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Novel epoxysuccinyl peptides

A selective inhibitor of cathepsin B, in vivo

Takae Towatari[1], Takeshi Nikawa[1], Mitsuo Murata[2], Chihiro Yokoo[2], Masaharu Tamai[2], Kazunori Hanada[2] and Nobuhiko Katunuma[1]

[1] *Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770* and [2] *Research Center, Taisho Pharmaceutical Co. Ltd., 1-403 Yoshino-cho, Ohmiya, Saitama 330, Japan*

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New derivatives of E-64 (compound CA-030 and CA-074) were tested in vitro and in vivo for selective inhibition of cathepsin B. They exhibited 10000–30000 times greater inhibitory effects on purified rat cathepsin B than on cathepsin H and L: their initial K_i values for cathepsin B were about 2–5 nM, like that of E-64-c, whereas their initial K_i values for cathepsins H and L were about 40–200 μM. In in vivo conditions, such as intraperitoneal injection of compound CA-030 or CA-074 into rats, compound CA-074 is an especially potent selective inhibitor of cathepsin B, whereas compound CA-030 does not show selectivity for cathepsin B, although both compounds CA-030 and CA-074 show complete selectivity for cathepsin B in vitro.

Cysteine proteinase inhibitory E-64 derivative; Cathepsin B; Cathepsin L; Cathepsin H; Cysteine proteinase

1. INTRODUCTION

Lysosomal cathepsins B, H and L are well-characterized cysteine proteinases that are important in intracellular protein degradation [1,2]. Furthermore, cathepsins B and L may be involved in muscular dystrophy [3–5], bone resorption [6], pulmonary emphysema [7,8] and tumor invasion [9]. In establishing the individual roles of lysosomal cathepsins in these and other biological phenomena, the discovery of selective inhibitors has been and will be of critical importance. E-64 is an irreversible inhibitor of cysteine proteinases isolated by Hanada et al. [10]. E-64 and its derivatives cause rapid inactivation of almost all cysteine proteinases [11–13]. Previously we have examined the mechanism of inhibition of cathepsins B and L by E-64 and the potent inhibitory activities of E-64 and its derivatives in vivo [14,15]. We found that these compounds inactivated cathepsins B and L more powerfully than cathepsin H, but did not cause their selective inhibition in vitro and in vivo. Therefore, we searched for selective inhibitors of lysosomal cysteine proteinases including calpain and discovered new selective inhibitors, derivatives of E-64, of cathepsin B in vitro. The characteristics of these inhibitors are described by Murata et al. [16]. In this paper, we report the specificity of the novel derivatives of E-64, compound CA-030 and CA-074, for cathepsins B, H and L in vitro and in vivo.

Correspondence address: N. Katunuma, Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan

Abbreviations: Z, benzyloxycarbonyl; MCA, methylcoumarylamide; BCA, bicinchoninic acid; E-64-a, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-4-aminobutylamide; E-64-c, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-3-methylbutylamide; CA-028, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-isoleucyl-L-proline; CA-030, *N*-(L-3-*trans*-Ethoxycarbonyloxirane-2-carbonyl)-L-isoleucyl-L-proline; CA-074, *N*-(L-3-*trans*-Propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; tES, L-*trans*-epoxysuccinyl; IAA, isoamylamide; Pr^{n}NH, *n*-propylamide

Enzymes: cathepsin B, EC 3.4.22.1; cathepsin H, EC 3.4.22.16; cathepsin L, EC 3.4.22.15

2. MATERIALS AND METHODS

2.1. *Materials*

Wistar rats, weighing 200–220 g were used for in vivo studies. Rat liver cathepsins B, H and L were purified as described previously [11,17,18] with the additional purification steps on HPLC on TSK gel G3000SW (Tokyo Soda) and Con A-Sepharose. Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA were purchased from the Peptide Institute (Osaka, Japan). All other chemicals were of analytical grade. E-64-a and E-64-c were products of Taisho Pharmaceutical Co. (Tokyo, Japan). The new derivatives of E-64, compounds CA-028 = *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-isoleucyl-L-proline, CA-030 = *N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-isoleucyl-L-proline and CA-074 = *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline, were prepared by M. Murata et al. as described previously [16].

2.2. *Methods*

2.2.1. *Enzyme asays*

Cathepsin activities were assayed with Z-Arg-Arg-MCA at pH 6.0 for cathepsin B, Arg-MCA at pH 7.0 for cathepsin H and Z-Phe-Arg-

Table I

Initial K_i values of E-64-c and compounds CA-030 and CA-074 for rat cathepsins B, H and L

Compound	Structure	K_i ($\times 10^{-9}$ M) Cathepsin B	Cathepsin H	Cathepsin L
E-64-c	HO-tES-Leu-IAA	8.70	111	3.5
CA-028	HO-tES-Ile-Pro-OH	140.00	1 353	625
CA-030	EtO-tES-Ile-Pro-OH	4.38	42 900	40 000
CA-074	Pr^n NH-tES-Ile-Pro-OH	1.94	75 000	233 000

Initial K_i values (after 5 min of incubation of enzyme with the inhibitor) were determined by Lineweaver-Burk double-reciprocal plots as described in the Materials and Methods.

MCA at pH 5.5 for cathepsin L as substrates by the method of Barrett and Kirschke [19]. For studies of inhibitor kinetics, the activity of each proteinase was adjusted to 0.3 units (one unit of enzyme activity is defined as that releasing 1 nmol of 7-amino-4-methylcoumarin per min at 37°C). Inhibitors were preincubated with enzyme for 5 min and then reactions were started by addition of the substrate. The fluorescence of 7-amino-4-methylcoumarin liberated from the substrate was monitored in a Hitachi fluorescence spectrometer, model 650-10S equipped with a recorder. Emission at 460 nm was measured with excitation at 370 nm. The K_i values for inhibitors were determined from double-reciprocal Lineweaver-Burk plots. Five or six concentrations of substrate that gave evenly distributed values of reciprocal values were used.

2.2.2. *Inhibition of cathepsin B, H and L by compounds CA-074, CA-030 and E-64-a in vivo*

Compound CA-074 or CA-030 or E-64-a was injected intraperitoneally as a solution in saline containing DMSO at the doses indicated in the legends to Figure and Tables. In in vivo studies, a dose of 8 mg/100 g body weight was injected. The rats were killed by a blow to the head 6 h after the injection, and their liver was perfused with saline, removed, weighed and chilled on ice. Samples of 4 g of liver were minced and homogenized in 7 vols of cold 0.25 M sucrose. The homogenate was centrifuged at 800 × *g* for 15 min and the supernatant was centrifuged at 12 000 × *g* for 30 min. The precipitate (crude mitochondrial-lysosomal fraction; ML fraction) was suspended in 2 ml of 0.05 M acetate buffer, pH 5.0, and freeze-thawed for measurements of cathepsin B, H and L activities.

2.2.3. *HPLC profiles of cathepsins B, H and L in ML fractions treated with compounds CA-074 and E-64-a*

ML fractions from 4 g of liver of rats treated i.p. with saline, E-64-a or compound CA-074, were freeze-thawed twice in 3.5 ml of 0.05 M acetate buffer, pH 4.0, containing 0.5 M NaCl. Each sample was dialyzed overnight against 1 liter of 0.05 M acetate buffer, pH 4.0, adjusted to 5.5 ml with the same buffer and centrifuged for 10 min at 16 000 × *g*. Then volumes of 0.9 ml of the supernatant were applied to TSK SP 5PW columns (8 × 75 mm) that had been equilibrated with 0.05 M acetate buffer, pH 4.0. Each column was washed with the same buffer (5 ml) and then the bound protein was eluted with a linear gradient formed with 15 ml of equilibration buffer and 15 ml of 0.9 M NaCl, 0.05 M acetate buffer, pH 4.0 at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and their cathepsin B, H and L activities were assayed.

Protein concentrations were determined by the BCA method (Pierce Chemical Co.) with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

3.1. *Inhibition kinetics of purified cathepsins B, H and L by compounds CA-028, CA-030, CA-074 and E-64-c*

In Table I, the inhibitory activities of compounds CA-028, CA-030 and CA-074 on purified rat cathepsins B, H and L are compared with those of E-64-c which is also a synthetic derivative of E-64 and has been characterized [20]. Compounds CA-030 and CA-074 were stronger inhibitors of cathepsins B than E-64-c. Moreover, they inhibited cathepsin B 10 000–30 000 times more than cathepsin H or L. Inhibition after 5 min was dose-dependent: the initial K_i values for

Table II

Inhibitory effects of E-64-a and compounds CA-074 and CA-030 on Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA hydrolyzing activities in vivo

Compound	Activities Z-Phe-Arg-MCA (pH 5.5) (Units/mg)	Z-Arg-Arg-MCA (pH 6.0) (Units/mg)	Arg-MCA (pH 7.0) (Units/mg)
Exp 1			
Control	78.65 ± 5.96 (100%)	8.38 ± 0.59 (100%)	6.35 ± 0.72 (100%)
E-64-a	6.03 ± 1.81 (7.7%)	0.18 ± 0.10 (2.1%)	0.31 ± 0.16 (5.0%)
CA-074	46.83 ± 13.29 (59.5%)	0.74 ± 0.30 (8.0%)	7.87 ± 1.60 (123.9%)
Exp 2			
Control	62.53 ± 2.58 (100%)	4.19 ± 0.45 (100%)	4.12 ± 0.34 (100%)
E-64-a	2.81 ± 0.37 (4.5%)	0.08 ± 0.03 (1.9%)	0.15 ± 0.04 (3.6%)
CA-030	17.27 ± 2.22 (27.6%)	0.54 ± 0.17 (12.9%)	1.43 ± 0.35 (34.7%)

Rats were treated i.p. with 8 mg of E-64-a or compound CA-074 or CA-030 and 6 h later, ML fractions of livers were prepared for assays of Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA hydrolyzing activities. Values are means for 5 rats.

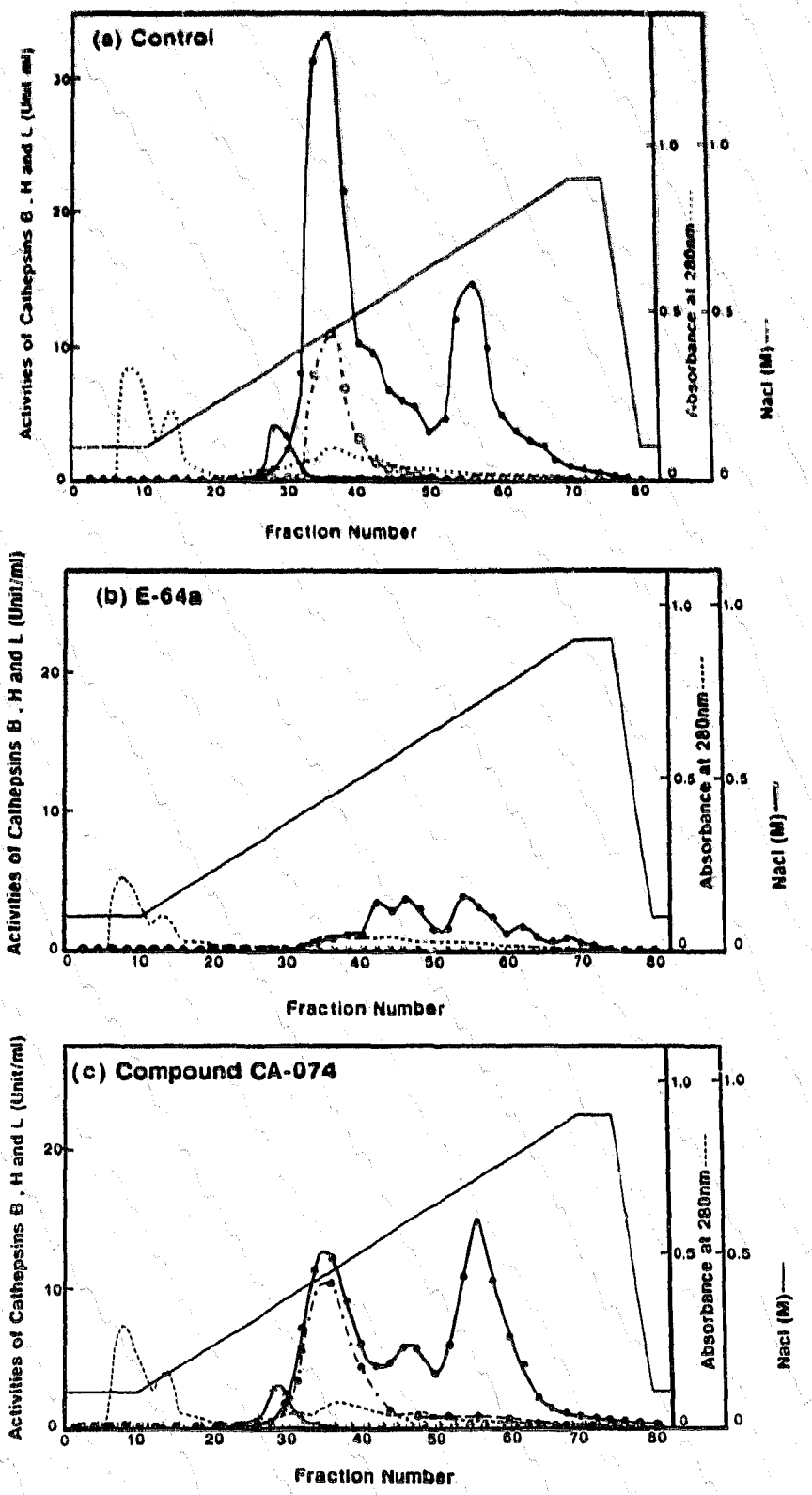

Fig. 1. HPLC profiles of cathepsin B, H and L activities in ML fractions of liver of rats treated with saline, E-64-a or compound CA-074. Rats were treated i.p. with 8 mg of E-64-a or compound CA-074 and killed 6 h later and ML fractions were prepared as for Table II. After dialysis, samples from (a) a control rat (2.8 mg protein in 0.9 ml) and rats treated with (b) E-64-a (3.0 mg protein in 0.9 ml) and (c) compound CA-074 (2.8 mg protein in 0.9 ml) were subjected to HPLC on TSK SP 5PW (Toyo Soda) and Z-Phe-Arg-MCA (•—•), Z-Arg-Arg-MCA (○—○) and Arg-MCA (X—X) hydrolyzing activitites were assayed as described in the Materials and Methods, except that in (c) Z-Phe-Arg-MCA hydrolyzing activities were assayed in the presence (•—•) and absence (○—•) of 100 nM E-64-c. Absorbance at 280 nm (----) is also shown.

cathepsin B were about 2–5 nM, like that of E-64-c, whereas initial K_i values for cathepsin H and L were about 40–200 μM. These results suggest that compounds CA-030 and CA-074 are potent selective inhibitors of cathepsin B in vitro.

3.2. *Inactivation of cathepsins B, H and L by compounds CA-074, CA-030 and E-64-a in vivo*

In in vivo studies using E-64, E-64-a and E-64-c, cathepsins L and B were inactivated very effectively [15]. On the other hand, it was predicted that compound CA-030 might be converted to compound CA-028 by hydrolysis of the ethylester bond in vivo. Practically, compound CA-030 did not show selective inactivation of cathepsin B in vivo (Table II). Compound CA-074 was designed with a modification to protect its attack by esterases. We examined whether it is actually incorporated in the lysosomal fraction of the liver as the intact form and inhibits lysosomal cathepsin B specifically.

Compounds CA-074, CA-030 and E-64-a (8 mg/100 g rat) were injected i.p. into rats. (A dose of 1 mg of E-64 per 100 g body weight has been found to be effective on rat cathepsin B [15].) Six hours later, the animals were killed for measurements of cathepsin B, H and L activities in the crude ML fraction of the liver and the HPLC profiles of cathepsin B, H and L activities in ML fractions as described in Materials and Methods. As can be seen from Table II at doses of 8 mg per 100 g body weight, E-64-a and compound CA-030 inactivated cathepsin B, H and L, whereas compound CA-074 inactivated cathepsin B selectively. Thus compound CA-074 can be used as an effective selective inactivator of cathepsin B both in vivo and in vitro.

3.3. *HPLC profiles of cathepsins B, H and L in ML fractions of rats treated with compounds CA-074 and E-64-a*

As Z-Phe-Arg-MCA is a substrate for both cathepsin B and L, to determine whether the remaining Z-Phe-Arg-MCA hydrolyzing activity in rats treated with compound CA-074 was due only to cathepsin L activity, we examined the HPLC profiles of cathepsins B, H and L as described in Materials and Methods. As shown in Fig. 1, in the preparation from the control rat, the main peak of cathepsin B activity was eluted with 0.44 M NaCl and those of cathepsin H and L were eluted with 0.33 M and 0.70 M NaCl, respectively. The preparation from the rat treated with E-64-a gave very small peaks of Z-Phe-Arg-MCA hydrolyzing activity eluted with various concentrations of NaCl but no peaks in the positions of cathepsin B and cathepsin L, and Z-Arg-Arg-MCA and Arg-MCA hydrolysing activities were inactivated completely. In contrast, the preparation from the rat treated with compound CA-074 gave peaks of Z-Phe-Arg-MCA hydrolyzing activity eluted in the positions of cathepsins B and L. Recently, we have purified a novel high molecular cysteine proteinase located in lysosomes that has low affinity for E-64 [21]. To determine whether the Z-Phe-Arg-MCA hydrolyzing activity remaining in the cathepsin B fraction is due to this high molecular cysteine proteinase or cathepsin B, we examined the inhibitory effect of E-64-c on remaining Z-Phe-Arg-MCA hydrolyzing activities in rats treated with compound CA-074 by in vitro assay. Results showed that 100 nM E-64-c caused complete inhibition of cathepsin L, but only partial inhibition of remaining Z-Phe-Arg-MCA hydrolyzing activity in the cathepsin B fraction. Thus we concluded that in the preparation from rats treated with compound CA-074 one peak of remaining Z-Phe-Arg-MCA hydrolyzing activity was attributable to the novel high molecular weight cysteine proteinase and the other to cathepsin L. Under these in vivo conditions, the Arg-MCA hydrolysing activity was not inactivated, as shown in Table II and Fig. 1c. Recently, the synthesis of selective cysteine proteinase inhibitors has been attempted [22–24]. Our results show that compound CA-074 is the first potent selective inhibitor of cathepsin B both in vivo and in vitro. Therefore it could be useful in studies on the selective role of cathepsin B in biological phenomena and intracellular protein degradation processes.

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