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The design of peptidyldiazomethane inhibitors to distinguish between the cysteine proteinases calpain II, cathepsin L and cathepsin B

Catherine CRAWFORD,*‡ Robert W. MASON,* Peter WIKSTROM† and Elliott SHAW†

* Department of Biochemistry, Strangeways Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K., and
† Friedrich Miescher Institut, P.O. Box 2543, CH-4002 Basel, Switzerland

A series of peptidyldiazomethanes was synthesized and tested as inactivators of the cysteine proteinases calpain II, cathepsin L and cathepsin B. Inactivators that react rapidly and that show a degree of selectivity between the enzymes were identified. Z-Tyr(I)-Ala-CHN_2 (where Z represents benzyloxycarbonyl) reacts rapidly with cathepsin L and more slowly with cathepsin B, but does not inhibit calpain II. Z-Leu-Leu-Tyr-CHN_2 reacts rapidly with cathepsin L and calpain II but very slowly with cathepsin B. Boc-Val-Lys(ε-Z)Leu-Tyr-CHN_2 (where Boc represents t-butyloxycarbonyl) reacts more rapidly with calpain II than with cathepsin L or cathepsin B. The discriminating inhibitory effects of these compounds make them potentially useful for investigation of enzyme functions *in vivo*. The data presented also provide insights into the subsite specificity of calpain.

INTRODUCTION

The well-characterized cysteine proteinases found in mammalian cells can be divided into two groups, one comprising the lysosomal enzymes cathepsins B, H and L (Barrett & Kirschke, 1981), and the other the cytoplasmic Ca^{2+}-dependent enzymes, calpains (Murachi, 1983). The lysosomal enzymes have a major role in protein turnover within cells and are also important in the breakdown of extracellular-matrix proteins including collagen and elastin (Kirschke *et al.*, 1982; Mason *et al.*, 1986). The role of the calpains is less clear. They may also be involved in protein turnover, for example in muscle (Kay, 1984). In addition, a variety of more specific functions for calpains have been postulated, including activation of protein kinase C (Melloni *et al.*, 1986), cleavage of membrane proteins before cell fusion (Glaser & Kosower, 1986), cytoskeletal modification during platelet activation (Fox *et al.*, 1985) and cleavage of epidermal-growth-factor receptor after stimulation of A431 cells (Yeaton *et al.*, 1983).

Several classes of inhibitors have been developed for cysteine proteinases. These include peptide aldehyde, chloromethane, epoxide and diazomethane derivatives (for review see Rich, 1986). Three of these classes have disadvantages as specific inhibitors of calpains. The aldehyde inhibitors form only reversible complexes with cysteine proteinases and also inhibit serine proteinases. Peptidylchloromethanes similarly react with both serine proteinases and cysteine proteinases. The peptide epoxides are irreversible inhibitors specific to cysteine proteinases, but they react more slowly with calpains than with other cysteine proteinases (Barrett *et al.*, 1982; Parkes *et al.*, 1985). The remaining class of compounds, the peptidyldiazomethanes, are specific inactivators of cysteine proteinases (Leary *et al.*, 1977; Shaw, 1984). They inhibit the enzymes by alkylation of the reactive-site cysteine residue (Leary *et al.*, 1977) and react very slowly with simple thiols such as 2-mercaptoethanol and cysteine (Green & Shaw, 1981). Investigation of various peptidyldiazomethanes as inactivators of the lysosomal cysteine proteinases identified Z-Phe-Thr(OBzl)-CHN_2 as an effective inactivator of cathepsin B (Shaw *et al.*, 1983) and Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 as very rapid inactivators of cathepsin L (Barrett *et al.*, 1982; Kirschke & Shaw, 1981; Mason *et al.*, 1985). The inactivators also function *in vivo* (Grinde, 1983; Shaw & Dean, 1980).

The peptidyldiazomethanes have not been extensively studied as calpain inactivators. When Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 were investigated, they were found to be ineffective, presumably because the peptide portion of the inhibitors bound poorly to the calpain active site (Parkes *et al.*, 1985). The subsite specificity of calpains is not well characterized, although development of peptide substrates demonstrated that the enzymes have a preference for Leu in the P_2 position (Sasaki *et al.*, 1984).

The main aim of the work described in the present paper has been to find inhibitors that react rapidly and irreversibly with calpains but that are poor inhibitors of the lysosomal cysteine proteinases. Inhibitors with these

Abbreviations used. The names of amino acids, peptides and their derivatives are abbreviated in accordance with IUPAC–IUB Recommendations [Biochem. J. (1984) **219**, 345–373]. Additional abbreviations are: NHMec, 4-methyl-7-coumarylamide; Fmoc, 9-fluorenylmethoxycarbonyl; homoPhe, C_6H_5–CH_2–CH_2–$CH(NH_2)$–CO_2H; Tyr(I), iodotyrosine; γ-MeLeu, $(CH_3)_3C$–CH_2–$CH(NH_2)$–CO_2H; t-Leu, $(CH_3)_3C$–$CH(NH_2)$–CO_2H; f.a.b.-m.s., mass spectroscopy by fast atom bombardment. The abbreviations used for enzyme kinetic parameters are: [I], total inhibitor concentration; [S], total substrate concentration; K_i, inhibition constant; K_m, Michaelis constant; $k_{obs.}$, observed pseudo-first-order rate constant for inactivation in the presence of substrate; k'_{+2}, apparent second-order rate constant for inactivation in the presence of substrate; k'_{+2}, apparent second-order rate constant for inactivation, independent of substrate concentration; k_{+2}, rate constant for irreversible inhibition of enzyme–inhibitor complex.

‡ Née Parkes. To whom correspondence should be sent, at present address: Laboratory of Molecular Biophysics, University of Oxford, The Rex Richards Building, South Parks Road, Oxford OX1 3QU, U.K.

characteristics would be invaluable in studying the role of calpains *in vivo*. A second aim has been to look for inhibitors that would clearly differentiate the actions of cathepsins L and B. To find selective inactivators of calpain it was decided to investigate a series of peptidyldiazomethanes with Leu in P_2 and to extend our knowledge of the subsite specificity of calpain by introducing different residues in P_1, P_3 and P_4.

MATERIALS AND METHODS

Materials

Z-Phe-Arg-NHMec and Suc-Leu-Tyr-NHMec were obtained from Bachem (U.K.), Saffron Walden, Essex, U.K; blocked peptides were obtained from Bachem, Bubendorf, Switzerland.

Synthesis of inactivators

Peptidyldiazomethanes. These inhibitors were synthesized by completing the blocked peptide structure by standard coupling procedures, following which conversion into the diazomethane was carried out by reaction of the blocked peptide, as the mixed anhydride, with ethereal diazomethane (Shaw & Green, 1981). The products were purified by chromatography on silica gel with a solvent mixture of 1–10% (v/v) methanol in dichloromethane, followed by crystallization if possible. Composition of crystalline products was confirmed by elementary analysis, whereas the structure of the others was confirmed by f.a.b.-m.s. (Table 1).

It was possible to carry out some transformations on the peptidyldiazomethanes provided that acidic conditions were avoided. The compounds Leu-Leu-Tyr-CHN_2 and Boc-Lys-Leu-Tyr-CHN_2 were prepared from the parent compounds Fmoc-Leu-Leu-Tyr-CHN_2 and Boc-Lys(Fmoc)-Leu-Tyr-CHN_2 by deblocking with piperidine (8 ml/mmol) at room temperature for 1 h. The solvent was removed with reduced pressure and the crude product was applied to silica gel. The by-product from the protecting group was washed off with ethyl acetate, after which the peptide was eluted with chloroform containing 20–50% (v/v) methanol. After removal of the organic solvent, the residue was converted into the acetate salt by solution in 1 equiv. of 0.01 M-acetic acid containing 20% (v/v) methanol and freeze-dried. Purity of the product was demonstrable on t.l.c. in methylene chloride/methanol (4:1, v/v), h.p.l.c. chromatography (acetonitrile/trifluoroacetic acid gradient) and i.r. spectroscopy, which revealed a characteristic sharp peak of the diazomethane band at 2100 cm^{-1}.

Ac-Leu-Leu-Tyr-CHN_2 was prepared from the amino compound obtained as described in the preceding paragraph by solution of the acetate salt (15 mg) in tetrahydrofuran (0.5 ml) followed by *N*-methylmorpholine (3.4 μl) and 3 ml of a solution of acetic anhydride in tetrahydrofuran (10 μl/ml). After 2 h the solvent was removed and the residue was taken up in ethyl acetate, washed with water and saturated aqueous NaCl and dried over anhydrous $MgSO_4$. The residue in ethyl acetate (2 ml) was treated with hexane to yield a white precipitate (9 mg). A single spot was observed on t.l.c. in methylene chloride/methanol (9:1, v/v) and the structure was supported by f.a.b.-m.s. analysis.

Peptidylchloromethanes. These inhibitors were synthesized as described by Kettner & Shaw (1981).

All the inhibitors were prepared for use in the kinetic studies as solutions in dimethyl sulphoxide, diluted with buffer before use.

Purification of proteins

Human liver cathepsin L was purified as described by Mason *et al.*, (1985), human liver cathepsin B was purified as described by Rich *et al.* (1986) and chicken gizzard calpain II was purified as described by Parkes *et al.* (1985).

Determination of rate constants for inactivation

Inactivation rate constants were determined by use of continuous assays in the presence of inhibitor and substrate as described by Tian & Tsou (1982). The appearance of product progress curves for the reactions were analysed by the Guggenheim method as described by Knight (1986) to obtain a pseudo-first-order rate constant of inactivation, $k_{obs.}$. Briefly, an even number of values of the product concentration, $[P]_1$, $[P]_2 \ldots [P]_{2n}$ corresponding to time t_1, $t_2 \ldots t_{2n}$, where t values are constant time increments, was obtained from the reaction progress curves. The value of $k_{obs.}$ is given by the slope of a plot of $\ln([P]_{n+m} - [P]_m)$ versus t_m, where m is an integer in the range $1 < m < n/2$. The apparent second-order rate constant for inactivation, k''_{+2}, was calculated as $k_{obs.}/[I]$. This value was corrected for the presence of substrate to give the apparent second-order rate constant, k'_{+2}, calculated as $k'_{+2} = k''_{+2}(1 + [S]/K_m)$.

k'_{+2} is equivalent to k_{+2}/K_i only when $[I] \ll K_i$. When this is the case k'_{+2} will be independent of [I]. To determine whether this was the case, a range of inhibitor concentrations was adopted. This was not the case for some inactivators of cathepsin L (Tables 3 and 4), and therefore a different method of analysis based on that used by Stone & Hofsteenge (1985) was used (Table 5). This procedure used weighted non-linear-regression analysis of the progress curve for each concentration of inhibitor used. Values of $k_{obs.}$ were weighted according to the squared inverse of their standard errors and fitted to eqn. (1) to obtain k_{+2} and K_i:

$$k_{obs.} = \frac{k_{+2}[I]}{[I] + K_i([I] + [S]/K_m)} \quad (1)$$

To enable comparisons with values obtained with inhibitors whose concentrations were very much less than K_i, k'_{+2} was calculated as k_{+2}/K_i.

For calpain the reactions were done at 22 °C in 3 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-$CaCl_2$, 10 mM-dithiothreitol, 0.1% Triton X-100 and 0.083 mM-Suc-Leu-Tyr-NHMec as the substrate. Calpain was added at 80 nM final concentration followed by the inactivator. The range of inactivator concentrations used is shown in the Tables. The appearance of the aminomethylcoumarin product was recorded continuously with a Perkin–Elmer LS-3 spectrofluorimeter, standardized with either 1 μM- or 0.2 μM-aminomethylcoumarin, with excitation at 360 nm and emission at 460 nm. The total amount of substrate consumed during the assays was below 2%. The K_m value used was 0.4 mM. The reaction progress curves were analysed over at least 3 half-lives and the Guggenheim plots had linear-regression coefficients generally greater than 0.994.

There are limits to the magnitude of the apparent second-order rate constants that can be measured by

Table 1. Analytical data for peptidyldiazomethanes

(1) Elementary analysis

Peptidyldiazomethane	M_r	Elementary analysis (%) C	H	N	M.p. (°C)
Z-Leu-Tyr-CHN_2 ($C_{24}H_{28}N_4O_5$)	452.5	63.70 63.84	6.24 6.44	12.38 12.58	128–129
Z-Leu-Tyr(I)-CHN_2 ($C_{24}H_{27}IN_4O_5$)	578.4	49.84 50.07	4.71 4.82	9.69 9.35	142–143
Z-Leu-Trp-CHN_2 ($C_{26}H_{29}N_5O_4$)	475.5	65.67 65.04	6.15 6.15	14.73 14.73	175–176
Z-Leu-Leu-CHN_2 ($C_{21}H_{30}N_4O_4$)	402.4	62.63 62.76	7.51 7.65	13.92 12.99	Amorph.
Z-Ile-Leu-CHN_2 ($C_{21}H_{30}N_4O_4$)	402.4	62.63 62.76	7.51 7.66	13.92 13.71	118–122
Z-Leu-homoPhe-CHN_2 ($C_{25}H_{30}N_4O_4$)	450.5	66.44 66.44	6.71 6.75	12.44 12.40	122–123
Z-Leu-Met-CHN_2 ($C_{20}H_{28}N_4O_4S$)	420.5	57.12 57.41	6.71 6.81	13.32 12.93	90–91
Z-γ-MeLeu-Tyr-CHN_2 ($C_{25}H_{30}N_4O_5$)	466.5	64.36 63.75	6.48 6.45	12.01 11.66	Amorph.
Z-Tyr-Ala-CHN_2 ($C_{21}H_{22}N_4O_5$)	410.4	61.46 61.31	5.41 5.40	13.65 13.35	157–158
Z-Tyr(I)-Ala-CHN_2 ($C_{21}H_{21}IN_4O_5$)	536.3	47.03 47.38	3.95 4.12	10.45 10.32	144–146
Fmoc-Leu-Leu-Tyr-CHN_2 ($C_{37}H_{43}N_5O_6$)	653.8	67.47 67.10	6.63 6.80	10.71 10.20	159–160
Boc-Val-Lys(ε-Z)-Leu-Tyr-CHN_2 ($C_{40}H_{57}N_7O_9$)	779.9	61.60	7.37	12.57	138–140
Boc-Lys(ε-F_3Ac)-Leu-Tyr-CHN_2 ($C_{29}H_{41}F_3N_6O_7$)	642.7	54.20 54.16	6.43 6.52	13.08 12.80	149–150
Z-Leu-Leu-Tyr-CHN_2 ($C_{30}H_{39}N_5O_6$)	565.7	63.70 63.47	6.95 6.93	12.38 12.21	166–167

(2) F.a.b.-m.s.

Peptidyldiazomethane	M_r	Mass of parent ion by f.a.b.-m.s.*
Z-t-Leu-Tyr-CHN_2 ($C_{24}H_{28}N_4O_5$)	452.5	425
Ac-Leu-Leu-Tyr-CHN_2 ($C_{24}H_{35}N_5O_5$)	473.6	446
Boc-Lys(ε-Fmoc)-Leu-Tyr-CHN_2 ($C_{24}H_{52}N_6O_8$)	768.9	741
Z-Phe-Ser(OBzl)-Leu-Tyr-CHN_2 ($C_{34}H_{48}N_6O_8$)	776.9	749

* A loss of nitrogen has been consistently found with peptidyldiazomethanes.

using the procedure outlined above. Slowly reacting inactivators are tested with high inhibitor concentrations and long assay times. Since there are problems of solubility and introducing large volumes of dimethyl sulphoxide when high inhibitor concentrations are used, and problems of loss of calpain activity as a result of autolysis at long assay times, the lowest rate constant of inactivation that can be measured is 10 $M^{-1} \cdot s^{-1}$. Rapidly reacting inhibitors are tested with low inhibitor concentrations and short assay times. Since for the kinetic analysis the inhibitor concentration should be at least 5 times the enzyme concentration, the substrate concentration ideally should be below K_m and the assay should be monitored for at least 2–3 min, the highest rate constant of inactivation that can be measured is approx. 100 000 $M^{-1} \cdot s^{-1}$.

Table 2. Inactivation rate constants for specific inhibitors of cathepsins L and B

Inactivator	Calpain			Cathepsin L			Cathepsin B		
	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1} \cdot s^{-1}$)	S.D. (n)	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1} \cdot s^{-1}$)	S.D. (n)	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1} \cdot s^{-1}$)	S.D. (n)
Z-Phe-Phe-CHN_2	20–80	< 10	– (2)	0.1–0.5	136000*	15000 (8)	1.0–6.0	185†	– (3)
Z-Phe-Ala-CHN_2	20–80	< 10	– (2)	0.1–0.5	160000*	18000 (6)	1.0–6.0	1220†	– (3)
Z-Phe(I)-Ala-CHN_2	20–50	< 10	– (2)	0.1–0.5	125900	18400 (4)	1.0–5.0	980	100 (3)
Z-Tyr-Ala-CHN_2	20–80	< 10	– (2)	0.1–0.5	176600	24000 (4)	0.5–2.0	1180	80 (3)
Z-Tyr(I)-Ala-CHN_2	20–50	< 10	– (2)	0.01–0.05	1128000	112000 (4)	0.2–1.0	27800	2480 (3)

* Data from Mason (1986).
† Data from Barrett *et al.* (1982).

For cathepsins B and L the enzymes were preincubated with 1 mM-dithiothreitol for 2 h at 4 °C to activate the enzymes. The inactivation reactions were done at 30 °C in 3 ml of 100 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA, 1 mM-dithiothreitol, 0.01% Brij 35 and 5 μM-Z-Phe-Arg-NHMec as the substrate (Mason *et al.*, 1985). Inactivator was added to the reaction mixture, within the range of concentrations shown in the Tables, and the solution was preincubated for 5 min in a cuvette placed in a holder thermostatically controlled at 30 °C. The reactions were started by the addition of enzyme at 0.1 nM final concentration. The appearance of the aminomethylcoumarin product was recorded with a spectrofluorimeter as described for the calpain assays. The total amount of substrate consumed during the assays was below 15%. The K_m values used were 2.4 μM for cathepsin L (Mason *et al.*, 1985) and 150 μM for cathepsin B (Barrett & Kirschke, 1981). The reaction progress curves were analysed over at least 3 half-lives and the Guggenheim plots had linear regression coefficients generally greater than 0.998.

Limitations for rates by cathepsins B and L are less restricting than those for calpain because the higher $k_{cat.}$ value for the synthetic substrate used enabled us to use lower concentrations of enzyme and inhibitor. However, rate constants greater than 1000000 $M^{-1} \cdot s^{-1}$ are very high, even with 1 nM inhibitor, and are therefore less accurate. The lower limit is similar to that of calpain for the same reasons.

RESULTS AND DISCUSSION

The first group of inhibitors studied were based on Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2, which were originally found to inactivate cathepsins L and B (Table 2). The results confirm the previous finding that, although they are good inactivators of the two cathepsins, they have no effect on calpain. Substitution of Tyr for Phe in P_2 gave an equally effective inhibitor of cathepsins B and L while remaining unreactive with calpain. The iodinated analogue of this proved to be an even better inactivator of cathepsins B and L. The radioiodinated compound is capable of detecting these enzymes in cell-culture systems (R. W. Mason & D. Wilcox, unpublished work).

The second group of inhibitors tested were a series of diazomethanes based upon the Suc-Leu-Tyr-NHMec calpain substrate. The apparent second-order rate constants (k'_{+2}) found for these inactivators are shown in Table 3. Comparing the series of inactivators Z-Leu-Xaa-CHN_2 gives an indication of the P_1 specificity for calpain inactivators. In order of increasing rate of inactivation the P_1 amino acid residues are Leu < Trp < Tyr(I) < Tyr < Met < homoPhe. The fact that Tyr(I) at P_1 gives much slower inactivation than Tyr suggests that iodination of the tryosine would not be a useful way of obtaining a radiolabelled inactivator.

Some modifications of Leu in P_2 and longer inhibitors with residues in P_3–P_5 were then tested (Table 4). Modification of the leucine to give γ-MeLeu or t-Leu did not increase the rate constant for calpain. Ile is not a good substitute for Leu, since Z-Ile-Leu-CHN_2 is a slower inactivator than Z-Leu-Leu-CHN_2 (see Table 3). These observations confirm the preference for Leu in P_2 found for calpain substrates (Sasaki *et al.*, 1984), and thus alternative modifications of the P_2 position of the inactivators were not investigated further. Extending the

Table 3. Inactivation rate constants for inhibitors of the type Z-Leu-Xaa-CHN_2

The rate constants were independent of inhibitor concentration except that marked with an asterisk (*). In this case the rate constant decreased as the inhibitor concentration increased. The value was calculated as k_{+2}/K_i as shown in Table 5. Abbreviation: N.D., not determined.

	Calpain			Cathepsin L			Cathepsin B		
Inactivator	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1}\cdot s^{-1}$)	S.D. (n)	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1}\cdot s^{-1}$)	S.D. (n)	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1}\cdot s^{-1}$)	S.D. (n)
Z-Leu-homoPhe-CHN_2	6–11	1860	60 (3)	0.1–0.5	198320	4300 (3)	0.1–0.5	8700	130 (3)
Z-Leu-Met-CHN_2	0.7–2.0	1600	80 (4)	0.1–0.5	211800	3140 (3)	1–5	4060	10 (3)
Z-Leu-Tyr-CHN_2	1–6	1470	140 (5)	1–3	14960*	– (3)	50	< 50	– (3)
Z-Leu-Tyr(I)-CHN_2	4–17	350	40 (5)		N.D.			N.D.	
Z-Leu-Trp-CHN_2	8–14	260	30 (2)	0.25–1.0	11890	493 (4)	50	< 50	– (3)
Z-Leu-Leu-CHN_2	8–34	200	50 (4)		N.D.			N.D.	
Z-Ile-Leu-CHN_2	80–330	10	2 (4)		N.D.			N.D.	

Table 4. Inactivation rate constants for inhibitors with a modified Leu in P_2 or further residues in P_3–P_5

The rate constants were independent of inhibitor concentration except those marked with an asterisk (*). In these cases the rate constant decreased as the inhibitor concentration increased. The values were calculated as k_{+2}/K_i as shown in Table 5. Abbreviation: N.D., not determined.

	Calpain			Cathepsin L			Cathepsin B		
Inactivator	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1}\cdot s^{-1}$)	S.D. (n)	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1}\cdot s^{-1}$)	S.D. (n)	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1}\cdot s^{-1}$)	S.D. (n)
Z-γ-MeLeu-Tyr-CHN_2	4–17	300	20 (3)		N.D.			N.D.	
Z-t-Leu-Tyr-CHN_2	100–400	< 10	– (2)		N.D.			N.D.	
Leu-Leu-Tyr-CHN_2	0.8–2	2300	130 (4)	0.25–1.0	55000*	– (3)	50	< 50	– (3)
Ac-Leu-Leu-Tyr-CHN_2	1.7–3.3	10570	790 (3)	0.1–0.5	90600	860 (3)		N.D.	
Z-Leu-Leu-Tyr-CHN_2	0.1–0.2	230000	113000 (3)	0.005–0.050	1500000	82000 (6)	0.5–1.5	1300	90 (3)
Boc-Lys-Leu-Tyr-CHN_2	0.4–1.7	1570	240 (3)	1–3	5784*	– (3)	2–3	570	20 (3)
Boc-Lys(ϵ-Fmoc)-Leu-Tyr-CHN_2	3–7	3450	750 (4)	1–5	32550*	– (4)	1–5	450	50 (3)
Boc-Lys(ϵ-F_3Ac)-Leu-Tyr-CHN_2	0.8–1.7	12980	1100 (7)	1–3	27300*	– (3)	1–5	1100	120 (3)
Boc-Val-Lys(ϵ-Z)-Leu-Tyr-CHN_2	0.5–1.7	20640	2900 (6)	1–4	13500*	– (9)	0.8–2.5	900	110 (6)
Z-Phe-Ser(OBzl)-Leu-Tyr-CHN_2	2.5–6.0	610	40 (3)		N.D.			N.D.	

Table 5. Calculated individual rate constants for inactivation of cathepsin L by peptidyldiazomethanes

The individual rate constants were calculated as described in the Materials and methods section. The values were used in calculating the rate constants quoted in Tables 3 and 4.

Inactivator	Concn. range (μM)	$K_i \pm$S.E.M. (μM)	$k_{+2} \pm$S.E.M. (s^{-1})
Z-Leu-Tyr-CHN_2	1–3	0.74±0.09	0.0111±0.0007
Leu-Leu-Tyr-CHN_2	0.25–1.0	0.621±0.023	0.034±0.001
Boc-Lys-Leu-Tyr-CHN_2	1–3	2.06±0.56	0.0119±0.0024
Boc-Lys(ϵ-Fmoc)-Leu-Tyr-CHN_2	1–5	0.323±0.013	0.0105±0.0001
Boc-Lys(ϵ-F_3Ac)-Leu-Tyr-CHN_2	1–3	2.16±1.58	0.0590±0.0317
Boc-Val-Lys(ϵ-Z)-Leu-Tyr-CHN_2	1–4	1.63±0.17	0.0220±0.0019

peptide chain for inactivators of the type R-Leu-Tyr-CHN_2 (Table 4) generally gave higher rate constants for calpain inactivation, presumably because of greater binding potential. The only exception was Z-Phe-Ser(OBzl)-Leu-Tyr-CHN_2, which inactivated more slowly than Z-Leu-Tyr-CHN_2, possibly because the additional peptide chain is bulky. The fastest-reacting peptidyldiazomethane inactivator of calpain is Z-Leu-Leu-Tyr-CHN_2. This compound has an inactivation rate constant greater than those observed previously for the peptide epoxides (Parkes *et al.*, 1985). Blocking the *N*-terminal amino acid residue of the inactivator appears to be important for rapid inactivation, since Ac-Leu-Leu-Tyr-CHN_2 gave faster inhibition than Leu-Leu-Tyr-CHN_2. The Z blocking group gave a much better inhibition rate than Ac, indicating that the Z group probably makes a significant contribution to binding in the S_4 binding pocket.

Although Leu in P_3 clearly gave a better inhibitor of calpains, this was also favourable to cathepsin L (Table 4, including data derived from Table 5). In order to find a discriminating inhibitor a different approach is required. When lysine was introduced into P_3 a poorer inhibitor of calpain was produced, suggesting that the Boc-Lys moiety is not contributing to binding. Blocking the lysine in Boc-Lys-Leu-Tyr-CHN_2 as in Boc-Lys(ϵ-F_3Ac)-Leu-Tyr-CHN_2 or Boc-Lys(ϵ-Fmoc)-Leu-Tyr-CHN_2 gave much faster-reacting inactivators, demonstrating that the positively charged group in P_3 is unfavourable. However, once again the increase in rate of inactivation for calpain was paralleled with an increased rate for cathepsin L. Further extension of the peptide chain with a blocked lysine residue in P_3 gave the very effective calpain inactivator Boc-Val-Lys(ϵ-Z)-Leu-Tyr-CHN_2. This was not such a good inhibitor of cathepsin L and in fact was the only inhibitor tested that reacted more slowly with cathepsin L than with calpain.

Data for two peptidylchloromethanes are given in Table 6. Z-Leu-Leu-Phe-CH_2Cl and Leu-Leu-Phe-CH_2Cl inactivated the enzymes tested very rapidly. The rate constants for calpain inactivation could not be calculated as they were outside the range of the assay. In agreement with the results in Table 6, Sasaki *et al.* (1986) obtained rapid inhibition of calpain by Leu-Leu-Phe-CH_2Cl; Leu-Leu-Tyr-CH_2Cl and Leu-Leu-Lys-CH_2Cl; however, they found a rate constant (k'_{+2}) of 10200 $M^{-1} \cdot s^{-1}$ for Leu-Leu-Phe-CH_2Cl. Despite the high reaction rates observed, the peptidylchloromethanes are not particularly useful for work *in vivo* because they do not discriminate between the calpains and the cathepsins, nor are they specific for cysteine proteinases.

Cathepsin L and cathepsin B show rather different inactivation characteristics from calpain. In general cathepsin B reacted much more slowly with the inactivators than did cathepsin L. This is not surprising, as cathepsin B has a specificity for Arg in P_1 and P_2 for substrates and compounds of this type are not represented in the Tables. The fastest-reacting inactivator for both cathepsin L and cathepsin B was Z-Tyr(I)-Ala-CHN_2 (Table 2). In contrast, Z-Leu-Leu-Tyr-CHN_2 reacted extremely rapidly with cathepsin L and rather slowly with cathepsin B. Thus inactivators that would differentiate between cathepsins B and L have emerged from the present work and also from a series of inhibitors in which the bulk of the P_1 side chain is varied (Kirschke *et al.*, 1988).

Direct comparison of the rate constants of inactivation observed for the cathepsins with those for calpain is not possible, as the rate constants were by necessity determined under different conditions and at different temperatures. However, comparison of relative rates for various inactivators is possible. The results presented identify several useful inactivators: firstly Z-Tyr(I)-Ala-CHN_2, which is a rapid inactivator of cathepsins L and B but is a poor inactivator of calpain, secondly Z-Leu-Leu-Tyr-CHN_2, which very rapidly inactivates calpain and cathepsin L but which reacts slowly with cathepsin B, and thirdly Boc-Val-Lys(ϵ-Z)-Leu-Tyr-CHN_2, which reacts more rapidly with calpain than with the cathepsins, the only inactivator to have this useful characteristic. These compounds could be used to study the functions of calpain, cathepsin L and cathepsin B *in vivo*.

To date, examination of the subsite specificity of calpain has shown a preference for Arg, Lys, Met, Tyr or Phe in P_1 and Leu (or less favourably Val) in P_2 (Sasaki *et al.*, 1984, 1986). Thus the data in Tables 3 and 4 extend current knowledge of the subsite specificities of calpain. Our results suggest that of the naturally occurring amino acids Met contributes most significantly to binding in S_1 (althogh homoPhe is better and Tyr is almost as good) and Leu binds strongly in S_2 and S_3. Occupancy of S_4 and possibly S_5 also contributes to binding, and further studies are required to determine the most suitable amino acid residues for these subsites. Our studies

Table 6. Inactivation rate constants for some peptidylchloromethanes

Abbrevation: N.D., not determined.

Inactivator	Calpain			Cathepsin L			Cathepsin B		
	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1} \cdot s^{-1}$)	S.D. (n)	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1} \cdot s^{-1}$)	S.D. (n)	Concn. range (μM)	Rate constant, k'_{+2} ($M^{-1} \cdot s^{-1}$)	S.D. (n)
Z-Leu-Leu-Phe-CH_2Cl	0.2–1.0	> 100 000	– (2)	0.001–0.002	21 500 000	490 000 (4)	0.005–0.020	190 000	36 600 (3)
Leu-Leu-Phe-CH_2Cl	0.2–1.0	> 100 000	– (2)		N.D.			N.D.	

indicate that the strategy employed to determine the subsite specificity of calpain should not necessarily be the same as that for finding a specific inhibitor. The specificity of other cysteine proteinases, cathepsin L in particular, needs to be considered.

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