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# Carbonic anhydrase inhibitors. Inhibition of cytosolic isoforms I, II, III, VII and XIII with less investigated inorganic anions

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ABSTRACT

An inhibition study of the cytosolic carbonic anhydrase (CA, EC 4.2.1.1) isoforms I, II, III, VII and XIII 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. Trithiocarbonate was the best inhibitor detected, showing affinities of 8.7–9.9 μM for CA I–III, of 36.15 mM for CA VII and of 0.43 mM for CA XIII. Considering trithiocarbonate as lead, we show that compounds incorporating the new zinc-binding group $CS_2^-$, such as among other the dithiocarbamates, are even more active inhibitors, with submicromolar inhibitory activity. New classes of CA inhibitors are being detected based on the $CS_2^-$ zinc-binding group.



Inorganic anions constitute a well-known class of inhibitors of the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1).[1–4] Our group investigated[1–3] the interaction between all mammalian CA isozymes with simple inorganic anions, including the physiological ones (such as chloride, bicarbonate, carbonate and sulfate), as well as 'metal poisons' (cyanide, cyanate, thiocyanate, azide, hydrogen sulfide, bisulfite, nitrite, etc.) or anions with less affinity for metal ions in solution (tetrafluroborate, perchlorate, nitrate, fluoride and heavier halides, among others).[1–4] 16 CA isoforms are presently known in mammals, CA I–CA XV, of which 13 show catalytic activity, that is, CA I–CA VII, CA IX, CA XII–CA XV (there are two type 'V' isoforms, CA VA and CA VB).[3] Such studies are helpful for better understanding the CA catalytic/inhibition mechanism,[3] the physiological role of some CA isoforms which may work in tissues where high concentrations of inorganic anions are present,[5] but also for the design of novel zinc-binding groups to be incorporated in new classes of tight-binding CA inhibitors (CAIs).[3,6] Indeed, inorganic anions are usually weak CAIs, with affinities in the millimolar–submillimolar range, with few anions arriving to be low micromolar inhibitors for some isoforms (e.g., cyanide, azide and hydrogen sulfide).[1–3] Still, some of these anions may be used to design much better CAIs incorporating a zinc-binding group (ZBG) attached to an organic scaffold, which usually lead to enhanced affinity for the enzyme active site and whence much better inhibitory activity as compared to the starting inorganic anion. Indeed, considering $HS^-$ as lead, organic thiols (of the type Ar-SH or Het-SH where Ar is an aromatic, substituted benzene, and Het a heterocyclic 1,3,4-thiadiazole or 1,3,4-triazole scaffold) were shown to act as potent CAIs, with low nanomolar affinity being detected in some cases.[7]

Except the simple anions mentioned above, few other such compounds have been investigated for their inhibitory capacity against CAs. In one such study Innocenti et al.[8] reported the interactions of the human isoforms hCA I, II, IV, VA and IX with complex anions such as hexafluorosilicate, hexafluorophosphate, hexafluoroarsenate(V), hexafluoroaluminate(III), hexafluoroferrate(III), heptafluroniobate(V), tetrachloroplatinate(II), hexachloroplatinate(IV) as well as complex cyanides of Cu(I), Ag(I), Au(I), Pd(II), Fe(II) and Fe(III). The investigated 5 CA isoforms showed a very different inhibition profile with these anions, some of which were acting as low micromolar inhibitors.[8] Continuing our work in exploring diverse anions with CA inhibitory activity, we report here an inhibition study of the five cytosolic mammalian isoforms (CA I, II, III, VII and XIII, of murine (m) or human (h) origin) 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]

Table 1 shows in vitro inhibition data of recombinant, purified CA isozymes hCA I, II, III, VII and mCA XIII with these less investigated inorganic anions mentioned above, obtained by a stopped-flow assay, monitoring the physiologic reaction catalyzed by CAs,

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**Table 1**
Inhibition constants of anionic inhibitors and *N,N*-diethyl-dithiocarbamate against cytosolic isozymes hCA I, II, III, VII and mCA XIII, for the $CO_2$ hydration reaction, at 20 °C[12]

| Inhibitor[b] | $K_I$[a] (mM) | | | | |
|---|---|---|---|---|---|
| | hCA I | hCA II | hCA IIII | hCA VII | mCA XIII |
| $SnO_3^{2-}$ | 0.57 | 0.83 | 0.64 | 0.67 | 0.93 |
| $SeO_4^{2-}$ | 118 | 112 | 138 | 215 | 484 |
| $TeO_4^{2-}$ | 0.66 | 0.92 | 0.27 | 0.46 | 0.38 |
| $OsO_5^{2-}$ | 0.92 | 0.95 | 1.06 | 1.02 | 0.96 |
| $S_2O_7^{2-}$ | 0.99 | 0.97 | 1.10 | 0.76 | 0.71 |
| $P_2O_7^{4-}$ | 25.77 | 48.50 | 1.01 | 0.87 | 0.73 |
| $V_2O_7^{4-}$ | 0.54 | 0.57 | 0.29 | 0.60 | 0.39 |
| $B_4O_7^{2-}$ | 0.64 | 0.95 | 0.69 | 0.68 | 0.69 |
| $ReO_4^-$ | 0.110 | 0.75 | 0.92 | 36.54 | 21.18 |
| $RuO_4^-$ | 0.101 | 0.69 | 1.06 | 17.50 | 20.45 |
| $S_2O_8^{2-}$ | 0.107 | 0.084 | 1.00 | 3.11 | 4.31 |
| $SeCN^-$ | 0.085 | 0.086 | 0.85 | 1.10 | 26.51 |
| $NH(SO_3)_2^{2-}$ | 0.31 | 0.76 | 1.00 | 39.10 | 0.98 |
| $FSO_3^-$ | 0.79 | 0.46 | 39.66 | 1.31 | 61.83 |
| $CS_3^{2-}$ | 0.0087 | 0.0088 | 0.0099 | 36.15 | 0.43 |
| $Et_2NCS_2^-$ | 0.00079 | 0.0031 | 0.0065 | 1.47 | 0.056 |

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

that is, $CO_2$ hydration to bicarbonate and a proton.[10] The following should be noted regarding data of Table 1:

(i) The slow, ubiquitous isoform hCA I was weakly inhibited by selenate and pyrophosphate ($K_I$s of 25.77–118 mM), whereas most of the investigated anions (e.g., stannate, tellurate, perosmate, pyrosulfate, pyrovanadate, tetraborate, iminodisulfonate and fluorosulfate) were weak, submicromolar inhibitors, with inhibition constants in the range of 0.31–0.99 mM. Several anions, among which perrhenate, perrutenate, persulfate and selenocyanate showed much better hCA I inhibitory activity compared to the previous anions, with $K_I$s in the range of 85–110 μM. The best hCA I inhibitor was trithiocarbonate ($K_I$ of 8.7 μM), which is an anion isostructural to carbonate (and bicarbonate), which are CA substrates. Trithiocarbonate is in fact one of the best anion hCA I inhibitor ever detected up to now, which is indeed a remarkable result.[1–3]

(ii) The physiologically dominant, rapid isoform hCA II showed a rather similar inhibition profile with hCA I, with anions investigated here. Thus, selenate and pyrophosphate were the weakest inhibitors ($K_I$s of 48.50–112 mM), and stannate, tellurate, perosmate, pyrosulfate, perrhenate, perrutenate, pyrovanadate, tetraborate, iminodisulfonate and fluorosulfate showed a moderate inhibitory capacity, with inhibition constants in the range of 0.46–0.97 mM. Pyrosulfate and selenocyanate were again effective inhibitors ($K_I$s of 84–86 μM), whereas trithiocarbonate was the best, low micromolar hCA II inhibitor in this series of anions ($K_I$ of 8.8 μM).

(iii) A completely different inhibition profile with these anions was observed for the very slow, muscle cytosolic isozyme hCA III (Table 1). Thus, the least effective inhibitors were selenate and fluorosulfate, with $K_I$s of 39.66–138 mM, whereas all the remaining anions except trithiocarbonate, showed a compact behaviour of weak inhibitors with $K_I$s of 0.29–1.10 mM. As for hCA I and II, trithiocarbonate effectively inhibited hCA III, with a $K_I$ of 9.9 μM, As this isozyme is not easily inhibited by sulfonamides (the main class of organic CAIs)[11,12] due to the presence of the very bulky Phe198 in the middle of the enzyme active site, which interferes with the binding of organic, bulky inhibitors,[13] this is indeed a significant result, as it evidences a low micromolar hCA III inhibitor, possibly useful in physiologic studies in which the activity of CA III should be inhibited.

(iv) The brain cytosolic isoform hCA VII was weakly inhibited by selenate, perrhenate, perrutenate, iminodisulfonate and unexpectedly, trithiocarbonate, with $K_I$s in the range of 17.50–215 mM. On the other hand, all the remaining anions showed more effective inhibitory activity, with inhibition constants in the range of 0.46–3.11 mM. The best hCA VII inhibitor in this series was tellurate ($K_I$ of 0.46 mM). It is interesting to note the important difference of activity between the isostructural, tetrahedral anions selenate, tellurate, perrhenate and perrutenate (Table 1). Thus, tellurate is 467 times a better hCA VII inhibitor than selenate; a 79.4 times better inhibitor than perrhenate and a 38 times better inhibitor than perrutenate, respectively.

(v) The last cytosolic isoform, mCA XIII also showed a distinct inhibition profile with these anions, compared to the remaining four other CAs investigated here. Thus, selenate was an exceedingly weak inhibitor ($K_I$ of 484 mM) whereas perrhenate, perrutenate, selenocyanate and fluorosulfate were also weak ones ($K_I$ in the range of 20.45–61.83 mM). The remaining anions showed again a compact behaviour, with a low affinity for this isozyme ($K_I$ in the range of 0.39–4.31 mM). The best mCA XIII inhibitor was pyrovanadate. A remarkable fact is the lack of susceptibility of mCA XIII to be inhibited by selenocyanate ($K_I$ of 26.51 mM) whereas all other cytosolic isoforms were generally easily inhibited by this anion ($K_I$s of 85 μM–1.10 mM, Table 1).

(vi) The most interesting finding of this work regards the strong inhibitory activity against several cytosolic CA isozymes of the simple anion trithiocarbonate, which presumably binds to the Zn(II) ion within the active site in a bidentate manner, similarly to carbonate (Fig. 1A).[1–3] Indeed, this anion represents a new ZBG for obtaining CAIs, which has never before been considered for such a purpose,[6] although literature data show that disubstituted organic trithiocarbonates possessing the general formula RS–C(=S)–SR′ act as efficient inhibitors of another zinc enzyme, the histone deacetylase.[14]

Such disubstituted trithiocarbonates are on the other hand too bulky to fit well in the restricted active site bottom of CAs, where the Zn(II) ion is placed.[11,12,15] However, compounds possessing a monodentate ZBG based on the trithiocarbonate, of the type R–S–C(=S)–S$^-$ or even R–C(=S)S$^-$ might in principle also act as efficient CAIs, with a binding similar to the one shown schematically in Figure 1B. In fact it is rather easy to check our hypothesis, since the widely used reagents in analytical chemistry dialkyl-dithiocarbamates[16] (of which sodium *N,N*-diethyl-dithiocarbamate is a well-known example) are available commercially. Thus, we tested sodium *N,N*-diethyl-dithiocarbamate for the inhibition of these cytosolic CAs, in the same conditions as the other anions (Table 1). The obtained data strongly confirm our hypothesis, i.e., it is possible to design potent CAIs possessing a new ZBGs, based on the $CS_2^-$ motif present in trithiocarbonate. Thus, *N,N*-diethyl-dithiocarbamate was an even more potent CAI as compared to the lead trithiocarbonate, inhibiting hCA I with a $K_I$ of 0.79 μM (11 times

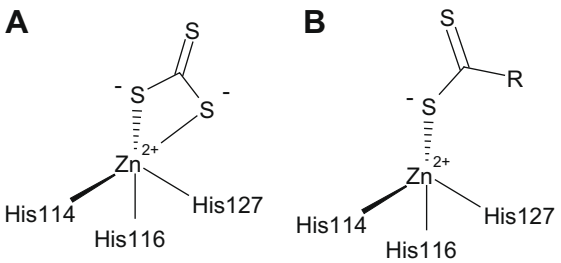


**Figure 1.** Putative bidentate binding mode of trithiocarbonate to the CA active site, based on the binding of carbonate/bicarbonate[3,15] (A), and, proposed monodentate binding for derivatives possessing the new ZBG based on the trithiocarbonate one (R = SR′ for monosubstituted trithiocarbonates or $R'_2N$ for dithiocarbamates, an example of which is shown in Table 1, R = $Et_2N$), (B).

better than trithiocarbonate which had a $K_I$ of 8.7 μM); hCA II with a $K_I$ of 3.1 μM (2.8 times better than trithiocarbonate); hCA III with a $K_I$ of 6.5 μM (1.5 times better than trithiocarbonate), hCA VII with a $K_I$ of 1.47 mM (24.6 times better than trithiocarbonate); and mCA XIII with a $K_I$ of 56 μM (7.6 times better than trithiocarbonate), respectively (Table 1).

In conclusion, we explored here less investigated inorganic anions for their interactions with the five cytosolic CA isozymes, I, II, III, VII and XIII. Selenocyanate was an effective inhibitor of CA I and II but showed less affinity for the other isoforms. Selenate was the worst inhibitor among the investigated anions, against all five isoforms. Trithiocarbonate was the best inhibitor detected here, showing affinities of 8.7–9.9 μM for CA I–III, of 36.15 mM for CA VII and of 0.43 mM for CA XIII. Considering trithiocarbonate as lead, we demonstrate that compounds incorporating the new zinc-binding group $CS_2^-$, such as among other the *N*,*N*-dialkyl-dithiocarbamates, are even more active inhibitors, with submicromolar activity against some isoforms. These new classes of CAIs can be further enlarged and developed due to ease of synthesis of both monosubstituted trithiocarbonates as well as dialkyl-dithiocarbamates possessing a wealth of diverse scaffolds.

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