Comparative behaviour of calpain and cathepsin B toward peptidyl acyloxymethyl ketones, sulphonium methyl ketones and other potential inhibitors of cysteine proteinases

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Peptidyl acyloxymethyl ketones, previously established as potent inactivators of the lysosomal cysteine proteinase cathepsin B, were evaluated against smooth-muscle calpain, a member of the family of Ca^{2+} -dependent cysteine proteinases. Only modest rates of time-dependent inhibition could be achieved, even with peptidyl affinity groups optimized for calpain and linked to a carboxylate leaving group of very low pK_a [$2,6\text{-(CF}_3)_2\text{PhCOO}^-$, pK_a 0.58]. Selective inactivation of cathepsin B versus calpain was consistently observed with this type of inhibitor. Examination of other potential inhibitors revealed a rank order of potency against calpain to be: peptidyl sulphonium methyl ketones > fluoromethyl ketones, diazomethyl ketones > acyloxymethyl ketones, an order which differs sharply from that found for cathepsin B.

INTRODUCTION

Calpains are a class of eukaryotic non-lysosomal Ca^{2+} -dependent cysteine proteinases (Johnson, 1990; Mellgren & Murachi, 1990) that exist in mammalian tissues as two subclasses: calpain I (micromolar- Ca^{2+} -requiring form) and calpain II (millimolar- Ca^{2+} -requiring form). The chemical and kinetic properties of these two forms have been studied extensively, and molecular-genetics studies reveal that calpains have evolved from gene-fusion events involving cysteine proteinases, calmodulin-like genes and the incorporation of two additional domains (Suzuki, 1990). The putative role of calpains in a variety of phenomena, including muscular dystrophy, haemostasis, memory formation, cataract development and hypertension (Johnson, 1990; Mellgren & Murachi, 1990) has stimulated interest in the design of potent and selective calpain inhibitors with potential therapeutic value. A number of inhibitors have been specifically targeted for calpains (Crawford, 1990) which bear functional groups known in the art to be potentially reactive with cysteine proteinases. Peptidyl epoxides (Sugita *et al.*, 1980; Parkes *et al.*, 1985), diazomethyl ketones (Crawford *et al.*, 1988), chloromethyl ketones (Sasaki *et al.*, 1986; Crawford *et al.*, 1988) and fluoromethyl ketones, when combined with an appropriate affinity group, can serve as inactivators of calpain enzymes. Of particular note, efforts by Shaw and co-workers (Crawford *et al.*, 1988; Anagli *et al.*, 1991; Angliker *et al.*, 1992) have yielded diazomethyl ketone and fluoromethyl ketone inhibitors with considerable potency against calpain.

Dipeptidyl acyloxymethyl ketones, of general structure $\text{Z-AA}_2\text{[AA}_1\text{]-CH}_2\text{-OCOAr}$ (where Z is benzyloxycarbonyl, AA is an amino acid and Ar is an aryl group), are powerful inactivators of the lysosomal cysteine proteinases cathepsin B, with second-order inactivation rate constants exceeding $2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the most potent members of this series (Krantz *et al.*, 1991). These inhibitors were designed as affinity labels in which the dipeptidyl moiety serves as an affinity group and the aryl carboxylate moiety offers variable structural elements in the departing group to permit control over the potency and chemical

reactivity of the affinity label. We therefore decided to determine whether calpains exhibited a similar sensitivity to changes in the affinity and departing groups and if mutually exclusive inactivators of these two families of cysteine proteinases could be identified. The present paper reports our observations with peptidyl acyloxymethyl ketones as potential calpain inhibitors and compares them with other classes of cysteine-proteinase inhibitors (Shaw, 1988).

METHODS AND MATERIALS

Inhibitors

Peptidyl acyloxymethyl ketones (Krantz *et al.*, 1991), diazomethyl ketones (Shaw & Green, 1981; Crawford *et al.*, 1988) and sulphonium methyl ketones (Shaw, 1988) were synthesized by the methods described previously. All compounds incorporated amino acids with L chirality, and were characterized by n.m.r., i.r., mass spectra, and/or elemental analysis. Elemental analyses were correct ($\pm 0.4\%$) for the elements and formulae as indicated. Z-Phe-Ala- CH_2F (14; see Tables 1 and 2 below for the structures of the various inhibitors) was a mixture of LL and LD diastereomers and was obtained from Dr. David Rasnick of Enzyme Systems Products, Dublin, CA, U.S.A. (Rasnick, 1985). *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (epoxide E-64; 1) was obtained from Sigma Chemical Co. Compounds prepared: 2 (HCl salt), m.p. 155–158 °C, liquid secondary-ion mass spectrum (LSIMS) m/z 634 (MH^+ , 52%), 43 (100%); 3 (HCl salt), m.p. 163–165 °C, LSIMS m/z 682 (MH^+ , 38%), 241 (40%), 91 (100%); 4 (HCl salt), m.p. 174–176 °C, anal. C,H,N for $\text{C}_{34}\text{H}_{42}\text{N}_3\text{O}_6\text{Cl} + 1.5 \text{ H}_2\text{O}$, LSIMS m/z 588 (MH^+ , 38%), 147 (100%); 5, m.p. 131–133 °C, anal. C,H,N for $\text{C}_{33}\text{H}_{32}\text{N}_2\text{O}_6$, LSIMS m/z 667 (MH^+ , 100%), 533 (30%), 427 (38%), 241 (74%); 6, m.p. 141–142 °C, anal. C,H,N for $\text{C}_{30}\text{H}_{34}\text{N}_2\text{O}_6\text{F}_6$, LSIMS m/z 663 (MH^+ , 100%), 499 (16%), 386 (12%), 241 (60%); 7, m.p. 174–177 °C, anal. C,H,N for $\text{C}_{32}\text{H}_{32}\text{N}_2\text{O}_6\text{F}_6$, LSIMS m/z 653 (MH^+ , 8%), 241 (88%), 91 (100%); 8, m.p. 127–131 °C, anal. C,H,N for $\text{C}_{39}\text{H}_{43}\text{N}_3\text{O}_7\text{F}_6$, LSIMS m/z 780 (MH^+ , 35%), 420 (40%), 241 (100%), 91

Abbreviations used: E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; Z, benzyloxycarbonyl; LSIMS, liquid secondary-ion mass spectrum; Boc, *tert*-butoxycarbonyl; Ph, phenyl; AA, amino acid; Ar, a residue belong to the aromatic series; M^+ , molecular ion.

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Table 1. Potency of acyloxymethyl ketones against chicken smooth-muscle calpain and bovine spleen cathepsin B

No.	Inhibitor Structure	$k_{\text{inact.}}/K_{\text{inact.}} (\text{M}^{-1} \cdot \text{s}^{-1})$	
		Calpain*	Cathepsin B†
1	E-64	1600 3700‡	43 000
2	Z-Val-Lys-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	1200	> 2 × 10 ⁶
3	Z-Phe-Lys-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	200 500‡	> 2 × 10 ⁶ §
4	Z-Phe-Lys-CH ₂ OCO[2,4,6-(CH ₃) ₃]Ph	< 40	230 000§
5	Z-Leu-Phe-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	< 600	< 200
6	Z-Leu-Leu-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	N.I.¶	270 000
7	Z-Val-Phe-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	N.I.	600
8	Z-Leu-Leu-Phe-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	< 1000	2500

* pH 7.5, 20 °C, 10 % dimethyl sulphoxide, except as noted.

† pH 6.0, 25 °C.

‡ 5 % dimethyl sulphoxide.

§ Krantz *et al.* (1991).

|| Poor solubility and poor dose response observed.

¶ N.I., no time-dependent inhibition observed at 5 μM -inhibitor.

(70 %); 9, 10, see Krantz *et al.*, (1991); 11, m.p. 172.5–173.5 °C, anal. C₂₈H₂₇N₃O₈; 12, m.p. 134–136 °C, anal. C₂₄H₂₈N₂O₇; 13, m.p. 134.5–135 °C (decomp.) (literature m.p. 132–133 °C; Shaw & Green, 1981); 15 (bromide salt), m.p. 133–134.5 °C (decomp.) (literature m.p. 103–104 °C; Shaw, 1988), LSIMS m/z 429 (M^+ , 48 %), 367 (14 %), 91 (100 %); 16, m.p. 148–150 °C, LSIMS m/z 395 ($MH^+ - N_2$, 56 %), 91 (100 %); 17 (bromide salt), m.p. 165–166 °C (decomp.), LSIMS m/z 457 (M^+ , 100 %), 395 (16 %); 18, m.p. 169–170 °C (literature m.p. 166–167 °C, Crawford *et al.*, 1988), LSIMS m/z 538 ($MH^+ - N_2$, 30 %), 176 (22 %), 91 (100 %); 20 (bromide salt), m.p. 94–97 °C (decomp.), LSIMS m/z 584 (M^+ , 100 %), 522 (42 %).

Enzyme assays

Purified chicken smooth-muscle calpain II was generously given by Professor Peter Johnson, Department of Chemistry and College of Osteopathic Medicine, University of Ohio, Athens, OH, U.S.A. A continuous fluorometric assay (Mehdi *et al.*, 1988) was adopted to monitor the calpain-mediated hydrolysis of Boc-Val-Leu-Lys-7-amido-4-methylcoumarin (Peninsula Laboratories) ($K_m = 7.1 \text{ mM}$; Sasaki *et al.*, 1984). The enzymic activity was monitored by measuring the increase in fluorescence at 460 nm ($\lambda_{\text{excitation}} = 370 \text{ nm}$) during hydrolysis of 0.18 mM-substrate in 50 mM-Tris/HCl, pH 7.5, containing 5 mM-CaCl₂, 10 mM-2-mercaptoethanol and 10 % dimethyl sulphoxide at 20 °C. Enzyme concentrations were typically 25–50 nM. After the initial uninhibited rate of calpain activity had been recorded, inhibitor (0.5–10 μl of a stock solution in dimethyl sulphoxide) was added and fluorescence monitoring continued, usually for an additional 10–20 min. Inhibitor concentrations were typically 0.25–5 μM . Under these assay conditions the uninhibited control samples provided linear increases in fluorescence for 30 min. Upon addition of inhibitor the rate of fluorescence increase was observed to decrease exponentially, and the inhibition curves (fluorescence units versus time data) were analysed by non-linear regression to the exponential equation:

$$\text{Fluorescence} = A_e^{-(k_{\text{obs.}})t} + B$$

where $k_{\text{obs.}}$ is the pseudo-first-order rate constant for inactivation and A and B are the limit and offset constants respectively. The second-order rate constants, $k_{\text{inact.}}/K_{\text{inact.}}$, were generally obtained as $k/[I]$ from the dependence of $k_{\text{obs.}}$ on inhibitor con-

centration ($[I]$). Saturation kinetics could not be observed for these compounds, owing to the limited solubility or limitations of very rapid inactivation rates. An exception was Z-Phe-Ala-CH₂F (14), for which saturation kinetics parameters $K_{\text{inact.}} = 2.1 \mu\text{M}$ and $k_{\text{inact.}} = 7.0 \times 10^{-4} \text{ s}^{-1}$ were determined.

Bovine spleen cathepsin B mediated hydrolysis of Z-Phe-Arg-7-amido-4-methylcoumarin (Peninsula Laboratories) was monitored by the methods reported by Krantz *et al.* (1991) (pH 6.0, 25 °C). For E-64 (1), saturation-kinetics parameters $K_{\text{inact.}} (= 0.96 \mu\text{M})$ and $k_{\text{inact.}} (= 0.041 \text{ s}^{-1})$ were determined.

RESULTS AND DISCUSSION

Calpains I and II exhibit similar substrate specificity, defined primarily by the nature of the amino acid residues at the P₂ and P₁ positions which precede the scissile bond (Takahashi, 1990). These intracellular proteinases discriminate in favour of Tyr, Phe, Arg or Lys at the P₁ position, whereas Val or Leu are 'preferred' at P₂ (Sasaki *et al.*, 1984). Accordingly, di- and tripeptidyl acyloxymethyl ketones incorporating the preferred peptidyl sequences were prepared as calpain inhibitors.

Krantz *et al.* (1991) established that the apparent second-order rate constants for inactivation of cathepsin B by acyloxymethyl ketones are critically dependent on the nature of the carboxylate leaving group, with 2,6-bis(trifluoromethyl)benzoates providing the most powerful inactivators. Coupling of this leaving group to peptidyl affinity moieties preferred by calpain provided relatively weak time-dependent inhibitors of calpain (Table 1). The limited aqueous solubility of some of these inhibitors and the modest potency against calpain required the introduction of 10 % (v/v) dimethyl sulphoxide as the organic co-solvent. Calpain activity was moderately sensitive to dimethyl sulphoxide concentration, with < 20 % activity lost at 10 % organic co-solvent compared with the activity in the absence of co-solvent. The observed second-order rate constants, $k/[I]$, decreased 2-fold as the dimethyl sulphoxide concentration increased from 5 to 10 % (see, e.g., 1 and 3, Table 1).

Most notably the acyloxymethyl ketones in Table 1, even those with affinity groups preferred by calpain, are more potent against cathepsin B. Indeed, the best dipeptidyl acyloxymethyl ketone inhibitor of calpain, 2, with potency comparable with that of E-64 against this enzyme, was at least three orders of magnitude more potent against cathepsin B. Selective inhibition of calpains versus the family of lysosomal thiol proteinases has also been difficult to realize with other classes of inactivators (Crawford *et al.*, 1988). For example, although Z-Leu-Leu-Phe-CH₂Cl inactivates calpain with a second-order rate constant of > 100 000 $\text{M}^{-1} \cdot \text{s}^{-1}$, this inhibitor is also very potent against lysosomal thiol proteinases. Other classes of thiol proteinase inactivators, including the diazomethyl ketones, suffer from a similar lack of selectivity for calpain. Only Boc-Val-Lys(ϵ -Z)-Leu-Tyr-CHN₂ was a faster inactivator of calpain ($k/K = 2.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) than cathepsin L ($k/K = 1.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$), but even in this case the selectivity was marginal (Crawford *et al.*, 1988).

The calpains have been reported to be less reactive than the lysosomal thiol proteinases, particularly cathepsin B, towards alkylating agents such as E-64, chloromethyl ketones and diazomethyl ketones. Our screening of several acyloxymethyl ketones against calpain shows them to be relatively poor inhibitors of calpains (Table 2), and thus quite selective for the lysosomal proteinase cathepsin B. Sulphonium methyl ketones have been reported to be potent inhibitors of both cathepsin B and papain (Shaw, 1988). We have observed that these compounds are more powerful inhibitors of calpain than the corresponding diazomethyl ketones (Table 2) and appear to be the

Table 2. Comparison of potency of various classes of inhibitors against calpain and cathepsin B

N.I. is defined in Table 1.

No.	Inhibitor Structure	$k_{\text{inact.}}/K_{\text{inact.}} (\text{M}^{-1} \cdot \text{s}^{-1})$	
		Calpain	Cathepsin B
9	Z-Phe-Ala-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	N.I.	1.6×10^6 *
10	Z-Phe-Ala-CH ₂ OCO[2,4,6-(CH ₃) ₃]Ph	N.I.	14000*
11	Z-Phe-Ala-CH ₂ OCO(4-NO ₂)Ph	N.I.	610*
12	Z-Phe-Ala-CH ₂ OCOCH ₂ OCH ₃	N.I.	240*
13	Z-Phe-Ala-CHN ₂	< 10 (< 10†)	1100‡
14	Z-Phe-Ala-CH ₂ F	350	21000§
15	Z-Phe-Ala-CH ₂ S ⁺ (CH ₃) ₂ ·Br ⁻	1100	5800
7	Z-Val-Phe-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	N.I.	600
16	Z-Val-Phe-CHN ₂	230	< 100
17	Z-Val-Phe-CH ₂ S ⁺ (CH ₃) ₂ ·Br ⁻	3500	1200
8	Z-Leu-Leu-Phe-CH ₂ OCO[2,6-(CF ₃) ₂]Ph	< 1000	2500
18	Z-Leu-Leu-Tyr-CHN ₂	> 2.0 (2.3†) $\times 10^5$	1300†
19	Z-Leu-Leu-Tyr-CH ₂ F	29000¶	
20	Z-Leu-Leu-Phe-CH ₂ S ⁺ (CH ₃) ₂ ·Br ⁻	≥ 200000	3500
21	Z-Leu-Leu-Phe-CH ₂ Cl	> 100000†	190000†

* Krantz *et al.* (1991) (pH 6.0, 25 °C).† Crawford *et al.* (1988) (calpain: pH 7.5, 22 °C; cathepsin B: pH 5.5, 30 °C).‡ Shaw *et al.* (1983) (pH 5.4).§ Smith *et al.* (1988) (pH 6.0, 25 °C).

|| Shaw (1988) (pH 5.4, 25 °C).

¶ Anglikier *et al.* (1992) (pH 7.5, 37 °C).

most potent class of calpain inactivators reported to date. The trends that we have observed (Table 2) indicate that, for a given peptidyl recognition moiety, the descending order of potency against calpain is sulphonium methyl ketones > fluoromethyl ketones, diazomethyl ketones > acyloxymethyl ketones. Although we report results for only one fluoromethyl ketone (14) (Table 2), Shaw and co-workers have recently determined that Z-Leu-Leu-Tyr-CH₂F is more potent than the corresponding diazomethyl ketone (18) against calpain in intact platelets (Anagli *et al.*, 1991), but less potent *in vitro*, whereas Z-Leu-Tyr-CH₂F was > 10-fold more potent than Z-Leu-Tyr-CHN₂ *in vitro* (Anglikier *et al.*, 1992). The rank order of potency that we have observed for calpain is in sharp contrast with the order observed for Z-Phe-Ala-CH₂X inhibitors versus cathepsin B wherein certain acyloxymethyl ketones > fluoromethyl ketones > sulphonium methyl ketones > diazomethyl ketones (Table 2).

Several reasons for the relatively poor potency of acyloxymethyl ketones against calpain come to mind.

(a) **The active site of calpain may not tolerate the steric bulk of the arylacyloxy leaving group.** Sequencing studies around the scissile bond in various protein substrates have shown that there is a marked preference for basic amino acids, particularly Arg, and also aliphatic amino acids, particularly Leu, in the P₁' position (Takahashi, 1990). Aromatic amino acids are not preferred residues, and, for perhaps similar reasons, the S₁' subsite may not accommodate the steric bulk of the arylacyloxy leaving group. It should be noted, however, that a sterically less demanding acyloxymethyl ketone, 12, was also found to be ineffective as a calpain inhibitor, even though the leaving group ability of this carboxylate ($pK_a = 3.57$) is comparable with that of fluoride. Alkanoyloxymethyl ketones were also found to be relatively poor inhibitors of cathepsin B (Krantz *et al.*, 1991).

(b) **The nucleophilicity of the active-site thiolate in calpain may be insufficient to enable facile displacement of the carboxylate**

leaving group. The calpains are thought to be considerably less reactive to alkylating agents (e.g. E-64) than are the lysosomal cysteine proteinases. The possibility of lower intrinsic nucleophilicity is difficult to establish without a generally accepted standard leaving group with minimal binding potential. It is, however, noteworthy that fluoromethyl ketones are effective calpain inhibitors (Table 2; Anagli *et al.*, 1991) and fluoride, based on its pK_a , is expected to be a poorer leaving group than 2,6-bis(trifluoromethyl)benzoate (Krantz *et al.*, 1991). Therefore the possibility of weak nucleophilicity of the calpain active-site thiolate would appear to be an untenable explanation of the poor calpain inhibition observed for acyloxymethyl ketones.

(c) **Expulsion of the arylcarboxylate group may require the participation of an additional active-site residue.** The participation of active-site residues in catalysing the inactivation of cathepsin B by facilitating the expulsion of carboxylate leaving groups is an intriguing possibility (e.g., the transfer of a proton to the acyloxy group from a protonated histidine to enable expulsion of carboxylic acid rather than carboxylate anion). An analogous active-site residue may not exist in calpain, or the conformation(s) required to exploit this interaction may not be available in the inhibitor-calpain complexes. It is perhaps noteworthy that very potent calpain inactivation can be achieved by diazomethyl and sulphonium methyl ketones, 18 and 19; this presumably occurs via expulsion of neutral unprotonated leaving groups (N₂ and Me₂S).

Although crystallographic structural data is available for papain (Kamphuis *et al.*, 1984) and cathepsin B (Musil *et al.*, 1991), the large size of calpains (80 kDa for the catalytic subunit), coupled with the propensity to undergo autolytic inactivation, has made structural studies on calpains challenging. The role of active-site residues and the topological complementarity at the S₁' subsite are difficult to assess in the absence of structural data.

Heretofore, dipeptidyl acyloxymethyl ketones were designed as quiescent affinity labels specifically for the cysteine proteinase cathepsin B. In the most favourable circumstances the dipeptidyl moiety serves as an affinity group (complementary to the S₁ and S₂ subsites of the enzyme), whereas the carboxylate ester of the acyloxymethyl ketone provides the departing group. The apparent second-order rate constants for inhibition of cathepsin B span several orders of magnitude, depending on the nature of the carboxylate leaving group, and in certain cases exceed $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. By contrast, even with the incorporation of peptidyl affinity groups optimized for calpain, and a potentially labile carboxylate leaving group of very low pK_a [2,6-bis(trifluoromethyl)benzoate, pK_a 0.58], rapid inactivation of calpain was not observed. Clearly, although calpain is considered to be related to papain-like cysteine proteinases (Ohno *et al.*, 1984), specific differences in the catalytic mechanism and/or active-site structure have allowed us to exploit the variable structure elements in acyloxymethyl ketones to attain specific, potent inactivators of cathepsin B which discriminate against calpain enzymes. Indeed, the very potent and selective inhibition of cathepsin B by inhibitors such as 3 and 4 make these compounds important pharmacological tools for differentiating the role of cathepsins and calpains in pathophysiological conditions.

Finally, for a series of potential inactivators with different labelling groups (Table 2), we have established differences for the rank order of potency of calpain versus cathepsin B. Indeed, we have identified sulphonium methyl ketones as the most potent class of calpain inhibitors reported to date.

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