An orally active cathepsin K inhibitor, furan-2-carboxylic acid (1-{1-[4-fluoro-2-(2-oxo-pyrrolidin-1-yl)-phenyl]-3-oxo-piperidin-4-ylcarbamoyl}-cyclohexyl)-amide (OST-4077), inhibits osteoclast activity in vitro and bone loss in ovariectomized rats.


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ALP, alkaline phosphatase; BMD, bone mineral density; CK, cathepsin K; CTx, C-telopeptide
of type 1 collagen; DPD, deoxypyridinoline; PBMCs, peripheral blood mononuclear cells; RANKL, receptor activated nuclear factor kappa B ligand; OST-4077, furan-2-carboxylic acid (1-[1-[4-fluoro-2-(2-oxo-pyrrolidin-1-yl)-phenyl]-3-oxo-piperidin-4-ylcarbamoyl]-cyclohexyl)-amide; TPTX, thyroparathyroidectomized; TRAP, tartrate-resistant acid phosphatase

e) Endocrine and Diabetes
ABSTRACT

Human cathepsin K, cysteine proteinase of the papain family, has been recognized as a potential drug target for the treatment of osteoporosis. The predominant expression of cathepsin K in osteoclasts has rendered the enzyme into a major target for the development of novel anti-resorptive drugs. Now, we report the pharmacological properties of OST-4077 as a novel selective cathepsin K inhibitor. Human and rat cathepsin K were inhibited in vitro by OST-4077 with the IC_{50} values of 11 nM and 427 nM, respectively. OST-4077 suppressed bone resorption induced by rabbit osteoclasts (IC_{50}, 37 nM), but did not affect bone mineralization or cellular alkaline phosphatase activity in MC3T3-E1 cells. PTH-induced bone resorption was inhibited in a dose-dependent manner in thyroparathyoidectomized (TPTX) rats gavaged with a single dose of OST-4077 (ED_{50}, 69 mg/kg). When given orally twice daily for 4 weeks to 3-month-old OVX rats, OST-4077 dose-dependently prevented bone loss, as monitored by bone densitometry, ash content and urinary excretion of deoxypyridinoline. No change in serum osteocalcin in the OVX rats by OST-4077 suggested that bone formation might not be affected by the agent. In summary, OST-4077 selectively inhibited bone resorbing activities of osteoclasts and prevented bone loss induced by estrogen deficiency, but did not affect bone formation. OST-4077, an orally active selective human cathepsin K inhibitor, may have the therapeutic potential for the treatment of diseases characterized by excessive bone loss including osteoporosis.
INTRODUCTION

Osteoporosis, a skeletal disorder characterized by decreased bone mass as a consequence of enhanced bone resorption relative to bone formation, may lead to bone fracture (Einhorn, 1996). Bone resorption results from secretion of acid and proteolytic enzymes by specialized cells called osteoclasts. The acid dissolves the mineral, hydroxyapatite and the proteolytic enzymes degrade the protein matrices of the bone. The major bone resorbing enzymes in osteoclasts include cathepsin K, a cysteine proteinase (Kamiya et al., 1998).

Evidence that cathepsin K is a major proteinase in humans can be found in a rare autosomal recessive condition known as pycnodysostosis, which is characterized by dwarfism, cranial anomalies and increased bone mineral density (Gelb et al., 1996). Cathepsin K-deficient mice show osteopetrotic characteristics with increased trabeculation of the bone marrow space and no overt phenotypic abnormalities until the age of 10 months (Saftig et al., 1998). From these observations, cathepsin K has been focused on as a novel target for the treatment of osteoporosis.

In fact, the chemical inhibitors of cathepsin K have been shown to inhibit bone resorption in ovariectomized rats (Lark et al., 2002). Potent and efficacious inhibition of bone resorption markers by the chemical inhibitors have also been studied in nonhuman primates (Stroup et al., 2001). However, the cathepsin K inhibitors studied so far have been applied...
only parenterally. Furthermore, the effects of long term treatment with the agents on bone mineral density and bone turnover markers have not been examined in osteopenic animal models.

The researchers in our laboratories have synthesized a series of potential chemical inhibitors for human cathepsin K and examined their pharmacological efficacy. In this study, we describe the anti-resorptive effects of OST-4077 \textit{in vitro} and the long term effects on bone loss \textit{in vivo}. To our knowledge, this is the first report of an orally active cathepsin K inhibitor, which potently inhibits bone resorption in acute and chronic animal models.
METHODS

Materials

Cloning and expression vectors for human and rat cathepsin K were pQE31 (QIAGEN, Germany), TA and pBlueBac4.5 (Invitrogen, Carlsbad, CA), respectively. Tissue culture media and supplements were purchased from Invitrogen (Carlsbad, CA). Bovine spleen cathepsin B was obtained from Sigma-Aldrich (St. Louis, MO). Human liver cathepsin L and bovine spleen cathepsin S were purchased from Calbiochem (La Jolla, CA). Recombinant human RANKL was purchased from Pepprotech (London, UK). Rat osteocalcin EIA kit was from Biomedical Technologies (Stoughton, MA). Serum CrossLaps™ One Step ELISA kit was purchased from Osteometer (Herlev, Denmark). Urinary DPD EIA kit was purchased from Metra Biosystems (Mountain View, CA). Leukocyte acid phosphatase kit and other reagents without special mention were obtained from Sigma-Aldrich (St. Louis, MO). A reference compound of cathepsin K inhibitor, SB357114, \((N-[3-Methyl-1(S)-[N-[3-oxo-1-(pyridin-2-yl)sulfonyl]perhydroazepin-4(S)-yl]carbamoyl]butyl]-1-benzofuran-2-carboxamide)\) was synthesized at our laboratory.

Animals

This study was performed in accordance with the institutional “Standard Procedure for Animal Care and Experiments” of Dong-A Pharmaceutical and the “Guide for the Care and Use of Laboratory Animals” from the National Institutes of Health. Sprague-Dawley rats were
obtained from Orient Co., Ltd. (Seoul, Korea). After ovary removal, the animals were housed 2 heads per polycarbonate cage with wood shavings, and were maintained under a controlled environment with temperature at 23 ± 2°C, relative humidity at 55 ± 5%, and a 12 h/12 h light/dark cycle throughout the experiment. The rats were given food and ultraviolet-sterilized tap water *ad libitum*.

**Cloning, expression, and purification of human cathepsin K**

Construction of human cathepsin K vector was carried out using the standard cloning procedure. The full-length cDNA of cathepsin K was obtained by PCR amplification with sense and antisense primers derived from the cDNA of MCF-7 human breast cancer cells (Littlewood-Evans et al., 1997). The PCR product was gel-purified and ligated into the pQE31 expression vector. The resulting construct encoding human cathepsin K gene was transformed into the *E. coli* BL21 (DE3) pLysS expression cells. Transformed cells were grown in LB broth containing 100 µg/ml ampicillin for about 16 h, diluted 1/100 with the same medium and grown to an A600 of 1.0. Then, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and the incubation was continued for another 3 h. Cells were collected and human cathepsin K protein was purified by Ni²⁺ affinity column chromatography. After renaturation, protein was used in enzyme inhibition assay of human cathepsin K (Bromme et al., 1996; Bossard et al., 1996; Linnevers et al., 1997).

**Cloning and expression of rat cathepsin K**
The full-length cDNA encoding rat procathepsin K from a lung cDNA library (Takara, Shiga, Japan) was engineered for expression in baculovirus by PCR modification of the original cDNA cloned into the TA cloning vector. The oligonucleotide primers 5'-TGC-TGG-ATC-CAT-GCC-GGA-GGA-AAC-GCT-G-3' (containing a BamHI restriction site, underlined) and 5'-CTG-GTC-TAG-ATG-AAT-CAC-ATC-TTG-GGG-3' (containing an XbaI restriction site, underlined) were used for amplification of a 1kb fragment. The PCR product was subcloned into the baculovirus vector, pBlueBac4.5 to create the plasmid pBlueBac-RCK (McQueney et al., 1998). To test the pBlueBac-RCK composite viruses for the expression of rat cathepsin K, Sf9 cells were infected at varying multiplicities of infection with pBlueBac-RCK, and the cell lysate and medium were collected every 24-96 h (Mitsudo et al., 2003). Collected crude proteins were used for the rat cathepsin K enzyme assay (Lark et al., 2002).

Cathepsin K assays

The enzyme inhibition assay of human and rat cathepsin K was carried out according to the previously described method with minor modifications (Bromme et al., 1996). In brief, the substrate for both human and rat cathepsin K was 25 µM of Z-Phe-Arg-AMC, and the each composition of the final reaction buffer is comprised of the following buffers; 100 mM sodium acetate buffer (pH 5.5) containing 20 mM cysteine and 5 mM EDTA for human CK, and 50 mM MES buffer (pH 5.5) containing 4 mM DTT and 2 mM EDTA for rat CK, in the composition of the final 2% DMSO solution (Aibe et al., 1996). For the initiation of reaction, an enzyme
solution was added to the substrate and inhibitor mixture, followed by incubation for 1 h at 37°C. Substrate hydrolysis was monitored with a SPECTRAFluor (Tecan, Austria) at excitation and emission wavelengths of 360 and 465 nm, respectively. The percent inhibition of the reaction was calculated from a control reaction containing only vehicle.

**Selectivity Assays**

To measure enzyme activity of cathepsin B, L, and S, enzymatic assays were carried out in the respective assay buffers containing 1% DMSO. Each reaction buffer was a 45 mM phosphate buffer (pH 6.0) containing 0.9 mM DTT for cathepsin B, 50 mM sodium acetate (pH 5.5) containing 1 mM EDTA, 2 mM DTT for cathepsin L, and 100 mM phosphate buffer (pH 6.5) containing 5 mM EDTA, 5 mM DTT, 0.01% Triton X-100 for cathepsin S. Inhibitory activities of compounds were determined using 90 µM of Z-Arg-Arg-pNA for bovine cathepsin B, 5 µM and 15 µM of Z-Phe-Arg-AMC for human cathepsin L and bovine cathepsin S as a substrate. Prior to the addition of substrate, the inhibitors were preincubated at 25°C for 10 min with the enzyme to allow the establishment of the enzyme-inhibitor complex. Substrate was then added and incubated at 25°C for 1 h. The enzyme activity was measured from the increase of absorbance (405 nm) for p-nitroanilide or fluorescence ($\lambda_{ex}$ 360 nm, $\lambda_{em}$ 465 nm) for aminomethylcoumarin with a SPECTRAFlour (TECAN, Austria). All experiments were carried out in duplicate. IC$_{50}$ values were determined by four parameter fits from the percentage of inhibition versus inhibitor concentration using the software program SigmaPlot.
Rabbit bone resorption assay

Unfractionated bone cells were isolated from rabbit long bones as described previously (Kaneko et al., 2000). The femora and tibiae of 1-day-old rabbits were dissected out. After removal of adherent soft tissues, the bones were minced in α-MEM (pH 6.8) containing 5% FBS for 5 min. The cells were dissociated from the bone fragments by brief vortexing, and the fragments were then allowed to sediment for 2 min. The cells in the supernatant were collected and used as unfractionated bone cells.

For the bone resorption assay, isolated bone cells (5x10^5 cells) were plated and attached on a sterilized dentine slice (6 mm in diameter, 100-200 µm thickness) in α-MEM supplemented with 5% FBS in each well of 96-well plate. After 2 h incubation in humidified air at 37°C in 5% CO₂, the medium was replaced with either fresh media containing the test compound or vehicle. The cells were then cultured for 48 h under the same conditions, thereafter the supernatant was harvested and the CTx levels in the media were measured using ELISA kit. The bone cells on dentine slices were fixed with 60% acetone in a citrate buffer for 30 seconds and washed with PBS and stained for TRAP activity using a kit. All experiments were carried out in triplicate.

For the quantification of TRAP-positive multinucleated cells, isolated bone cells were plated in each well of a 96-well plate and treated with the same method as described above.
The number of TRAP-positive multinucleated cells (> 2 nuclei per cell) in each well of a 96-well plate, were measured using an Olympus IX 71 microscope (Olympus Co., Tokyo, Japan).

Data was represented as mean ± S.E.M. from five determinants.

**Human bone resorption assay**

Human peripheral blood was obtained from healthy adult volunteers and osteoclast-like cells were generated using slight modifications of previously described methods (Fujikawa et al., 2001; Massey et al., 2001). In brief, heparinized whole blood was collected and diluted 1:1 in α-MEM, and layered over Ficoll-Paque. After centrifugation, isolated PBMCs (1×10⁵) were plated on dentine slices in a 96-well plate in a final volume of 200 µl of a medium containing reagents. Media contained 25 ng/ml of M-CSF and test compounds throughout the experiments, 10 ng/mL of TGF-β for the first 3 days, and 30 ng/mL of RANKL for day 3-19. Cultures were maintained at 37°C in 5% CO₂ humidified incubator. On day 3, 90% of the medium was removed and replaced with fresh medium containing cytokines and compounds. Thereafter, half of the medium was exchanged every three days. On day 19, whole media was removed, washed twice with fresh media and replenished with fresh media containing cytokines and test compounds. After incubation for an additional 24 h, the conditioned media was harvested and then the CTx level was measured. All experiments were carried out in triplicate. IC₅₀ values were calculated by the non-linear regression method using Sigma Plot 4.0.
Bone mineralization and alkaline phosphatase assay

Bone mineralization was induced as previously described (Spinella-Jaegle et al., 2001). Briefly, MC3T3-E1 mouse preosteoblast-like cells were plated in 24-well plates at a density of 1x10^4/well and incubated in α-MEM supplemented with 10% FBS. Bone mineralization was induced by the addition of 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate to the medium. Cells were either left untreated or treated with BMP-2 or OST-4077 for 19 days. At the end of each experiment, the cultures were rinsed with PBS and fixed with 10% formalin. Fixed cultures were rinsed three times with DW, and stained with 2% alizarin red S for 5 min at room temperature with gentle agitation. Cultures were then washed three times with DW and photographed. For cellular alkaline phosphatase assay, on day 17, the medium was replenished with serum-deprived α-MEM. After starved for 48 h, cell layers were detached and disrupted by ultrasonicator (Sonics & Materials, Danbury, CN). Ten microliter of cytosolic supernatant was incubated with 100 µl of 2 mg/ml p-nitrophenyl phosphate in alkaline buffer for 15 min at 37°C. The reaction was terminated by adding 100 µl of ice-cold 0.5 N NaOH. The increase of released p-nitrophenol was measured at 405 nm using the SPECTRAFluor (Tecan, Austria). Total protein concentration of lysate was determined by BCA method according to the manufacturer’s instruction (Pierce Biotechnology, Rockford, IL). The data was expressed as nmole of p-nitrophenol/mg protein.

PTH-induced bone resorption in TPTX rats
The TPTX rat model was used according to the previously described methods (Votta et al., 1997). Male SD rats were allowed to acclimate for at least 7 days. The animals had free access to tap water and standard rat chow until the day of TPTX. Surgical TPTX was performed under anesthesia with pentotal sodium (40 mg/kg, iv) on rats weighing 300-350g. TPTX rats were allowed to recover under fasting conditions with free access to tap water. On the day following the surgery, femoral artery and vein were catheterized under ether anesthesia. The plasma calcium concentration was measured and success of TPTX (plasma calcium < 7.5 mg/dl) was confirmed before animals were allocated to study groups. OST-4077 or 0.5 % methylcellulose was orally administered to rats and 30 minutes later, rhPTH(1-34) or vehicle (0.025 % BSA in saline) was infused at a rate of 0.3 nmole/kg/h. Plasma calcium levels were measured just before rhPTH(1-34) infusion and every 1 h for 6 h thereafter, using an automated clinical chemistry analyzer, Konelab 20i (Thermo Electron Co., Finland) according to manufacturer’s instructions. ED$_{50}$ was mathematically calculated by detecting the compound-induced reduction of AUC$_{0-6h}$ at several doses compared to the rhPTH-treated group.

**Estrogen deficiency-induced bone loss in rats**

Sixty 3-month-old SD rats were used for this experiment. Rats were bilaterally ovariectomized by dorsal approach under anesthesia with pentotal sodium (40 mg/kg, iv). The animals were kept in pairs and fed a standard laboratory diet (Charles River, Japan)
containing 0.85% calcium and 0.62% phosphorus. Diet was available *ad libitum* to the rats.

The fifty OVX rats were allocated into five groups receiving vehicle alone or twice daily doses of 10, 20, 50 and 100 mg/kg. OST-4077 or vehicle was orally administered starting on the day after the surgery and continuing throughout the remainder of the experimental period.

Four weeks after OVX, for urine collection, the rats were placed into metabolic cages without food for 16 h. After urine collection, blood was obtained from the abdominal artery. Urine and serum samples were stored in the refrigerator. Success of OVX was confirmed by markedly reduced uterus weight.

**Measurement of BMD and bone mineral contents**

Femora were isolated and the adherent tissues were removed. BMD was measured by Lunar PIxImus II densitometer (GE Medical Systems, CA) using dual energy x-ray absorptiometric technique. The coefficient of variation (CV) of the machine was 0.84%.

Paired femora in crucibles were ashed using furnace (600°C, 24 h) and weighed. Femur ash weights were divided by the length of femurs, measured from great trochanter to the external condyle (Caselli et al., 1997; Rodan et al., 1993). Data was expressed as milligrams per millimeters.

**Bone turnover marker**

The level of serum osteocalcin was measured by EIA specific for rat osteocalcin according to the manufacturer's instruction. The concentration of urinary total
deoxypyridinoline (DPD) was determined using EIA kit and normalized with the concentration of urinary creatinine concentration.

**Statistical analysis**

Statistics were computed using SigmaStat 5.0 software program (SPSS, Chicago, IL). The data was analyzed using one-way analysis of variance (ANOVA). When the ANOVA performed over all groups indicated a significant (p<0.05) difference among the groups, statistical differences between groups were subsequently evaluated with Student-Newman-Keuls multiple comparison test. A value of p less than 0.05 was considered significant. The data is presented as the mean ± S.E.M.
RESULTS

Cathepsin K inhibition and selectivity

OST-4077 is a potent inhibitor of human cathepsin K (IC\textsubscript{50}=11 nM), and the inhibitory activity against human CK is 39-fold more potent than against rat CK (Fig. 1). SB357114 showed concentration-dependent inhibition with an IC\textsubscript{50} of 3.5 nM (Fig. 2A). OST-4077 is more selective for inhibition of cathepsin K than cathepsin B (IC\textsubscript{50} = 239 nM), L (IC\textsubscript{50} = 981 nM) or S (IC\textsubscript{50} = 196 nM) (Fig. 2B).

Inhibition of bone resorbing activity by rabbit osteoclasts

TRAP-positive osteoclast-like cells were identified in unfractionated bone cells in the absence (Fig. 3A) or presence of OST-4077 (Fig. 3B). There were no significant changes in the number of TRAP-positive multinucleated cells between groups treated with vehicle (266 ± 11 cells/well) and 300 nM of OST-4077 (285 ± 15 cells/well, p > 0.05). Addition of CK inhibitors to rabbit bone cells on dentine slices resulted in a concentration-dependent inhibition of the CTx release in the culture media (Fig. 3C). In this culture condition, the released CTx from bone slices by osteoclasts was in the range of 450–2,000 pg/ml. OST-4077 and SB357114 showed potent inhibitory activity against bone resorption with IC\textsubscript{50} of 37 and 20 nM, respectively.

Inhibition of bone resorbing activity by human osteoclasts
Treatment with hM-CSF and hTGFβ/hRANKL differentiated human PBMCs into TRAP-positive multinucleated osteoclast-like cells (Fig. 4B and D). These cells on bone slices resorbed bone and left resorption pits on the surface (Fig. 4D). Concentration-dependent inhibition of the pit formation by OST-4077 was observed under microscopy (Fig. 4E and F). OST-4077 and SB357114 inhibited the CTx release from dentine slices with IC₅₀ of 205 and 113 nM, respectively (Fig. 4G). The relative potency between OST-4077 and SB-357114 coincided with the results of human CK inhibition assay and rabbit bone resorption assay.

**Effects on bone mineralization and alkaline phosphatase activity**

MC3T3-E1 cells differentiated into osteoblast and formed bone-like nodules in the presence of ascorbic acid and β-glycerophosphate. BMP-2 at 50 ng/ml significantly stimulated the formation of bone nodules (Fig. 5A). Under this culture condition, OST-4077 did not induce the increase of bone nodules (Fig. 5B). The increase of alkaline phosphatase activity is an early sign of osteoblastic differentiation. In this study, OST-4077 at concentrations of 10-1,000 nM did not increase cellular ALP activity whereas BMP-2 at 50 ng/ml significantly increased ALP activity (Fig. 5C and D).

**Anti-resorptive effects in TPTX rats**

In the preliminary pharmacokinetic study, OST-4077 showed a good oral availability (>80%) and favorable terminal half life (>4 h) in rats. Because of its favorable pharmacokinetic characteristics as well as relatively potent inhibitory activity against rat CK, anti-resorptive
effects of OST-4077 were evaluated in the TPTX rat model. PTH infusion was started 0.5 h after oral treatment of OST-4077 considering $T_{\text{max}}$ of 0.5 h. Dose-related inhibition of the calcemic response by rhPTH(1-34) was observed with an ED$_{50}$ of 69 mg/kg (Fig. 6).

**Anti-resorptive effects in OVX rats**

Anti-resorptive effects of OST-4077 were evaluated in the OVX rats to determine if inhibition of cathepsin K would prevent estrogen deficiency-induced osteopenia. In the previous study, OVX rats were orally dosed at 50 mg/kg, bid or 100 mg/kg, uid. Preventive effects on BMD were found in both treatment groups but the efficacy of 50 mg/kg, bid was superior to that of 100 mg/kg, uid (data not shown). Based on these results, the compound was treated twice daily throughout the experimental period. Four-week treatment of OST-4077 preserved OVX-induced bone loss dose-dependently in 3-month-old rats. OST-4077, 100 mg/kg showed complete prevention of bone loss and similar results were also found in the change of ash contents of femora (Fig. 7A and B). Bone resorption marker, urinary DPD, was increased by OVX and diminished by OST-4077 dose-dependently (Fig. 7C). Osteocalcin was also increased by OVX but this increased bone formation marker was not changed by OST-4077 treatment (Fig. 7D).
DISCUSSION

OST-4077 is a potent inhibitor of human and rat CK. This compound inhibited human CK activity at nanomolar concentrations with 30-fold more selective to other cysteine cathepsin subtypes. The compound directly inhibited rabbit osteoclast-mediated bone resorption in vitro. These data were consistent with the results that inhibition of CK retarded bone matrix degradation in CK knockout mice (Hou et al., 1999). However, the potencies of CK inhibitors are greater in rabbit osteoclast-mediated bone resorption than those in osteoclast-like cells differentiated from human PBMCs. SB357114 has been reported to have potent inhibitory activity against human osteosarcoma-induced bone resorption comparable to rabbit osteoclast-induced bone resorption (Stroup et al., 2001). It is possible that the apparent reduction of potency in human PBMC-derived osteoclasts assay reflects the differences of CK levels between assay systems (Lark et al., 2002). Therefore, herein results suggest that OST-4077 effectively inhibits osteoclast-mediated bone resorption in vitro.

To ascertain whether the CK inhibitor cause any changes in osteoblasts, preosteoblast MC3T3-E1 cells were treated with OST-4077. In this study, OST-4077 did not induce any significant changes in bone-like nodule formation or ALP activities whereas BMP-2 significantly increased these osteoblast differentiation markers. It is well documented that the CK is mainly localized within osteoclasts and is absent or at low frequency in other tissues (Drake et al., 1996). Therefore these results mean that the pharmacological activities of
selective cathepsin K inhibitors such as OST-4077 are restricted within osteoclasts.

It was reported that catalytic sites of CK are different between humans and rats (Tepel et al., 2000). Because of this species difference, \textit{in vivo} elucidation of the pharmacological activities of CK inhibitor has been hampered. Although OST-4077 is a weak rat CK inhibitor with an $IC_{50}$ of 427 nM, its pharmacokinetic profile is favorable enough to reach about 20 $\mu$mole/ml of $C_{\text{max}}$ when administered at 50 or 100 mg/kg, orally. These characteristics enabled us to study the pharmacological activities of this CK inhibitor \textit{in vivo} as well as \textit{in vitro}.

Oral administration of OST-4077 partially prevented PTH-induced calcemic response in the TPTX rat model. Because the results are expressed as changes of plasma calcium concentration, these results mean that this selective CK inhibitor can partially prevent demineralization. This coincides with the results that peptide aldehyde CK inhibitors can prevent calcium release in a fetal rat long bone assay \textit{in vitro} (Yasuma et al., 1998). In this acute bone resorption model, the inhibitory efficacy of OST-4077 is comparable to that of another CK inhibitor, SB357114 that showed a maximum inhibition of 57% at plasma level 10 $\mu$mole/ml (Lark et al., 2002).

OST-4077 prevented bone resorption in the OVX rats, resulting in prevention of the OVX-induced bone loss. The compound inhibited OVX-induced decreases of BMD and mineral contents in femora dose-dependantly. Furthermore, OST-4077 inhibited excretion of
the bone resorption marker (urinary DPD), but did not affect the bone formation marker (serum osteocalcin). It is well known that bone formation and resorption are tightly coupled. However these results indicate that this selective CK inhibitor reduced the bone resorption rate without modulating one of the bone formation markers in the OVX rats characterized by the increased bone turnover rate for at least 4 weeks. According to the previous evidence, alendronate as an anti-resorptive, completely blocked bone resorption with bone formation rate at the periosteal surface still increased (Pennypacker et al., 2004). This indicates alendronate partly uncoupled the bone turnover. However data for the histomorphometrical change was not made, observations to these markers influenced by OST-4077 could not be interpreted directly as the explanation for the bone turnover uncoupling.

From these in vivo results, it appears that CK inhibitors can inhibit demineralization as well as matrix degradation as evaluated by urinary DPD. Because bone demineralization and protein matrix degradation are linked, inhibition of CK-mediated proteolysis would result in maintaining an intact mineralized matrix. Furthermore, CK knockout mice showed increased bone mass and strength (Pennypacker et al., 1999), suggesting that prevention of matrix degradation does not cause negative effects on bone integrity.

Taken together, this data suggests that a potent and selective CK inhibitor such as OST-4077 can be effective in preventing bone loss in vivo, and that such a compound may be therapeutically beneficial for the treatment of diseases with accelerated bone loss such as
postmenopausal osteoporosis.
REFERENCES


Yasuma T, Oi S, Choh N, Nomura T, Furuyama N, Nishimura A, Fujisawa Y, and Sohda T
FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Chemical structures of OST-4077 and SB357114.

Figure 2. (A) OST-4077 (■) and SB357114 (●) inhibited human cathepsin K activities in a concentration-dependent manner. Rat cathepsin K inhibition (▲) by OST-4077 was found at 39-fold higher concentration than human cathepsin K. Assay conditions are described under Methods section. (B) OST-4077 inhibited enzyme activities of cathepsin B (○), cathepsin L (▲) and cathepsin S (□).

Figure 3. After 48 h cultivation of unfractionated rabbit bone cells on dentine slices, TRAP-positive osteoclast-like cells were identified. Arrows indicate TRAP-positive multinucleated cells on dentine slices. Original magnification, x200. There were no significant changes in the number of TRAP-positive multinucleated cells between vehicle (A) or 300 nM of OST-4077 (B) treated groups. OST-4077 and SB357114 inhibited osteoclast-mediated CTx release in a concentration-dependent manner (C). Data from five different experiments was expressed as mean ± SEM of % inhibition compared to vehicle control. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs. vehicle control.

Figure 4. TRAP positive osteoclast-like cells on plastic plates (A and B) and dentine slices (C~F) in the absence (A and C) or presence of hM-CSF and hTGFβ/hRANKL (B and D~F). Isolated human PBMCs were treated with or without compounds throughout the whole
experimental period (20 days): 100 nM (E) and 1000 nM (F) of OST-4077. Original magnifications, x200. Arrows indicate the TRAP positive multinucleated cells and arrowheads for traces that remained after bone resorption by multinucleated osteoclast-like cells. (G), OST-4077 inhibited the CTx release by human PBMC-derived osteoclast-like cells in a concentration-dependent manner. The IC₅₀ values of OST-4077 and SB357114 were calculated as 205 and 113 nM, respectively. The CTx level in the last 24h-conditioned media was measured using Serum CrossLaps™ One Step ELISA kit. Data from five different experiments was expressed as mean ± SEM of % inhibition compared to vehicle control. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs. vehicle control.

**Figure 5.** MC3T3-E1 cells were cultured in mineralization medium containing 10% FBS and ascorbic acid for 19 days. Cells were treated with BMP-2 (A) or OST-4077 (B) at the indicated concentrations. The mineralized matrix was stained with Alizarin Red-S for calcium. Experiments were performed in triplicate. OST-4077 did not increase cellular ALP activities, early marker of osteoblast differentiation (D). But, BMP-2 at 50 ng/ml significantly stimulated the expression of ALP (C). **, p < 0.01 vs. vehicle control.

**Figure 6.** (A) PTH-induced bone resorption was suppressed by OST-4077 in TPTX rats. PTH was infused 0.5 h after oral dosing of OST-4077 or vehicle. Blood samples were collected over the 6 h duration. Data is represented as mean±SEM of net change in plasma calcium level from each determinant (N=7-10/group). O, 0.5% methylcellulose; ●, PTH; ▲, 10 mg/kg;
▼, 30 mg/kg; ■, 100 mg/kg of OST-4077. (B) OST-4077 significantly reduced the PTH-induced rise in AUC$_{0-6h}$ by plasma calcium levels in a dose-dependent manner. ). ###, p < 0.001 vs. vehicle control; *, p < 0.05; ***, p < 0.001 vs. PTH control.

**Figure 7.** Effects of OST-4077 on bone mass and turnover in 3-month-old OVX rats (n=10/group). When orally administered by 10, 20, 50, and 100 mg/kg twice daily, OST-4077 preserved bone mineral density (A) and bone ash contents (B) in paired femora comparable to the level of sham control. Increased urinary DPD excretion by OVX was significantly inhibited at high doses of OST-4077 (C) without affecting the level of serum osteocalcin (D). #, p < 0.05 vs. sham control; *, p < 0.05 vs. OVX control.
Figure 1

OST-4077

SB357114
Figure 2

A) 

B) 

% of Inhibition

Concentration (nM)

% of Inhibition

Concentration (nM)
Figure 3

A) DMSO

B) OST-4077

C) Bar graph showing the percentage inhibition of CTx release with varying concentrations of SB357114 and OST-4077. The graph compares the effects at different concentrations against a control.
Figure 5

A) Control
0.5 ng/mL
5 ng/mL
50 ng/mL

BMP-2

B) Control
10 nM
100 nM
1000 nM

OST-4077

C) ALP activity (n mole/mg protein)

D) ALP activity (n mole/mg protein)

BMP-2

OST-4077
Figure 6

A) Change of plasma calcium (mg/dl) over time (h) after PTH infusion. The graph shows the effect of OST-4077 or vehicle on plasma calcium levels. The data is represented by different symbols and lines for each group.

B) AUC0-6h (h x mg/dl) for OST-4077 and PTH treatment. The bar graph compares the AUC0-6h for control (C), PTH, and different doses of OST-4077 (10, 30, 100 mg/kg), with statistical significance indicated by asterisks.
Figure 7

A) BMD (g/cm³)

B) Bone ash (mg/mm)

C) DPD/creatinine (nM/mM)

D) Osteocalcin (ng/ml)

C  O VX  10  20  50  100 (mg/kg)

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