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Development of Peptidyl α-keto-β-aldehydes as New Inhibitors of Cathepsin L — Comparisons of Potency and Selectivity Profiles with Cathepsin B

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Abstract—We have utilized previously known substrate and inhibitor specificity profiles for the lysosomal cysteine protease, cathepsin L, to design a new series of putative inhibitors of this enzyme, based on di- and tri-peptidyl α-keto-β-aldehydes. Kinetic evaluation of these compounds revealed Z-Phe-Tyr(OBut)-COCHO, with a $K_i = 0.6$ nM, to be the most potent, synthetic reversible inhibitor of cathepsin L reported to date.

Cathepsin L, a lysosomal cysteine protease has been implicated in the progression of a number of important pathophysiological processes, including rheumatoid arthritis,[1] tumour invasion and metastasis[2] and bone resorption and remodelling.[3] In the latter process, cathepsin L has been reported to degrade bone type I collagen in tandem with another cysteine protease, cathepsin K.[4]

A number of compounds have been shown to act as inhibitors of cathepsin L, including diazomethyl ketones,[5] acyloxymethyl ketones,[6] epoxysuccinyl derivatives (such as E-64)[7] and peptidyl aldehydes, such as leupeptin.[8] We have previously reported the inhibitory activity of compounds analogous to peptidyl aldehydes, termed peptidyl glyoxals (peptidyl α-keto-β-aldehydes) which generally display enhanced potency and selectivity for the cysteine proteases.[9]

With this in mind, we decided to synthesize a number of putative cathepsin L inhibitors based on the α-keto-β-aldehyde motif and evaluate such new compounds for their selectivity for this protease versus cathepsin B. As a result, we now wish to report the discovery of an exceptionally potent, low molecular weight cathepsin L inhibitor, Z-Phe-Tyr(OBut)-COCHO, which acts as a slow, tight-binding reversible inhibitor of cathepsin L with a $K_i = 0.6$ nM. In addition, this compound shows some 360-fold selectivity for cathepsin L compared with cathepsin B.

Chemistry

A representative synthetic protocol is outlined in Figure 1. It has been previously demonstrated that α-keto-β-aldehydes can be easily obtained, in almost quantitative yield, by the oxidative cleavage of the diazo-group of peptidyl diazomethyl ketones, using dimethyldioxirane.[10] The peptidyl diazoketones used in this study were prepared, in turn, from their corresponding orthogonally protected C-terminal free acids, using a rapid, combined solid-/solution-phase methodology.

Inhibitor design was based on the simple use of known peptide targeting sequences, previously shown to have either selectivity for cathepsins L and B (Z-Phe-Tyr(OBut)-[11] and Ac-Arg-Arg-,[12] respectively) or broader spectrum specificity for either protease (Z-Phe-Arg-[12] and Ac-Leu-Leu-Arg-[8]). In brief, the desired target peptide

Abbreviations: Z-: benzyloxycarbonyl; -COCHO: α-keto-β-aldehyde; DIPEA: *N*,*N*-diisopropylethylamine; Fmoc-: 9-fluorenylmethoxycarbonyl; HBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt: 1-hydroxybenzotriazole; -NHMec: 7-amido-4-methylcoumaryl; SASRIN: super acid sensitive resin.

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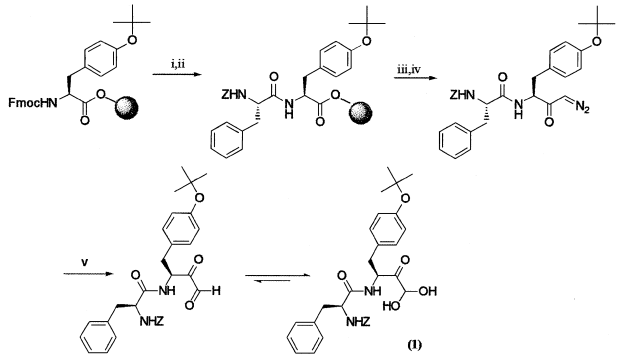

Figure 1. Solid-/solution-phase synthesis of Z-Phe-Tyr(OBut)-COCHO. (i) 20% (v/v) piperidine/DMF, 45 min. (ii) Z-Phe-OH:HBTU: HOBt:DIPEA (1:1:1:2), 1.5 h. (iii) 1% (v/v) TFA/DCM, 2×30 min. (iv) *N*-methylmorpholine (1.1 equiv), isobutyl chloroformate (1.1 equiv) at 0 °C, 15 min, then add excess ethereal diazomethane, stir to room temp. (v) dimethyldioxirane in moist acetone, 1.5 h.

sequences were prepared (typically on a 0.25 mmol scale) on a SASRIN® polystyrene-based resin containing a super acid-sensitive linker (2-methoxy-4-alkoxybenzyl alcohol) employing standard Fmoc-/*t*Bu-based solid phase peptide synthesis methodologies.[13] Upon completion of the synthesis, the peptides were cleaved from the support by treatment with trifluoroacetic acid in dichloromethane (1%, v/v).

After neutralization and standard work up, the peptide free acids were then converted into the corresponding diazomethyl ketone derivatives by reaction of their unsymmetrical anhydrides (formed by reaction with isobutyl chloroformate) with ethereal diazomethane.[14] Finally, the peptidyl diazoketones were treated with a solution of dimethyldioxirane in moist acetone[10] (for preparation of α-keto aldehydes). The identity and purity of the final products were confirmed by a combination of TLC, ^{1}H NMR and electrospray mass spectrometry. Invariably, the peptidyl α-keto-β-aldehydes were obtained as their hydrates.

Inhibition Studies

Recombinant human cathepsin L was a kind gift from Dr Maurice Pagano (Faculté de Médecine, Université Pierre et Marie Curie, Paris, France). Bovine cathepsin B, Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec were purchased from Sigma Chemical Co. (Poole, Dorset, UK). All other reagents were of analytical grade and were purchased from either Sigma or Aldrich (Poole, Dorset, UK).

Compounds were examined for their ability to block cathepsin B- or cathepsin L-catalysed hydrolysis of the fluorogenic substrates, Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec, respectively, in the presence of varying amounts of the peptidyl α-keto-β-aldehydes. Progress curves obtained from these studies were typical of inhibitors exhibiting reversible, slow-binding kinetics.[15,16] A comparison of the final K_i values determined for both of the peptidyl α-keto aldehyde sequences synthesized in the present study, against cathepsins L and B are shown in Table 1.

Table 1. Comparison of the inhibition of cathepsins L and B by peptidyl α-keto aldehydes

Enzyme	Cathepsin L	Cathepsin B
	K_i (nM)[a]	
Z-Phe-Tyr(OBut)-COCHO	0.6±0.054	214±22.5
Ac-Leu-Leu-Arg-COCHO	970±85	1250±113
Ac-Arg-Arg-COCHO	N.I.[b]	640±61
Z-Phe-Arg-COCHO	90±8.7	40±3.6

[a]Values are S.E.M for 4 determinations.
[b]N.I. = no inhibition (at 100 μM).

Discussion

The peptidyl α-keto-β-aldehydes synthesized for the present study contained previously described targeting sequences which fell into three categories:

1. selective for cathepsin L (Z-Phe-Tyr(OBut)-COCHO)
2. selective for cathepsin B (Ac-Arg-Arg-COCHO)
3. exhibiting activity against both cathepsin B and L (Ac-Leu-Leu-Arg-COCHO and Z-Phe-Arg-COCHO)

Such an approach was developed to examine if peptidyl α-keto-β-aldehydes exhibited conventional binding characteristics and as such could be utilized for the specific inhibition of cathepsin L.

A number of features are immediately apparent from the final K_i values presented in Table 1. First, with the exception of Ac-Leu-Leu-Arg-COCHO, the remainder of the peptidyl α-keto-β-aldehydes prepared in this study exhibit sub-micromolar final K_i values against both cathepsins B and L. Secondly, the compounds behaved as expected with regard to their substrate specificity. Ac-Arg-Arg-COCHO had no activity against cathepsin L, even at concentrations as high as 100 μM. Ac-Leu-Leu-Arg-COCHO was the least potent and selective inhibitor, which was somewhat surprising as the analogous compound leupeptin is a good inhibitor of both proteases. Initially, we believed this may be due to cyclization of the C-terminal α-keto-β-aldehyde moiety with the side chain guanidino group of Arg, however subsequent NMR and mass spectral analysis have not shown this to be the case (results not shown). In addition, the observation that, as expected, Ac-Arg-Arg- possesses activity against cathepsin B (but not cathepsin L) and Z-Phe-Arg- exhibits activity against both species suggests that it is unlikely that cyclization occurs in assay buffers, as this would render the inhibitors inactive.

We believe that P′ hydrogen-bonding interactions, between active site residues and the hydrated β-aldehyde hydroxyl groups of peptidyl α-keto-β-aldehydes, are crucial for the effectiveness of these compounds. In the absence of crystallographic/NMR data of cathepsin-α-keto-β-aldehyde complexes, we would wish to draw some tentative comparisons, for example, with vinyl sulphone and vinyl ester-based inhibitors where the

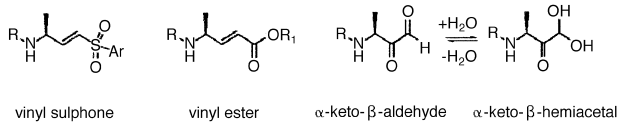

Figure 2. Comparison of the structures of cysteine protease inhibitors.

importance of P′ hydrogen bonding interactions has previously been demonstrated. Vinyl sulphone-based inhibitors,[17] which possess *two* sulphone oxygens, both of which have been shown to interact with active site residues close to the catalytic apparatus, are substantially more potent than the closely related vinyl esters.[18] The fact that vinyl esters have only *one* carbonyl oxygen capable of interacting with P′ site residues may explain the reduced potency of these compounds (Fig. 2). In addition to hydrogen-bonding potential of the β-hydroxyl groups of the hydrated β-aldehyde, the high potency of α-keto-β-aldehydes may be attributable to the presence of the α-carbonyl group, and its probable interaction, as the tetrahedral transition state, with the oxyanion binding site.

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