Antiosteoporotic Activity of the Stems of *Sambucus sieboldiana*

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We previously found that a methanolic extract of the stems of *Sambucus sieboldiana* inhibited bone resorption in organ culture. In this study, we further fractionated the methanol extract guided by the activity towards bone resorption stimulated by parathyroid hormone (PTH) *in vitro*. The ethyl acetate fraction (EtOAc Fr.) of the methanolic extract inhibited PTH-stimulated bone resorption of neonatal mouse bones, and the inhibitory activity was more potent than that of those other fractions. Oral administration of the EtOAc Fr. (50 and 100 mg/kg/d) to ovariectomized (OVX) rat prevented the decrease in bone mineral density (BMD) of the lumbar (L2-L4) vertebra, indicating that the EtOAc Fr. is effective *in vivo*. Furthermore, the EtOAc Fr. (50, 100 and 150 mg/kg/d) decreased the serum calcium level elevated in low calcium dietary rats. The phenolic constituents of the EtOAc fraction were examined for their inhibitory effect on bone resorption stimulated by PTH in neonatal mouse bone. Among them, vanillic acid, vanillin and coniferyl alcohol showed significant inhibitory effects on bone resorption. Of the compounds examined, vanillic acid was found to have a significant inhibitory effect on the decrease of BMD in OVX mice. Therefore, the EtOAc Fr. of *S. sieboldiana* showed a suppressive effect on bone resorption *in vitro* and *in vivo*. In addition, the inhibitory effects of the EtOAc Fr. on bone resorption may be at least partly due to the inhibitory action of vanillic acid.

**Key words** antiosteoporosis; osteoporosis; *Sambucus sieboldiana*; parathyroid hormone (PTH); ovariectomy (OVX); vanillic acid

Osteoporosis occurs because of an imbalance between bone formation and bone resorption and is the main causal factor of bone fractures in elderly persons. This disorder has been increasing remarkably in frequency along with the increase in life expectancy. Many agents such as calcium and estrogen (the first-generation antiosteoporotic drugs) and calcitomin and vitamin D₃ (second-generation) and ifipilavone, a synthetic compound belonging to the isoflavone group (third-generation) have been used clinically in the treatment of the disease. [1, 2]

In a search for natural crude drugs which have inhibitory activity on bone resorption, we have screened a number of medicinal plants for their inhibitory activity on the resorption induced by parathyroid hormone (PTH) in organ culture. [3] Among them, the methanol extract of *Sambucus sieboldiana* (Caprifoliaceae) was found to inhibit bone resorption and has been targeted for the present study. This plant is distributed in various regions of China, Korea and Japan [4] and is widely used as a traditional medicine in analgesic, anti-inflammatory, homeostatic and diuretic drugs which act on bruises, fractures and edema. [5] In the present work, we wish to report the inhibitory activity of various fractions of *S. sieboldiana* on bone resorption, together with the major constituents of the ethyl acetate fraction (EtOAc Fr.) of *S. sieboldiana*.

**MATERIALS AND METHODS**

**General** Mice (ddy) and rats (Wistar) were purchased from Shizuoka Animal Center (Shizuoka, Japan). Parathyroid hormone (human, 1—34; PTH) was purchased from Peptide Institute, Inc. (Osaka, Japan). Ham's F-12 medium was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). ⁴⁵CaCl₂ was purchased from NEN Research Products (Boston, MA, U.S.A.). Ifipilavone was extracted and purified from commercially available Osten TM™ (TC-80) (Takeda Pharmaceutical Co., Ltd., Osaka, Japan). Coniferyl alcohol was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *S. sieboldiana* was purchased from Tochimoto Tenkaido (Osaka, Japan).

**Extraction and Fractionation** The stems of *S. sieboldiana* (4 kg) were extracted twice with methanol (91× 3) by refluxing them for 3 h. The hot extracts were filtered through filter paper and the combined filtrate was concentrated under reduced pressure, then lyophilized. The MeOH extract (130.57 g) was suspended in water and fractionated by successive extraction with hexane, EtOAc and butanol (BuOH) to give hexane- (25.06 g), EtOAc- (11.24 g), BuOH- (15.00 g) and water-soluble (75.88 g) fractions, respectively.

**Measurement of Bone-Resorbing Activity** Bone-resorbing activity was assessed using the method of Shigeno et al. [6] Briefly, 2-days-old mice were injected with 2 μCi of ⁴⁵Ca. Parietal bones from 4-days-old mice were preincubated for 1 d at 37°C in Ham's F-12 medium containing 5% heat-inactivated horse serum. The bones were cultured in fresh medium with or without PTH (2×10⁻⁹ M) plus test substances for 6 d. The medium was changed every 3 d. The ⁴⁵Ca in the medium and in the bone was counted separately. Bone resorption was quantified on the basis of the percentage of ⁴⁵Ca released into the medium compared to the total ⁴⁵Ca.

**Measurement of Bone Mineral Density (BMD)** Female rats underwent ovariectomy (*n*=24) or a sham operation (*n*=8, group 1) at the age of 8 months. Ovariectomized (OVX) rats were assigned to three groups (groups 2—4). Each group included eight animals. Group 2 was the OVX group; groups 3 and 4 were given the EtOAc Fr. The EtOAc Fr. was suspended in distilled water, then orally administered for 6 weeks. At the end of the experimental period, BMD

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values were measured in the lumbar (L2-4) vertebra using dual energy X-ray absorptiometry (DXA).

The female mice underwent ovariectomy or a sham operation (n=8, group 1) at the age of 8 weeks. OVX mice were assigned to eight groups (groups 2—9). Each group had five to eight animals. Groups 2 and 3 were OVX and ethinyl estradiol treated groups, respectively; whereas groups 4—9 were given the test compounds.

Each compound suspended in distilled water was administered orally for 4 weeks. At the end of the experimental period, BMD values were measured in the lumbar vertebra using DXA.

Measurement of Bone Ca Mobilization Male Wistar rats fed an insufficient Ca (0.003%) diet for 1 week were used. These animals were given test substances. After oral administration of the test substances to the animal for 1 week, blood samples were collected from the heart. The serum Ca level was measured by the method of Gitelman H. J. using orthocresolphthalain complexon (OCPC).

Recovery Assay A recovery assay was performed as described by Klein-Nulend et al.5) Bones alone were cultured with PTH and the compounds for 72 h. After 72 h, the compounds were removed and the bones were cultured with PTH only.

Statistical Analysis All values were expressed as means±S.E. for n experiments. The significance of the mean differences in each experiment was analyzed by Student's t-test, and the p value <0.05 was considered significant.

RESULTS

Effects of Various Fractions of the Methanolic Extract on PTH-Stimulated Bone Resorption The methanolic extract was partitioned into hexane, EtOAc, BuOH and water-soluble fractions. At a dose of 44 μg/ml, no fraction showed any inhibitory effect, whereas three fractions at 440 μg/ml except H2O Fr., showed an inhibitory effect. Among the fractions examined, the EtOAc Fr. showed the most potent inhibitory effect on PTH-stimulated bone resorption (Fig. 1).

Effects of the EtOAc Fr. on BMD of OVX Rats To evaluate the effect of EtOAc Fr. on BMD in the OVX rat, the fraction was given to the rats for 6 weeks. The OVX rat exhibited a significant decrease in BMD (0.167±0.0031 g/cm² in sham-operated rats vs. 0.154±0.0022 g/cm² in OVX rat, p<0.01) (Fig. 2). Oral administration of the EtOAc Fr. (50 and 100 mg/kg/d) significantly increased the BMD values compared with the OVX rat. However, there were no significant changes in plasma calcium or phosphorus levels among the treated and untreated groups (data not shown).

Effects of the EtOAc Fr. on Plasma Calcium Content in Insufficient-Calcium Dietary Rats When rats ingested an insufficient calcium diet for 7 d, the serum calcium levels were increased (9.600±0.163 mg/ml in insufficient calcium diet rats vs. 9.254±0.130 mg/ml in normal diet rats, p<0.05). However, oral administration of the EtOAc Fr. (50, 100 and 150 mg/kg/d) with an insufficient calcium diet showed a significant decrease in the serum calcium level (Fig. 3).

Effects of Phenolic Compounds on PTH-Stimulated Bone Resorption GC-MS analysis of the EtOAc Fr. showed various phenolic compounds, and vanillic acid (1, 21.5%), protocatechuic acid (2, 1.0%), vanillin (3, 2.8%), hydroxycinnamic acid (4, 1.9%) and coniferyl alcohol (5, 1.1%) were considered to be the major constituents of the EtOAc Fr. They were purchased and examined for their inhibitory effect on PTH-stimulated bone resorption in vitro.

Fig. 1. Effects of Various Fractions Obtained from MeOH Extract of S. sieboldiana on PTH-Stimulated 45Ca Release from Neonatal Mouse Parterial Bones
Normal: cultured without PTH and each compound, PTH: cultured with PTH (2×10−7 M). Samples: cultured with PTH (2×10−7 M) and each fraction. Each value represents the mean±S.E., n=6—7. Significant decrease in 45Ca release compared to PTH group, †: p<0.05, ‡: p<0.01. Significant decrease in 45Ca release compared to normal group, †: p<0.01.

Fig. 2. Effect of EtOAc Fraction on BMD of OVX Rat
Sham: sham operated group, OVX: ovariectomized group. Samples: OVX mice p.o. administered with EtOAc fraction (mg/kg/d) for 6 weeks. Values are expressed as mean±S.E., n=8. Significantly different from OVX group, †: p<0.01. Significantly different from sham group, ‡: p<0.01.

Fig. 3. Effects of EtOAc Fraction on Serum Ca2+ Levels in Low Calcium Dietary Rats
Normal: normal diet group, control: low Ca diet group. Samples: p.o. administered (mg/kg/d) in low Ca dietary rats for 7 d. Values are expressed as mean±S.E., n=5. Significantly different compared to control group, **: p<0.01. Significantly different compared to normal group, †: p<0.05.
As shown in Fig. 4, 1, 3 and 5 showed an inhibitory effect on PTH-stimulated bone resorption at concentrations of 20 and 200 \( \mu \)M. Their effects compared to that of iprilavone. It has been reported that iprilavone has a suppressive effect on PTH-stimulated bone resorption in a rat long bone culture system. Thus, the inhibitory effects of 1, 3 and 5 were comparable to that of iprilavone at a concentration of 200 \( \mu \)M (Fig. 4).

### Reversibility of the Inhibitory Effect of the Phenolic Compounds on Bone Resorption

It is pertinent to consider that the inhibitory activity of drugs is sometimes a result of their toxic effect, and consequently, an erroneous conclusion might occur in screening studies. Thus, to avoid such confusion, a recovery experiment was performed. As shown in Table 1, the suppressive effect on bone resorption by 1, 3 and 5 at a maximal concentration (200 \( \mu \)M) could be reversed by removing these compounds at 72 h. These results indicate that in PTH-induced bone culture, the inhibitory effects of 1, 3 and 5 did not appear to cause irreversible toxicity.

#### BMD of O VX Mice at 2 and 4 Weeks after Surgery

BMD at consecutive scan sites of the lumbar was measured in a separate experiment at 2 or 4 weeks after surgery. The OVX mice showed a significant decrease in BMD compared with sham-operated mice at 4 weeks after surgery (0.060 \( \pm \) 0.0013 g/cm\(^2\) in sham-operated vs. 0.0546 \( \pm \) 0.0016 g/cm\(^2\) in the OVX mice, \( p < 0.05 \)) (Fig. 5), indicating that bone loss due to estrogen deficiency occurred at 4 weeks.

#### Effect of 5 on BMD of OVX Mice

The effect of 5 on BMD in the OVX mice for 4 weeks was investigated. The OVX caused a significant decrease in BMD (0.0643 \( \pm \) 0.0023 g/cm\(^2\) in sham-operated vs. 0.0573 \( \pm \) 0.0011 g/cm\(^2\) in OVX mice, \( p < 0.01 \)) (Fig. 6). On the other hand, coniferyl al-
cohab (10 and 30 mg/kg/d) increased the BMD values insignificantly.

Effects of 1, 3 and Sodium Vanillate (6) on BMD of OVX Mice  The effects of 1, 3 and 6 on BMD in the OVX mice for 4 weeks were evaluated. Compound 6 was used to determine whether its inhibitory potency differs from that of 1 suspension. As in the previous case, the OVX caused a significant decrease in BMD (0.0646±0.0006 g/cm²) in sham-operated vs. 0.0532±0.0012 g/cm² in OVX mice, p<0.05), and this bone loss was prevented by estrogen replacement (Fig. 7). The BMD of the OVX mice treated with 1 at doses of 50 and 100 mg/kg/d was significantly greater than that of the OVX group, but not dose dependently. Compound 6, on the other hand, showed a significant effect at a dose of 100 mg/kg/d, dose dependently.

DISCUSSION

In the principal actions of PTH on bone and kidney, PTH is responsible for the minute-to-minute regulation of calcium levels in the blood and extracellular fluid. It acts on the kidney to enhance the reabsorption of calcium and to diminish the reabsorption of phosphate. In the skeleton, PTH stimulation leads to the resorption of bone and to the subsequent release of calcium and phosphate into the circulation. PTH stimulates the formation of 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂D₃), which in turn has direct biological effects on the intestine, the result being an increased absorption efficiency of dietary calcium. The overall effect is an increase of calcium level and a decrease of phosphate level in the blood. On the other hand, the excretion of calcium and phosphate into the urine is increased. In vitro, the addition of PTH causes calcium release from the bones with continuous exposure.

In this study, we have examined the inhibitory effect of various fractions obtained from the MeOH extract of S. sieboldiana on PTH-stimulated ⁴⁵Ca release from neonatal mouse parietal bones (Fig. 1). Among these fractions, the EtOAc Fr. showed the most potent activity on bone resorption induced by PTH, and therefore its further investigation was undertaken.

In osteoporosis, postmenopausal osteoporosis is major. Postmenopausal osteoporosis is a disorder characterized by a progressive loss of bone tissue which begins after natural or surgical menopause and leads to the occurrence of spontaneous fractures. The causal role of estrogen deficiency in this condition is well established. Nowadays, an ovariectomized animal is commonly used as a postmenopausal osteoporosis model. It has been reported that, in an ovariectomized animal, the levels of PTH together with cytokine, acting as a bone-resorbing factor, like interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α), were increased. Our experimental results showed that the EtOAc Fr. inhibited the decreased BMD in the OVX rat, indicating that it has a preventive activity on bone loss.

Insufficient calcium intake is one of the well known osteoporotic risk factors that can reduce the amount of absorbed calcium in the intestines. As a consequence, PTH secretion becomes high to maintain the calcium level. Thus, the overall effect is bone resorption. In this study, the EtOAc Fr. decreased the serum calcium concentration in hypercalcemic rat, indicating that it may have inhibitory effects on bone resorption both in vitro and in vivo.

In order to investigate the active constituents of the EtOAc fraction, five major constituents, 1, 2, 3, 4 and 5 were tested for their inhibitory activity against PTH-induced bone resorption. Among them, 1, 3 and 5 showed significant inhibitory effects on bone resorption (Fig. 4). Furthermore, 1 showed a preventive activity on bone loss in terms of BMD in OVX mice. The effects of 1 on osteoclast-induced bone resorption (pit formation) and osteoclast formation were evaluated. Compound 1 inhibited pit formation and caused a decrease in the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) (unpublished result). Study of the detailed inhibitory mechanism of 1 is underway and will be reported elsewhere. In conclusion, our results suggest that the inhibitory effects of the EtOAc Fr. on bone resorption may be at least partly due to the inhibitory action of vanillic acid.

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