Selective Inhibition of the Collagenase Activity of Cathepsin K*  

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Cathepsin K, the main bone degrading protease, and chondroitin 4-sulfate (C4-S) form a complex with enhanced collagenase activity. In this report, we demonstrate the specific inhibition of the collagenase activity of cathepsin K by negatively charged polymers without affecting the overall proteolytic activity of the protease. Three different mechanisms to interfere with cathepsin-catalyzed collagen degradation are discussed: 1) inhibition of the formation of the cathepsin K/C4-S complex, 2) inhibition of the attachment of C4-S to collagen, and 3) masking of the collagenase cleavage sites in collagen. By targeting these interaction sites, collagen degradation can be modulated while the non-collagenolytic activities of cathepsin K remain intact. The main inhibitory effect on collagen degradation is due to the impeding effect on the active cathepsin K/C4-S complex. Essential structural elements in the inhibitor molecules are negative charges which compete with the sulfate groups of C4-S in the cathepsin K/C4-S complex. The inhibitory effect can be controlled by length and charge of the polymers. Longer negatively charged polymers (e.g. polyglutamates, oligonucleotides) tend to inhibit all three mechanisms, whereas shorter ones preferentially affect the cathepsin K/C4-S complex.

An imbalance between bone formation and resorption can cause various bone diseases such as osteoporosis, certain forms of arthritis, and Paget disease. Type I collagen represents an essential part (90%) of the organic bone mass (1). It has been shown that cathepsin K, a cysteine protease predominantly expressed in osteoclasts (2–4), is an efficient collagenase that cleaves type I collagen at multiple sites in its helical domain (5, 6). Based on the physiological role of cathepsin K in bone resorption, cathepsin K inhibitors are being developed for the treatment of osteoporosis (7). Those inhibitors may also serve as drugs for rheumatoid arthritis (8). Presently, conventional cathepsin K inhibitors target the active site, thus causing a complete inhibition of enzymatic activity. An active site inhibition is associated with the loss of other physiological functions of cathepsin K activity which may result in undesirable side effects. Ablation of the matrix-degrading function in tandem with preservation of its non-collagenolytic protease activity would be greatly advantageous and will be addressed in this report.

Collagen consists of three intertwining α-chains of ~1000 residues each (9). The three-dimensional structures of triple-helical collagen and cathepsin K suggest that the active cleft of cathepsin K does not provide sufficient space to accommodate intact triple-helical collagen. The entrance to the catalytic site of cathepsin K is only 5 Å wide (10), while triple-helical collagen has a diameter of 15 Å. This leads to the assumption that prior to hydrolysis by cathepsin K, the triple-helical collagen has to be unwound to expose its single chains. It was recently demonstrated that bone- and cartilage-resident glycosaminoglycans such as chondroitin sulfate (C4-S)² enhance degradation of type I collagens by cathepsin K (11), suggesting a specific interaction between C4-S and cathepsin K. These glycosaminoglycans are released as peptidyl glycosaminoglycans by cathepsin activity from proteoglycans such as aggregan and can subsequently form the complex (12). In a previous report the formation of an oligomeric cathepsin K/C4-S complex has been described (13).

The existence of a collagenolytically active cathepsin K/C4-S complex offers a new target and a novel approach to inhibit excessive bone degradation. We assume that compounds which specifically interfere with the formation of an active cathepsin K/C4-S complex and/or its binding to collagen would only inhibit collagen breakdown without affecting the proteolytic function of the enzyme.

In this report, we also introduce a novel method using fluorescence polarization (FP) to discover compounds which are capable of selectively inhibiting the collagenase function of cathepsin K. The FP technique allows both the study of the carbohydrate-protein interaction which takes place in the formation of an active cathepsin K/C4-S complex, as well as the binding of the complex to collagen. The impact of a disturbed carbohydrate-protein interaction (e.g. cathepsin K/C4-S complex) is additive.

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plex) on collagen breakdown is elucidated by an in vitro collagen degradation assay. The selectivity of potential inhibitors with respect to collagen degradation is confirmed by the unabated proteolytic activity of the protease toward gelatin. Based on findings presented in this report, we propose novel drug design strategies for cathepsin K mediated bone degradation.

MATERIALS AND METHODS

Chondroitin 4-sulfate (C4-S) and polyamino acids were purchased from Sigma-Aldrich (Oakville, Ontario, Canada) with the exception of polyglutamates of 3–20 residues (E3 to E20), which were synthesized using standard automated peptide synthesis methods. Briefly, peptides Glu3, Glu5, and Glu20 were prepared on an ABI 431A synthesizer (Applied Biosystems, Roissy, France), using a 4-(2′,4′-dimethoxyphenyl-Fmoc-(N-(9-fluorenyl)methoxycarbonyl)-aminomethylphenoxyacetamido-norculeyl)-4-methylbenzydylamine resin (Novabiochem, Vancouver, British Columbia, Canada). Dipeptides Glu13, Glu14, and Glu15 were prepared on an automated solid phase peptide synthesizer (Pioneer, Applied Biosystems) using a PAL-PEG-PS resin (Applied Biosystems) and tetramethylfluorooromamidium hexafluorophosphate as activator in the presence of diisopropylethylamine according to the procedure reported elsewhere (15). Glu10, Glu11, Glu12, Glu13, Glu14, and Glu15 were prepared on an automated solid phase peptide synthesizer (Pioneer, Applied Biosystems) using a PAL-PEG-PS resin (Applied Biosystems) and tetramethylfluorooromamidium hexafluorophosphate as activator in the presence of diisopropylethylamine according to the procedure reported elsewhere (15).

Cathepsin K/C4-S Complex Formation

The cathepsin K complex was generated by mixing purified human cathepsin K and C4-S in 1/1 or 4/1 molar ratios in 100 mM sodium acetate buffer (pH 5.5), containing 2.5 mM DTT and EDTA. The complex solution was excited at 485 nm and the fluorescence polarization was quantified at 520 nm. All measurements were conducted in a total volume of 100 μl in a 96-well plate using fluorescence polarimeter Fluostar optima (BMG LABTECH, Offenburg, Germany). The relationship between protease concentration and FP was plotted and analyzed using non-regression analysis. The plot of fluorescence polarization (FP = FP\(_{\text{catK/C4-S*}}\) – FP\(_{\text{C4-S*}}\)) was calculated by subtracting the blank FP\(_{\text{C4-S*}}\) from FP\(_{\text{catK/C4-S*}}\). The blank FP\(_{\text{C4-S*}}\) corresponds to 20 nm fluorescence-tagged C4-S measured at 37 °C.

Cathepsin K/C4-S Competition Experiments

Potential inhibitors of the complex formation such as polyamino acids and oligonucleotides of different chain lengths were added at various concentrations to the cathepsin K\(_{\text{A}}\)/C4-S\(_{\text{A}}\) complex (20 nm). The change of the fluorescence polarization was monitored (excitation, 485 nm; emission, 520 nm).

Collagen/C4-S Competition Experiments

The collagen/C4-S complex was generated by combining 0.05 mg/ml collagen and 20 nm C4-S. Polyamino acids (poly-L-Ala, poly-D-Lys, poly-L-Lys, poly-L-Asp, poly-D-Glu, poly-L-Glu) and oligonucleotides of different chain lengths were added to the collagen/C4-S complex, and the mixtures were incubated for 20 min. The change of the fluorescence polarization was monitored (excitation, 485 nm; emission, 520 nm).

Collagen Degradation by Cathepsin K

0.6 mg/ml type I collagen was incubated with cathepsin K (200 nm) and C4-S (200 nm) in 100 mM sodium acetate buffer (pH 5.5), containing 2.5 mM DTT and EDTA. The collagen digest was performed at 28 °C in the absence or presence of NaCl, different polyamino acids, or oligonucleotides in a total volume of 30 μl. The degradation reaction was stopped with E-64 (Sigma-Aldrich) after 4 or 5 h. The degradation mixtures were subjected to SDS-PAGE using 4–20% Tris/glycine gels (Invitrogen). The gels were stained with Coomassie Blue (Sigma-Aldrich).

Collagen Degradation by Cathepsin L

0.6 mg/ml type I collagen was incubated with cathepsin L (2 μM) in 100 mM sodium acetate buffer (pH 5.5), containing 2.5 mM DTT and EDTA. The collagen digest was performed at 28 °C in the presence and absence of different polyamino acids.

\[3 \text{ D. Brömme, unpublished data.}\]
and with random sequence oligonucleotides in a total volume of 30 μl. The degradation reaction was stopped with E-64 after 9 h and subsequently subjected to SDS-PAGE using 4–20% Tris/glycine gels.

**Gelatin Degradation by Cathepsin K**

0.4 mg/ml heat-denatured type I collagen (gelatin) was incubated with 2 nm cathepsin K for 4 h at 28 °C. The gelatin degradation was performed in the presence of oligonucleotides of 15- to 30-mers (random sequence), poly-L-Asp, poly-L-Lys, and poly-DL-Ala.

**Statistics**

All measurements were performed in 2 or more replicates, and error bars represent the standard deviations from at least two independent experiments.

**RESULTS**

FP is a method allowing the evaluation of the binding between a molecule of small molecular mass and an acceptor molecule due to their molecular movement in solution (16). Besides protein-protein interactions (16), FP can be applied to the analysis of carbohydrate-protein interactions (17). In this study, we applied FP on the analysis of the carbohydrate-protein interaction between C4-S and cathepsin K or collagen and monitored the displacement of C4-S from the cathepsin K/C4-S complex by potential inhibitors.

**Binding Studies of Cathepsin K and Cathepsin L to C4-S**

A concentration-dependent change in polarization was found for cathepsin K and C4-S. As cathepsin K (0–200 nm) was gradually added to 20 nm C4-S, the polarization value increased (Fig. 1A). Since fluorescence polarization is considered to be proportional to the molecular size, an increasing polarization indicates the binding of cathepsin K to C4-S. Saturation of the polarization signal was reached at 160–200 nm cathepsin K in the presence of 20 nm C4-S, suggesting the average binding of 8–10 cathepsin K molecules to 1 molecule C4-S. In contrast to cathepsin K, addition of 0–125 nm cathepsin L to 20 nm C4-S did not significantly alter the polarization signal of free C4-S, signifying that cathepsin L did not interact with C4-S (Fig. 1B).

For subsequent inhibitor experiments, the binding curve of cathepsin K to C4-S was evaluated with respect to cathepsin K/C4-S ratios versus FP values. A high FP value of the cathepsin K/C4-S complex is required to receive a significant signal response as the complex is inhibited. At a 1/1 ratio, the protease-C4-S complex produced only a low polarization signal (Fig. 1A, a) and consequently a low signal response when inhibited. A stronger FP value was obtained at a 4/1 ratio between cathepsin K and C4-S (Fig. 1A, b) leading consistently to a signal response as an inhibitor was added. Therefore, the following experiments for inhibitor screening have been carried out using a cathepsin K/C4-S ratio of 4:1 (cathepsin K4/C4-S1).

**Inhibition of the Cathepsin K/C4-S**

—the goal of this study was to find inhibitors which selectively target the collagenolytic activity of cathepsin K without affecting its proteolytic activity. C4-S is a long, unbranched polymer consisting of the repeating disaccharide unit of β-glucuronic acid-(1,3)-N-acetyl-β-galactosamine, which contains numerous negative charges (–SO42– and –COO–). A putative inhibitor of the complex should possess similar physicochemical properties to compete with C4-S in the complex. In the following we describe the inhibitory effects of various polyamino acids and oligonucleotides.

**Polyamino Acids**—Polyamino acids of different physicochemical properties were studied with respect to their inhibitory effect on the collagenolytically active cathepsin K/C4-S complex (Fig. 2A). A selection of neutral and positively and negatively charged polyamino acids was chosen to explore the significance of charge in a putative inhibitor molecule. To determine whether the absolute configuration of the polyamino acid plays a role in the specific carbohydrate-protein interaction, a set of optical antipodes of poly-Lys and poly-Glu was included in the experiment. 20 nm cathepsin K4/C4-S1 was incubated with 0.5 μM polyamino acid for 20 min. The polarization was measured according to the experimental section and compared with the polarization value of the intact cathepsin K4/C4-S1 complex, which was taken as 100%. The FP assay revealed that negatively charged polyamino acids (poly-Asp and poly-Glu) cause a strong decrease in the polarization signal indicating an inhibition of the cathepsin K4/C4-S1 complex, whereas larger size 32.6–38-kDa poly-D- and poly-L-Glu show a slightly stronger effect than 10 kDa poly-L-Asp. The same magnitude of inhibition was found for poly-D- and poly-L-Glu underlining the marginal role of the absolute configuration in the displacement of C4-S. Positively charged polyamino acids led to a statistically unchanged FP signal as found for poly-L-Lys and poly-D-Lys. No effect on the complex was found for neutral poly-DL-Ala, which is presumably due to a lack of charges in the molecule (Fig. 2A).
The identical set of polyamino acids was applied to the \textit{in vitro} collagen degradation assay (Fig. 2B) to study whether the complex-inhibitory properties is associated with a suppressing effect on the collagenase function of cathepsin K. In this experiment, poly-Asp and poly-Glu showed a strong inhibition of the collagen degradation which correlates to the inhibitory effect that we observed on the complex. In the presence of poly-Asp and poly-Glu, monomeric $\alpha$-chains were generated, at the expense of the dimeric $\beta$- and trimeric $\gamma$-chains, which is due to a cleavage of telopeptides in collagen. This cleavage within non-helical telopeptides is characteristic for cysteine proteases and also evidence for the unimpeed proteolytic activity of cathepsin K. When incubating type I collagen with the active cathepsin K/C4-S complex in the absence of inhibitors (poly-Asp and poly-Glu), the degradation pattern shows a ladder of low molecular weight fragments.

An intriguing result was obtained for positively charged poly-L-Lys, which is capable of moderately inhibiting collagen degradation despite having no effect on the cathepsin K/C4-S complex as demonstrated in the FP assay. Neutral poly-DL-Ala did not affect collagen degradation.

\textbf{Chain Length of Poly-L-Glu Versus Inhibitory Effect—}To address the significance of polyamino acids chain length in the process of complex inhibition (Fig. 3A) and collagen degradation (Fig. 3B), the effect of poly-L-Glu of increased chain length (3–60 residues) was examined. For this experiment, poly-L-Glu solutions of equal percentage (0.001%) were used. This ensured that the amount of glutamate residues, regardless of whether they were part of longer or shorter chains, was kept constant while only the chain length varied. This allowed a direct comparison of shorter and longer poly-Glu specimens. The results show that poly-Glu consisting of 3–15 residues (E3 to E10) did not significantly affect the complex formation of cathepsin K$_4$/C4-S$^*$1. At least 15 residues (E15) were required to partially inhibit the complex formation and 60 residues were sufficient to suppress its formation almost completely (Fig. 3A). The effect of chain length is probably also the reason that both poly-L-Glu and poly-D-Glu were more effective than poly-L-Asp as the later polymer had a three to four times lower molecular weight than the poly-Glu derivatives (Fig. 2A).

In the collagen degradation assay we found that the inhibition of the cathepsin K$_4$/C4-S$^*$1 complex correlated directly to the inhibition of collagen degradation by cathepsin K (Fig. 3, A and B). Only polyglutamates of 15 or more residues exhibited an inhibition of collagen degradation, resulting in the preservation of undigested $\alpha$1 and $\alpha$2 bands and only a few low molecular weight degradation products. Incubation of the protease-C4-S complex with 20-mer or larger oligomers did not generate any collagen fragments, suggesting a complete inhibition of the triple-helical collagenase activity of the protease complex and its dependence from the chain length of the inhibitory specimen.

\textbf{Study of Chain Length and Sequence of Oligonucleotides—}Oligonucleotides of varying chain lengths (5-, 15-, 25-, and 30-mer) and sequence (random or arbitrarily defined ATCTG repeats) were studied regarding their potency to inhibit the cathepsin K$_4$/C4-S$^*$1 complex formation as well as its associated collagen degradation. Based on the poly-Glu experiments, the
The total number of mononucleotide building blocks was kept constant in the oligonucleotide solutions, while only the chain length varied. Analogous to the poly-Glu specimens, oligonucleotides were found to interfere with the cathepsin K/C4-S*1 complex formation (Fig. 4A) and collagen degradation (Fig. 4B) with respect to their increasing chain length (5-mer to 25-mer). Oligonucleotides of both, defined ATCTG-repeating and random, sequences complied with the rule of the chain length versus inhibitory effect, with no major difference in the magnitude of inhibition between the sequence-defined and randomized specimens based on the FP assay. Interestingly, the randomized oligonucleotides of longer chain length revealed a somewhat different pattern of collagen fragments in the collagenase assay. This may indicate an additional inhibitory effect independent from the inhibition of the cathepsin K/C4-S complex formation.

Dose Dependence—The concentration-dependent inhibitory effect of oligonucleotides was studied with the 30-mer. The study showed that an increasing concentration of the 30-mer oligonucleotide was associated with an increasing inhibition of complex formation (Fig. 5A). In the collagen degradation assay we obtained a significant inhibition with a 50-fold molar excess of the 30-mer oligonucleotide (10 μM 30-mer oligonucleotide/200 nM cathepsin K), which is comparable with the findings derived from the polarization studies (Fig. 5B). Interestingly, at lower concentrations of the 30-mer oligonucleotide, the effect on collagen degradation weakened whereas the oligonucleotide retained its potency to displace C4-S from cathepsin K at those low concentrations (Fig. 5A).

Collagen/C4-S Interaction; Influence of Polyamino Acids and Oligonucleotides—Since C4-S can potentially interact with positive charges present on collagen, the effect of polyamino acids and oligonucleotides was also tested on the collagen/C4-S binding using the FP assay. Preliminary polarization data for a mixture of type I collagen and C4-S* (data not shown) confirmed a binding of C4-S to type I collagen. For the FP competition assay a collagen/C4-S ratio similar to the in vitro collagen degradation assay was chosen, using 20 nM C4-S and 0.05 mg/ml type I collagen. To compare the results for the collagen/C4-S interaction (C of Figs. 2–4) with the cathepsin K/C4-S interaction (A of Figs. 2–4), identical concentrations of polyamino acids (0.001%) and oligonucleotides (0.00045%) were used in both competition experiments.

An impeding effect on the collagen/C4-S interaction was observed for negatively charged polyamino acids such as poly-L-Asp and poly-L-Glu (Fig. 2C) and for oligonucleotides (Fig. 4C). Analogous to the cathepsin K/C4-S competition experiments, the inhibitory effect of poly-Glu (Fig. 3C) and oligonucleotides (Fig. 4C) depends on charge and chain length. However, the ability of poly-Glu and oligonucleotides to inhibit the collagen/C4-S complex is apparently lesser when compared with the cathepsin K/C4-S complex (C and A of Figs. 2–4). The competitive binding of the charged ligands to both cathepsin K and collagen may affect the overall potency of the ligands to inhibit the degradation of collagen. This may explain why in the absence of collagen, the 30-mer oligonucleotide sufficiently inhibits the formation of the cathepsin K/C4-S complex at low ligand concentration whereas the inhibition in the collagen
degradation assay is decreased (Fig. 5, A and B). Interestingly, positively charged poly-L-Lys interferes with the collagen/C4-S binding which is, in magnitude, similar to poly-L-Asp (Fig. 2C), while the cathepsin K/C4-S complex is not affected (Fig. 2A).

Gelatinolytic Activity of Cathepsin K in the Presence of Potential Inhibitors—To verify that the tested molecules do not inhibit the collagenolytic activity by interacting with the active site of the enzyme, the proteolytic activity of cathepsin K was examined by degrading gelatin in the presence of polyamino acids and oligonucleotides. Heat-treated type I collagen (gelatin) was incubated with 2 nM cathepsin K in the presence or absence of 5 μM 15- to 30-mers of oligonucleotides with random sequence and a representative for each type of polyamino acid (negatively charged poly-L-Asp, positively charged poly-L-Lys, neutral poly-DL-Ala). The gelatin degradation assay revealed that the fragmentation pattern of gelatin generated by cathepsin K in the presence or absence of all tested compounds was very similar, leaving only small traces of undigested gelatin (Fig. 6). Consequently, neither of the tested compounds had an effect on the general proteolytic activity of cathepsin K.

Characterization of the Cathepsin L Recognition Site on Collagen—To investigate whether a part of the observed in vitro inhibition of collagen degradation is due to a masking of collagenase cleavage sites on collagen by the tested inhibitors, the digest of collagen by cathepsin K would have to be carried out in the absence of C4-S. This is rather difficult due to the fact that commercially available collagen preparations of type I collagen contain ~0.2 μg/ml C4-S (13), which can always interact with cathepsin K to form an active complex. The complete removal of C4-S from collagen is difficult to achieve when potentially denaturing the native triple-helical collagen structure. Therefore, cathepsin L was used as it does not interact with C4-S (Fig. 1A). Provided, a
compound masks cathepsin L cleavage sites in collagen, the collagenolytic activity of cathepsin L should be abrogated. For this study, type I collagen was incubated without C4-S in the presence of polyamino acids of varying charge and length, as well as random sequence oligonucleotides. As cathepsin L has a significantly lower collagenolytic activity than cathepsin K (18), we used a high concentration of cathepsin L (2 μM) in the cleavage assay and increased the digest time to 9 h. We found that only negatively charged compounds inhibited collagen degradation while neutral and positively charged polyamino acids showed no effect. The fragmentation pattern shows that the collagenase activity of cathepsin L vanished in the presence of negatively charged poly-L-Asp and poly-D- and poly-L-Glu (Fig. 7A). Regarding chain length, longer polymers (polyglutamate of 60 residues, 30-mer random sequence oligonucleotide) clearly inhibited cathepsin L-mediated degradation, whereas shorter chains (polyglutamate of 3 residues, 5-mer random sequence oligonucleotide) had no effect (Fig. 7B). Since none of the cathepsin L-inhibiting substances interfered with the active site of cathepsin L (no effect on gelatin degradation; data not shown), it can be concluded that they block the cleavage sites of cathepsin L in collagen.

DISCUSSION

Cathepsin K is a very potent collagenase (18) with the ability to cleave triple-helical collagens at multiple sites (5, 6). It has been shown previously that glycosaminoglycans such as C4-S can modulate the collagenolytic activity of cathepsin K (19) and that it forms a collagenolytically active protease/C4-S complex (13). Exploiting the fluorescence polarization between labeled C4-S and cathepsin K, we demonstrated the binding of cathepsin K to C4-S.

The cathepsin K molecule is characterized by a unique and high density of positively charged residues (Lys, Arg) located on the opposite side of the catalytic center (Fig. 8). Presumably, these positive charges allow for electrostatic interactions with negatively charged C4-S, contributing to the formation of the active complex. Contrary to cathepsin K, cathepsin L (which lacks comparable positively charged patches on its protein surface) does not interact with C4-S* in the FP assay emphasizing that positive charges on cathepsin K are involved in the binding with C4-S*.

To inhibit the formation of a collagenolytically active complex between cathepsin K and C4-S, a selection of potential C4-S competitor compounds was tested. Molecules featuring similar ionic properties to C4-S were chosen as initial lead structures. Polymers of polyamino acids seemed to be appropriate candidates for studying the structural requirements for a potent complex inhibitor, with a focus on charge, absolute configuration and molecular size. The FP competition studies revealed that neither neutral (poly-Ala) nor positively charged polymers (poly-Lys) had an inhibitory effect on the cathepsin K/C4-S* complex. Apparently, only polymers carrying negative charges (−COO−), such as poly-Asp and poly-Glu, are capable of impeding the cathepsin K/C4-S* complex. These findings clearly revealed that negative charges are essential for competitively preventing the sulfate groups of C4-S from binding the positively charged surface residues on cathepsin K. The results also demonstrated that the nature of this interaction is mainly electrostatic. The configuration of the polyamino acids played no role in their inhibitory potential as no differences were found between D and L isomers.
The significance of chain length in negatively charged polymers was investigated in more detail by testing poly-Glu species consisting of 5–60 residues. An unambiguous correlation between chain length and complex inhibition was found. This implies that besides negative charges, the size of the molecule is also relevant for the disruption of the active complex. For example, an inhibitory effect seen with \( \frac{1}{11003} \) E20 did not occur with \( \frac{4}{11003} \) E5. As the number of charges is the same in the \( \frac{1}{11003} \) E20 and \( \frac{4}{11003} \) E5, the inhibitory effect depends on the chain length. Interestingly, a size of at least 15 Glu residues (E15) is required to inhibit complex formation. A prediction of the secondary structure for poly-Glu of varying length based on an implementation of the Bayesian prediction formalism in the modeling program MOE (20) suggests a helix as a secondary structure for molecules sized E15 and above, which agrees with inhibitory potentials for complex inhibition. It can be speculated that the formation of a helix optimally organizes the spatial orientation of carboxylate groups for an efficient interaction with the Lys and Arg residues of cathepsin K, thus resulting in a potent complex inhibition.

Analogous to poly-Glu, the inhibition profile of oligonucleotides revealed that the binding affinity, which is related to the size of oligonucleotides and their charges, is the defining factor for complex inhibition. Since there was no significant difference between the magnitude of complex inhibition for random and defined oligonucleotides, it can be ascertained that the sequence of oligonucleotides associated with a certain secondary structure plays a subordinate role to the protein-nucleotide interaction.

By means of in vitro collagen degradation assays, we could confirm that an inhibition of the cathepsin \( K/\text{C4-S}^* \) by negatively charged polyamino acids and oligonucleotides was consistently associated with a suppression of type I collagen degradation. As polyamino acids and oligonucleotides did not affect the proteolytic function of cathepsin K (as determined by the gelatin degradation assay, it can be concluded that (i) the tested polyamino acids and oligonucleotides do not block or interact with the active site of cathepsin K, (ii) the observed inhibition of collagen degradation mainly stems from an inhibition of the formation of an active cathepsin K/C4-S complex, and (iii) the degradation of noncollagenous proteins (such as gelatin) does not require a cathepsin K/C4-S complex, which confirms earlier findings (13). We have hypothesized that the cathepsin K/C4-S complex is required for the unfolding of the collagen triple-helix prior to the proteolytic cleavage of its alpha-chains. Thus, the prevention of complex formation or its maintenance would specifically preclude the unwinding of the collagen helix and thereby its subsequent hydrolysis. On the other hand, the inhibition of complex formation does not affect the catalytic site of the protease and thus does not inhibit the hydrolysis of non-helical substrate (11, 13, 19).

We have previously described that the collagenase activity of cathepsin L is inhibited in the presence of C4-S, while its proteolytic activity is not affected (19). Moreover, as shown in this report, no complex formation between C4-S and cathepsin L was observed. This raises the question at which stage C4-S interferes with cathepsin L-mediated collagen degradation. Potentially, C4-S could prevent the binding of cathepsin L to collagen by binding to collagen itself and disguising the putative binding site for cathepsin L. This proposed collagen/C4-S binding has been demonstrated by chromatographic experiments by others (21). Ionic association takes place between negatively charged groups on the C4-S and positively charged groups on collagen. Consequently, C4-S could on one hand prevent the binding of cathepsin L to collagen and on the other hand may enhance the binding of cathepsin K by acting as a linker between collagen and the protease. This would effectively increase the local concentration of cathepsin K on the surface of collagen. In general, we have observed that compounds which impeded the cathepsin \( K/\text{C4-S}^* \) complex were also able to
does not affect cathepsin K-mediated collagen degradation (Fig. 7A), it can be considered as a cathepsin K-specific inhibitor.

In summary, selective inhibitors of collagen degradation can target three different inhibition sites without affecting the proteolytic function of the protease itself: (i) cathepsin K/C4-S interaction, (ii) collagen/C4-S(protease) interaction, and (iii) the protease cleavage sites on collagen (Scheme 1). This study also suggests that inhibitors may use more than one pathway of inhibition.

This work demonstrates that it is possible to specifically inhibit the collagenase function of cathepsin K without affecting its proteolytic activity. In prospective work, we will focus on elucidating the structure of a cathepsin K/inhibitor complex by means of crystallographic studies. The structural knowledge of the binding site and mechanism of interaction of negatively charged polymers to cathepsin K will support the design of small molecular inhibitors which will be superior in their specificity when compared with classical active site-directed inhibitors as they specifically target the collagenase activity of this therapeutically important protease.

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REFERENCES

1. Krane, S. M., and Simon, L. (1994) in Scientific American Medicine (Rubenstein, E., and Federman, D. D., eds) Vol. 3, pp. 1–26, Scientific American, Inc., New York

2. Tezuka, K., Tezuka, Y., Maeljima, A., Sato, T., Nemoto, K., Kamioka, H., Hakeda, Y., and Kumeawga, M. (1994) J. Biol. Chem. 269, 1106–1109

3. Bromme, D., and Okamoto, K. (1995) Biol. Chem. Hoppe Seyler 376, 379–384

4. Drake, F. H., Dodds, R. A., James, I. E., Connor, J. R., Debouck, C., Richardson, S., Lee-Ryjakczewski, E., Coleman, L., Rieman, D., Barthlow, R., Hastings, G., and Gowen, M. (1996) J. Biol. Chem. 271, 12511–12516

5. 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) J. Biol. Chem. 273, 32347–32352

6. Kafienah, W., Bromme, D., Batle, D. J., Croucher, L. J., and Hollander, A. P. (1998) Biochem. J. 331, 727–732

7. Yamashita, D. S., and Dodds, R. A. (2000) Curr. Pharm. Des. 6, 1–24

8. Yasuda, Y., Kaleta, J., and Bromme, D. (2005) Adv. Drug Delivery Rev. 57, 973–993

9. Ramachandran, G. N., and Kartha, G. (1955) Nature 176, 593–595

10. McGrath, M. E., Klaus, J. L., Barnes, M. G., and Bromme, D. (1997) Nat. Struct. Biol. 4, 105–109

11. Li, Z., Hou, W. S., and Bromme, D. (2000) Biochemistry 39, 529–536

12. Hou, W. S., Li, Z., Buttner, F. H., Bartnik, E., and Bromme, D. (2003) Biol. Chem. 384, 891–897

13. Li, Z., Hou, W. S., Escalante-Torres, C. R., Gelb, B. D., and Bromme, D.
14. Godat, E., Lecaillle, F., Desmazes, C., Duchene, S., Weidauer, E., Saftig, P., Bromme, D., Vandier, C., and Lalmanach, G. (2004) Biochem. J. 383, 501–506
15. Linnevers, C. J., McGrath, M. E., Armstrong, R., Mistry, F. R., Barnes, M., Klaus, J. L., Palmer, J. T., Katz, B. A., and Bromme, D. (1997) Protein Sci. 6, 919–921
16. Jameson, D. M., and Seifried, S. E. (1999) Methods 19, 222–233
17. Ingham, K. C., Brew, S. A., Migliorini, M. M., and Busby, T. F. (1993) Biochemistry 32, 12548–12553
18. Brömme, D., Okamoto, K., Wang, B. B., and Biroc, S. (1996) J. Biol. Chem. 271, 2126–2132
19. Li, Z., Yasuda, Y., Li, W., Bogyo, M., Katz, N., Gordon, R. E., Fields, G. B., and Bromme, D. (2004) J. Biol. Chem. 279, 5470–5479
20. Thompson, M. J., and Goldstein, R. A. (1997) Protein Sci. 6, 1963–1975
21. Obrink, B., and Wasteson, A. (1971) Biochem. J. 121, 227–233