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Carbonic anhydrase inhibitors. Inhibition of transmembrane isoforms IX, XII, and XIV with less investigated anions including trithiocarbonate and dithiocarbamate

Alessio Innocenti, Andrea Scozzafava, 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

An inhibition study of the transmembrane carbonic anhydrase (CA, EC 4.2.1.1) isoforms IX, XII (tumor-associated), and XIV with anions such as stannate(IV), selenate(VI), tellurate(VI), perosmate(VIII), persulfate, pyrophosphate(V), pyrovanadate(V), tetraborate, persulfate, perrhenate(VII), perrutenate(VII), selenocyanate, iminodisulfonate, fluorosulfate, and trithiocarbonate is reported. Selenate, perosmate, and pyrophosphate were ineffective inhibitors, whereas most of these anions inhibited the three enzymes in the millimolar–submillimolar range. Trithiocarbonate and diethyldithiocarbamate were the best CA IX inhibitors (K_Is of 1.4–9.7 μM), but trithiocarbonate showed less affinity for CA XII and XIV (K_Is of 0.12–0.66 mM). *N*,*N*-Diethyldithiocarbamate was a low micromolar inhibitor also against CA XII and XIV (K_Is of 1.0–1.1 μM), suggesting that this new zinc-binding group (CS_2^-) may lead to efficient inhibitors targeting transmembrane CAs.

In a recent contribution from our laboratory[1] we reported the inhibition of the cytosolic isoforms I, II, III, VII, and XIII of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) with less investigated inorganic anions, such as stannate(IV), selenate(VI), tellurate(VI), perosmate(VIII), persulfate, pyrophosphate(V), pyrovanadate(V), tetraborate, perrhenate(VII), perrutenate(VII), persulfate, selenocyanate, iminodisulfonate, fluorosulfate, and trithiocarbonate.[9] Furthermore, in a subsequent paper[2] we have reported the X-ray crystal structure for the adduct of human (h) hCA II with trithiocarbonate, one of the best enzyme inhibitors detected in the first work,[1] and found a very interesting binding mode of this low micromolar anion inhibitor to the enzyme (Fig. 1). It has also been observed that *N*,*N*-diethyldithiocarbamate, a compound containing the CS_2^- fragment also present in trithiocarbonate, is an even stronger, low micromolar CA inhibitor (CAI).

These studies thus afford interesting hints for the design of CAIs possessing a new zinc-binding group (ZBG), of the CS_2^- type, scarcely investigated up to now for its interaction with metalloenzymes.[1,2] As there are 16 CA isoforms described in mammals, that is, CA I–CA XV (with two V type isoforms, CA VA and VB), of which 13 show catalytic activity,[3] and are involved in many physiological processes,[3–5] it appeared of interest to study the interaction of these less investigated inorganic (and organic) anions mentioned above[1] also with other isoforms playing important physiological/pathologic functions, such as the transmembrane

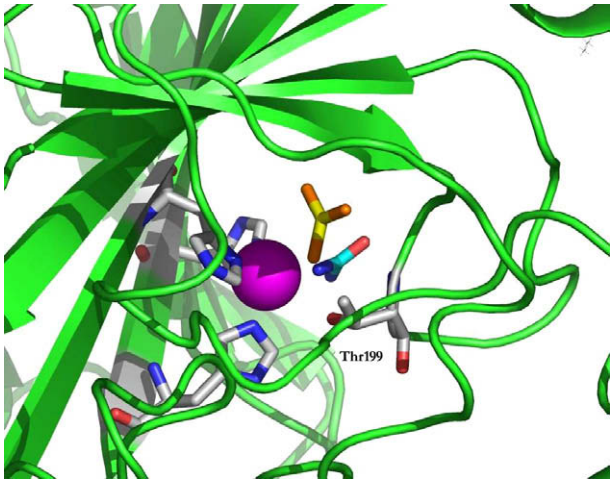

Figure 1. Binding of the tritiocarbonate (yellow and gold) and ureate (sky, blue, and red) to hCA II as determined by X-ray crystallography.[2] The Zn(II) ion of the enzyme (violet sphere), its three protein ligands (His94, 96, and 119) and residue Thr199 participating in interactions with the inhibitors are also shown, whereas the protein backbone is represented as green ribbon.[2,3]

* Corresponding author. Tel.: +39 055 4573005; fax: +39 055 4573385 (C.T.S.).
E-mail address: claudiu.supuran@unifi.it (C.T. Supuran).

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ones CA IX, XII, and XIV.[6–8] In fact, the presently clinically used inhibitors, such as the sulfonamides and sulfamates indiscriminately inhibit all these 13 CA isoforms, and as a consequence such agents have many side effects when used therapeutically.[3–5]

Indeed, two of the transmembrane CA isoforms, CA IX and XII, are over-expressed in many solid tumors in response to the hypoxia inducible factor (HIF-1α) pathway, and research on their involvement in cancer has progressed in recent years, being shown that they are attractive targets for the development of antitumor drugs/diagnostic tools with a novel mechanism of action.[3,6,7,9–12] CA IX and CA XII are strongly over-expressed in many hypoxic tumors, and their high CO_2 hydrase activity leads to a pH imbalance in such tumor tissues, with most hypoxic tumors having acidic external pH values (pHe) of around 6.5, in contrast to the normal tissue which has characteristic pHe values around 7.4.[9] Poussegur's group[10] showed recently that in hypoxic LS174Tr tumor cells expressing either CA IX or both CA IX and XII isoforms, in response to a CO_2 load, both enzymes contribute to extracellular acidification and to maintaining a more alkaline resting intracellular pH (pHi), an action that preserves ATP levels and cell survival in a range of acidic outside pH (6.0–6.8) conditions. In vivo experiments showed that silencing of CA IX alone leads to a 40% reduction in xenograft tumor volume, with up-regulation of the gene encoding for CA XII. Silencing of both CA IX and CA XII gave an impressive 85% reduction of tumor growth.[10] Thus, hypoxia-induced CA IX and CA XII are major tumor prosurvival pH-regulating enzymes, and their combined targeting has great potential for the design of anticancer drugs.[3,10] The in vivo proof-of-concept study that sulfonamide CA IX inhibitors may indeed show antitumor effects, has been only very recently published by Neri's group.[11] By using membrane-impermeant sulfonamide CAIs this group demonstrated the strong tumor growth retardation (in mice with xenografts of a renal clear cell carcinoma line, SK-RC-52) in animals treated for one month with inhibitors.[11] Very recently, the proof-of-concept study showing that targeting of CA IX with specific sulfonamide inhibitors can be used also for imaging hypoxic tumors, has been published by this group.[12] Fluorescent sulfonamides with a high affinity for CA IX developed by our group were shown to bind to cells only when CA IX protein was expressed and while cells were hypoxic.[12] NMRI-nu mice subcutaneously transplanted with HT-29 colorectal tumors were used in these experiments. Accumulation of the fluorescent CAI was monitored by non-invasive fluorescence imaging and could be observed only in delineated tumor areas in which CA IX was present. Furthermore, the bound inhibitor fraction was rapidly reduced upon tumor reoxygenation. Such in vivo imaging results confirmed previous in vitro data[13] demonstrating that CAI binding and retention require exposure to hypoxia. Fluorescent labeled sulfonamides were thus shown to provide a powerful tool to visualize hypoxia response in solid tumors.

CA XIV is not present in tumors, but similarly to CA IX and XII it is a transmembrane protein sharing high sequence homology with the tumor-associated isozymes CA IX and XII.[6,8] All these data clearly show the relevance of these three transmembrane CA isoforms for the drug design of inhibitors with biomedical applications.

Continuing our work in exploring diverse anions with CA inhibitory activity, we report here an inhibition study of the three transmembrane human isoforms (hCA IX, XII, and XIV) with the less investigated inorganic anions mentioned earlier, such as stannate(IV), selenate(VI), tellurate(VI), perosmate(VIII), persulfate, pyrophosphate(V), pyrovanadate(V), tetraborate, perrhenate(VII), perrutenate(VII), persulfate, selenocyanate, iminodisulfonate, fluorosulfate, trithiocarbonate, and *N*,*N*-diethyl-dithiocarbamate.[14,15]

Table 1 shows in vitro inhibition data of recombinant, purified human (hCA) isozymes hCA IX, XII, and XIV with these less investigated inorganic anions mentioned above, obtained by a stopped-flow assay, monitoring the physiological reaction catalyzed by CAs, that is, CO_2 hydration to bicarbonate and a proton.[15–18] Inhibition data of these anions against the ubiquitous, physiologically dominant cytosolic isozyme hCA II are also reported in Table 1, for comparison reasons, as they were recently reported.[1]

Table 1
Inhibition constants of anionic inhibitors and sodium *N*,*N*-diethyl-dithiocarbamate against cytosolic isozymes hCA II, and transmembrane isoforms hCA IX, XII, and XIV, for the CO_2 hydration reaction, at 20 °C[15]

Inhibitor[c]	K_I[b] (mM)			
	hCA II[a]	hCA IX	hCA XII	hCA XIV
SnO_3^{2-}	0.83	0.74	1.02	0.30
SeO_4^{2-}	112	183	138	49.6
TeO_4^{2-}	0.92	0.82	0.87	0.79
OsO_5^{2-}	0.95	184	130	72.5
$S_2O_7^{2-}$	0.97	0.69	1.15	1.33
$P_2O_7^{4-}$	48.5	46.3	63.9	0.76
$V_2O_7^{4-}$	0.57	0.45	0.57	0.43
$B_4O_7^{2-}$	0.95	0.82	0.61	0.59
ReO_4^-	0.75	0.81	0.53	0.64
RuO_4^-	0.69	0.62	0.80	0.65
$S_2O_8^{2-}$	0.084	1.43	0.72	0.93
$SeCN^-$	0.086	1.10	0.66	0.87
$NH(SO_3)_2^{2-}$	0.76	1.35	1.24	1.24
FSO_3^-	0.46	0.70	0.68	1.17
CS_3^{2-}	0.0088	0.0097	0.12	0.66
$Et_2NCS_2^-$	0.0031	0.0014	0.0011	0.0010

[a] From Ref. 1.
[b] Errors were in the range of 3–5% of the reported values, from three different assays.
[c] As sodium salts.

The following should be noted regarding data of Table 1:

(i) The tumor-associated isoform hCA IX was weakly inhibited by selenate, perosmate(VIII), and pyrophosphate (K_Is of 46.3–184 mM) whereas most of the remaining anions, such as stannate, tellurate, persulfate, pyrovanadate(V), tetraborate, perrhenate(VII), perrutenate(VII), selenocyanate, iminodisulfonate, and fluorosulfate were more effective, millimolar inhibitors, with K_Is in the range of 0.45–1.43 mM. The best inhibitors were trithiocarbonate (K_I of 9.7 μM) and diethyldithiocarbamate (K_I of 1.4 μM) (Table 1). It may be observed that there is a rather close parallelism between the inhibition of the transmembrane isozyme hCA IX and the cytosolic one hCA II[1] with this set of anions (except perosmate, which is a very weak hCA IX inhibitor and a submillimolar one against hCA II), although there are also differences between the two isozymes (e.g., diethyldithiocarbamate was 2.2 times a stronger hCA IX than hCA II inhibitor, which is an important finding for the drug design of CA IX inhibitors possessing this new ZBG discovered recently).[1]

(ii) hCA XII, similarly to hCA II and IX, was weakly inhibited by selenate and pyrophosphate (K_Is of 63.9–138 mM), and highly inhibited by diethyldithiocarbamate (K_I of 1.1 μM). However trithiocarbonate was a weaker inhibitor, with a K_I of 0.12 mM, being thus 12.4–15 times less effective as hCA XII inhibitor compared to its inhibition of hCA IX or hCA II (Table 1). Diethyldithiocarbamate on the other hand was 1.3–2.8 times a better hCA XII than hCA II/IX inhibitor. All the remaining anions were weak hCA XII inhibitors, with inhibition constants close to 1 mM (K_Is in the range of 0.53–1.24 mM). It should be noted that perosmate was a very weak hCA XII inhibitor (K_I of 130 mM), a situation similar to that observed for the inhibition of hCA IX.

(iii) hCA XIV showed an inhibition profile with these anions slightly different from those of hCA IX and XII discussed above. Thus, selenate and perosmate were the weakest inhibitors (K_Is of 49.6–72.5) but they seem to be at least an order of magnitude more effective against hCA XIV compared to hCA IX and XII (K_Is of 130–184 mM against these isoforms). Furthermore, pyrophosphate which was a highly ineffective inhibitor of hCA II, IX, and XII (K_Is of 46.3–63.9 mM) was much more effective against hCA XIV (K_I of 0.76 mM). Except *N*,*N*-diethyldithiocarbamate (K_I of 1.0 μM), the best hCA XIV inhibitor among the investigated anions, all remaining inorgnic anions investigated here showed weak, millimolar or submillimolar inhibitory activity, with K_Is in the range of 0.30–1.33 mM (Table 1). It should be noted that stannate was the second most effective hCA XIV inhibitor after diethyldithiocarbamate (K_I of 0.30 mM), being more than two times better inhibitor compred to trithiocarbonate (K_I of 0.66 mM).

It is impossible to rationalize the important differences of activity observed for these three transmembrane isozymes with some of the investigated anions (e.g., stannate, pyrophosphate, trithiocarbonate, etc.) without extensive X-ray crystal data, but these findings may be relevant to obtain isoform-selective CAIs diverse of the sulfonamides and their bioisosteres.[3]

In conclusion, we explored here less investigated inorganic anions for their interactions with the transmembrane CA isozymes hCA IX and XII (tumor-associated enzymes, recently shown to be drug targets for obtaining antitumor or antiglaucoma agents)[3] and hCA XIV, an isoform having a high degree of sequence homology with hCA IX and XII, but not associated with tumors and present in the normal kidneys, brain, and lungs. Selenate, perosmate, and pyrophosphate were ineffective inhibitors of all three isozymes, whereas most of these anions inhibited the three enzymes in the millimolar–submillimolar range. Trithiocarbonate and diethyldithiocarbamate were the best CA IX inhibitors (K_Is of 1.4–9.7 μM), but trithiocarbonate showed less affinity for CA XII and XIV (K_Is of 0.12–0.66 mM). *N*,*N*-Diethyldithiocarbamate was a low micromolar inhibitor also against CA XII and XIV (K_Is of 1.0–1.1 μM), proving that this new zinc-binding group (CS_2^-) may lead to efficient inhibitors targeting the transmembrane isoforms.

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