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Species Differences Between Human and Rat in the Substrate Specificity of Cathepsin K

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Cathepsin K is known to play an important role in bone resorption, and it has the P2 specificity for proline. Rat cathepsin K has 88% identity with the human enzyme. However, it has been reported that its enzymatic activity for a Cbz-Leu-Arg-MCA substrate is lower than that of human cathepsin K, and that the rat enzyme is not well inhibited by human cathepsin K inhibitors. For this study, we prepared recombinant enzyme to investigate the substrate specificity of rat cathepsin K. Cleavage experiments using the fragment of type I collagen and peptidic libraries demonstrated that rat cathepsin K preferentially hydrolyses the substrates at the P2 Hyp position. Comparison of the S2 site between rat and human cathepsin K sequences indicated that two S2 residues at Ser134 and Val160 in rat are varied to Ala and Leu, respectively, in the human enzyme. Cleavage experiments using two single mutants, S134A and V160L, and one double mutant, S134A/V160L, of rat cathepsin K showed that all the rat mutants lost the P2 Hyp specificity. The information obtained from our comparative studies on rat and human cathepsin K should make a significant impact on developing specific inhibitors of human cathepsin K since rat is usually used as test species.

Key words: cathepsin K, hydroxyproline, S2–P2 interaction, species difference, substrate specificity.

Abbreviations: Abz, *o*-aminobenzoic acid; Cbz, carbobenzyloxy; Hyp, hydroxyproline; MCA, 4-methylcoumaryl-7-amide; Phe(NO2), *p*-nitro-l-phenylalanine.

Cathepsin K (EC 3.4.22.38) was first identified in rabbit osteoclasts as a novel and predominantly expressed cysteine protease (*1*). In recent years, it has been found that cathepsin K is highly and selectively expressed in human osteoclasts and has a high collagenolytic activity against type I and II collagen (*2–8*). Also, it has been reported that cathepsin K-antisense oligonucleotides inhibit osteoclastic bone resorption *in vitro* (*9*) and that cathepsin K-deficient mice manifest an impaired osteoclastic bone resorption, leading to osteopetrosis (*10*, *11*). In humans, mutations of the cathepsin K gene are known to be associated with pycnodysostosis, an autosomal recessive osteochondrodysplastic disease (*12–14*). These lines of evidence strongly suggest that cathepsin K plays a major role in bone resorption.

Since identification of cDNA encoding cathepsin K in rabbit osteoclasts (*1*), the corresponding cDNAs have been isolated from various species: human (*2*, *3*, *15*, *16*), monkey (*17*, *18*), rat (*19*, *20*) and mouse (*21*). Comparison of cathepsin K pre-proprotein sequences consisting of 329 amino acids between various species reveals that the sequence of the enzymes has a high degree of identity without any insertions or deletions. The identity to human cathepsin K pre-proprotein is 98.5% for monkey; 94.2% for rabbit; 87.5% for rat; and 86.6% for mouse, respectively (*17*).

The enzymatic characterization of human cathepsin K using the recombinant enzyme has been performed by several laboratories (*2*, *3*, *15*, *16*). As for substrate specificity, human cathepsin K prefers Pro at the P2 position with type I collagen, and Leu at the P2 position with short substrate peptides or inhibitors (*22–26*). In papain-like cysteine proteases, it is known that the S2–P2 interaction substantially contributes to defining the specificity for substrates and/or inhibitors (*27*). The positions of amino acids from the N-terminal side of the scissile bond in the substrate are denoted as P1, P2 and P3 sites, and the corresponding binding spaces in the enzyme are numbered S1, S2 and S3 subsites. The overall high homology among cathepsin K enzymes across the species suggests that the enzymatic characteristic of cathepsin K among the species is likely to be similar. In fact, monkey cathepsin K was shown to have a profile similar to the human enzyme (*18*).

However, rat cathepsin K, which has an 88.4% sequence identity to human cathepsin K in the mature

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protein, was less inhibited by human cathepsin K inhibitors than the human enzyme in most cases (*28–30*). Also, it was reported that the kinetic parameter k_{cat}/K_M of rat cathepsin K for the Cbz-Leu-Arg-MCA substrate is 12- to 35-fold lower than that of human cathepsin K (*30*). Despite these differences in the enzymatic characters from human cathepsin K, rat cathepsin K most likely contributes to bone resorption in rats, because the symptoms of osteopetrosis are observed in knockout mice that also belong to the rodent species (*10*, *11*); the sequence identity of rat cathepsin K to the mouse enzyme is 95.4%.

Here, we investigate the enzymatic character of rat cathepsin K and of three mutants of rat enzyme to comprehensively elucidate the differences between rat and human cathepsin K.

EXPERIMENTAL PROCEDURES

Fluorogenic Peptides—Cbz-Leu-Arg-MCA was purchased from Calbiochem-Novabiochem (Läufelfingen, Switzerland). Two peptidic substrate libraries having Abz-Gly-Pro-Ser-Gly-Phe(NO_2)-Gly-OH as a parent sequence were synthesized in our laboratory (*31*). All the fluorogenic substrates were stored frozen at −30°C as 10 mM stock solution in 100% (v/v) DMSO.

Preparation of Rat and Human Recombinant Cathepsin K—We have isolated cDNAs encoding procathepsin K from a human bone cDNA library (*2*). cDNAs encoding procathepsin K were also isolated from rat spleen by the RT–PCR method, using specific primers designed from the DNA sequence of rat cathepsin K (*19*). Procathepsin K expression was carried out basically according to the method described previously (*32*).

Culture medium of Tn cells infected with recombinant viruses carrying the rat or human cathepsin K gene was collected by low-speed centrifugation after 4 or 2 days of infection, respectively. The supernatant was adjusted to pH 5.5 by adding 0.1 N HCl, and applied to an SP-Sepharose fast flow column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 100 mM sodium acetate (pH 5.5) containing 1 mM EDTA (buffer A). Procathepsin K was eluted using a 0–1.5 M NaCl linear gradient in buffer A. Fractions were analysed by dot blotting, using a monoclonal anti-human cathepsin K. The pooled fractions containing procathepsin K were adjusted to pH 5.0 with 5 mM HCl, and concentrated using Centriprep-10 (Amicon, Danvers, USA) in the presence of 0.01% Tween-20, and then stored at −30°C until the next activation step. The procathepsin K was activated by incubation at 37°C in the presence of DTT and dextran sulphate (average MW: 50,000, Sigma, St. Louis, USA) at final concentrations of 1 mM and 100 μg/ml, respectively. The activation was monitored every 20 min using 20 μM Cbz-Leu-Arg-MCA as a fluorogenic substrate measured for 5 min at 37°C in 100 mM sodium phosphate (pH 7.0) containing 1 mM EDTA and 20 μM Tween-80. When the level of enzyme activity reached a plateau, the reaction was stopped by passing the activation mixture through a Sephadex G-25 column (Amersham Pharmacia Biotech, NAP-10) equilibrated with buffer A. To remove dextran sulphate, the activated solution was applied to a HiTrap Q column (Amersham Pharmacia Biotech) equilibrated with buffer A. The enzyme activity was eluted using a linear gradient between 0.4 and 0.75 M NaCl in buffer A. The final enzyme preparations were stored at −80°C until use. The purity of the enzymes was confirmed by SDS–PAGE analysis. N-terminal amino acid sequences of the purified mature cathepsin K were determined by automated Edman degradation with a PSQ-2 protein sequencer (Shimadzu, Kyoto, Japan).

Expression and Purification of Recombinant Rat Cathepsin K Mutants—Site-directed mutagenesis was performed by using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, USA) according to the manufacturer's instruction. The mutagenic primers 5′-GAC CCGTCTCTGTGGCCATCGATGCAAGC-3′ and 5′-GCTTG CATCGATGGCCACAGAGACGGGTC-3′ were used for generating the S134A mutant, and 5′-GCGACCGTGATA ATTTAAACCATGCCGTG-3′ and 5′-CACGGCATGGTTTA AATTATCACGGTCGC-3′ were used for generating the V160L mutant. The mutations introduced were confirmed by DNA sequencing. The mutated coding fragments of rat cathepsin K were subcloned into the pMBac vector (Stratagene) and the recombinant baculoviruses were prepared according to the standard procedures. The mutated rat cathepsin K was purified using the same methods as for the recombinants of rat and human cathepsin K.

Identification of Cleavage Sites in a Synthetic Peptide of the Type I Collagen Fragment—A 36-mer peptide was synthesized, based on the conserved sequence found in the rat, human and bovine type I collagens, as previously reported (*33*). The synthetic peptide (20 nmol) was incubated with cathepsin K (20 pmol) at 30°C for 10, 20 and 60 min. The reaction was stopped by adding 10 μM E-64. Samples were applied at various times to reverse-phase high performance liquid chromatography on an ODS-120T column (Tosoh, Tokyo, Japan), and the resultant peptides were subjected to N-terminal amino acid sequencing with a PSQ-2 protein sequencer (Shimadzu). The preference of cleavage sites was determined by comparing the yield of each digested peptide, separated by reverse-phase chromatography.

Enzyme Assay—Cathepsin K activity was determined fluorometrically by measuring the rate of increase in fluorescence of 4-methylcoumaryl-7-amide (MCA) produced by enzymatic hydrolysis of Cbz-Leu-Arg-MCA. Excitation and emission wavelengths used were 355 and 460 nm, respectively. The reaction mixture contained 100 mM sodium phosphate (pH 7.0), 1 mM EDTA, 1 mM DTT, 20 μM Tween-80 and 20 μM Cbz-Leu-Arg-MCA in a volume of 100 μl. The final concentration of DMSO was 1%. The reaction was started by the addition of the enzyme, and the increase in the intensity of fluorescence was recorded continuously at 37°C with a Fluoroskan Ascent (Labsystems, Helsinki, Finland). One unit of enzyme activity was defined as the amount of enzyme hydrolysing 1 μmol of Cbz-Leu-Arg-MCA per min.

When using the quenched fluorogenic peptides of the peptidic library, the enzyme activity was determined fluorometrically by measuring the rate of increase in fluorescence of *o*-aminobenzoic acid (Abz) liberated from the quenching *p*-nitro-L-phenylalanine [Phe(NO_2)] moiety

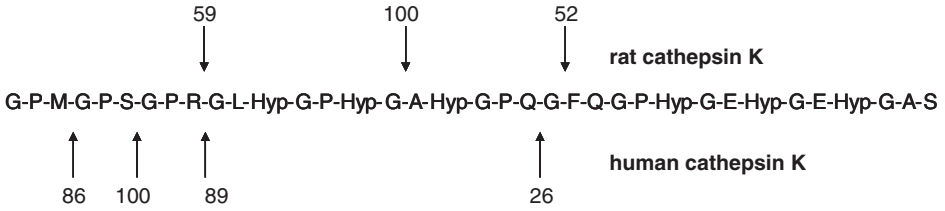

Fig. 1. **Cleavage of the collagen fragment by rat and human cathepsin K.** The sequence of a 36-mer peptide corresponding to the sequence from Gly1 to Ser36 in the mature form of rat, human and bovine type I collagens (α1) is shown with the cleavage sites by rat cathepsin K (upper) and human cathepsin K (lower). Cleavage sites with relative digestion rates are indicated by arrows.

by enzymatic hydrolysis of the substrate. The assay was carried out by the same method as that with Cbz-Leu-Arg-MCA, except for the substrate concentration of 100 μM. The fluorescence was monitored at 30°C at 460 nm (excitation wavelength 370 nm) using a Shimazdu RF-5000 spectrofluorophotometer. The initial velocity of the substrates was determined from the linear increase of the fluorescence observed from the beginning of the reaction, and shown as relative fluorescence unit (r.f.u.)/second by dividing by the reaction time.

Collagenolytic activity—Rat tail type I collagen was purchased from Becton Dickinson Labware (Bedford, MA, USA). The collagen (0.77 mg/ml) was incubated with cathepsin (0.5 μM) in 100 mM buffer containing 1 mM EDTA and 1 mM DTT at 25°C for 16 h. The incubation was performed in 100 mM sodium acetate (pH 5.5). The final molar ratio of cathepsin to collagen was 1:5. The reaction was stopped by adding 50 μM E-64, and then subjected to SDS–PAGE analysis (6% polyacrylamide gel, TEFCO).

RESULTS

Cleavage of a 36-mer Fragment Peptide of Type I Collagen by Rat Cathepsin K—To examine the enzymatic profiles of human and rat cathepsin K, we prepared the active forms of the recombinant enzymes. The proteolytic activity of the recombinant enzymes were examined using a 36-mer fragment peptide of type I collagen, which was designed from a conserved sequence among rat, human and bovine type I collagens (Fig. 1). In the sequence, the positions of hydroxyproline (Hyp) residues were deduced from the positions of rat skin type I collagen (*34*).

Human cathepsin K, as we have already reported (*33*), selectively cleaved the Xaa-Gly bond in a repeating motif, Pro-Xaa-Gly, of the type I collagen fragment, except for the case where Xaa is Hyp. On the other hand, rat cathepsin K showed a cleavage pattern completely different from human cathepsin K. The rat enzyme cleaved the Gly-Ala bond in the Hyp-Gly-Ala sequence, with which the human enzyme did not react.

Cleavage of the Quenched Fluorogenic Peptidic Libraries—The results shown in the previous section strongly suggest that the S2 specificity of the rat and human enzymes may differ. To further characterize the substrate preference of the rat enzyme on each subsite in substrates, cleavage experiments using peptidic libraries with quenched fluorogenic moieties were conducted.

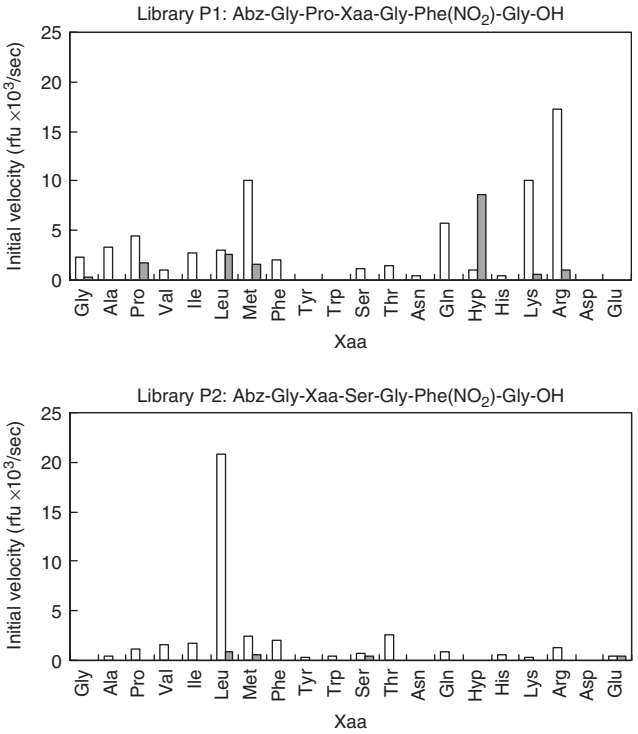

Fig. 2. **Hydrolytic activity of human and rat cathepsin K for quenched fluorogenic peptidic libraries.** Blank and filled bars are for human and rat cathepsin K, respectively. Cathepsin K was incubated with each substrate (100 μM) for 40 min at 37°C. Initial velocity is shown as relative fluorescence units (r.f.u.)/second.

The parent sequence of the fluorogenic peptide, Abz-Gly-Pro-Ser-Gly-Phe(NO_2)-Gly-OH, was designed from the sequences cleaved in the collagen fragment by human or rat cathepsin K. The following two peptidic libraries were prepared; library P1 [Abz-Gly-Pro-Xaa-Gly-Phe(NO_2)-Gly-OH] examining for the S1-subsite preference and library P2 [Abz-Gly-Xaa-Ser-Gly-Phe(NO_2)-Gly-OH] examining for the S2-subsite preference, respectively.

Rat cathepsin K showed a significantly different cleavage profile to the peptidic library from human cathepsin K (Fig. 2). Especially, the rat enzyme hydrolysed the substrate Abz-Gly-Pro-Hyp-Gly-Phe(NO_2)-Gly-OH in the library P1 approximately three times more preferentially than the second best substrate including

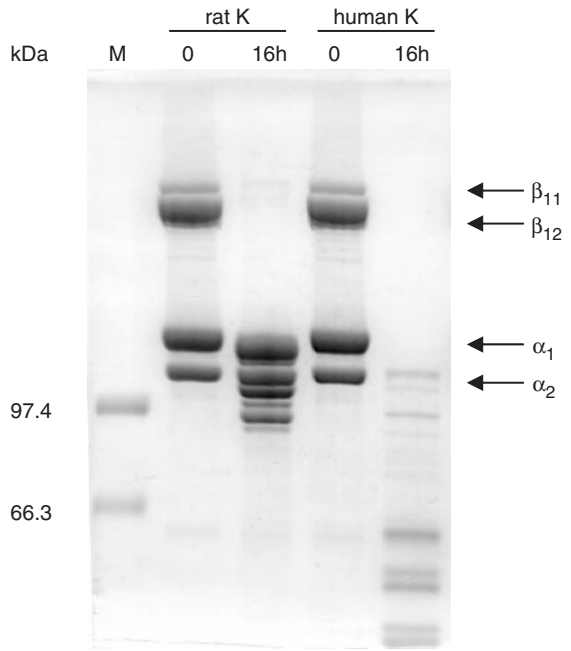

Fig. 3. **Cleavage of native type I collagen by cathepsin K.** The position of molecular mass markers (lane M) is indicated on the left. Positions of α and β chains are indicated on the right. Native rat type I collagen was incubated with rat cathepsin K (rat K) or human cathepsin K (human K) at 25°C for 16 h at pH 5.5. Samples (15 mg of collagen) at the reaction time of 0 and 16 h were separated on 6% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue.

Leu at the Xaa position of the sequence, whereas this profile was not observed for the human enzyme. MALDI-MS analysis for the fragments cleaved by rat cathepsin K confirmed that the cleavage occurred at the C terminus of Abz-Gly-Pro-Hyp-Gly (data not shown), indicating that the rat enzyme utilizes Hyp as the P2 residue.

Collagenolytic Activity of Rat Cathepsin K—We next examined the collagenolytic activity against native type I collagen, which forms a triple-stranded helix and is the main component of bone matrix. The reaction was performed under pH 5.5 at 25°C, a temperature at which the collagen triple helix is reported to be resistant to the attack by most proteinases (*35–38*). Both rat and human cathepsin K extensively degraded the type I collagen (Fig. 3). This indicates that both enzymes cleaved native type I collagen under acidic conditions corresponding to the environment in the resorption lacuna (*39*). However, the collagenolysis of the native type I collagen by the rat enzyme was much slower than that by the human enzyme.

Sequence Comparison of the S2 Site Between Rat and Human Cathepsin K and Construction of Rat Mutant Enzymes—The cleavage experiments using the collagen fragment and quenched fluorogenic libraries indicated that the S2 specificity of rat cathepsin K differs from the human enzyme. Cysteine proteases are known to predominantly determine their substrate specificity by the S2–P2 interaction (*27*, *40*). From the X-ray structures of human cathepsin K (*25*, *41–44*), amino acid residues possibly forming the S2 site in rat cathepsin K were predicted, and compared with those of human cathepsin K (Table 1). It was thus found that the S2 residues at the positions 134 and 160 are different between rat and human cathepsin K. In rat cathepsin K, the positions 134 and 160 are serine and valine, while human cathepsin K has alanine and leucine, respectively (numbering of the mature protein is used in this article). We assumed that these differences of the S2 residues between rat and human cathepsin K are relevant to the unique S2 specificity of rat cathepsin K.

Table 1. **Putative amino acid residues in the S2 and S3 subsites of human and rat cathepsin K[a].**

Subsite	Position	Human	Rat
S3	61	Asp	Tyr
	67	Tyr	Tyr
S2	68	Met	Met
	134	Ala	Ser
	160	Leu	Val
	163	Ala	Ala
	209	Leu	Leu

[a]Sources: human [*Homo sapiens*; GenBank accession No. X82153 (*2*)]; rat [*Rattus norvegicus*; GenBank accession No. AF010306 (*19*)].

To prove our assumption, we constructed two single mutants (S134A and V160L mutants) and one double mutant (S134A/V160L mutant) of rat cathepsin K by altering the S2 residue(s) of the rat enzyme to that of human cathepsin K, and investigated their substrate specificities.

Cleavage Experiments of the Type I Collagen Fragment and the Rat Mutant Enzymes—The hydrolytic preferences of the three rat mutant enzymes were examined using the collagen fragment. Figure 4 summarizes the results of the cleavage patterns for the three mutant enzymes. The cleavage patterns by the mutant enzymes were quite different from that by the wild-type rat cathepsin K (Fig. 1). The cleavage sites by the two single mutants, S134A and V160L, were identical, and the relative digestion rates were similar each other. Both cleaved the Xaa-Gly bonds in the repetitive Pro-Xaa-Gly sequences in the collagen fragment, except for the case where Xaa is Hyp. The cleavage of the Gly-Ala bond in the Hyp-Gly-Ala sequence, which was seen in the wild-type rat enzyme, was not observed for the single mutants. In addition, the single mutants hydrolysed the Gly-Phe bond in the Gln-Gly-Phe sequence as cleaved by the wild-type rat enzyme. The cleavage pattern by the double mutant was the same as that by human cathepsin K, although the quantitative profile of the digestion was not identical (Fig. 1).

Cleavage Experiments Using the Quenched Fluorogenic Peptidic Libraries and the Rat Mutant Enzymes—The cleavage patterns of the collagen fragment by the mutant enzymes suggested that those substrate specificities are changed from that of the wild-type rat cathepsin K. To confirm the alteration of the specificity, the cleavage experiments were also performed using the two fluorogenic peptidic libraries. As a result, the specificity for the Hyp residue was diminished in all the rat mutant enzymes (Fig. 5). Between the single mutants, the difference of the cleavage selectivity was unclear for

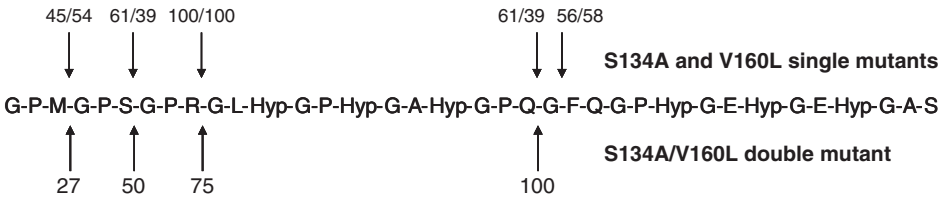

Fig. 4. **Cleavage sites of the collagen fragment by the rat cathepsin K mutants.** The amino acid sequence of the 36-mer fragment of type I (α1) collagen is shown with the cleavage sites by the single mutants (upper) and the double mutant (lower). Cleavage sites with relative digestion rates are indicated by arrows. The digestion rates of the single mutants are shown by the order of S134A and V160L mutants, respectively.

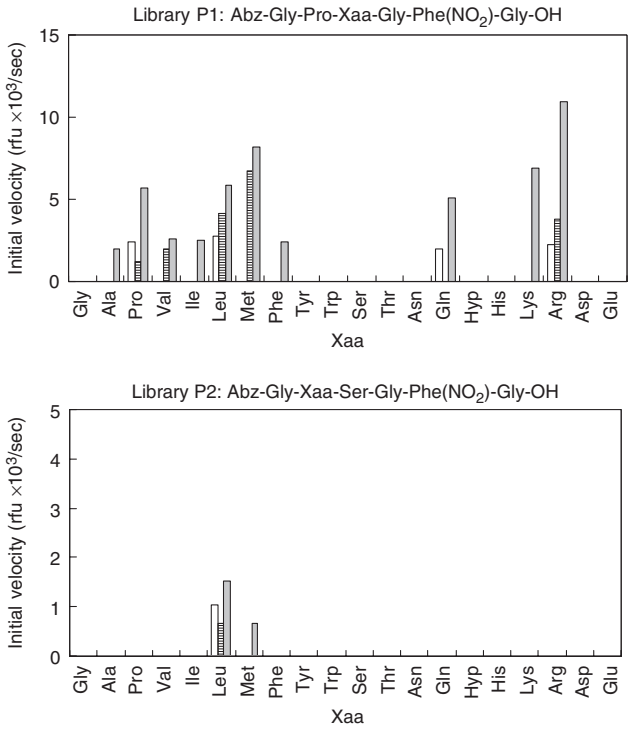

Fig. 5. **Cleavage results of quenched fluorogenic peptidic libraries by the rat mutant enzymes.** Blank, striped and filled bars are for S134A, V160L and S134A/V160L mutant enzyme, respectively. Initial velocity is shown as relative fluorescence units (r.f.u.)/second.

the collagen fragment. However, for the shorter substrates, these single mutants showed distinct specificity; for instance, the V160L and the double mutant cleaved the substrate Abz-Gly-Pro-Met-Gly-Phe(NO_2)-Gly-OH in the library P1.

Determination of Kinetic Parameters for the Substrate Cbz-Leu-Arg-MCA—To quantitatively analyse effects on catalytic activity by the replacement of the S2 residues in rat cathepsin K, the kinetic parameters of the rat mutant enzymes were determined and compared with those of rat and human cathepsin K using the substrate Cbz-Leu-Arg-MCA. All the rat mutant enzymes showed an increase of the catalytic activity over the rat enzyme by a factor of 5–19 in the k_{cat}/K_M values (Table 2). These increments were mainly achieved by an 11- to 57-fold decrease of the K_M values. The K_M value of the double mutant was the lowest among the mutants and almost

Table 2. **Kinetic parameters of cathepsin K enzymes for the Cbz-Leu-Arg-MCA substrate.**

	K_M (μM)	k_{cat} (s^{-1})	$k_{cat}/K_M \times 10^{-3}$ (M^{-1} s^{-1})
Rat cathepsin K	292.7	95.7	326.9
S134A	16.1	97.5	6,055.3
V160L	26.2	46.3	1,765.3
S134A/V160L	5.1	21.1	4,144.3
Human cathepsin K	7.5	56.1	7,476.7

equal to that of human cathepsin K. The V160L and the double mutant decreased their turn-over values by a factor of 2–5, while the k_{cat} value of the S134A mutant was similar to that of the wild-type rat enzyme.

DISCUSSION

In this study, the enzymatic character of rat cathepsin K was investigated and an unique substrate specificity was found from the results of enzymatic cleavage experiments using the type I collagen fragment and fluorogenic quenched substrates: the rat cathepsin K has a P2 specificity for Hyp residue. The catalytic activity of the rat enzyme for the substrate Cbz-Leu-Arg-MCA was shown to be smaller than that of the human enzyme: K_M(rat) = 292.7 μM, K_M(human) = 7.5 μM; k_{cat}/K_M of rat enzyme was 23 times lower than that of human enzyme. These values are similar to the kinetic parameters of rat cathepsin K for the same substrate reported by Marquis and colleagues (*29*): K_M(rat) = 99 μM, K_M(human) = 6 μM; k_{cat}/K_M of rat is 12–35 times lower.

In papain-like cysteine proteases, it is known that the S2–P2 interaction substantially contributes to defining the specificity for substrates and/or inhibitors (*27*). Therefore, the P2 Hyp specificity of rat cathepsin K suggests that there is a difference in the S2 site between rat and human cathepsin K. Sequence comparison of the S2 site between rat and human cathepsin K showed a difference of residues at the 134th and 160th positions: alanine and valine in the rat enzyme versus serine and leucine in the human enzyme, respectively (Table 1). The mutant enzymes of rat cathepsin K, S134A, V160L and S134A/V160L, were thus prepared by altering one or both residue(s), in order to investigate the effect of the S2 residues on the unique P2 Hyp specificity. The cleavage experiments using these rat mutants with the collagen fragment and fluorogenic substrates showed that both Ser134 and Val160 are needed to retain the P2 Hyp specificity.

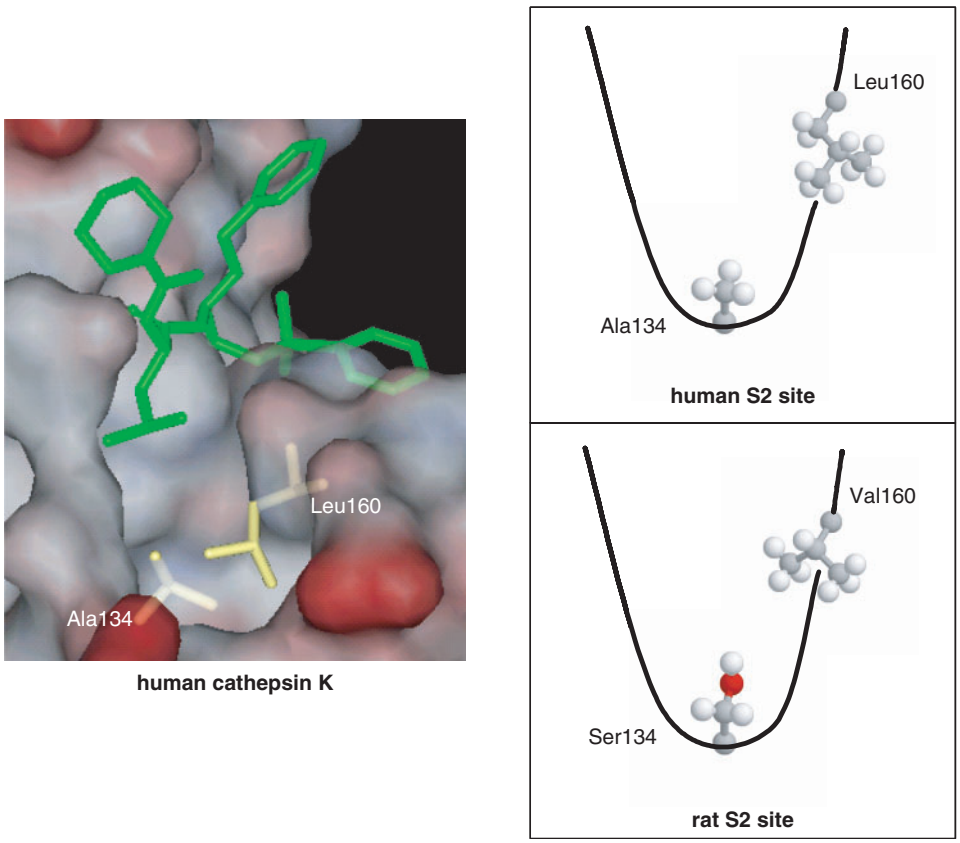

Fig. 6. **The S2 sites of the X-ray structure of human cathepsin K [PDB ID: 1MEM (*40*)] and of schematic model of human and rat cathepsin K.** The surface of the S2 site of the human enzyme is drawn by solvent accessible surface model using a probe with 1.4 angstrom in radius coloured by electropotential representation. The side chain atoms of Ala134 and Leu160, and inhibitor molecule are drawn by stick model in yellow and green, respectively. In the human and rat models, the side chain atoms at the positions 134 and 160 are represented by ball-and-stick model. The oxygen atom of Ser134 is coloured red.

Although the 3D structure of rat cathepsin K has not been solved yet, the position 134 of rat cathepsin K is most likely located at the bottom of the S2 pocket in analogy to the X-ray structures of human cathepsin K complexed with various inhibitors (Fig. 6). Also, the X-ray structure of the human enzyme reveals that the S2 site is composed only of hydrophobic amino acids such as Met68, Ala134, Leu160, Ala163 and Leu209 (Table 2). In the case of rat cathepsin K, a hydrophilic serine with the hydroxyl group in the side chain is placed at the position 134. This hydrophilic Ser134 should alter the S2 configuration of the rat enzyme toward more hydrophilic or hydrogen bondable compared with the hydrophobic S2 site of the human enzyme, and favour hydrophilic and/or hydrogen-bonding interactions with the hydroxyl group of the P2 Hyp.

The 160th position in human cathepsin K is located at the side-wall of the S2 pocket, as is presumed for the case of Val160 in rat cathepsin K (Fig. 6). Compared with leucine, valine has a sterically bulkier dimethyl group at the β-position in the side chain. Therefore, this steric bulkiness of Val160 may narrow the width of the S2 pocket in the rat enzyme. Judging from the lesser activity of rat cathepsin K for the P2 Pro, it is possible to predict that the P2 Pro residue in the substrates may not deeply bind to the narrowed S2 pocket due to lack of space and/or imperfect complementarity. On the contrary, hydroxyproline has a protruding portion, a hydroxyl group, on its proline scaffold and is the best P2 residue for rat cathepsin K. This suggests that the hydroxyl group of the P2 Hyp will be able to enter into the narrowed S2 space of rat cathepsin K to interact favourably with the hydroxyl group of Ser134. In summary, it was found that the results obtained with the rat mutants support our assumption that Ser134 and Val160 are key residues in the S2 site of the rat cathepsin K to determine the P2 Hyp specificity.

The double mutation of Ser134Ala and Val160Leu of the rat cathepsin K showed a human cathepsin K-like specificity; it had a cleavage pattern for the collagen fragment similar to that of the human enzyme and strong catalytic activity to the Cbz-Leu-Arg-MCA substrate. However, there are some differences from the human enzyme in the catalytic activity for the substrates used in this study. This could be due to differences in other important residues such as the S3 site for substrate specificity of papain-like cysteine protease: tyrosine for rat cathepsin K at the position 61, as opposed to asparatic acid for the human enzyme.

Another important finding in this study for rat cathepsin K was that the rat enzyme also has a collagenolytic activity for native type I collagen with

triple helical structure (Fig. 3). This finding is in line with the unique P2 Hyp specificity of the rat enzyme because hydroxyproline is known as one of the major components in type I collagen as well as proline. These results suggest that rat cathepsin K plays an important physiological role in bone resorption and/or in the bone remodelling cycle in rats.

Although the specificity of cysteine proteases is generally considered less strict than that of serine proteases (*27*), the findings in this study suggest that the S2–P2 interaction is characterized by very high selectivity even in cysteine proteases. In conclusion, our findings with rat cathepsin K in this study—distinct species differences in the substrate specificity and the weakened collagenolytic activity—should make a significant impact on for developing specific inhibitors of human cathepsin K since rat is usually used as a test species.

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CONFLICT OF INTEREST

The Authors, Sachiyo Tada, Kae Tsutsumi, Hideki Ishihara, Kenji Suzuki, Keigo Gohda and Naoki Teno are the employees of Novartis Pharma K.K.

REFERENCES

1. Tezuka, K., Tezuka, Y., Maejima, A., Sato, T., Nemoto, K., Kamioka, H., Hakeda, Y., and Kumegawa, M. (1994) Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts. *J. Biol. Chem.* **269**, 1106–1109
2. Inaoka, T., Bilbe, G., Ishibashi, O., Tezuka, K., Kumegawa, M., and Kokubo, T. (1995) Molecular cloning of human cDNA for cathepsin K: novel cysteine proteinase predominantly expressed in bone. *Biochem. Biophys. Res. Commun.* **206**, 89–96
3. Shi, G.-P., Chapman, H.A., Bhairi, S.M., DeLeeuw, C., Reddy, V.Y., and Weiss, S.J. (1995) Molecular cloning of human cathepsin O, a novel endoproteinase and homologue of rabbit OC2. *FEBS Lett.* **357**, 129–134
4. Brömme, D., Okamoto, K., Wang, B.B., and Biroc, S. (1996) Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. *J. Biol. Chem.* **271**, 2126–2132
5. Drake, F.H., Dodds, R.A., James, I.E., Connor, J.R., Debouck, C., Richardson, S., Lee-Rykaczewski, E., Coleman, L., Rieman, D., Barthlow, R., Hastings, G., and Gowen, M. (1996) Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. *J. Biol. Chem.* **271**, 12511–12516
6. Littlewood-Evans, A., Kokubo, T., Ishibashi, O., Inaoka, T., Wlodarski, B., Gallagher, J.A., and Bilbe, G. (1997) Localization of cathepsin K in human osteoclasts by in situ hybridization and immunohistochemistry. *Bone* **20**, 81–86
7. Kafienah, W., Bromme, D., Buttle, D.J., Croucher, L.J., and Hollander, A.P. (1998) Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix. *Biochem. J.* **331**, 727–732
8. Garnero, P., Borel, O., Byrjalsen, I., Ferreras, M., Drake, F.H., McQueney, M.S., Foged, N.T., Delmas, P.D., and Delaisse, J.M. (1998) The collagenolytic activity of cathepsin K is unique among mammalian proteinases. *J. Biol. Chem.* **273**, 32347–32352
9. Inui, T., Ishibashi, O., Inaoka, T., Origane, Y., Kumegawa, M., Kokubo, T., and Yamamura, T. (1997) Cathepsin K antisense oligodeoxynucleotide inhibits osteoclastic bone resorption. *J. Biol. Chem.* **272**, 8109–8112
10. Saftig, P., Hunziker, E., Wehmeyer, O., Jones, S., Boyde, A., Rommerskirch, W., Moritz, J.D., Schu, P., and von Figura, K. (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc. Natl Acad. Sci. USA* **95**, 13453–13458
11. Gowen, M., Lazner, F., Dodds, R., Kapadia, R., Feild, J., Tavaria, M., Bertoncello, I., Drake, F., Zavarselk, S., Tellis, I., Hertzog, P., Debouck, C., and Kola, I. (1999) Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. *J. Bone Miner. Res.* **14**, 1654–1663
12. Gelb, B.D., Shi, G.-P., Chapman, H.A., and Desnick, R.J. (1996) Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* **273**, 1236–1238
13. Johnson, M.R., Polymeropoulos, M.H., Vos, H.L., Ortiz de Luna, R.I., and Francomano, C.A. (1996) A nonsense mutation in the cathepsin K gene observed in a family with pycnodysostosis. *Genome Res.* **6**, 1050–1055
14. Motyckova, G. and Fisher, D.E. (2002) Pycnodysostosis: role and regulation of cathepsin K in osteoclast function and human disease. *Curr. Mol. Med.* **2**, 407–421
15. Brömme, D. and Okamoto, K. (1995) Human cathepsin O2, a novel cysteine protease highly expressed in osteoclastomas and ovary molecular cloning, sequencing and tissue distribution. *Biol. Chem. Hoppe-Seyler* **376**, 379–384
16. Li, Y. -P., Alexander, M., Wucherpfennig, A.L., Yelick, P., Chen, W., and Stashenko, P. (1995) Cloning and complete coding sequence of a novel human cathepsin expressed in giant cells of osteoclastomas. *J. Bone Miner. Res.* **10**, 1197–1202
17. McQueney, M.S., Feild, J., Hanning, C.R., Brun, K., Ramachandran, K., Connor, J., Drake, F., Jones, C.S., and Amegadzie, B.Y. (1998) Cynomolgus monkey (Macaca fascicularis) cathepsin K: cloning, expression, purification, and activation. *Protein Expr. Purif.* **14**, 387–394
18. Guay, J., Riendeau, D., and Mancini, J.A. (1999) Cloning and expression of rhesus monkey cathepsin K. *Bone* **25**, 205–209
19. Payne, J.A. and Miller, L.R. (1997) Direct submission of Rattus norvegicus cathepsin K mRNA sequence (Accession No. AF010306).
20. Feild, J.A. and Brun, K. (1998) Rat cathepsin K polynucleotide and polypeptide sequence encoding it. Patent EP861898.
21. Rantakokko, J., Aro, H.T., Savontaus, M., and Vuorio, E. (1996) Mouse cathepsin K: cDNA cloning and predominant expression of the gene in osteoclasts, and in some hypertrophying chondrocytes during mouse development. *FEBS Lett.* **393**, 307–313
22. Lecaille, F., Choe, Y., Brandt, W., Li, Z., Craik, C.S., and Bromme, D. (2002) Selective inhibition of the collagenolytic activity of human cathepsin K by altering its S2 subsite specificity. *Biochemistry* **41**, 8447–8454
23. Lecaille, F., Weidauer, E., Juliano, M.A., Bromme, D., and Lalmanach, G. (2003) Probing cathepsin K activity with a selective substrate spanning its active site. *Biochem. J.* **375**, 307–312
24. Alves, M.F, Puzer, L., Cotrin, S.S., Juliano, M.A., Juliano, L., Bromme, D., and Carmona, A.K. (2003) S3 to S3′ subsite specificity of recombinant human cathepsin K and development of selective internally quenched fluorescent substrates. *Biochem. J.* **373**, 981–986
25. Thompson, S.K., Halbert, S.M., Bossard, M.J., Tomaszek, T.A., Levy, M.A., Zhao, B., Smith, W.W.,

Abdel-Meguid, S.S., Janson, C.A., D'Alessio, K.J., McQueney, M.S., Amegadzie, B.Y., Hanning, C.R., DesJarlais, R.L., Briand, J., Sarkar, S.K., Huddleston, M.J., Ijames, C.F., Carr, S.A., Garnes, K.T., Shu, A., Heys, J.R., Bradbeer, J., Zembryki, D., Lee-Rykaczewski, L., James, I.E., Lark, M.W., Drake, F.H., Gowen, M., Gleason, J.G., and Veber, D.F. (1997) Design of potent and selective human cathepsin K inhibitors that span the active site. *Proc. Natl Acad. Sci. USA* **94**, 14249–14254

26. Hernandez, A.A. and Roush, W.R. (2002) Recent advances in the synthesis, design and selection of cysteine protease inhibitors. *Curr. Opin. Chem. Biol.* **6**, 459–465
27. Storer, A.C. and Ménard, R. (1996) Recent insights into cysteine protease specificity: lessons for drug design. *Perspect. Drug Discovery Des.* **6**, 33–46
28. Tavares, F.X., Boncek, V., Deaton, D.N., Hassell, A.M., Long, S.T., Miller, A.B., Payne, A.A., Miller, L.R., Shewchuk, L.M., Wells-Knecht, K., Willard, , D.H. Jr, , Wright, L.L., and Zhou, H.Q. (2004) Design of potent, selective, and orally bioavailable inhibitors of cysteine protease cathepsin K. *J. Med. Chem.* **47**, 588–599
29. Lark, M.W., Stroup, G.B., James, I.E., Dodds, R.A., Hwang, S.M., Blake, S.M., Lechowska, B.A., Hoffman, S.J., Smith, B.R., Kapadia, R., Liang, X., Erhard, K., Ru, Y., Dong, X., Marquis, R.W., Veber, D., and Gowen, M. (2002) *Bone* **30**, 746–753
30. Marquis, R.W., Ru, Y., LoCastro, S.M., Zeng, J., Yamashita, D.S., Oh, H.J., Erhard, K.F., Davis, L.D., Tomaszek, T.A., Tew, D., Salyers, K., Proksch, J., Ward, K., Smith, B., Levy, M., Cummings, M.D., Haltiwanger, R.C., Trescher, G., Wang, B., Hemling, M.E., Quinn, C.J., Cheng, H.Y., Lin, F., Smith, W.W., Janson, C.A., Zhao, B., McQueney, M.S., D'Alessio, K., Lee, C.P., Marzulli, A., Dodds, R.A., Blake, S., Hwang, S.M., James, I.E., Gress, C.J., Bradley, B.R., Lark, M.W., Gowen, M., and Veber, D.F. (2001) Azepanone-based inhibitors of human and rat cathepsin K. *J. Med. Chem.* **44**, 1380–1395
31. Teno, N., Kometani, M., Betschart, C., Toriyama, K., Niwa, S., Sakaki, J., Altmann, E., Buhl, T., Gamse, R., Gasser, J.A., Lattmann, R., and Missbach, M. (2004) *Proceedings of the 12th Annual Meeting of Division of Medicinal Chemistry.* Tsukuba, Japan, Nov24–26, pp. 1–28
32. Summers, M.D. and Smith, G.E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*. Texas Agricultural Experiment Station and Texas A&M University, College Station, TX. Bulletin No. 1555.
33. Nosaka, A.Y., Kanaori, K., Teno, N., Togame, H., Inaoka, T., Takai, M., and Kokubo, T. (1999) Conformational studies on the specific cleavage site of type I collagen (α-1) fragment (157-192) by cathepsins K and L by proton NMR spectroscopy. *Bioorg. Med. Chem.* **7**, 375–379
34. Bornstein, P. (1967) Comparative sequence studies of rat skin and tendon collagen. I. Evidence for incomplete hydroxylation of individual prolyl residues in the normal proteins. *Biochemistry* **6**, 3082–3093
35. Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J.A. (1993) Matrix metalloproteinases: a review. *Crit. Rev. Oral. Biol. Med.* **4**, 197–250
36. Aimes, R.T. and Quigley, J.P. (1995) Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4- length fragments. *J. Biol. Chem.* **270**, 5872–5876
37. Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1997) Membrane type I matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.* **272**, 2446–2451
38. Kafienah, W., Buttle, D.J., Burnett, D., and Hollander, A.P. (1998) Cleavage of native type I collagen by human neutrophil elastase. *Biochem. J.* **330**, 897–902
39. Silver, I.A., Murrills, R.J., and Etherington, D.J. (1988) Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp. Cell Res.* **175**, 266–276
40. Brömme, D., Bonneau, P.R., Lachance, P., and Storer, A.C. (1994) Engineering the S2 subsite specificity of human cathepsin S to a cathepsin L- and cathepsin B-like specificity. *J. Biol. Chem.* **269**, 30238–30242
41. McGrath, M.E., Klaus, J.L., Barnes, M.G., and Brömme, D. (1997) Crystal structure of human cathepsin K complexed with a potent inhibitor. *Nat. Struct. Biol.* **4**, 105–109
42. Yamashita, D.S., Smith, W.W., Zhao, B., Janson, C.A., Tomaszek, T.A., Bossard, M.J., Levy, M.A., Oh, H.-J., Carr, T.J., Thomson, S.K., Ijames, C.F., Carr, S.A., McQueney, M., D'Alessio, K.J., Amergadzie, B.Y., Hanning, C.R., Abdel-Meguid, S., DesJarlais, R.L., Gleason, J.G., and Veber, D.F. (1997) Structure and design of potent and selective cathepsin K inhibitors. *J. Am. Chem. Soc.* **119**, 11351–11352
43. Zhao, B., Janson, C.A., Amegadzie, B.Y., D'Alessio, K., Griffin, C., Hanning, C.R., Jones, C., Kurdyla, J., McQueney, M., Qiu, X., Smith, W.W., and Abdel-Meguid, S.S. (1997) Crystal structure of human osteoclast cathepsin K complex with E-64. *Nat. Struct. Biol.* **4**, 109–111
44. DesJarlais, R. L., Yamashita, D. S., Oh, H.-J., Uzinskas, I. N., Erhard, K. F., Allen, A. C., Haltiwanger, R. C., Zhao, B., Smith, W. W., Abdel-Meguid, S., D'Alessio, K., Janson, C. A., McQueney, M. S., Tomaszek, T. A., Levy, M. A., and Veber, D. F. (1998) Use of x-ray co-crystal structures and molecular modeling to design potent and selective non-peptide inhibitors of cathepsin K. *J. Am. Chem. Soc.* **120**, 9114–9115