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Inhibition of rat liver cathepsins B and L by the peptide aldehyde benzyloxycarbonyl-leucyl-leucyl-leucinal and its analogues

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Abstract

Cathepsins B and L belong to the papain superfamily of cysteine proteases and play important roles in various physiological and pathological processes. In the course of studies on their inhibitors, we examined the inhibitory effects of the peptide aldehyde benzyloxycarbonyl-leucyl-leucyl-leucinal (ZLLLal) and its analogues. As a result, rat liver cathepsins B and L were shown to be strongly inhibited by them. The concentration required for 50% inhibition (IC_{50}) by ZLLLal was 88 nM for cathepsin B and 163 nM for cathepsin L. Moreover, various analogues of ZLLLal, including 2-furancarbonyl-, nicotinyl-, isonicotinyl- and 4-morpholinylsuccinyl-LLLals, and some acetyl-Pro (AcP)-containing analogues, AcPLLLal and AcPPLLLal, were shown to inhibit both enzymes more strongly than ZLLLal. Among them, isonicotinyl-LLLal was most inhibitory against both cathepsins B (IC_{50}, 12 nM) and L (IC_{50}, 20 nM). Several of these inhibitors were indicated to be somewhat more soluble in aqueous media than ZLLLal.

Keywords: *Cathepsin B, cathepsin L, cysteine protease inhibitor, IC_{50}, peptide aldehyde, inhibition*

Introduction

Among the low molecular weight inhibitors of cysteine proteases, most potent inhibitors are irreversible inhibitors such as certain nitriles, halomethyl ketones, acyloxymethyl ketones, vinyl sulfones, and epoxysuccinates, which covalently alkylate the active site thiol groups of the enzymes [1]. Alternatively, certain aldehyde-based inhibitors reversibly inhibit them by reacting with the active site thiol groups to form tetrahedral hemithioacetal intermediates [1]. Among them, benzyloxycarbonyl-leucyl-leucyl-leucinal (ZLLLal) draws special attention for its physiological effects. It was shown to be a strong inhibitor of calpain and proteasome [2], thus inhibiting the membrane fusion of erythrocytes in the presence of exogenous Ca^{2+} through inhibition of the autolytic activation of μ-calpain [3], or initiating neurite outgrowth and induce p53-dependent apoptosis through inhibition of proteasome [4–6]. So far, however, its inhibitory effects on other important intracellular cysteine proteases such as cathepsins B and L have not been investigated. Cathepsins B and L are members of the papain superfamily of cysteine proteases and suggested to play important biological roles in various physiological and pathological processes, including lysosomal proteolysis, tumor invasion and metastasis and arthritis [7,8]. Therefore, it is important to examine the inhibitory effects of ZLLLal and its analogues on these enzymes. Moreover, ZLLLal appears to have rather low solubility in water, and therefore it is thought to be useful to develop analogue inhibitors which might have higher solubility in aqueous media than ZLLLal.

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In the present study, we synthesized ZLLLal and its various analogues possessing an LLal or LLLal moiety and investigated their inhibitory effects on rat liver cathepsins B and L. As a result, ZLLLal was shown to be a strong inhibitor of these enzymes. Moreover, several of them proved to be stronger inhibitors against cathepsins B and L, and indicated to be more soluble in aqueous media than ZLLLal.

Materials and methods

Materials

Cathepsins B and L were purified from the crude extract supernatant of rat liver, as described previously [9]. HPLC was performed on a TSK-GEL CM (carboxymethyl)-2SW column (4.6 × 250 mm) (Toso Co., Tokyo) using a 1h-NaCl linear gradient (0 to 1.0 M) in 10 mM sodium acetate buffer, pH 5.0, containing 10 mM 2-mercaptoethanol at a flow rate of 1.0 ml/min in a Shimadzu LC-8A HPLC system. Elution was monitored at 280 nm and 1-ml fractions were collected. The major peak fractions of cathepsins B and L were eluted at around 0.18 M and 0.35 M NaCl, respectively, well separated from each other. Reagents for peptide synthesis and analyses were largely obtained from Wako Pure Chem. Ind., Tokyo. Z-Arg-Arg-4-methyl coumaryl-7-amide (MCA) and Z-Phe-Arg-MCA were obtained from Peptide Institute, Osaka. All other reagents used were of analytical grade.

Enzyme assay

Cathepsins B and L were assayed as described previously [10] with some modifications using Z-Arg-Arg-MCA and Z-Phe-Arg-MCA, respectively, as substrates. The assay mixture contained 50 μL of 0.1 M sodium acetate buffer (pH 5.0)-5 mM EDTA, 10 μL of 0.1 M 2-mercaptoethanol, 20 μL of water, 10 μL of an enzyme solution and 10 μL of the 100 μM substrate solution. After reaction at 37°C for 6 min, 100 μL of 10% SDS solution and 1.3 mL of 0.2 M Tris-HCl buffer (pH 9.0) were added to the reaction mixture to stop the reaction, and the fluorescence intensity at 450 nm was measured with excitation at 370 nm in a Jasco FP-6300 fluorescence spectrometer. The blank sample contained no enzyme. The effect of each inhibitor was measured in the same manner except that the enzyme in the reaction mixture without the substrate was preincubated for 30 min with the inhibitor at various concentrations.

Synthesis of peptide derivatives

The syntheses of peptide semicarbazones and their conversion to the corresponding aldehydes were performed as described previously [11–14] with some modifications. Figure 1 shows schematically the synthetic procedures for Z-Leu-Leu-Leu-al (ZLLLal) (7) and 2-furancarbonyl-Leu-Leu-Leu-al (FuLLLal) (**11**) as typical examples together with that of Leu-semicarbazone (*p*-toluenesulfonic acid salt) (Lsc(TosOH)) (**3**) used for the synthesis of ZLLLal (7). The detailed synthetic procedures for these compounds are described below. Except ZLLLal (7), all other peptide aldehydes, including nicotinyl-(Ni), isonicotinyl-(isoNi) and 4-morpholinylsuccinyl-(MoSu)LLLals, and Pro-containing analogues (see Table I) were synthesized in essentially the same manner as described for FuLLLal (**11**).

*Synthesis of Lsc(TosOH) (**3**).* Z-Leu-OMe (**1**) (21.0 g, 75 mmol) was dissolved in tetrahydrofuran (THF) (60 mL) and to this was added 1.0 M diisobutylaluminum hydride in THF (150 mL, 150 mmol) over 1 h under N_2 at −50°C. The mixture was stirred for further 5 h, then 4 M HCl (93.7 mL, 375 mmol) was carefully added. After gradually raising the temperature to room temperature, the mixture was extracted with ethylacetate, washed with water, then dried overnight

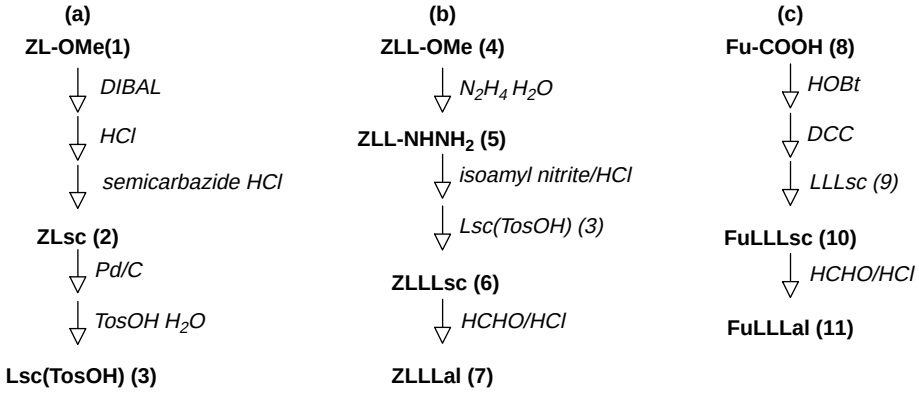
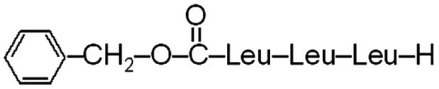
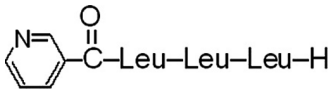
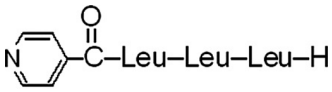
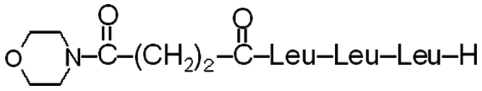
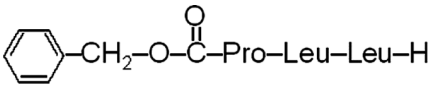
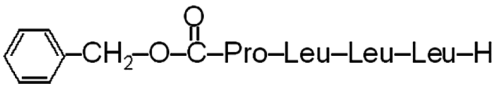
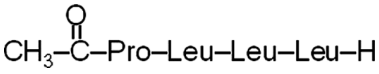
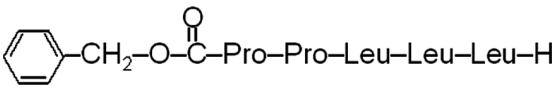
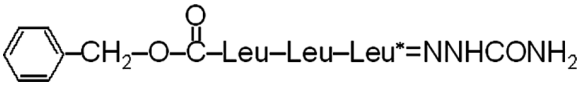
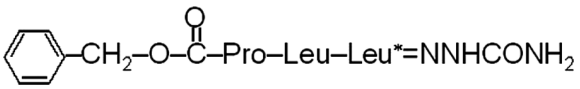


Figure 1. Synthetic procedures of ZLLLal and Its analogues. Synthetic procedures of Lsc(TosOH) (a), ZLLLal (b) and FuLLLal (c) are schematically shown. DIBAL, diisobutylaluminum hydride; Pd/C, activated palladium/charcoal; N_2H_4 H_2O, hydrazine hydrate; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide.

Table I. Structures of ZLLLal and its analogues synthesized in this study.

	Inhibitor*	Structure†
a	ZLLLal	
b	FuLLLal	
c	NiLLLal	
d	isoNiLLLal	
e	MoSuLLLal	
f	ZPLLLal	
g	ZPLLLal	
h	AcPLLLal	
i	APPLLLal	
j	AcPPLLLal	
k	ZLLLsc	
l	ZPLLsc	

* Inhibitors a, b and k are identical with compounds (**7**), (**11**) and (**6**), respectively, described in the text. † All the amino acid residues are in the L-form. Leu*: Leu residue which has a hydrogen atom, but no oxygen atom on the carbonyl carbon (i.e. —CH═NNHCONH$_2$).

with anhydrous sodium sulfate. The desiccant was removed by filtration and the filtrate was concentrated under reduced pressure below 40°C. To this was added 70% ethanol (100 mL), semicarbazide HCl (9.16 g, 82.5 mmol) and sodium acetate (6.77 g, 82.5 mmol), and the mixture was refluxed at 80°C for 10 min. The solution was concentrated under reduced pressure below 40°C, and the residue was mixed with ethylacetate (300 mL) and dried overnight with anhydrous sodium sulfate. After removing the desiccant by filtration, the filtrate was concentrated under reduced pressure below 40°C, and the residue was washed with petroleum ether by decantation and dried. Yield, 19.1 g (83%). ZLsc (**2**) thus prepared was converted to Lsc(TosOH) (**3**) as follows: To 10% palladium-charcoal (10 g) was added methanol (40 mL) under nitrogen. After replacing the reaction vessel with nitrogen, then hydrogen, the mixture was stirred for 2 h to activate palladium. To this was added ZLsc (**2**) (20.0 g, 65 mmol), and after nitrogen, then hydrogen replacement, the mixture was stirred for 48 h. The activated palladium-charcoal was removed by filtration, and the filtrate was mixed with TosOH monohydrate (11.4 g, 60 mmol) and concentrated under reduced pressure. To the residue was added ether to crystallize Lsc(TosOH) (**3**). Recrytallization was performed with methanol and ether. Yield, 5.33 g (24%).

Synthesis of ZLLLal (7). ZLL-OMe (**4**) (9.80 g, 25 mmol) was dissolved in methanol (25 mL) and to this was added hydrazine monohydrate (12.5 g, 250 mmol). The mixture was stirred for 1 h at room temperature, and then concentrated under reduced pressure. To the residue was added water to crystallize ZLL-$NHNH_2$ (**5**). Yield, 9.63 g (98%). ZLL-$NHNH_2$ (**5**) (7.84 g, 20 mmol) was dissolved in dimethylformamide (40 mL) and to this was added 3.5 N HCl/dioxane (17.2 mL, 60 mmol) and isoamyl nitrite (2.50 g, 22 mmol) at −50°C. After 2 h at −50°C, when the mixture became negative to the hydrazine test, it was brought to −60°C and neutralized with triethylamine (6.06 g, 60 mmol). To this was added Lsc(TosOH) (**3**) (7.56 g, 22 mmol) and N-ethylmorpholine (2.22 g, 22 mmol), and the mixture was stirred for 48 h at 0°C. After filtration of the reaction mixture, the filtrate was concentrated under reduced pressure. To the residue was added a small volume of methanol and water for crystallization. The separated crystal of ZLLLsc (**6**) was collected by filtration, washed with 5% citric acid and saturated NaCl aq. solution and dried in a desiccator. Recrystallization was performed with methanol and ether. Yield, 9.54 g (90%). To convert ZLLLsc (**6**) to ZLLLal (7), ZLLLsc (**6**) (100 mg) dissolved in methanol (3 mL) was mixed with 37% formalin (1 mL) and 4 M HCl (5 equivalents) and the mixture was stirred for 2 h at room temperature.

*Synthesis of FuLLLal (**11**)*. To 2-furan carboxylic acid (**8**) (0.11 g, 1 mmol) dissolved in THF (2 mL) was added 1-hydroxybenzotriazole (0.14 g, 1 mmol) and the mixture was stirred for 20 min at ice-cold temperature. To this was added dicyclohexylcarbodiimide (0.24 g, 1.2 mmol) and the mixture was stirred further for 30 min. LLLsc (**9**) (0.40 g, 1 mmol) prepared from ZLLLsc (**6**) was dissolved in THF (3 mL) and added to this and the mixture was stirred for 30 min at ice-cold temperature, then for 48 h at room temperature. Then a few drops of glacial acetic acid were added and the mixture was stirred for 30 min. After removing dicyclohexylurea by filtration, the filtrate was concentrated under reduced pressure and the residue was dissolved in dichloromethane (100 mL). After washing successively with 5% citric acid, 5% sodium bicarbonate and saturated sodium chloride, the solution was dried overnight with anhydrous sodium sulfate. The desiccant was removed by filtration and the filtrate was concentrated under reduced pressure. To the residue was added ether for crystallization of FuLLLsc (**10**). Recrystallization was performed with methanol and ether. Yield, 0.20 g (80%). Conversion of FuLLLsc (**10**) to FuLLLal (**11**) was performed in the same manner as for ZLLLal (**7**).

Purification of synthetic peptide derivatives by HPLC

Each peptide derivative was purified to homogeneity by HPLC on a Capcell Pak C_{18} ODS column (4.6 × 150 mm) (Shiseido Co., Tokyo) in a Shimadzu LC-10A HPLC system using a 1 h-gradient elution of acetonitrile (20–60%) in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min, monitored at 210 nm. To obtain a sufficient amount of each peptide derivative, HPLC was repeated several times. Some physico-chemical properties of the peptide derivatives thus prepared are shown in Table II.

Molecular weight determination

The molecular weight was determined by mass spectrometry using a JEOL (JMS-SX102/JMA-DA6000) mass spectrometer.

Determination of IC_{50} values

IC_{50} was determined by fitting each inhibition data set to the four-parameter IC_{50} equation for nonlinear regression analysis $[y = (range)/[1 + (x/IC_{50})^S] + background]$ using GraFit, ver. 4.0 [15].

Results and discussion

The structures of ZLLLal (Inhibitor a, (**7**)) and its analogues synthesized in this study are shown in Table I. In the first series (Inhibitors b–e) of the

Table II. Physico-chemical properties of ZLLLal and its analogues.

	Inhibitor	MS (m/z)	Mp (dec.) (°C)	$[\alpha]_D$ (°) (c(%), MeOH)	Rf value*	Retention time (min)†
a	ZLLLal	475	80–84	−63.9 (1.13)	0.76	37.1
b	FuLLLal	435	108–114	−55.5 (0.94)	0.72	20.1
c	NiLLLal	446	144–150	−66.7 (0.87)	0.74	19.3
d	isoNiLLLal	446	143–147	nd	0.68	21.9
e	MoSuLLLal	510	109–116	nd	0.68	11.7
f	ZPLLal	459	55–65	−85.4 (0.93)	0.64	27.1
g	ZPLLLal	572	150–155	−127.3 (0.94)	0.83	36.9
h	AcPLLLal	480	95–102	−84.5 (0.81)	0.65	12.7
i	ZPPLLLal	669	78–84	−151.8 (0.88)	0.75	36.3
j	AcPPLLLal	577	80–88	−174.2 (0.92)	0.77	14.5
k	ZLLLsc	532	110–117	−58.6 (1.02)	0.60	31.7
l	ZPLLsc	516	105–110	−88.3 (0.98)	0.53	26.8

* The Rf values were obtained by TLC using CH_3Cl:MeOH (5:1 by vol.) as a solvent.; † Retention time on HPLC. nd, not determined.

ZLLLal analogues, the Z group was substituted with somewhat less hydrophobic groups containing a heterocyclic ring. In the second series (Inhibitors f–j), the Z group of ZLLLal was replaced with Z-Pro, Z-Pro-Pro, Ac-Pro or Ac-Pro-Pro, or the first Leu of ZLLLal was replaced with a Pro. Some of their physico-chemical properties are listed in Table II, including those of two peptide semicarbazones, ZLLLsc (Inhibitor k, (**6**)) and ZPLLsc (Inhibitor l). Each peptide derivative gave a molecular weight essentially identical with the theoretical value upon mass-spectorometric analysis. The retention times on the ODS column in HPLC for FuLLLal (Inhibitor b, (**11**)), NiLLLal, isoNiLLLal, MoSuLLLal, ZPLLal, AcPLLLal, AcPPLLLal and ZPLLsc were much shorter than that of ZLLLal, suggesting that they have reduced hydrophobicities as compared with ZLLLal, hence higher solubilities in aqueous media, although their solubilities were not directly determined in this study.

The inhibition profiles of the peptide derivatives toward cathepsin B are shown in Figures 2 and their concentrations required for 50% inhibition (IC_{50}) in Table III. ZLLLal was shown to be a fairly strong

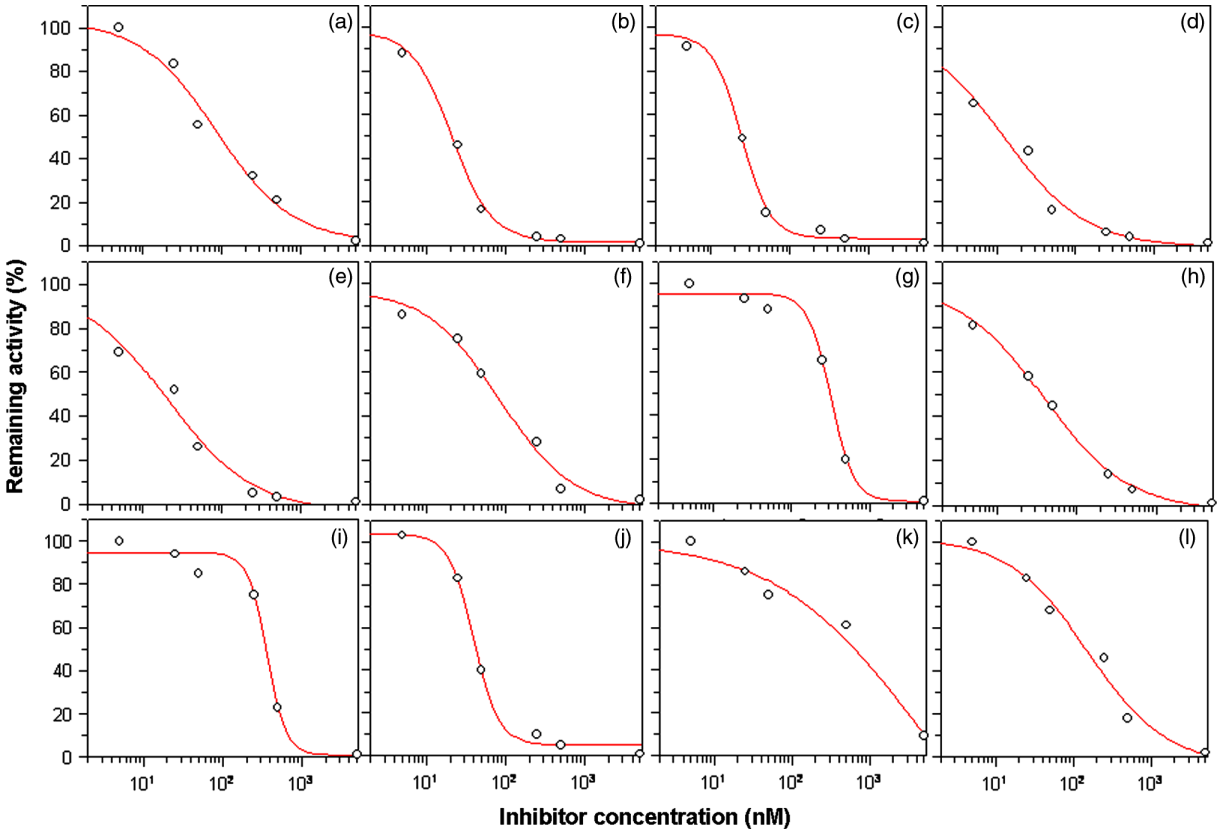


Figure 2. Inhibition of rat liver cathepsin B by ZLLLal and its analogues. The curve fitting was performed using GraFit 4.0 for determination of IC50 values [15].

Table III. IC_{50} values for the inhibition of cathepsins B and L with ZLLLal and its analogues. The IC_{50} values were obtained from Figures 2 and 3 using GraFit 4.0 for determination of IC_{50} values [15].

	Inhibitor	IC_{50} (nM)	
		Cathepsin B	Cathepsin L
a	ZLLLal	88	163
b	FuLLLal	21	44
c	NiLLLal	24	27
d	isoNiLLLal	12	20
e	MoSuLLLal	20	48
f	ZPLLal	84	116
g	ZPLLLal	319	205
h	AcPLLLal	39	57
i	ZPPLLLal	360	208
j	AcPPLLLal	40	19
k	ZLLLsc	690	407
l	ZPLLsc	154	79

inhibitor of cathepsin B, the IC_{50} value being 88 nM. The first four ZLLLal analogues (Inhibitors b–e), in which the Z group was replaced with a less hydrophobic group, were more inhibitory, the IC_{50} values being 12–24 nM. In addition, the inhibitors AcPLLLal and AcPPLLLal were somewhat more inhibitory than ZLLLal to cathepsin B, presumably due to introduction of a less hydrophobic group in place of the Z group in ZLLLal. On the other hand, the inhibition of cathepsin B by ZPLLal was similar to that by ZLLLal, but ZPLLLal and ZPPLLLal were much less inhibitory than ZLLLal.

Figures 3 and Table III also show the inhibitory effects of the above peptide derivatives toward cathepsin L. ZLLLal was less inhibitory to cathepsin L than to cathepsin B. The inhibitors b–e were more inhibitory than ZLLLal, but less inhibitory to cathepsin L than to cathepsin B, the IC_{50} values being 20–48 nM. ZPLLal, AcPLLLal and AcPPLL-Lal were more inhibitory than ZLLLal to cathepsin L, whereas the inhibition of cathepsin L by ZPLLLal and ZPPLLLal were somewhat weaker than that by ZLLLal.

The inhibition profiles for ZLLLsc and ZPLLsc show that they were less inhibitory than the corresponding aldehyde derivatives toward both cathepsins B and L except for the inhibition of cathepsin L by ZPLLsc (Table III). The lower inhibitory activities of these semicarbazones seem to be consistent with the fact that the inhibition of cysteine proteases by a peptide aldehyde is primarily due to the hemithioacetal formation between the catalytic thiol group in the enzyme and the aldehyde group of the inhibitor. The above semicarbazones may also form hemithioacetal with the thiol group in the enzyme, but somewhat more slowly.

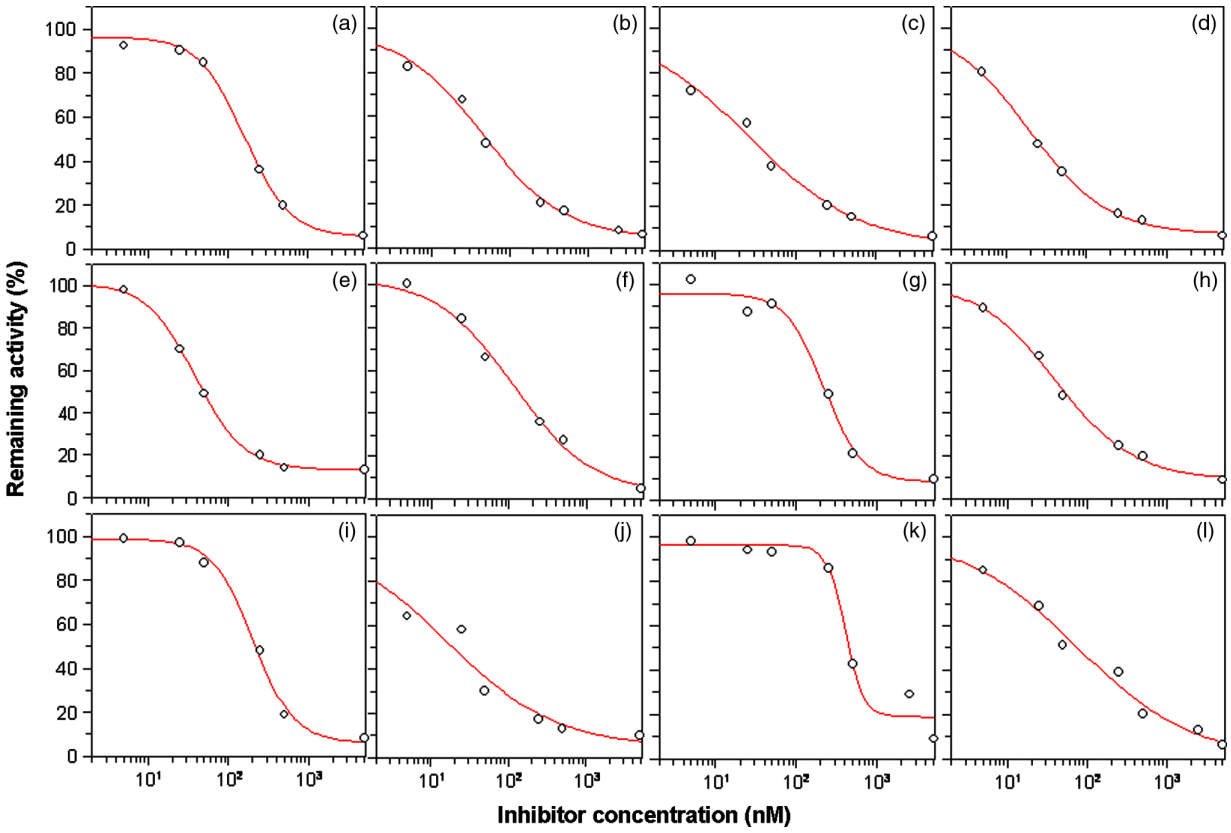


Figure 3. Inhibition of rat liver cathepsin L by ZLLLal and its analogues. The curve fitting was performed using GraFit 4.0 for determination of IC50 values [15].

Most peptide aldehyde inhibitors prepared in this study contain an LLL sequence corresponding to the P3-P1 residues of a substrate, and this sequence is thought to be accommodated well by the S3-S1 subsites of each enzyme as judged from the crystal structures [16–19] and kinetic studies [20]. Furthermore, ZPLLal showed a similar or somewhat stronger inhibition toward both enzymes as compared with ZLLLal. Thus, Pro may be also accommodated at the S3 subsite of both enzymes as reported previously [20]. On the other hand, little information is available from X-ray crystallographic studies on the interactions at the S4 subsites and beyond them. In many cases, however, the inhibitory potency appeared to increase with the decrease in hydrophobicity of the inhibitor due to the substitution of the Z group. This suggests at least the occurrence of P4-S4 interaction within the enzymes. Indeed, the existence of the P4-S4 interaction within them were suggested previously from kinetic studies [20]. The reason for the increase in the inhibitory potency is not clear, but the groups with somewhat less hydrophobicity than the Z group as used in the present study might interact more effectively with a certain group(s) in the S4 subsite of the enzymes. Moreover, AcPLLLal and AcPPLLLal were much more potent inhibitors than ZPLLLal and ZPPLLLal, respectively. This suggests a favorable interaction of the acetyl group and/or an unfavorable interaction of the Z group of these compounds with the active sites of the enzymes. Thus, ZLLLal and several of its analogues, FuLLLal, NiLLLal, isoNiLLLal, MoSucLLLal, AcPLLLal and AcPPLLLal, were shown to be potent inhibitors of both cathepsins B and L.

These peptide aldehydes are analogues of the cysteine/serine protease inhibitor leupeptin (acetyl- or propionyl-Leu-Leu-arginal), which was reported to inhibit, among others, cathepsin B (K_i, 7 nM) [21] and cathepsin L (K_i, 37 nM) [10]. Furthermore, several di- and tripeptidyl aldehyde inhibitors were synthesized and their inhibitory effects investigated toward cysteine proteases including cathepsins B and L [1]. Among these, the most potent inhibitors were reported to be acetyl-Leu-Val-lysinal toward cathepsins B (IC_{50}, 4 nM) [22] and acetyl-Leu-Leu-norleucinal toward cathepsin L (K_i, 0.5 nM) [23], respectively. Thus, several of the present peptide aldehydes appear to possess inhibitory potencies toward cathepsins B and L comparable with or somewhat weaker than those inhibitors.

On the other hand, the IC_{50} value of ZLLLal for the casein-degrading activity of m-calpain was reported to be 1200 nM and those for succinyl-Leu-Leu-Val-Tyr-MCA- and ZLLL-MCA-degrading activities of proteasome to be 850 nM and 100 nM, respectively [2]. Therefore, ZLLLal appears to be a stronger inhibitor toward cathepsins B and L than toward m-calpain, whereas the inhibition seems to be comparable or stronger than that on the activities of proteasome depending on the substrate used for the assay of the activities of proteasome. The inhibitory effects of the present inhibitors other than ZLLLal on calpain and proteasome remain to be determined. Since several of the new inhibitors were indicated to be somewhat more soluble in aqueous media than ZLLLal, they may be useful when a higher concentration of the inhibitor is needed for studies using crude biological samples or in vivo. In addition, the Pro-containing inhibitors might hopefully be more resistant to degradation by other endopeptidases when used in crude biological samples or in vivo. It should be interesting and important to examine the physiological effects of the new peptide aldehydes prepared in this study in comparison with ZLLLal.

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