Characterization of CAA0225, a Novel Inhibitor Specific for Cathepsin L, as a Probe for Autophagic Proteolysis

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We screened a series of new epoxysuccinyl peptides for the development of a lysosomal cathepsin L-specific inhibitor. Among the compounds tested, (2S,3S)-oxirane-2,3-dicarboxylic acid 2-{[(S)-1-benzylcarbamoyl-2-phenyl-ethyl]-amide}-3-{[2-(4-hydroxy-phenyl)-ethyl]-amide} (compound CAA0225) was the most potent inhibitor of cathepsin L. CAA0225 inhibited rat liver cathepsin L with IC50 values of 1.9 nM, but not rat liver cathepsin B (IC50 > 1000–5000 nM). To assess the contribution of cathepsin L to lysosomal proteolysis, we evaluated autophagy, which is the process of lysosomal self-degradation of cell constituents. In HeLa and Huh-7 cells cultured under nutrient-deprived conditions CAA0225 significantly inhibited degradation of long-lived proteins; however, the magnitude of inhibition was comparable to that in the presence of CA-074-OMe, which is a cathepsin B-specific inhibitor. Thus the contributions of cathepsin L and cathepsin B to autophagic protein degradation of cytoplasmic proteins are nearly equal. During autophagy, microtubule-associated protein 1A/1B light chain 3-II (LC3-II) and γ-aminobutyric acid (GABA) receptor-associated protein (GABARAP)-II, which are specific markers of autophagosomal membranes that engulf cytoplasmic components, also undergo degradation upon fusion of autophagosomes with lysosomes. CAA0225 effectively inhibited degradation of LC3-II and GABARAP, whereas CA-074-OMe had only a marginal effect on their levels. Therefore we conclude that cathepsin L does not play a general role in the degradation of proteins in the lumen of autophagosomes, but rather is involved specifically in the degradation of autophagosomal membrane markers.

Key words cathepsin L; autophagy; protein degradation; lysosome; microtubule-associated protein 1A/1B light chain 3; γ-aminobutyric acid (A) receptor-associated protein

Lysosomes play an essential role in cellular protein catabolism, participating in both the degradation of extracellular proteins that are transported through endocytic-phagocytic pathways and the turnover of intracellular constituents via autophagy, a process with unique membrane dynamics. Among the lysosomal proteinases, which comprise a diverse group of enzymes, the cysteine proteinases, including cathepsin B and cathepsin L, make a significant contribution to non-specific lysosomal proteolysis. Target deletion of cathepsins B and L in mice has shed light on the specific role of each cathepsin. While seeking a type-specific cathepsin B-specific inhibitor, CA-074-OMe had only a marginal effect on their levels. Therefore we conclude that cathepsin L does not play a general role in the degradation of proteins in the lumen of autophagosomes, but rather is involved specifically in the degradation of autophagosomal membrane markers.

Autophagy is a cellular catabolic process responsible for the turnover of cell organelles including mitochondria, endoplasmic reticulum, and peroxisomes, as well as cytosolic proteins. Autophagy is enhanced during starvation due to the presence of catabolic hormones such as glucagon. Upon nutrient deprivation, a unique double-membrane structure, which is called an isolation membrane, forms and non-selectively surrounds a portion of the cytoplasm. The two edges of the expanding isolation membrane seal to form an autophagosome with cytoplasmic components sequestered in the lumen. The autophagosome then fuses with a lysosome to form an autophagosome with cytoplasmic components sequestered in the lumen. The autophagosome then fuses with a lysosome to form a phagolysosome, in which sequestered cytoplasmic components are degraded by lysosomal proteases. It should be noted that the inner autophagosomal membranes are also degraded after fusion of autophagosomes and lysosomes. An unequivocal marker of autophagosomal membranes is phosphatidylethanolamine-conjugated microtubule-associated protein 1A/1B light chain 3 (LC3). LC3 is synthesized as a soluble precursor form (proLC3), which is cleaved by Atg4B, a cysteine

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proteinase, to expose the carboxyl-terminal glycine. The processed form, LC3-I, is subsequently conjugated with phosphatidylethanolamine to form LC3-II, which has the characteristics of an integral membrane protein that is then recruited onto autophagosomal membranes. In the presence of (L-3-trans-ethoxycarbonyloxirane-2-carbonyl)-L-leucine (E64d) and peptatin A, which are inhibitors of lysosomal cysteine proteases and cathepsin D, respectively, autolysosomal degradation of LC3-II is effectively inhibited and LC3-II accumulates. Therefore ongoing autophagy activity can be assessed by quantifying cellular levels of LC3-II with and without inhibitors using immunofluorescence and immunoblotting. In the present study, we used cathepsin-specific inhibitors to compare the relative contributions of cathepsin L and cathepsin B to both autophagic proteolysis of sequestered cytoplasmic proteins and autolysosomal degradation of LC3-II.

MATERIALS AND METHODS

Reagents All reagents used were of analytical grade and purchased from the following sources: Phe-Arg-methylcoumarylamide (MCA), Arg-Arg-methylcoumarylamide (MCA), MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Arg-Leu-Lys-(Dnp)-NH2, [L-3-trans- (propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline, (CA-074), [L-3-carbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA-074-Me), pepstatin, (L-3-trans-ethoxycarbonyloxirane-2-carbonyl)-L-leucine (E64c), and (L-trans-3-ethoxycarbonyloxirane-2-carbonyl)-L-leucine (E64d) from Peptide Institute (Osaka, Japan); BCA protein assay kit, SuperSignal West Pico Chemiluminescent Substrate, and SuperSignal West Dura Extended Duration Substrate (Pierce) as the substrate for the horseradish peroxidase conjugate of the secondary antibodies.

Cell Culture Huh-7 and HeLa cells were cultured and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (DMEM/10% FCS). Autophagic protein degradation was induced as follows. Cells grown in 6-cm dishes were washed with 20 mM Na-phosphate (pH 7.5)–0.15 M NaCl (PBS) and supplemented with Hank’s balanced salt solution containing 10 mM HEPES–NaOH (pH 7.4) (HBSS–Hepes). The cells were incubated at 37°C for 4 h in the presence or absence of 10 μg/ml E64d, CA-074-Me, or CAA0225. The cells were removed from dishes using cell scrapers and centrifuged at 800 rpm for 5 min. The pelleted cells were suspended in PBS containing proteinase inhibitors (Nakarai Tesque) and lysed by sonication at 0°C for 10 s. The cell lysates were mixed with SDS-PAGE sample buffer, incubated at 100°C in a water bath, and subjected to immunoblotting analyses.

Protein Degradation Assay Huh-7 and HeLa cells grown in 24-well microplates were incubated with DMEM/10% FCS containing [14C]-leucine (0.5 μCi/ml) for 22 h to label long-lived proteins. The cells were then washed with DMEM/10% FCS containing 2 mM unlabelled leucine and incubated with the medium for 2 h to allow degradation of short-lived proteins and to minimize re-incorporation of labeled leucine released by proteolysis. The cells were then washed with 20 mM Na-phosphate (pH 7.4) and 0.15 M NaCl (PBS), and incubated at 37°C with either HBSS–Hepes or DMEM/10% FCS in the presence or absence of protease inhibitors. During the next 3 h of the chase period, aliquots of the medium were removed and a one-tenth volume of 100% trichloroacetic acid was added to each aliquot. The mixtures were centrifuged at 12000×g for 5 min, and acid-soluble radioactivity was determined by liquid scintillation counting. At the end of the experiment, the cultures were washed with PBS, and 1 ml of cold trichloroacetic acid was added to fix the cellular proteins. The fixed-cell monolayers were washed with trichloroacetic acid and dissolved in 0.5 ml of 1 N NaOH at 37°C. The amount of radioactivity in each aliquot of 1 N NaOH was determined by liquid scintillation counting. Percent protein degradation was calculated using a previously published procedure.

Preparation of Mitochondrial/Lysosomal (ML) Frac-
RESULTS

Previously, our group developed a novel cathepsin B-specific inhibitor, CA-074.3-6 While developing the inhibitor, we noticed that the t-trans-epoxy-succinic acid group was important for specific interaction with cathepsin B; therefore we designed >30 different compounds that have this moiety in common. However, except for CA-074, these compounds did not specifically inhibit purified cathepsin B. At that time, we did not examine these compounds further. However, we hypothesized that some of these compounds might be cathepsin L-specific inhibitors, despite their lack of specificity for cathepsin L. We therefore systematically reexamined the effects of these compounds on cathepsin L and cathepsin B, with the aim of identifying a potential inhibitor for cathepsin L.

To confirm the specificity of the inhibitor, we next investigated the effects of the inhibitors on lysosomal protein degradation in vivo. HeLa and Huh-7 cells were cultured in DMEM/10% FCS containing [14C]-leucine for 22 h to label long-lived proteins. The cells were chased for 2 h with DMEM/10% FCS containing unlabeled 2 mM leucine then were incubated with HBSS–10 mM Hepes to induce autophagy by nutrient deprivation. Figure 2A summarizes the data for the degradation of long-lived proteins in HeLa cells. Autophagic proteolysis of long-lived proteins was inhibited significantly by E64d (approximately 3%), which is a general inhibitor of lysosomal cysteine proteinases. CA-074-Me, a membrane permeant form of CA-074, and CAA0225 inhibited autophagic protein degradation partially and moderately (approximately 2%). There were no significant differences in the magnitude of inhibition between CA-074-Me and CAA0225. Similarly, degradation of long-lived proteins of Huh-7 cells was partially inhibited by CA-074-Me and CAA0225 (Fig. 2B). The data clearly show that the contributions of cathepsin L and cathepsin B to autophagic protein degradation are independent and nearly equal.

During starvation-induced autophagy in mammals, LC3-II is recruited to autophagosomal membranes, which engulf the cytoplasmic components to be degraded via autophagy.16-19 LC3-II is then degraded upon fusion of autophagosomes with lysosomes.18,19 It has been established that E64d, a general cysteine proteinase inhibitor, effectively inhibits degradation of LC3-II. Therefore during nutrient deprivation, accumulation of LC3-II in the presence of E64d is a convenient marker for autophagosomal turnover.18-20 It is of interest to

Table 1. Composition Formulas and Molecular Structures of CAA0225, a Cathepsin L-Specific Inhibitor

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<thead>
<tr>
<th>Inhibitor</th>
<th>Molecular structure</th>
<th>IC50 (nM)</th>
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<tbody>
<tr>
<td>CAA0225</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td>1.9 L, 1070 B</td>
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The activities of purified rat cathepsin L and cathepsin B were determined separately using z-Phe-Arg-MCA (cathepsin L) and z-Arg-Arg-MCA (cathepsin B) in the presence of various concentrations of the inhibitors, as described in Materials and Methods. Inhibition versus inhibitor concentration was plotted and the profile of activity plotted and IC50 (nM) determined.

**The effects of these compounds on cathepsin L and cathepsin B, whereas z-Arg-Arg-MCA is cleaved only by cathepsin B. As clearly shown in Fig. 1, the activity of cathepsins B+L was inhibited substantially by CA-074 and partially by CAA0225. CA-074 inhibited cathepsins B+L activity by approximately 90%, while inhibition by CAA0225 was approximately 10% (Fig. 1; upper panel). Inhibition of cathepsin B activity in liver ML fraction by CA-074 was nearly complete (Fig. 1; middle panel). In contrast, CAA0225 did not inhibit cathepsin B or cathepsin D (Fig. 1; middle and bottom panels). These data are consistent with those obtained using purified cathepsin B and cathepsin L.

**The results for the degradation of long-lived proteins in HeLa cells.**
determine whether cathepsin L and cathepsin B contribute equally to LC3-II degradation.

When HeLa cells were incubated with HBSS–10 mM Hepes for 4 h, the band detected in immunoblots corresponding to LC3-II was faint (Fig. 3; top panel). In the presence of E64d, marked accumulation of LC3-II was detected (Fig. 3; HeLa, E64d), indicating substantial activity of autophagic turnover in HeLa cells under these conditions. Intriguingly, LC3-II accumulated when the cells were treated with CAA0225, but not with CA-074. These results indicate that cathepsin L preferentially contributes to LC3-II turnover. In the case of Huh-7 cells, however, small amounts of LC3-II accumulated in the presence of E64d and CAA0225, and the inhibitor-dependent increase in LC3-II was less than that observed in HeLa cells (Fig. 3; third panel). We found that in Huh-7 cells, expression of GABARAP, which is another homologue of LC3 not expressed in HeLa cells, was significant. As clearly shown in Fig. 3 (fourth panel), GABARAP-II, a membrane-bound form of GABARAP, accumulated markedly in the presence of E64d and CAA0225, but not in the presence of CA-074-Me. Thus autophagic turnover of GABARAP-II, another autophagosomal membrane marker, is strongly dependent on cathepsin L.

**DISCUSSION**

The results of the present study clearly indicate that CAA0225, a specific inhibitor of cathepsin L, can be used to assess the contribution of cathepsin L to lysosomal protein degradation. 1-trans-epoxy-succinic acid group of E64c, a well-known inhibitor for cysteine proteinases including cathepsin B and cathepsin L, is critical for the inhibitor to bind to the active-site cysteine of these enzymes. In an earlier study, we systematically synthesized derivatives of E64c and developed a new 1-trans-epoxysuccinyl peptide (CA-074), a specific inhibitor for cathepsin B.3) We found that the n-propylamide moiety of CA-074 is critical for specific inhibition of cathepsin B (Fig. 4; box).3) CAA0225 was also synthesized as one of these derivatives. Recently, a series of cathepsin L-specific inhibitors were developed and named the cathepsin L inhibitor Katunuma (CLIK) series.8,9) These inhibitors share the essential structure N-(trans-carbamoyloxy)-l-phenylalanine dimethylamide that is necessary for formation of a thioester bond with the active site of cathepsin L. Due to N-terminal pyridine part, the CLIK series of inhibitors do not fit into the active site of cathepsin B.8) Instead, the C-terminal phenylalanine moiety of the CLIK series that is also present in CAA0225 is important for specific interaction with cathepsin L (Fig. 4; dotted box).8) CAA0225 has a structural feature that is similar to the characteristic moiety of the CLIK series. The difference is that the phenylalanine dimethylamide moiety of the CLIK series is substituted with a phenylalanine phenyl-ethyl-amide moiety in CAA0225. It is likely that, because of this moiety,
CAA0225 is hydrophobic enough to penetrate biological membranes, resulting in efficient inhibition of cathepsin L in vivo.

Incubation of HeLa and Huh7 cells with CAA0225 caused partial inhibition of autophagic proteolysis of 14C-leucine-labeled long-lived proteins. The magnitude of the inhibitory effect of CAA0225 is comparable to that of CA-074-Me, a cathepsin B-specific inhibitor. The majority of 14C-leucine-labeled proteins constitute long-lived cytoplasmic proteins sequestered in autophagosomes. Hence our results indicate that cathepsin L and cathepsin B contribute equally to autophagic protein degradation of HeLa and Huh-7 cytoplasmic proteins.

Intriguingly, the turnover of LC3-II and GABARAP-II, two autophagosomal membrane markers, was more sensitive to the cathepsin L-specific inhibitors than were cytoplasmic primary autophagosomal membrane marker in Huh-7 cells. It indicate that GABARAP-II, rather than LC3-II, appears the topographic protein degradation of HeLa and Huh-7 cytoplasmic proteins.

REFERENCES