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Carbonic anhydrase inhibitors: Cloning, characterization, and inhibition studies of the cytosolic isozyme III with sulfonamides

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Abstract—The cytosolic human carbonic anhydrase (hCA, EC 4.2.1.1) isozyme III (hCA III) has been cloned and purified by the GST-fusion protein method. Recombinant pure hCA III had the following kinetic parameters for the CO_2 hydration reaction at 20 °C and pH 7.5: k_{cat} of 1.3×10^4 s^{-1} and k_{cat}/K_M of 2.5×10^5 M^{-1} s^{-1}, being a slower catalyst for the physiological reaction as compared to the genetically related cytosolic isoforms hCA I and II. An inhibition study with a library of sulfonamides and one sulfamate, some which are clinically used compounds, is reported. hCA III is less prone to be inhibited by these compounds as compared to hCA I and II for which many low nanomolar inhibitors were detected earlier. The best hCA III inhibitors were prontosil, sulpiride, indisulam, benzolamide, aminobenzolamide, and 4-amino-6-chloro-benzene-1,3-disulfonamide which showed K_Is in the range of 2.3–18.1 μM. Clinically used compounds such as acetazolamide, methazolamide, ethoxzolamide, dorzolamide, brinzolamide, topiramate, zonisamide, celecoxib, and valdecoxib were less effective hCA III inhibitors, with affinities in the range of 154–2200 μM. This is the first study in which low micromolar hCA III inhibitors are reported.

1. Introduction

There are 16 carbonic anhydrase (CA, EC 4.2.1.1) isoforms described so far in mammals.[1–4] The third one, CA III, is the least understood and investigated at this moment, in addition to being the worst catalyst for CO_2 hydration as compared to other cytosolic, mitochondrial or membrane-associated isozymes.[5,6] Similarly to the highly abundant CA I and II, CA III is a cytosolic isoform,[1–5] but its CO_2 hydration catalytic activity was reported to be of around 0.3–1% that of CA II (depending on the organisms from which the enzyme has been isolated and purified) for the physiologic reaction catalyzed by these enzymes.[7] In fact, unlike the ubiquitous isozymes I and II, CA III is mainly present in slow skeletal muscles (10% of the cytosolic protein content), adipocytes (24% of the soluble protein), and liver (8% of the soluble protein), where its primary functions remain largely unknown.[7,8] Recent studies with CA III knockout mice showed CA III to be involved in mitochondrial ATP synthesis,[8] whereas its levels were found to be significantly decreased in mutant mice lacking the gene SULT1E1, indicating a role of CA III in cystic fibrosis liver disease.[9] CA III is also considered as one of the proteins involved in oxidative stress response both in liver[10] and skeletal muscle,[11] probably acting as a scavenger of reactive oxygen species (ROS) and thus protecting cells from oxidative damage.[12] CA III seems to play an important role (together with E-cadherin) also in disruption of the intercellular barrier associated with the down-regulation of E-cadherin in the laryngopharyngeal reflux disease.[13]

These physiologic/pathophysiologic functions of CA III are poorly understood, except for the antioxidant role of this enzyme, which has been shown to be modulated by the *S*-glutathionylation of two cysteine residues (Cys181 and Cys186) present on the surface of the protein (but not within its active site).[14,15] Indeed, oxidants such as hydrogen peroxide, peroxy radicals or hypochlorous acid were shown to oxidize these two cysteine residues to sulfinic/sulfenic acids (in the absence of glutathione),

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but when this tripeptide was present in the medium, the *S*-glutathionylation of the two Cys residues occurred, without damage to the protein.[14,15] It is thus probable that one of the main in vivo functions of CA III is that of protecting proteins from irreversible oxidation processes with subsequent cellular damage.[4,14,15]

Another research line showed some interesting connections between obesity and CA III. Thus, Lynch et al.[16] reported a decrease in CA III expression in obese Zucker rats, possibly related to hyperinsulinemia, whereas Keha's group[17,18] showed that leptin, another protein involved in the genesis of obesity, decreased CA III expression (whereas insulin increased it). Since some CA inhibitors are known to act as effective agents for the management of obesity,[1,19,20] mainly targeting the mitochondrial isoforms CA VA and CA VB, it is of great interest to better understand the biochemical/physiologic processes connecting obesity and various CAs, including the less investigated CA III.

Up to now CA III from various organisms (e.g., bovines,[5,6] rodents,[7] or humans[21]) was obtained by extracting/purifying the enzyme from muscles or liver. Here we report the first cDNA cloning, purification and characterization of recombinant human CA III (hCA III), as well as a detailed inhibition study of the enzyme with sulfonamides/sulfamates, the main class of CA inhibitors,[1–3] as no detailed such investigations were reported up to now in the literature.

2. Results and discussion

2.1. hCA III cloning, sequence and purification

To date, the complete amino acid sequences in the open reading frame of three hCA III clones have been deposited in GenBank (Accession Nos. BC004897, NM_005181, and AK096880). The amino acid sequence of our clone was identical to those of the other three clones mentioned above, except for one amino acid substitution at position 70, that is, Phe(TTT):Ser(TCT). Another amino acid substitution was found in the NM_005181 clone at position 31, that is, Ile(ATT): Val(GTT). Considering the fact that these amino acids are not within the active site cavity of this enzyme, they are probably normal (neutral) polymorphic substitutions.

The amino acid sequence deduced from the cDNA sequence of our hCA III clone, was aligned with that of other cytoplasmic CA isozymes, that is, hCA I and hCA II (Fig. 1). hCA III shows a sequence similarity of 55% with hCA I and of 58% with hCA II. In Figure 1, the previously defined 36 CA active site amino acid residues[22] are marked with 'asterisk', 'plus', and 'z' signs above the hCA III sequence, where: * = not hydrogen bonded; + = hydrogen bonded, and z = zinc ligand amino acid. Among these residues, 24 amino acids are conserved between hCA III and hCA II. These two isozymes are those with the lowest and highest activity for the CO_2 hydration,[1–5] respectively, among the mammalian CAs (Table 1). Among these active site residues 18 are known to form a network of hydrogen bonds important for the binding of the substrates, inhibitors, and activators.[22,23] Fifteen of these amino acids are conserved between hCA III and hCA II. However, two of the remaining three amino acids (i.e., the residue 64, which is His in CA II and I, and Lys in CA III, and 198, which is Leu in CA II and I, and Phe in CA III, respectively) play a very important role in catalysis/binding of inhibitors, and they may explain the tremendous differences between these proteins.[1,2,4–7,23] Thus, His64 acts as a proton shuttle residue in the catalytically active CA isoforms (such as among others CA I, II, IV, VI, VII, IX, XII, XIII, and XIV),[1–4,22,23] favoring the

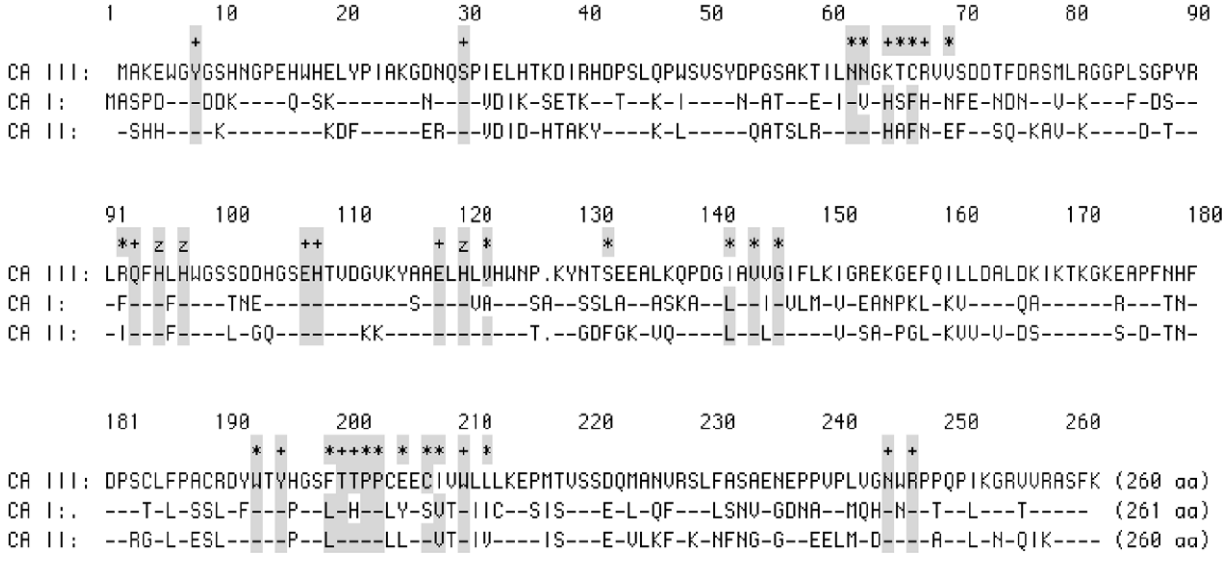


Figure 1. Alignment of the amino acid sequence of isoform CA III with those of isozymes CA I and II (CA I numbering system used). Thirty-six active site residues previously defined as forming the active site[22] are indicated by a mixture of *, +, and z signs above the CA III sequence. Seventeen residues known to participate in a network of hydrogen bonds and being involved in the binding of inhibitors/activators[23] are indicated by 'plus' and 'z' above the sequence; the latter sign indicating the three zinc-liganded histidine residues (His94, 96, and 119). Conserved amino acids in all three isoforms are indicated by a closed box. Dashes signify the same amino acid as in the sequence above the one of the considered isoforms.

Table 1. Kinetic parameters for the CO_2 hydration reaction catalyzed by the recombinant cytosolic hCA isozymes I-III, at 20 °C and pH 7.5,[31a] 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 III[a]	Very low	1.3×10^4	2.5×10^5	2.36×10^5
hCA III[b]	Very low	3.0×10^5	7.5×10^5	2.00×10^5
hCA III[c]	Very low	1.5×10^4	5.0×10^5	nd

[a] This study.
[b] Wistrand (Ref. 7a).
[c] An et al. (Ref. 7b); nd, not determined.

transfer of a proton from the zinc-bound water molecule to the reaction medium, with formation of the nucleophilic, zinc-hydroxide species of the enzyme (this is the rate-determining step of the entire catalytic cycle for the CO_2 hydration reaction catalyzed by these enzymes).[4] Lys64 present in CA III is less efficient as a proton shuttling residue as compared to His, due to the inappropriate pK_a of the ε-NH_2 moiety of this residue (pK_a around 9) as compared to the imidazole of a histidine (pK_a around 7).[1–5] On the other hand, the residue in position 198 is situated just in the middle of the active site cavity.[23–26] When this residue is a relatively compact Leu (such as in CA I and II), there is enough space for the binding of inhibitors (and activators), as shown by detailed X-ray crystallographic studies from this and other laboratories.[23–26] However, the bulky Phe198 present in CA III, unlike Leu198, probably interferes with the binding of most inhibitors, due to the steric impairment engendered by the phenyl moiety of the Phe198 residue. As a consequence of these two factors, CA III presents a quite low catalytic activity as compared to CA II (and also CA I), and is difficultly inhibited by most sulfonamide CA inhibitors, although no detailed inhibition studies were published up to now.[1,2,4–7]

A GST-hCA III fusion protein construct has been then obtained by the procedure already described by us for the production of other isoforms such as hCA VB, VI; IX and XII among others.[27–29] This fusion protein with the molecular weight of 50 kDa (data not shown) has been thereafter purified in two steps by affinity chromatography: the first one involved a Glutathione Sepharose 4B column (which binds the GST part of the fusion protein with high affinity), followed by cleavage of the GST part by thrombin.[27–29] The second step consisted in sulfonamide affinity chromatography, which was actually complicated by the low affinity of hCA III for the normally used columns for purification of other CA isoforms, based on 4-aminomethyl-benzenesulfonamide derivatized columns.[27–29] However, hCA III has high enough affinity for prontosil-based affinity columns (see later in the text),[30] and we used such a column for purification of our protein. Elution of hCA III from the column was then achieved with sodium azide (which is a rather strong CA III inhibitor, with a K_I of 80 μM),[5a] and extensive dialysis in Hepes buffer afforded the pure protein in rather good yield (4.5 mg protein/L of culture).

2.2. hCA III catalytic activity

Since all CA III preparations reported so far in the literature were isolating the enzyme from various organs, such as muscle or liver,[5–7] sometimes involving rather harsh treatments which potentially lead to protein denaturation/unfolding, we were interested to measure the kinetic parameters for the physiologic reaction (CO_2 hydration to bicarbonate) catalyzed by our recombinant enzyme, which has been produced in much milder, non-denaturating conditions. These parameters are shown in Table 1, where data for the other cytosolic, recombinant isozymes hCA I and II are also included for comparison.

Data of Table 1 show that hCA III produced by us is indeed a less effective catalyst for CO_2 hydration (k_{cat} of $1.3\times10^4\,s^{-1}$) as compared to the highly active hCA II (k_{cat} of $1.4\times10^6\,s^{-1}$) or the slower hCA I (k_{cat} of $2.0\times10^5\,s^{-1}$). Furthermore, the K_m for CO_2 of hCA III is higher as compared to the values of the other two related isozymes, which is clearly reflected in the k_{cat}/k_m values presented in Table 1. Considering these values, hCA III roughly shows 0.16% of the catalytic activity of hCA II (the best catalyst among all known CAs[1–4]) and 0.50% of the catalytic activity of hCA I. Our data are also in good agreement with those published by An et al.[7b] but slightly different of those of Wistrand[7a] on the enzyme purified from liver. It is difficult to explain why nature preserved during evolution such a 'bad' catalyst for CO_2 hydration, when the much more efficient and highly abundant hCA I and II were clearly available in many tissues and cell types (in addition to the remaining nine other catalytically active human CAs). Thus, our data reinforce the idea that probably CA III has different physiological functions, probably not connected to its catalytic function for CO_2 hydration.[1–4] Data of Table 1 also show that whereas hCA I and II are inhibited by the clinically used sulfonamide CA inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with inhibition constants in the range of 12–250 nM, hCA III has a much weaker affinity for this compound, with an inhibition constant of 236 μM. As outlined above, this is probably due to the presence of the bulky Phe198 in the middle of the hCA III active site, which interferes with the binding of compounds possessing an organic scaffold attached to the sulfonamide zinc-binding moiety (see the following paragraph for a detailed discussion).

2.3. hCA III inhibition with sulfonamides/sulfamates

A library of simple aromatic/heterocyclic sulfonamides and one sulfamate of types **1**–**24** and **AAZ-VLX** were investigated for their inhibitory activity against the newly cloned hCA III (Table 2), since very few such data are available in the literature.[1–5] Inhibition data for the related cytosolic isoforms hCA I and II are also provided for comparison, as all of them except the prontosil inhibition data have been published earlier.[25,27,29,32–34] The investigated sulfonamides include both relatively simple benzenesulfonamide, benzene-1,3-disulfonamide, 1,3,4-thiadiazole-2-sulfonamide as well as benzothiazole-2-sulfonamide derivatives, previously used to design potent

Table 2. Inhibition of recombinant isozymes hCA I, II, and III with sulfonamides **1**–**24** and the clinically used derivatives **AAZ-VLX** by a stopped-flow kinetic assay monitoring the CO_2 hydration reaction, at 20 °C and pH 7.5.[31a] The inhibition data with the dye prontosil are also included as this sulfonamide was used for hCA III purification on an affinity chromatography column

		K_I[c]	
Inhibitor	hCA I[a] (nM)	hCA II[a] (nM)	hCA III[b] (μM)
1	45,400	295	887
2	25,000	240	988
3	40	5	1060
4	78,500	320	7.9
5	25,000	170	681
6	21,000	160	1600
7	8300	60	2075
8	9800	110	710
9	9650	73	10.7
10	3700	29	19.0
11	5800	63	1276
12	8400	75	9.7
13	8600	60	148
14	9300	19	198
15	6	2	18.1
16	164	46	114
17	185	50	140
18	109	33	2633
19	1.4	0.3	80.5
20	690	12	14.6
21	55	80	4890
22	21,000	125	1187
23	23,000	133	11.3
24	24,000	125	1995
AAZ	250	12	236
MZA	50	14	770
EZA	25	8	1113
DCP	1200	38	684
DZA	50,000	9	770
BRZ	45,000	3	110
BZA	15	9	14.3
IND	31	15	10.4
SLP	12,000	40	10.6
TPM	250	10	780
ZNS	56	35	2200
SAC	18,540	5950	1000
CLX	50,000	21	154
VLX	54,000	43	788
Prontosil	63,000[b]	13[b]	2.3

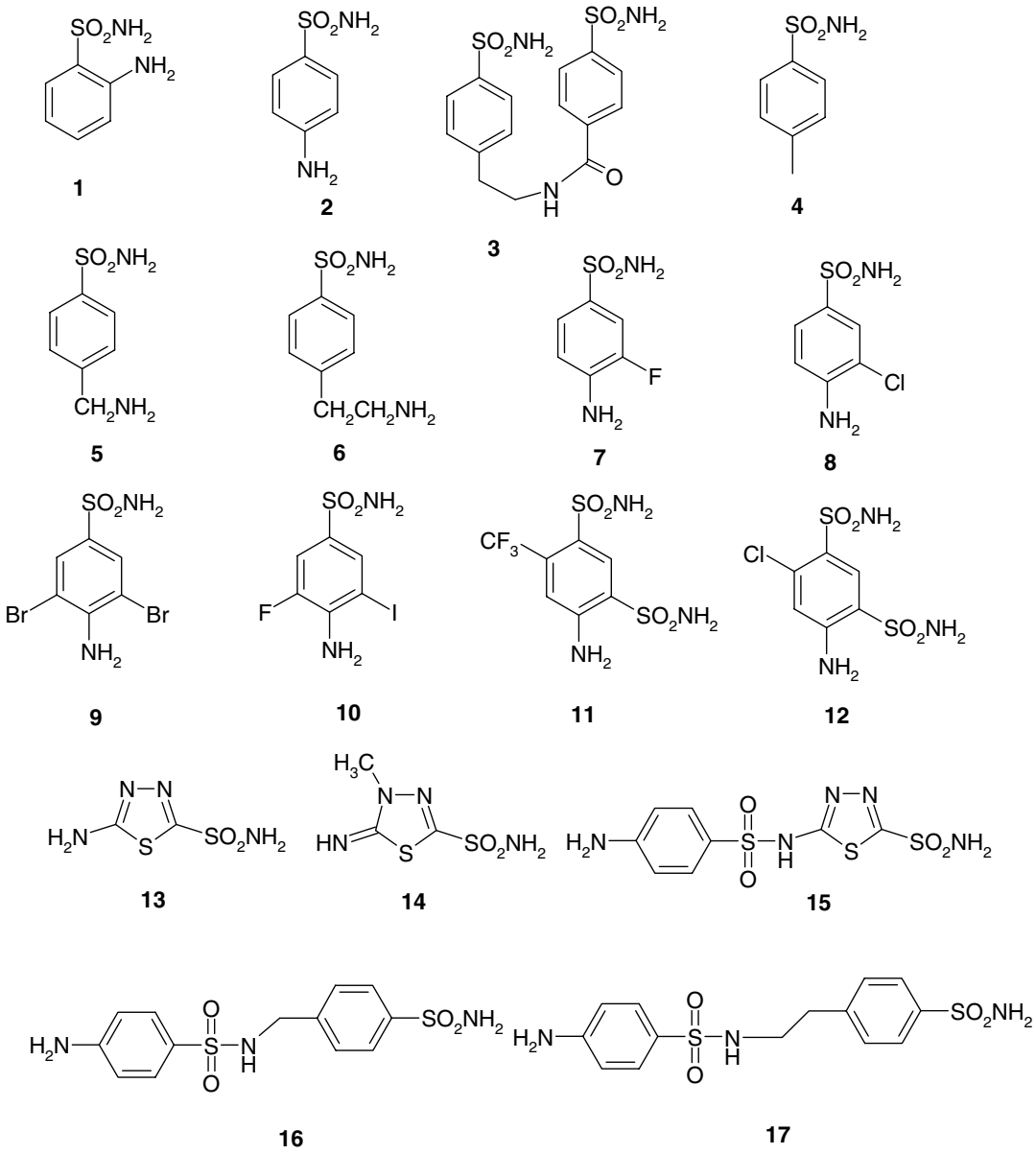
[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[31a] Inhibition data from Refs. 25,27,29,32–34.

[b] Human recombinant isozyme, inhibition data reported here for the first time.

[c] Errors in the range of 5–10% of the shown data, from three different assays.

CA inhibitors with various applications by means of the tail approach,[35] but also most of the clinically used drugs from this class of pharmacological agents, such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ** (the last two compounds are topically acting antiglaucoma agents)[1–4]; benzolamide **BZA** (an orphan drug),[1–4] the antitumor drug in phase II clinical trials indisulam **IND**,[36] the antipsychotic sulpiride **SLP**,[37] the antiepileptics topiramate **TPM**[38] and zonisamide **ZNS**,[25] the sweetener saccharin **SAC**,[39] as well as the COX-2 selective inhibitors celecoxib **CLX**[40] and valdecoxib **VLX**.[41] The azo-dye prontosil (a sulfanilamide derivative)[1–4] has also been included in this study, since the only sulfonamide affinity column that could be used for the purification of hCA III was the one based on this compound (see discussion above).[30]

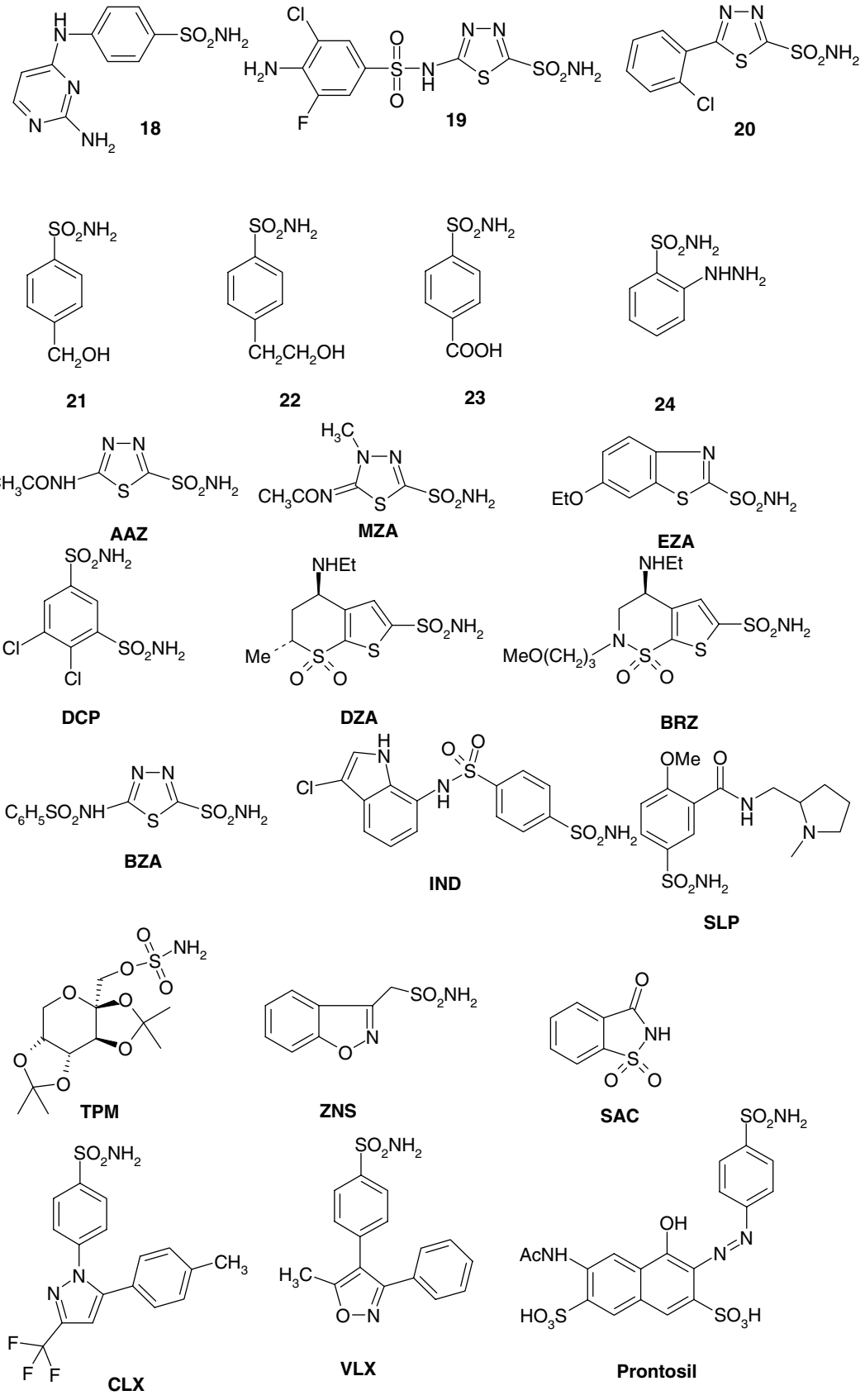
The following structure–activity relationship (SAR) can be observed by analyzing data of Table 2: (i) a large group of derivatives, including compounds **1**–**3**, **6**–**8**, **11**, **18**, **21**, **22**, **24**, **MZA**, **DZA**, **TPM**, **ZNS**, **SAC**, and **VLX**, show very weak hCA III inhibitory activities, with inhibition constants in the range of 710–4890 μM. From the chemical point of view, this is a very heterogeneous class of inhibitors, since both sulfonamides as well as the only investigated sulfamate cluster here. For sulfonamides, both simple benzenesulfonamide as well as various heterocyclic, aliphatic or more complex derivatives are present in this subgroups of weak hCA III inhibitors. It may be also observed that some of these compounds are quite bulky (e.g., **3**, **18**, **EZA**, and **TPM**), whereas others are relatively simple, less bulky derivatives (e.g., **1**, **2**, **6**, **21**, etc.); (ii) a second group of compounds, including sulfonamides **5**, **13**, **14**, **16**, **17**, **19**, **AAZ**, **DCP**, **BRZ**, and **CLX** show better hCA III inhibitory activity, with K_Is in the range of 80.5–684 μM. Again SAR is not easy to evidence, since both aromatic and heterocyclic sulfonamides show this behavior, with compact (**5**, **13**, **14**) as well as bulkier compounds (**16**, **17**, **19**, **BRZ**) having comparable activities; (iii) the most effective hCA III inhibitors evidenced so far were compounds **4**, **9**, **10**, **12**, **15**, **20**, **23**, **BZA**, **IND**, **SLP**, and prontosil, which showed K_Is in the range of 2.3–19.0 μM (Table 2). Again we want to stress the rather unexpected results we obtained, especially considering the fact that a very bulky derivative, such as the azo-dye prontosil, was the best hCA III inhibitor detected so far, with an inhibition constant of 2.3 μM, together with the quite simple and less bulky benzenesulfonamides **4**, **12**, **23**, **IND**, and **SLP**. This may explain in fact the empirical report of Chegwidden[30] that hCA III can be purified on affinity columns derivatized with this compound, technique that we also used to purify our recombinant hCA III. However, it is difficult to rationalize the binding mode of this compound within the enzyme active site, also considering that sulfanilamide **2**, the parent compound from which the dye is obtained, behaves as a 430 times less effective hCA III inhibitor as compared to prontosil. However, we note that several other such cases of very 'discrepant' inhibitory power between structurally quite similar compounds were evidenced during our study. For example, the amino- and methyl-substituted benzenesulfonamide derivatives **2** and **4** possess a very similar geometry/molecular shape, but their hCA III inhibitory powers differ by a factor of 125 (Table 2). Important differences were also observed between the two benzene-1,3-disulfonamide derivatives **11** and **12**, with the last one being 131 times a better hCA III inhibitor than the first one (when the chlorine atom substitutes the trifluoromethyl group). Compounds **21** and **23** also constitute a pair of structurally related derivatives with a difference of activity of 432 times in favor of the carboxy-substituted over the hydroxymethyl-substituted benzenesulfonamide. Again, the structurally



related **DZA** and **BRZ** show a rather big difference of activity against hCA III, although their activities against hCA I and II are quite similar. A more straightforward behavior was on the other hand observed for benzolamide **BZA** and its amino-derivative **15**, both of them acting as good hCA III inhibitors. It is impossible to rationalize these observations, since adducts of hCA III with sulfonamides were not yet characterized by X-ray crystallography (only one modeling such study, for the interaction of **15** with bovine CA III, has been reported so far)[42]; (iv) whereas the other cytosolic isoforms (hCA I and II) are easily inhibited by sulfonamides/sulfamates, with many low nanomolar inhibitors detected (e.g., **15**, **19**, **EZA**, **BZA**, **IND** against hCA I; **3**, **15**, **18**, **19**, **20**, **AAZ-ZNS**, **CLX**, **VLX**, and prontosil against hCA II), no nanomolar hCA III inhibitors were detected so far. It is in fact typical to observe inhibition constants in the low nanomolar–micromolar range with most of the investigated sulfonamides/sulfamate against hCA I and II; whereas very few micromolar hCA III inhibitors were detected, most of these compounds showing an affinity for this isoform in the range of 100–4890 μM. As already mentioned above, this is probably done to the presence of a bulky Phe residue in the middle of the active site cavity of hCA III.[5,6] However, we wish to stress that additional work is warranted in order to explain the rather efficient hCA III inhibitory power of some derivatives investigated by us here, some of which are clinically used drugs or pharmacological agents in clinical development (indisulam, sulpiride, etc.).

3. Conclusions

In conclusion, we report here the cloning and purification of hCA III. The enzyme shows lower catalytic activity as compared to other cytosolic isoforms (such as



hCA I and II), and a very characteristic inhibition profile with sulfonamides. The best hCA III inhibitors were prontosil, sulpiride, indisulam, benzolamide, aminobenzolamide, and 4-amino-6-chloro-benzene-1,3-disulfonamide which showed K_Is in the range of 2.3–18.1 μM. Clinically used compounds such as acetazolamide,

methazolamide, ethoxzolamide, dorzolamide, brinzolamide, topiramate, zonisamide, celecoxib, and valdecoxib were less effective hCA III inhibitors, with affinities in the range of 154–2200 μM. This is the first study in which low micromolar hCA III inhibitors are reported.

4. Experimental

4.1. Chemistry

Buffers were from Sigma–Aldrich (Milan, Italy) of highest purity available and were used without further purification. Compounds **1**, **2**, **4**–**6**, **11**, **12**, **18**–**20**, **23**, and **AAZ-SLP** and **SAC** are commercially available from Sigma–Aldrich, except **DZA** which was a gift from Merck, **BRZ** (a gift from Alcon), and **TPM** which was extracted from the Topamax^{R} pills (from Johnson & Jonson). **ZNS** was from DaiNippon, **CLX** and **VLX** from Pfizer. Derivatives **3**,[32] **7**–**10**,[33] **13**–**17**,[34,35] **21**,[34] **22**,[34] and **24**,[34] were prepared as reported earlier by this group.

4.2. CA III cloning

The cDNA fragment encoding the open-reading frame of hCA III was amplified from polyA(+) RNA obtained from human pancreas (Clontech, Palo Alto, CA, USA) by using a commercial RT-PCR kit (Takara, Kyoto, Japan) with adopter primers including EcoRI and SalI recognition sequences (underlined in the following sequences, respectively): 5′-CGGAATTCCC ATGGCCAAGGAGTGGGGC-3′ and 5′-GCAGTCGG ACCCTCATTTGAAGGAAGCTCT-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 EcoRI and SalI, purified and cloned in-frame into the pGEX-4T2 vector (Amersham). The cDNA sequence of the hCA III 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 III fusion protein, similar to the procedure already described for hCA VB, IX, and XII.[25–27] Following induction of the protein expression by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 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 III 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.[25–27] The advantage of this method is that hCA III is purified easily and the procedure is quite simple. The obtained hCA III was further purified by prontosil affinity column chromatography,[30] the elution being achieved with sodium azide 5 mM in 50 mM Hepes–HCL, pH 7.5 buffer. The amount of enzyme was determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[31] Human CA I and CA II cDNAs were expressed in *E. coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II as described earlier.[25–27]

4.3. CA catalytic/inhibition assay

An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA I, II, and III CO_2 hydration activity.[31a] 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 $NaClO_4$ (for maintaining constant the ionic strength—this anion is not inhibitory anyhow),[31b] following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10–50 mM (in the assay buffer) and dilutions up to 1 nM done with the assay buffer mentioned above. Enzyme concentrations were 0.09 μM for CA I, 0.06 μM for CA II, and 0.10 μM for CA III. Kinetic parameters and inhibition constants were calculated as described in Refs. 25,26,27,32–34

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