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Structure based development of novel specific inhibitors for cathepsin L and cathepsin S in vitro and in vivo

N. Katunuma[a,*], E. Murata[a], H. Kakegawa[a], A. Matsui[a], H. Tsuzuki[a], H. Tsuge[a], D. Turk[c], V. Turk[c], M. Fukushima[b], Y. Tada[b], T. Asao[b]

[a] *Institute for Health Sciences, Tokushima Bunri University, Yamasirocho, Tokushima 770-8514, Japan*
[b] *Research Institute for New Drug Design, Taiho Pharmaceutical Company, Hanno, Japan*
[c] *Department of Biochemistry, J. Stefan Institute, Jamova, Ljubliana, Slovenia*

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Abstract Specific inhibitors for cathepsin L and cathepsin S have been developed with the help of computer-graphic modeling based on the stereo-structure. The common fragment, *N*-(L-*trans*-carbamoyloxyrane-2-carbonyl)-phenylalanine-dimethylamide, is required for specific inhibition of cathepsin L. Seven novel inhibitors of the cathepsin L inhibitor Katunuma (CLIK) specifically inhibited cathepsin L at a concentration of 10^{-7} M in vitro, while almost no inhibition of cathepsins B, C, S and K was observed. Four of the CLIKs are stable, and showed highly selective inhibition for hepatic cathepsin L in vivo. One of the CLIK inhibitors contains an aldehyde group, and specifically inhibits cathepsin S at 10^{-7} M in vitro.

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Key words: Cathepsin L; Cathepsin S; Cathepsin inhibitor; Epoxysuccinate; X-ray crystallography

1. Introduction

Several mammalian lysosomal cysteine proteases, such as cathepsins B, H, L, S, C and K, have been well characterized. Specific inhibitors of individual cathepsins would be powerful tools for clarifying the different functions of each cathepsin. An irreversible specific inhibitor of cysteine proteases, derived from epoxysuccinate, was first isolated by Hanada et al. and named E-64 [1]. This E-64, however, inhibits all cysteine proteases except cathepsin C. Derivatives of L-*trans*-epoxysuccinyl-Ile-Pro derivatives were synthesized by Katunuma et al., in which the right side chain of the dipeptides was changed from the leucylagmatine of E-64 to isoleucylproline, named CA-030 and CA-074, showed strict specific inhibition of cathepsin B [2–5]. However, specific inhibitors for cathepsin L and cathepsin S have not been reported. New derivatives of L-*trans*-epoxysuccinate and aldehyde were designed as specific inhibitors of cathepsins L and S based on the differences in their substrate binding pockets using computer-graphic analysis. They were named the cathepsin L inhibitor Katunuma (CLIK) series, and were chemically synthesized. The chemical structures are shown in Fig. 1. The inhibitory selectivities were tested in vitro and in vivo.

2. Materials and methods

2.1. Materials

All epoxysuccinate derivatives of the CLIK group were synthesized in our laboratory by methods previously described for E-64 and CA-074, with some modifications [5,6]. They were fully characterized by infrared spectroscopy (IR), proton-nuclear magnetic resonance spectroscopy (proton-NMR) and fast atom bombardment mass spectroscopy (MS), and gave single spots on TLC. CLIK-060, *N*-{L-3-*trans*[(1-phenylcarbamoyl-5-amino) pentylcarbamoyl]oxirane-2-carbonyl}-L-phenylalanine-dimethylamide hydrochloride, was synthesized in five steps. The condensation of N^{ε}-acetyl-N^{α}-benzoyl-L-lysine and L-phenylalaninol with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 1-hydroxy benzotriazole yielded N^{ε}-Acetyl-N^{α}-benzolyl-L-phenylalaninol, which was oxidized by sulfur trioxide pyridine complex in DMSO to yield the desired compound with m.p. 128–130°C. Computer graphics were used to analyze the binding of CLIKs with cathepsin L using the method for analyzing cathepsin B-CA-030 complex [3,7] and the structure of cathepsin L [8]. The programs used were QUANTA Version 4.1 and CHARMM Version 23.1, Molecular Simulation, San Diego, CA, USA and MAIN [9].

2.2. Cathepsin assays in vitro and in vivo

Rat liver cathepsins B, L and C were purified as reported previously [10–13] and recombinant human cathepsins S [14] and K were expressed by *Escherichia coli* and Sf21 insect cells and were purified by the methods of Inaoka and Bossard, respectively [15,16]. Cathepsin activities were assayed with Z-Arg-Arg-MCA for cathepsin B, and Z-Phe-Arg-MCA for cathepsins L, S, K and C using the method described by Barrett and Kirschke [17]. Intracellular cathepsin L activity was assayed in mice splenocytes (10^6 cells/ml) incubated with inhibitors in Hanks buffer for 3 h at 37°C, and cathepsin L activity in the mitochondrial-lysosomal (ML) fraction was assayed by Inubushi's method [18]. Selective inhibition of intracellular cathepsin L in mouse liver was assayed by intraperitoneal injection of the inhibitors. At 45 and 120 min after injection, the ML fraction was prepared and the individual cathepsin activities were assayed.

2.3. Separation and determination of degradative products of CLIKs by high performance liquid chromatography (HPLC)

After 0.1 mM of each CLIK was incubated with organ homogenate at 37°C for 1 h at pH 5.5, the reactions were stopped by methanol. The remaining CLIKs and degradation products were separated by HPLC using an Inertsil ODS-3 column (4.6–150 mm), a mixture of 75% 10 mM (pH 6.0) phosphate buffer and 25% acetonitrile was used for the elution phase. CLIKs-088, -112 and -121 were converted to common products of CLIK-079 through enzymatic degradation by organ homogenates. The degradation product, CLIK-079, was separated from the original compounds in different eluted fractions from the column.

*Corresponding author. Fax: (81) (88) 622-3217.

Abbreviations: E-64, 1-[L-*N*-(*trans*-epoxysuccinyl) leucyl] amino-4-guanidinobutane; CA-030, ethyl-ester of epoxysuccinyl-*L*-isoleucyl-L-proline; CA-074, propyl-amide of epoxysuccinyl-L-isoleucyl-L-proline; MCA, methyl coumaryl amide; CLIK, cathepsin L inhibitor Katunuma; MS, mass spectroscopy; IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; KPB, potassium phosphate buffer; HPLC, high performance liquid chromatography

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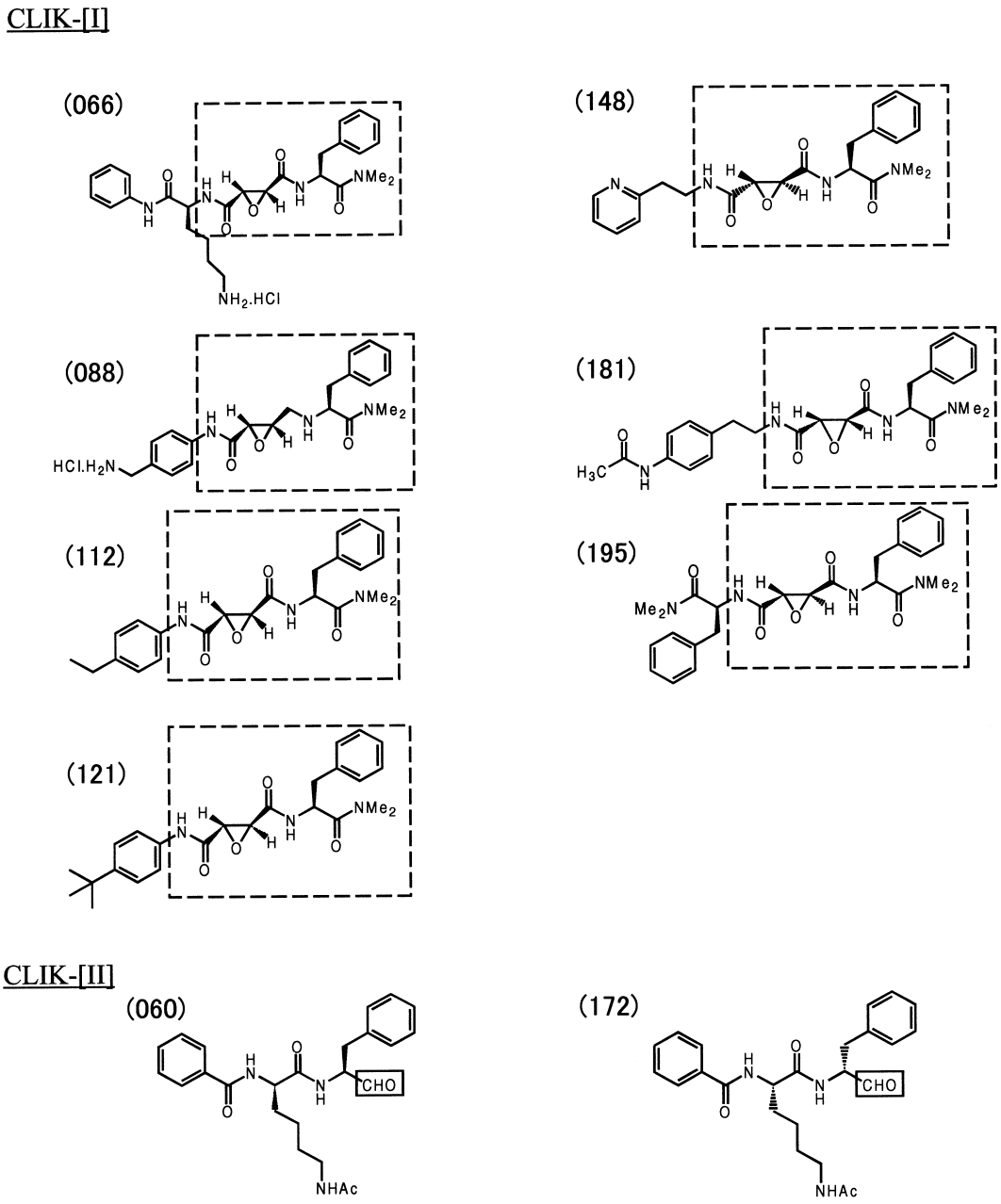


Fig. 1. Essential common structure for expression of specific inhibition of cathepsin L and cathepsin S. The essential common structure for specificity to cathepsin L inhibition is illustrated in the enclosed circle in the CLIK group [I]. Group [II], CLIK-060, shows specificity for cathepsin S. The aldehyde residue is located in the right end domain, and is available to react with -SH of cathepsin S.

3. Results

3.1. Essential structure of CLIK group for selective inhibition of cathepsin L

The following two CLIK groups for inhibition of cathepsin L and cathepsin S were developed and their chemical structures are shown in Fig. 1. CLIK group [I] is composed of CLIKs-066, -088, -112, -121, -148, -181 and -195, these inhibitors share the common essential structure, *N*-(*trans*-carbamoyloxyrane-2-carbonyl)-L-phenylalanine-dimethylamide, for forming a thioether specifically with the active site cysteine-SH of cathepsin L. Only CLIK-066 contains a lysine residue attached to the epoxysuccinate fragment. The characteristic aromatic derivatives in the left hand domain are bound directly to the epoxysuccinate-amide of the common fragment. CLIK-112, which is an S-S stereo-structure specific for epoxysuccinate rings, showed strong inhibition of cathepsin L, but CLIK-141, which is the R-R stereo-isomer of CLIK-112, showed no inhibition, indicating that the S-S stereo-structure is required for inhibition of cathepsin L. CLIK-060 and CLIK-172 in group [II] contain an aldehyde group at the right hand end of the molecule as a binding radical to the cysteine -SH of cathepsin S, as shown in Fig. 1, panel [II]. CLIK-172, a stereo-isomer of CLIK-060, showed some specificity for cathepsin S, but the inhibition potency for cathepsin S was weak. CLIK-173, in which the aldehyde of CLIK-060 mole-

cule is replaced by alcohol, showed no inhibition. Therefore, the aldehyde might be the active group reacting with the -SH of cathepsin S to form a semithioacetal.

3.2. Selective inhibition for purified cathepsin L and cathepsin S by CLIK groups in vitro

Selective inhibition profiles of CLIK groups for various cathepsins are compared in Table 1. CLIK group [I], CLIKs-066, -088, -112, -121, -148, -181 and -195, specifically inhibited cathepsin L, at 10^{-7} M, while no inhibition of cathepsins B and C was observed at 10^{-6} M, and CLIKs-148, -181 and -195 inhibited cathepsins S and K at 10^{-5} M. CLIK-060 in group [II] exhibited strong specificity for cathepsin S at a concentration of 10^{-7} M, although almost no inhibition of cathepsins B, L, K and C was observed at 10^{-6} M.

3.3. Degradation of some of the CLIK group [I] by enzymes in liver and small intestine and changes in inhibitory specificities

CLIK group [I] is stable in phosphate buffer at pH 4.0 ~ 8.0 for 24 h at room temperature. Enzymatic degradation of the CLIKs was calculated from the residual percentage of their original compounds, as Fig. 2 shows. The small intestine and liver were homogenized with three volumes of potassium phosphate buffer (KPB) of pH 5.5 and sonicated. Some of the CLIK group [I] inhibitors were degraded by an unknown organ enzyme. CLIKs-066, -148, -181 and -195 showed strong resistance to the enzymatic degradation, therefore, they have the potential to be used as stable and specific inhibitors in vivo. CLIKs-088, -112 and -121 were degraded and formed the common product, CLIK-079, which showed no inhibitory selectivity for cathepsin L, but also decreased the inhibitory potency for cathepsin L. This enzymatic degradation was inhibited by co-addition of chymostatin (data not shown).

Table 1
Selective inhibition profile of CLIKs for various cathepsins in vitro

	CLIK No.	10^x M	Inhibition of cathepsins (%)					Usable for
			B	L	K	S	C	
CLIK [I]	066	−5	0	100	27	28	0	
		−6		100	11	0		in vitro
		−7		76	0			in vivo
		−8		26				
	088	−5	0	100	23	64	17	in vitro
		−6		79	15	0	0	
		−7		30	0			
	112	−5	6	100	50	77	15	in vitro
		−6	0	100	30	14	0	
		−7		100	10	0	0	
		−8		90	0			
		−9		30	0			
	121	−5	15	100	25	100	29	in vitro
		−6	0	100	17	72	0	
		−7		74	0	35		
		−8		41	0	10		
	148	−5	0	100	40	100	35	
		−6		100	0	30	0	in vitro
		−7		63		0		in vivo
		−8		20				
	181	−5	0	100	90	80	50	
		−6		100	15	10	5	in vitro
		−7		80	0	0	0	in vivo
		−8		50				
	195	−5	20	100	80	100	40	
		−6	0	100	0	25	0	in vitro
		−7		80		0		in vivo
		−8		20				
		−9		0				
CLIK [II]	60	−6	25	30	10	100	13	
		−7	0	0	0	86	7	in vitro
		−8				40	0	in vivo
		−9				0		

CLIK group [I]: CLIKs-066, -088, -112, -121, -148, -181 and -195 are specific inhibitors for cathepsin L. CLIK [II] is a specific inhibitor for cathepsin S. Rat liver cathepsin B, L and C were purified as reported previously [10–13] and recombinant human cathepsins S and K were expressed by *E. coli* and Sf21 insect cells and purified according to the method of Zhao and Inaoka, respectively [14–16]. Cathepsin activities were assayed using Z-Phe-Arg-MCA as the substrate by the method of Barrett and Kirschke [17].

CLIK Numbers	% Remaining activities after one hour	
	Small Intestine	Liver
CLIK-066	99.1	80.6
CLIK-088	91.0	44.3
CLIK-112	88.0	11.7
CLIK-121	85.0	42.2
CLIK-148	124.7	95.1
CLIK-181	92.2	100.0
CLIK-195	100.0	100.0

Degradation product: CLIK-079

CLIK-079

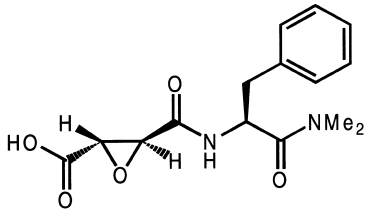


Fig. 2. Stability of CLIK group [I] in the presence of enzymes from liver and small intestine. Each inhibitor (0.1 mM) was incubated with mouse organ homogenates at 37°C for 1 h (pH 5.5), and the reaction was stopped by adding three volumes of methanol. The remaining original compound and the product formed, CLIK-079, were separated and assayed by HPLC.

3.4. Selective inhibition of lysosomal cathepsin L in cultured splenocytes and liver in vivo

Selective inhibition of cathepsin L was tested in mice hepatic lysosomes by intraperitoneal injection of CLIK-148, -181 or -195 in vivo, and also in cultured mice splenocytes. The cathepsin L activity in the ML fraction of cultured splenocytes in Hanks medium with and without inhibitor was assayed after a 3 h incubation. A 50% inhibition of cathepsin L activity in the splenocytes was observed by addition of 10^{-6} M CLIK-148 or -195 into the medium, almost complete inhibition was observed by 10^{-5} M. At 45 and 120 min after intraperitoneal injection of 3 mg/kg of CLIK-148, -181 or -195, the activities of cathepsins B and L in hepatic ML fractions were assayed, cathepsin L was inhibited selectively as illustrated in the upper panel of Fig. 3, while cathepsin B activity was not.

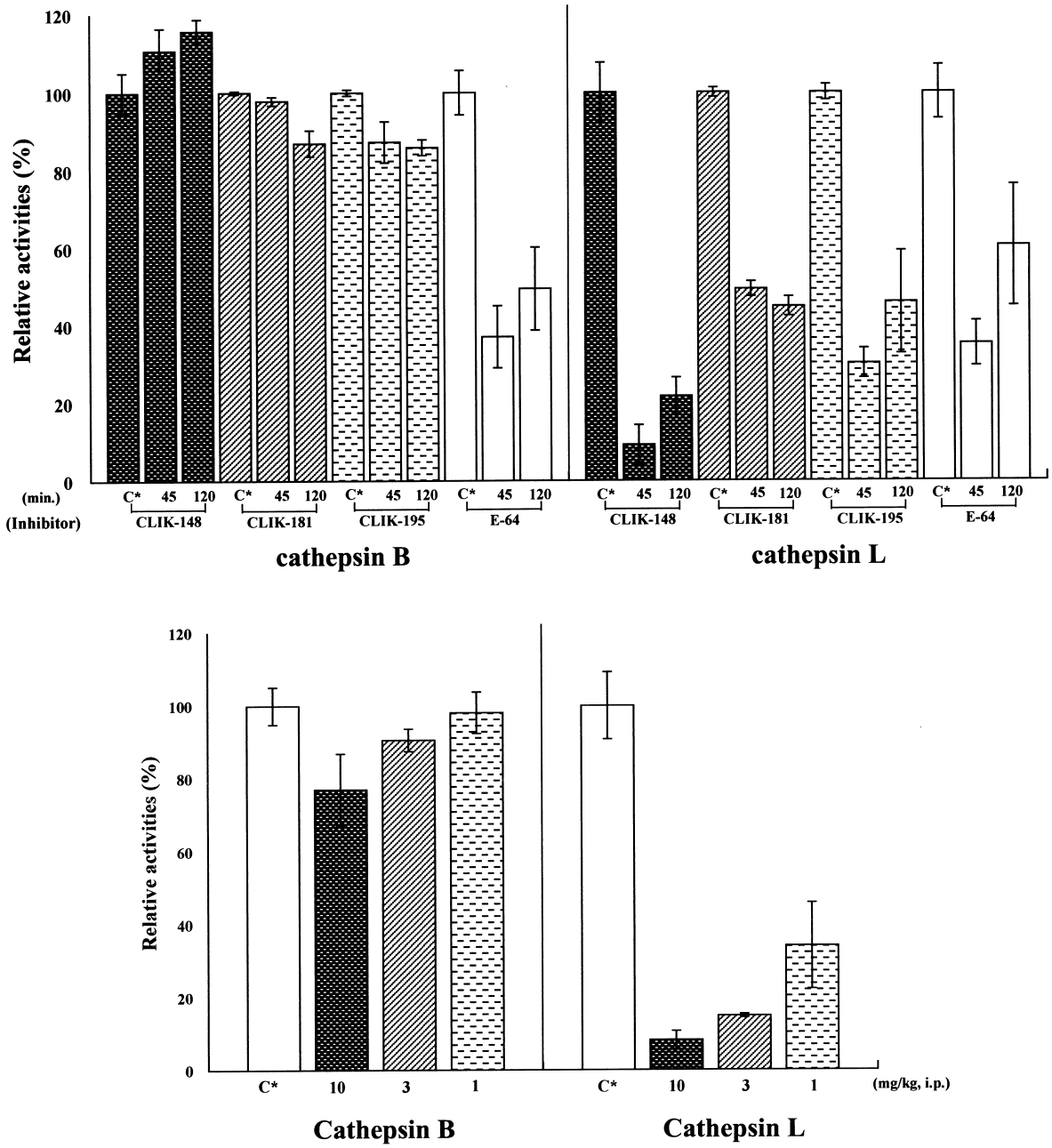


Fig. 3. Selective inhibition of cathepsins B and L in hepatic ML fraction by intraperitoneal injection of CLIK-148, -181 or -195. Each inhibitor (3 mg/kg) was injected intraperitoneally into mice. At 45 or 120 min after injection, hepatic ML fractions were prepared. The supernatants of the sonicated ML fractions were used as enzyme samples. The vertical axis illustrates the % remaining activities, calculated as 100% in the case without inhibitor. Inubushi's selective inhibition method for cathepsins B and L was used [18]. The upper panel illustrates the selective inhibition of hepatic cathepsins B and L by CLIK-148, -181 or -195. The lower panel shows the dose-dependent inhibition of hepatic cathepsin L by administration of 1.0, 3.0 or 10.0 mg/kg of CLIK-195.

CLIK-195 inhibited cathepsin L in a dose dependent manner at 120 min after injections of 1, 3 or 10 mg/kg inhibitor, as shown in the lower panel of Fig. 3. Therefore, CLIKs-148, -181 and -195 were effectively incorporated into the lysosomes and showed powerful selective inhibition of hepatic intralysosomal cathepsin L in vivo.

4. Discussion

The design of CLIK series inhibitors was based on structural and kinetic data. The crystal structures of papain-E-64 have shown that E-64 binds along the protein surface in the reverse direction to the peptide chain of substrate analogues in the non-prime site [19,20]. The crystal structure of cathepsin B [7] and the complex between CA-030 and cathepsin B [3] showed that an epoxysuccinyl analogue can also bind to the prime site of a cysteine protease active site cleft, leading to the obvious suggestion that an epoxysuccinyl fragment can be used as a building block that enables access to both the prime and non-prime substrate binding sites, in contrast to chloromethyl, fluoromethyl or aldehyde based inhibitors that bind only the non-prime subsites. Cathepsin B is the only papain

superfamily protease that contains an occluding loop from Ile-105 to Glu-112. The carboxyl group of CA-030 or C-terminus of substrates is locked by His-110 and His-111 on the loop. CLIKs were designed not to inhibit cathepsin B using the structural information. With the help of molecular models and, when they became available, crystal structures of cathepsins L [8] and K [21,22], binding pockets on the surface of cathepsins were compared and utilized in the design of the CLIK series. The modeling studies suggested that the fragment of CLIK group [I] can presumably fit optimally in the S2 non-prime site of the protease, whereas the selectivity of the series is achieved by the fragments extending into the prime binding region. As seen from Table 1, the CLIK group [I] compounds exhibit better selectivity of cathepsin L than cathepsins B and C, having an exopeptidase nature, than against endopeptidase cathepsins S and K. The specificity of the CLIK group [I] for cathepsin L is thus achieved by the rigidity and bulkiness of the phenyl-ring attached directly to the amino group of the common fragment. Recently we determined the crystal structure of the papain-CLIK-148 complex [23]. The structure confirmed that the phenyl-ring occupied the S2 non-prime site, and that the N-terminal pyridine-ring was located in the prime site by an aromatic-aromatic interaction with the Trp-177. The CLIK group [I] series of cathepsin L inhibitors and CLIK group [II] type of cathepsin S inhibitors constitute novel selective tools for investigating the role of these enzymes in various physiological processes and disease states.

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