Active-Site Inhibitors

Disclosing New Inhibitors by Finding Similarities in Three-Dimensional Active-Site Architectures of Polynuclear Zinc Phospholipases and Aminopeptidases**

Albert González-Roura, Isabel Navarro, Antonio Delgado, Amadeu Llebaria, and Josefina Casas*

Phosphatidylcholine-specific phospholipase C from *Bacillus cereus* (PC-PLC$_{Bc}$) is a bacterial enzyme that catalyzes the hydrolysis of choline phospholipids to form diacylglycerols and phosphorylcholine. This enzyme has been the subject of intense study as a model for the elusive mammalian PLC enzymes.[1,2] Several PC-PLC$_{Bc}$ inhibitors are known, including substrate analogues,[3] xanthate D609, and other related xanthate salts.[4] The interesting in vivo biological activities of D609[5] are counterbalanced by the fact that xanthates display limited chemical stability in water, especially at acidic pH values. Furthermore, D609 also inhibits other enzymes such as sphingomyelin synthase.[6] For these reasons, the development of more chemically stable and specific inhibitors for PC-PLC$_{Bc}$ was considered to be of interest.

Enzymatic catalysis is the result of a cooperative concatenation of several factors, which takes place in the enzyme active site under defined chemical and geometric constraints and specific conditions that have been evolutionarily optimized. The disruption of any of these essential elements for catalysis usually results in reduced or even abolished enzyme activity. Therefore, an enzyme inhibitor can be anticipated by finding a molecule that interferes with one (or several) of these cooperative factors. With this reasoning in mind, and since X-ray structural studies on PC-PLC$_{Bc}$ have led to a good knowledge of the three-dimensional structure of this enzyme,[7] we decided to look for structural similarities between PC-PLC$_{Bc}$ and other enzymes that may help us to define new PC-PLC$_{Bc}$ inhibitors. A prominent structural element in PC-PLC$_{Bc}$ is the presence of zinc ions, which are known to play a dominant role in catalysis,[8] as has been reported in related hydrolytic enzymes with zinc-containing active sites. We hypothesized that molecules interfering with

[*] A. González-Roura, Dr. I. Navarro, Dr. A. Llebaria, Dr. J. Casas
RUBAM, Department of Biological Organic Chemistry (IIQAB-CSIC)
Jordi Girona 18–26, 08034-Barcelona (Spain)
Fax: (+34) 93-2045904
E-mail: allebaria@iiqab.csic.es
Dr. A. Delgado
University of Barcelona, Faculty of Pharmacy
Unit of Medicinal Chemistry (CSIC Associated Unit)
Avgda. Joan XXIII, s/n, 08028 Barcelona (Spain)

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the essential binding of substrates to zinc ions in enzymes with active sites structurally similar to PC-PLC$_{Bc}$ might also behave as inhibitors of this phospholipase.[9]

The PC-PLC$_{Bc}$ active site contains three zinc ions (Figure 1). Zn1 and Zn3 appear to define a dinuclear center bridged by the carboxyl group of Asp122 and a water (or hydroxide) molecule, with an internuclear distance of 3.5 Å, whereas Zn2 is not bridged to either Zn1 or Zn3.[7] Dinuclear centers are common in different metallohydrolases[10] and (μ-carboxylato)dizinc(II) catalytic cores are found in several hydrolytic enzymes.[11] Several aminopeptidases, the enzymes involved in the hydrolysis of the N-terminal amino acid of polypeptides, contain a dizinc carboxylate-bridged center in the active site.[12] Although none of these proteins have sequence homology or tertiary-structure similarity with PC-PLC$_{Bc}$, comparison of the structures of *Streptomyces griseus* aminopeptidase (AP$_{Sg}$) and *Aeromonas proteolytica* aminopeptidase (AP$_{Ap}$)[13] with the available structure of PC-PLC$_{Bc}$ revealed a close structural relationship in the dizinc centers (Figure 2). Although the nature of the zinc ligands in the dinuclear centers of PC-PLC$_{Bc}$ and the aminopeptidases is not identical, the coordination geometries and distances are surprisingly similar.[14] This structural analogy does not appear to have been previously noted. The similarities between these enzymes were not only structural but also functional. An exceptionally high activity of AP$_{Sg}$ in phosphodiester hydrolysis was previously described.[15] Moreover, tris(hydroxymethyl)aminomethane, a common buffer solute, is a weak inhibitor for both dizinc aminopeptidases[16] and PC-PLC$_{Bc}$.[17] All this evidence led us to hypothesize that PC-PLC$_{Bc}$ inhibitors could be found among the reported inhibitors of these zinc-containing aminopeptidases.

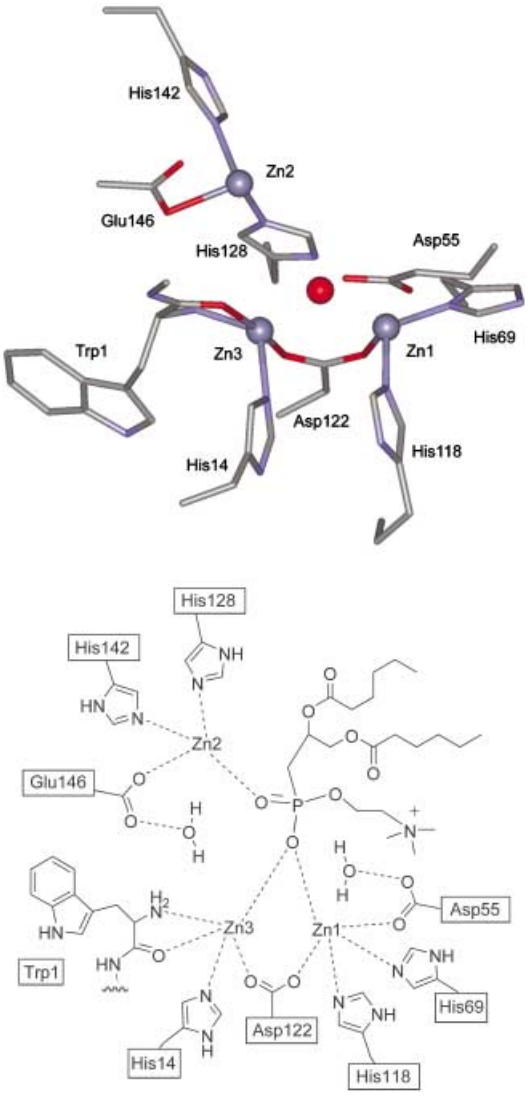


Figure 1. a) Native PC-PLC$_{Bc}$ active site (PDB file: 1AH7) showing amino acid side chains involved in zinc coordination, the bridging water molecule (red sphere), and the three zinc ions (blue spheres). Coordinative bonds of amino acid residues to zinc are displayed as covalent bonds. b) Schematic diagram of the binding mode of substrate analogue inhibitor (3*S*)-3,4-dihexanoylbutyl-1-phosphonylcholine to the active site of PC-PLC$_{Bc}$. (Adapted from Ref. [3].)

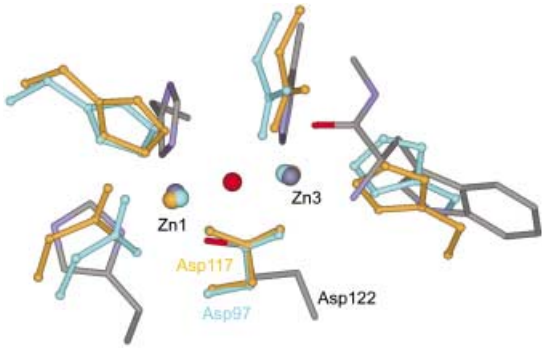
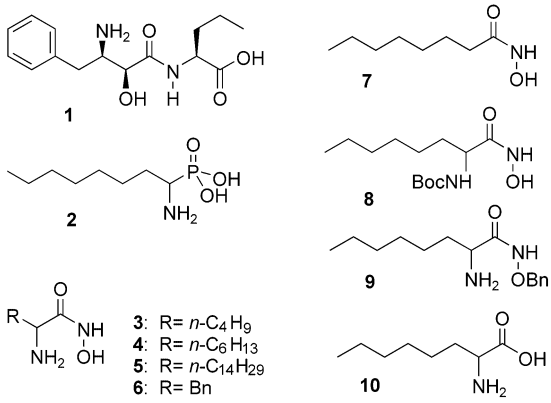
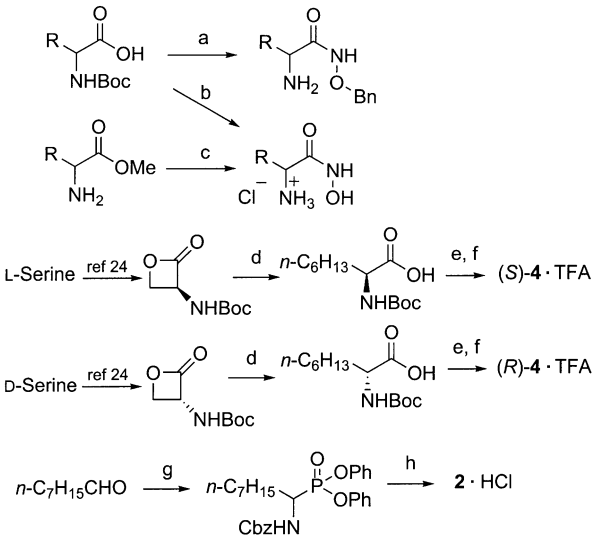


Figure 2. Superposition of dizinc catalytic centers of PC-PLC$_{Bc}$ (gray = carbon backbone, red = oxygen, dark blue = nitrogen, red sphere = μ-aqua ligand; PDB file: 1AH7), AP$_{Ap}$ (orange; 1AMP), and AP$_{Sg}$ (light blue; 1CP7). This arrangement results from overlay of the zinc atom and bridging carboxylate in the corresponding X-ray crystal structures.

Therefore, the effect of bestatin (**1**),[18] α-aminophosphonic acid[19] **2**, and α-aminohydroxamic acids[20] **3**–**6** (Scheme 1) on PC-PLC$_{Bc}$ was determined. Compounds **2**–**6** were obtained as depicted in Scheme 2. Gratifyingly, D,L-norleucine hydroxamic acid **3** showed a strong inhibition of



Scheme 1. Structures of the compounds used in this study. Bn = benzyl, Boc = *tert*-butoxycarbonyl.



Scheme 2. Synthesis of compounds **2**–**6**. a) $NH_2OBn{\cdot}HCl$, EDC, THF/H_2O (1:2), 24 h; then HCl/MeOH, 25–32%; b) R = *n*-$C_{14}H_{29}$, $NH_2OH{\cdot}HCl$, *N*,*N*-carbonyldiimidazole, 48 h; then HCl/MeOH, 18%; c) $NH_2OH{\cdot}HCl$, NaOMe/MeOH, 25 °C, 72 h, 68%; d) *n*-$C_5H_{11}MgBr$, $CuBr{\cdot}Me_2S$, 5% Me_2S in THF, −23 °C, 2 h, 55–65%; e) $NH_2OtBu{\cdot}HCl$, DCC, HOBt, RT, 12 h, 95–98%; f) $B(OCOCF_3)_3$, TFA, 0 °C, 3 h, 90–94%; g) $BnOCONH_2$, $P(OPh)_3$, 2 h, 50 °C, 31%; h) HBr, AcOH; then conc. HCl, reflux, 60%. DCC = *N*,*N*′-dicyclohexylcarbodiimide, EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, HOBt = 1-hydroxy-1*H*-benzotriazole, TFA = trifluoroacetic acid, THF = tetrahydrofuran.

$PC\text{-}PLC_{Bc}$, although neither bestatin (**1**) nor (±)-α-aminooctylphosphonic acid (**2**) were inhibitors at 0.5 mM. The reasons for this different behavior are not clear at present. In any case, we have found that α-aminohydroxamates efficiently inhibit $PC\text{-}PLC_{Bc}$ with IC_{50} values in the low micromolar range at substrate/inhibitor ratios of 200 or higher (Table 1). A Lineweaver–Burk plot of inhibition for **4** established that it is a competitive inhibitor for $PC\text{-}PLC_{Bc}$, with an inhibition constant (K_i) of 3 μM (data not shown). This result is in agreement with our starting structural correlation, which would imply competition between the substrate and inhibitor for binding to the active-site zinc ions. Although only structural studies will fully confirm the binding mode of α-aminohydroxamic acids to $PC\text{-}PLC_{Bc}$, the present evidence would suggest a similar arrangement to that of the aminopeptidase–inhibitor complex. Further support for this was found by comparing the active-site structure of $PC\text{-}PLC_{Bc}$ with the dizinc center of AP_{Ap} complexed with *p*-iodophenylalanine hydroxamate[20a] (Figure 3). When the zinc ions and the bridging carboxylate carbon atom of the dizinc centers are overlapped, the bridging water molecule of the $PC\text{-}PLC_{Bc}$ dizinc center and the hydroxamate inhibitor oxygen atom on AP_{Ap} are placed in a close spatial arrangement (Figure 3). Interestingly, the α-amino group of the inhibitor is located near Zn2, which suggests a possible interaction.

Table 1: In vitro inhibitory concentration values on $PC\text{-}PLC_{Bc}$.

Compound	IC_{50} [μM][a]	Compound	IC_{50} [μM][a]	Compound	IC_{50} [μM][a]
1	>1000[b]	(*R*)-**4**	5	**7**	1200
2	>500[c]	(±)-**4**	5	**8**	250
3	7	**5**	45	**9**	750
(*S*)-**4**	5	**6**	4	**10**	>500[c]

[a] With 2 mM dihexanoylphosphatidylcholine as the substrate. [b] No inhibition at 1 mM. [c] No inhibition at 0.5 mM.

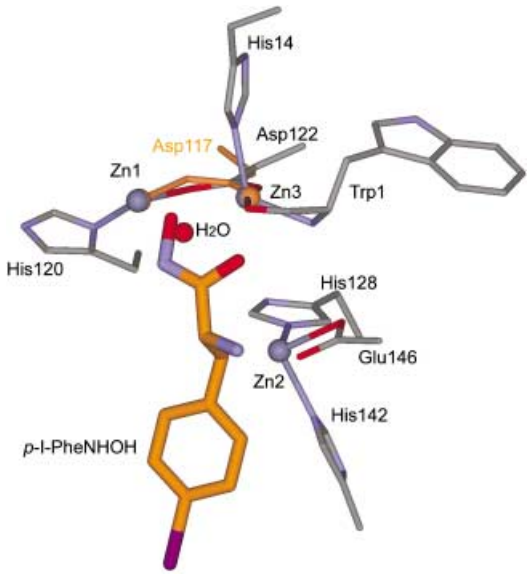


Figure 3. Superposition of $PC\text{-}PLC_{Bc}$ active site (gray = carbon, red = oxygen, dark blue = nitrogen, red sphere = μ-aqua ligand; PDB file: 1AH7) with the dizinc catalytic center of the AP_{Ap} (orange = Zn atoms and μ-carboxylato ligand; 1IGB) complexed with *p*-iodoPhe-NHOH (orange = carbon, red = oxygen, dark blue = nitrogen).

To find out about the influence of the different functional groups present in the active inhibitors we introduced some modifications on the α-aminohydroxamic acids (Scheme 1, **7**–**10**), which were obtained as depicted in Scheme 2 and assayed with $PC\text{-}PLC_{Bc}$ (Table 1). When the α-amino group was removed (**7**) or blocked as NHBoc (**8**), the inhibitory power of the compounds was considerably reduced. The importance of this group for strong inhibition is in agreement with the presumed interaction of the amino group with Zn2 (Figure 3). A similar result was found after *O*-benzylation (**9**) or removal of the *N*-hydroxyamino group (**10**). Finally, both (*R*)-**4** and (*S*)-**4** displayed very similar inhibitory potencies, a result indicating that $PC\text{-}PLC_{Bc}$ inhibition by α-aminohydroxamic acids is not enantioselective. This result is in sharp contrast with the inhibition of aminopeptidases, where the D-α-aminohydroxamic acids display higher potencies than their corresponding enantiomers.[20] In summary, the presence of both the *N*-hydroxyamino and α-amino groups is essential for strong inhibition of $PC\text{-}PLC_{Bc}$ by α-aminohydroxamic acids.[21] These results highlight the high degree of correlation between AP_{Sg}, AP_{Ap}, and $PC\text{-}PLC_{Bc}$.

The $PC\text{-}PLC_{Bc}$ activity was measured according to a described method[22] based on the quantification of phosphate ions arising from alkaline phosphatase (AlkP) catalyzed

hydrolysis of the initially formed phosphorylcholine. AlkP is a zinc phosphoesterase that has been traditionally related to PC-PLC$_{Bc}$ because it also includes a trinuclear active site, although structural differences are evident. It is noteworthy that this enzyme was not inhibited by compounds **3**–**10** (data not shown) even at concentrations two orders of magnitude higher (0.5 mM) than those required for 50% inhibition of PC-PLC$_{Bc}$; this indicates that the presence of a dinuclear zinc active site is not the only element required for inhibition by α-aminohydroxamates. These results could be related to the different catalytic mechanism operating in AlkP, which involves a serine phosphate intermediate[23] that is subsequently hydrolyzed to the final phosphate product. In contrast, the accepted mechanism for PC-PLC$_{Bc}$ and related aminopeptidases involves a direct attack of water or a hydroxide ion on the phosphorus atom of phosphatidylcholine or on the carbonyl amide group. To stress the structural differences of AlkP and PC-PLC$_{Bc}$, the absence of a carboxylate group bridging the zinc ions in the AlkP active site and the presence of a magnesium ion in the place of the third zinc center is noteworthy.

In summary, a new family of PC-PLC$_{Bc}$ inhibitors has been disclosed by finding similarities among fold- and sequence-unrelated proteins, which, however, possess structurally and chemically related active sites. It is interesting to note the evolutionary convergence of the active-site architecture in dinuclear zinc aminopeptidases and lipid phosphohydrolases that leads to a similar solution for catalyzing the hydrolysis of amides and phosphodiesters. Appropriate modifications of the inhibitors to selectively address either phospholipase or aminopeptidase enzymes are to be found in future work. The results obtained show that when the structural determinants of the desired biological activity are available, alternative lead discovery procedures can be envisaged. With all necessary caution, we consider that the approach presented here may be useful not only for the identification of new inhibitors for a particular enzyme but also to unravel structurally related targets for known enzyme inhibitors.

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