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Carbonic anhydrase inhibitors. Inhibition of isozymes I, II, IV, V, and IX with anions isosteric and isoelectronic with sulfate, nitrate, and carbonate

Alessio Innocenti, Daniela Vullo, Andrea Scozzafava and Claudiu T. Supuran*

Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy

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Abstract—The inhibition of five human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes; the cytosolic hCA I and II, the membrane-bound hCA IV, the mitochondrial hCA V, and the tumor-associated, transmembrane hCA IX, with anions isosteric and isoelectronic with sulfate, nitrate, and carbonate; such as chlorate, perchlorate, bromate, iodate, periodate, silicate, bismuthate, vanadate, molybdate, and wolframate is reported. Apparently, the geometry of the inhibitor (tetrahedral or trigonal) does not influence its binding to the Zn(II) ion of the enzyme active site, but the nature of the central element is the most important factor influencing potency. Isozymes hCA I and II are best inhibited by chlorate, perchlorate, and silicate, together with the anions structurally related to sulfate, sulfamate, and sulfamidate, but sulfate itself is a weak inhibitor (inhibition constant of 74 mM against hCA I and 183 mM against hCA II). Molybdate is a very weak hCA I inhibitor (K_I of 914 mM) but it interacts with hCA II (K_I of 27.5 mM). Isozyme IV is well inhibited by sulfate (K_I of 9 mM), sulfamate, and sulfamidate (in the low micromolar range), but not by perchlorate (K_I of 767 mM). The mitochondrial isozyme V has the lowest affinity for sulfate (K_I of 680 mM) and carbonate (K_I of 95 mM) among all the investigated isozymes, suggesting on one hand its possible participation in metabolon(s) with sulfate anion exchanger(s), and on the other hand an evolutionary adaptation to working at higher pH values (around 8.5 in mitochondria) where rather high amounts of carbonate in equilibrium with bicarbonate may be present. Metasilicate, isosteric to carbonate, is also about a 10 times weaker inhibitor of this isozyme as compared to other CAs investigated here (K_I of 28.2 mM). Surprisingly, the tumor-associated isozyme IX is resistant to sulfate inhibition (K_I of 154 mM) but has affinity in the low micromolar range for carbonate, sulfamate, and sulfamidate (K_I in the range of 8.6–9.6 μM). This constitutes another proof that this isozyme best works at acidic pH values present in tumors, being inhibited substantially at higher pH values when more carbonate may be present. Bromate and chlorate are quite weak CA IX inhibitors (K_I s of 147–274 mM).

1. Introduction

The interaction of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) with small molecules, such as inorganic anions, is critical for several reasons, among which: (i) understanding the catalytic/inhibition mechanism of the many CA isozymes presently known all over the phylogenetic tree;[1–4] (ii) for the design of potent, clinically useful inhibitors (sulfonamides[1–5] and sulfamates,[6] the most potent CA inhibitors (CAIs), are clinically used for a long period for the treatment or prevention of a multitude of diseases involving CA isozymes disregulation);[1–5] and (iii) for understanding the physiological/physiopathological role of many of these isozymes. In fact, in humans, 15 α-CA isozymes were described so far, 12 of which possess catalytic activity.[1–5] The physiological function of some of them is little understood at this moment, but it became clear recently that in addition to acting as efficient catalysts for CO_2 hydration to bicarbonate and H^+ ions, these enzymes are frequently associated with other proteins, forming metabolons that possess critical physiological roles in a multitude of processes. For example, a physical interaction has been identified between the widely distributed cytosolic isozyme CA II, and the erythrocyte membrane Cl^-/HCO_3^- anion exchanger, AE1, mediated by an acidic motif in the AE1 carboxy-terminus.[7–10] It has been proven that the presence of CA II attached to AE1 accelerates AE1 bicarbonate transport activity, as AE1 moves bicarbonate either into or out of the

* Corresponding author. Tel.: +39 055 4573005; fax: +39 055 4573385; e-mail: claudiu.supuran@unifi.it

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cell.[7–9] Functional and physical interactions were also shown to occur between isozymes CA II or CA IV and Na^+/HCO_3^- co-transporter isoforms NBC1 and NBC3 by the same group.[7–10] Recently, a CA II-sulfate/anion exchanger metabolon has been described too,[11] which was shown to speed both the secretory and reabsorptive processes in the renal proximal tubule of vertebrates. Thus, the interaction between different anions and diverse CA isozymes is of great importance for understanding some fundamental biochemical/physiological processes.

Inhibition of CAs by anions is also important from the physiological point of view, considering the high concentrations of some anions (bicarbonate, chloride, phosphate, sulfate, etc.) present in different tissues.[13–16] Simple inorganic anions usually act as weak inhibitors of CAs (usually in the milli- to micromolar range), due to their binding to the metal ion at the active site, but as some of these anions are present in different tissues in high enough concentrations (i.e., millimolar range), their interaction with some isozymes may lead to diverse degrees of inhibition, which may have physiological consequences.[12–16]

Among such anions, carbonate and nitrate are quite important, since they mimic some of the intermediates in the catalytic cycle for the CO_2 hydration reaction (nitrate is isosteric and isostructural with bicarbonate, whereas carbonate is obviously in chemical equilibrium with bicarbonate), whereas sulfate is important since it mimics rather well the sulfamoyl moiety present in most of the potent, organic inhibitors (of the sulfonamide and sulfamate types).[1–5] Sulfate inhibition of isozymes IV and V has recently been investigated by this group,[13–16] whereas older work from Maren's laboratory dealt with the inhibition of isozymes I and II.[17] Nitrate and carbonate data for isozymes I, II, IV, and V were also reported by us recently.[13–16] It is also interesting to note the fact that different isozymes show highly diverse affinities for these three anions, as well as for structurally very similar anions, such as bicarbonate, hydrogensulfite, sulfamate, or sulfamide, which are important for the drug design of CAIs.[13–16,18] Since no detailed studies are available regarding the interaction of different α-CA isozymes with more complex anions, which are isoelectronic/isosteric with sulfate, nitrate, and carbonate, here we report the first such study, including 5 different human isozymes (hCA I, II, IV, V, and IX) and 10 different anions of the type XO_3^{n-} and XO_4^{n-} (X = halogen, Si, Bi, V, Mo, and W; $n = 1$ and 2).

2. Chemistry

Buffers and sodium/potassium salts (chlorate, perchlorate, bromate, iodate, periodate, metasilicate, bismuthate, metavanadate, molybdate, and wolframate) were of highest purity available, from Sigma–Aldrich (Milan, Italy) and were used without further purification. CA isozymes were prepared as previously reported by our group.[13–16]

3. CA inhibition data

Inhibition data against five CA isozymes involved in critical physiological/pathological processes, that is, hCA I, hCA II (cytosolic forms), hCA IV (membrane associated), hCA V (mitochondrial), and hCA IX (transmembrane, tumor associated),[19–21] with the above mentioned anions are shown in Table 1. Inhibition data

Table 1. Inhibition constants of anionic inhibitors against human isozymes hCA I, II, IV, and V, for the CO_2 hydration reaction, at 20 °C[19]

Inhibitor	K_I [mM][#]				
	hCA I[a]	hCA II[a]	hCA IV[b]	hCA V[c]	hCA IX[d]
SO_4^{2-}	74 (63)[e]	183 (>200)[e]	9.0[*]	680	154
$H_2NSO_3^{-}$[*]	0.021	0.39	9.3×10^{-4}	0.12	9.2×10^{-3}
$H_2NSO_2NH^{-}$[*]	0.31	1.13	8.8×10^{-4}	0.84	9.6×10^{-3}
NO_3^{-}[*]	7.0	35	58.7	16	46
CO_3^{2-}[*]	15	73	5.7	95	8.6×10^{-3}
ClO_3^{-}	0.54	0.85	64.9	7.12	147
ClO_4^{-}	0.69	1.26	767	13.6	11.6
BrO_3^{-}	4.79	12.0	128	11.3	274
IO_3^{-}	95	48.9	108.7	13.0	49.7
IO_4^{-}	14.6	75.8	226	15.7	30.3
SiO_3^{2-}	0.57	2.68	2.15	28.2	2.75
BiO_3^{-}	19.0	50.4	5.16	687	22.8
VO_3^{-}	22.5	11.6	44.8	38.6	42.6
MoO_4^{2-}	914	27.5	165	17.2	24.7
WO_4^{2-}	112	31.0	15.9	17.1	23.9

[a] Human recombinant isozymes.
[b] Truncated human isozyme lacking the first 20 amino acid residues.[16]
[c] Full length human isozyme.[13]
[d] Catalytic domain of human, recombinant isozyme.[14]
[e] From Ref. 17.
[*] Data from Refs. 13,14,16 except hCA IX inhibition data with sulfate, sulfamate, sulfamidate, and carbonate, which are reported here for the first time.
[#] Errors were in the range of 3–5% of the reported values, from three different assays.

for sulfate, sulfamate, sulfamide, nitrate, and carbonate (some of the anions structurally related with the CA substrates CO_2 and bicarbonate) are also provided for comparison, as they were recently reported by this group (in fact the hCA IX inhibition data with sulfate, sulfamate, sulfamide, and carbonate are published here for the first time, as they were not available in the literature).[13–16]

Data of Table 1 allow us to draw the following conclusions regarding CA isozymes interaction with the anions investigated here: (i) The slow cytosolic isozyme hCA I, shows a moderate sensitivity to sulfate inhibition (a K_I of 74 mM has been obtained with our stopped-flow assay method, as compared to the old data from Maren's laboratory[17] of 63 mM, obtained with a manual, non-computerized assay), being also modestly inhibited by iodate and wolframate (inhibition constants in the range of 95–112 mM). Molybdate was the weakest CA I inhibitor, with a K_I of 914 mM. Better inhibitors were periodate, bismuthate, and vanadate, which showed inhibition constants in the range of 14.6–22.5 mM, which is of the same order of magnitude as that of carbonate (15 mM) (Table 1). Even better inhibitors were nitrate and bromate (K_I in the range of 4.79–7.0 mM), whereas chlorate, perchlorate, silicate, and sulfamide showed an increased affinity for this isozyme, with K_I values in the range of 0.31–0.69 mM. The best hCA I inhibitor was sulfamic acid (as sulfamate anion) with an inhibition constant of 21 μM. Several interesting facts must be stressed here: the geometry of the complexing anion, whether tetrahedral (as for sulfamate, perchlorate, or chlorate among others) or planar, trigonal (as for nitrate, carbonate, or metasilicate) is rather irrelevant, all equally promoting efficient binding to the Zn(II) ion within the enzyme active site. Secondly, the nature of the central element X in the XO_3^{n-} and XO_4^{n-} anions seem to be the most important factor influencing the inhibitory capacity of these compounds, but no direct correlation with the atomic weight of X or the geometry of the anion can be drawn; (ii) The rapid, major cytosolic isozyme hCA II is generally considered to be less susceptible to inhibition by anions,[1–5,17] and more prone to be inhibited by sulfonamides, as compared to hCA I. Indeed, data in Table 1 generally show this to be the trend. Thus, sulfate is a weak hCA II inhibitor (with a K_I of 183 mM, determined here for the first time, as the early[17] data only provided an approximate value), in contrast to the structurally related sulfamate and sulfamide, which are much more potent inhibitors (K_I s in the range of 0.39–1.13 mM). Weak inhibitors are also nitrate, carbonate, iodate, periodate, bismuthate, molybdate, and wolframate, with K_I s in the range of 27.5–75.8 mM, whereas bromate and vanadate are more potent inhibitors (K_I s in the range of 11.6–12.0 mM). As for hCA I, the best hCA II inhibitors were chlorate, perchlorate, and silicate, with K_I s in the range of 0.85–2.68 mM. It should be noted that molybdate may be considered as a kind of CA II-'specific' inhibitor, considering only the cytosolic isozymes investigated here. Indeed, this compound is a 33.2 times more potent on hCA II than hCA I inhibitor, being together with iodate, vanadate, and wolframate, one of the few anions with higher affinity for isozyme II than for isozyme I; (iii) Sulfate is a much better hCA IV inhibitor (K_I of 9 mM) as compared to its activity against all other CA isozymes investigated here, and in fact inhibition measurements with this isozyme have been done in the presence of 0.1 M perchlorate for maintaining the ionic strength constant (and not in the presence of 0.1 M sulfate as for all other isozymes).[19] Indeed, perchlorate is an extremely weak CA IV inhibitor (K_I of 767 mM, Table 1). Weak hCA IV inhibitors were also nitrate, chlorate, bromate, iodate, periodate, vanadate, and molybdate, which showed K_I s in the range of 44.8–226 mM. More efficient inhibitors were carbonate, silicate, bismuthate, and wolframate, with inhibition constants in the range of 2.15–15.9 mM. On the other hand, as already shown earlier,[16b] sulfamate and sulfamide act as very potent hCA IV inhibitors, with affinities in the low micromolar range (Table 1); (iv) The mitochondrial isozyme hCA V has also a very different affinity for these anion inhibitors, as compared to the cytosolic (CA I and II) and membrane-associated isozymes (CA IV) discussed earlier. Thus, among all CAs investigated up to now, CA V has the lowest affinity for sulfate, with an inhibition constant of 680 mM, being 3.7 times less sensitive that isozyme II. This finding may have consequences, since sulfate is an important physiological anion, and at this point we may hypothesize that similarly with CA II (also resistant to sulfate inhibition), which participates in a metabolon with the sulfate/anion exchanger,[11] CA V may also take part in such processes involving the energy-producing organellae in which this isozyme is very abundant and plays important physiological functions.[3b] Another very weak hCA V inhibitor was bismuthate, with a K_I of 687 mM. Carbonate, silicate, and vanadate, with K_I s in the range of 28.2–95.0 mM showed weak inhibitory activity, whereas nitrate, perchlorate, bromate, iodate, periodate, molybdate, and wolframate were stronger inhibitors (K_I s in the range of 11.3–17.2 mM). The most potent inhibitor among these inorganic anions was chlorate, with a K_I of 7.12 mM, but sulfamate and sulfamide were much better inhibitors (K_I s in the range of 0.12–0.84 mM). The most interesting data of this isozyme are those of carbonate and silicate. Thus, CA V is the most resistant isozyme to carbonate inhibition, and this may be due to the fact that this isozyme works in mitochondria at a pH value close to 8.5, when much more carbonate is present (as compared to other body fluids, which generally are at pH 7.4). Thus, CA V resistance to inhibition by this anion may represent an evolutionary adaptation to the higher pH values present in mitochondria, which are thus enriched in carbonate over bicarbonate. As metasilicate is isosteric with carbonate, this may also explain why silicate is on average a 10 times less efficient inhibitor of CA V than of CA I, II, IV, and IX (Table 1); (v) CA IX, the tumor associated isozyme, is also rather resistant to inhibition with sulfate, with a K_I value of 154 mM, being more susceptible than CA V and CA II, and less susceptible than CA I and CA IV. Chlorate and bromate have a very similar affinity with sulfate for this isozyme, with K_I values in the range of 147–274 mM, being the least potent CA IX inhibitors in this series of anions. Just a contrary effect is observed

for carbonate, sulfamate, and sulfamide, which all behave as low micromolar CA IX inhibitors, with inhibition constants in the range of 8.6–9.6 μM. This finding is very surprising, especially considering the CA V susceptibility to inhibition by carbonate mentioned above. The difference of affinity for carbonate between CA IX and CA V is of 1.1×10^6, which may be the highest ever observed for a simple inhibitor of two CA isozymes. This means that the tumor associated isozyme IX, which usually works in hypoxic conditions at lower pH values than the physiological one (i.e., at pH of 6.5–6.9) is unable to catalyze CO_2 hydration in the presence of even very small amounts of carbonate, which acts as a very potent inhibitor, of the same order of magnitude as sulfamic acid and sulfamate. Since organic sulfamates/sulfamides were shown to acts as low nanomolar hCA IX inhibitors,[6,22] this finding may have as a consequence the fact that organic carbonates might be developed as CA IX-specific inhibitors, possessing a completely novel zinc-binding function. We are presently testing this hypothesis, since CA IX-targeted inhibitors may represent a new strategy in the management of cancer.[2,6,20] On the other hand, nitrate, iodate, periodate, bismuthate, vanadate, molybdate, and wolframate behave as moderate CA IX inhibitors, with inhibition constants in the range of 22.8–49.7 mM. Perchlorate was a more efficient inhibitor, with a K_I of 11.6 mM, which is more than 12.6 times lower than that of chlorate, a rather weak inhibitor as mentioned above. It is difficult to rationalize these tremendous differences of activity for such similar anions (i.e., chlorate and perchlorate), and this proves again that even one atom in an enzyme–inhibitor complex can make the difference between a strong and a weak inhibitor. Silicate was an effective hCA IX inhibitor, with a K_I value very similar to that shown against hCA II (2.75 mM for CA IX, 2.68 mM for CA II).

4. Conclusion

We report here a detailed inhibition study of five CA isozymes with anions, which are isosteric/isoelectronic with sulfate, nitrate, and carbonate. The inhibition of five human isozymes; the cytosolic hCA I and II, the membrane-bound hCA IV, the mitochondrial hCA V and the tumor-associated, transmembrane hCA IX; with chlorate, perchlorate, bromate, iodate, periodate, silicate, bismuthate, vanadate, molybdate, and wolframate has been investigated. Apparently, the geometry of the inhibitor (tetrahedral or trigonal) does not influence its binding to the Zn(II) ion of the enzyme active site, but the nature of the central element is the most important factor influencing potency. Isozymes hCA I and II are best inhibited by chlorate, perchlorate, and silicate, together with the anions structurally related to sulfate, sulfamate, and sulfamidate, but sulfate itself is a weak inhibitor (inhibition constant of 74 mM against hCA I and 183 mM against hCA II). Molybdate is a very weak hCA I inhibitor (K_I of 914 mM) but it interacts with hCA II (K_I of 27.5 mM). Isozyme IV is inhibited greatly by sulfate (K_I of 9 mM), sulfamate, and sulfamidate (in the low micromolar range), but not by perchlorate (K_I of 767 mM). The mitochondrial isozyme V has the lowest affinity for sulfate (K_I of 680 mM) and carbonate (K_I of 95 mM) among all the investigated isozymes, suggesting on one hand its possible participation in metabolon(s) with sulfate anion exchanger(s), and on the other hand an evolutionary adaptation to working at higher pH values (around 8.5 in mitochondria) where rather high amounts of carbonate in equilibrium with bicarbonate may be present. Metasilicate, isosteric to carbonate, is also about a 10 times weaker inhibitor of this isozyme as compared to other CAs investigated here (K_I of 28.2 mM). Surprisingly, the tumor-associated isozyme IX is resistant to sulfate inhibition (K_I of 154 mM) but has affinity in the low micromolar range for carbonate, sulfamate, and sulfamidate (K_I in the range of 8.6–9.6 μM). This constitutes further proof that this isozyme works best at acidic pH values present in tumors, being inhibited substantially at higher pH values when more carbonate may be present. Bromate and chlorate are quite weak CA IX inhibitors (K_I s of 147–274 mM).

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