L-*trans*-Epoxy succinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L

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1. L-*trans*-Epoxy succinyl-leucylamido(4-guanidino)butane (E-64) at a concentration of 0.5 mM had no effect on the serine proteinases plasma kallikrein and leucocyte elastase or the metalloproteinases thermolysin and clostridial collagenase. In contrast, 10 μ M-E-64 rapidly inactivated the cysteine proteinases cathepsins B, H and L and papain ($t_{0.5}$ = 0.1–17.3 s). The streptococcal cysteine proteinase reacted much more slowly, and there was no irreversible inactivation of clostripain. The cysteine-dependent exopeptidase dipeptidyl peptidase I was very slowly inactivated by E-64. 2. The active-site-directed nature of the interaction of cathepsin B and papain with E-64 was established by protection of the enzyme in the presence of the reversible competitive inhibitor leupeptin and by the stereospecificity for inhibition by the L as opposed to the D compound. 3. It was shown that the rapid stoichiometric reaction of the cysteine proteinases related to papain can be used to determine the operational molarity of solutions of the enzymes and thus to calibrate rate assays. 4. The apparent second-order rate constants for the inactivation of human cathepsins B and H and rat cathepsin L by a series of structural analogues of E-64 are reported, and compared with those for some other active-site-directed inhibitors of cysteine proteinases. 5. L-*trans*-Epoxy succinyl-leucylamido(3-methyl)butane (Ep-475) was found to inhibit cathepsins B and L more rapidly than E-64. 6. Fumaryl-leucylamido(3-methyl)butane (Dc-11) was 100-fold less reactive than the corresponding epoxide, but was nevertheless about as effective as iodoacetate.

Inhibitors are important in the study of proteinases; they give the clearest evidence as to the type of catalytic site, information that forms the basis for the classification of the enzymes (Barrett,

1980a); they also allow the identification of individual proteinases, provide quantification through active-site titration and facilitate investigation of the biological functions of the proteinases.

Abbreviations used: names of amino acids, peptides and their derivatives are abbreviated in accordance with IUPAC-IUB Recommendations [Biochem. J. (1972) 126, 773–780]. An additional abbreviation is: -NMec, 4-methyl-7-coumarylamide. The inhibitors (for which the structures are also indicated in Table 2) are Dc-11, fumaryl-leucylamido(3-methyl)butane; E-64, *trans*-epoxy succinyl-L-leucylamido(4-guanidino)butane (L-isomer unless otherwise stated); Ep-174, L-*trans*-epoxy succinyl-leucine; Ep-420, benzyl-DL-*trans*-epoxy succinylisoleucyltyrosine methyl ester; Ep-429, L-*trans*-epoxy succinyl-leucyl-leucine; Ep-459, L-*trans*-epoxy succinyl-leucylamido(4-amino)butane; Ep-460, L-*trans*-epoxy succinyl-leucylamido(4-benzoyloxycarbonylamino)butane; Ep-475, L-*trans*-epoxy succinyl-leucylamido(3-methyl)butane; Ep-479, L-*trans*-epoxy succinyl-leucylamido(7-amino)heptane.

The cysteine proteinases, of which papain is the best known example, contain an essential highly reactive thiol group, and therefore are inhibited by thiol-blocking reagents such as iodoacetate and mercuribenzoate. These reagents are reactive with low- M_r thiol compounds too, so that they are not easy to use under the usual conditions of assay of the cysteine proteinases, i.e. in the presence of thiol activators, and certainly cannot be relied on to show stoichiometric inhibition of the proteinases under these conditions. Reagents more specific for the active-site cysteine residue have been described; these include 2,2'-dipyridyl disulphide used at pH 4 (Brocklehurst & Little, 1973), and more recently the peptidyl diazomethanes (Green & Shaw, 1981). The diazomethanes are unreactive with free cysteine,

but react irreversibly with cysteine proteinases, and not with a variety of serine, aspartic and metallo-proteinases. A high degree of specificity for individual cysteine proteinases has been obtained by use of appropriate peptide sequences, just as was done previously with the peptidylchloromethanes for serine proteinases (Kettner & Shaw, 1981).

A new class of compounds that show promise of acting as class-specific inhibitors for the cysteine proteinases are the *L-trans*-epoxysuccinylpeptides related to the compound E-64 [*L-trans*-epoxy-succinyl-*L*-leucylamido(4-guanidino)butane] (Fig. 1), isolated from cultures of *Aspergillus japonicus* (Hanada *et al.*, 1978c). E-64 was shown to inhibit papain, ficin and the fruit and stem bromelains, with disappearance of the thiol group of papain. In contrast, the serine proteinases trypsin, chymotrypsin, tissue kallikrein, plasmin and pancreatic elastase, and the aspartic proteinases pepsin and *Paeclomyces* acid proteinase were unaffected. E-64 did not react at an appreciable rate with 100 mM-cysteine and did not inactivate lactate dehydrogenase, a non-proteolytic thiol-dependent enzyme (Hanada *et al.*, 1978a). More recently, E-64 has been reported to inhibit two other mammalian cysteine proteinases: cathepsin L (Towatari *et al.*, 1978) and a proteinase from human breast-tumour tissue (Mort *et al.*, 1980), and the calcium-dependent proteinase, calpain, from chicken muscle (Sugita *et al.*, 1980). All of these characteristics suggested that E-64 might be a valuable inhibitor for the study of cysteine proteinases, and we have now examined the possibility in more detail. We have also been able to look at structure-function relationships for this class of compounds.

Materials and methods

Enzymes

Cathepsins B and H from human liver were prepared as described by Schwartz & Barrett (1980), and rat cathepsin L by a modification of the

method of Kirschke *et al.* (1977). Papain (type IV), ficin (crystalline), clostripain, clostridial collagenase (chromatographically purified) and thermolysin (protease type X) were from Sigma, bromelain (crystalline) was from Boehringer Corp., Lewes, East Sussex BN7 1LG, U.K. Streptococcal cysteine proteinase (Elliott & Liu, 1970) was kindly given by Dr. S. Elliott, Department of Pathology, University of Cambridge, Cambridge, U.K. Leucocyte elastase and plasma kallikrein were prepared in our laboratory (Saklatvala & Barrett, 1980; Nagase & Barrett, 1981). Bovine spleen dipeptidyl peptidase I was kindly given by Dr. J. K. McDonald, University of South Carolina, Charleston, SC 29403, U.S.A.

Substrates and inhibitors

Z-Phe-Arg-NMec, Arg-NMec, Gly-Phe-NMec and Z-Lys-OPhNO₂ were purchased from Bachem Feinchemikalien AG, CH-4416 Bubendorf, Switzerland. Suc-Ala-Ala-Pro-Val-NMec was the gift of Dr. M. Baggiolini, Sandoz, Basle, Switzerland. Azocoll was from Calbiochem, and azocasein was prepared as described by Barrett & Kirschke (1981).

Leupeptin was from the Peptide Institute Inc., 476 Ina, Minoh-Shi, Osaka 562, Japan. Optically active E-64 and its analogues were prepared by the method of Tamai *et al.* (1981), except that *L-trans*-epoxy-succinic acid was obtained stereoselectively from D-tartaric acid by a method based on that of Mori & Iwasawa (1980). Table 1 summarizes the physicochemical properties of the new compounds, and the structures of the compounds are shown in Table 2. Z-Phe-Ala-CHN₂, Z-Phe-Phe-CHN₂ and Pro-Phe-Arg-CH₂Cl were kindly given by Dr. Elliott Shaw, Brookhaven National Laboratory, New York, NY, U.S.A.

Enzyme assays

Cathepsin B, bromelain and ficin. The method was slightly modified from that of Barrett (1980b). The stock buffer/activator was 0.4 M-sodium potas-

Fig. 1. Structure of *L-trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64)

Table 1. Physicochemical properties of the new E-64 analogues

Melting points are uncorrected, and optical-rotation measurements ($c = 1$) were made under the conditions indicated. The structures of the compounds are shown in Table 2.

Compound	M.p. (°C)	$[\alpha]_D$ (degrees)
Ep-475		
(LL)	157–160	+45.5 (ethanol, 20°C)
(LD)	82–84	+86.7 (ethanol, 27°C)
(DL)	83–85	–88.0 (ethanol, 29°C)
(DD)	157–158	–39.0 (ethanol, 28°C)
Ep-479	270–280	+23.0 (water, 26°C)
Ep-420	72–73	–19.3 (methanol, 26°C)
Dc-11	171–172	–40.4 (methanol, 23°C)

sium phosphate, pH 6.0, containing 8 mM-dithiothreitol and 4 mM-EDTA. Each assay tube contained 0.25 ml of buffer/activator and 0.50 ml of enzyme in 0.1% Brij 35. After 5 min preincubation to 40°C, 0.25 ml of 20 μ M-Z-Phe-Arg-NMec in water was introduced to start the reaction. Precisely 10 min later the reaction was stopped with 1.0 ml of 100 mM-sodium monochloroacetate in 100 mM-sodium acetate, pH 4.3.

Fluorescence of the liberated aminomethylcoumarin was measured in a Mk. 4 single-sided Locarte fluorimeter, fitted in the excitation light path with an LF/2 (340–380 nm) filter and a 15 mm-path-length liquid filter containing acetone, and with the emission monochromator set at 458 nm. The instrument was zeroed against water, and set to read 1000 arbitrary units (1.0 V output) with 0.5 μ M-aminomethylcoumarin standard.

Cathepsin B was also assayed with Bz-dl-Arg-NNap as described by Barrett (1976), and with Z-Arg-Arg-NNap at a final concentration of 0.5 mM in the same procedure (Barrett & Kirschke, 1981).

Papain. The method was as for cathepsin B, except that the pH of the buffer/activator was 6.8.

Cathepsin H. The method was as for papain, but the substrate was Arg-NMec.

Cathepsin L. The assay was as for cathepsin B, except that the pH was 5.5, and the temperature 30°C.

Clostripain. The method was as for cathepsin B, except that the buffer/activator stock was 20 mM-Tris/HCl, pH 7.5, containing 10 mM-CaCl₂ and 8 mM-cysteine.

Streptococcal proteinase. The enzyme (1.5 mg/ml) was activated by preincubation at 25°C during 1 h in 0.2 M-sodium acetate (pH 5.5)/10 μ M-EDTA/50 μ M-dithiothreitol. A sample containing 7.5 μ g was diluted to 1 ml in the same buffer/activator, and the reaction was started by introduction of 20 μ l of 10 mM-Z-Lys-OPhNO₂ in dimethyl sulphoxide. After 10 min at 25°C, 1 ml of the buffered chloroacetate stopping reagent was added, and A_{340} was measured.

Leucocyte elastase. The procedure was similar to that used for cathepsin B, but the buffer was 0.2 M-Tris/HCl (pH 7.5)/1 M-NaCl, with no activator. The substrate was 20 μ M-Suc-Ala-Ala-Pro-Val-NMec, and the stopping reagent was 0.1 mg of soya-bean trypsin inhibitor/ml in water.

Plasma kallikrein. Again the procedure was as for cathepsin B, but the buffer stock was 20 mM-Tris/HCl (pH 8.0)/0.4 M-NaCl, with no activator. The stopping reagent was 5% acetic acid.

Thermolysin. The enzyme in 0.5 ml of 0.1% Brij 35 was mixed with 0.25 ml of buffer containing 0.4 M-Tris/HCl, pH 7.5, and 0.25 ml of 6% (w/v) azocasein, to start the reaction. After 15 min at 40°C the reaction was stopped with 5 ml of 3%

trichloroacetic acid and A_{366} of the filtrates was determined.

Clostridial collagenase. Azocoll (20 mg/ml) was suspended in 0.5 M-sucrose/0.2 M-Tris/HCl (pH 7.5)/20 mM-CaCl₂, and 1 ml portions were transferred to assay tubes. A collagenase sample diluted to 1.0 ml with 0.1% Brij 35 was added to each tube, and incubation was for 30 min at 37°C, with shaking. The reaction was stopped with 1 ml of 100 mM-disodium EDTA, and the A_{520} of the filtrate was measured.

Dipeptidyl peptidase I. The assay was as for cathepsin B, except that the substrate was Gly-Phe-NMec (250 μ M) and the activators were dithiothreitol (5 mM) and NaCl (10 mM) in 0.10 M-sodium citrate buffer, pH 5.5, at 37°C.

Determination of rate constants for inactivation of the cysteine proteinases

The molar concentration of solutions of papain and cathepsins B, H and L was determined by titration with E-64 (see below). The inhibitor under test (about 10 μ mol) was dissolved in 0.1 ml of dimethyl sulphoxide, and diluted with water to make a 1.0 mM stock solution, which was stable for some days at 4°C. This was further diluted with 0.1% Brij 35. (It should be noted that the use of Triton X-100 in place of Brij 35 has given somewhat different rates of inhibition.)

The reaction of each enzyme with the inhibitors was in the buffer/activator used for its assay (see above). The enzyme concentration was generally 1×10^{-8} – 2×10^{-8} M (papain, cathepsin B, cathepsin L) or 1×10^{-7} M (cathepsin H), and the inhibitor concentration was normally at least 5-fold greater (see below). The precise inhibitor concentration was chosen to give a conveniently measurable rate of inactivation of the enzyme. The enzyme and inhibitor stock solutions were separately pre-equilibrated to 40°C and mixed at zero time. Samples (25 μ l) were removed at suitable times (typically 30, 60, 120, 180, 240, 300, 600 s), and immediately diluted into assay mixtures. These, also at 40°C, comprised 250 μ l of 20 μ M-Z-Phe-Arg-NMec, 250 μ l of buffer/activator and 475 μ l of diluent (0.1% Brij 35). The assays were then completed as described above for cathepsin B.

In most experiments, we were able to measure rates with at least a 5-fold molar excess of inhibitor (I, concn. i) over enzyme (E, concn. e). When $e \ll i$, the kinetics of inactivation follow eqn. (1) (Kitz & Wilson, 1962):

$$\ln \left(\frac{a_t}{a_0} \right) = -k_{+2}t / (1 + K_i/i) \quad (1)$$

in which the reaction scheme is assumed to be as in

eqn. (2), where a_0 is activity at zero time, and a_t is activity at time t .

Semilogarithmic plots of a_t against time were found to be linear, and the observed (pseudo-first-order) rate of inactivation, $k_{obs.}$, was calculated as $0.693/t_{0.5}$. The apparent second-order rate constant for inactivation, k'_{+2} (eqn. 3), was calculated as $k_{obs.}/i$ (Thompson & Blout, 1973).

For papain, the rate of inactivation by E-64(L) was so high that it was necessary to allow the enzyme and inhibitor to react at equal molar concentrations. The rate constant, k'_{+2} , was then calculated from eqn. (4).

$$k'_{+2} = (1/t) \cdot [(a_0 - a_t)/a_0 a_t] \quad (4)$$

Active-site titration of papain and the cathepsins with E-64

This was much as described by Barrett & Kirschke (1981). Working solutions of E-64 at 1, 2, 3 ... 10 μ M concentration were prepared as required from a 1.0 mM-stock solution (see above).

To 50 μ l of buffer/activator (as used in assays of the respective enzymes, see above) was added 25 μ l of enzyme solution (approx. 10 μ M, 0.25 mg/ml, in 0.1% Brij 35) and 25 μ l of 1, 2, 3 ... 10 μ M-E-64. After 30 min at 30°C each mixture was diluted to 5.0 ml by addition of 4.9 ml of 0.1% Brij 35. Samples (usually 10 μ l) were assayed for residual activity against the appropriate methylcoumarylamide substrate (see above), and the results were plotted against inhibitor concentration.

Results

Specificity of inhibition of proteinases by E-64

The results of Hanada *et al.* (1978c) (see the introduction) were extended by examination of reaction with a wider range of proteinases.

Non-cysteine proteinases. The non-reactivity of serine proteinases generally with E-64 is already quite well established, but we considered it important to determine whether plasma kallikrein is affected by E-64, since this serine proteinase is extremely active on the substrate (Z-Phe-Arg-NMec) used for cathepsins B and L and papain. Pure human plasma kallikrein (100 μ g/ml) was incubated for 60 min at 22°C with 0.5 mM-E-64 in the buffer used for the assay (see the Materials and methods section), and then a sample containing 2 μ g was assayed for activity (see the Materials and methods section). There was no loss of activity. A similar experiment was performed with leucocyte

elastase at 50 μ g/ml; 1.25 μ g was assayed, and the result was the same.

There has not been any report of an examination of the activity of E-64 against metalloproteinases. We therefore tested it with thermolysin and *Clostridium histolyticum* collagenase. The enzymes (50 μ g and 10 μ g/ml respectively) were treated with 0.5 mM-E-64 as described above, and samples containing 1 and 2 μ g were taken for assay. Again, there was no loss of activity of either enzyme.

Cysteine proteinases. Each enzyme was tested for inactivation by 10 μ M-E-64(L) during 60 min at 40°C, in the buffer used for their assay (see the Materials and methods section). All of the cysteine-proteinase preparations were completely inactivated by 1 mM-iodoacetate (under the same conditions) during 60 min.

Complete inactivation of papain, and cathepsins B, H and L was obtained with 10 μ M-E-64. These enzymes react relatively rapidly, and it has been possible to measure the rates of reaction (see below).

Stem bromelain and ficin were also completely inactivated by 10 μ M-E-64 under the conditions used. Time-course experiments intended to establish a rate constant for the reaction of ficin with E-64 (not shown) yielded a complex curve not explicable by a single second-order rate constant. The data would have been consistent with about 20% of the activity being due to an enzyme with a rate of reaction of about 250 000 $M^{-1} \cdot s^{-1}$, and the remainder having a rate about half of this. Several distinct forms of ficin exist (Leiner & Friedenson, 1970), and the commercial preparation may well have been heterogeneous.

Clostripain (0.25 μ g/ml) showed no time-dependent inactivation by E-64 at 1 mM-concentration during 60 min at 25°C. There was, however, 81% reversible inhibition at the final concentration of 100 μ M-E-64 in the assay for activity. Further examination of the reversible inhibition indicated a K_i in the range 10–20 μ M. The inhibition was probably due to the leucylamido(4-guanidino)butane moiety of E-64 acting as a substrate analogue, in view of the affinity of clostripain for Sepharose-arginine (Emöd & Keil, 1977). This impression was confirmed by the finding that Ep-475 caused no time-dependent inactivation at 1 mM, or any reversible inhibition at 25 μ M.

The streptococcal cysteine proteinase was much less reactive with E-64 than were papain and the cathepsins. An inactivation occurred with $t_{0.5}$ 222 s in 5 μ M-E-64, so that k'_{+2} was 624 $M^{-1} \cdot s^{-1}$.

Dipeptidyl peptidase I. Dipeptidyl peptidase I was inactivated slowly by 0.5 mM-E-64, the second-order rate constant being approx. 100 $M^{-1} \cdot s^{-1}$.

Active-site-directed nature of the action of E-64

Previous work has indicated that E-64 inactivates

papain and rat cathepsins B and L by stoichiometric reaction with the cysteine residue essential for catalytic activity. Nevertheless, Lineweaver-Burk plots of inhibition data seemed to show that the action of E-64 was not competitive with substrate (Hanada *et al.*, 1978c, 1980; Hashida *et al.*, 1980). Moreover, the optical isomerism of the epoxy-succinyl moiety seemed to have no effect on the activity of E-64 as an inhibitor of papain (Hanada *et al.*, 1978a,b). We reasoned that if E-64 were indeed acting by covalent reaction at the active site, its rate of reaction would be decreased by the presence of leupeptin, a tight-binding reversible inhibitor (Knight, 1980).

Cathepsin B ($0.012\mu\text{M}$) was incubated in the buffer/activator used for its assay (see the Materials and methods section) with or without $0.25\mu\text{M}$ -leupeptin, and with $0.0375\mu\text{M}$ -E-64. Samples were withdrawn at 1, 2, 3, 4, 6, 8 and 10 min, and immediately diluted 40-fold into assay mixtures with substrate. Semilogarithmic plots of activity (as a percentage of the zero-time activity) against time were linear, showing that inactivation was first-order in enzyme. The decrease in the rate of inactivation by E-64 in the presence of leupeptin was 90%. This concentration of leupeptin decreased activity against $5\mu\text{M}$ -Z-Phe-Arg-NMec by 88%. Leupeptin similarly inhibited the reaction of the active site of papain equally for substrate and E-64. These results clearly confirmed the active-site-directed nature of the reaction with E-64.

Further evidence came from the comparison of rates for the D- and L-isomers of E-64 (Table 2). The rates were decreased by one to three orders of magnitude with E-64 in which the *trans*-epoxy-succinyl residue was in the D-configuration, showing that the covalent reaction is strongly stereospecific. In the previous work cited above the method of determination of inhibitory potency was too insensitive to distinguish a rate of reaction as high as that of E-64(D) with papain from rates much higher still.

Rate constants for inactivation of the cysteine proteinases

Although Hanada and co-workers (Hanada *et al.*, 1978a,b,c, 1980) obtained evidence that the reaction of E-64 with papain is irreversible, their data on the extent of inhibition were presented in terms of 50%-inhibitory dose (ID_{50}), which is not appropriate for the quantification of rapid irreversible inhibition.

We incubated the proteinases with E-64, structurally related epoxides and some other inhibitors. Samples were removed at appropriate times for dilution and assay (see the Materials and methods section). The calculated k'_{+2} values are given in Table 2.

Kitz & Wilson (1962) have shown that if $i \ll K_i$,

then k'_{+2} is independent of i , and can be taken to represent k_{+2}/K_i . We were not able to measure rate constants for all of the enzymes with all of the inhibitors at several i values, but several i values were used with five of the inhibitors for cathepsin B. The results are shown in Table 3. It was concluded that the measured rates were unaffected by saturation effects, so that the k'_{+2} values in Table 2 can indeed be taken to represent k_{+2}/K_i .

Active-site titration with E-64

The finding that E-64 reacts rapidly with several of the cysteine proteinases (see below), taken with previous evidence for stoichiometric reaction (Hanada *et al.*, 1980), suggested that it might be useful as an active-site titrant.

Solutions of papain and cathepsins B, H and L were treated with a series of increasing amounts of E-64 (see the Materials and methods section), and assayed for activity. With cathepsin B (Fig. 2), cathepsin L and papain, we found that activity declined linearly with increasing amount of E-64, so that it was easy to draw a straight line through the points to the abscissa. The intercept was taken to give the molar concentration of the enzyme, and the slope of the line gave the specific activity of the enzyme in the rate assay, in terms of molar concentration. For cathepsin L, a titration was also made by the same method, but with Z-Phe-Phe-CHN₂ as inhibitor (Kirschke & Shaw, 1981); the titration curve obtained was indistinguishable from that with E-64.

The titration of cathepsin H was less satisfactory, in that below 30% activity the curve deviated markedly from linearity, tending to become asymptotic with the abscissa. This was attributable to incompleteness of reaction of the enzyme with E-64 at the low concentrations of both reactants as stoichiometric reaction was approached, in view of the relatively low rate constant for the reaction of E-64 with cathepsin H (Table 2). Despite this, it was found that inactivation fitted a linear relationship down to at least 50% inactivation, and a straight line could be drawn through the upper points and extrapolated to the baseline with reasonable confidence.

The molar concentrations of solutions of cathepsins B and H indicated by titration with E-64 were typically 30–50% of those calculated from the protein concentration of the solution (Lowry *et al.*, 1951) and the M_r of the enzymes (e.g. 53% in the experiment shown in Fig. 2). The corollary of this is that the true specific activities of the enzymes in the rate assays are two to three times the values we have previously accepted. For example, purification of human cathepsin B to apparent homogeneity and constant specific activity gives material of specific activity 5.2–6.4 units/mg of protein (Barrett, 1973)

Table 2. *Relative rate constants of inactivation of papain and cathepsins B, H and L by E-64, its synthetic analogues, and other irreversible inhibitors*

The rates (apparent second-order, $M^{-1} \cdot s^{-1}$) were measured at 40°C, pH 6.0, for cathepsin B, 40°C, pH 6.8, for cathepsin H, and 30°C, pH 5.5, for cathepsin L. Non-standard abbreviations used: Fum, fumaryl; Eps, epoxysuccinyl.

Inhibitor	Structure	Relative rate constants			
		Papain	Cathepsin		
			B	H	L
E-64					
L	HO-Eps-Leu-NH[CH ₂] ₄ NHCH(NH ₂) ₂	638 000	89 400	4000	96 250
D	HO-Eps-Leu-NH[CH ₂] ₄ NHCH(NH ₂) ₂	60 900	1900	65	2700
Ep-459	HO-Eps-Leu-NH[CH ₂] ₄ NH ₂	—*	69 500	3080	27 500
Ep-479	HO-Eps-Leu-NH[CH ₂] ₇ NH ₂	874 000	339 000	2070	142 600
Ep-460	HO-Eps-Leu-NH[CH ₂] ₄ NH-Z	471 000	175 000	778	231 000
Ep-174	HO-Eps-Leu	207	388	—	—
Ep-429	HO-Eps-Leu-Leu	122 000	64 500	—	—
Ep-475	HO-Eps-Leu-NH[CH ₂] ₂ CH(CH ₃) ₂				
	LL	357 000	298 000	2018	206 000
	LD	32 500	3790	28	—
	DL	38 000	6900	6	—
	DD	110	24	—	—
Ep-420†	Bzl-DL-Eps-Ile-Tyr-OMe	11 340	49 400	1970	12 660
Dc-11	HO-Fum-Leu-NH[CH ₂] ₂ CH(CH ₃) ₂	—	625	11	2272
	Z-Phe-Ala-CHN ₂	35 000	1220	—	70 000
	Z-Phe-Phe-CHN ₂	3000	185	—	160 400
	Tos-Lys-CH ₂ Cl	747	520	177	783
	Pro-Phe-Arg-CH ₂ Cl	—	5.12×10^6	—	30×10^6
	Iodoacetic acid	2300	444	117	—
	N-Ethylmaleimide	—	1	—	—

* — = not done.

† Rates were calculated on the assumption that the preparation contained equimolar D and L isomers, and that only the reaction of the L-isomer was significant.

Table 3. *Independence of inhibitor concentration (i) and apparent second-order rate constant (k'_{+2})*

The rates were measured as described in the text, with human cathepsin B ($1 \times 10^{-8} M$) in incubations at 40°C. It can be seen that there was no significant effect of i on k'_{+2} . Non-standard abbreviations used: Fum, fumaryl; Eps, epoxysuccinyl.

Inhibitor	Concn. (M)	$t_{0.5}$ (s)	k'_{+2} ($M^{-1} \cdot s^{-1}$)
E-64 (L)	5×10^{-8}	155	89 400
	1×10^{-7}	80	86 600
	2×10^{-7}	39	88 800
E-64 (D)	1×10^{-6}	360	1920
	3×10^{-6}	110	2100
	6×10^{-6}	60	1920
Ep-475 (LL){HO-Eps-Leu-NH[CH ₂] ₂ CH(CH ₃) ₂ }	2×10^{-8}	168	206 250
	4×10^{-8}	84	206 250
	8×10^{-8}	48	190 000
Ep-420 (Bzl-DL-Eps-Ile-Tyr-OMe)	1×10^{-7}	242	28 640
	2×10^{-7}	122	28 400
	3×10^{-7}	87	26 551
	6×10^{-7}	36	32 083
DC-11 {Fum-Leu-NH[CH ₂] ₂ CH(CH ₃) ₂ }	5×10^{-6}	236	587
	1×10^{-6}	114	592
	2×10^{-5}	66	558
	4×10^{-5}	27	625

Fig. 2. Titration of human cathepsin B with E-64. For each point, 25 μ l of cathepsin B solution (280 μ g/ml) was allowed to react with an equal volume of 0–10 μ M-E-64 in a total volume of 100 μ l. A 0.5 μ l sample of each reaction mixture was then assayed for activity against Z-Phe-Arg-NMec. The measured fluorescences (left ordinate) were adjusted for the sampling factor to give the residual activities/ml indicated on the right ordinate. From this plot it can be calculated that (a) the enzyme solution had an operational molarity of 5.3 μ M (53% of that expected on a protein basis), and (b) the absolute specific activity of cathepsin B in this assay is 238 units/ μ mol. In the same experiment (but not shown for clarity) assays were made also with Bz-DL-Arg-NNap (found 402 units/ μ mol) and Z-Arg-Arg-NNap (found 9160 units/ μ mol), as described in the text.

in our assay with Bz-DL-Arg-NNap (Barrett, 1976), whereas titration now gives a value of about 14.5 unit/mg of completely active enzyme. The revised specific activity for Z-Phe-Arg-NMec in the assay of Barrett (1980b) is 8.5 units/mg.

The discovery that protein concentration is not a reliable indication of the concentration of active cathepsin B in preparations of the enzyme from human liver (and probably other sources) necessitates correction of k_{cat} values previously obtained in this and other laboratories (Barrett & Kirschke, 1981). We do not know the chemical nature of the inactive, unactivatable component of preparations of cysteine proteinases made by most, if not all, of the methods currently in use.

Discussion

Specificity of E-64 for cysteine proteinases

Since E-64 had been found to inhibit all of the cysteine proteinases tested, but none of the other enzymes, we started this investigation thinking that this might be a general inhibitor for the whole class of cysteine proteinases. The impression that E-64 inhibits only cysteine proteinases has been confirmed (except for the slow inactivation of dipeptidyl peptidase I), but we have found that it does not inhibit all of the cysteine proteinases. We therefore thought it worthwhile to consider the characteristics of the cysteine proteinases that are readily inhibited in contrast with those that are not.

Papain showed a particularly high reactivity with E-64, and good rates were also obtained with the other plant enzymes and the lysosomal cysteine proteinases. There is structural evidence that these enzymes form an homologous group (Takio *et al.*, 1980), and they resemble each other in having M_r about 25 000, no (detected) zymogens and no distinct requirement for calcium. The specificity of E-64 and its analogues for enzymes within the papain family was investigated in some detail (discussed below).

The streptococcal cysteine proteinase reacted only very slowly with E-64. This enzyme is probably a very distant member of the papain family; it has an M_r of 32 000, but differs from papain in having a proteolytically activatable zymogen (M_r 44 000), and in lacking disulphide bonds. Nevertheless, the general positioning of the essential cysteine, histidine and tryptophan residues is similar, and there are slight indications of sequence homology around these residues (Tai *et al.*, 1976). Perhaps more decisive is the fact that the primary specificity is for a hydrophobic residue in P_2 (in the terminology of Berger & Schechter, 1970), just as it is for papain and cathepsin B (Gerwin *et al.*, 1966; Kortt & Liu, 1973); this type of specificity is very unusual, and seems most unlikely to have arisen by convergent evolution.

Clostripain, the cysteine proteinase from *Clostridium histolyticum* that showed no reactivity with E-64, is a remarkably trypsin-like cysteine proteinase with a strong specificity for the cleavage of arginyl bonds, susceptibility to many trypsin inhibitors, and stimulation by Ca^{2+} (Mitchell & Harrington, 1971). The M_r is about 50 000, and the partial sequence shows no homology with papain (A.-M. Gilles & B. Keil, personal communication).

Finally, the specificity and structure of the calpains, mammalian calcium-dependent cysteine proteinases, are unknown, but their M_r of about 80 000 and strong requirement for Ca^{2+} do nothing to suggest that they are of the papain family (Murachi *et al.*, 1981). Chicken skeletal-muscle

calpain is reported to be inhibited by E-64, but the rate constant has not been determined (Sugita *et al.*, 1980).

Mode of binding of the inhibitors

As was pointed out in the introduction, we now have two families of irreversible inhibitors of cysteine proteinases that have the important property of negligible reactivity with thiol compounds of low M_r . They are the peptidyl diazomethanes and the epoxysuccinylpeptides. In both groups, the covalently reacting structure is inherently of low reactivity and specificity, and the selectivity and potency of the inhibitors are due to the affinity of the appropriate peptide components (typically dipeptides) for the specificity sites of the proteinases. There is little doubt that the peptide component of the peptidyl diazomethanes is bound by specificity subsites S_1 , S_2 , etc. of the susceptible proteinase, whereas that of E-64 is bound in S'_1 , S'_2 , etc. (Fig. 3). The characteristics of the sites on the amino side of the catalytic sites of several cysteine proteinases are known, and were exploited by Green & Shaw (1981) in the design of peptidyl diazomethanes. In contrast, very little is known about the sites on the carboxy side, but it now seems they may be equally useful in the design of inhibitors. It is noteworthy that although the S-subsites of the streptococcal proteinase are generally similar to those of papain, and Z-Phe-Ala-CHN₂ accordingly shows a similar order of reactivity with the two enzymes (Green & Shaw, 1981), E-64 was 1000-fold more reactive with papain, suggesting that the S'-sites differ importantly. We would guess that clostripain, too, might well be inactivated by epoxysuccinylpeptides of different sequence from E-64.

The conclusion that the S'-sites show significant specificity, as well as the high affinity for suitable peptides that must be responsible for the high rates of reaction with the enzymes, suggested that epoxysuccinylpeptide analogues of E-64 might have interesting selectivity amongst cysteine proteinases.

Fig. 3. Proposed binding modes of E-64 and its analogues

(a) E-64; (b) Z-Phe-Ala-CHN₂. Abbreviations used: -Agm, agmatine [4-amido(4-guanidino)butane]; Eps, epoxysuccinyl.

Specificity of E-64 and its analogues among proteinases of the papain family

Table 2 shows that E-64(L) reacted very rapidly with papain, distinctly more slowly with cathepsins B and L, and much more slowly still with cathepsin H. In fact, all of the inhibitors tested reacted much more slowly with cathepsin H than with the other enzymes, and E-64 proved to be the fastest inhibitor for this enzyme.

Two of the analogues of E-64 retained the positively charged side chain on R₂ (Ep-459 and Ep-479). If R₂ of E-64 is thought of as arginine residue lacking the carboxy group, Ep-459 would be the corresponding lysine compound; the reaction rates were not very different from those of E-64; Increasing the length of the R₂ side chain of Ep-459 by three carbon atoms to produce Ep-479 greatly increased reactivity, and the chain length rather than the positive charge seems to be the important factor, since Ep-475, with a neutral R₂, was about as reactive as Ep-479. The C-terminal carboxy group was unfavourable, as is shown by the comparison of Ep-429 with Ep-475.

Ep-475 has proved to be substantially more reactive than E-64 with cathepsins B and L, and indeed it can well be used in place of E-64 as an active-site titrant of cysteine proteinases. The importance of the optical isomerism of the Eps (epoxysuccinyl) and R₁ residues was shown by the low reactivity of the LD, DL and DD isomers.

The compound Dc-11 is the analogue of Ep-475 in which the epoxysuccinyl group is replaced by a fumaric acid residue. The k'_{+2} values for reaction of *trans*-epoxysuccinate and fumarate with cysteine are similar (0.01–0.04 M⁻¹·s⁻¹; K. Hanada, unpublished results). Dc-11 reacted at least 100-fold more slowly with the proteinases than Ep-475 presumably because the configuration of the epoxysuccinyl group is more favourable than that of the fumaric acid residue for the reaction at the active site. Nevertheless, the reactivity of Dc-11 is appreciable, being comparable with that of iodoacetate with cathepsin B, and much greater than that of the other unsaturated inhibitor, *N*-ethylmaleimide.

The diazomethanes we have tested are relatively poor inhibitors of human and rat cathepsin B (see also Kirschke & Shaw, 1981), but inhibit rat cathepsin L at rates comparable with those obtained with E-64.

The simple halomethylketo compounds, Tos-Lys-CH₂Cl and iodoacetate, were surprisingly unreactive, in contrast with the exceptionally high rates obtained with Pro-Phe-Arg-CH₂Cl. This compound could almost certainly be used as a stoichiometric titrant for the cathepsins in the presence of cysteine, in spite of the fact that the chloromethanes react much more rapidly with low- M_r thiol compounds than the epoxides or diazomethanes.

Active-site titration

The most obvious practical application of E-64 is in the active-site titration of the papain-related cysteine proteinases. Active-site titration as a method of determining enzyme concentration has the advantage over rate assays of being insensitive to reaction conditions, and giving a result in active-site molarity (Bender *et al.*, 1966). When a 'burst assay' is available, such as that with *p*-nitrophenyl *p*'-guanidinobenzoate for trypsin (Chase & Shaw, 1967), it provides a particularly elegant form of titration in which the concentration of the reagent is not critical and the product quantified is equal in molar concentration to the enzyme. Several milligrams of enzyme are needed for such a spectrophotometric titration, but sensitivity may be increased 100–1000-fold by use of fluorimetric or radiochemical methods. A spectrophotometric 'burst substrate', Z-Tyr-OPhNO₂, has been described for papain (Bender *et al.*, 1969), but not widely used because it has a high turnover rate. A better self-indicating titrant is the inhibitor, 2,2'-dipyridyl disulphide, which reacts preferentially with the catalytic cysteine residue in the presence of other thiols at pH 3.8 (Brocklehurst & Little, 1973), but again, large amounts of enzyme are required.

The approach we have exploited, of using an active-site-directed inhibitor in conjunction with a rate assay, is much more generally applicable and much more sensitive than the use of any of the self-indicating titrants. The requirements are an inhibitor reacting rapidly and stoichiometrically with the enzyme but not with other thiols, and a rate assay sensitive enough to allow full advantage to be taken of the reactivity of the inhibitor. The concentration of the inhibitor must be known accurately. An incidental advantage of the approach is that it provides a direct calibration of the rate assay in enzyme molarity.

In order to calculate the minimum concentration at which solutions of cysteine proteinases can be accurately titrated, we have assumed that an incubation period of up to 1 h is usually acceptable, and that the requirement is for at least 49% inhibition by a 0.50 molar proportion of inhibitor, so that an essentially linear relationship from 100–50% activity can be extrapolated accurately to the abscissa. On this basis, a rate constant of $4000\text{ M}^{-1}\cdot\text{s}^{-1}$ allows titration of a $5 \times 10^{-7}\text{ M}$ enzyme solution, 40000 allows $5 \times 10^{-8}\text{ M}$, and 400000, $5 \times 10^{-9}\text{ M}$. In our experience, these would be concentrations of the order commonly dealt with in work on mammalian proteinases, and one could not conveniently use inhibitors reacting much more slowly.

E-64 is an excellent titrant for papain and cathepsin B, and adequate for cathepsin H; for each of these enzymes it is preferable to any known

peptidyl diazomethane. On the other hand, Z-Ala-Phe-Ala-CHN₂ is better than E-64 for the streptococcal proteinase, with a rate of $29500\text{ M}^{-1}\cdot\text{s}^{-1}$ (Green & Shaw, 1981), and the diazomethanes also seem more promising for clostripain. For cathepsin L, both E-64 and Z-Phe-Phe-CHN₂ (Kirschke & Shaw, 1981) are excellent titrants. It thus seems that the epoxysuccinyl compounds and the diazomethanes have approximately equal potential for further development as powerful and selective inhibitors of cysteine proteinases.

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