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Authors: Jean-Marc Lion, Romuald Mentaverri, Stéphanie Rossard, Nathalie Jullian, Bernard Courtois, Josiane Courtois, Michel Brazier, Jean-Claude Mazière, Said Kamel

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**Oligogalacturonic acid Inhibit Bone Resorption and Collagen Degradation
through its interaction with type I Collagen.**

Jean-Marc Lion¹, Romuald Mentaverri¹, Stéphanie Rossard², Nathalie Jullian², Bernard
Courtois², Josiane Courtois², Michel Brazier¹, Jean-Claude Mazière¹ and Said Kamel¹

1- Unité INSERM ERI-12, Université de Picardie Jules Verne. 1, rue des Louvels, 80037
Amiens, France. 2- Laboratoire des Glucides – EPMV (UMR-CNRS-6219), Université de
Picardie Jules Verne, avenue des Facultés, 80025 Amiens, France.

Running Title: Oligogalacturonic acid reduces osteoclastic bone resorption.

The correspondence should be addressed to: Said Kamel, Pharm D. PhD.

Unité de Recherche sur les Mécanismes de la Résorption Osseuse et INSERM, ERI-12.

Université de Picardie Jules Verne, 1, rue des Louvels, 80037 Amiens, France.

Tel : (+33) 3 22 82 77 90; Fax : (+33) 3 22 82 74 69; Email : said.kamel@sa.u-picardie.fr

Abbreviations: oligogalacturonic acid : OGA; Tartrate Resistant Acid Phosphatase: TRAP;
type I collagen C-telopeptides fragment: CTX; Response Unit: RU; Surface Plasmon
Resonance : SPR ;

ABSTRACT

In this study, we showed that oligogalacturonic acid (OGA) purified from flax pectin inhibit *in vitro* osteoclastic bone resorption in a dose-dependent manner. The OGA inhibitory effect was neither linked to an effect on osteoclast apoptosis, nor to an inhibition of cathepsin K activity. By means of an *in vitro* collagen degradation assay we demonstrated that OGA prevented triple helical type I collagen cleavage by cathepsin K in a dose and chain length dependent manner. This inhibition was not restricted to cathepsin K, since collagenolytic activity of other lysosomal cysteine proteases, such as cathepsin B and cathepsin L, as well as matrix metalloproteinases such as MMP-9 were also inhibited. Interestingly, using non-collagen substrates we demonstrated that OGA does not inhibit the proteolytic activity of cathepsin B and L, suggesting that OGA inhibits collagen degradation without affecting the lysosomal cysteine enzyme proteolytic activity. Finally, preliminary study using Surface Plasmon Resonance (SPR) showed that OGA binds to type I collagen but not to albumin, consistent with a specific effect on collagen. These results suggest that the observed inhibition of collagen degradation by OGA may be due to its ability to bind to the collagen molecule. By masking the collagen surface, OGA may render the collagen cleavage site less accessible to enzymes and thus prevent its enzymatic degradation.

Key words : osteoclast, bone resorption, type I collagen, collagen breakdown, lysosomal cysteine protease, matrix metalloproteinase.

1- INTRODUCTION

The osteoclast is the unique cell type capable of resorbing the mineralized bone matrix. Osteoclasts arise from the proliferation, differentiation and the fusion of mononuclear hematopoietic precursor cells which originate from the Granulocyte Macrophage – Colony Forming Units (GM-CFU) lineage (1-3). Bone resorption is a multistep process which includes osteoclast adhesion to the bone surface, cell polarization and formation of an extracellular sub-osteoclastic compartment where bone degradation occurs (4). In order to resorb the bone matrix, osteoclasts secrete protons through vacuolar H^+ -ATPases located in the extracellular membrane facing the sub-osteoclastic compartment delimited by the sealing zone, the so called “ruffled border”. The resulting acidification dissolves the mineral part of the bone, exposing the collagenous matrix to both lysosomal cysteine proteases such as Cathepsin K and matrix metalloproteinases such as MMP-9 (5-7) secreted by mature osteoclasts. At the end of the bone resorption process, osteoclasts die through an apoptotic cell death (8).

The bone remodelling process consists of both osteoclastic bone resorption and osteoblastic bone formation. Under physiological conditions, osteoclast and osteoblast activities are controlled by both systemic and local factors, maintaining bone mineral density and bone micro-architecture. An imbalance in the regulation of osteoclast and osteoblast activities, mostly in favor of a hyper-osteoclastic activity, is responsible for a decrease in bone mineral density leading to osteoporosis. During the past decade, several effective compounds have been developed for the treatment of osteoporosis. Most of them have shown the capacity to reduce bone resorption *e.g.* estrogens (9), bisphosphonates (10), calcitonin (11), selective oestrogen receptor modulators (12). However, to date, none of these agents has been able to completely abolish the development of new bone fractures. Therefore, new therapeutic agents

continue to be sought. The protective and inhibitory influence of dietary components on the development of osteoporosis is a topic of major interest. Indeed, various compounds extracted from plants and/or ingested during feeding have already been proven to be capable of reducing bone resorption and therefore able to prevent and treat postmenopausal osteoporosis. Recently, it has been shown that dietary consumption of chicory fructans and other non-digestible oligosaccharides such as inulin (oligofructose), increased the bone mineral content in growing rats (13,14) and prevented bone loss in ovariectomized rats (15) mainly by stimulating intestinal calcium absorption. Pectin, a highly complex branched polysaccharides fiber, rich in galactoside residues, is also an important component of human diet. Modified pectins administered orally (16) or intravenously (17) have demonstrated a protective effect against cancer cells. However, the effect of such compounds on bone cells is not yet known. Herein, we therefore focused our attention on the effect of oligogalacturonic acid (OGA), with various degrees of polymerization on osteoclast activities. Using mature rabbit osteoclasts, we demonstrated that OGA reduced the osteoclastic bone resorption in a dose dependent manner. We gathered evidences suggesting that OGA inhibits the ability of lysosomal cysteine proteases to cleave type I collagen thanks to an interaction between OGA and the collagen molecule.

2 - MATERIELS AND METHODS

2-1 Materials

Corning well plates were provided by Fisher Bioblock Scientific (France). Human cathepsin K, B and L were purchased from WVR-Calbiochem (Germany). Recombinant MMP-9 was purchased from R&D systems (France). Type I collagen from rat tail was obtained from SERVA (Heidelberg, Germany). All other chemical reagents, such as the medium and E-64

(L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane), were supplied by SIGMA-ALDRICH (St Quentin Fallavier, France).

2-2 Preparation of Oligogalacturonic acid

All saccharides tested were provided by Pr J. Courtois, (Laboratoire des Glucides-LPMV; AMIENS, FRANCE), except for digalacturonic acid, which was provided by SIGMA-ALDRICH (St Quentin Fallavier, France). Briefly, saccharides were obtained after thermal degradation of isolated flax pectin. The oligogalacturonic fraction (OGA), which was obtained after purification of the crude oligopectin, consisted of a mix of saccharides of 2 to 25 unit length, with an average size of 13 units. OGA were also purified by size and we selected three purified fractions with a degree of polymerization (dp) of 5, 8 or 11-16 unit length (dp5, dp 8 or dp11-16).

2-3 Primary osteoclast isolation and culture

Mature osteoclasts were isolated from 10 day old New-Zealand male rabbits (Charles River Lab., France) as previously described (18). Briefly, rabbit long bones and scapula were dissected and harvested into α -MEM (alpha-modified Minimum Essential Medium) supplemented with 10% heat-inactivated Foetal Calf Serum (FCS, Dominic Dutscher, France) prior to being minced with scissors. Cells were then separated from the bone fragment by vigorously vortexing, followed by centrifugation. Isolated cells were finally seeded on bovine bone slices (for bone resorption assay) or 24 well plate (for apoptosis evaluation) and incubated overnight. The osteoclast population used hereafter to assess osteoclast apoptosis was obtained using a 0.1% (w/v) solution of collagenase-dispase (ROCHE, France) in Phosphate Buffered Saline without calcium (20 min, 37°C). Tartrate-Resistant Phosphatase Alkaline (TRAP) staining (Leukocyte Acid Phosphatase Kit, SIGMA, France) confirmed the purity of our osteoclast population, which was close to 99%. After a 2 hour rest, the medium

was exchanged for the testing medium (α -MEM + 10% FBS for bone resorption and α -MEM + 1% FBS for apoptosis) and cells were cultured for the indicated length of time.

2-4 Bone resorption assay

To evaluate the bone resorbing activity of osteoclasts, extracted cells were seeded on bovine bone slices as previously described (19). After an overnight sedimentation, cells were gently washed and incubated for 48 hours with testing media containing various concentrations of oligosaccharides. Slices were then gently washed to remove cells and were double stained using an acid hematoxylin solution and a 1% toluidin-blue - 1% borate solution. Pit (resorption lacuna) area was evaluated using an image analysis system (Biochim les Ulis, France) linked to a light microscope (Olympus BH-2, France). Results are expressed as percentage of control. Bone resorption was also assessed by type I Collagen C-telopeptide fragment (CTX) measurement. Bone resorption products contained in the culture supernatant were collected and CTX were quantified using an ELISA based technology (CrossLapsTM for culture, Nordic Bioscience, Denmark), following the manufacturer's instructions. Results are expressed in nmol/L.

2-5 Osteoclast Apoptosis Assay

Evaluation of apoptosis was carried out as previously described (9). After a 48 hour treatment, cells were fixed in 3.7 % formaldehyde solution for 5 min and stained with 0.2 mM Hoechst 33258 (SIGMA, France) for 10 min. Osteoclasts were examined with a fluorescence microscope to visualize any change in chromatin morphology. At least 100 TRAP-positive multinucleated cells were scored to evaluate the rate of apoptosis, which is defined as the ratio of the number of apoptotic osteoclasts to the total number of osteoclasts (apoptotic plus non-apoptotic cells).

2-6 Cathepsin K inhibition

Inhibition of Cathepsin K activity was assessed using a fluorimetric assay as previously described (20). Z-Phe-Arg-AMC (Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride, SIGMA, France) at 10 μM was used as cathepsin K substrate. The reaction was carried out at 37°C in 100 mM Sodium Acetate, 20 mM L-Cystein and 5 mM EDTA buffer (pH 5.5) in the presence of human recombinant cathepsin K at the concentration of 0.5 $\mu\text{g.mL}^{-1}$. Fluorescence intensity was monitored by a Shimadzu RF-1501 spectrofluorimeter (Shimadzu, Japan) with λ_{ex} : 365nm and λ_{em} : 440nm. A standard curve with AMC was used in the conversion of fluorescence to molar units. Cathepsin K activity was measured in the absence (control) or in the presence of either OGA (200 $\mu\text{g.mL}^{-1}$) or E-64 (10 μM).

2-7 In vitro Type I collagen degradation by lysosomal cysteine enzymes and MMP 9

Type I collagen solution at a final concentration of 1.4 mg.mL^{-1} was incubated either with cathepsin K (2.86 $\mu\text{g.mL}^{-1}$) cathepsin B (33.3 $\mu\text{g.mL}^{-1}$) and cathepsin L (1.1 $\mu\text{g.mL}^{-1}$) in 100 mM sodium acetate buffer (pH 5.5) containing 20 mM L-cystein and 5 mM EDTA (6) or with MMP 9 (4.5 $\mu\text{g.mL}^{-1}$) in 50 mM Tris HCl pH 7.5 additioned with 10 mM CaCl_2 . The collagenic digestion was performed at 37°C in the absence or in the presence of either OGA at various concentrations or E64 at 10 μM . After incubation, the reactions were stopped by heating the samples for 5 minutes at 95°C prior to be subjected to 8 % SDS-PAGE electrophoresis under denaturing condition. The polyacrylamide gel was then fixed with a methanol-acetic acid solution (40:10, v/v) and stained using 0.25 % coomassie blue solution in order to reveal proteins bands.

2-8 Degradation of non-collagenic proteins by Cathepsin B and L.

Bovine serum albumin (BSA, 1.4 mg.mL^{-1}), haemoglobin (4 mg.mL^{-1}), casein (2 mg.mL^{-1}) and type 2A histone (2 mg.mL^{-1}) were incubated with either cathepsin B (33.3 $\mu\text{g.mL}^{-1}$) or cathepsin L (1.1 $\mu\text{g.mL}^{-1}$) for 2 to 4 hours at 37°C. The incubation buffer used was the same

as that used for type I collagen degradation as described above. The protein degradation was performed in the absence or in the presence of either OGA at 100 $\mu\text{g.mL}^{-1}$ or E64 at 10 μM . After incubation, the reactions were stopped by heating the samples for 5 minutes at 95°C prior to subjection to a SDS-PAGE electrophoresis under denaturing condition.

2-9 Binding assay

The ability of OGA to bind type I collagen or BSA was evaluated using Surface Plasmon Resonance (SPR, BIAcore). OGA was immobilized on the sensor chip surface at 25°C with a constant flow rate of 5 $\mu\text{L}/\text{min}$ in HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM ethylenediamine tetra-acetic acid, 0.005% polysorbate 20 [v/v], pH 7.4), the amount of OGA fixed on the sensor chip was at least of 1500 RU. To assess binding of type I collagen or BSA on the OGA coated sensor chip, we injected 40 μL of a solution containing either BSA (2 mg.mL^{-1}) or type I collagen (0.1 mg.mL^{-1}) at a flow rate of 5 $\mu\text{L.min}^{-1}$, followed by 5 min washing with HBS-EP buffer. Results are expressed in Response Unit (RU). Binding was thought to be selective when the RU value was over 100.

2-10 Analysis and Statistics

All data were analysed using Prism 3 software (GraphPad software). The results are expressed as mean $\pm$ SEM. The statistical differences among groups were evaluated using the Kruskal-Wallis test. The Mann-Whitney U-test was then used to identify differences between the groups when the Kruskal-Wallis test indicated a significant difference ($p < 0.01$). Statistical significance was marked as follows * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3- RESULTS

3-1 OGA inhibits mature osteoclast bone resorption.

To evaluate the activity of OGA on bone resorption, we used mature osteoclasts isolated from rabbit long bone and scapula. Cells were seeded on bovine bone slices and treated for 48 hours, and then the level of resorption was evaluated by assessment of the pit area. As seen in Figure 1A, after the removal of cells, bone slices were stained and pit surface (resorption area) was revealed and measured using CountScan software. OGA were tested at 50, 100 and 200 $\mu\text{g.mL}^{-1}$ demonstrating a dose dependant decrease in bone resorption. At a concentration of 200 $\mu\text{g.mL}^{-1}$, an inhibition level of approximately 60% was reached. Inhibition of bone resorption was also assessed by measuring the amount of type I collagen C-telopeptide fragments (CTX) released into the culture medium (figure 1C), which confirmed the inhibitory effect of the OGA. Because OGA are a complex mix of various chain lengths, we tested the effect of size using OGA purified fractions of 2, 5, 8 and 11-16 units chain lengths. The results (figure 1 D) showed that the minimum size required for a significant effect was 5 units, reaching a level of inhibition of approximately 40%.

3-2 OGA have no effect on mature osteoclast survival.

In order to explain the inhibition of bone resorption, we first tested the effect of OGA on the rate of mature osteoclast apoptosis. Osteoclasts displaying characteristics of apoptosis such as chromatin condensation and DNA fragmentation can be easily distinguished from normal cells by Hoechst staining (figure 2A and 2B). Quantitative evaluation of apoptosis of cells treated with OGA at concentration ranging from 50 to 200 $\mu\text{g.mL}^{-1}$ during a culture period of 48 hours showed that OGA have no significant effect on mature osteoclast apoptosis (figure 2C). Calcium used as a positive control (at 20 mM) (19) demonstrated an increase in apoptosis, producing nearly two time more apoptosis than the untreated culture.

3-3 OGA does not affect cathepsin K activity but does affect cathepsin K mediated collagenolysis.

Another possible explanation for the mature osteoclast-induced inhibition of bone resorption is that OGA can reduce bone matrix degradation, for example by inhibiting cathepsin K activity (6). We thus evaluated the effect of OGA on the *in vitro* cathepsin K activity in a cell-free enzyme assay using a synthetic substrate Z-Phe-Arg-AMC and recombinant cathepsin K. OGA at 200 $\mu\text{g.mL}^{-1}$ had no significant effect on cathepsin K activity (figure 3A), while E-64 (10 μM), used as inhibitory control, reduced cathepsin K activity by nearly 99 %. This observation may suggest that OGA are not cathepsin K inhibitors but may act by protecting collagen from further degradation. We thus evaluated the effect of OGA on the cathepsin K collagenolytic activity. We showed (figure 3B) that OGA used at concentrations ranging from 25 to 200 $\mu\text{g.mL}^{-1}$ were able to reduce the ability of cathepsin K to cleave type I collagen in a dose-dependent manner. As was done in the bone resorption studies, we tested different sizes of OGA on cathepsin K mediated collagenolysis (figure 3C). Inhibition level clearly increased with chain length. These results suggest that inhibition of the cathepsin K mediated collagenolysis might be one of the mechanisms underlying OGA inhibition of bone resorption. In order to determine whether the OGA effects are specific or not for cathepsin K, we investigated the effect of OGA on collagenolysis mediated by other lysosomal cysteine enzymes such as cathepsin B and cathepsin L and a non lysosomal enzyme, MMP 9. As shown in figure 4A, 4B and 4C, both enzymes were able to cleave the collagen molecule. When OGA were added, the collagen degradation was also reduced in a dose-dependent manner for both enzymes, meaning that OGA effect on collagenolysis were not specific for cathepsin K.

Finally, to see whether OGA effects are collagen specific, we tested their effect on the proteolytic activity of cathepsin B and L using non collagenic substrates. In this assay, we

selected cathepsin B and L because they have a broader range of substrates than cathepsin K. We thus tested the activity of cathepsin B and L on albumin (figure 5A), haemoglobin (figure 5B), alpha-casein (figure 5C) and type II-A histone (figure 5E). All the substrates tested were at least partially degraded by the enzymes, but OGA in contrast to E-64, were unable to inhibit their proteolytic activity.

3-4 OGA interact with type I collagen.

The results presented so far, demonstrated that OGA inhibited collagenolysis by a specific effect on the collagen molecule, rather than by affecting enzyme activity. In order to explain this inhibition, we hypothesized that OGA may bind to collagen type I by interacting with specific sites on the molecule. In a preliminary study, we used Surface Plasmon Resonance (SPR), to assess the possible interaction between BSA and type I collagen with the OGA coated sensor chips. When a BSA solution was injected on the OGA coated sensor chip (figure 6A), the difference between the baseline response after washing and the baseline response before injection reached a value lower than 100 RU, meaning that no interaction occurred. The large jump in RUs obtained with BSA may result from a difference in refraction index between the buffer and the BSA solution. By contrast, when type I collagen was injected (figure 6B), the difference value between the baseline levels was determined to be at around 400 RU, which reflects an interaction between collagen I and the OGA sensor chips. The residual signal obtained with collagen after wash shows that dissociation is very slow.

4- DISCUSSION

Identifying new inhibitors of bone resorption and understanding their mechanism of action is of great importance for the treatment of post-menopausal osteoporosis, a worldwide health

problem. In this study, we used a mix of oligogalacturonic acids obtained after degradation and purification of flax pectin. This mix, composed of OGA from 2 to 25 units, was tested in *in vitro* bone resorption, using a well established model of primary osteoclast cells prepared from rabbit long bone. Using two different techniques for the assessment of bone resorption, we demonstrated that OGA dose-dependently inhibited the bone resorbing activity of osteoclasts. The inhibitory effect was dependent on the chain length of the OGA and at least five residues of galacturonic acid (dp 5) were necessary to obtain a significant inhibitory effect on bone resorption. In our cell model of fully differentiated osteoclasts, inhibition of bone resorption can be achieved mainly in two ways. Firstly by reducing the osteoclast life span by inducing osteoclast apoptosis, as can be done by the use of bisphosphonates (21). However we have shown that inhibition of bone resorption by OGA was independent of any effect on osteoclast apoptosis. Another way to inhibit bone resorption within our cell model is by interfering with bone matrix degradation. It is now clearly established that in the sub-osteoclastic compartment, both lysosomal cysteine enzymes and metalloproteases, act in concert to degrade type I collagen (7), the main component (90%) of the organic bone matrix. Among the lysosomal cysteine enzymes, cathepsin K is the key regulator in the osteoclast-mediated bone resorption (22) and several cathepsin K inhibitors have been recently developed as inhibitor of bone resorption (23). These conventional cathepsin K inhibitors target the active site, thus causing a complete inhibition of enzymatic activity. In the present report, using a synthetic substrate Z-Phe-Arg-AMC, we demonstrated that OGA, by contrast to E64 a classical lysosomal cathepsin inhibitor, did not modulate cathepsin K activity. This result indicates that OGA do not interact with the cathepsin K active site. Although no inhibition of cathepsin K activity was observed, we demonstrated by means of an *in vitro* collagen degradation assay that OGA dose-dependently inhibited the ability of cathepsin K to cleave triple-helical collagen. This inhibition was also dependent on the chain length of the

OGA, efficient inhibition being evident with a degree of polymerization greater than 5. The effect of OGA on collagenolysis was not specific to cathepsin K, since other lysosomal cysteine enzymes known to cleave collagen molecules, such as cathepsin B and L were also inhibited as well as the MMP 9 an other key enzyme of osteoclast activity. Of particular interest, using non-collagenic substrates, we demonstrated that OGA do not inhibit the proteolytic activity of cathepsin B and L. These findings clearly revealed that OGA inhibits collagen degradation mediated by proteases without affecting their proteolytic activity. From these results, we hypothesized that the observed inhibition of collagen degradation may be due to the ability of OGA to bind to the collagen molecule. By masking the collagen surface, OGA may render the cleavage site less accessible to the proteases and thus prevent collagen breakdown. To address this issue, we tried to evaluate the possible interaction between OGA and collagen by Surface Plasmon Resonance, a method that can be applied to the study of protein-ligand interactions. In the present study, our preliminary SPR data clearly showed that OGA bind to collagen, while they don't bind to a non-collagenic substrate such as albumin, consistent with a specific effect on collagen. It is surprising to note that all the lysosomal cysteine proteases tested in this study were inhibited even though the cleavage sites in type I collagen targeted by the different proteases are not identical (6). The nature of the non-covalent interaction between collagen and OGA and the structural knowledge of the binding sites remain to be elucidated. A more detailed study with SPR will enable us to obtain more information about the specificity of the binding.

In order to treat pathological conditions characterized by excessive collagen degradation, several approaches have been developed to block the activity of proteases, including small-molecule inhibitors, antibodies and increased production of endogenous inhibitors. Specific inhibition of the collagenase function of proteases by compounds that are capable of masking the cleavage site of the collagen substrate may represent a new strategy in the treatment of

those pathological conditions. To date, very few compounds that fulfil these requirements have been identified. In a recent study, Selent et al. (24) have demonstrated that negatively charged polymers (*e.g.* polyglutamates and oligonucleotides) inhibited the collagenase activity of cathepsin K, while the non collagenolytic activity remained intact. It has been reported that this inhibitory effect is mainly due to the impeding effect of polymers on the active complex formed between cathepsin K and chondroitin sulphate, a glycosaminoglycan which enhances the degradation of type I collagen by cathepsin K. In our study, such a mechanism is unlikely, since the inhibitory effect was also observed with other proteases, which do not require chondroitin sulfate to degrade collagen.

In summary, in this work we identified OGA as inhibitors of collagen degradation, acting through a non-catalytic inhibitory pathway. These compounds, which specifically target the collagenase function of proteases without affecting their proteolytic activity, may have therapeutic advantages over classical active site inhibitors regarding their undesirable side effects. Due to the physiological role of human cathepsins and metalloproteases in bone collagen degradation, OGA may be active as inhibitors of bone resorption and may serve in the treatment of osteoporosis and other disorders characterized by increased bone resorption such as rheumatoid arthritis and osteolytic bone metastasis. These inhibitors may also serve in the treatment of other pathological conditions due to excessive collagen degradation such as aneurysms of blood vessels and tumor invasion. Future studies aiming to identify the binding sites and the precise mechanism of interaction between OGA and collagen will help in the development of efficacious molecular inhibitors.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure Legends

Figure 1: Oligogalacturonic acid (OGA) with various degree of polymerization (dp) inhibit the osteoclastic bone resorption. Figure 1A represents an illustration of resorption pits on bovine bone slices. Unfractionated bone cells were cultured on bovine bone slices for 48 hours. Thereafter, cells were wiped off and excavated pits were detected with 1% toluidine blue-1% borate. (Arrows indicate the pits, Gx100). Figure 1 B: Dose-dependent effect of OGA on osteoclastic bone resorption. Osteoclasts were cultured on bovine bone slices for 48 hours, in the absence (control = Ct) or in the presence of various concentrations of OGA. Pits area measurement were done and the results were expressed in percentage of the control. Figure 1 C: Dose-dependent effect of OGA on collagen type I C-telopeptide fragment released into the culture medium. Osteoclasts were cultured on bovine bone slices for 48 hours and bone resorption was assessed by CTX measurement in culture medium. Figure 1 D: effect of OGA with various dp on bone resorption. Osteoclasts were cultured on bovine bone slices for 48 hours in the absence (Ct) or in the presence of 200 $\mu\text{g.mL}^{-1}$ of OGA with various dp.

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared to control.

Figure 2: OGA doesn't modify the osteoclast apoptosis rate. A represents a photomicrograph of a non apoptotic osteoclast with normal nuclei after Hoechst 33258 staining (Gx200). B represents a photomicrograph of an apoptotic osteoclast with fragmented nuclei and condensed chromatin after Hoechst 33258 staining (Gx200). C: Effect of various concentrations of OGA on mature osteoclast apoptosis rate. Purified rabbit osteoclasts were cultured for 48 hours in the absence (control) or in the presence of increasing concentrations of OGA. Osteoclast apoptosis was detected by Hoechst staining and percentages of apoptotic

cells were evaluated by calculating the ratio of apoptotic osteoclasts to the total number of osteoclasts. Calcium at 20 mM (Ca 20) was used as positive control. *** $p < 0.001$ as compared to control.

Figure 3: OGA inhibit collagenolytic activity of cathepsin K by a mechanism independent of site-active inhibition. A: Effect of OGA on cathepsin K activity. Inhibition of cathepsin K activity was assessed at pH 5.5 using Z-phe-Arg-AMC as synthetic substrate (10 μ M) and human recombinant cathepsin K (0.5 μ g.mL⁻¹). The amount of AMC released was evaluated by fluorimetric measurement. A standard curve with AMC was used in the conversion of fluorescence to molar units. Cathepsin K activity was measured in the absence (control) or in the presence of either E64 (10 μ M) or OGA (200 μ g.mL⁻¹). B: Dose dependent inhibitory effect of OGA on the cathepsin K mediated collagenolysis. Triple helical type I collagen was degraded by cathepsin K in the absence or in the presence of either various OGA concentrations or E-64 and then subjected to SDS PAGE. C: Influence of the OGA chain length on the inhibition of cathepsin K mediated collagenolysis (OGA of different size were used at the concentration of 100 μ g.mL⁻¹)

Figure 4: OGA also inhibit the collagenolytic activity of cathepsin B and L as well as MMP 9 in a dose-dependent manner. Triple helical type I collagen degradation by cathepsin B (A), L (B) was carried out at 37°C during 4 hours in the absence or in the presence of either various OGA concentrations or E-64. The MMP 9 degradation (C) was carried out at 37°C during 15 hours in the presence or not of OGA at various concentrations. The samples were then subjected to a SDS-PAGE electrophoresis under denaturing condition.

Figure 5: OGA do not inhibit non-collagenic protein degradation by cathepsin B or L. BSA (A and B), haemoglobin (C), alpha-casein (D) and Type II-A histone, were degraded by cathepsin B or L in the absence or in the presence of either OGA (100 ng.ml⁻¹) or E-64 (10µM) and then subjected to a SDS-PAGE electrophoresis under denaturing condition.

Figure 6: Sensorgram for the interaction of BSA (A) and collagen I (B) obtained after injection over an OGA coated chips.

Bibliography

1. Suda, T., Takahashi, N., and Martin, T. J. (1992) *Endocr Rev* **13**(1), 66-80
2. Roodman, G. D. (1996) *Endocr Rev* **17**(4), 308-332
3. Miyamoto, T., and Suda, T. (2003) *Keio J Med* **52**(1), 1-7
4. Vaananen, K. (2005) *Adv Drug Deliv Rev* **57**(7), 959-971
5. Inaoka, T., Bilbe, G., Ishibashi, O., Tezuka, K., Kumegawa, M., and Kokubo, T. (1995) *Biochem Biophys Res Commun* **206**(1), 89-96
6. 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**(48), 32347-32352
7. Everts, V., Korper, W., Hoeben, K. A., Jansen, I. D., Bromme, D., Cleutjens, K. B., Heeneman, S., Peters, C., Reinheckel, T., Saftig, P., and Beertsen, W. (2006) *J Bone Miner Res* **21**(9), 1399-1408
8. Manolagas, S. C. (2000) *Endocr Rev* **21**(2), 115-137
9. Kameda, T., Mano, H., Yuasa, T., Mori, Y., Miyazawa, K., Shiokawa, M., Nakamaru, Y., Hiroi, E., Hiura, K., Kameda, A., Yang, N. N., Hakeda, Y., and Kumegawa, M. (1997) *J Exp Med* **186**(4), 489-495
10. Mayahara, M., and Sasaki, T. (2003) *Anat Rec A Discov Mol Cell Evol Biol* **274**(1), 817-826
11. Reginster, Y., and Reginster, J. Y. (1998) *BioDrugs* **10**(4), 295-300
12. Michael, H., Harkonen, P. L., Kangas, L., Vaananen, H. K., and Hentunen, T. A. (2007) *Br J Pharmacol* **151**(3), 384-395
13. Nzeusseu, A., Dienst, D., Haufroid, V., Depresseux, G., Devogelaer, J. P., and Manicourt, D. H. (2006) *Bone* **38**(3), 394-399
14. Takahara, S., Morohashi, T., Sano, T., Ohta, A., Yamada, S., and Sasa, R. (2000) *J Nutr* **130**(7), 1792-1795
15. Zafar, T. A., Weaver, C. M., Zhao, Y., Martin, B. R., and Wastney, M. E. (2004) *J Nutr* **134**(2), 399-402
16. Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Bresalier, R., and Raz, A. (2002) *J Natl Cancer Inst* **94**(24), 1854-1862
17. Platt, D., and Raz, A. (1992) *J Natl Cancer Inst* **84**(6), 438-442

18. Tezuka, K., Sato, T., Kamioka, H., Nijweide, P. J., Tanaka, K., Matsuo, T., Ohta, M., Kurihara, N., Hakeda, Y., and Kumegawa, M. (1992) *Biochem Biophys Res Commun* **186**(2), 911-917
19. Lorget, F., Kamel, S., Mentaverri, R., Wattel, A., Naassila, M., Maamer, M., and Brazier, M. (2000) *Biochem Biophys Res Commun* **268**(3), 899-903
20. Votta, B. J., Levy, M. A., Badger, A., Bradbeer, J., Dodds, R. A., James, I. E., Thompson, S., Bossard, M. J., Carr, T., Connor, J. R., Tomaszek, T. A., Szewczuk, L., Drake, F. H., Veber, D. F., and Gowen, M. (1997) *J Bone Miner Res* **12**(9), 1396-1406
21. Parfitt, A. M., Mundy, G. R., Roodman, G. D., Hughes, D. E., and Boyce, B. F. (1996) *J Bone Miner Res* **11**(2), 150-159
22. Lecaille, F., Bromme, D., and Lalmanach, G. (2008) *Biochimie* **90**(2), 208-226
23. Grabowski, U., Chambers, T. J., and Shiroo, M. (2005) *Curr Opin Drug Discov Devel* **8**(5), 619-630
24. Selent, J., Kaleta, J., Li, Z., Lalmanach, G., and Bromme, D. (2007) *J Biol Chem* **282**(22), 16492-16501

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