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Novel epoxysuccinyl peptides

Selective inhibitors of cathepsin B, in vitro

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A series of new epoxysuccinyl peptides were designed and synthesized to develop a specific inhibitor of cathepsin B. Of these compounds, *N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-isoleucyl-L-proline (compound CA-030) and *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (compound CA-074) were the most potent and specific inhibitors of cathepsin B in vitro. The carboxyl group of proline and the ethyl ester group or *n*-propylamide group in the oxirane ring were necessary, the ethyl ester group or the *n*-propylamide group being particularly effective for distinguishing cathepsin B from other cysteine proteinases such as cathepsins L and H, and calpains.

Epoxysuccinyl peptide; Cathepsin B; Cysteine proteinase; Specific inhibitor

1. INTRODUCTION

Cathepsins B, H and L, and calpains, well-characterized cysteine proteinases in mammalian cells, play major roles in intracellular protein breakdown [1] and in the degradation of extracellular-matrix proteins such as collagen and elastin [2,3]. Specific inhibitors of these proteinases would be useful in studying their individual biological roles. Hanada et al. discovered a new type of irreversible inhibitor of cysteine proteinases [4]. The first inhibitor of this type was isolated from *Aspergillus japonicus*, identified as *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-4-guanidinobutylamide, and named E-64 for simplicity. It inhibits cathepsins B and L [5], papain [6] and calpains specifically [7]. Hashida et al. reported the in vivo mechanisms of inhibition of cathepsin B and L by E-64 and its derivatives [8,9]. But E-64 and its derivatives are not, however, selective inhibitors of cathepsins B and L either in vitro or in vivo. As specific inhibitors of the various cysteine proteinases are required for clarifying the individual roles of these cysteine proteinases, the main aim of this study was to find a specific inhibitor of cathepsin B. X-ray crystal structure analysis of papain and the papain-E-64-c complex [10], together with those of previous extensive studies on E-64 [4,5] and its derivatives [6,11,12] showed that an L-*trans*-epoxysuccinic acid group is advantageous in design of an inhibitor that can distinguish cathepsin B from cathepsins H and L and calpains. Therefore, we designed new L-*trans*-epoxysuccinyl peptides that could fit the active site of cathepsin B, and developed two new selective inhibitors of cathepsin B, compound CA-030 *N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-isoleucyl-L-proline and compound CA-074 (*N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline. The present paper reports the selective inhibitory activities of these new epoxysuccinyl peptides on cathepsin B in vitro.

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Abbreviations: Z, benzyloxycarbonyl; MCA, methylcoumarylamide; E-64-c (Ep-475), *N*-(L-3-*trans*-Carboxyoxirane-2-carbonyl)-L-leucine-3-methylbutylamide; E-64-d (EST or Loxistatin), *N*-(L-3-*trans*-Ethoxycarbonyloxirane-2-carbonyl)-L-leucine-3-methylbutylamide

Enzymes: cathepsin B, EC 3.4.22.1; cathepsin H, EC 3.4.22.16; cathepsin L, EC 3.4.22.15; calpain, EC 3.4.22.17

2. MATERIALS AND METHODS

2.1. *Materials*

All derivatives of E-64 were synthesized in our laboratory by the method described previously [13] with some modifications. They were fully characterized by IR, proton NMR and fast atom bombardment MS and gave single spots on TLC.

Rat liver cathepsins B, H and L were purified as described previously [5,14,15] with additional purification steps of HPLC on TSK gel G3000 SW (Tosoh) and Con A-Sepharose. Calpain II from porcine kidney was purchased from Nakarai Tesque, Kyoto, Japan. Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA were from the Peptide Institute Inc., Osaka, Japan.

Table I

IC_{50} values of epoxysuccinyl peptide derivatives for cysteine proteinases

Compound	Structure	IC_{50} (nM)			
		Cathepsin			Calpain II
		B	L	H	
1 (CA-030)	EtO-tES-Ile-Pro-OH	2.28	32 000	240 000	200 000
2	EtO-tES-Pro-Pro-OH	25.0	47 000	1 000 000	200 000
3	EtO-tES-Thr-Ile-OH	13.5	540 000	1 000 000	200 000
E-64-c	HO-tES-Leu-IAA	3.36	0.09	1 640	3 000

tES, L-*trans*-epoxysuccinyl; IAA, isoamylamide

2.2. *Methods*

2.2.1. *Determination of inhibitory activities.* The inhibitory activities of new compounds prepared in this study are shown as their 50% inhibitory concentrations (IC_{50}) and their specificities as relative IC_{50} values.

2.2.2. *Assays of cathepsins B, H and L.* For determination of IC_{50} values, the activity of each cathepsin was adjusted to 0.3 U (1 U of enzyme activity is defined as that releasing 1 nmol of 7-amino-4-methylcoumarin per min at 37°C). Cathepsin activities were assayed at pH 5.5 with Z-Arg-Arg-MCA as substrate for cathepsin B, Arg-MCA for cathepsin H and Z-Phe-Arg-MCA for cathepsin L by the method of Barrett and Kirschke [16]. The reaction was initiated by addition of substrate (10 μM final concentration) after preincubation with the test compound for 3 min at 37°C. The fluorescence of the liberated 7-amino-4-methylcoumarin was measured in a Hitachi fluorescence spectrometer, model 650-10S equipped with a recorder. Emission at 460 nm was measured with excitation at 370 nm.

2.2.3. *Assay of calpain.* Calpain II (15 μg/tube) was assayed with 0.24% alkaline-denatured casein as substrate at pH 7.5 by the method of Ishiura et al. [17]. After preincubation with the test compound for 5 min at 30°C, the reaction was started by adding $CaCl_2$ at a final concentration of 5 mM. After 20 min, the reaction was stopped by adding 10% trichloroacetic acid solution. The mixture was then centrifuged, and the absorbance at 280 nm of the supernatant was measured in a Hitachi spectrophotometer, model U3210.

3. RESULTS AND DISCUSSION

Recent X-ray crystal structure analyses by two separate groups [10,18] indicated that E-64 and E-64-c bind to S subsites of papain. Therefore, in a computer-simulated study, epoxysuccinyl peptides such as E-64 were also assumed to bind to S subsites of cathepsin B. Of the compounds designed on the basis of this hypothesis, compounds **1**, **2** and **3**, shown in Table I, were found to be much stronger inhibitors of cathepsin B, but much weaker inhibitors of cathepsins L and H and calpain II than E-64-c. The common structures of these 3 compounds are a free carboxyl group at the C-terminal of the peptide and an ethyl ester group in the oxirane ring. Of these compounds, compound **1** (CA-030), which was the strongest inhibitor of cathepsin B, was further modified focusing on these common structures.

First, we converted the carboxyl group of proline to an amide group, an ester group, a hydroxymethyl group or a hydrogen atom. As shown in Table II, the inhibitory activities of these compounds **4–7** on cathepsin B were much weaker than that of compound **1** (CA-030) with a carboxyl group, suggesting that the carboxyl group is necessary for inhibition of cathepsin B.

Next, we replaced the ethyl ester group in the oxirane ring by a carboxyl group or other ester groups. As summarized in Table III, compounds **8** (CA-028) and **9**, with a carboxyl group and a methyl ester group, respectively, had weaker inhibitory activities and lower specificities for cathepsin B than compound **1** (CA-030), but compounds **10–12** with bulky alkyl ester groups, such as isopropyl, isobutyl and cyclohexyl ester

Table II

IC_{50} values and relative IC_{50} values of CA-030 derivatives for cysteine proteinases

EtO-tES-Ile-N—R

Compound	R	IC_{50} (nM)				Relative IC_{50}		
		Cathepsin			Calpain II	Cathepsin		
		B	L	H		B :	L :	H
1 (CA-030)	COOH	2.28	32 000	240 000	200 000	1:	14 000:	105 000
4	$CONH_2$	5600	18 000	112 000	200 000	1:	3:	20
5	COOMe	4100	18 300	10 900	200 000	1:	4:	3
6	CH_2OH	6300	18 000	43 000	200 000	1:	3:	7
7	H	2500	20 500	57 000	200 000	1:	8:	23

tES, L-*trans*-epoxysuccinyl

Table III

IC_{50} values and relative IC_{50} values of CA-030 derivatives for cysteine proteinases

RO-tES-Ile-Pro-OH

Compound	R	IC_{50} (nM)				Relative IC_{50}		
		Cathepsin			Calpain II	Cathepsin		
		B	L	H		B :	L :	H
8 (CA-028)	H	30.4	530	15 400	82 000	1:	17:	500
9	Me	20.0	2 460	40 000	200 000	1:	120:	2 000
1 (CA-030)	Et	2.28	32 000	240 000	200 000	1:	14 000	105 000
10	i-Pr	1.45	27 500	46 000	200 000	1:	19 000:	32 000
11	i-Bu	1.41	22 500	58 000	200 000	1:	16 000:	41 000
12	c-Hex	1.11	5 600	19 000	200 000	1:	5 000:	17 000

tES, L-*trans*-epoxysuccinyl; i-Pr, isopropyl; i-Bu, isobutyl; c-Hex, cyclohexyl

groups, caused strong inhibition of cathepsin B. These compounds also showed marked specificity for cathepsin B, although compound **12** with a cyclohexyl ester group, showed lower ability to distinguish between cathepsins B and L than the other compounds. Judging by comparison of compounds **8** (CA-028), **9** and **1** (CA-030), the presence of a bulkier ester group than ethyl ester in *trans*-epoxysuccinic acid seemed indispensable for specific inhibition of cathepsin B. However, when these compounds are given to animals, they are probably readily hydrolyzed like E-64-d, one of the E-64 analogs [19]. Therefore, to obtain analogs that were stable in vivo, we examined the effect of replacing the ethyl ester group of compound **1** (CA-030) by various amide groups that should be more resistant to hydrolysis than ester groups. As shown in Table IV, replacements of the esters by the corresponding amides did not affect the specific inhibitory activities against cathepsin B, except in the case of compound **13**, the diethyl amide derivative. The amide derivatives seemed to have somewhat weaker inhibitory activities than the corresponding ester derivatives, but considerably higher specificities for cathepsin B. Of these compounds, compound **16** (CA-074), the *n*-propylamide derivative, showed the highest inhibitory activity and specificity for cathepsin B. This compound specifically inactivated cathepsin B in rats in vivo as expected. Details of its inhibitory activity in vivo are described by Towatari et al. [20].

The computer simulation study indicated the presence of a fairly large pocket around the thiol group of the active site of cathepsin B. Its presence may distinguish cathepsin B from other cysteine proteinases. Compounds such as CA-030 with ethyl ester group and CA-074 with *n*-propylamide group may fit into this pocket and strongly inactivate cathepsin B, but hardly affect other cysteine proteinases.

CA-030 and CA-074 are the first compounds found

Table IV

IC_{50} values and relative IC_{50} values of CA-030 derivatives for cysteine proteinases

R-tES-Ile-Pro-OH

Compound	R	IC_{50} (nM)				Relative IC_{50}		
		Cathepsin			Calpain II	Cathepsin		
		B	L	H		B :	L :	H
13	Et_2N	2080	180 000	1 000 000	200 000	1:	87:	480
14	EtNH	6.88	186 000	1 000 000	200 000	1:	27 000:	145 000
15	i-PrNH	4.64	260 000	1 000 000	200 000	1:	56 000:	216 000
16 (CA-074)	*n*-PrHN	2.24	172 000	420 000	200 000	1:	77 000:	188 000
17	*i*-BuNH	1.78	122 000	92 000	200 000	1:	69 000:	52 000
18	*n*-BuNH	2.26	106 000	220 000	200 000	1:	47 000:	97 000
19	i-AmNH	2.40	68 000	238 000	200 000	1:	28 000:	99 000
20	*n*-AmNH	3.16	51 000	200 000	200 000	1:	16 000:	63 000
21	*n*-HexNH	3.92	38 000	214 000	200 000	1:	10 000:	55 000
22	$PhCH_2NH$	5.48	51 200	192 000	100 000	1:	9 000:	35 000
23	PhNH	12.2	35 000	190 000	200 000	1:	3 000:	16 000
24	c-HexNH	2.20	20 000	33 000	200 000	1:	9 000:	15 000

tES, L-*trans*-epoxysuccinyl; i-Pr, isopropyl; i-Bu, isobutyl; *n*-Pr, *n*-propyl; *n*-Bu, *n*-butyl; i-Am, isoamyl; *n*-Am, *n*-amyl; *n*-Hex, *n*-hexyl; Ph, phenyl; c-Hex, cyclohexyl

to inhibit cathepsin B selectively in vitro. In in vitro studies, these compounds should be useful for the identification of cathepsin B and the study of inhibition characteristics of cysteine proteases.

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