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# Microbial Serine Carboxypeptidase Inhibitors

## —Comparative Analysis of Actions on Homologous Enzymes Derived from Man, Yeast and Wheat—

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The actions of peptidase inhibitors derived from *Streptomycete* on human cathepsin A (hCath A), yeast carboxypeptidase Y (CPY), and wheat carboxypeptidase II (CPW) were analyzed comparatively. Lactacystin and omuralide (*clasto*-lactacystin $\beta$-lactone), well-known cytoplasmic proteasome inhibitors, both had a potent and non-competitive inhibitory effect on these homologous serine carboxypeptidases, although they inhibited CPW and hCath A more effectively than CPY *in vitro*. Ebelactone B exhibited a mixed non-competitive inhibitory effect and selectivity for CPY. Piperastatin A showed competitive inhibition of CPY and hCath A but had little effect on CPW. In contrast, chymostatin inhibited CPW efficiently, while it had less effect on hCath A and CPY. In cell culture system, lactacystin was the most potent as to inactivation of the intralysosomal recombinant hCath A activity expressed in a genetically engineered fibroblastic cell line with galactosialidosis (hCath A deficiency). These results suggest that the specific inhibitory effects of lactacystin and its derivatives on hCath A might be applicable to elucidate the pathophysiological roles in the human deficinecy.

Serine carboxypeptidases catalyze the hydrolysis of carboxyl terminal amino acids of peptides and proteins, which are widely distributed in eukaryotes and are classified into two groups; C-type carboxypeptidases (EC 3.4.16.5), with a preference for a hydrophobic amino acid residue at the P1′ position, and D-type carboxypeptidases (EC 3.4.16.6), with high affinity for positively charged (basic) P1′ residues[1]. All these enzymes have a S-H-D "catalytic triad" in their active sites, and are inactivated by specific inhibitors of serine proteases including diisopropyl fluorophosphate (DFP). Among them, yeast carboxypeptidase Y (CPY)[2] and wheat carboxypeptidase II (CPW)[3] are the well-characterized C- and D-type enzymes, respectively. Cathepsin A (Cath A; EC 3.4.16.1) is a mammalian enzyme that exerts serine carboxypeptidase activity at acidic pH and deamidase/esterase activities at neutral pH toward artifical substrates and a set of bioactive peptides, including endothelin-1 and tachykinins[4,5]. It preferentially hydrolyzes N-blocked dipeptides having aromatic and large hydrophobic amino acids at the positions P1 and P1′ as well as positively charged residues at the P1′ position at acidic pH[6]. The enzyme activity is strongly inhibited by DFP and mercurial compounds that react with free SH groups[7]. Although these serine

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carboxypeptidases exhibit only about 25% overall primary sequence identity, their three-dimensional structures, especially that of the active-site cleft, are very similar[2,8], and they belong to the so-called $\alpha/\beta$-hydrolase fold family[8,9]. Cath A is also known as a lysosomal protective protein that has protective function as to lysosomal glycosidases including $\beta$-galactosidase (EC3.2.1.23) and neuraminidase (EC3.2.1.18), forming a multimeric complex in lysosomes to protect the former enzyme from proteolytic degradation, and activating the latter one[10,11]. A human lysosomal protective protein/cathepsin A (PPCA) is synthesized as a catalytically inactive precursor/zymogen and is proteolytically processed to the active mature two-chain form[11,12].

The mature form (Cath A) is also secreted from human platelets and lymphoid cells[4,13], suggesting that the enzyme has not only an intralysosomal function but also an extracellular role. A primary defect of the PPCA gene that is located on chromosome 20q13.1, causes an autosomal inherited metabolic disorder (galactosialidosis) with simultaneous loss of these enzyme activities, and accumulation of their substrates and a variety of clinical manifestations[14,15]. The pathophysiological significance of Cath A has not been established, although an abnormal distribution of endothelin-1 as a possible natural substrate has been revealed in brain tissues from galactosialidosis patients[16]. Recently, it was also demonstrated that Cath A activity is up-regulated during the malignant progression of human melanomas[17]. The relation between the malignancy and the increase in Cath A activity in tumor tissues remains to be elucidated. Therefore, it is worth developing a specific inhibitor of Cath A as a probe for analysis of pathophysiological roles and as a new drug for the treatment of some types of malignant cancer.

In this study we analyzed the action of serine carboxypeptidase inhibitors of microbial origin, including lactacystin[18~20], omuralide[18~20], ebelactone B[21,22], piperastatin[23,24], chymostatin[25], and poststatin[26]. The specificity and action mechanism of the inhibitors as to human Cath A *in vitro* and in a culture system are discussed.

## Experimental

### Materials

Piperastatin A, poststatin, and chymostatin were provided by the Institute of Microbial Chemistry (Tokyo, Japan). Lactacystin and omuralide were kindly donated by Prof. HATAKEYAMA (Graduate School of Biomedical Scineces, Nagasaki University, Nagasaki, Japan). Carboxypeptidase Y (CPY), ebelactone B, *N*-benzyloxycarbonyl-L-phenylalanyl-L-leucine (*Z*-Phe-Leu), RPMI1640, and fetal calf serum (FCS) were purchased from Sigma (St. Louis, MO, USA). Wheat carboxypeptidase II (CPW) was purchased from Wako Pure Chemicals (Osaka, Japan). *N*-benzyloxycarbonyl-L-glycyl-L-glycyl-L-leucyl-7-amino-4-methylcoumarin (*Z*-Gly-Gly-Leu-AMC) was from CALBIOCHEM (San Diego, CA, USA). *Z*-lle-Glu-(*O-t*-butyl)-Ala-leucinal (PSI)[27] was from the Peptide Institute (Osaka, Japan). Stock solutions (10 mM) of the serine carboxypeptidase inhibitors (piperastatin A, poststatin and ebelactone B) and proteasome substrate (*Z*-Gly-Gly-Leu-AMC) were made up in ethanol, and those of chymostatin, *Z*-Gly-Gly-Leu-AMC and PSI were made with dimethyl sulfoxide (DMSO), which were stored at −30°C. Geneticin (G418), neomycin sulfate, and Ham's F-10 were purchased from GIBCO/BRL (Grand Island, NY, USA). The type I collagen solution (0.1%) was purchased from the Koken Institute (Tokyo, Japan). The reagents and enzymes for molecular biology were from Nippon Gene (Osaka, Japan) and Takara (Tokyo, Japan).

### Cells and Culture Conditions

A human fibroblastic cell line ASVGS-1[15] derived from a Japanese galactosidosis patient was maintained in Ham's F-10 medium supplemented 10% FCS at 37°C in the presence of 5% $CO_2$. The research was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the ethical committee of the institution in which the work was performed. A human neuroblastoma GOTO cell line stably expressing human PPCA cDNA[28] was cultured in RPMI 1640 containing 5% FCS at 37°C in the presence of 5% $CO_2$ on 150 mm-dishes (Nunc, Naperville, IL, USA), which were coated with 0.05% of the type I collagen solution.

### Establishment of the ASVGS-1 Cell Line Stably Expressing Human PPCA cDNA

ASVGS-1 cells ($4\times10^5$ cells) were seeded onto 60-mm plastic dishes 24 hours prior to the addition of vector plasmid DNA. Lipofection of 5 $\mu$g $pCXN_2$ or pCXPPCA[15] with cationic lipid reagent Ultrafector (B-bridge International Inc., San Jose, CA, USA) was performed. 6 hours after transfection, the cells were washed with phosphate-buffered saline (PBS) and then cultured in fresh Ham's F-10 medium containing 10% FCS. After 3 days, the cells were trypsinized and split 1 : 5 into 100-mm dishes, and then neomycin-resistant cell lines were selected with Ham's F-10 with 10% FCS and a final 400 $\mu$g/ml of G418.

Sources of Serine Carboxypeptidases and Proteasome

Partially purified yeast carboxypeptidase Y (CPY) was purchased from Sigma (St. Louis, MO, USA) and the purified wheat carboxypeptidase II (CPW) was from Wako Pure Chemicals (Osaka, Japan). As a source of hCath A, we used cell extracts of ASVGS-1 and GOTO cell lines stably expressing human PPCA cDNA, which are designated as GS-1-PPCA and GOTO-PPCA, respectively. About $1\times10^8$ cells (packed volume, 0.3 ml) of GOTO-PPCA were homogenized by sonication in nine volumes of 50 mM sodium acetate buffer (pH 5.6) or 50 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl, followed by centrifugation at 15,000×*g* for 15 minutes. The resultant supernatant (cell extract) was used for *in vitro* analysis of the mode of inhibition of hCath A by peptidase inhibitors. Cell extracts were also prepared from about $1\times10^7$ cells of GS-1-PPCA in the same manner for *in vitro* analysis of the dose-effects of inhibitors on hCath A and proteasome except that distilled water was used in preparation of the extract for proteasome assay.

Immunoblotting of Human Cath A

Cell extracts were prepared from $4\times10^5$ cells of ASVGS-1, GOTO-PPCA and GS-1-PPCA. Aliquots of the cell extracts were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% acrylamide gels. Proteins were subjected to SDS-PAGE after reduction with 25 mM mercaptoethanol, and then to immunoblotting with anti-PP32N12 and anti-PP20C12 antibodies as described previously[29]. Detection of the immunoreactive bands was performed with a chemiluminescence kit and a horseradish peroxidase-conjugated anti-rabbit IgG (CLONETECH, Palo Alto, CA, USA). Biotinylated molecular standards (B-bridge International Inc.) were used as molecular mass standard proteins.

Enzyme Assays

$\beta$-Galactosidase ($\beta$-Gal), neuraminidase (Neur), and $\beta$-hexosamindase ($\beta$-Hex) activities were measured fluorometrically with 4-methylumbelliferyl glycosides as substrates[30]. Cath A activity was measured with *Z*-Phe-Leu as the substrate at pH 5.6 and 6.5, as previously reported with a slight modification[31]. The pH of the substrate solution was also adjusted to pH 5.6 or 6.5. Proteasome activity was assayed with *Z*-Gly-Gly-Leu-AMC as the substrate at pH 7.5 as described previously[32]. Protein determination was performed with a D*c* protein assay kit (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as the standard.

Inhibition Study on Human Cath A, CPY and CPW with Microbial Inhibitors *in Vitro*

The stock solutions of microbial peptidase inhibitors were diluted with ethanol before inhibition assays. The inhibition assays were performed under two different pH conditions using 50 mM sodium acetate buffer (pH 5.6) and 50 mM sodium phosphate buffer (pH 6.5), each containing 50 mM NaCl. For analysis of the dose-dependency of inhibitors, various doses of inhibitors were added to aliquots of CPY, CPW, and a cell extract derived from GS-1-PPCA including hCath A, and then the mixtures were incubated for 30 minutes at 25°C before the enzyme assays. The concentrations (1~2%) of ethanol and DMSO as solvents in the mixture did not affect the Cath A activity. In the kinetic study, CPY and a cell extract derived from GOTO-PPCA were used as enzyme sources. The concentration of *Z*-Phe-Leu, as a substrate, and the incubation time were varied during measurement of the Cath A activities in the kinetic experiments. At the end of the incubation, the reaction was stopped by boiling for 2 minutes, and then the release of the degradation product was measured as described above. The *Km* and Vmax values, as kinetic parameters, were determined from a Lineweaver-Burk plot. For determination of the action modes of inhibitors, the same assay was performed in the presence of various doses of each inhibitor.

Inhibition Assay for Intracellular Recombinant Cath A in Human Fibroblastic Cells

GS-1-PPCA cells at subconfluency on 60-mm dishes were washed with PBS, and then further cultured in Ham's F-10 medium containing 10% FCS supplemented with various doses of inhibitors or ethanol, as a control. After incubation for 24 hours, the cells were washed PBS twice and harvested, and then cell extracts were prepared. Aliquots of the cell extracts were assayed for Cath A and other lysosomal enzyme activities. Note that almost all of the intracellular Cath A activity is due to the gene product of the exogenously introduced human PPCA cDNA.

## Results

### Inhibitory Effects of Microbial Inhibitors on Peptidases *In Vitro*

The structures of the peptidase inhibitors derived from *Streptomyces* strains and used in this study are summarized in Fig. 1. All the compounds including lactacystin, omuralide (*clasto*-lactacystin $\beta$-lactone), ebelactone B, chymostatin, piperastatin A and poststatin exhibited

Fig. 1. Microbial serine carboxypeptidase inhibitors used in this study.

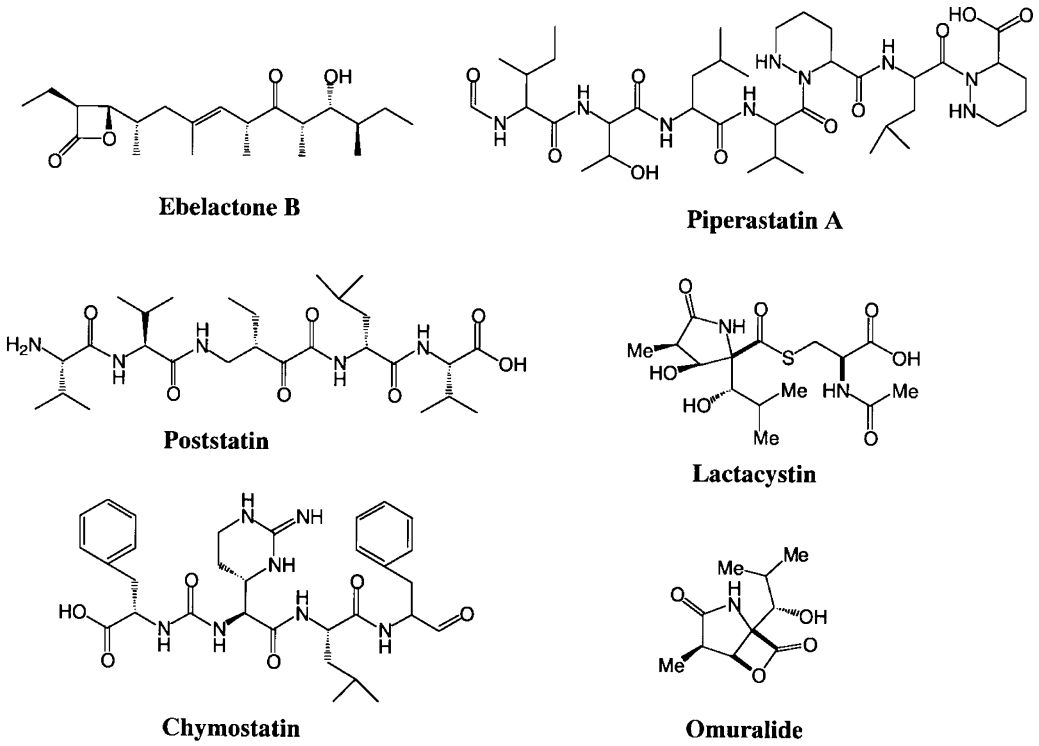


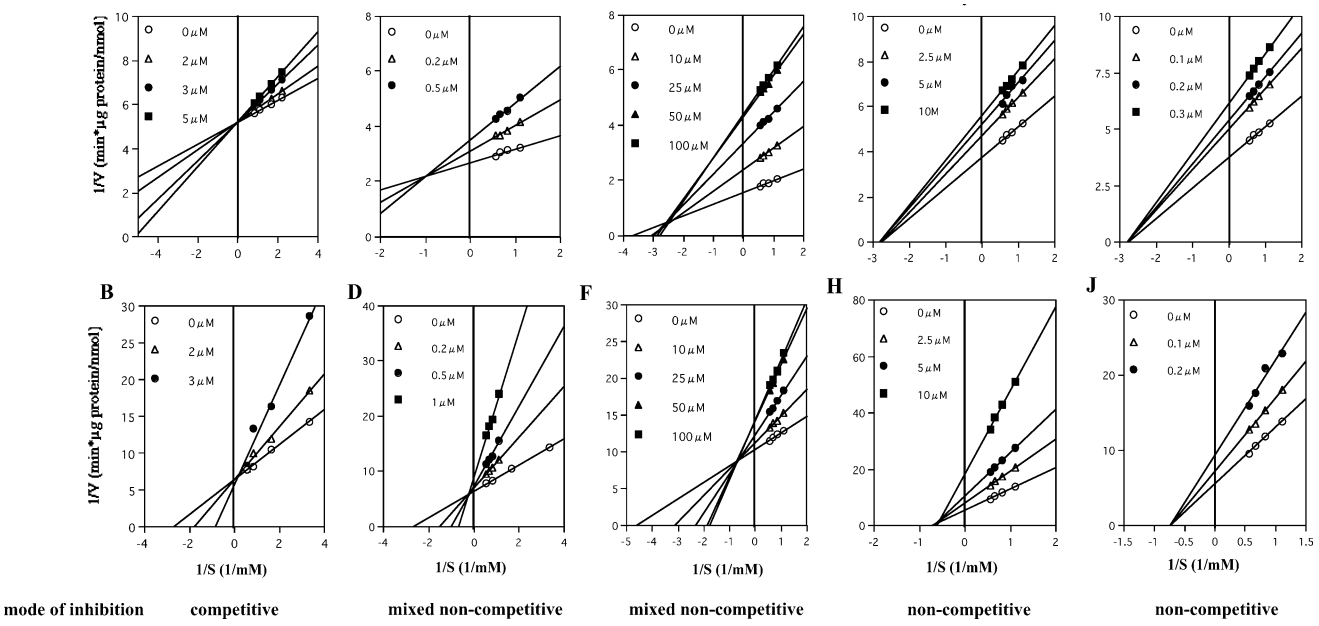

Table 1. Inhibitory activities of microbial inhibitors against peptidases[a].

| | $IC_{50}$ (μM) | | | | | | |
|---|---|---|---|---|---|---|---|
| | hCath A | | CPY | | CPW | | Proteasome |
| | pH 5.6 | pH 6.5 | pH 5.6 | pH 6.5 | pH 5.6 | pH 6.5 | pH7.5 |
| Omuralide | 0.099 | 0.012 | 0.096 | 0.078 | 0.0048 | 0.0020 | 0.10 |
| Lactacystin | 5.2 | 1.8 | 48 | 31 | 1.4 | 4.3 | 0.66 |
| Ebelactone B | 1.6 | 2.0 | 0.02 | 0.03 | 2.1 | 1.5 | >100 |
| Piperastatin A | 18 | 32 | 4.1 | 4.5 | >100 | >100 | >100 |
| Chymostatin | 92 | >100 | >100 | >100 | 13 | 2.2 | 21 |
| Poststatin | >100 | >100 | 89 | 29 | >100 | >100 | >100 |

[a]Preincubation of inhibitors with the enzyme at 25℃ for 30 min was performed as described under "Experimental".

inhibitory effects on the recombinant human Cath A expressed in fibroblastic cells, CPY and CPW *in vitro*, although the enzyme specificity, dose-dependency and degree of inhibition differed among them at different pHs. Table 1 summarizes the concentrations of the compounds necessary to inhibit the serine carboxypeptidases and proteasome by 50% ($IC_{50}$) at pH 5.6, 6.5 and 7.5, respectively. Among these inhibitors, omuralide, the active form of lactacystin, had the most potent effect on each enzyme, and inhibited their activities by more than 90% at below 5 $\mu$M (data not shown). The selectivity of CPW and hCath A for omuralide was relatively higher than that of

Fig. 2. Lineweaver-Burk plot of inhibition of hCath A by peptidase inhibitors under different pH conditions.




(A) by piperastain A at pH 5.6, (○) 0 $\mu$M; (△) 2 $\mu$M; (●) 3 $\mu$M; (■) 5 $\mu$M. (B) by piperastatin A at pH 6.5, (○) 0 $\mu$M; (△) 2 $\mu$M; (●) 3 $\mu$M. (C) by ebelactone B at pH 5.6, (○) 0 $\mu$M; (△) 0.2 $\mu$M; (●) 0.5 $\mu$M. (D) by ebelactone B at pH 6.5, (○) 0 $\mu$M; (△) 0.2 $\mu$M; (●) 0.5 $\mu$M; (■) 1 $\mu$M. (E) by chymostatin at pH 5.6, (○) 0 $\mu$M; (△) 10 $\mu$M; (●) 25 $\mu$M; (▲) 50 $\mu$M; (■) 100 $\mu$M. (F) by chymostatin at pH 6.5, (○) 0 $\mu$M; (△) 10 $\mu$M; (●) 25 $\mu$M; (▲) 50 $\mu$M; (■) 100 $\mu$M. (G) by lactacystin at pH 5.6, (○) 0 $\mu$M; (△) 2.5 $\mu$M; (●) 5 $\mu$M; (■) 10 $\mu$M. (H) by lactacystin at pH 6.5, (○) 0 $\mu$M; (△) 2.5 $\mu$M; (●) 5 $\mu$M; (■) 10 $\mu$M. (I) by omuralide at pH 5.6, (○) 0 $\mu$M; (△) 0.1 $\mu$M; (●) 0.2 $\mu$M; (■) 0.3 $\mu$M. (J) by omuralide at pH 6.5, (○) 0 $\mu$M; (△) 0.1 $\mu$M; (●) 0.2 $\mu$M. V=velocity. S=substrate. The modes of inhibition were also dipicted under the figures.

CPY at pH 6.5. Lactacystin exhibited more evident selectivity for proteosome, CPW and hCath A, albeit that the $IC_{50}$ values were two- or three-orders higher than those of omuralide. However, PSI, a specific inhibitor of the proteasome, had little effect on the recombinant hCath A activity, even at 100 $\mu$M (data not shown). Ebelactone B showed efficient inhibition of hCath A, CPY and CPW as well as selectivity for CPY, but had no effect on proteasome. The $IC_{50}$ value of ebelactone B at pH 5.6 for CPY (0.02 $\mu$M) was one-order smaller than those for hCath A (1.6 $\mu$M) and CPW (2.1 $\mu$M), respectively. Significant pH dependency of the ebelactone B action was not observed. Piperastatin A also inhibited the CPY activity effectively, and the $IC_{50}$ value for CPY was one-order lower than that for hCath A. Interestingly, this substance did not show any inhibitory effect on CPW and proteasome. On the other hand, chymostatin showed selective inhibition of CPW, although the proteasome activity was significantly inhibited. In contrast, chymostatin had little inhibitory effect on CPY and hCath A. Poststatin exhibited less effectiveness for these enzymes, albeit that the sensitivity of CPY was rather higher than those of the other enzymes.

## Modes of Inhibition by Peptidase Inhibitors of hCath A and CPY *In Vitro*

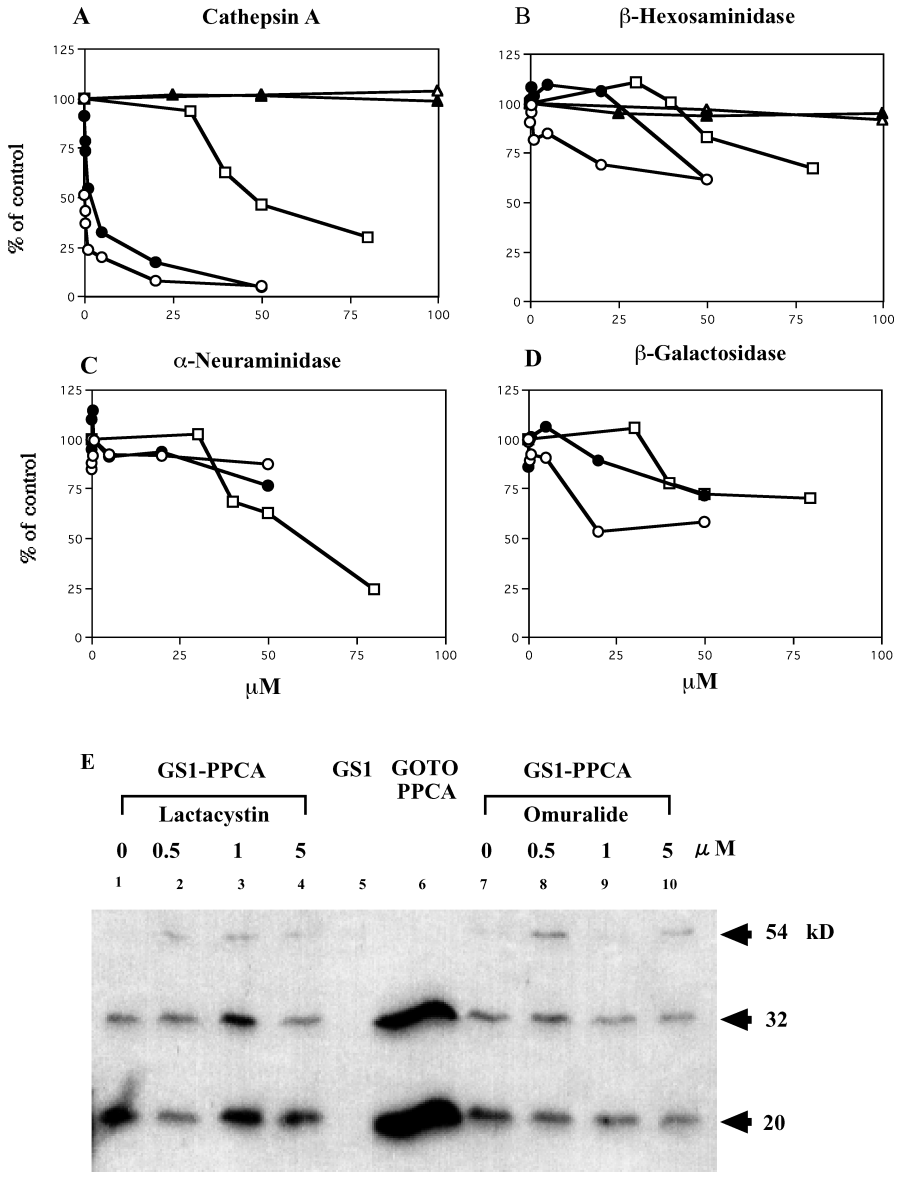
The modes of action of the inhibitors for hCath A were analyzed by means of a Lineweaver-Burk plot in comparison with those for CPY at pH 5.6 and 6.5. Fig. 2 summarizes the results. Piperastatin A exhibited competitive inhibition of hCath A (panel A and B) and CPY (data not shown). Ebelactone B (panel C and D) and chymostatin (panel E and F) showed mixed non-competitive inhibition of hCath A and CPY (data not shown). On the other hand, lactacystin (panel G and H) and omuralide (panel I and J) showed non-competitive

inhibition of hCath A. Modes of inhibition by these inhibitors were the same under both pH conditions.

## Effects of Microbial Serine Carboxypeptidase Inhibitors on Intracellular Recombinant hCath A Activity in Cultured Fibroblastic Cells

Microbial inhibitors were added to the culture medium of a human fibroblastic cell line (GS1-PPCA), in which almost

Fig. 3. Dose dependency of inhibitors for intracellular lysosomal enzymes in the culture system.




The GS1-PP cell line was treated for 24 hours with microbial peptidase inhibitors at the indicated doses added to the culture medium, and then harvested. Lysosomal enzyme activities [Cath A (A); $\beta$-Hex (B); Neur (C); $\beta$-Gal (D)] in the cell extracts were measured. The cells were treated with lactacystin (○), omuralide (●), ebelactone B (□), piperastatin A (▲), and poststatin (△) at the indicated doses. Aliquots of the extracts after treatment with lactacystin or omuralide were analyzed by immunoblotting using anti-PP32N12 and anti-PP20C12 antibodies as described under "Experimental". (E) Immunoblot pattern of the recombinant hCath A expressed in the fibroblastic cell line treated with lactacystin (lanes 1 to 4) and omuralide (lanes 7 to 10) at the indicated concentrations. Lane 5, fibroblastic cells derived from a galactosialidosis (PPCA deficiency patient) as a negative control. Lane 6, human neuroblastoma cell line GOTO stably expressing the recombinant human PPCA gene as a positive control. Arrows indicate the 54-kDa PPCA precursor, and the 32-kDa and 20-kDa subunits of mature PPCA (Cath A), respectively.

all of the Cath A activity was due to expressed recombinant human PPCA. After 24 hours the cells were harvested, and then cell extracts were assayed for lysosomal enzyme activities. As shown in Fig. 3, lactacystin and omuralide inhibited the intracellular Cath A activity dose-dependently, and the $IC_{50}$ values were estimated to be 0.5 $\mu$M and 5 $\mu$M, respectively. At these concentrations, these compounds had less effect on other lysosomal enzymes including $\beta$-hexosaminidase ($\beta$-Hex, panel B), $\alpha$-neuraminidase (Neur, panel C) and $\beta$-galactosidase ($\beta$-Gal, panel D), although $\beta$-Gal and $\beta$-Hex were inhibited by 40% of the control level when above 10 $\mu$M of these substances was added. Ebelactone B also showed a dose-dependent, inhibitory effect on the intracellular hCath A activity. The hCath A activity was reduced to about 50% of the control level when 50 $\mu$M was added. However, the other lysosomal enzyme activities were also dose-dependently inhibited at above 30 $\mu$M, although a preference of the ebelactone B for Cath A was observed. On the other hand, piperastatin A and poststatin had no effect on intracellular Cath A activity or $\beta$-Hex activity.

To determine whether the apparent decrease in intracelluar hCath A activity was due to degradation of the protein or not, immunoblotting for PPCA with anti-PP32N12 and anti-PP20C12 was performed before and after GS1-PPCA cells had been treated with lactacystin or omuralide. As shown in Fig. 3 (panel E), the intensity of the immunoreactive 32-kDa and 20-kDa bands corresponding to the two-chain mature form of PPCA (Cath A) (lane 6) did not significantly change for GST1-PPCA cells on treatment with various doses of lactacystin (lanes 1 to 4) and omuralide (lanes 7 to 10), indicating that these compounds did not cause the decrease in the intracellular mature PPCA but inhibited the catalytic activity of hCath A. In contrast, these bands were not detected for extracts derived from ASVGS-1 cells. Another cell-permeable proteasome inhibitor, PSI, also did not inhibit the intracellular Cath A activity (data not shown).

## Discussion

In this study the effects of microbial peptidase inhibitors derived from *Streptomyces* strains on recombinant human cathepsin A (Cath A) were compared with those on CPY and CPW. These substances were demonstrated to exhibit different inhibitory effects on the homologous enzymes in spite of the structural similarity in their active-site clefts[8].

Lactacystin and omuralide (*clasto*-lactacystin $\beta$-lactone) are well-known specific inhibitors of cytoplasmic multicatalytic proteasomes[18~20] that exhibit three peptidase activities: chymotrypsin-like, trypsin-like, and peptidylglutamylpeptidase activities. The substances inhibit the chymotrypsin-like peptidase activity *via* covalent binding between the $\beta$-lactone rings of active derivatives, and the threonine residues of the $\beta$- and X-subunits of the yeast and mammalian 20S proteasomes, which elicit many biological phenomena including the induction of neurite outgrowth from murine neuroblastoma cell line Neuro2a[18~20]. On the other hand, OSTROWSKA *et al.* have reported that the Cath A-like enzyme in human platelets was separable from proteasomes, and was also inhibited by lactacystin and omuralide[32]. The results indicated that these inhibitors are not necessarily specific for proteasomes, and suggest that some of the biological effects of these inhibitors may be due to the inhibition of Cath A. In this study we demonstrated directly that the recombinant human Cath A expressed in human fibroblastic cells could be inactivated by treatment with these inhibitors. Lactacystin and omuralide showed a non-competitive mode of inhibition of the Cath A activity, suggesting that these substances might inhibit the activity irreversibly. Another inhibitor, PSI, specific for proteasomes[27], did not show any inhibitory effect on the Cath A activity (data not shown). Although the inhibition mechanism for Cath A has not been elucidated in detail, the active form of lactacystin (omuralide) with the $\beta$-lactone moiety is also considered to bind covalently and irreversibly to the active serine. Another possibility is that these compounds may abolish Cath A activity through binding to the free cysteine residue located in the active site, because of the reaction on heavy metal ions and iodoacetamide reacting with the free cysteine residue was demonstrated previously[7]. However, in this comparative analysis these substances exhibited the efficient, inhibitory effects on CPW, which has no free cysteine residue in its active site. Therefore, it was suggested that omuralide could inhibit serine carboxypeptidases *via* direct interaction with the active serine residue but not necessarily with the free cysteine residues in their active sites.

Lactacystin and omuralide were also demonstrated in this study to selectively abolish the intracellular recombinant Cath A activity at relatively low concentrations when added to the culture medium of GS1-PPCA cells. Even though these substances have been reported to be cell-permeable to inactivate the cytoplasmic proteasomes[18,20], the present results suggest that these inhibitors could also be efficiently incorporated into lysosomes. Furthermore, lactacystin exhibited a more potent inhibitory effect on the intracellular Cath A activity than omuralide did in the cultured cell

system. One possible explanation for this is that omuralide itself with $\beta$-lactone may be more reactive with other components than lactacystin, and may be inactivated before transport to lysosomes when added to the culture medium. Alternatively, lactacystin is considered to be more stable in the culture medium and to be intracellularly activated after it has been incorporated into cells like a pro-drug. It would be interesting to determine whether any specific cell surface receptor molecules for lactacystin are present or not, which are delivered to lysosomes after binding with the substance.

Piperastatin A (*N*-formyl-allo I-T-L-V-Pip-L-Pip, Pip; hexahydropyridadine-3-carboxylic acid) showed competitive inhibition of hCath A and CPY *in vitro*, but the *Ki* value for hCath A (4.5 $\mu$M at pH 5.6) was one-order higher than that for CPY (0.11 $\mu$M at pH 5.6)(data not shown). In constrast, this substance did not show any inhibitory effect on CPW, suggesting that the active-site cleft of CPW can not adopt the structure of piperastatin A. Piperastatin A has been reported to selectively inhibit serine carboxypeptidases including CPY ($IC_{50}$, 0.064 $\mu$M at pH 6.5) and human platelet deamidase, possibly identical to hCath A ($IC_{50}$, 10.4 $\mu$M at pH 7.0)[23,24]. However, this substance had no effect on the intracellular Cath A activity in the culture system, indicating that it could not be taken up by the human fibroblastic cells.

Ebelactones are potent inhibitors of serine esterases derived from *Streptomyces* strains and are structually mycolic acid $\beta$-lactones[21,22]. They have been reported to inhibit pancreas lipase, liver esterase, and many other esterases[22]. This inhibition is believed to be irreversible because the active serine residues of the enzymes are expected to bind covalently to ebelactones by reacting with their $\beta$-lactone moiety. In the present study ebelactone B exhibited efficient inhibition of serine carboxypeptidases, in particular, CPY ($IC_{50\%}$, 0.02 $\mu$M at pH 5.6 and 0.03 $\mu$M at pH 6.5), possibly due to the structural preference for the active-site cleft. However, the substance showed a mixed non-competitive mode of inhibition of hCath A and CPY, suggesting that it might not abolish these enzyme activities irreversibly. Ebelactone B also inhibited the intracellular Cath A activity in the culture system, but the effect was suggested to be rather non-specific and cytotoxic because the other lysosomal enzyme activities also decreased depending on the dose of the agent. As ebelactone B has the ability to act on various serine esterases including lipase, it possibly inhibits multiple intracellular enzymes to cause some cytotoxic side-effect.

Poststatin had little effect on hCath A and CPW *in vitro*, or on the intracellular Cath A activity when added to the culture system. This peptidic inhibitor has been reported to be specific for prolylendopeptidases[26]. Therefore, the specificity of the inhibitor was suggested to be relatively low for serine carboxypeptidases.

RUDENKO *et al.* demonstrated the X-ray crystal structure of the precursor form of hCath A and the structural similarity to CPY and CPW, and the conformations of the conserved amino acid residues in the active sites, including the S-H-D catalytic triad, and carboxylate binding were predicted to be very similar among these carboxypeptidases[8]. Nevertheless, the specificity and preference of serine carboxypeptidase inhibitors for CPY, CPW and hCath A were shown to be rather different in the present study. The unconserved amino acid residues in the active sites of serine carboxypeptidases were considered to contribute to determination of the specificity and preference of inhibitors as well as peptidic substrates.

As the pathophysiological roles of the Cath A activity *in vivo* have not been fully elucidated, it is necessary to develop a specific inhibitor for Cath A as a probe. Most of the microbial inhibitors used in this study exhibited inhibitory effects on serine carboxypeptidases, and some of them showed selectivity for hCath A among these enzymes. However, these compounds have the ability to inhibit other enzymes, including esterases, lipase, proteasome and so on. On the other hand, PPCA gene knock-out mice have been utilized as a human galactosialidosis model for elucidating the pathogenic mechanism and for developing therapeutic methods[33]. However, as the protective function for regulating glycosidases as well as the catalytic function were disrupted in the model mice, the pathophysiological effects due to the catalytic defect of PPCA were indistinguishable from those due to the defect of its protective function. Recently, evidence has been presented that the catalytic (proteolytic) activity of PPCA triggers the degradation of lysosome-associated membrane protein type 2a (lamp2a), a receptor for chaperone-mediated autophagy[34], suggesting that unknown functions of Cath A remain to be elucidated. Therefore, it is worthwhile discovering and developing a more specific and potent inhibitor for Cath A activitiy to analyze the pathophysiological roles of Cath A in various experimental systems including human cultured cell lines. The accumulation of structural information concerning the homologous serine carboxypeptidase family is expected to facilitate the development of specific inhibitors of human Cath A.

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