesulfonamides were ineffective hCA VB inhibitors, with inhibition constants in the range of 6.21−12.3 μM, whereas better inhibitors were detected among such derivatives bearing 4-amino, 4-hydrazino, 4-methyl, or 4-carboxy moieties or among halogenated sulfanilamides (K_i's in the range of 1.56−4.3 μM). The 10 clinically used compounds, acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, topiramate, sulpiride, and indisulam, showed effective hCA VB inhibitory activity, with inhibition constants in the range of 18−62 nM. The new mitochondrial isoform shows higher affinity for these inhibitors as compared to hCA VA, but the most sulfonamide-avid isozyme remains hCA II. Only three compounds showed better activity against hCA VB over hCA II, among which were sulpiride and ethoxzolamide, which were almost 2 times more effective inhibitors of the mitochondrial over the cytosolic isozyme. hCA VB is thus a druggable target and some of its inhibitors may lead to the development of novel antiobesity therapies.

Experimental Section

Chemistry. Compounds **1**, **2**, **4**−**6**, **11**, **12**, **18**−**20**, **23**, and AAZ through SLP are commercially available from Sigma-Aldrich, Merck, Alcon, or Johnson & Jonson, whereas **3**, **7**−**10**,[35] **13**−**17**,[36,37] **21**, **22**,[38] and **24**[39] were prepared as reported earlier by this group.

The GST-hCA VB Fusion Protein. The cDNA fragment encoding the open reading frame of hCA VB was amplified from the cDNA clone previously obtained[6] (Accession No. AB021660) by PCR using adopter primers including *EcoR* I and *Sal* I recognition sequences (italic in the following sequences, respectively): 5′-CCC*GAATTC*TCAAGCTAAAGATGGTGGTGA-3′ and 5′-TT*GTCGAC*GGGGTTGCTTGGCTGGTGGC-3′. The PCR reaction was hot-started with incubation for 1 min at 94 °C and consisted of 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C. The PCR products were cleaved with *EcoR* I and *Sal* I, purified and cloned in-frame into the pGEX-4T2 vector (Amersham). The proper cDNA sequence of the hCA VB 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 VB fusion protein, similarly to the procedure already described for hCA IX and XII.[41,46] Following induction of the protein expression by adding 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 VB 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 VB is purified easily and the procedure is quite simple. The obtained hCA VB was further purified by sulfonamide affinity chromatography,[47] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[48]

CA Inhibition Assay. An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[48] 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 determin-

ing 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 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.[40,41]

References

(1) Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T., Carbonic anhydrases: Current state of the art, therapeutic applications and future prospects. *J. Enzyme Inhib. Med. Chem.* **2004**, *19* (3), 199−229.

(2) 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., Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family. *J. Biol. Chem.* **2004**, *279* (4), 2719−27.

(3) Supuran, C. T.; Scozzafava, A.; Casini, A., Carbonic anhydrase inhibitors. *Med. Res. Rev.* **2003**, *23* (2), 146−89.

(4) Supuran, C. T.; Vullo, D.; Manole, G.; Casini, A.; Scozzafava, A. Designing of novel carbonic anhydrase inhibitors and activators. *Curr. Med. Chem. Cardiovasc. Hematol. Agents* **2004**, *2* (1), 49−68.

(5) Tashian, R. E.; Venta, P. J.; Nicewander, P. H.; Hewett-Emmett, D., Evolution, structure, and expression of the carbonic anhydrase multigene family. *Prog. Clin. Biol. Res.* **1990**, *344*, 159−75.

(6) Fujikawa-Adachi, K.; Nishimori, I.; Taguchi, T.; Onishi, S., Human mitochondrial carbonic anhydrase VB. cDNA cloning, mRNA expression, subcellular localization, and mapping to chromosome x. *J. Biol. Chem.* **1999**, *274* (30), 21228−33.

(7) Nishimori, I.; Onishi, S., Carbonic anhydrase isozymes in the human pancreas. *Dig. Liver Dis.* **2001**, *33* (1), 68−74.

(8) (a) Fujikawa-Adachi, K.; Nishimori, I.; Taguchi, T.; Onishi, S., Human carbonic anhydrase XIV (CA14): cDNA cloning, mRNA expression, and mapping to chromosome 1. *Genomics* **1999**, *61* (1), 74−81; (b) Hilvo, M.; Tolvanen, M.; Clark, A.; Shen, B.; Shah, G. N.; Waheed, A.; Halmi, P.; Hanninen, M.; Vihinen, M.; Sly, W. S.; Parkkila, S. Characterization of CA XV, a new GPI-anchored form of carbonic anhydrase. *Biochem. J.* **2005**, in press (Epub August 8, 2005).

(9) Dodgson, S. J.; Forster, R. E., 2nd; Storey, B. T.; Mela, L., Mitochondrial carbonic anhydrase. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77* (9), 5562−6.

(10) Dodgson, S. J.; Forster, R. E., 2nd; Schwed, D. A.; Storey, B. T., Contribution of matrix carbonic anhydrase to citrulline synthesis in isolated guinea pig liver mitochondria. *J. Biol. Chem.* **1983**, *258* (12), 7696−701.

(11) Dodgson, S. J.; Forster, R. E., 2nd; Storey, B. T., The role of carbonic anhydrase in hepatocyte metabolism. *Ann. N. Y. Acad. Sci.* **1984**, *429* 516−24.

(12) Dodgson, S. J.; Forster, R. E., 2nd, Carbonic anhydrase: Inhibition results in decreased urea production by hepatocytes. *J. Appl. Physiol.* **1986**, *60* (2), 646−52.

(13) Carter, N. D.; Dodgson, S. J.; Quant, P. A., Expression of hepatic mitochondrial carbonic anhydrase V. *Biochim. Biophys. Acta.* **1990**, *1036* (3), 237−41.

(14) Heck, R. W.; Tanhauser, S. M.; Manda, R.; Tu, C.; Laipis, P. J.; Silverman, D. N., Catalytic properties of mouse carbonic anhydrase V. *J. Biol. Chem.* **1994**, *269* (40), 24742−6.

(15) Boriack-Sjodin, P. A.; Heck, R. W.; Laipis, P. J.; Silverman, D. N.; Christianson, D. W., Structure determination of murine mitochondrial carbonic anhydrase V at 2.45-A resolution: Implications for catalytic proton transfer and inhibitor design. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (24), 10949−53.

(16) Supuran, C. T.; Scozzafava, A.; Conway, J., *Carbonic Anhydrase − Its Inhibitors and Activators*. CRC Press: Boca Raton, FL, 2004; pp 1−363.

(17) Vullo, D.; Franchi, M.; Gallori, E.; Antel, J.; Scozzafava, A.; Supuran, C. T., Carbonic anhydrase inhibitors. Inhibition of mitochondrial isozyme V with aromatic and heterocyclic sulfonamides. *J. Med. Chem.* **2004**, *47* (5), 1272−9.

(18) Winum, J. Y.; Innocenti, A.; Gagnard, V.; Montero, J. L.; Scozzafava, A.; Vullo, D.; Supuran, C. T., Carbonic anhydrase inhibitors. Interaction of isozymes I, II, IV, V, and IX with organic phosphates and phosphonates. *Bioorg. Med. Chem. Lett.* **2005**, *15* (6), 1683−1686.

(19) Innocenti, A.; Vullo, D.; Scozzafava, A.; Casey, J. R.; Supuran, C., Carbonic anhydrase inhibitors. Interaction of isozymes I, II, IV, V, and IX with carboxylates. *Bioorg. Med. Chem. Lett.* **2005**, *15* (3), 573−8.

(20) Innocenti, A.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T., Carbonic anhydrase inhibitors: Inhibition of human cytosolic isozyme II and mitochondrial isozyme V with a series of benzene sulfonamide derivatives. *Bioorg. Med. Chem. Lett.* **2004**, *14* (22), 5703−7.

(21) Supuran, C. T., Carbonic anhydrase inhibitors in the treatment and prophylaxis of obesity. *Expert Opin. Ther. Pat.* **2003**, *13*, 1545−1550.

(22) Winum, J. Y.; Scozzafava, A.; Montero, J. L.; Supuran, C. T., Sulfamates and their therapeutic potential. *Med. Res. Rev.* **2005**, *25*, 186−228.

(23) Hazen, S. A.; Waheed, A.; Sly, W. S.; LaNoue, K. F.; Lynch, C. J., Differentiation-dependent expression of CA V and the role of carbonic anhydrase isozymes in pyruvate carboxylation in adipocytes. *Faseb. J.* **1996**, *10* (4), 481−90.

(24) Supuran, C. T.; Scozzafava, A., Carbonic anhydrase inhibitors and their therapeutic potential. *Expert Opin. Ther. Pat.* **2000**, *10*, 575−600.

(25) Supuran, C. T.; Scozzafava, A. Applications of carbonic anhydrase inhibitors and activators in therapy. *Expert Opin. Ther. Pat.* **2002**, *12*, 217−242.

(26) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T., Modulation of carbonic anhydrase activity and its applications in therapy. *Expert Opin. Ther. Pat.* **2004**, *14*, 667−702.

(27) Abbate, F.; Casini, A.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors: X-ray crystallographic structure of the adduct of human isozyme II with the perfluorobenzoyl analogue of methazolamide. Implications for the drug design of fluorinated inhibitors. *J. Enzyme Inhib. Med. Chem.* **2003**, *18* (4), 303−8.

(28) 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, FL, 2004; pp 1−23.

(29) 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, FL, 2004; pp 67−147.

(30) Shah, G. N.; Hewett-Emmett, D.; Grubb, J. H.; Migas, M. C.; Fleming, R. E.; Waheed, A.; Sly, W. S., Mitochondrial carbonic anhydrase CA VB: Differences in tissue distribution and pattern of evolution from those of CA VA suggest distinct physiological roles. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (4), 1677−82.

(31) Liddell, N. E.; Maren, T. H. CO2 retention as a basis for increased toxicity of salicylate with acetazolamide: Avoidance of increased toxicity with benzolamide. *J. Pharmacol. Exp. Ther.* **1975**, *195* (1), 1−7.

(32) Casini, A.; Antel, J.; Abbate, F.; Scozzafava, A.; David, S.; Waldeck, H.; Schafer, S.; Supuran, C. T., Carbonic anhydrase inhibitors: SAR and X-ray crystallographic study for the interaction of sugar sulfamates/sulfamides with isozymes I, II and IV. *Bioorg. Med. Chem. Lett.* **2003**, *13* (5), 841−5.

(33) Abbate, F.; Coetzee, A.; Casini, A.; Ciattini, S.; Scozzafava, A.; Supuran, C. T., Carbonic anhydrase inhibitors: X-ray crystallographic structure of the adduct of human isozyme II with the antipsychotic drug sulpiride. *Bioorg. Med. Chem. Lett.* **2004**, *14* (2), 337−41.

(34) Abbate, F.; Casini, A.; Owa, T.; Scozzafava, A.; Supuran, C. T., Carbonic anhydrase inhibitors: E7070, a sulfonamide anticancer agent, potently inhibits cytosolic isozymes I and II, and transmembrane, tumor-associated isozyme IX. *Bioorg. Med. Chem. Lett.* **2004**, *14* (1), 217−23.

(35) Ilies, M. A.; Vullo, D.; Pastorek, J.; Scozzafava, A.; Ilies, M.; Caproiu, M. T.; Pastorekova, S.; Supuran, C. T. Carbonic anhydrase inhibitors. Inhibition of tumor-associated isozyme IX by halogenosulfanilamide and halogenophenylaminobenzolamide derivatives. *J. Med. Chem.* **2003**, *46* (11), 2187−96.

(36) Scozzafava, A.; Briganti, F.; Mincione, G.; Menabuoni, L.; Mincione, F.; Supuran, C. T., Carbonic anhydrase inhibitors: Synthesis of water-soluble, aminoacyl/dipeptidyl sulfonamides possessing long-lasting intraocular pressure-lowering properties via the topical route. *J. Med. Chem.* **1999**, *42* (18), 3690−700.

(37) Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G.; Supuran, C. T., Carbonic anhydrase inhibitors. Synthesis of water-soluble, topically effective, intraocular pressure-lowering aromatic/heterocyclic sulfonamides containing cationic or anionic moieties: Is the tail more important than the ring? *J. Med. Chem.* **1999**, *42* (14), 2641−50.

(38) Supuran, C. T.; Clare, B. W., Quantum theoretic QSAR of benzene derivatives: Some enzyme inhibitors. *J. Enzyme Inhib. Med. Chem.* **2004**, *19* (3), 237−48.

(39) Winum, J. Y.; Dogne, J. M.; Casini, A.; de Leval, X.; Montero, J. L.; Scozzafava, A.; Vullo, D.; Innocenti, A.; Supuran, C. T., Carbonic anhydrase inhibitors: Synthesis and inhibition of cytosolic/membrane-associated carbonic anhydrase isozymes I, II, and IX with sulfonamides incorporating hydrazino moieties. *J. Med. Chem.* **2005**, *48* (6), 2121−5.
(40) Vullo, D.; Voipio, J.; Innocenti, A.; Rivera, C.; Ranki, H.; Scozzafava, A.; Kaila, K.; Supuran, C. T., Carbonic anhydrase inhibitors. Inhibition of the human cytosolic isozyme VII with aromatic and heterocyclic sulfonamides. *Bioorg. Med. Chem. Lett.* **2005**, *15* (4), 971−6.
(41) Vullo, D.; Innocenti, A.; Nishimori, I.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T., Carbonic anhydrase inhibitors. Inhibition of the transmembrane isozyme XII with sulfonamides-a new target for the design of antitumor and antiglaucoma drugs? *Bioorg. Med. Chem. Lett.* **2005**, *15* (4), 963−9.
(42) Winum, J. Y.; Pastorekova, S.; Jakubickova, L.; Montero, J. L.; Scozzafava, A.; Pastorek, J.; Vullo, D.; Innocenti, A.; Supuran, C. T., Carbonic anhydrase inhibitors: Synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, and IX with bis-sulfamates. *Bioorg. Med. Chem. Lett.* **2005**, *15* (3), 579−84.
(43) Okuyama, T.; Sato, S.; Zhu, X. L.; Waheed, A.; Sly, W. S., Human carbonic anhydrase IV: cDNA cloning, sequence comparison, and expression in COS cell membranes. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89* (4), 1315−9.
(44) Tashian, R. E. The carbonic anhydrases: Widening perspectives on their evolution, expression and function. *Bioessays* **1989**, *10* (6), 186−92.
(45) Fiore, A. D.; Simone, G. D.; Menchise, V.; Pedone, C.; Casini, A.; Scozzafava, A.; Supuran, C. T., Carbonic anhydrase inhibitors: X-ray crystal structure of a benzenesulfonamide strong CA II and CA IX inhibitor bearing a pentafluorophenylaminothioureido tail in complex with isozyme II. *Bioorg. Med. Chem. Lett.* **2005**, *15* (7), 1937−1942.
(46) Svastova, E.; Hulikova, A.; Rafajova, M.; Zat'ovicova, M.; Gibadulinova, A.; Casini, A.; Cecchi, A.; Scozzafava, A.; Supuran, C. T.; Pastorek, J.; Pastorekova, S., Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. *FEBS Lett.* **2004**, *577* (3), 439−45.
(47) Khalifah, R. G.; Strader, D. J.; Bryant, S. H.; Gibson, S. M., Carbon-13 nuclear magnetic resonance probe of active-site ionizations in human carbonic anhydrase B. *Biochemistry* **1977**, *16* (10), 2241−7.
(48) Khalifah, R. G., The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **1971**, *246* (8), 2561−73.

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