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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 3139–3143

Carbonic anhydrase inhibitors: Inhibition of the cytosolic human isozyme VII with anions

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Received 24 February 2006; revised 17 March 2006; accepted 18 March 2006
Available online 18 April 2006

Abstract—An inhibition study of the cytosolic carbonic anhydrase (CA, EC 4.2.1.1) isozyme VII (hCA VII) with anions has been conducted. Cyanate, cyanide, and hydrogensulfite were weak hCA VII inhibitors (K_Is in the range of 7.3–15.2 mM). Cl^- and HCO_3^- showed good inhibitory activity against hCA VII (K_Is of 0.16–1.84 mM), suggesting that this enzyme is not involved in metabolons with anion exchangers or sodium bicarbonate cotransporters. The best inhibitors were sulfamate, sulfamide, phenylboronic, and phenylarsonic acid (K_Is of 6.8–12.5 μM).

Among the 16 presently known mammalian carbonic anhydrase (CA, EC 4.2.1.1) isozymes, five are found in the cytosol.[1–5] In humans, these isozymes are hCA I, II, III, VII, and XIII, and many of them are required for pH homeostasis, gas/fluid/ion exchange processes in respiration, digestion, bone resorption, renal function, fertilization, sperm motility, and many other fundamental physiological processes.[1–5] The fact that these zinc enzymes exist in such a high number of isoforms, showing various degrees of enzymatic activity, subcellular localization, tissue distribution, abundance, kinetic properties, and sensitivity to inhibitors, reflects the very large spectrum of their biological roles.[1–5]

As catalysts for the reversible conversion between carbon dioxide and bicarbonate (with release of a proton during the hydration reaction), these enzymes possess a wide range of catalytic activities, from the evolutionarily 'perfect' CA II which is one of the fastest enzymes known (k_{cat}/K_m of 1.5×10^8 M^{-1} s^{-1}), to the very inefficient CA III, which possesses around 1% of the activity of the isozyme II.[1–6] Some of the cytosolic CAs are ubiquitous (CA I and II) or broadly distributed (CA VII and CA XIII), whereas others are confined to only few tissues, such as the muscles and adipocytes (CA III).[1,7,8]

CA VII appears to be the least studied and understood among the cytosolic CAs. Recently, we found hCA VII to be catalytically highly effective (k_{cat} of 9.5×10^5 s^{-1} and k_{cat}/K_m of 8.3×10^7 M^{-1} s^{-1} for the carbon dioxide hydration reaction at pH 7.5 and 20 °C)[9] and inhibited by the classical inhibitors of these enzymes, the aromatic and heterocyclic sulfonamides/sulfamates (these included among others, the clinically used drugs acetazolamide, ethoxzolamide, methazolamide, dichlorophenamide, topiramate or zonisamide).[9–11] In the CNS CA VII is primarily expressed in neurons.[12,13] The postnatal up-regulation of intrapyramidal CA VII in rat hippocampus has been shown to closely parallel the generation of high-frequency stimulation (HFS)-induced GABAergic excitation.[13] Such results point to a crucial role of the developmental expression of CA VII activity in shaping long-term plasticity and promoting epileptogenesis.[13] In addition, its potent inhibition by sulfonamides and sulfamates showed that this isoform is a potential target for the design of anticonvulsants or antiepileptic drugs.[9]

In addition to the sulfonamides and their derivatives (sulfamates, sulfamides, etc.),[4,10] all CAs are also

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doi:10.1016/j.bmcl.2006.03.078

inhibited by metal-complexing anions, such as the typical metal poisons cyanide, (thio)cyanate, hydrogen sulfide, azide, etc., but also by some anions which show less propensity to complex metal ions in solution (such as sulfate, nitrate, perchlorate, etc.).[14] Indeed, anion inhibition studies of the human isozymes I, II, IV, VA, IX, and XIII have been reported by our group in recent years.[15–21] However, no anion inhibition data of hCA VII are available in the literature. Such data are important, since it has been shown that in metabolically active tissues, and especially in situations requiring efficient ion transport, many CA isoforms interact with bicarbonate transporters or biosynthetic enzymes to form spatially and functionally orchestrated protein complexes called metabolons.[22] CAs can improve the transmembrane movement of bicarbonate or other membrane-impermeable anions which are transported by integral membrane proteins including the Cl^-/HCO_3^- anion exchangers (AEs), Na^+-coupled HCO_3^- co-transporters (NBCs), and SLC26A transporters.[23–25] The role of CAs (which physically interact with these anion transporters) is to increase the local availability of bicarbonate (or other anions, such as sulfate or carboxylates) and thereby accelerate their transmembrane flux.[21–23] Via this mechanism, various CA isoforms contribute to modulation of pH at both sides of the plasma membranes. Such transport metabolons have been first described in red blood cells, where the cytosolic CA II binds to the erythrocyte Cl^-/HCO_3^- exchanger (previously known as band 3 protein).[22] Functional and physical interactions between CA II and the Cl^-/HCO_3^- exchangers (isoforms AE1-3), the Na^+/HCO_3^- co-transporter (NBC1), and SLC26A6 have been then demonstrated in kidney cells.[23–25] Furthermore, AE1-3 and NBC1 were also shown to bind to the membrane-anchored isozyme CA IV simultaneously with the cytosolic CA II, providing the cells with a push–pull mechanism that maximizes the bicarbonate transport.[24] It is thus clear that in addition to their catalytic activity, most CA isoforms are also involved in much more complex processes in which they interact with anions present in rather high concentrations, as well as with their transporters. Continuing our investigations on the interactions of CAs with various classes of modulators of their activity, we report here the first anion inhibition study of the cytosolic isozyme hCA VII.

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

Inhibition data against the four main cytosolic CA isozymes, that is, the human hCA I, hCA II, hCA VII, and murine mCA XIII, with the above-mentioned anions are shown in Table 1.[26] CA III is (as mentioned above) a low-activity isozyme which has not been investigated and no detailed anion inhibition data are available.[1,14] This is the reason why such data are not present in Table 1. In addition to the physiological anions (chloride, bicarbonate, and sulfate) and the metal-complexing anions, we also investigated sulfamic acid and sulfamide,[27,28] as these are the simplest compounds incorporating a sulfonamido moiety, present in the potent CA inhibitors (the sulfonamides and sulfamates) previously investigated.[9] It has also been shown[28] that they bind to the Zn(II) ion of the human isozyme hCA II in a way that allows their use as lead molecules for the development of potent, non-sulfonamide CA inhibitors (the X-ray structure of the adducts of these two compounds with hCA II has been reported by our group).[28] Furthermore, phenylboronic and phenylarsonic acids were also included in our study, as we recently showed that these two compounds act as potent inhibitors of archaeal CAs belonging to the β- and γ-class.[29,30] Inhibition data for the other major cytosolic isozymes, hCA I and II, and mCA XIII are also provided in Table 1 for comparison, in order to allow a better rationalization of our results.

Table 1. Inhibition constants of anionic inhibitors against the cytosolic human isozymes hCA I, II, VII and murine isozyme mCA XIII, for the CO_2 hydration reaction, at 20 °C

Inhibitor	K_I[a] (mM)			
	hCA I[b]	hCA II[b]	hCAVII[c]	mCA XIII[d]
F^-	>300	>300	1.24	3.0
Cl^-	6	200	1.84	138
Br^-	4	63	1.06	45.0
I^-	0.3	26	0.25	5.4
CNO^-	0.0007	0.03	15.2	0.00025
SCN^-	0.2	1.6	0.17	0.74
CN^-	0.0005	0.02	9.2	0.065
N_3^-	0.0012	1.5	1.41	4.8
HCO_3^-	12	85	0.16	140
CO_3^{2-}	15	73	0.27	5.5
NO_3^-	7	35	0.19	36
NO_2^-	8.4	63	1.78	12.6
HS^-	0.0006	0.04	1.24	5.2
HSO_3^-	18	89	7.3	75.5
SO_4^{2-}	63	>200	1.38	267
H_2NSO_3H[e]	0.021	0.39	0.0095	21.5
$H_2NSO_2NH_2$	0.31	1.13	0.0068	0.14
$PhB(OH)_2$	58.6	23.1	0.0083	2.85
$PhAsO_3H_2$[e]	31.7	49.2	0.0125	1.65

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] Human recombinant isozyme, data from Ref. 15.
[c] Human recombinant isozyme, this work.
[d] Mouse recombinant isozyme, data from Ref. 15.
[e] As sodium salt.

The data in Table 1 allow us to draw the following conclusions regarding hCA VII inhibition with anions: (i) cyanate, cyanide, and hydrogensulfite show relatively weak hCA VII inhibitory properties, with inhibition constants (K_Is) in the range of 7.3–15.2 mM. These data are quite surprising from at least two points of view: cyanide and cyanate-anions possessing a high capacity to form coordination compounds with a variety of metal ions in metallo-enzymes or in solution—show this weak affinity for the zinc ion at the hCA VII active site, although they act as very potent inhibitors of the other

cytosolic isozymes, hCA I, hCA II, and mCA XIII (for which these anions act as micromolar inhibitors). On the other hand, bisulfite is a much more potent hCA VII inhibitor (K_I of 7.3 mM) than an inhibitor of the other cytosolic isozymes, for which its inhibition constant is in the range of 18–89 mM; (ii) a group of anions, including the halogenides fluoride, chloride, and bromide, the pseudohalogenide azide, nitrite, hydrogensulfide, and sulfate, show stronger hCA VII inhibitory activity, with K_Is in the range of 1.06–1.84 mM (Table 1). It may be observed that again the inhibition profile of hCA VII with these anions is completely different from that of the other cytosolic isozymes. Thus, fluoride is a potent hCA VII and mCA XIII inhibitor, whereas it does not bind at all to the active site of hCA I and II. On the contrary, the physiological anion chloride inhibits well hCA I and especially hCA VII (K_Is in the range of 1.84–6 mM), whereas it is much less inhibitory for hCA II (K_I of 200 mM) and mCA XIII (K_I of 138 mM), probably because these last two isoforms (CA II and XIII) unlike the first ones (CA I and VII) are partners in metabolons with AEs involved in chloride/bicarbonate transport processes. Thus, both CA II and XIII must show catalytic activity in the presence of high concentrations of bicarbonate and chloride, and indeed, they are less sensitive to inhibition by these anions (Table 1). Our data constitute the first evidence that hCA VII may be not involved in metabolons with AEs or NBCs, because it has a high susceptibility to be inhibited by both chloride and especially bicarbonate (K_I of 0.16 mM). For the halogenides, it may be observed that starting with chloride, an increase in the halogen atomic weight leads to enhanced hCA VII inhibitory properties, with iodide as the best inhibitor (K_I of 0.25 mM). Hydrogensulfide needs also a special mention, as this anion is a very strong hCA I and hCA II inhibitor (K_Is of 0.6–40 μM) but it shows weaker affinity for hCA VII and especially mCA XIII. Sulfate on the other hand is a very weak inhibitor of hCA I, II and mCA XIII, but appreciably inhibits hCA VII (K_I of 1.38 mM), making this isoform the most sensitive to inhibition by this anion. In fact, most kinetic CA activity measurements are made in the presence of 10 mM sodium sulfate for maintaining constant the ionic strength.[26] In the case of hCA VII this was not meaningful (due to the strong inhibition observed with sulfate), and sodium perchlorate has been used (10 mM) for maintaining constant the ionic strength, as perchlorate has an inhibition constant around 150 mM for hCA VII (data not shown). Nitrite is also a rather efficient hCA VII inhibitor, whereas its affinity for the other cytosolic isozymes is much lower; (iii) several other anions, such as iodide, thiocyanate, bicarbonate, carbonate and nitrate, behaved as submillimolar hCA VII inhibitors, with K_Is in the range of 0.16–0.27 mM. It may be observed that there is a huge difference of activity between thiocyanate and cyanate, with the first anion being almost 90 times better as an hCA VII inhibitor than the second one. It should be also noted that the isosteric bicarbonate, carbonate and nitrate show very similar hCA VII inhibition data, being, as already mentioned above, potent hCA VII inhibitors and much weaker inhibitors for the other cytosolic isozymes (hCA I, II and mCA XIII); (iv) sulfamate, sulfamide (presumably as sulfamidate anion),[28] and phenylboronic and phenylarsonic acid showed very potent hCA VII inhibitory activity, with K_Is in the range of 6.8–12.5 μM. Among all cytosolic isozymes, hCA VII is the most sensitive to inhibition by these anions, some of which may be really considered as CA VII-selective inhibitors. For example, phenylboronic acid is 343 times a better hCA VII than mCA XIII inhibitor, whereas its selectivity ratio for hCA II is of 2783, and for hCA I of 7060. Undoubtedly, these four last compounds may act as excellent leads for the possible design of CA VII-selective, potent inhibitors.

It is rather difficult to rationalize these results, since the X-ray crystal structure of hCA VII is not known at the moment (the structure of CA XIII is also unknown). However, the homology of the active-site amino acid sequence in hCA VII is rather high with that of the well-investigated (by X-ray crystallography) isoforms hCA I and II. Thus, except for the zinc ligands which are identical, also the other important amino acid residues for the catalytic/inhibition mechanisms are identical in CA I, II, and VII. These are His64 involved in proton transfer processes between the active site and the environment, Thr199, Glu106 involved in a network of hydrogen bonds with the zinc ligand, and Thr200, which in many cases participates in the stabilization of inhibitors bound to the zinc ion, by means of a hydrogen bond involving its OH moiety.[1,4,6,28] However, there are several amino acid residues in the active site of hCA VII which are characteristic only of this isozyme and which may explain the inhibition data reported here. These amino acids (which were shown for hCA II to be involved in the binding of inhibitors)[28,31–33] are: Asp67 (which is histidine in CA I and Asn in CA II) and Asp69 (which is Asn is CA I and Gln in CA II). Another important difference in the sequence between CA VII and the other cytosolic isozymes regards the fragment comprising the amino acid residues 144–148, which is GVFLE in CA VII, IGVLM in CA I, LGIFL in CA II, and LGVFL in CA XIII, respectively.

Physiologically the high sensitivity of CA VII to anions, especially to Cl^- and HCO_3^-, is intriguing. Developmentally regulated changes in intraneuronal Cl^- concentration may have an effect on the catalytical activity of CA VII.[34] It is also tempting to speculate that the sensitivity of CAVII to Cl^- reflects a molecular-level control mechanism of cell volume regulation: in situations where neurons are exposed to excessive Cl^- and/or HCO_3^- load (e.g., in epilepsy), inhibition of CA VII would tend to reduce neuronal swelling.

In conclusion, we report here the first inhibition study of the newly isolated cytosolic isozyme CA VII with anions. This cytosolic CA shows an inhibition profile by anions quite different from the cytosolic isozymes CA I, II, and XIII. Cyanate, cyanide and hydrogensulfite are weak CA VII inhibitors (K_Is in the range of 7.3–15.2 mM). Chloride and bicarbonate showed good inhibitory activity against hCA VII (K_Is of 0.16–1.84 mM), suggesting that this enzyme is not involved in metabolons with anion exchangers or sodium bicar-

bonate cotransporters. Sulfate was also a good inhibitor (K_I of 1.38 mM), whereas it has no inhibitory action on CA I, II, and XIII. The best isoform VII inhibitors were sulfamate, sulfamide, and phenylboronic, and phenylarsonic acid, which showed K_Is in the range of 6.8–12.5 μM. Phenylboronic acid was 343 times a better hCA VII than mCA XIII inhibitor, whereas its selectivity ratio for hCA II was of 2783, and for hCA I of 7060. Undoubtedly, such compounds may act as excellent leads for the possible design of CA VII-selective, potent inhibitors.

Acknowledgment

This research was financed in part by an Italian FIRB project (MIUR/FIRB RBNE03PX83_001).

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26. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, An SX.18MV-R Applied Photophysics stopped-flow instrument has been used. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength these anions are not inhibitory in the used concentration),[15–21] following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 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 DMSO/water 1:1, v/v) and dilutions up to 0.1 μM done with the assay buffer mentioned above. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are means of such results. K_I-s were obtained from Lineweaver–Burk plots, as reported earlier.[15–21]
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