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Carbonic anhydrase inhibitors. Interaction of isozymes I, II, IV, V, and IX with organic phosphates and phosphonates

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Abstract—The interaction of five human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes, that is, hCA I, II, IV, V, and IX with a small library of phosphonic acids/organic phosphates, including methylphosphonic acid, MPA; phenylphosphonic acid, PPA; *N*-(phosphonoacetyl)-L-aspartic acid, PALA, methylene diphosphonic acid MDPA, the *O*-phosphates of serine (Ser-OP) and threonine (Thr-OP) as well as the antiviral phosphonate foscarnet has been studied. hCA I was activated by all these compounds, with the best activators being MPA and PPA (K_As of 0.10–1.20 μM). MPA and PPA were on the other hand nanomolar inhibitors of hCA II (K_Is of 98–99 nM). PALA showed an affinity of 7.8 μM, whereas the other compounds were weak, millimolar inhibitors of this isozyme. The best hCA IV inhibitors were PALA (79 nM) and PPA (5.4 μM), whereas the other compounds showed K_Is in the range of 0.31–5.34 mM. The mitochondrial isozyme was weakly inhibited by all these compounds (K_Is in the range of 0.09–41.7 mM), similarly to the transmembrane, tumor-associated isozyme (K_Is in the range of 0.86–2.25 mM). Thus, phosphonates may lead to CA inhibitors with selectivity against two physiologically relevant isozymes, the cytosolic hCA II or the membrane-bound hCA IV.

1. Introduction

In a recent study [1] we have investigated the interaction of several carbonic anhydrase (CA, EC 4.2.1.1) isozymes with inorganic phosphates, carbamoyl phosphate as well as the antiviral drug foscarnet, a carboxylate phosphonate derivative, evidencing a very interesting behavior and different affinities of the various human CA isozymes (hCA) to these anion inhibitors. Indeed, anions represent the second class of inhibitors of these metallo-enzymes, in addition to the sulfonamides/sulfamates/sulfamides with clinical applications as antiglaucoma, antiepileptic, antiobesity or antitumor drugs.[2–6] Recently we have investigated in detail the binding of both metal-complexing anions such as cyanide, cyanate, thiocyanate, halides, azide, hydrogensulfide, etc., as well as anions with a lower tendency to bind metal ions, such as perchlorate, tetrafluoroborate, or sulfate among others,[6–11] to many of the CA isozymes isolated so far in diverse organisms all over the phylogenetic tree (14 such α-CA isozymes are presently known in humans, and many representatives belonging to the β-, γ-, δ-, and ε-CA families have recently been described in other organisms).[2–5]

In the previous work we observed that the membrane-associated isozyme hCA IV was the most sensitive to inhibition by phosphates/phosphonates, showing a K_I of 84 nM for PO_4^{3-}, of 9.8 μM for HPO_4^{2-} and of 9.9 μM for carbamoyl phosphate.[1] Foscarnet was the best inhibitor of this isozyme (K_I of 0.82 mM) highly abundant in the kidneys, which probably explains the renal side effects of this antiviral drug.[12] The mitochondrial isozyme hCA V was weakly inhibited by all phosphates/phosphonates, except carbamoyl phosphate which behaved as a potent inhibitor, with a K_I of 8.5 μM.[1] Based on these observation, it has been concluded that hCA V cannot be the isozyme involved in the carbamoyl phosphate synthetase I biosynthetic reaction, as hypothesized earlier by Chegwidden et al.[13] The relative resistance of hCA V to inhibition by inorganic phosphates suggested an evolutionary adaptation of

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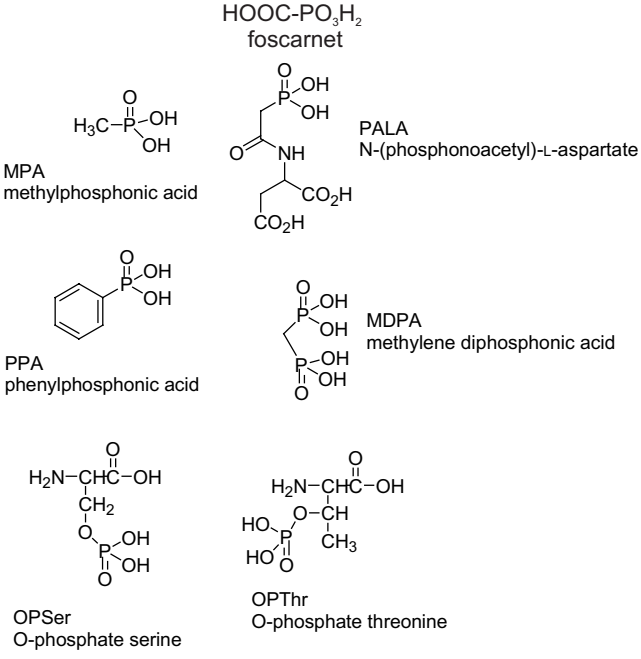
the mitochondrial isozyme to the presence of high concentrations of such anions in these energy-converting organelles, where high amounts of ATP are produced by ATP synthetase, from ADP and inorganic phosphates.[1,14] Furthermore, in the previous study it has also been shown that the cytosolic ubiquitous isozyme hCA II and the transmembrane, tumor associated isozyme hCA IX were slightly inhibited by phosphates/phosphonates, whereas the cytosolic isozyme hCA I was activated by most of them.[1] It is obvious that CA inhibition studies with anions may lead on one side to a better understanding of the physiological role(s) of some isozymes, and on the other one to the design of inhibitors selective for various CAs as well as to inhibitors incorporating novel zinc binding functions, except the classical ones based on the sulfonamide motif.[2–4] Here we extend our previous work,[1] presenting a study on the interactions of five hCA isozymes with four simple phosphonates (methylphosphonic acid, MPA; phenylphosphonic acid, PPA; *N*-(phosphonoacetyl)-L-aspartic acid, PALA, and methylene diphosphonic acid MDPA) and two organic phosphates, that is, the *O*-phosphates of serine (Ser-OP) and threonine (Thr-OP).



2. Chemistry

Buffers and phosphates/phosphonates were of highest purity available, from Sigma–Aldrich (Milano, Italy) and were used without further purification, except foscarnet (trisodium salt) which was from Pharmacia Co. and PALA which was prepared as described by Montero and Imbach.[15] Recombinant CA isozymes used in this work were obtained as reported previously.[1]

3. CA inhibition data

Inhibition data against five CA isozymes, that is, hCA I, hCA II (cytosolic forms), hCA IV (membrane-associated), hCA V (mitochondrial), and hCA IX (transmembrane), with the above-mentioned phosphonates/phosphates are shown in Table 1.[16] Data of foscarnet, previously investigated[1] are also presented for comparison.

The following should be noted regarding the interaction of phosphonates/phosphates with the CA isozymes investigated here: (i) the slow cytosolic isozyme hCA I was activated by all the investigated derivatives, as already evidenced earlier for foscarnet and some inorganic phosphates.[1] Thus, MPA and PPA behaved as potent activators, with activation constants in the range of 0.10–1.2 μM, of the same order of magnitude as histamine or other investigated CA activators (CAAs) for which the X-ray crystal structures in complexes with the enzyme have been reported by our group.[17–20] Foscarnet was a somehow weaker CAA (K_A of 12 μM), whereas PALA, MDPA, Ser-OP, and Thr-OP showed modest CA I activatory properties, with K_A values in the range of 0.1–0.5 mM (Table 1). As shown earlier,[17–20] CAAs participate in the catalytic cycle by favoring the rate-limiting step in catalysis, which is a proton transfer reaction between the active site and the medium. Probably the phosphonates/phosphates investigated here bind at the entrance of the enzyme active site, similarly to histamine[19] or phenylalanine[20] (the best investigated CAAs) and shuttle protons between the active site and the reaction medium by means of their phosphonic acid moieties (it is presumed that at the pH of our experiments, 7.5, these compounds bind to the CAs in ionized, phosphonate form); (ii) against the major cytosolic isozyme hCA II, the phosphonates/phosphates investigated here showed a very interesting behavior as CA inhibitors (CAIs). Thus, the very simple aliphatic- and aromatic derivatives MPA and PPA showed very effective inhibition of hCA II, with inhibition constants of 98 and 99 nM, respectively. This is indeed a remarkable result, proving that the phosphonate group can be considered as an efficient zinc-binding function also for the design of CAIs (it was demonstrated previously that the phosphonate moiety is an efficient zinc-binding function for the design of matrix metalloproteinase, as well as other protease inhibitors[21]). Less efficient as a CAI was PALA, with a K_I of 7.8 μM, whereas the diphosphonate MDPA and the two phosphates Ser-OP and Thr-OP were very weak hCA II inhibitors, with K_Is in the range of 0.42–1.25 mM. It is interesting to note that foscarnet which theoretically possesses a much better zinc-coordinating moiety (with both the carboxylate as well as the phosphonate moieties present in its molecule) than the other compounds investigated here, is in fact the weakest CA II inhibitor (K_I of 14.2 mM); (iii) The membrane-associated isozyme hCA IV also showed an interesting behavior toward this class of inhibitors. PALA was the most efficient CA IV inhibitor, with a K_I of 79 nM, whereas PPA showed efficient inhibition too (K_I of 5.4 μM). All other investigated derivatives were only weakly active, showing inhibition constants in the range of 0.31–5.34 mM. Thus, there is a dramatic difference of activity of these derivatives against hCA IV as compared to hCA II, with the most diverse behavior of MPA, which is a

Table 1. Inhibition/activation constants of foscarnet and organic phosphonates/phosphates against isozymes hCA I, II, IV, V, and IX, for the CO_2 hydration reaction, at 20 °C and pH 7.5[16]

Inhibitor	K_I [mM][a]				
	hCA I[b]	hCA II[b]	hCA IV[b]	hCA V[c]	hCA IX[d]
$HOOC–PO_3H_2$[e]	A	14.2	0.82	41.7	2.21
MPA	A	98 nM	0.31	0.11	1.26
PPA	A	99 nM	5.4 μM	0.09	2.21
PALA	A	7.8 μM	79 nM	0.37	2.25
MDPA	A	1.25	5.34	0.73	0.86
Ser-OP	A	0.42	4.26	0.36	0.92
Thr-OP	A	1.08	3.18	0.85	1.23

A = activator: K_A (foscarnet) = 12 μM; K_A (MPA) = 1.2 μM; K_A (PPA) = 0.10 μM; K_A (PALA) = 0.1 mM; K_A (MDPA) = 0.2 mM; K_A (OPSer) = 0.1 mM; K_A (OPThr) = 0.5 mM; (K_A = activation constant).

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

[b] Human cloned isozymes.

[c] Recombinant, full-length form of hCA V.

[d] Catalytic domain of the human, recombinant isozyme.

[e] From Ref. 1.

nanomolar inhibitor of the cytosolic isozyme and a millimolar inhibitor of the membrane-bound one. This is an encouraging result for the design of isozyme-specific inhibitors, a goal rarely achieved up to now;[22] (iv) the mitochondrial isozyme hCA V was also inhibited by all the investigated phosphonates/phosphates, but less as compared to the previously mentioned isozymes, hCA II and hCA IV. The best inhibitor was PPA, with a K_I of 90 μM, whereas all the other derivatives were less effective, with K_I values in the range of 0.11–0.85 mM. Foscarnet was the least effective hCA V inhibitor (K_I of 41.7 mM); (v) the tumor-associated, transmembrane isozyme hCA IX was also weakly inhibited by all these derivatives, with inhibition constants in the range of 0.86 – 2.21 nM.

All these data show that some of the CA isozymes investigated here, such as hCA II and hCA IV are potently inhibited by some phosphonates, whereas isozyme hCA I is strongly activated, and hCA V and hCA IX are only weakly inhibited. Thus, phosphonates may lead to CAIs with selectivity against hCA II or hCA IV. The two phosphates investigated here were on the other hand quite weak inhibitors of all isozymes except hCA I against which they showed weak CA activatory properties.

4. Conclusion

We report here a detailed inhibition study of five CA isozymes with organic phosphates and phosphonates, including methylphosphonic acid, MPA; phenylphosphonic acid, PPA; *N*-(phosphonoacetyl)-L-aspartic acid, PALA, methylene diphosphonic acid MDPA, the *O*-phosphates of serine (Ser-OP) and threonine (Thr-OP) as well as the antiviral phosphonate foscarnet. hCA I was activated by all these compounds, with the best activators being MPA and PPA (K_As of 0.10–1.20 μM). MPA and PPA were on the other hand nanomolar inhibitors of hCA II (K_Is of 98–99 nM). PALA showed an affinity of 7.8 μM, whereas the other compounds were weak, millimolar inhibitors of this isozyme. The best hCA IV inhibitors were PALA (79 nM) and PPA (5.4 μM), whereas the other compounds showed K_Is in the range of 0.31–5.34 mM. The mitochondrial isozyme was weakly inhibited by all these compounds (K_Is in the range of 0.09–41.7 mM), similarly to the transmembrane, tumor-associated isozyme (K_Is in the range of 0.86–2.25 mM). Thus, phosphonates may lead to CA inhibitors with selectivity against two physiologically relevant isozymes, the cytosolic hCA II or the membrane-bound hCA IV.

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