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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[☆]

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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).

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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