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Carbonic anhydrase inhibitors. Inhibition of transmembrane isozymes XII (cancer-associated) and XIV with anions

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Abstract—Metal complexing anions represent an important class of inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). The first inhibition study of the transmembrane isozymes CA XII (tumor-associated) and XIV with anions is reported. These isozymes showed inhibition profiles with physiologic/non-physiologic anions quite distinct from any other cytosolic (CA I and II) or transmembrane isoforms (e.g., CA IX) investigated earlier. hCA XII has a good affinity for fluoride and bicarbonate but is not inhibited by heavier halides, perchlorate, nitrate, and nitrite. The best hCA XII inhibitors were cyanide (K_I of 1 μM) and azide (K_I of 80 μM). hCA XIV was on the other hand weakly inhibited by fluoride and not at all inhibited by perchlorate, but showed good affinity for most other anions investigated here. Chloride and bicarbonate showed K_Is in the range of 0.75–0.77 mM for this isoform. The best hCA XIV anion inhibitors were sulfate, phenylarsonic, and phenylboronic acid (K_I in the range of 10–92 μM).

Similarly to many metalloenzymes, the carbonic anhydrases (CAs, EC 4.2.1.1), which catalyze the interconversion between carbon dioxide and bicarbonate, are inhibited by metal complexing anions such as cyanide, cyanate, halides, etc., but in many cases, also by anions with lower capacity to bind metal ions in solution (such as perchlorate, tetrafluoroborate or sulfate among others).[1–8] The inhibition is achieved by coordination of the anion to the catalytically critical Zn(II) ion from the enzyme active site.[1–8] The interaction of various CAs (among the 16 isoforms described until now in mammals)[1–8] with such inhibitors is crucial from the physiologic point of view, since many CAs participate in metabolons in which various anion exchangers (AEs) or sodium bicarbonate cotransporters (NBCs) interact physically and functionally with the enzyme.[9–11] Indeed, for allowing the control of their pH and bicarbonate levels, cells express various anion and bicarbonate transport proteins that rapidly and selectively move bicarbonate across the plasma membrane (Fig. 1).[9]

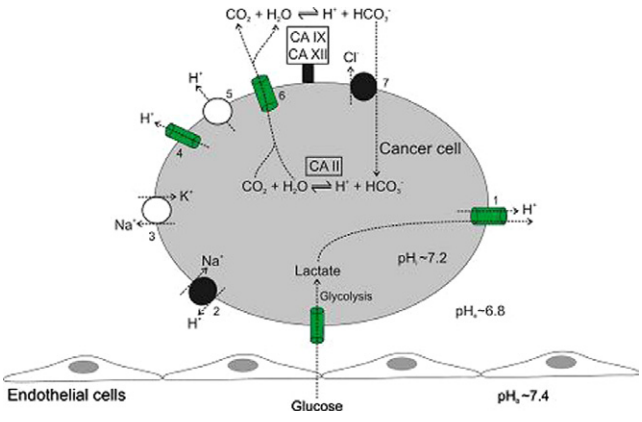

Figure 1. Interaction of cytosolic (CA II) and transmembrane (CA IX and XII) isozymes with other proteins involved in pH homeostasis and anion transport such as (1) the monocarboxylate transporter; (2) the Na^+–H^+-antiporter; (3) the ATP-dependent Na^+–K^+-antiporters; (4) the H^+–ATP-ase; (5) acquaporins ; (6) membrane-bound CAs (CA IX, XII or XIV); (7) bicarbonate/chloride anion exchangers (AEs).[21]

Keywords: Carbonic anhydrase; Tumor-associated isozyme; Isoforms IX, XII, XIV; Anion; Enzyme inhibitor.
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Physical interactions have been identified between the ubiquitous cytosolic isoform CA II and the erythrocyte membrane Cl^-/HCO_3^- anion exchanger AE1, mediated by an acidic motif in the AE1 C-terminus.[9] In a seminal research, Casey's group has found that the presence of CA II attached to AE1 accelerates AE1 bicarbonate transport activity, as AE1 moves bicarbonate either into or out of the cell.[9] In efflux mode the presence of CA II attached to AE1 increases the local concentration of bicarbonate at the cytoplasmic AE1 transport site. In the opposite direction, when bicarbonate is transported into the cell, the presence of CA II on the cytosolic surface accelerates transport by consumption of bicarbonate, thereby maximizing the transmembrane bicarbonate concentration gradient experienced by the AE1 transporter. It should be mentioned that CA II is highly resistant to inhibition by bicarbonate.[4] The same interactions also occur between CA II and Na^+/HCO_3^- cotransporter isoforms NBC1 and NBC3.[9–11] All examined bicarbonate transport proteins, except the DRA (SLC26A3) Cl^-/HCO_3^- exchange protein, have a consensus CA II binding site in their cytoplasmic C-terminus.[10,11] Casey's group also identified extracellular regions of AE1 and NBC1 that directly interact with the membrane-bound isoform CA IV,[1] to form a physical complex (metabolon) between these proteins.[10,11] SLC26A6, a plasma membrane Cl^-/HCO_3^- exchanger with a suggested role in pancreatic bicarbonate secretion, was also recently found to bind the cytoplasmic enzyme CA II.[11] Mutation of the identified CA II binding site greatly reduced SLC26A6 activity, demonstrating the importance of the interaction. Taken together, these data of Casey's group support a mechanism for acute regulation of membrane transport, denominated 'metabolon disruption', and allow the design of therapeutic applications for such phenomena, as for example in the management of cardiomyocyte hypertrophy.[12]

Many mammalian CA isozymes have been investigated in the last years for their interaction with a multitude of physiologic (such as among others bicarbonate, chloride, sulfate, etc.) as well as non-physiologic anions, such as CA I, II, IV, VA, VI, VII, IX, and XIII.[13–19] Some of the isozymes mentioned above, such as CA IX, are predominantly found in cancer cells.[20,21] CA IX is a transmembrane protein with a critical function in tumorigenesis, being involved among others in maintaining the acid–base balance and intercellular communication.[20a,21a] CA IX consists of an N-terminal proteoglycan-like domain unique among the CAs, an active CA catalytic domain, a single transmembrane region, and a short intracytoplasmic tail,[20a,21] being particularly interesting for its ectopic expression in a multitude of carcinomas derived from cervix uteri, kidney, lung, esophagus, breast, colon, etc., contrasting with its restricted expression in normal tissues, namely in the epithelia of the gastrointestinal tract.[20,21a,22]

CA XII is another transmembrane, tumor-associated CA isozyme with a more diffused expression in some normal tissues,[23] which displays several characteristics in common with CA IX but also with CA XIV,[24] another transmembrane isoform which is normally not found in tumors: (i) all these three enzymes are multidomain proteins with the CA domain situated outside the cell; (ii) their CO_2 hydrase catalytic activity is medium–high,[1–8] being intermediate between that of the very effective catalyst which is CA II (one of the fastest enzymes known in nature)[4] and that of the slow cytosolic isozyme CA I, a highly abundant protein in red blood cells and the gastrointestinal tract, whose physiologic function is little understood at this time;[1–8] (iii) all these three enzymes are highly inhibited by sulfonamides and their derivatives (sulfamates, sulfamides, etc.), making them interesting candidates for various medicinal chemistry applications.[25]

The inhibition of CA IX with anions has been investigated earlier by this group,[13a] but no such data are available for the other two transmembrane isozymes, that is, CA XII and XIV. Here we report the first CA XII and XIV inhibition study with a group of physiologic as well as non-physiologic anions.

Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, perchlorate, nitrate, hydrogen sulfide, and arsenate) were from Sigma–Aldrich (Milan, Italy) of highest purity available, and were used without further purification. Inhibition data of the cytosolic human (h) isozymes hCA I and II, as well as the three transmembrane ones hCA IX, XII, and XIV are shown in Table 1. Data for hCA I, II, and IX were reported earlier[13–19] and are presented in Table 1 for the sake of comparison.

Data of Table 1 show the following regarding CA XII and XIV interaction with these anion inhibitors: (i) halides (except fluoride), perchlorate, nitrate, and nitrite behave as very weak hCA XII inhibitors, with inhibition constants in the range of 73–300 mM (a precise value was not possible to estimate for perchlorate since no appreciable inhibition has been achieved at 300 mM and higher concentrations led to ionic strength changes that alter the enzyme assay).[26] On the other hand, only hydrogen sulfide behaved as a moderate hCA XII inhibitor (K_I of 4.85 mM), whereas most of the anions investigated here, such as fluoride, cyanate, thiocyanate, bicarbonate, carbonate, hydrogen sulfite, sulfate, sulfamide, sulfamate (deprotonated sulfamic acid),[27] phenylarsonic acid, and phenylboronic acid, showed good hCA XII inhibitory activity, with K_Is in the range of 0.56–0.84 mM. The best hCA XII inhibitors were the metal complexing anions cyanide and azide, which showed inhibition constants in the range of 1.0–80 μM. (ii) Several important differences between hCA XII and all other investigated CA isoforms regarding their inhibition by anions can be observed from these data. Thus, similarly to CA II (involved in a metabolon with an anion exchanger transporting chloride and bicarbonate)[9–11] CA XII is resistant to inhibition by chloride (as well as the other heavy halogenides, such as bromide and iodide, with K_Is in the range of 73–215 mM), but unlike CA II, which is also insensitive to inhibition by bicarbonate/carbonate, CA XII is well inhibited by these two anions/substrates of all CAs

Table 1. Inhibition constants of anion inhibitors against isozymes CA I, II, IX, XII, and XIV for the CO_2 hydration reaction, at 20 °C[26]

Anion	K_I (mM)[a]				
	hCA I[b]	hCA II[b]	hCA IX[b, c]	hCA XII[c,e]	hCA XIV[d,e]
F^-	>300[b]	>300	48	0.56	37
Cl^-	6	200	33	73	0.77
Br^-	4	63	16	82	0.77
I^-	0.3	26	7	215	0.78
CNO^-	0.0007	0.03	0.043	0.73	0.73
SCN^-	0.2	1.6	0.13	0.80	1.19
CN^-	0.0005	0.02	0.004	0.001	0.77
N_3^-	0.0012	1.5	0.005	0.08	0.76
HCO_3^-	12	85	13	0.75	1.10
CO_3^{2-}	15	73	29	0.64	0.98
HSO_3^-	18	89	75	0.84	0.76
SO_4^{2-}	63	>300	>300	0.77	0.010
ClO_4^-	3.6	1.3	8	>300	>300
NO_3^-	7	35	46	79	0.81
NO_2^-	8.4	63	42	94	0.92
HS^-	0.0006	0.04	0.007	4.85	0.74
$H_2NSO_2NH_2$	0.31	1.13	0.096	0.83	0.75
H_2NSO_3H	0.021	0.39	0.092	0.70	1.09
$PhAsO_3H_2$	31.7	49.2	0.055[e]	0.84	0.084
$PhB(OH)_2$	58.6	23.1	0.12[e]	0.80	0.092

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] From Ref. 13a, against the human recombinant isozyme.
[c] Catalytic domain of the human, recombinant isozyme.
[d] Full length, human recombinant isozyme.
[e] This work.

(K_Is of 0.64–0.75 mM). Thus, from such divergent data, it is rather unclear whether CA XII can participate in metabolons with AEs/NBCs, since it is strongly inhibited by one of the anions involved in these transport processes, that is, bicarbonate. However, we cannot rule out this hypothesis, due to the high resistance of CA XII to chloride inhibition. Thus, it may be possible that in tissues in which the enzyme must work in pH conditions in which bicarbonate/carbonate are not very stable (i.e., acidic pH), an isoform such as CA XII, which is not inhibited by chloride, could in principle participate in a metabolon involving the exchange of this anion for another one (such as for example bicarbonate). This hypothesis warrants further physiological experiments for better understanding this very interesting behavior of CA XII, for the first time evidenced for any CA isoform, that is, resistance to inhibition by chloride, but sensitivity to bicarbonate/carbonate. Another striking result is the very good inhibition of hCA XII by fluoride (K_I of 0.56 mM), considering the fact that hCA I and II are not at all inhibited by it, whereas hCA IX and XIV (see latter in the text) have affinities in the range of 37–48 mM for this anion (Table 1). The same is true for sulfate, an anion which does not inhibit hCA I, II, and IX, but acts as a good inhibitor of hCA XII (and XIV too). Among all isozymes investigated here, hCA XII was the least inhibited by hydrogen sulfide (K_I of 4.85 mM), whereas all other isozymes showed K_Is in the range of 0.6 μM–0.74 mM for this anion. Perchlorate on the other hand was a good hCA I, II, and IX inhibitor, but did not inhibit at all hCA XII and XIV; (iii) hCA XIV was weakly inhibited only by fluoride (K_I of 37 mM) and was not inhibited appreciably by perchlorate even at concentrations as high as 300 mM. All other anions investigated here, except sulfate, phenylarsonic acid, and phenylboronic acid, showed good inhibitory activity against hCA XIV, with K_Is in the range of 0.74–1.19 mM (Table 1). The best hCA XIV inhibitors were just the three anions/compounds mentioned above, that is, sulfate, phenylarsonic acid, and phenylboronic acid (K_Is in the range of 10–92 μM). Such compounds (the phenylarsonic and phenylboronic acids), together with the simplest derivatives incorporating a sulfonamide moiety, that is, sulfamide and sulfamic acid,[27] may formally be considered as anion inhibitors of CAs, since generally their inhibition profile against most isozymes is very similar to that of the inorganic anions (i.e., K_Is in the milli-micromolar range), and distinct from that of the 'organic' inhibitors, such as the aromatic/heterocyclic sulfonamides, which typically show K_Is in the micro–nanomolar range.[1,5] It has also been proven by X-ray crystallography that both sulfamide and sulfamic acid bind as mono-/dianions to hCA II.[27] (iv) there are again some quite surprising results regarding CA XIV inhibition by anions. Thus, the best anion inhibitor of hCA XIV was unexpectedly sulfate (K_I of 10 μM), which appreciably inhibits only hCA XII among other investigated isoforms,[13–19] and does not bind at all (or binds quite weakly) isoforms I, II, and IX (Table 1). The isoelectronic/isosteric anions with sulfate, sulfamide (in deprotonated form, as monoanion)[27], and sulfamate[27] were much weaker hCA XIV inhibitors (K_Is of 0.75–1.09 mM) as compared to sulfate, although these compounds generally act as better hCA I, II, and IX inhibitors (again as compared to sulfate—Table 1). Phenylboronic acid and phenylarsonic acid also showed much higher affinity for hCA XIV as compared to hCA XII (by a factor of 8.7–10), these compounds being

rather weak hCA I and II inhibitors, but more efficient hCA IX inhibitors (Table 1). Thus, both derivatives (phenylarsonic and phenylboronic acids) may be considered as interesting lead molecules for the design of inhibitors with diverse zinc-binding functions of the classical sulfonamide anchor, for the potential design of isozyme-selective CA XIV inhibitors. Another very interesting difference between hCA XII and XIV regards their behavior toward halides. As mentioned above, hCA XII is resistant to inhibition by halides (except fluoride to which it is highly susceptible), whereas hCA XIV is well inhibited by all halides heavier than fluoride, the difference of affinity of these two isoforms being quite appreciable, that is, 94.8-fold for chloride, 106.5-fold for bromide, and 275.5-fold for iodide, respectively. It is rather difficult to rationalize these data, considering the fact that the active sites of hCA XII and XIV are highly similar (Fig. 2).[28,29] However, as both chloride and bicarbonate are rather good hCA XIV inhibitors, we can state that probably this isozyme is not involved in metabolons with AEs/NBCs, in contrast to hCA II. The same situation as for halides can be noted regarding the interaction of these isoforms with nitrate or nitrite, with hCA XIV being 97–102 times more susceptible to be inhibited as compared to hCA XII.

The amino acid sequences of a CA domain of three transmembrane CA isozymes (hCA IX, XII, and XIV) were aligned with those of two cytosolic CA isozymes (hCA II and I) manually[24a] (Fig. 2). The three transmembrane CAs showed an overall similarity of 34.1–35.4% with hCA II. Among hCA IX, XII, and XIV, 39.3–46.3% of similarity were seen in amino acid sequences of the CA domain. The highest similarity was observed between hCA XII and hCA XIV. A phylogenetic analysis also revealed that these two transmembrane CAs were most closely related to each other,[24a] which is consistent with the findings observed in this study that a panel of anionic inhibitors revealed inhibition profiles comparable between hCA XII and hCA XIV, but differing from those of hCA I, hCA II, and hCA IX. In Figure 1, the 36 amino residues that were previously defined[24b] to participate in the active site architecture are indicated by a mixture of asterisk, plus sign, and 'z' above the hCA II sequence. Among these active site residues, 26 amino acids were conserved between all three transmembrane CAs and hCA II, the enzyme showing the highest CA catalytic activity among the CA family. Interestingly, hCA IX, hCA XII, and hCA XIV exhibit specific amino acid usages which are conserved among transmembrane CAs but not seen in cytosolic CAs (indicated by light-gray box in Figure 1), including two cysteine residues at residue numbers 23 and 202 (the numbering was based on hCA II sequence) that probably enables a more rigid molecular structure of extracellular CA isozyme.

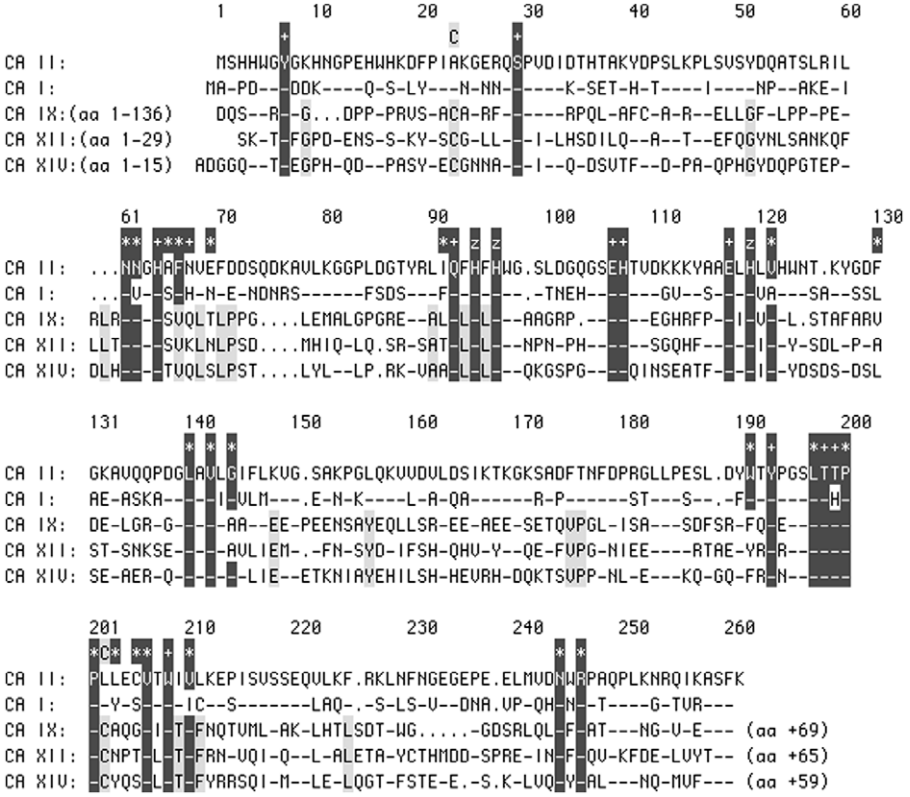

Figure 2. Sequence alignment of the CA domain sequence of three transmembrane CA isozymes (hCA IX, XII, and XIV) with that of two cytosolic isoforms (hCA II and I). Conserved amino acid residues (as compared to hCA II) are indicated by a bar. Residues (36 amino acids) previously defined to participate in the active site are indicated by a mixture of asterisk, plus sign, and 'z' above the hCA II sequence. Seventeen residues known to form a hydrogen-bond network within active site are indicated by plus and 'z'; the latter indicates the three zinc-liganded histidines. Amino acid residues conserved among the three transmembrane CAs but not seen in cytosolic CAs are indicated by light-gray boxes, including two cysteine residues (indicated by 'C', above the hCA II sequence).

In conclusion, the first anion inhibition studies of the transmembrane isozymes hCA XII and XIV are reported here. These enzymes showed inhibition profiles with physiologic/non-physiologic anions quite distinct from any other cytosolic (CA I and II) or transmembrane isoforms (e.g., CA IX) investigated earlier. hCA XII has a good affinity for fluoride and bicarbonate but is not inhibited by heavier halides, perchlorate, nitrate, and nitrite. The best hCA XII inhibitors were cyanide (K_I of 1 μM) and azide (K_I of 80 μM). hCA XIV was on the other hand weakly inhibited by fluoride and not at all inhibited by perchlorate, but showed good affinity for most other anions investigated here. Chloride and bicarbonate showed K_Is in the range of 0.75–0.77 mM for this isoform. The best hCA XIV anion inhibitors were sulfate, phenylarsonic, and phenylboronic acid (K_I in the range of 10–92 μM).

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557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4/$NaClO_4$ (for maintaining constant the ionic strength; for hCA I, II, and IX Na_2SO_4 has been used, whereas for hCA XII and XIV $NaClO_4$—see Table 1 for the inhibition data of these anions toward the corresponding isoform), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 μM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier,[13–19] and represent the mean from at least three different determinations.

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