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A Novel Strategy for Designing Irreversible Inhibitors of Metalloproteases: Acetals as Latent Electrophiles That Interact with Catalytic Nucleophile at the Active Site

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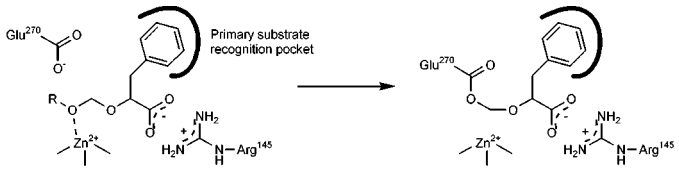
ABSTRACT



A new strategy for design of irreversible inactivators for carboxypeptidase A (CPA), a prototypic zinc protease, has been developed by exploiting the property of acetals to generate an oxacarbenium ion intermediate in the conversion into the corresponding carbonyl compounds. The design strategy is exemplified by 2-benzyl-5-alkyl-3,5-dioxapentanoic acids (1a–c). Interestingly, (*R*)-1b is slightly more potent than an (*S*)-1b as an inactivator of CPA.

Excessive production of metabolites in the living system results in diseases. The overproduction of metabolite may be moderated through inhibition of the catalytic activity of the enzymes that are involved in the chemical transformation processes. In fact, a majority of therapeutic agents that we are using currently are inhibitors of enzymes. Thus, development of inhibitors that are effective for the specific enzyme that is involved in the pathologically or physiologically important metabolic processes constitutes a viable approach for new drug discovery.

Zinc-containing enzymes constitute the family of the most widely distributed enzymes, for which carboxypeptidase A (CPA) serves as a prototypic enzyme.[1] Thus, CPA has been intensively studied, especially with respect to the active site structure and catalytic mechanism.[2] The enzyme has also been used as a model enzyme for the development of enzyme inhibitor design strategies that can be transferred to other zinc-containing enzymes that are less well understood but of medicinal importance such as angiotensin converting enzyme or matrix metalloproteases.[3,4] Inhibitors of these enzymes are valuable as potential therapeutic agents. We report herein a novel inhibitor design strategy that we have developed with CPA. In this strategy, we have exploited the unique property of acetals to generate an oxacarbenium ion intermediate in their conversion into the corresponding carbonyl compounds under acidic conditions.

Hydrolysis of acetals under aqueous acidic conditions starts with equilibrium protonation on the acetal oxygen. The protonated acetals undergo unimolecular breakdown to form resonance-stabilized oxacarbenium ions and alcohols. The oxacarbenium ion intermediates then interact with water to yield aldehydes and alcohols.[5] It is conceivable that the breakdown of a C−O bond of an acetal may also be effected by a metallic Lewis acid such as zinc ion. It was thought

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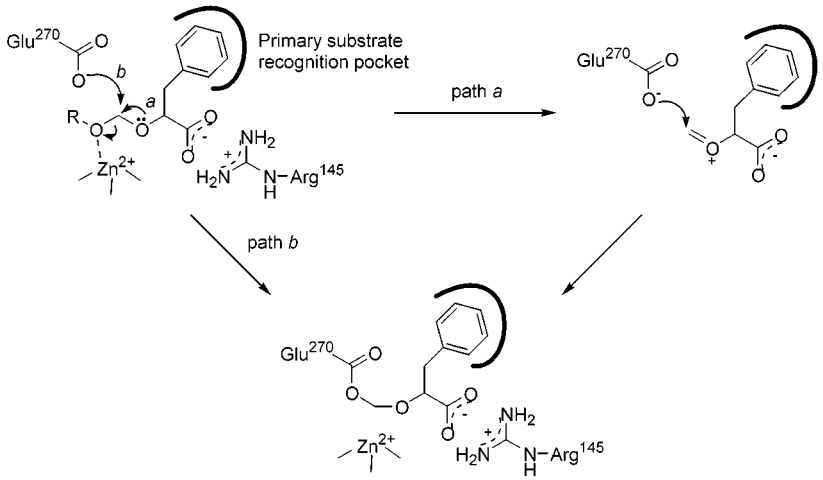
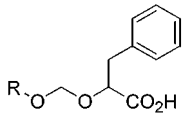
Scheme 1



that such a reaction, if it would occur at the active site of CPA, may lead to covalent modification of an active site nucleophile, resulting in inactivation of the enzyme. Compounds **1a**−**c** are potential inactivators for CPA designed on the basis of the foregoing rationale.



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a; R = CH_3
b; R = CH_2Ph
c; R = CH_2CH_2Ph

In order for a compound to undergo a chemical reaction at the active site of an enzyme, the compound should first bind to the enzyme at the active site to form a complex with the enzyme. The terminal carboxylate of the inhibitors (**1**) was thought to hydrogen bond to the guanidinium moiety of Arg-145 and the phenyl ring of the benzyl side chain to anchor in the S_1·subsite pocket of CPA.[6] Such a mode of binding would place the O-5 in **1** in close proximity to the zinc ion at the active site of CPA, leading to the formation of a coordinative bond to the metal ion. Just as protonated acetals break down to oxacarbenium ions in the acetal hydrolysis, the ether oxygen coordinated to the metallic Lewis acid may undergo the C−O bond cleavage with the generation of an oxacarbenium ion intermediate. The oxacarbenium ion thus generated would undergo a bond formation reaction with the carboxylate of Glu-270 to result in the covalent modification of the catalytic nucleophile (path *a* in Scheme 1). The covalently modified enzyme can no longer perform its enzymatic function. Alternatively, the reaction path that involves nucleophilic attack of the carboxylate at the C-4 of **1** in an S_N2 fashion may also be possible (path *b* in Scheme 1). Precedences for this type of acetal reaction are found in the literature.[7,8] The reaction paths leading to the covalent modification of the enzyme are analogous to the reaction mechanism proposed for the enzymic cleavage of the substrate, in which the carboxylate of Glu-270 attacks at the scissile ester carbonyl carbon to generate an anhydride intermediate that hydrolyzes to products.[9−11]

The inhibitors were synthesized readily by allowing alkoxymethyl chloride to react with phenyllactic acid in the presence of 2 molar equiv of *n*-butyllithium. The alkoxymethyl chloride was obtained by bubbling HCl gas into a mixture of paraformaldehyde and the corresponding alcohol at 0 °C.[12]

The inhibitory activity of these compounds toward CPA was evaluated using *O*-(*trans-p*-chlorocinnamoyl)-L-phenyllactic acid (ClCPL)[13] as substrate. Compounds **1a**−**c** inhibited CPA in a time-dependent manner as can be seen in Figure 1, suggesting that the inhibition occurs in an irreversible fashion. Figure 1 exemplifies plots for all three inhibitors

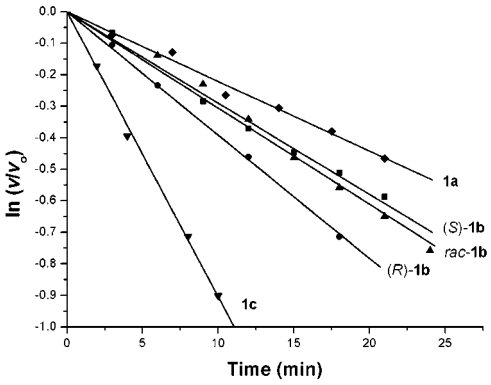

Figure 1. Plot of ln(v/v_o) vs incubation time, suggesting irreversible inhibition of CPA by each inhibitor (20 mM).

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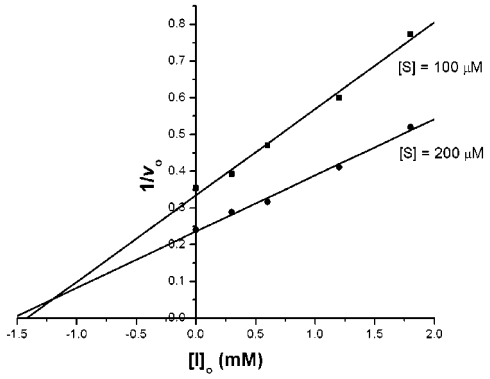


Figure 2. The Dixon plot for the inhibition of CPA-catalyzed hydrolysis of ClCPL by *rac*-**1b** at $[CPA]_0 = 3.80 \times 10^{-8}$ M.

evaluated in the present study. We have chosen inhibitor **1b** for an extensive kinetic study.

CPA that is inactivated by **1b** was dialyzed for 24 h. The enzyme activity did not return by the dialysis, which suggests strongly that a covalent bond formed between the inhibitor and the nucleopbilic residue, most probably the carboxylate of Glu-270 at the active site of CPA. The survival of the newly formed linkage under dialysis conditions may be envisioned on the grounds that the bond is shielded by the bulk of the enzyme molecule and is not exposed to the aqueous buffer solution.[14] The dissociation constants (K_i) for the reversible enzyme·inhibitor complexes were determined by the method of Dixon[15] (Figure 2) and are listed in Table 1.

Table 1. Kinetic Parameters for the Inhibition of Caboxypeptidase A

inhibitor	$k_{obs}/[I]_o$ (M^{-1} min^{-1})	K_i (mM)
rac-**1a**	1.13 ± 0.03	1.14 ± 0.10
rac-**1b**	1.53 ± 0.03	1.15 ± 0.15
(*S*)-**1b**	1.45 ± 0.03	2.47 ± 0.30
(*R*)-**1b**	1.96 ± 0.02	0.98 ± 0.15
rac-**1c**	4.52 ± 0.07	0.54 ± 0.23
2	NI[a]	0.57 ± 0.05

[a] NI represents no irreversible inhibition at 15 mM.

An active site protection experiment was performed. Thus, protection of the CPA inactivation by **1b** was noted when CPA was preincubated with 2-benzylsuccinic acid, a known competitive inhibitor that binds CPA at the active site,[16] suggesting that the inactivation chemistry takes place at the active site of the enzyme (Figure 3).

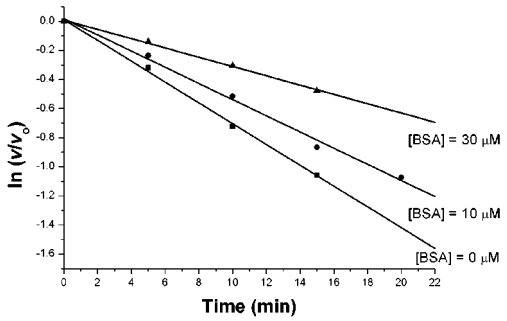
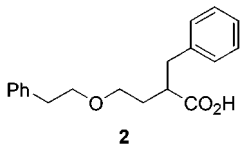


Figure 3. Time-dependent loss of CPA activity in the hydrolysis of **1b** (15 mM) in the presence of 2-benzylsuccinic acid at concentrations of 0, 10, and 30 μM, respectively.

Compound **2** in which the oxygen at the 3-position of **1b** is replaced with a methylene unit was synthesized: 2-Benzyl-



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4-iodobuatanoic acid *tert*-butyl ester that was prepared from 3-benzyl-γ-lactone by a literature method[17] was allowed to react with phenylethanol in a concentrated sodium hydroxide solution in the presence of tetrabutylammomnium hydrogen sulfate to give **2** as a *tert*-butyl ester, which was then treated with trifluoroacetic acid.[18] Compound **2** failed to inactivate CPA up to a concentration of 15 mM, but it is only a poor reversible inhibitor, indicating that the oxygen atom at the 3-position in **1b** is required for **1b** to have CPA inactivating activity. This observation tends to eliminate path *b* in Scheme 1 and furthermore supports the acetal moiety in the inhibitors being responsible for the inactivation of CPA.

The second-order inhibitory rate constants (k_{inact}/K_I) for the inactivation of an enzyme may be represented by eq 1

$$k_{inact}/K_I = k_{obs}/[I]_0 \qquad (1)$$

when the K_I value is much greater than the inhibitor

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(13) Suh, J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1976**, *98*, 1940.
(14) Furthermore, alkoxymethyl esters are, in general, stable in neutral aqueous solutions such as the dialysis medium, although they are readily hydrolyzed by strong acid (Weinstock, L. M.; Karady, S.; Roberts, F. E.; Hoinowski. A. M.; Brenner, G. S.; Lee, T. B. K.; Lumma, W. C.; Sletzinger, M. *Tetrahedron Lett.* **1975**, 3979.).
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concentration.[19] Table 1 also lists the second-order inhibitory rate constants expressed in terms of $k_{obs}/[I]_0$. It is interesting to note that (*R*)-**1b** which belongs to the D-series is slightly more potent than its enantiomer as an inactivator of CPA.

In the inactivation of CPA with **1b**, the graph of k_{obs} against the concentration of the inhibitor at fixed concentration of the enzyme bears a sigmoidal shape (Figure 4), which

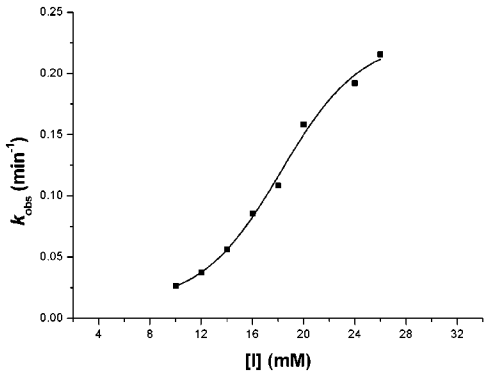

Figure 4. Plot of k_{obs} vs [**1b**].

suggests that under the assay conditions CPA behaves as an allosteric enzyme showing a positive cooperativity effect.[20] It has been postulated by Bunting and Kibir,[21] Sebastian and Lo,[22,23] and others[24] that an apolar binding pocket exists located adjacent to the primary binding pocket of CPA. The binding pocket was proposed to consist of Tyr-198, Phe-279, and Arg-71 and bear lower binding affinity compared with that of the primary binding site and is implicated to effect the catalytic activity of CPA.[23] Thus, the rate of CPA-catalyzed hydrolysis of Hipp-L-Phe was found to be enhanced by about 2-fold in the presence of 3,3-diphenypropanoic acid and the rate enhancement was attributed to the binding of the compound to the secondary binding pocket probably in the form of hydrophobic interactions.[22] We are suggesting that, owing to its hydrophobic nature, **1b** may bind at the secondary binding site when the concentration reaches a high enough level and this binding causes an accelerate in the inactivation chemistry.

In conclusion, a new strategy for designing irreversible inactivators of CPA, a prototypic zinc protease, has been developed by exploiting the property of acetals to generate oxacarbenium ion intermediates in the conversion into the corresponding carbonyl compounds under acidic conditions. It was envisioned that the formation of the oxacarbenium ion from acetals could also be effected by the zinc ion (Lewis acid) at the active site of CPA, and the oxacarbenium ion intermediate thus generated would be trapped by the catalytic nucleophile of Glu-270 carboxylate. The design concept that is illustrated with 2-benzyl-5-alkyl-3,5-dioxapentanoic acids (**1a**−**c**) may be applied to other zinc proteases. Interestingly, (*R*)-**1b** is slightly more potent than (*S*)-**1b** as an inactivator of CPA.

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Supporting Information Available: Detailed experimental procedures for the preparations of **1a**−**c** and **2**, spectral data and $[\alpha]_D$ of the inhibitors, and details of kinetic studies. This information is available free of charge via the Internet at http://pbus.acs.org.

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