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Carbonic anhydrase inhibitors: The membrane-associated isoform XV is highly inhibited by inorganic anions

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ABSTRACT

The membrane-associated mouse isozyme of carbonic anhydrase XV (mCA XV), has been investigated for its interaction with anion inhibitors. mCA XV is an isoforms possessing a very particular inhibition profile by anions, dissimilar to that of all other mammalian CAs investigated earlier. Many simple inorganic anions (thiocyanate, cyanide, azide, bicarbonate, hydrogen sulfide, bisulfite and sulfate) showed low micromolar inhibition constants against mCA XV (K_Is of 8.2–10.1 μM), whereas they acted as much weaker (usually millimolar) inhibitors of other isoforms. Halides, nitrate, nitrite, carbonate, sulfamate, sulfamide and phenylboronic/arsonic acid were weaker inhibitors, with inhibition constants in the range of 27.6–288 μM. Our data may be useful for the design of more potent inhibitors of mCA XV (considering various zinc binding groups present in the anions investigated here, e.g., the sulfonate one) and for understanding some physiologic/pharmacologic consequences of mCA XV inhibition by anions such as bicarbonate or sulfate which show quite high affinity for it.

In earlier contributions from our group, the inhibition of the carbonic anhydrase (CA, EC 4.2.1.1) isoforms I–XIV with simple, inorganic anions has been investigated in detail.[1,2] Indeed, both metal-complexing anions (such as cyanide, thiocyanate, hydrogen sulfide, etc.) as well as anions showing less affinity for metal ions in solution (such as nitrate, perchlorate, sulfate, etc.) are known to inhibit these metalloenzymes possessing a catalytically critical Zn(II) ion at the active site, which is involved in the interconversion between carbon dioxide and bicarbonate (the physiologic reaction catalyzed by CAs).[3,4] Interaction of various CAs with anions seems to be crucial for several physiologic processes, since CAs participate in metabolons (functional and physical associations with other proteins) with anion transporters (such as the anion exchangers AE1, AE2, AE3, which exchange bicarbonate for chloride), the sodium bicarbonate cotransporter (NBC) family of proteins,[5,6] and probably other anion transporters which have been less investigated up until now. Thus, physiologic anions (such as chloride, bicarbonate, carbonate, sulfate, etc.) may greatly influence the catalytic activity of the CA isoforms (due to the diverse inhibition profile of the 16 CA isozymes known up to now in mammals),[3,4] but investigation of their inhibitory activity may also give some hints regarding their physiologic function. For example, we have observed[1,2] that CA II and XIII are rather resistant to inhibition by bicarbonate (K_Is in the range of 85–150 mM) and chloride (K_Is in the range of 138–200 mM) probably because they interact with various anion exchangers which shuttle these anions across the plasma membranes.[1c] A strong inhibition by chloride and/or bicarbonate could compromise the catalytic function of these CAs in the metabolon with anion exchangers or other transporters. Another example is constituted by CA IX which is resistant to inhibition by lactate (although structurally related monocarboxylates show a rather effective inhibitory activity)[2f] which is probably due to the fact that this tumor-associated isoform is mainly present in hypoxic tumors where a rather high amount of lactic acid is produced due to the anaerobic glycolysis.[7]

The latest member of the mammalian CA family was characterized in 2005, when the isozyme XV was reported by Hilvo et al.[8] CA XV appeared to be an exceptional member of this family because it is expressed in several species in many vertebrates all over the phylogenetic tree, whereas in primates, such as humans and chimpanzees, it has become a non-functional pseudogene. CA XV is a glycosylphosphatidylinositol (GPI)-anchored enzyme, similarly to CA IV,[9] possessing an extracellular active site, and it is plausible that the highly abundant CA IV has taken the functional role of CA XV in primates. Nevertheless, CA XV has a high relevance for the biomedical research, because it is expressed[8] in widely used model organisms, such as rodents (mice and rats). This issue has to be taken into particular account when the results from these organisms are inferred to the human physiology/pharmacology. In the first publication on this enzyme,[8] the activity of mouse CA XV (mCA XV) was measured for a recombinant protein form

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produced in a bacterial expression system, and it appeared to be very low. The data showed only low enzymatic activity, which seems to result from defective processing of the recombinant protein in prokaryotic cells. Recently, we have determined the enzymatic activity of mCA XV produced in an eukaryotic expression system, and observed that it possesses a significant catalytic activity (for the physiologic, CO_2 hydration reaction), comparable to those of the physiologically relevant human isoforms hCA XII and XIV, with k_{cat} of 4.7×10^5 s^{-1} and k_{cat}/K_M of 3.3×10^7 M^{-1} s^{-1}.[10]

Since mCA XV inhibition with inorganic anions is totally unexplored, we report here an detailed such study, including both physiological anions (such as chloride, bicarbonate, sulfate) as well as "metal poisons" (cyanide, cyanate, thiocyanate, azide, etc.) were included in this study, together with sulfamide and sulfamate (as sodium salt), the two simplest anions incorporating the sulfonamide moiety present in the other class of potent CA inhibitors with clinical applications, that is, the aromatic/heterocyclic sulfonamides.[3,4] Inhibition data with a boronic acids and an arsonic acid are also presented here, as these compounds show an inhibition profile similar to that of inorganic anions against other mammalian CAs investigated earlier, such as CA I–XIV.[1,2]

Inhibition data against four CA isozymes, that is, hCA I, II, IV, and XV, with anions as well as sulfamic acid, sulfamide, phenylboronic and phenylarsonic acids are shown in Table 1. Data of hCA I, II and IV have been published previously,[1,2] and are presented here because they are useful for the discussion.

As seen from the data of Table 1, the membrane-associated isozyme mCA XV is highly inhibited by anions, with affinities for this class of inhibitors which are quite different from those of hCA I (an isozyme susceptible to this class of inhibitors) and hCA II or hCA IV (which are generally more resistant to inhibition by anions, but very susceptible to be inhibited by sulfonamides).[1,2] The following behavior of anion inhibitors against mCA XV has been observed:[11]

(i) Some of the investigated anions, such as thiocyanate, cyanide, azide, bicarbonate, hydrogen sulfide, bisulfite and to our greatest surprise, sulfate, showed very efficient mCA XV inhibitory properties with inhibition constants in the range of 8.2–10.1 μM. Some of these anions are well-known for their ability to complex metal ions present in metalloenzymes,[1,2] and for their strong inhibition of hCA I (K_Is of 0.5–1.2 μM for azide, cyanide and hydrogen sulfide, Table 1).[1] However, most of them are much weaker (millimolar) inhibitors of other CA isozymes, such as CA II and IV (Table 1). A special mentions should be made regarding the bicarbonate inhibition data against mCA XV. Indeed, bicarbonate, one of the CA substrates, is the most potent anion inhibitor detected in this work (K_I of 8.2 μM), which strongly indicates that this enzyme might be an inefficient catalyst for the bicarbonate dehydration reaction (due to its too high affinity for this substrate/inhibitor), whereas being an efficient one for the CO_2 hydration reaction (a turnover number of 3.3×10^7 M^{-1} s^{-1} has been measured in a previous study by this group).[10] Thus, our inhibition data indicate that mCA XV catalytic function may have been evolved in such a way as to act as an efficient catalyst for CO_2 hydration (and an inefficient one for bicarbonate dehydration) in the acidic environment present in the renal collecting ducts (where mCA XV is abundant, unpublished observations),[8] and in which bicarbonate is obviously not present due to the acidic pH. It may be also remarked that bicarbonate is several orders of magnitude a weaker inhibitor of other CA isoforms, such as CA I, II and IV (K_Is of 6.6–85 mM). Indeed, these enzymes show a good catalytic efficiency both for CO_2 hydration as well as bicarbonate dehydration to CO_2.[1–4]

A second very surprising finding of this study is the fact that sulfate acts as a very potent inhibitor of mCA XV (K_I of 9.5 μM), although this anions is usually a very ineffective inhibitor of most other CAs,[1,2,13] being in fact used (as sodium sulfate at a concentration of 10–20 mM) in the assay buffer for maintaining constant the ionic strength.[12] In the case of mCA XV this was obviously not possible and we found that only tetrafluoroborate has very weak inhibitory activity against this (and other) isozyme (Table 1), being thus possible to replace sodium sulfate by sodium tetrafluoroborate in the kinetic assay. Thus, mCA XV is thus unique in its very high affinity for this anion as well as the related bisulfite and hydrogen sulfide, although the three anions contain S(VI), S(IV) and S(II), respectively, and their binding to the enzyme should presumably be very different. It is impossible to rationalize these data at the moment, as the X-ray crystal structure of mCA XV is unknown, but we may infer that the weak activity of mCA XV originally reported by Hilvo et al.[8] may partially be due to the fact that the enzyme has been purified and stored in the presence of a rather high amount of Tris sulfate as buffer, and the sulfate was probably bound to the enzyme, inhibiting substantially its catalytic activity.

(ii) Another group of anions, including fluoride, chloride, cyanate, nitrate, nitrite, sulfamide, phenylboronic acid and phenylarsonic acid, showed slightly less effective inhibitory activity against mCA XV as compared to the anions discussed above, with inhibition constants in the range of 27.6–85.3 μM (Table 1). Again all these anions (except cyanate against hCA I and sulfamide, phenylboronic and phenylarsonic acid against hCA IV) are much weaker, usually millimolar inhibitors of other mammalian CAs, such as hCA I, II or IV. It is interesting to note that cyanate is a much better inhibitor than thiocyanate against CA I, II and IV, whereas the reverse is true for the inhibition of mCA XV.

(iii) Except for tetrafluoroborate mentioned earlier, which does not significantly inhibit mCA XV, the least effective anion inhibitors investigated here were bromide, iodide, carbonate and sulfamate, which showed inhibition constants in the range of 98.6–288 μM (Table 1). It is worth noting the huge difference in inhibitory activity between the isostructural anions sulfate and sulfamate, with the last one being roughly a 10-times weaker inhibitor than the first one.

How can we explain the anion inhibition data shown in Table 1, and the differences observed between mCA XV and other CA isoforms, such as for example the cytosolic hCA II (that has high affinity for sulfonamides), the cytosolic hCA I (that has low affin-

Table 1
Inhibition constants of anionic inhibitors against human isozymes hCA I, II, IV and mCA XV, for the CO_2 hydration reaction, at 20 °C[12]

Inhibitor	K_I[a]			
	hCAI (mM)	hCAII (mM)	hCAIV (mM)	mCAXV (μM)
F^-	>300	>300	0.07	54.4
Cl^-	6	200	0.09	85.3
Br^-	4	63	0.09	104
I^-	0.3	26	0.08	288
CNO^-	0.0007	0.03	0.61	27.6
SCN^-	0.2	1.6	39.0	10.1
CN^-	0.0005	0.02	0.77	9.4
N_3^-	0.0012	1.5	65.1	8.5
HCO_3^-	12	85	6.6	8.2
CO_3^{2-}	15	73	5.7	106
NO_3^-	7	35	58.7	68.7
NO_2^-	8.4	63	30.8	61.2
HS^-	0.0006	0.04	3.9	9.2
HSO_3^-	18	89	13.2	9.7
SO_4^{2-}	63	>200	9.0	9.5
BF_4^-	>200	>200	>200	>200,000
H_2NSO_3H[b]	0.021	0.39	0.00093	98.6
$H_2NSO_2NH_2$	0.31	1.13	0.00088	37.4
$PhB(OH)_2$	58.6	23.1	0.00088	67.8
$PhAsO_3H_2$[b]	31.7	49.2	0.00087	68.2

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] As sodium salt.

ity for sulfonamides), and the extracellular one CA IV? As mCA XV X-ray crystal structure is not yet available, we shall only consider an alignment of the amino acid sequence of these four isozymes (Fig. 1), as well as the extensive X-ray crystal data of many adducts of inhibitors with isozyme hCA II.[1,15–18] Thus, from Figure 1 it may be observed that the amino acid residues which are critical in the CA catalytic cycle are conserved in all these isozymes: (i) the three zinc ligands, His94, His96 and His119 (hCA II numbering system);[1] (ii) the "gate-keeping" residues Thr199 (which is not conserved only in hCA I, being a His for this isoform) and Glu106, which orient the substrate in the right position to be attacked by the zinc-bound hydroxide ion; and (iii) His64 (hCA II numbering system), the proton shuttle residue, which transfers protons from the zinc-bound water molecule towards the external medium, leading to the generation of the active form of the enzyme with hydroxide as the fourth zinc ligand (this is the rate-determining step for the entire catalytic cycle).[1–4] Thus, mCA XV has all the requisites to show a catalytic activity comparable to that of human isozymes I, II, or IV investigated in great detail earlier,[1] as mentioned above. There are however several amino acid residues which are characteristic only to CA XV among the α-CA isozymes, such as among others those in position 67 and 130 (corresponding to 130 in the hCA II numbering system), which have been shown earlier to be critically important for the binding of inhibitors and activators to several CA isozyme active sites.[15–18] In fact, in hCA II, the best investigated isoforms from the crystallographic point of view, the residue 67 is an Asn and the residue 130 is a Phe, and they were shown to participate to strong interactions (H-bonds for Asn67 and π-stacking for Phe130) with many inhibitors/activators for which the X-ray crystal structures in adducts with this isozyme have been resolved.[15–18] From data of Figure 1, it may be observed that in mCA XV there is a leucine in position 67, whereas in the other investigated isozymes the corresponding amino acid is not hydrophobic, namely His (CA I), Asn (CA II) and Met (CA IV). Also for the amino acid in the position 130, which is a Met in mCA XV, there is a great variability for the other isoforms (Leu in CA I, Phe in CA II, Val in CA IV, see Fig. 1). It may be hypothesized that also for mCA XV, these two amino acids may play an important role for the binding of inhibitors, and this may explain the completely different inhibition profile observed with the investigated anions (Table 1). We do not think that only these two amino acid residues are involved in the binding of inhibitors to the CA active site but, as they show a rather great variability between the different isozymes, their contribution to the selectivity profiles of inhibitors may be quite relevant.

In conclusion, mCA XV is an isoforms possessing a very particular inhibition profile by anions, dissimilar of that of all other mammalian CAs investigated earlier. Many simple inorganic anions (thiocyanate, cyanide, azide, bicarbonate, hydrogen sulfide, bisulfite and sulfate) show low micromolar inhibition constants against mCA XV (K_Is of 8.2–10.1 μM), but act as much weaker (usually millimolar) inhibitors of other isoforms. Halides, nitrate, nitrite, carbonate, sulfamate, sulfamide and phenylboronic/arsonic acid are weaker inhibitors, with inhibition constants in the range of 27.6–288 μM. Our data may be useful for the design of more potent inhibitors of CA XV (considering various zinc binding groups present in the anions investigated here, e.g., the sulfonate one) and for understanding some physiologic/pharmacologic consequences of CA XV inhibition by anions such as bicarbonate or sulfate which show quite high affinity for it.

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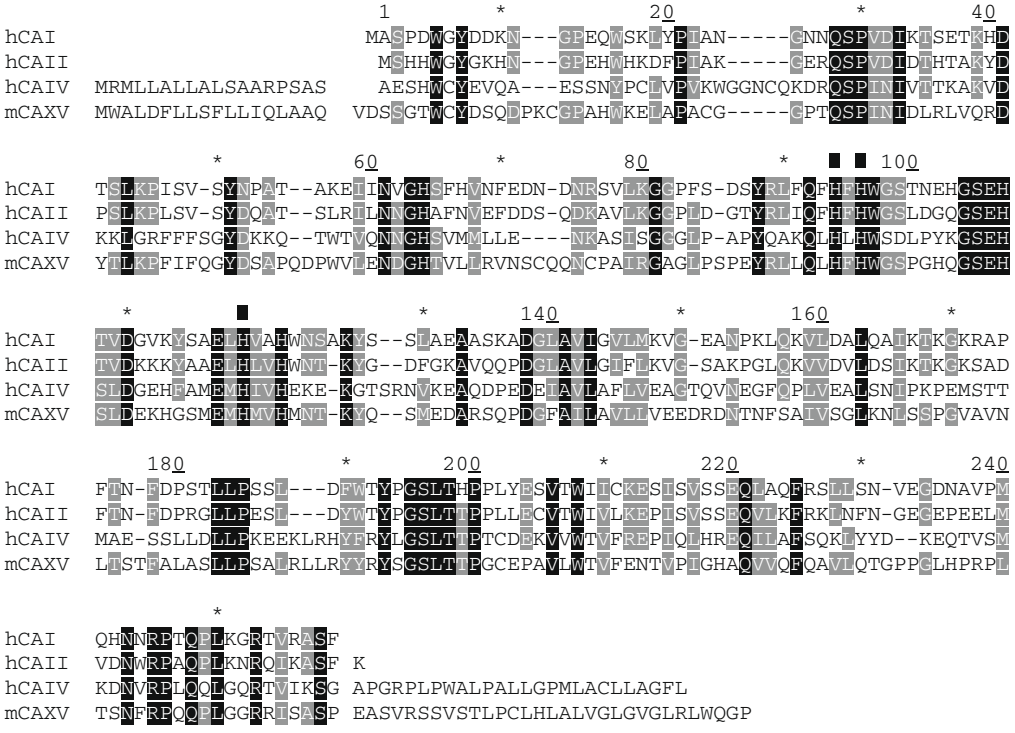

Figure 1. Alignment of human CAs I, II and IV and mouse CA XV. The alignment for the sequence of the CA domains was performed with TCoffee with regular settings,[14] and the alignment was visualized with Genedoc 2.6.02. The numbering is according to human CA II. The positions shaded with black color have identical residues in all four isozymes, and those positions that have similar residues at least in three isozymes are shaded with gray. The histidine residues that bind zinc ion and are crucial for the CA catalytic activity are pointed out by the ■ symbol.

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