In Vivo Inhibition of Cathepsin B by Peptidyl (Acyloxy)methyl Ketones§

Bonnie M. Wagner,*,† Roger A. Smith,*,‡,⊥ Peter J. Coles,‡ Leslie J. Copp,‡ Michael J. Ernest,† and Allen Krantz‡

Department of Lipid and Protease Biochemistry, Institute of Immunology and Biological Sciences, Syntex Research, Palo Alto, California 94304, and Syntex Research (Canada), 2100 Syntex Court, Mississauga, Ontario, L5N 3X4 Canada

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Peptidyl (acyloxy)methyl ketones, previously established as potent irreversible inhibitors of the cysteine proteinase cathepsin B *in vitro*, were investigated and optimized for their inhibitory activity *in vivo*. Incorporation of polar or charged functional groups in the inhibitor structure afforded effective cathepsin B inhibition, following dosing to rats. The most effective inhibitor, Z-Phe-Lys-CH_2OCO-(2,4,6-Me_3)Ph (8), was found to give ED_{50} values of 18 mg/kg po (orally) and 5.0 mg/kg ip (intraperitoneally) at 4–5 h postdose, and 2.4 mg/kg sc (subcutaneously) at 24 h postdose, for liver cathepsin B inhibition (measured *ex vivo*). The subcutaneous route of administration of (acyloxy)methyl ketone 8 also provided potent cathepsin B inhibition in certain peripheral tissues (e.g., ED_{50} 1.0 mg/kg for skeletal muscle, 0.1 mg/kg for heart). These investigations demonstrate that peptidyl (acyloxy)methyl ketones such as 8 have promise as tools for the characterization of *in vivo* biochemical processes and as therapeutic agents.

Introduction

The cysteine proteinase cathepsin B (EC 3.4.22.1)[1,2] is a major lysosomal enzyme which catalyzes normal protein degradation but has also been associated with several pathophysiological conditions such as inflammation,[3] tumor metastasis,[4,5] bone resorption,[6] muscular dystrophy,[7-10] and myocardial infarction.[11-13] In order to clarify the biochemical role that this cysteine proteinase plays in these conditions with the goal of developing novel therapeutics, there is a need for selective inhibitors of cathepsin B which are highly effective *in vivo*.

We recently described the development of peptidyl (acyloxy)methyl ketones as potent and specific inactivators of cathepsin B *in vitro*.[14-16] These derivatives, of general structure Z-[AA_1]-[AA_2]-CH_2OCOAr, were designed as affinity labels which incorporate variable structural elements in both the peptidyl affinity group and the carboxylate nucleofuge (leaving group). Inactivation rates were determined to span several orders of magnitude, in some cases exceeding 10^6 M^{-1} s^{-1}. As well, a strong relationship between the second-order inactivation rate (k/K) and pK_a of the carboxylate leaving group (as a measure of leaving group ability) was established. By utilizing a peptidyl affinity group (Z-Phe-Lys) with particularly high affinity for cathepsin B, effective inactivators could be obtained with acyloxy groups of very low nucleofugality. Recently, very high selectivity of peptidyl (acyloxy)methyl ketones for inhibition of cathepsin B, over the calcium-activated cysteine proteinase calpain, was demonstrated.[17a] Furthermore, the selectivity of this class of inhibitors for cathepsin B vs other cysteine proteinases has also been investigated.[17b]

In this article, we describe our systematic variation of the (acyloxy)methyl ketone structure to achieve cathepsin B inactivators with high potency *in vivo*, and our investigation of the effects of the route of administration on the time course and tissue distribution of cathepsin B inhibition.

Table 1. Rates of Cathepsin B Inactivation *in Vitro* by Peptidyl (Acyloxy)methyl Ketones[a]

compound	k/K (M^{-1} s^{-1})
1, Z-Phe-Ala-CH_2OCO-(2,4,6-Me_3)Ph	14 000[b]
2, MeO-Suc-Phe-Ala-CH_2OCO-(2,4,6-Me_3)Ph	4000
3, HO-Suc-Phe-Ala-CH_2OCO-(2,4,6-Me_3)Ph	1000
4, $PhSO_2NHCOC_6H_4CO$-Phe-Ala-CH_2OCO-(2,4,6-Me_3)Ph	2500
5, Z-Phe-Gly-CH_2OCO-(2,4,6-Me_3)Ph	9900[b]
6, HO-Suc-Phe-Gly-CH_2OCO-(2,4,6-Me_3)Ph	*ca* 700
7, Z-Phe-Ala-CH_2OCO-(2,3,5,6-Me_4-4-COOH)Ph	63 000
8, Z-Phe-Lys-CH_2OCO-(2,4,6-Me_3)Ph·HCl	230 000[b]
9, Z-Tyr(OMe)-Lys-CH_2OCO-(2,4,6-Me_3)Ph·CF_3COOH	170 000

[a] Conditions: bovine spleen cathepsin B, 100 mM potassium phosphate, 1.25 mM EDTA, 1 mM dithiothreitol, pH 6.0, 25 °C (ref 14). [b] Reference 14.

Results and Discussion

In the early stages of our investigation and development of peptidyl (acyloxy)methyl ketones as cathepsin B inhibitors, the Z-Phe-Ala mesitoyl derivative 1 was selected as a preferred, representative inhibitor. This selection was made on the basis of its relatively simple chemical structure and an *in vitro* cathepsin B inactivation rate (Table 1) comparable to those of other standard inhibitors determined under our assay conditions [E-64 (10), k/K 43 000 M^{-1} s^{-1};[17a] Z-Phe-Ala-CH_2F (11), k/K 21 000 M^{-1} s^{-1} [18]]. In addition, the flanking ortho methyls in the mesitoate group shield the ester carbonyl from nucleophilic attack,[19] and this compound was found to be very stable in the cathepsin B assay medium ($t_{1/2}$ >24 h, pH 6.0, 1 mM DTT[14]). Inhibitor 1 was therefore evaluated for activity *in vivo*.

It was determined in initial studies that administration of a single 100 mg/kg dose of 1 to rats achieved only 22% inhibition of liver cathepsin B activity by the oral route and 31% after intraperitoneal treatment (4–5 h postdose). The highly potent and chemically more reactive [2,6-bis-(trifluoromethyl)benzoyl]oxy analog[14] (k/K 1.6×10^6 M^{-1} s^{-1}) was found to provide no improvement in the activity (24% inhibition at 100 mg/kg po, 4–5 h postdose). Therefore, in efforts to enhance aqueous solubility, bioavailability, and *in vivo* activity, polar or charged functionalities

† Department of Lipid and Protease Biochemistry.
‡ Syntex Research (Canada).
§ Contribution No. 381 from the Institute of Bioorganic Chemistry.
⊥ Current address: Institute for Chemistry, Pharmaceutical Division, Miles Inc., 400 Morgan Lane, West Haven, CT 06516.
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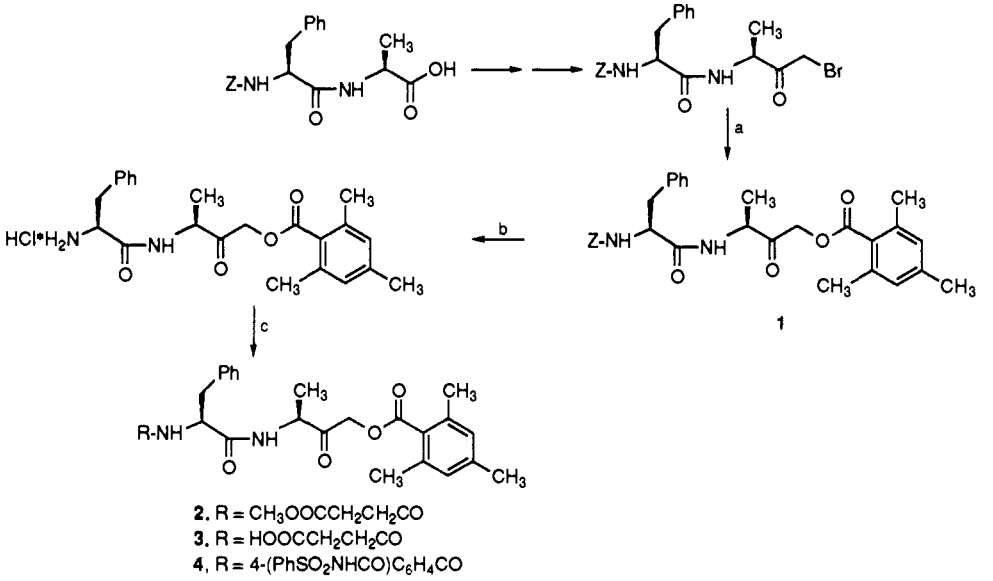
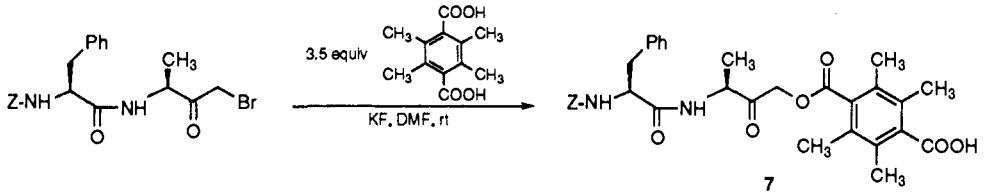
Scheme 1[a]



[a] (a) 2,4,6-Me_3PhCOOH, KF; DMF, room temperature; (b) H_2, Pd–C, HCl, EtOH, room temperature; (c) for **2**: (i) NMM, CH_2Cl_2, 0 °C; (ii) $MeOOCCH_2CH_2COOH$, EDCI, DMAP; for **3**: (i) NMM, DMF; (ii) succinic anhydride; for **4**: (i) NMM, CH_2Cl_2, 0 °C; (ii) 4-$(PhSO_2NHCO)C_6H_4COOH$, EDCI, DMAP.

Scheme 2[a]



were incorporated at the N-terminal group, acyloxy group, and P_1 side chain of our inhibitors.[20] Phenylalanine was retained at the P_2 position,[20] dictated by the high affinity of papain-like cysteine proteinases for aromatic amino acids such as Phe at this position in substrates and inhibitors.[14,20,21]

Chemistry and *in Vitro* Activity. Peptidyl (acyloxy)-methyl ketones were synthesized by the general method described previously,[14] involving potassium fluoride-mediated alkylation of a carboxylic acid by a peptidyl bromomethyl ketone. Hydrogenolysis of **1** provided the *N*-deprotected analog,[14] which could then be acylated to provide inhibitors **2–4** (Scheme 1). *N*-(Methoxysuccinyl)-, *N*-succinyl-, and *N*-4-(phenylsulfonamido)benzoyl groups have been used successfully in other types of proteinase inhibitors.[22–24] The Z-Phe-Gly analogs **5** and **6** were also investigated. Compounds **2–6** were all found to be effective as cathepsin B inactivators *in vitro* (Table 1), albeit to a lesser degree than the parent **1**.

Attempts to incorporate a carboxylate group into the acyloxy function, by base hydrolysis of Z-Phe-Ala-CH_2-OCO-(2,6-Me_2-4-COOMe)Ph,[14] were problematic in that decomposition of this inhibitor was competitive with the desired hydrolysis of the methyl ester function. As an alternative, 2,3,5,6-tetramethyl-1,4-benzenedioic acid was condensed with Z-Phe-Ala-CH_2Br to give the desired product **7** (Scheme 2), together with the dialkylation product formed as a minor product. As anticipated, the increased leaving group ability of the acyloxy function in **7**, relative to that in **1**, gave rise to a significantly enhanced cathepsin B inactivation rate (Table 1).

Use of lysine at the P_1 position enabled the incorporation of a charged side-chain functionality. In accord with the affinity of cathepsin B for positively charged P_1 residues, quite dramatic potency was observed for analog **8**.[14] In an initial synthesis, trifluoroacetyl was used as the ϵ-amino protecting group; deprotection to give the product **8** could be cleanly achieved by treatment with methanolic HCl. In an alternative synthesis, the N^{ϵ}-Boc-lysine analog could be readily prepared and then deprotected to **8** by standard methods.

Finally, derivatives with potentially improved metabolic stability were investigated. A sarcosine analog (Z-Phe-Sar-CH_2OCO-(2,4,6-Me_3)Ph) was, however, found to give no significant inactivation (time-dependent inhibition) of cathepsin B.[14] The use of *O*-methyltyrosine to reduce proteolytic degradation has been reported previously.[25] We previously reported[14] that an *O*-methyltyrosine analog (Z-Tyr(OMe)-Ala-CH_2OCO-(2,4,6-Me_3)Ph) had *in vitro* cathepsin B inhibitory activity that was comparable to the parent compound **1**. An *O*-methyltyrosine analog of **8** (i.e., **9**) was therefore prepared for evaluation and was found to be comparable in potency to **8**.

***Ex Vivo* Assay for Inhibition of Cathepsin B Activity after Intraperitoneal Administration.** A number of (acyloxy)methyl ketones in Table 1 were evaluated in comparison with the peptidyl epoxide standard, E-64 (**10**) [L-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane].[26] An intraperitoneal (ip) route of administration was chosen for E-64, since it had been reported[27] that a close analog of this compound (E-64c) had low activity when administered orally (po). Compounds were administered as suspensions in carboxymethyl cellulose (CMC) vehicle. At 4–5 h postdose, animals were sacrificed and tissues were removed for analysis (see the Experimental Section). This time period

Table 2. Effect of Inhibitors on Rat Liver Cathepsin B by a Single Intraperitoneal Dose[a]

compound	dose (mg/kg)	cathepsin B % control
1	100	69 ± 19
2	10	38 ± 14
3	10	23 ± 7
4	10	68 ± 23
7	10	53 ± 38
8	10	4 ± 3
E-64 (10)	10	24 ± 15

[a] Enzyme activities were determined *ex vivo* at 4–5 h postdose; for each experiment, enzyme activity was assayed at two dilutions (in triplicate) from each animal, using 4–5 animals per group.

was selected on the basis of the reported duration of action of "EST" (ethyl ester of E-64)[27] and our own similar studies with 11 (E-64) and 8. The 4–5 h postdose time period was confirmed to be near optimal in the duration of action study for the po route of administration (see below, Figure 3).

Following isolation of the enzyme, *ex vivo* proteolytic activity was measured spectrofluorometrically with the use of a substrate specific for cathepsin B. It was established, for both E-64 (**10**) (10 mg/kg ip) and (acyloxy)-methyl ketone 8 (10 or 100 mg/kg ip), that inhibition results obtained with liver lysosomal enzymes obtained from the frozen and homogenized whole organ were equivalent to those obtained with enzymes from purified lysosomes, isolated from the fresh organ by a modification of the classic differential centrifugation technique.[1,28] This result established the validity of the determinations made using frozen tissue homogenates. In a recent study of *in vivo* cathepsin B inhibition by peptidyl fluoromethyl ketones,[29] frozen liver and kidney homogenates were also used for the *ex vivo* enzyme assay, and their results are in accord with our findings.

From *in vitro* studies, Wilcox and Mason[30] concluded that E-64 does not readily diffuse across membranes and that uptake of E-64 can only occur via pinocytosis. In our *in vivo* studies (using an *ex vivo* assay of cathepsin B obtained from either a purified lysosomal pellet or tissue homogenate; *vide supra*), E-64 (**10**) was found to be a potent inhibitor of liver cathepsin B following intraperitoneal dosing to rats.

Following a single dose (10 mg/kg ip) of compound, liver cathepsin B activity was found to be significantly suppressed by a number of compounds. The Z-Phe-Lys analog 8 was clearly the most active inhibitor, equivalent in potency to E-64 (Table 2, Figure 1). In follow-up dose–response studies, compounds **3**, **4**, **7**, and **8** yielded comparable ED_{50} values of 4–5 mg/kg, while the methoxysuccinyl analog **2** was somewhat less effective (ED_{50} 21 mg/kg ip). These results suggest that the incorporation of charged functional groups in compounds **3**, **4**, **7**, and **8** improved intraperitoneal bioavailability. However, there was no distinct correlation between the *in vitro* cathepsin B inactivation rates (Table 1) and the *in vivo* inhibitory activities, suggesting that the differences in bioavailability, metabolism, and other such factors *in vivo* were of prime importance.

***Ex Vivo* Assay for Inhibition of Cathepsin B Activity after Oral Administration.** Initial oral dosing studies were conducted using a single 50 mg/kg dose. In these studies, several (acyloxy)methyl ketones (including the parent compound 1) were evaluated in comparison with the fluoromethyl ketone Z-L-Phe-D,L-Ala-CH_2F (**11**).[31]

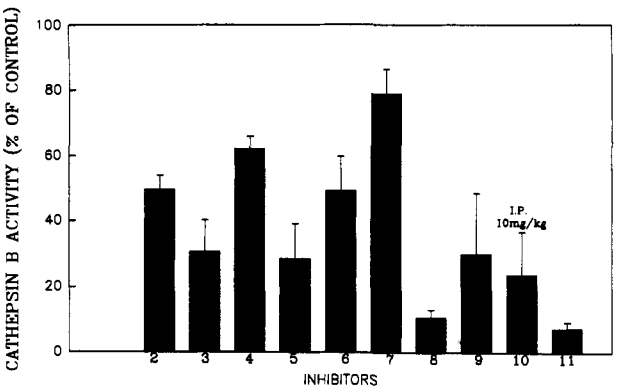


Figure 1. Effect of inhibitors on rat liver cathepsin B activity (4–5 h postdose) by a single oral dose. Inhibitors **2–9**, and 11 at 50 mg/kg po and inhibitor **10** at 10 mg/kg ip; all measured at 4–5 h postdose.

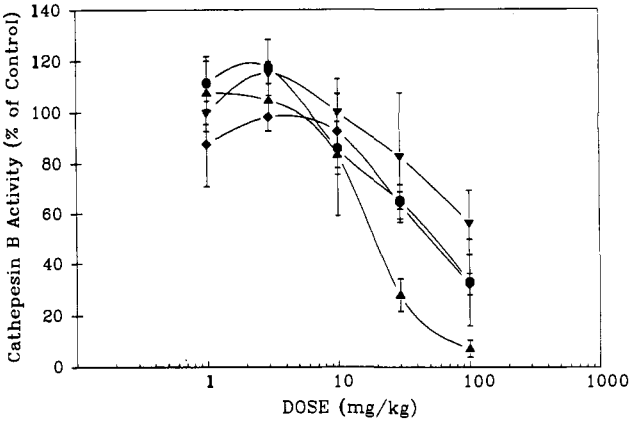


Figure 2. Dose–response of rat liver cathepsin B (4–5 h postdose) to oral administration of inhibitor. Symbol (compound, ED_{50}): (●) **2**, 130 mg/kg; (▼) **3**, 52 mg/kg; (◆) **5**, 44 mg/kg; (▲) **8**, 18 mg/kg (at 4–5 h postdose).

Table 3. Effect of Inhibitors on Rat Liver Cathepsin B by a Single Oral Dose[a]

compound	dose (mg/kg)	cathepsin B % control
1	100	78 ± 8
2	50	50 ± 4
3	50	31 ± 4
4	50	62 ± 4
5	50	29 ± 11
6	50	49 ± 11
7	50	79 ± 8
8	50	11 ± 3
9	50	30 ± 19
11	50	7 ± 2

[a] Enzyme activities were determined *ex vivo* at 4–5 h post dose; for each experiment, enzyme activity was assayed at two dilutions (in triplicate) from each animal, using 4–5 animals per group.

Compounds were administered by gavage suspended in carboxymethyl cellulose (CMC) vehicle. At 4–5 h postdose, the livers were removed and analyzed for cathepsin B activity. These studies revealed the fluoromethyl ketone 11 and the Z-Phe-Lys (acyloxy)methyl ketone 8 to be the most potent inhibitors of rat liver cathepsin B, each producing approximately 90% inhibition of enzyme activity (Table 3). Compounds **3**, **5**, and **9** were slightly less effective (~70% inhibition).

The dose dependence for cathepsin B inhibition was investigated for four of these compounds. As shown in Figure 2, the Z-Phe Lys inhibitor 8 was the most active with an ED_{50} of 18 mg/kg. The succinyl-Phe-Ala analog

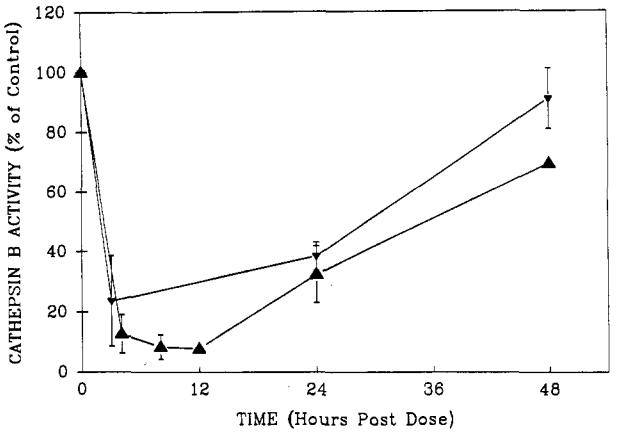


Figure 3. Duration of action of inhibitors on rat liver cathepsin B activity. Symbol (tissue, route): (▲) 8, 50 mg/kg po; (▼) 10, 10 mg/kg ip.

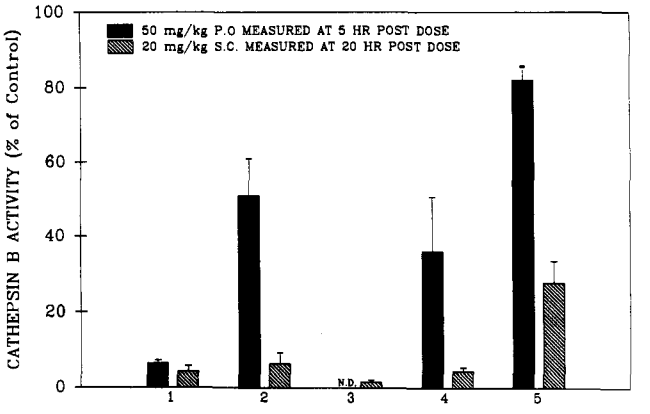


Figure 4. Effect of inhibitor 8 on rat tissue cathepsin B activity: (solid bars) 50 mg/kg po, measured at 5 h postdose; (hashed bars) 20 mg/kg sc, measured at 20 h postdose. (1) Liver, (2) heart, (3) spleen, (4) pancreas, (5) muscle; ND = not determined.

3 and Z-Phe-Gly analog 5 demonstrated modest activity with ED_{50} values of 52 and 44 mg/kg po, respectively (Figure 2).

The Z-Phe-Lys (mesitoyloxy)methyl ketone 8, demonstrated by these studies to be a potent inhibitor of rat liver cathepsin B following either ip or oral administration, was therefore selected for further investigation. The related compound 9, designed to have potentially improved metabolic stability, did not appear to provide a significant advantage over 8 (Table 3).

In a duration-of-action study, a single 50 mg/kg po dose of 8 produced a rapid decline in rat liver cathepsin B activity, followed by sustained inhibition for approximately 24 h. Between 24 and 48 h, enzyme activity gradually returned to normal levels. This time course of inhibition is similar to that obtained with 10 mg/kg ip dosing of E-64 (10) (Figure 3) and to that reported[27] for 100 mg/kg po dosing of EST (the ethyl ester of E-64c) to hamsters. Since these compounds are irreversible inhibitors of cathepsin B, the recovery phase presumably represents the time required by hepatocytes to synthesize new enzyme. With significant cathepsin B inhibition by 8 established in the liver, the effects of this compound on other tissues was investigated.

Cathepsin B Inhibition in Other Tissues. For application to the treatment of specific diseases, inhibitor 8 was tested for its effects on potential target tissues following a single 50 mg/kg dose (po). The tissues selected for analysis were (1) heart (myocardial infarction), (2) skeletal muscle (muscular dystrophy), (3) pancreas (pancreatitis), and (4) spleen and peritoneal macrophages (immune-based diseases).

Following a single oral 50 mg/kg dose of 8 to rats, the inhibition observed in heart and pancreas, although significant (50–70%), was not as great as that determined in the liver (~90%). There was virtually no effect on resident peritoneal macrophages. The weak inhibition (18%) of cathepsin B activity measured in skeletal muscle may reflect the difficulty in delivering the inhibitor to peripheral tissues.

Since structural variations might confer improved inhibitor bioavailability, compounds 6 and 9 were also tested for activity in target tissues, following a single 50 mg/kg oral administration. The succinyl-Phe-Gly analog 6 and Z-Tyr(OMe)-Lys analog 9 demonstrated similar patterns of inhibition of cathepsin B; although there was good inhibition in the liver (56 ± 11%, 70 ± 19%, respectively), little to no activity was demonstrated in other tissues (data not shown).

***Ex Vivo* Assay for Inhibition of Cathepsin B after Subcutaneous Administration.** In an attempt to further improve cathepsin B inhibition in tissues other than liver, compound 8 was formulated at 20 mg/kg in three different vehicles for sc administration: carboxymethyl cellulose (CMC), 50% PEG-400/saline, and a phosphatidyl choline (PC)-liposome vehicle. Since absorption from subcutaneous administration was expected to be slower than from an oral route of administration, tissues were harvested at 20 h postdose for analysis. Optimal activity in peripheral tissues such as heart and skeletal muscle following sc administration of 8 at 5 mg/kg was confirmed in a subsequent duration of action study to be 12–20 h postdose (see below, Figure 6).

Using a 20 mg/kg sc dose of 8, very strong inhibition of cathepsin B in liver and spleen was achieved, with the three vehicles providing equivalent results (92–96% inhibition in liver; 98–99% inhibition in spleen). Further experiments, using the CMC formulation (8, 20 mg/kg sc) provided very potent and sustained inhibition of cathepsin B in the liver (96 ± 1%), heart (94 ± 3%), spleen (98 ± 1%), and pancreas (96 ± 1%), with somewhat less inhibition obtained in skeletal tissue (72 ± 6%) (Figure 4). This indicates that employing the subcutaneous route of administration to avoid first-pass metabolism improves the activity of 8 in peripheral tissues.

Potent inhibitory activity in peripheral target tissues, at 20 h following sc administration, was confirmed by dose response studies. ED_{50} values were determined for liver, heart, and skeletal muscle as 2.4, ~0.1, and 1.0 mg/kg sc, respectively (Figure 5).

To further characterize the inhibitory action of 8 administered subcutaneously, the time course of inhibition was examined following a 5 mg/kg dose. Effective and long-lasting inhibition of cathepsin B was observed in the liver, heart, and skeletal muscle (Figure 6). Maximum levels of inhibition in the liver and heart were achieved within 6 h and were maintained for the 6–18-h time period. In skeletal muscle, the onset of inhibitory action was more gradual, but 40–50% inhibition was maintained from 12 to at least 48 h postdose (Figure 6).

A very gradual restoration of activity in these tissues was observed, between 24 and 48 h. However, the return of cathepsin B activity in the liver was slower than had been observed following oral administration. This may

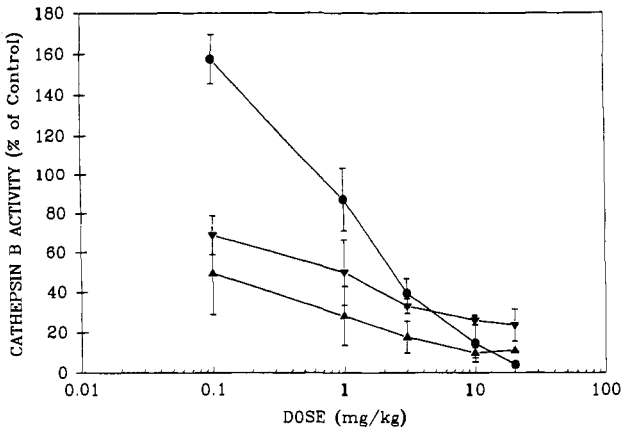


Figure 5. Dose-response of rat tissue cathepsin B (at 20 h postdose) to subcutaneous administration of inhibitor 8. Symbol (tissue, ED_{50}): (●) liver, 2.4 mg/kg; (▲) heart, 0.1 mg/kg; (▼) skeletal muscle, 1.0 mg/kg at 20 h postdose.

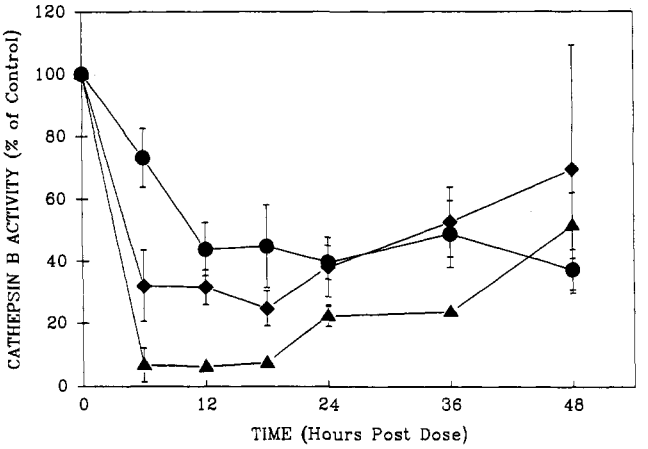


Figure 6. Duration of action of inhibitor 8 on rat peripheral tissue cathepsin B activity, dosed at 5 mg/kg sc. Symbol (tissue): (▲) liver; (◆) heart; (●) skeletal muscle.

reflect a gradual, prolonged release of the inhibitor from the subcutaneous site of administration (i.e., depot effect).

Stability of Inhibitor 8. Although inhibitor 1 is stable to the *in vitro* cathepsin B assay conditions ($t_{1/2}$ >24 h, pH 6.0, 25 °C, 1 mM dithiothreitol) and other media (e.g., MeOH; MeOH + catalytic CF_3COOH), it was found to undergo epimerization to a mixture of the L,L and L,D diastereomers in basic methanol (e.g., 95% aqueous MeOH, 4 mM K_2CO_3). Inhibitor 8 (as the HCl salt) was found to be more susceptible, apparently epimerizing in neat ethanol solution and more rapidly under basic conditions. Further stability studies, using *stable* aqueous suspensions of 8 in CMC dosing vehicle, indicated that (acyloxy)methyl ketone 8 was quite stable to chemical changes (human plasma $t_{1/2} \gg 33$ h; rat plasma $t_{1/2}$ 1.6 h), but was rapidly epimerized. The stability of 8 could be further demonstrated in that this inhibitor (at 2 μM) was unchanged for at least 90 min in human plasma (37 °C) containing added glutathione (3–20 mM) as a representative bionucleophile.

Conclusions

In a previous report,[14] we established peptidyl (acyloxy)-methyl ketones as *in vitro* cathepsin B inhibitors, with the inhibitory activity characterized as time-dependent and irreversible, active-site directed, stoichiometric, and selective for cysteine proteinases. Variation of the peptidyl affinity group and carboxylate leaving group provided

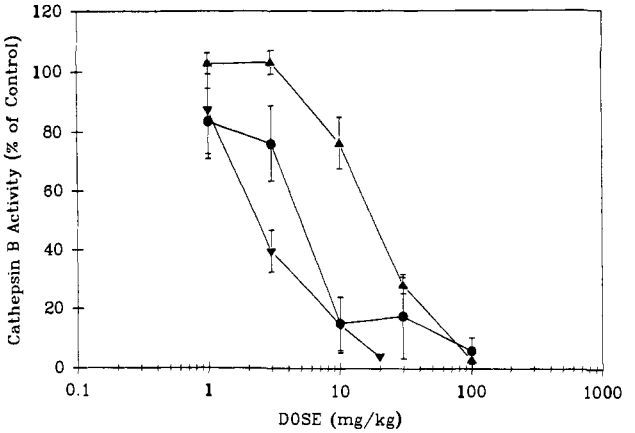


Figure 7. Effect of the route of administration of inhibitor 8 on rat liver cathepsin B activity. Symbol (route, ED_{50}): (▲) po, 18 mg/kg; (●) ip, 5.0 mg/kg; (▼) sc, 2.4 mg/kg. The confidence limit for 8 was 1.6–9.6 mg/kg for the ip route, 13.4–23.6 mg/kg for the po route, and 0.64–2.37 mg/kg for the sc route.

profound effects on the cathepsin B inactivation rate. In the present studies, our efforts were focused on investigating variations in inhibitor structure toward the optimization of bioavailability and *in vivo* cathepsin B inhibition. Further objectives included characterization of the effects of mode of administration on the *in vivo* inhibitory activity.

Testing of several inhibitors derived from 1, dosed ip or po to rats, indicated that the incorporation of polar or charged functional groups at the N-terminal group, acyloxy group, or P_1 side chain provided significantly enhanced *in vivo* cathepsin B inhibition (assayed *ex vivo*) (Table 2, Figure 2). There was no obvious correlation between the *in vitro* cathepsin B inactivation rates (Table 1) and the *in vivo* inhibitory activities, suggesting that the differences in bioavailability, metabolism, and other such factors *in vivo* were of prime importance. The peptidyl affinity group in the Z-Phe-Lys analog 8 gives rise to a high inactivation rate[14] and presumably enhanced bioavailability (compared to 1), thereby identifying this compound as our lead inhibitor for *in vivo* studies. Dose–response studies provided ED_{50} values for 8 as 18 mg/kg po, 5.0 mg/kg ip, and 2.4 mg/kg sc in liver (Figure 7). However, in other target tissues the inhibitory effect was significantly reduced. The relative lack of cathepsin B inhibitory activity observed in these peripheral target tissues was considered to reflect first-pass metabolism by the liver.

Parenteral routes of administration, such as subcutaneous and intramuscular, avoid this effect by initial absorption of the compound into the venous and/or lymphatic systems providing alternate routes to the heart and lungs, before reaching other peripheral tissues and the liver.[32] As expected, potent cathepsin B inhibition was obtained in five different tissues following sc administration of 8 (Figure 5). ED_{50} values of 2.4, 1.0, and 0.1 mg/kg were determined for the liver, skeletal muscle, and heart, respectively (Figure 4).

These investigations have demonstrated that peptidyl (acyloxy)methyl ketones such as 8 although subject to epemerization are otherwise chemically stable, yet potent inactivators of cysteine proteinases such as cathepsin B. Structural elements in both the peptidyl affinity group and carboxylate leaving group may be varied so as to control not only the inhibitor stability and *in vitro* enzyme inactivation rate, but also the *in vivo* activity. We have

identified 8 as a very effective *in vivo* cathepsin B inhibitor (in rats), with potent action observed against the liver enzyme by three different routes of administration (Figure 7). Inhibition in peripheral tissues was demonstrated only by the sc route of administration (Figures 4–6). These findings, and the selective nature of the enzyme inhibition by 8,[14,17a] establish this inhibitor as a valuable probe for the elucidation of biochemical processes and the potential for the development of a therapeutic utility.

Experimental Section

Chemistry. General experimental information, and compounds 1, 5, and 8 (as the CF_3COOH salt), have been described previously.[14,15,33] Z-Phe-Ala-CH_2F[31] was a mixture of L,L and L,D diastereomers and was obtained from Dr. David Rasnick of Enzyme Systems Products. Peptidyl epoxide E-64 was obtained from Sigma Chemical Co. Proton NMR were recorded with a Bruker WP80 at 80 MHz, with chemical shifts reported relative to tetramethylsilane at 0 ppm. HPLC analyses were performed by using a Perkin-Elmer Pecosphere 3X3C C-18 reverse-phase cartridge column (0.46 × 3.3 cm), with an elution gradient of 0–100% CH_3CN in 50 mM NH_4OAc buffer (pH 7) over 10 min, at 3.0 mL/min, with UV detection at 254 nm. Mass spectra (which were recorded with a Finnigan MAT-CH7, MAT-311A, or TSQ-70 instrument) and elemental analyses were determined by Syntex Analytical Research, Palo Alto, CA.

N-Methoxysuccinyl-L-phenylalanyl-L-alanyl [(2,4,6-Trimethylbenzoyl)oxy]methyl Ketone (2). A solution of L-phenylalanyl-L-alanyl [(2,4,6-trimethylbenzoyl)oxy]methyl ketone hydrochloride (1.15 mmol, 500 mg) (mp 132–133 °C[14,33]) in CH_2Cl_2 (30 mL) was cooled to 0 °C and then treated with N-methylmorpholine (2.3 mmol, 255 μL). Monomethyl succinate (1.27 mmol, 168 mg) was added, followed by 3-ethyl-1-[3-(dimethylamino)propyl]carbodiimide hydrochloride [EDCI] (1.27 mmol, 244 mg) and 4-(dimethylamino)pyridine [DMAP] (10 mg). The reaction mixture was stirred at 0 °C for 2 h and then at room temperature overnight (18 h). The reaction mixture was then rotary evaporated, and the residue was dissolved in ethyl acetate. The solution was washed with dilute HCl (1 N), water, saturated aqueous $NaHCO_3$, and brine; dried (Na_2SO_4); and rotary evaporated to give a light yellow solid residue. This residue was recrystallized (EtOAc/hexanes) and then further purified by silica gel column chromatography (EtOAc as eluent) to give 203 mg (35%) of the product 2 as a white powder: mp 185–186 °C; ^{1}H-NMR ($CDCl_3$) δ 7.3 (s, 5 H, Ph), 7.0 (br d, N*H*), 6.9 (s, 2 H, $Me_3C_6H_2CO$), 6.1 (br d, N*H*), 4.9 (app s, 2 H, $COCH_2O$), 4.7 (m, 2 H, 2 × NHC*H*CO), 3.6 (s, 3 H, CH_3OCO), 3.2 (app d, 2 H, $CHCH_2Ph$), 2.7 (m, 4 H, $COCH_2CH_2CO$), 2.4 and 2.3 (2 s, 9 H, $(CH_3)_3Ar$), 1.3 (d, 3 H, $CHCH_3$); HPLC t_R 6.09 min (>95%); electron impact mass spectrum, *m/z* 511 (MH^+, 10), 305 (30), 262 (100), 234 (100), 147 ($Me_3C_6H_2CO^+$, 100); exact mass calculated for $C_{28}H_{34}N_2O_7$ 510.2366, measured 510.2365. Anal. C, H, N (±0.4%) for $C_{28}H_{34}N_2O_7$.

N-(Hydroxysuccinyl)-L-phenylalanyl-L-alanyl [(2,4,6-Trimethylbenzoyl)oxy]methyl Ketone (3). A solution of L-phenylalanyl-L-alanyl [(2,4,6-trimethylbenzoyl)oxy]methyl ketone hydrochloride (2.3 mmol, 1.0 g) (as above) in anhydrous DMF (35 mL) was treated with *N*-methylmorpholine (2.3 mmol, 255 μL). After the mixture was stirred for 5–10 min, succinic anhydride (6.9 mmol, 693 mg) was added, and the reaction mixture was stirred overnight at room temperature. The mixture was then diluted with diethyl ether; washed with dilute HCl (1 N), water, and brine; dried (Na_2SO_4); and rotary evaporated to give an off-white solid residue. Recrystallization (EtOAc/hexanes) gave 528 mg (46%) of the product 3 as a white powder: mp 193–194 °C; ^{1}H-NMR (acetone-d_6) δ 7.8 (br d, N*H*), 7.5 (br d, N*H*), 7.3 (s, 5 H, Ph), 6.9 (s, 2 H, Me_3C_6HCO), 5.0 (app s, 2 H, $COCH_2O$), 4.7 (m, 2 H, 2 × NHC*H*CO), 3.1 (m, 2 H, $CHCH_2Ph$), 2.5 (m, 4 H, $COCH_2CH_2CO$), 2.3 (2 s, 9 H, $(CH_3)_3Ar$), 1.3 (d, 3 H, $CHCH_3$); HPLC t_R 4.52 min (>95%); electron impact mass spectrum, *m/z* 478 ($(M - H_2O)^+$, 5), 248 (10), 220 (15), 147 ($Me_3C_6H_2CO^+$, 50), 120 (100); exact mass calculated for ($C_{27}H_{32}N_2O_7 - H_2O$) 478.2104, measured 478.2111.

N-[4-[(Phenylsulfonamido)carbonyl]benzoyl]-L-phenylalanyl-L-alanyl [(2,4,6-Trimethylbenzoyl)oxy]methyl Ketone (4). In a similar manner to that described for the preparation of 2, a solution of L-phenylalanyl-L-alanyl [(2,4,6-trimethylbenzoyl)oxy]methyl ketone hydrochloride (2.3 mmol, 1.0 g) in CH_2Cl_2 (35 mL) was cooled to 0 °C and then treated with *N*-methylmorpholine (5.1 mmol, 560 μL). 4-[(Phenylsulfonamido)carbonyl]benzoic acid (2.54 mmol, 780 mg) (mp 267–270 °C[33]) was added, followed by EDCI (2.54 mmol, 440 mg) and DMAP (10 mg). The reaction mixture was stirred at 0 °C for 2 h and then at room temperature overnight (18 h). The reaction mixture was then rotary evaporated, and the residue was dissolved in ethyl acetate. The solution was washed with dilute HCl (1 N), water, and brine; dried (Na_2SO_4); and rotary evaporated to give an off-white solid residue. Recrystallization (EtOAc/hexanes) gave 817 mg (52%) of the product 4 as a white powder: mp 182–185 °C; ^{1}H-NMR (acetone-d_6 plus 1 drop CF_3COOH) δ 8.1 (m, 4 H, Ar*H*), 7.8 (m, 5 H, Ar*H*), 7.3 (m, 5 H, Ph), 6.9 (s, 2 H, $Me_3C_6H_2CO$), 5.0 (app s, 2 H, $COCH_2O$), 4.9 (m, 1 H, NHC*H*CO), 4.6 (m, 1 H, NHC*H*CO), 3.3 (m, 2 H, $CHCH_2Ph$), 2.3 (2 s, 9 H, $(CH_3)_3Ar$), 1.3 (d, 3 H, $CHCH_3$); HPLC t_R 5.16 min (≥93%); LSIMS/FAB MS-MS mass spectrum, *m/z* 684 (MH^+, 2), 250 (8), 147 (100). Anal. C, H, N (±0.3%) for $C_{37}H_{37}N_3O_8S \cdot 2H_2O$.

N-(Hydroxysuccinyl)-L-phenylalanylglycyl [(2,4,6-Trimethylbenzoyl)oxy]methyl Ketone (6). Using methodology similar to that used to prepare 3, Z-L-Phe-Gly-CH_2OCO-(2,4,6-Me_3)Ph (5) was N-deprotected to the HCl salt (mp 126–127 °C) and then acylated with succinic anhydride. The crude product was purified by silica gel column chromatography (10% MeOH/CH_2Cl_2) to give 6 in 37% yield, mp 114–118 °C. Further purification by preparative reverse-phase chromatography (70% MeCN/H_2O as eluent) afforded 8: mp 124–126 °C; HPLC t_R 4.30 min (>98%); LSIMS/FAB mass spectrum, *m/z* 483 (MH^+, 38), 147 (8). Anal. C, H, N (±0.4%) for $C_{26}H_{30}N_2O_7$.

N-(Benzyloxycarbonyl)-L-phenylalanyl-L-alanyl [(2,3,5,6-Tetramethyl-4-carboxybenzoyl)oxy]methyl Ketone (7). Anhydrous potassium fluoride (6.7 mmol, 390 mg) was added to a solution of Z-L-phenylalanyl-L-alanyl bromomethyl ketone (2.2 mmol, 1.0 g) in 30 mL of anhydrous DMF. The mixture was stirred for 3 min at room temperature, 2,3,5,6-tetramethylterephthalic acid (7.8 mmol, 1.74 g) [mp >300 °C; prepared by CrO_3/H_2SO_4 oxidation of 2,3,5,6-tetramethyl-*p*-xylene-α,α′-diol[33]] was added, and the mixture was stirred for 3 h at room temperature. The mixture was diluted with ethyl ether and then washed with dilute HCl (1 N), water (2×), and brine (2×). The crude product which precipitated from the organic phase was filtered, washing with acetone, to give a white powder characterized as the dialkylation product (43 mg, 10%): mp 210–211 °C; LSIMS/FAB mass spectrum, *m/z* 955 (MH^+, 9). Anal. Calcd for $C_{54}H_{58}N_4O_{12}$: C, 67.91; H, 6.12; N, 5.87. Found: C, 67.36; H, 6.12; N, 5.87. The filtrate was evaporated, and the residue obtained was recrystallized from hot ethanol to give 788 mg (60%) of 7 as a white powder: mp 198–200 °C; ^{1}H-NMR (acetone-d_6) δ 7.8 (br d, 1 H, N*H*), 7.3 (2 s, 10 H, 2 × Ph), 6.6 (br d, 1 H, N*H*), 5.0 (2 s, 4 H, $PhCH_2O$ and $COCH_2O$), 4.5 (m, 2 H, 2 × NHC*H*CO), 3.1 (m, 2 H, $CHCH_2Ph$), 2.23 and 2.25 (2 s, 12 H, $(CH_3)_4Ar$), 1.3 (d, 3 H, $CHCH_3$); HPLC t_R 4.40 min (≥94%); LSIMS/FAB mass spectrum, *m/z* 589 (MH^+, 3), 205 (28), 185 (100). Anal. C, H, N (±0.3%) for $C_{33}H_{36}N_2O_8$.

N-(Benzyloxycarbonyl)-L-phenylalanyl-L-lysyl [(2,4,6-Trimethylbenzoyl)oxy]methyl Ketone Hydrochloride Salt (8). Preparation of the CF_3COOH salt of 8 (mp 129–131 °C), by deprotection of the *N*-Boc analog by treatment with CF_3COOH in CH_2Cl_2, has been described previously.[14] To prepare the HCl salt form, a solution of *N*-(benzyloxycarbonyl)-L-phenylalanyl-N^{ϵ}-Boc-L-lysyl [(2,4,6-trimethylbenzoyl)oxy]methyl ketone (12 mmol, 8.0 g) (mp 161–162.5 °C[14]) in CH_2Cl_2 (400 mL) was cooled to 0 °C. Anhydrous HCl gas was gently bubbled into the stirred solution for 15 min. The reaction mixture was then allowed to warm to room temperature and was stirred for 2 h at that temperature. Solvent was removed by rotary evaporation; anhydrous diethyl ether was added to and then rotary evaporated from the residue twice to give a white solid residue. This residue was washed with ethyl acetate (2×) and hexane (3×) and dried at high vacuum to give 7.32 g (98%) of the hydrochloride salt 8 as a white powder: mp 176–178 °C; ^{1}H-NMR (CD_3OD) δ 7.5–7.1

(m, 10 H, 2 Ph), 6.9 (s, 2 H, Me_3C_6HCO), 5.1 (s, 2 H, $PhCH_2O$), 4.8 (s, exchangeable protons), 4.7 (app s, 2 H, $COCH_2O$), 4.7–4.3 (m, 2 H, 2 NHCHCO), 3.3–2.7 (m, 4 H), 2.4 and 2.3 (2 s, $(CH_3)_3$-Ar), 2.2–1.2 (br m, 6 H); LSIMS/FAB mass spectrum, m/z 588 (MH^+, 38), 570 (4), 147 ($Me_3C_6H_2CO^+$, 100), 126 (16), 91 (46); HPLC t_R 6.73 min (>97%). Anal. C, H, N (±0.2%) for $C_{32}H_{42}N_3O_6Cl\cdot H_2O$. In the *in vitro* cathepsin B inhibition assay, the HCl and CF_3COOH salts of 8 were found to be equivalent.

Alternatively, a solution of *N*-(benzyloxycarbonyl)-L-phenylalanyl-N^{ϵ}-(trifluoroacetyl)-L-lysyl [(2,4,6-trimethylbenzoyl)oxy]-methyl ketone (50 mg) (mp 182–183 °C[33]) in anhydrous methanol (25 mL), previously saturated with anhydrous hydrogen chloride, was stirred at room temperature overnight. The solution was then rotary evaporated, and the residue was washed with ethyl acetate and ether to give the hydrochloride salt product 8 as a white solid, mp 165–167 °C dec.

N-(Benzyloxycarbonyl)-O-methyl-L-tyrosyl-L-lysyl [(2,4,6-Trimethylbenzoyl)oxy]methyl Ketone Trifluoroacetate Salt (9). Using methodology similar to that used to prepare 8, the *N*-hydroxysuccinimide ester of *N*-(benzyloxycarbonyl)-*O*-methyl-L-tyrosine was prepared (75%, mp 106–108 °C) and then used to acylate N^{ϵ}-Boc-L-lysine to give Z-L-Tyr(OMe)-L-Lys(NHBoc)-OH (73%, mp 103–106 °C). This dipeptide derivative was then converted to the corresponding bromomethyl ketone (70%, mp 143–145 °C), which was used to alkylate 2,4,6-trimethylbenzoic acid to give the corresponding (acyloxy)methyl ketone in 90% yield, mp 130.5–133 °C, by our usual procedures.[14,15] Removal of the Boc protecting group, as described[14] for 8, gave the product as the CF_3COOH salt in 83% yield: mp 120–123 °C; HPLC t_R 6.67 min (>96%); LSIMS/FAB mass spectrum, m/z 618 (MH^+, 90), 600 (4), 456 (4), 240 (20), 147 ($Me_3C_6H_2CO^+$, 100), 91 (58). Anal. C, H, N (±0.1%) for $C_{37}H_{44}N_3O_9F_3$.

***In Vitro* Enzyme Kinetics.** Second-order inactivation rates (k/K, M^{-1} s^{-1}) were determined for purified bovine spleen cathepsin B, using a fluorometric continuous assay in 0.1 M potassium phosphate, 1.25 mM EDTA, and 1 mM dithiothreitol, pH 6.0, 25 °C, as described previously.[14]

***In Vivo* Methods.** Female Lewis Cr/Br rats (150–200 g each) were purchased from Charles River, Bloomington, MA. After 1 week of acclimation in-house, the animals (usually four per group) were dosed by the selected route of administration. Test compounds were suspended in carboxymethyl cellulose (CMC) just prior to dose, except as noted in the text. In control studies animals dosed with only CMC vehicle gave results essentially identical to untreated animals. Therefore, for studies in this report, the CMC-treated animals were used as the control group.

Tissue Homogenate Enzyme Preparation. At the appropriate time postdose, the treated animals were anesthetized with CO_2/O_2 (60/40), decapitated, and exsanguinated. The tissues of interest were removed, quickly frozen in liquid nitrogen, and then stored at −70 °C until ready for processing. All subsequent manipulations of the tissue samples were carried out at 4 °C. Liver and skeletal muscle were pulverized while still frozen and then homogenized, while other target tissues were homogenized without prior pulverization. The tissue homogenization, in distilled water or 0.1% Brig-35, was subsequently performed using three 15-s bursts with a 10 N probe on a Tekmar Tissuemizer set to 75–80% power. The samples were centrifuged at 15000*g* for 40 min, which partitioned into an upper lipid layer, a lower clarified layer, and a solid pellet. The clarified supernatant was carefully aspirated and transferred to clean polypropylene tubes for storage at −70 °C, until the fluorimetric assay for enzyme activity could be performed.

Purified Lysosomal Enzyme Preparation. The procedure was based on that reported by Bohley et al.[28] and Barrett and Kirshke.[1] At the appropriate time postdose, the treated animals were anesthetized with sodium barbital, and the livers were perfused *in situ* with ice-cold saline. The livers were then removed, rinsed in ice-cold saline, blotted, and weighed. The animals were sacrificed by CO_2 asphyxiation. All subsequent manipulations of the tissue samples were carried out at 4 °C. The livers were homogenized in 2 volumes of 0.25 M sucrose at 0 °C with a 30-mL Wheaton Teflon-on-glass homogenizer, using five full strokes with a motor setting at 55. Following centrifugation at 600*g* for 10 min, the supernatant was transferred to clean tubes for centrifugation at 3000*g* for 10 min. The resulting supernatant was centrifuged at 15000*g* for 15 min. The light mitochondrial pellet was washed twice with 0.25 M sucrose, lysed in 2.5 volumes of distilled water using a glass-on-glass homogenizer, and then centrifuged at 10000*g* for 60 min. The supernatant was stored at −70 °C until the fluorimetric assay for enzyme activity was performed.

***Ex Vivo* Fluorimetric Assays.** The fluorimetric assay for cathepsin B was adapted from methods published by Barrett and Kirschke.[1] The stock assay buffer was 0.1 M KH_2PO_4 (pH 6.0) containing 1 mM Na_2EDTA and 1 mM dithiothreitol, which was added just prior to use. The substrate used for cathepsin B was Z-Arg-Arg-NMec (Peninsula Laboratories), which was dissolved in dimethyl sulfoxide (10 mM) and then diluted to 20 μM in distilled water. The "enzyme samples" (isolated as above) were diluted with distilled water or 0.1% Brij-35 (Sigma) as necessary. The assay "stopping reagent" (buffer) consisted of 100 mM sodium chloroacetate, 30 mM sodium acetate, and 70 mM glacial acetic acid (pH 4.3). Appropriate blanks were prepared by starting with the stopping reagent.

Assay Technique. The fluorimetric assay was initiated by the addition of 250 μL of the substrate solution to duplicate tubes containing 500 μL of the diluted enzyme samples plus 250 μL of the assay buffer. After vortexing, these assay solutions were incubated at 37 °C for 30 min. The enzymatic reaction was then quenched by the addition of 1.0 mL of the stopping reagent. Fluorescence of these solutions was measured by using a Perkin-Elmer Model 650-40 fluorimeter (λ_{ex} 340 nm, λ_{em} 460 nm). A standard curve, prepared using 7-amino-4-methylcoumarin (Sigma) dissolved in 1:1 assay buffer/stopping reagent, was linear and optimized in the micromolar range.

The same diluted test solutions were also assayed for total protein, using the reagents obtained from Pierce.[34] Specific activity was calculated, and the values were reported as a percent of the vehicle control activity.

A microtiter plate modification of the fluorimetric assay was performed using a Flow Skan II instrument (λ_{ex} 355 nm, λ_{em} 460 nm). [Equivalent fluorescence readings were obtained with the two instruments (Perkin-Elmer and Flow Skan II), using the same λ_{ex} and λ_{em}.] In this case, the assay solution volumes were decreased proportionately to accommodate the size of the wells in the microtiter plate, and the assay was conducted in the plate.

For both fluorimetric assays, test enzyme solutions were prepared at several dilutions using distilled water or 0.1% Brij-35 to determine the appropriate concentration for the linear range of the standard curve. Two or more different concentrations were selected for fluorimetric analysis, with each of these measured in triplicate. In a typical experiment, this was carried out for each of four or five animals per tissue and treatment. Some biovariability was evident, as reflected by the standard deviations (indicated by the error bars in the figures and error values given in the text).

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