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Suppressive effect of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on bone resorption in vitro and in vivo

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Abstract

The suppressive effect of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on bone resorption was examined in vitro and in vivo. This synthetic peptidyl aldehyde was found to be a potent and selective cathepsin L inhibitor in our screening for cysteine protease inhibitors. In the pit formation assay with unfractionated rat bone cells, 1.5 nM of this compound markedly inhibited parathyroid hormone-stimulated osteoclastic bone resorption. In addition, intraperitoneal administration of this peptidyl aldehyde (2.5–10 mg/kg) for 4 weeks suppressed bone weight loss dose dependently in the ovariectomized mouse, experimental model of osteoporosis. Hydroxyproline measurement of the decalcified femurs from these ovariectomized mice suggested that this compound acts as a bone resorption suppressor through the inhibition of collagen degradation.

Keywords: Cathepsin L; Cysteine protease inhibitor; Bone resorption; Dipeptidyl aldehyde

1. Introduction

Bone resorption occurs mainly in the extracellular compartment sealed by osteoclasts at the surface of the bone matrix (Vaes, 1968; Baron et al., 1985). This compartment named Howships' lacuna is acidified by H^+ released through the H^+ pump (H^+/K^+-ATPase) of activated osteoclasts. Osteoclastic bone resorption includes mineral release and the degradation of organic components. Type-I collagen, one of the main organic components, is thought to be solubilized by protease digestion (Delaisse et al., 1980, 1984) and the identification of the key enzyme in bone-collagen degradation has been one of the important topics in the understanding of the mechanisms of bone metabolism and osteoporosis.

Although involvement of neutral collagenase in collagen degradation has been reported, these enzymes have not been found in osteoclasts or in the subosteoclastic resorption zone (Sakamoto and Sakamoto, 1984). Recently, involvement of lysosomal cysteine proteases in bone resorption was suggested by their presence in that zone, and a close correlation between the release of these enzymes and bone resorption was found in cultured bone explants (Vaes, 1980). Several inhibitors of these cysteine proteases have been shown to inhibit osteoclastic bone resorption effectively by inhibiting the degradation of collagen fibers in some in vitro experiments (Delaisse et al., 1980). These facts suggest that some cysteine proteases play essential roles in the effect of collagen degradation on bone resorption (Vaes, 1980).

Among the cysteine proteases, cathepsin B, L and N can degrade Type-I collagen in acidic conditions (Burleigh et al., 1974; Evans and Etherington, 1978; Kirschke et al., 1982; Maciewicz and Etherington, 1985). Comparison of their collagenolytic activities showed cathepsin L to be particularly potent (Maciewicz et al., 1987; Maciewicz and Etherington, 1988; Delaisse et al., 1991). CA-074 (Murata et al., 1991), a cathepsin B-specific inhibitor, and a proteinaceous cathepsin L-specific inhibitor from pig (PLCPI; pig leucocyte cysteine protease inhibitor) (Kopitar et al., 1989) were good tools for identification of the key enzyme

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in bone resorption. Using these specific inhibitors, Kakegawa et al. (1993) first proved that cathepsin L is the key enzyme for degradation of bone collagen. They reported that osteoclastic bone resorption induced in rat by parathyroid hormone was markedly inhibited by PLCPI but not by CA-074 (Kakegawa et al., 1993; Tagami et al., 1994). Therefore, a low-molecular mass cathepsin L-specific inhibitor is expected to be a good candidate as a new osteoporotic drug.

During our screening for cysteine protease inhibitors from dipeptidyl aldehyde derivatives, we found that a novel peptidyl aldehyde, *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal showed more potent inhibitory activity for cathepsin L than for the other cysteine proteases, for example, cathepsin B and calpain II. We have described the characterization of this compound previously (Woo et al., 1995). We now report on the suppressive effects of this compound on bone resorption in vitro and in vivo.

2. Materials and methods

2.1. Materials

Human parathyroid hormone-(1–34) was purchased from Sigma Chemical Co. α-Minimum essential medium and fetal bovine serum were purchased from Flow Laboratories, cathepsin L (EC 3.4.22.15), cathepsin B (EC 3.4.22.1) and calpain II (EC3.4.22.17) from Calbiochem Co., benzyloxycarbonyl-phenylalanyl-arginyl-methylcoumaryl amide, benzyloxycarbonyl-arginyl-arginyl-methylcoumaryl amide, and succinyl-leucyl-leucyl-valyl-tyrosyl-methylcoumaryl amide from Peptide Research Institute, Osaka, Japan. E-64 [*N*-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucine-4-guanidinobutylamide] was purchased from Cambridge Research Biochemicals. *N*-(Benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal was synthesized in the Sagami Chemical Research Center, Kanagawa, Japan. All the other chemicals were of analytical grade.

2.2. Methods

2.2.1. Measurement of enzyme inhibitory activity

The method of Barrett and Kirschke (1985) was employed for the measurement of the cathepsins' activities. Cathepsin L or B was dissolved in the reaction buffer [100 mM acetate buffer (pH 5.5)], containing 1 mM EDTA and 8 mM cysteine to be 20 mU. 1 U is defined as the concentration of the enzyme that gives 1 μmol of 7-amino-4-methylcoumarine per min at 37°C as a reaction product in this reaction mixture. After preincubation of the enzyme solution with a test sample for 5 min at 37°C, the reaction was initiated by adding substrates (benzyloxycarbonyl-arginyl-arginyl-methylcoumaryl amide for cathepsin B and benzyloxycarbonyl-phenylalanyl-arginyl-methylcoumaryl amide for cathepsin L). The final concentration of each substrate was 10 μM and the total volume of the reaction mixture was 0.1 ml. Five minutes' reaction at 37°C was terminated by adding 0.1 ml of 100 mM acetate buffer (pH 4.3) containing 100 mM monochrolacetate.

The modified method of Sasaki et al. (1984) was used for calpain II. 20 mU calpain II was dissolved in 100 mM imidazol-HCl buffer (pH 7.3) containing 5 mM cysteine, 5 mM $CaCl_2$, 2.5 mM mercaptoethanol and 3% dimethylsulfoxide. This enzyme solution was preincubated with a test sample for 5 min at 30°C and addition of 10 μM succinyl-leucyl-leucyl-valyl-tyrosyl-methylcoumaryl amide (substrate) initiated the reaction. The total reaction volume was 0.1 ml. After 10 min incubation, the reaction was terminated by adding 0.1 ml of 100 mM EGTA.

The fluorescence of the liberated 7-amino-4-methylcoumarine was measured at 460 nm for emission and at 370 nm for excitation. These experiments were repeated 5 times in duplicate.

2.2.2. Pit formation assay

The effect of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on osteoclastic pit formation was tested according to the method of McSheery and Chambers (1987) with some modifications. Unfractionated bone cells were prepared from tibiae, femurs and humeruses of 1 to 2-day-old Sprague-Dawley rats. The bones were dissected to be free from soft tissues in the iced tissue culture medium (α-minimum essential medium, pH 7.2, supplemented with 100 IU/ml of benzylpenicillin) containing 10 mM Hepes and were minced with scissors in the same medium. The suspension was then triturated with a wide-bore plastic pipette and 100 μl (10^6 cells/ml) was added to each well of a 96-well plate containing a sliced bovine femur derived from cortical bone (8 mm in diameter). The cells were incubated at 37°C in a CO_2 incubator (5% CO_2-95% air) for 1 h. The slices were then rinsed with α-minimum essential medium and transferred to fresh medium containing 10% fetal bovine serum, various concentration of the inhibitor and 100 nM of parathyroid hormone, and were incubated for 24 h. The cells were removed from the slices by vigorous washing with distilled water, and the slices were then stained with acid hematoxylin for 5 min. The total number of pits formed by osteoclastic bone degradation was counted under an optical microscope. Osteoclastic cell number was quantified by counting the adhesive tartrate-resistant acid phosphatase-positive cells on a femur slice cultured in the same way as in the pit formation assay.

2.2.3. In vivo suppressive effects on bone resorption stimulated by ovariectomy

Four-week-old female ddY mice were used for this experiment. Bilateral ovariectomies and sham operations were carried out under ketamine hydrochloride anesthesia. The mice were divided into six groups: sham-operated,

ovariectomized control, *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal-treated ovariectomized groups [for dry weight measurement; 2.5, 5, 10 mg/kg/day, for hydroxyproline measurement; 1.25, 5, 10 mg/kg] and a 17β-estradiol (0.3 mg/kg)-treated ovariectomized group. Each group contained five mice. Intraperitoneal injection of the vehicle or *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal dissolved in 5% dimethylsulfoxide was started from the day of operation and continued daily for 4 weeks. Four weeks after the operation, all the mice were killed, and the femurs were removed to measure dry weight.

Hydroxyproline content in the femur was measured as follows: the dried and weighed femur was decalcified in 1 N HCl overnight and dried again at 60°C. The dried decalcified femur was hydrolyzed in 4 N NaOH (1 ml) at 120°C for 20 min. The hydrolysate was neutralized with 5.6 N citric acid and filtered. Hydroxyproline in the hydrolysate was measured by the method of Gabor et al. (1986). In brief, chloramin T solution (100 μl) was added to 10 μl of the 5-fold diluted filtrate and the reaction mixture was kept at room temperature for 20 min. Aldehyde perchloric acid solution (100 μl) was added and the reaction mixture was heated at 65°C for 15 min in a water bath. After cooling, the absorbance of the solution at 550 nm was measured.

3. Results

Selectivity and potency of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal for cathepsin L inhibition were compared with those of the epoxysuccinyl peptide, E-64, known as a potent cysteine protease inhibitor and an anti-resorptive reagent in vitro. As shown in Fig. 1a, *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal showed the most potent inhibitory activity for cathepsin L among the three kinds of cysteine proteases, cathepsin L, B and calpain II. The inhibitory potency of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal for cathepsin L was about 100 times greater than that of the other two enzymes. On the other hand, E-64 did not show the selectivity for cathepsin L (Fig. 1b). Furthermore, the 50% inhibitory concentration of E-64 for cathepsin L was about 100 times higher than that of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal.

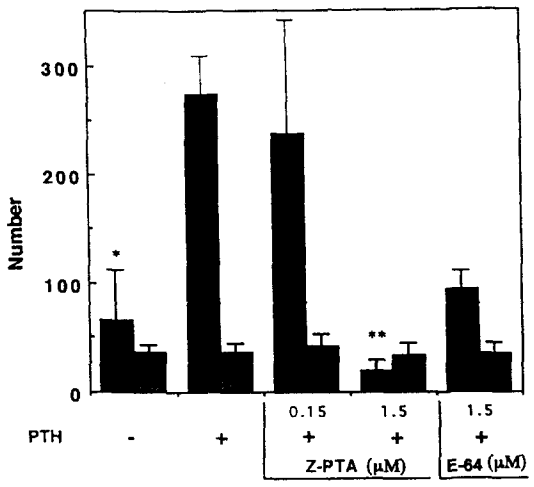

Fig. 2. Effects of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on osteoclastic pit formation. Closed and shaded columns indicate pit and adhesive osteoclastic cell number on bone slice, respectively. Z-PTA and PTH mean *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal and human parathyroid hormone-(1–34), respectively. Error bar means S.E.M. * $P < 0.05$ vs. parathyroid hormone alone. ** $P < 0.01$ vs. parathyroid hormone alone.

The effect of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on pit formation induced by parathyroid hormone is shown in Fig. 2. The pit number of the control (without parathyroid hormone) group was about 65, whereas that of the parathyroid hormone-treated group was as high as about 270. This result clearly shows that pit formation was enhanced by parathyroid hormone. Tagami et al. suggested that parathyroid hormone stimulates osteoclasts to secrete lysosomal enzymes including cathepsin L (Tagami et al.,

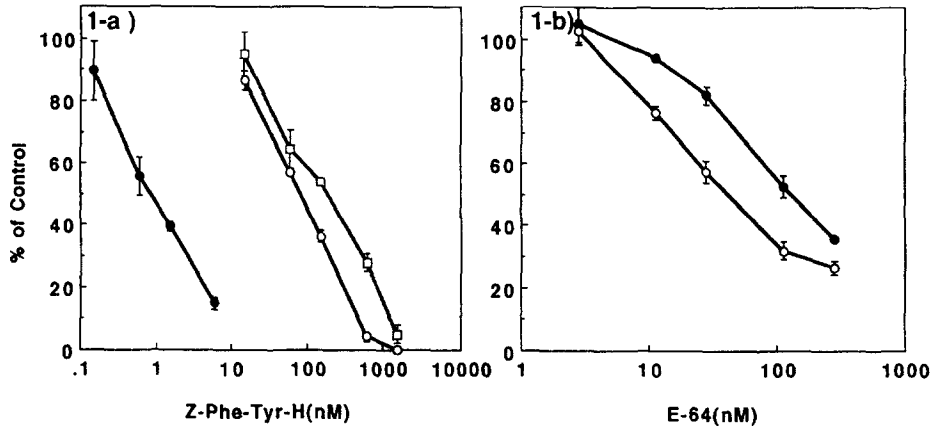

Fig. 1. In vitro enzyme inhibitory activities of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal (a) and E-64 (b). Vertical axis shows the ratio (%) of the remaining enzyme activity to full activity (without inhibitors). Closed circles, open circles and open squares in (a) and (b) indicate cathepsin L, B and calpain II, respectively.

1994) and our data for pit number increase in the parathyroid hormone-treated group are consistent with their report. On the other hand, *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal inhibited by 10–100% the pit formation stimulated by parathyroid hormone at the concentration of 0.15–1.5 μM in a dose-dependent manner. To elucidate the cytotoxicity of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal in this assay, the adhesive tartrate-resistant acid phosphatase-positive cells on the slice were counted. In many cases, cytotoxic samples reduced the number of tartrate-resistant acid phosphatase-positive cells on the slice. Although *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal seemed to reduce the adhesive osteoclastic cell number slightly at the concentration of 1.5 μM, no significant difference from the group with parathyroid hormone alone was observed at 0.15-1.5 μM. Therefore, the reduction of pit number observed in the group treated with 1.5 μM of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal is considered to be due to the suppression of collagen hydrolysis through cathepsin L inhibition. *N*-(Benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal was confirmed not to react with parathyroid hormone or the parathyroid hormone receptor in the other experiment (data not shown). On the other hand, 1.5 μM of E-64 showed about 60% inhibition in the same experiment. The tartrate-resistant acid phosphatase-positive cell number in the E-64 (1.5 μM)-treated group was almost the same as that in the *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal (1.5 μM)-treated group. The data shown in Fig. 2 are from one of the three experiments in duplicate. The effect of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on pit formation was not as potent as on cathepsin L inhibition. One possible explanation is that *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal is not stable in the assay medium for 24 h.

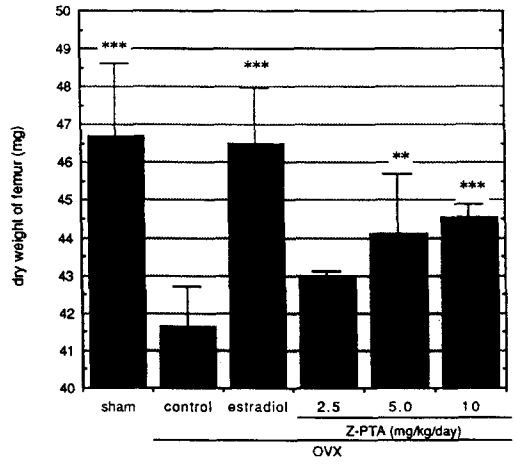

Fig. 3. Effect of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on bone weight loss in ovariectomized mice. Z-PTA and OVX mean *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal and ovariectomized mice, respectively. Error bar means S.E.M. ** $P < 0.05$ vs. ovariectomized control. *** $P < 0.01$ vs. ovariectomized control.

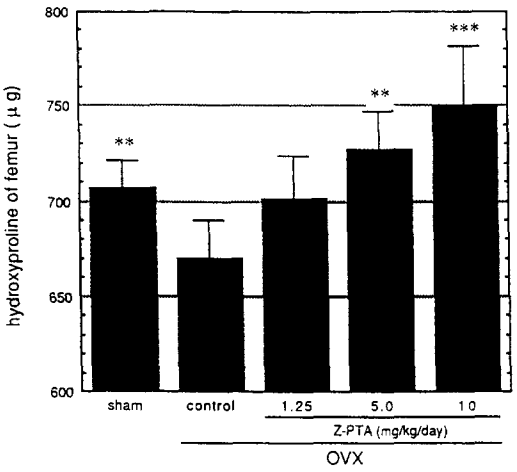

Fig. 4. Effect of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on hydroxyproline content of the femur in ovariectomized mice. Z-PTA and OVX mean *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal and ovariectomized mice, respectively. Error bar means S.E.M. ** $P < 0.05$ vs. ovariectomized control. *** $P < 0.01$ vs. ovariectomized control.

The effect of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal on bone resorption in vivo was examined for the ovariectomized mice. The dry weights of the femurs were measured 4 weeks after ovariectomy. The averaged dry weight of femurs from the sham-operated group was 46.7 mg but that from the ovariectomized group was 41.5 mg. This weight loss is thought to have resulted from bone resorption enhanced by estrogen deficiency. Intraperitoneal administration of 17β-estradiol (0.3 mg/kg/day) for 4 weeks completely prevented this bone weight loss as shown in Fig. 3. Four weeks' intraperitoneal administration of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal partially prevented the loss in bone weight in a dose-dependent manner. The maximum effect was obtained at the dose of 10 mg/kg/day and its suppressive effect was 60%.

The same experiment as above was repeated with the doses of 1.25, 5, 10 mg/kg/day for hydroxyproline measurement. The hydroxyproline content per femur is shown in Fig. 4. Compared with that in the sham-operated group, the hydroxyproline content for the ovariectomized group was significantly reduced. However, the hydroxyproline content of all the *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal-treated ovariectomized groups was greater than that of the sham-operated group. These data clearly show that *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal suppressed bone collagen hydrolysis in ovariectomized mice.

4. Discussion

Based on the idea that cathepsin L plays an essential role in the process of bone resorption, *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal, a potent and selective cathepsin L inhibitor was applied to the experimental

bone resorption system in vitro and in vivo. From the results obtained in this study, it is suggested that *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal acts as an anti-resorptive reagent through the inhibition of collagen hydrolysis and could be a good candidate for a new osteoporotic drug. Further study of the histology of bone treated with this compound will be needed and the effect of this compound on the other cathepsins distributed in the other organs in vivo should be clarified.

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