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Phosph(on)ate as a zinc-binding group in metalloenzyme inhibitors: X-ray crystal structure of the antiviral drug foscarnet complexed to human carbonic anhydrase I☆

Claudia Temperini,[a] Alessio Innocenti,[a] Annalisa Guerri,[a] Andrea Scozzafava,[a] Stefano Rusconi[b] and Claudiu T. Supuran[a,*]

[a]*Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy*
[b]*Istituto di Malattie Infettive e Tropicali, Università degli Studi di Milano, Ospedale Luigi Sacco, via G.B. Grassi 74, 20157 Milano, Italy*

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Abstract—Foscarnet (phosphonoformate trisodium salt), an antiviral used for the treatment of HIV and herpes virus infections, also acts as an activator or inhibitor of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). Interaction of the drug with 11 CA isozymes has been investigated kinetically, and the X-ray structure of its adduct with isoform I (hCA I-foscarnet complex) has been resolved. The first CA inhibitor possessing a phosphonate zinc-binding group is thus evidenced, together with the factors governing recognition of such small molecules by a metalloenzyme active site. Foscarnet is also a clear-cut example of modulator of an enzyme activity which can act either as an activator or inhibitor of a CA isozyme.

Metalloenzyme inhibition constitutes a widely used means for the regulation of enzymatic activity in a multitude of physiologically relevant biological systems, with important consequences for phenomena such as biosynthetic reactions, signal transduction, secretion of electrolytes, ovulation, embryonic development, blastocyst implantation, nerve growth, morphogenesis, apoptosis, inflammation, angiogenesis, cancer invasion and metastasis, tissue resorption and remodeling, bone remodeling, arthritis, atherosclerosis, aneurysm, breakdown of blood–brain barrier, etc.[1–4] The binding of an inhibitor molecule to the catalytic metal ion(s) leads to the reorganization of the active site geometry, with generation of enzyme-inhibitor adducts which are catalytically ineffective. Metalloenzyme inhibitors contain a metal-binding function attached to a scaffold that interacts with the remaining binding regions of the enzyme active site.[1–4]

Many of the most widespread metalloenzymes are zinc-containing ones.[1–4] The Zn(II) ion(s) present in such enzymes generally show a high affinity for sulfur and/or nitrogen-containing ligands, and as a consequence, most such enzyme inhibitors contain zinc-binding groups incorporating sulfur/nitrogen functionalities.[1–4] However, there are many examples of zinc-binding groups coordinating the metal ion by means of oxygen atom(s), most of which incorporate the carboxylate functionality. Examples of the most common zinc-binding functions contained in metalloenzyme inhibitors include quite heterogeneous chemical classes, such as carboxylates, hydroxamates, thiols, phosphorus-based compounds (phosphates/phosphonates), sulfonamides (and their variants such as the sulfamates/sulfamides), sulfodiimines, etc.[1–4] For metalloproteases (among which the matrix metalloproteinases represent the best studied case), usually the strongest inhibitors are the hydroxamates, followed by the thiols (which are 20–50 times less potent than the structurally related hydroxamates), whereas the carboxylates/phosphonates are 100-2000 times less potent inhibitors compared to the corresponding hydroxamates incorporating the same organic scaffold.[3,4]

Keywords: Carbonic anhydrase; Foscarnet; Isozymes; X-ray crystallography; Zinc-binding group; Enzyme inhibitor; Enzyme activator.
☆ Coordinates and structure factors are deposited within the Protein Data Bank (PDB Accession code 2IT4).
* Corresponding author. Tel.: +39 055 457 3005; fax: +39 055 457 3385; e-mail: claudiu.supuran@unifi.it

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Small inorganic anions also bind to the metal ion within the active site of zinc enzymes.[5–7] Two types of interactions have been observed so far in most zinc enzymes, exemplified here by the carbonic anhydrase (CA, EC 4.2.1.1): (i) substitution of the non-protein fourth zinc ligand (a hydroxide ion or water molecule) by the inhibitor, as anionic species, with formation of a tetrahedral zinc adduct, and (ii) addition of the inhibitor to the metal coordination sphere, with generation of trigonal-bipyramidal adducts, in which the metal ion within the enzyme active site is coordinated in addition to the three protein ligands (His94, 96, and 119 in the case of CAs) by a water molecule and the anion inhibitor.[5–7]

As many CA isoforms amongst the 16 presently known in vertebrates[8,9] are quite stable enzymes, that easily crystallize in complex with various classes of inhibitors, whereas their active site geometry is also shared by many other enzyme families which are more difficult to investigate,[1–4] studying the detailed interactions between such inhibitors and the CA active site may lead to novel developments for the better understanding of the catalytic/inhibition mechanism of metalloenzymes, and also to the development of pharmaceutically valuable compounds.[1–9]

In this paper, we report the interaction of the antiviral drug foscarnet (phosphonoformate trisodium salt, $^{2-}O_3P\text{-}COO^{-}\cdot 3Na^{+}$) used for the treatment of herpes and HIV infections[10,11] with 11 CA isozymes, and the X-ray crystal structure for the adduct of the drug with the human isoform I, hCA I. Two reasons prompted us to perform this study: (i) foscarnet contains two potential zinc-binding groups (the phosphonate and the carboxylate one) which have not been investigated up to now in detail (by means of X-ray crystallography) for the design of metalloenzyme inhibitors. Furthermore, there is a multitude of possible ways in which these two groups (alone, or both of them) could coordinate a metal ion from an enzyme active site (for example, the carboxylate can act as a mono- or bidentate ligand against many metal ions; the same could be achieved by the PO_3^{2-} moiety present in this drug); (ii) in a previous work[12] we showed by means of kinetic stopped-flow measurements that this antiviral drug acts as an activator of isoform hCA I and as a weak inhibitor of four other physiologically relevant isozymes, i.e., CA II, IV, VA, and IX. It appeared thus of interest to investigate in detail its interaction with all catalytically efficient CA isozymes (i.e., CA I–XIV) in order to better understand how such enzymes bind small molecule regulators of activity,[12,13] which may act either as activators or inhibitors, and which might lead to the drug design of interesting modulators of enzyme activity.[12,13]

Inhibition data against 11 CA isozymes, i.e., hCA I, hCA II, hCA VII and mCA XIII (cytosolic forms; h stands for human, m for murine isoform), hCA IV (membrane-associated), hCA VA and hCA VB (mitochondrial), hCA VI (secreted in the saliva), and hCA IX, hCA XII, and hCA XIV (transmembrane, tumor-associated isozymes in the case of CA IX and XII),[14] with foscarnet are shown in Table 1.[15]

Table 1. Inhibition constants of foscarnet ($^{-}OOC-PO_3^{2-}$) trisodium salt, against 11 CA isozymes, hCA I, II, IV, VA, VB, VII, IX, XII, mCA XIII, and hCA XIV (h, human; m, murine isoform), for the CO_2 hydration reaction, at 20 °C[15]

Isoform	K_I[e] (mM)
hCA I[a]	24.1
hCA II[b]	14.2
hCA IV[b]	0.82
hCA VA[b]	41.7
hCA VB[b]	11.8
hCA VI[b]	1.81
hCA VII[b]	0.56
hCA IX[c]	2.21
hCA XII[c]	1.29
mCA XIII[d]	0.87
hCA XIV[b]	3.60

[a] Human cloned isozyme, preincubation of enzyme with inhibitor for 5 days at 4 °C.
[b] Recombinant, human isoforms, preincubation of enzyme with inhibitor for 15 min at room temperature.[12]
[c] Catalytic domain of the human, recombinant isozymes, preincubation of enzyme with inhibitor for 15 min at room temperature.[14]
[d] Murine, recombinant isoform, preincubation of enzyme with inhibitor for 15 min at room temperature.[14]
[e] Errors were in the range of 3–5% of the reported values, from three different assays.

Data of Table 1 allow us to draw the following conclusions regarding the interaction of these CA isozymes with the phosphonate antiviral drug foscarnet: (i) four isozymes are weakly inhibited by foscarnet, with inhibition constants in the range of 11.8–41.7 mM. These isoforms are hCA I, hCA II (cytosolic) and hCA VA/VB (mitochondrial). It is interesting to note that as mentioned in our previous study,[12a] if foscarnet was preincubated with hCA I for 15 min (which is the standard procedure used for assaying CA inhibitors/activators)[15] the compound acts as an efficient hCA I activator, with an activation constant K_A of 12 μM (but is an inhibitor of all other investigated CA isozymes). This was after all not such a surprising result, considering the fact that many weak acids incorporating phosphate/phosphonate or carboxylate moieties act as efficient buffers.[12a] However, as shown later in the paper, the X-ray crystal data reported here showed foscarnet to bind to the metal ion within the hCA I active site, a binding site typical for CA inhibitors[8,9,12] and not CA activators, which do not directly bind to the metal ion, but to a diverse region of the enzyme cavity and participate thereafter in the proton shuttling between the enzyme active site and the reaction medium.[14,16,17] Thus, this 'discrepancy' between kinetic data[12a] and crystal structure prompted us to investigate in detail the interaction of this isoform (hCA I) with foscarnet. Thus, a longer incubation between the enzyme and the inhibitor, of the order of several days (2–5 days) allowed us to measure an inhibition constant of hCA I by foscarnet of 24.1 mM. This is a clear-cut example of a molecule which may act either as a CA activator as well as a CA inhibitor against the same isoform. Some evidences that 4-methylimidazole may act either as an activator or as an inhibitor for isozyme hCA II were furnished recently by Silverman's group.[17c] By means of X-ray crystallography it has been shown that this modulator of activity binds in the

activator binding site, at the entrance of the cavity of the wild-type, zinc enzyme (similarly to other CA activators),[13,17] whereas for the Co(II)-substituted CA II, by means of ^{1}H-NMR relaxivity measurements it has been calculated that 4-methylimidazole is coordinated to the metal ion (and thus acts as an inhibitor with an inhibition constant of around 200 mM),[17c] with a distance between Co(II) and one nitrogen atom of the azole of 4.8 Å.[17c] This is an unacceptably long distance for a coordination bond, even within an enzyme active site, and we appreciate that the inhibition data proposed by Silverman's group are artefactual, whereas it is clearly established that 4-methylimidazole acts as a CA activator similarly to histamine, histidine or other such derivatives investigated earlier by this group.[13] In fact, most CA inhibitors coordinated to Zn(II) ion in various CA isozymes show a distance between the metal ion and the inhibitor atom coordinated to it in a much shorter range of 1.7–2.2 Å.[5–7]

Thus, when hCA I and foscarnet were in contact for a short period (15 min[12a]–3 h, data not shown), an enzyme-activator complex is formed, in which foscarnet shuttles protons between the active site and the environment, presumably by its two protonatable moieties, the carboxylate and phosphonate one, facilitating the catalytic turnover of hCA I. In this way, the nucleophilic species of the enzyme (with hydroxide as the fourth Zn(II) ligand) is formed, which explains the activating properties of the compound, similarly to those of L-His, the first activator for which the X-ray crystal structure in adduct with hCA I was recently reported by this group.[17a] It should be also mentioned that the affinity of foscarnet for the activator binding site in the CA I active cavity is good, i.e., in the low micromolar range (K_A of 12 μM), whereas that of L-His is much higher, of around 30 nM. However, it may be probable that foscarnet binds in a similar way as L-His to the activator binding site within the hCA I cavity,[17a] but we were unable to characterize this interaction by means of X-ray crystallography due to the fact that when hCA I crystals and foscarnet were incubated for shorter periods (3 h–5 days), no formation of a crystallographically characterizable complex occurred.

When the enzyme and inhibitor were incubated for longer periods in solution (3–5 days), a totally different behavior was observed, with foscarnet acting as a CA I inhibitor (affinity in the millimolar range, K_I of 24.1 mM—see Table 1). The X-ray crystallographic data presented later in the text will thus resolve the apparent contradiction raised by us above, and also explain this rather low affinity of foscarnet for hCA I; (ii) isozymes hCA VI (secreted in the saliva), hCA IX, hCA XII, and hCA XIV were better inhibited by this compound as compared to the isoforms discussed above, with K_Is in the range of 1.81–3.60 mM. It should be mentioned that all these four isoforms are extracellular ones, being either secreted (in saliva or milk in the case of CA VI)[1b] or transmembrane ones (CA IX, XII, and XIV) with the active site situated outside the cell;[14] (iii) foscarnet showed submillimolar affinity for three CA isozymes, i.e., hCA IV (membrane-anchored), hCA VII and mCA XIII (cytosolic isozymes), with K_Is in the range of 0.56–0.87 mM (Table 1). What is indeed remarkable is the fact that although all these 11 isozymes have exactly the same coordination sphere of the catalytically critical zinc ion (where inhibitors bind), with three histidine residues (His94, 96, and 119) and a water molecule/hydroxide ion,[1,7,8,14] their affinity for this inhibitor varies by a factor of 75, if one considers the isozyme which is the best inhibited (CA VII, K_I of 0.56 mM) and the one with the worst inhibition profile, i.e., CA VA, with a K_I of 41.7 mM. Thus, even for this very small inhibitor, supplementary factors regulate the interaction between inhibitor and active site, in addition to the coordination to the metal ion. For having a better view of these phenomena we resolved the X-ray crystal structure of foscarnet complexed to hCA I.

The overall structure of the hCA I-foscarnet complex is similar to that of the native enzyme.[18] The global structure of the protein remained essentially unaltered by the binding of this ligand, with an rmsd of 0.4 Å (both for molecules A and B, see later in the text). The final model of the hCA I-foscarnet complex was constituted from two independently refined molecules in the asymmetric unit (molecule A and molecule B), related by a translation operator. It contained a total of 4038 protein atoms and 366 water molecules. Each molecule also contained one Zn(II) ion within the active site, critical for activity and binding of inhibitors. The thermal B factors were as follows: mean B (for the entire adduct) of 18.80; B for the main chains of 18.11; B for side chain and water molecules of 19.46; B for the foscarnet molecule of 36.70 (with an occupancy of 90%). The first four amino acids in both molecules A and B were missing due to the lack of electron density, as observed in other CA I X-ray crystallographic structures reported earlier.[18] The crystallographic parameters and refinement statistics are shown in Table 2.[19] Only molecule A of the asymmetric unit will be discussed and is shown in Figures 1 and 2, for the sake of simplicity.

Inspection of the electron density maps showed only one molecule of foscarnet bound within the active site of hCA I (Fig. 1). The inhibitor was found near the Zn(II) ion, being coordinated to it and interacting with amino acid residues nearby, more precisely with Thr199, His200, and Leu198. The electron density of two water molecules involved in the binding of foscarnet (i.e., Wat215 and 336) was also clearly observed in the density maps (Fig. 1). Unexpectedly, foscarnet coordinated to the Zn(II) ion by means of the phosphonate moiety, and not the carboxylate one (Figs. 1 and 2). This is in fact the first example of a CA inhibitor incorporating a phosphorus zinc-binding functionality investigated by crystallography, although many such complexes are available in PDB.[24] Thus, one of the oxygen atoms of the phosphonate moiety of foscarnet (O3, see the crystallographic numbering in Fig. 2a) is coordinated to the Zn(II) ion, at a distance of 1.73 Å (a short one as usually the Zn–X distance in other CA inhibitors is of the order of magnitude of 1.80–2.20 Å).[6,24] However, all inhibitors reported up to now were monoanions (for example RSO_2NH^- derivatives),[24] whereas foscarnet binds to Zn(II) with the phosphonate group

Table 2. Crystallographic parameters and refinement statistics for the hCA I-foscarnet adduct

Parameter	Value
Crystal parameter	
Space group	$P2_12_12_1$
Cell parameters	
a	62.58Å
b	69.51 Å
c	120.44 Å
α, *β*, *γ*	90°
Data collection statistics (20.0–2.0 Å)	
Temperature (K)	100
No. of total reflections	217,484
No. of unique reflections	36020
Completeness (%)[a]	97.4 (90.0)
$\langle I/\sigma(I)\rangle$	8.3 (2.03)
R-sym (%)	12.7
Refinement statistics (20.0–2.0 Å)	
R-factor (%)	24.9
R-free (%)[b]	31.0
Rmsd of bonds from ideality (Å)	0.014
Rmsd of angles from ideality (°)	1.53
Molecules/asymmetric unit	2

R-factor = $\Sigma|F_o - F_c|/\Sigma F_o$; *R*-free calculated with 5% of data.
[a] Values in parentheses relate to the highest resolution shell (2.1–2.0).
[b] *R*-sym = $\Sigma|I_i - \langle I\rangle|/\Sigma I_i$.

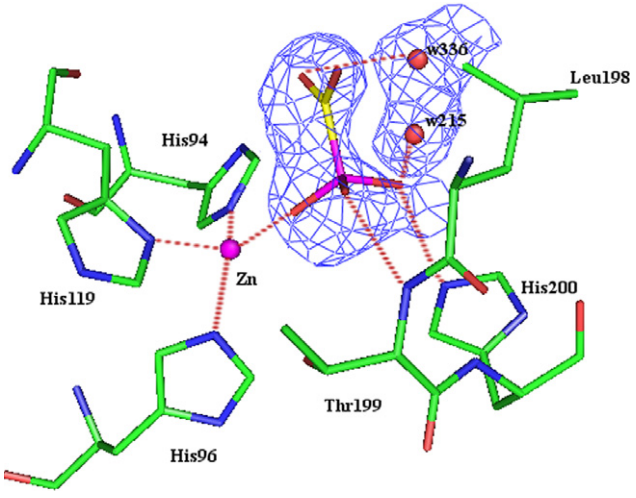

Figure 1. 2Fo-Fc electron density map contoured at 1σ level for foscarnet bound within the hCA I active site and water molecules w215 and w336 involved in binding. The zinc ion, its three histidine ligands (His94, 96, and 119) as well as amino acid residues 198–200 involved in the binding of the inhibitor are also evidenced. Only molecule A of the asymmetric unit is shown, for the sake of simplicity.

possessing two negative charges and this may lead to additional electrostatic attraction between the positively charged dication (Zn^{2+}) and the negatively charged dianion, with the consequent reduction of the Zn–O distance. Another oxygen atom of the phosphonate moiety (O1) is hydrogen bonded to the backbone NH of Thr199, an amino acid critically important for CA catalysis/inhibition, with a distance of 2.77 Å between the two atoms, whereas the third oxygen of this functionality, O2, participates in two weaker hydrogen bonds, one

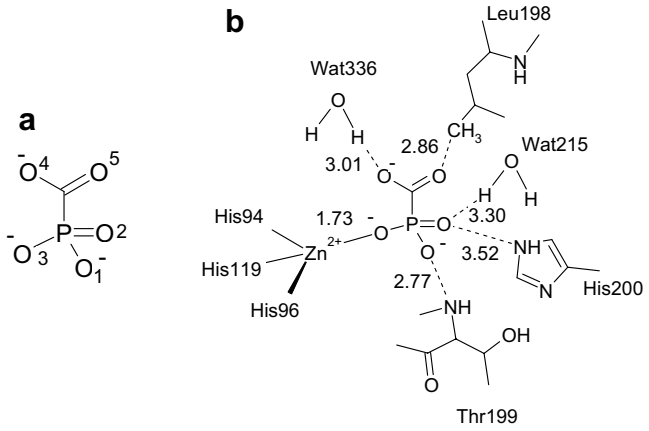

Figure 2. (a) Crystallographic numbering of foscarnet. (b) Schematic representation of the interactions between the inhibitor and the hCA I active site amino acid residues involved in its recognition. Figures represent distances in Å. Only molecule A of the asymmetric unit is shown, for the sake of simplicity.

(of 3.5 Å) with the NH of His200, and the second one (of 3.3 Å) with a water molecule (Wat330) (Figs. 1 and 2b). It should be also mentioned that in all other CA inhibitor adducts investigated up to now by means of X-ray crystallography, the OH moiety of Thr199 was observed to make a strong hydrogen bond to the X inhibitor atom directly coordinated to the Zn(II) ion in the Zn–X complex. However, in this case no such bond was evidenced, as the distance between the Thr199 OH and the O3 atom of foscarnet is of 3.88 Å (the distance between O1 of foscarnet and the same OH moiety of Thr199 is on the other hand of 3.76 Å, and again no hydrogen bond is formed). The carboxylate moiety of foscarnet also interacts with a water molecule (hydrogen bond between O4 and Wat336 of 3.0 Å) and an amino acid residue in the vicinity of the zinc ion, i.e., Leu198. However, this is a repulsive interaction, as one methyl group of the side chain of Leu198 clashes with the O5 atom of foscarnet, the two groups being at a distance of only 2.86 Å (Fig. 2b). This phenomenon was already reported earlier by us for other CA inhibitors,[25] and may explain why foscarnet is a relatively weak CA I inhibitor (and also lead to the design of isozyme-selective inhibitors), with a K_I of around 24 mM (however, much stronger as compared to 4-methylimidazole, claimed by Silverman's group to act either as an activator, as well as an inhibitor of CA II, with a K_I of around 200 mM).[17c] On the other hand, only hCA I has the bulky His200 and thus a rather constrained active site cavity near the Zn(II) ion, which may explain why other CA isoforms show much better inhibition (up to 75-fold times) with this compound, as compared to hCA I (Table 1).

In conclusion, we report here the first X-ray crystal structure of a compound possessing a phosphonate zinc-binding group in complex with a metalloenzyme, hCA I. The hCA I-foscarnet adduct data reported here may be relevant for better understanding the interactions between small molecules and metalloenzymes'

active sites. Foscarnet is also the first clear-cut example of modulator of an enzyme activity which can act either as an activator or inhibitor of a CA isozyme.

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19. hCA I was crystallized at 22 °C by the hanging drop vapor diffusion method.[18] Drops containing 5 μl of 20-30 mg/ml hCA I in 100 mM Tris.HCl buffer, pH 9.0, were mixed with 5 μl of precipitant buffer (25% (w/v) PEG 4000, LiCl 0.4 M, 10% ethylene glycol in 100 mM Tris–HCl, pH 9.0) and equilibrated over a reservoir of 1 ml of precipitant buffer. Crystals appeared after 5–6 weeks. The hCA I-foscarnet complex was prepared by soaking these crystals in 100 mM Tris–HCl buffer (pH 9.0) solution containing 50 mM drug for 1 week. When crystals were maintained in contact with foscarnet for shorter periods (2h–5 days), no binding of the drug to the enzyme has been evidenced by means of X-ray crystallography (data not shown). Crystals of the adduct were mounted in nylon loop and exposed to a cold (100 K) nitrogen stream. Diffraction data were collected on a CCD Detector KM4 CCD/Sapphire using CuKα radiation (1.5418 Å). The unit cell dimensions were determined to be $a = 62.58$ Å, $b = 69.51$ Å, $c = 120.44$ Å, and $\alpha = \beta = \gamma = 90°$, in the space group $P2_12_12_1$. Data were processed with CrysAlis RED (Oxford Diffraction 2006).[20] The structure was analyzed by difference Fourier technique, using the PDB file 2FW4 as starting model. The refinement was carried out with the program REFMAC5[21]; the model building and map inspections were performed using the COOT program.[22] The final model of the complex had an *R*-factor of 24.9% and *R*-free 31.0% in the resolution range 20.0–2.0 Å, with a rms deviation from standard geometry of 0.014 Å in bond lengths and 1.53° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[23] Coordinates and structure factors have been deposited within the Protein Data Bank (accession code 2IT4). Crystallographic parameters and refinement statistics are summarized in Table 2.
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