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Use of heteroatom-containing small cyclic compounds for enzyme inhibitor design

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Abstract : Varied mode of inhibitors for carboxypeptidase A were designed using heteroatom-containing small rings which interact with the catalytic center to modify it covalently. Substrate analogs having an oxirane ring and those having 2-oxo-1,3-oxazolidine moiety inhibited the enzyme irreversibly, but those bearing β-lactam ring were shown to be reversible competitive inhibitors.

Enzyme inhibitors are important not only as therapeutic agents but also as tools for studying enzymic action. A majority of medicinal agents which are being used clinically manifest their therapeutic effects by inhibiting the catalytic action of the enzyme which is involved in the physiology related to the particular disease. Bioorganic and medicinal chemists are increasingly successful in *de novo* designing of ever potent and efficient inhibitors. Chemistry of heteroatom-containing small ring compounds is characterized by their enhanced chemical reactivity mostly arising from their large ring strain. Furthermore, they are in general small in size enough to be fit in the active site of enzyme. These chemical and physical properties of heteroatom-containing small rings are thought to be of value for designing inhibitors of enzyme by incorporating them in substrate analogs as a reactive group which interacts with the catalytic center in the enzyme.

We have successfully employed an oxirane ring as an alkylating species in the design of inhibitors which inactivate carboxypeptidase A (CPA, EC 3.4.17.1)(1), a prototypic enzyme for a class of zinc containing proteolytic enzymes important from both the mechanistic and biological standpoint(2). CPA preferentially cleaves off the C-terminal amino acid residue that bears an aromatic side chain from polypeptide substrate with L-stereospecificity. Three principal binding sites, *i.e.*, Arg-145, primary recognition pocket, and Zn^{2+}, and one catalytic site have been identified in the active site of the enzyme(3). The recognition pocket which has the shape complementary to an aromatic ring accommodates the aromatic side chain of P_1' residue of the substrate, and the Arg-145 is involved in forming a salt link with the terminal carboxylate of the substrate. The active site zinc which is coordinated to the backbone amino acid residues of His-69, Glu-72, and His-196 is essential not only for binding substrate but also for activating the scissile peptide bond of the substrate through ligation. The carboxylate of Glu-270 is intimately involved with the enzymic action as the catalytic site, serving as a nucleophile which attacks the activated carbonyl carbon with the generation of a highly unstable anhydride intermediate (anhydride pathway)(4). However, there has been proposed an equally important alternative mechanism (general base mechanism)(5). In the latter mechanism, the carboxylate serves as a general base, activating the nearby zinc bound water molecule which in turn attacks at the peptide carbonyl to form a tetrahedral intermediate(5).

Oxirane derivatives having structural feature that can bind to the active site of CPA, *i.e.*, oxiranes carrying a carboxylate group and a benzyl moiety in such a way that they can interact with their respective binding site in the active site of the enzyme were thought to be potential inhibitors for the enzyme. 2-Benzyl-3,4-epoxybutanoic acid (BEBA) was obtained employing such designing principle(1). Its carboxylate would interact with the guanidium moiety of Arg-145 and the phenyl ring would be accommodated by the recognition pocket. In such a mode of binding, the epoxide ring is expected to be positioned in the vicinity of the active site zinc, thus its oxygen atom ligates to the zinc, causing the ring to be an effective electrophile. The carboxylate of Glu-270 may then successfully attack the ring, resulting in an alkylation to form an ester linkage (Figure 1).

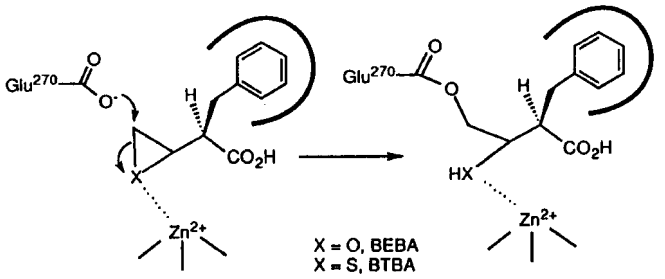

Figure 1. Schematic representation of the rationale used for designing 2-benzyl-3,4-epoxybutanoic acid (BEBA) as well as 2-benzyl-3,4-epithiobutanoic acid (BTBA) as irreversible inhibitors of CPA.

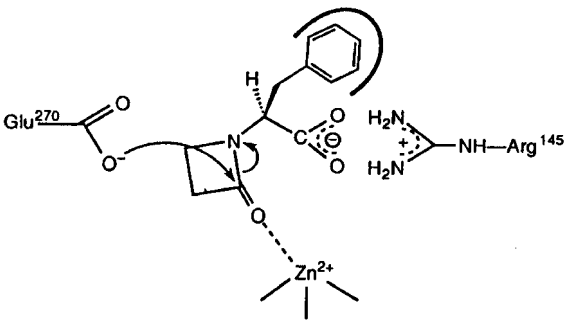

Figure 2. Schematic representation showing a possible interaction of carboxypeptidase A with 2-(2-oxoazetidin-1-yl)-3-phenylpropanoic acid.

Indeed, BEBA was found to be a potent inactivator of CPA(1). Interestingly, however, of four possible stereoisomers only two isomers were effective in inactivating the enzyme. Furthermore, to our surprise the most potent inhibitor has (2*S*,3*R*)-configuration which belongs to the "D" series at the 2-position. This inhibitory stereochemistry of the enzyme by BEBA was confirmed by the single crystal X-ray crystallographic analysis of the inactivated CPA obtained by cocrystallizing it with racemic BEBA(6). The other isomer which modified the carboxylate of Glu-270 has (2*R*,3*S*)-configuration. Kinetic parameters for these inhibitors are found in Figure 4. These inhibitors were referred to as pseudomechanism-based inactivators because they are converted to chemically active species upon binding to the enzyme not by a chemical means but through ligation to the active site zinc. Importantly, these inhibitors are extremely efficient inactivators whose partition ratios are shown to have values of 0.53 and 1.01 for (2*S*,3*R*)- and (2*R*,3*S*)-BEBA, respectively: Nearly every turnover results in inactivation of the enzyme.

Thiirane analogs of BEBA were thought to be interesting candidates for inhibitors of CPA since the thiol group that would be generated when the thiirane moiety undergoes an electrophilic reaction with the catalytic carboxylate of CPA is expected to ligate to the active site zinc with a higher affinity than the hydroxyl from BEBA. Thiol group is known to have a high ligating propensity to the active site zinc(7). However, contrary to the expectation, (2*R*,3*R*)-2-benzyl-3,4-epithiobutanoic acid (BTBA) which corresponds to the most potent stereoisomer in the case of BEBA showed no appreciable improvement in its K_i value ($K_i = 2.02 \times 10^{-4}$ M) over that of (2*S*,3*R*)-BEBA. It appears that in the case of BTBA there are operating simultaneously two opposing factors: the stronger ligating property of the sulfhydryl group to the zinc and the lowered reactivity of the thiirane ring toward the carboxylate nucleophile(8).

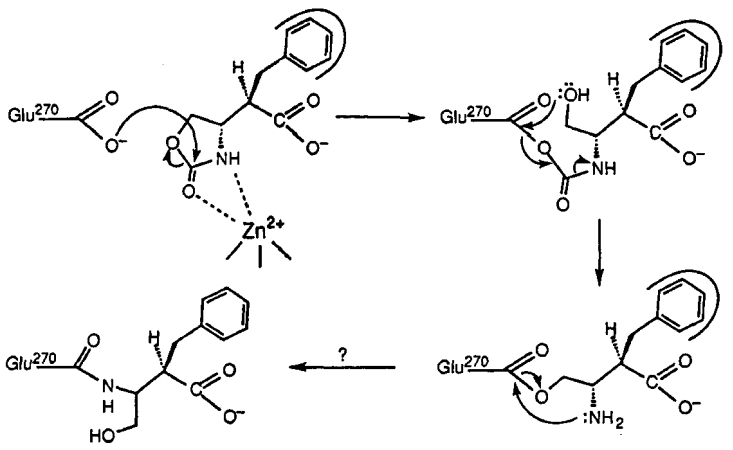

Figure 3.
A Possible pathway for the irreversible inhibition of carboxypeptidase A by (2*S*,3*R*)-2-(2- oxo-1,3-oxazolidin-4-yl)-3-phenylpropanoic acid.

β-Lactam is an enchanted ring which has received enormous attention as being an essential structural constituent of therapeutically invaluable antibiotics such as penicillins and cephalosporins. These antibiotics manifest their antibacterial activity *via* inhibition of D-alanyl-D-alanine carboxypeptidase transpeptidase which plays a critical role in the bacterial cell wall formation, and the highly reactive β-lactam ring is known to be responsible for the inhibitory action of the antibiotics for the enzyme. It has been believed that the high chemical reactivity of the β-lactam ring is due to its high degree of ring strain and the reduced resonance stabilization, but recently Page *et al* raised a question on the validity of the notion(9). Accordingly, 2-(2-oxoazetidin-1-yl)-3-phenylpropanoic acid was thought to be an interesting compound to evaluate as a potential inhibitor of CPA. The following three possibilities are anticipated upon the designed inhibitor is exposed to the enzyme: (i) The β-lactam derivative simply behaves as a substrate of the enzyme. (ii) The anhydride intermediate that is generated upon its reaction with the enzyme may be reasonably stable to hydrolysis, resulting in inactivation of the enzyme transiently. Lastly, (iii) the designed compound may be a reversible competitive inhibitor for the enzyme just by binding to the active site of CPA (Figure 2).

The substitution of the oxirane moiety in BEBA with 2-oxo-1,3-oxazolidine ring afforded an irreversible inhibitor for the enzyme. Thus (2*S*,3*R*)- and (2*R*,3*S*)-2-(2-oxo-1,3-oxazolidin-4-yl)-3-phenylpropanoic acid inactivated the enzyme in a time-dependent fashion with K_i value of 0.67 mM and 1.2 mM, respectively. Values of k_{inact} were found to be 0.075 min^{-1} and 0.15 min^{-1}, respectively.

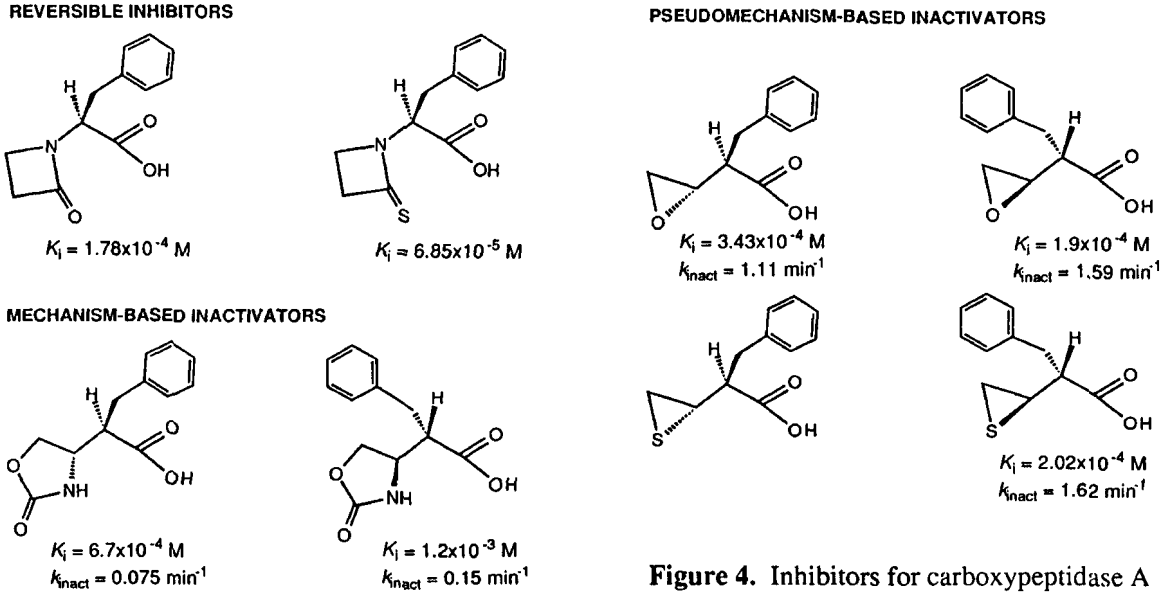

Figure 4. Inhibitors for carboxypeptidase A

In the inhibitory kinetic study *S*-2-(2-oxoazetidin-yl)-3-phenylpropanoic acid did not exhibit the time-dependent loss of the enzymic activity but rather was shown to be a reversible competitive inhibitor having K_i value of 1.78×10^{-4} M for CPA. There was no indication of the β-lactam ring being cleaved even under incubation conditions. Hence, our study tends to support the view of Page(9) that the β-lactam ring is fairly stable and does not undergo ring opening reactions as readily as may be anticipated.

The inactivated CPA failed to restore its enzymic activity upon dialysis, demonstrating that there occurs a covalent modification at the catalytic center, mostly likely at the carboxylate of Glu-270. A plausible pathway for the inactivation reaction suggesting the oxazolidine derivative to be a mechanism-based inactivator is depicted in Figure 3.

In summary, heteroatom-containing small rings have been successfully utilized in rational designing of inhibitors for CPA, a prototypic zinc-containing protease. In this way, inhibitors having varied mode of inhibition were obtained (Figure 4): Substrate analogs having an oxirane ring and those having 2-oxo-1,3-oxazolidine moiety inhibit the enzyme in a irreversible fashion modifying covalently the catalytic carboxylate of Glu-270, but to our surprise the substrate analogs in which an 2-oxoazetidine ring is incorporated was shown to be a reversible competitive inhibitor. The substrate analog containing an azetin-2-thione ring also inhibited the enzyme in a reversible competitive manner. The enzyme inhibitor designing methodology that was developed making use of the unique properties of heteroatom-containing small rings may be applicable for the design of therapeutically useful inhibitors which are effective against physiologically important zinc-containing enzymes.

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