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Effects of ONO-5334, a novel orally-active inhibitor of cathepsin K, on bone metabolism

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ABSTRACT

In the present study, we examined the *in vitro* and *in vivo* pharmacological effects of ONO-5334, a novel inhibitor of cathepsin K, on bone metabolism. *In vitro* experiments indicated that ONO-5334 is a potent inhibitor of cathepsin K with Ki value of 0.1 nM. Although this compound inhibited other cysteine proteases, such as cathepsin S, L and B, its inhibitory activity for these enzymes was 8 to 320 fold lower than that for cathepsin K. ONO-5334 also inhibited human osteoclasts bone resorption *in vitro* at a concentration more than 100 fold lower than that of alendronate, a bisphosphonate. While alendronate disrupted actin ring and induced pyknotic nuclei in osteoclasts, ONO-5334 did not have such effects, suggesting that this compound does not affect osteoclasts viability. In *in vivo* experiments, oral administration of ONO-5334 dose-dependently reduced plasma calcium level increased by parathyroid hormone related peptide in thyroparathyroidectomized rats. Furthermore, *in vivo* experiment using normal monkeys demonstrated that ONO-5334 decreases serum and urine C-telopeptide of type I collagen level, a bone resorption marker, soon after oral dosing. These levels were consistently decreased below pre-dose levels by repeated oral dosing with ONO-5334 for 7 days. ONO-5334 on the other hand did not affect bone formation markers, serum osteocalcin and bone specific alkaline phosphatase. These findings indicate that ONO-5334 is a specific inhibitor for cathepsin K and thus may be a novel therapeutic agent for metabolic bone diseases.

Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength with increased risk of fracture [1]. In postmenopausal osteoporosis, estrogen deficiency increases bone turnover with an imbalance between bone resorption and formation, which results in low bone mass, and subsequent increase in bone fragility. The most commonly-used agents for osteoporosis are the bisphosphonates, which suppress accelerated bone turnover, increase bone mineral density (BMD), and reduce the incidence of fracture in postmenopausal women with osteoporosis. While bisphosphonates have been shown to reduce the risk for vertebral fractures by 40% to 70%, their reduction of the risk for non-vertebral fractures is as low as 20% to 40% [2–5]. There is also concern that bisphosphonates may cause complications, such as atypical fracture of the femur, due to their prolonged decrease in bone turnover [6]. Thus, more effective and safer anti-osteoporosis agents with novel modes of action are highly needed.

Cathepsin K (EC 3.4.22.38) is a member of the papain cysteine protease superfamily known to be implicated in osteoclasts bone resorption [7–10]. This enzyme, which is secreted along with protons, is specifically, but abundantly, expressed in osteoclasts. Upon bone resorption, cathepsin K degrades type I collagen, a major organic component of the bone, in an acidic microenvironment sealed by osteoclasts. It has been shown that loss of function mutation in cathepsin K gene is associated with a number of complications in human, including increase in bone density, short stature, acro-osteolysis of the terminal distal phalanges, and skull deformities [11,12]. In animals, mice deficient in cathepsin K exhibit osteopetrotic phenotype and show increased trabecular bone volume, trabecular number, trabecular thickness, and cortical bone mass [13–15]. Conversely, transgenic mice with over-expressed cathepsin K show accelerated bone turnover and decreased bone mass [16,17]. These lines of evidence support the importance of cathepsin K in osteoclasts bone resorption.

It has been proposed that new anti-resorptive agents that can allow reduction of osteoclasts bone resorption without affecting bone formation would be therapeutically useful for patients with osteoporosis [18]. Inhibitors of cathepsin K may be of particular interest as they would allow reduction of bone resorption, via suppression of collagen degradation, without affecting osteoclasts viability. In the present study, we examined the *in vitro* and *in vivo* pharmacological effects of ONO-5334, a novel cathepsin K inhibitor, on bone metabolism.

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Materials and methods

Compounds

ONO-5334, N-((1S)-3-{(2Z)-2-[(4R)-3,4-Dimethyl-1,3-thiazolidin-2-ylidene] hydrazino}-2,3-dioxo-1-(tetrahydro-2H-pyran-4-yl) propyl) cycloheptanecarboxamide was synthesized in our laboratories (ONO Pharmaceutical Co., Ltd., Osaka, Japan). Alendronate, a bisphosphonate, was purchased from Dr. Reddy's Laboratories (India).

Enzymatic assays

Inhibition of human cathepsin K activity was measured using an enzymatic assay with Z-Phe-Arg-MCA (Peptide Institute, Inc., Osaka, Japan) as a substrate [19]. Inhibition of rabbit [20] and rat [19] cathepsin K activity was measured using Z-Leu-Arg-MCA substrate (Peptide Institute, Inc., Osaka, Japan). Inhibition of human cathepsin S, L and B activity was measured using Z-Val-Val-Arg-MCA, Z- Phe-Arg-MCA and Z-Arg-Arg-MCA substrates (Peptide Institute, Inc., Osaka, Japan), respectively [21–23]. Porcine calpain I and II [24], and bovine cathepsin C [25] activity was measured as described in the literature. Enzymatic reaction velocity was determined from the increase in fluorescence intensity of AMC (aminomethylcoumarin). ONO-5334 inhibition constant; Ki was determined by a graphical method (Dixon plot).

Human osteoclasts culture and assay

Whole blood was collected from healthy human male volunteers in heparinized tubes (syringes). Isolation of peripheral blood mononuclear cells (PBMCs) and generation of osteoclasts were conducted as described by Susa et al. [26]. PBMCs were cultured on bovine cortical bone slices in a medium (α-MEM containing 10% FBS and penicillin-streptomycin, Invitrogen, USA) containing 25 ng/mL M-CSF (R&D Systems, Minneapolis, USA), 50 ng/mL human RANKL (Santa Cruz Biotechnology, USA), 5 ng/mL human TGF-β1 (R&D Systems, Minneapolis, USA) and 1 μM dexamethasone (Sigma-Aldrich, St. Louis, USA). After 14 days of culture, the cells were further cultured for 24 hours in the presence of ONO-5334 or alendronate. C-telopeptide of type I collagen (CTX) that was released into the conditioned medium was quantified using CrossLaps for Culture (Nordic Biosciences, Herlev, Denmark). Bone slices were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were stained with Alexa Fluor 488 phalloidin (Molecular Probes), mounted using SlowFade light antifade kit with DAPI (Molecular probes), and examined with confocal laser microscope system TCS-SP2 AOBS; objective lens magnification: 40× (Leica Microsystems).

Thyroparathyroidectomized (TPTX) rat model

Female Crl:CD(SD) rats (Charles River Laboratories Japan Inc., Japan) aged 7 weeks were used in this experiment. They were assigned to the following seven groups ($n = 14$ per group): sham, TPTX, control, and ONO-5334 (0.12, 0.6, 3 and 15 mg/kg) groups. Animals were anesthetized with interperitoneal injection of a mixture of 10-volume 50 mg/mL ketamine HCl (Ketalar 50, Sankyo Lifetec Co., Ltd., Japan) and 1-volume 20 mg/mL xylazine HCl (Celactar 2% injection solution, Bayer, Japan). The right and left thyroid glands and parathyroid glands attached to the trachea were removed using an electric cautery. In the sham group, only the skin of the throat was incised. After surgery, each animal was fed 2 pieces (approximately 6 to 7 g) of standard laboratory chow (CRF-1, Oriental Yeast Co., Ltd., Japan). One day after surgery, blood samples were collected prior to administration of parathyroid hormone related peptide (PTHrP). PTHrP (PTHrP 1–34, American Peptide Company Inc., USA) at a dose of 0.5 mg/kg was administered subcutaneously to each animal in the control and ONO-5334 groups, while the vehicle (physiological saline containing 0.1 w/v% bovine serum albumin) was administered to animals in the sham and TPTX groups. Immediately after PTHrP administration, animals were orally given ONO-5334 (0.12, 0.6, 3, and 15 mg/kg) or its vehicle (0.5%w/v methylcellulose). Animals in the sham and TPTX groups were given the vehicle. Blood samples were collected 3 hours after PTHrP administration, and plasma calcium concentration was measured with a Calcium E-HA Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma CTX concentration was also measured using RatLaps ELISA (Nordic Bioscience, Herlev, Denmark). Plasma was filtered with Ultrafree-MC (30,000 NMWL, Millipore, USA), and the filtrate was used for CTX measurement.

Assessment of bone turnover markers in monkey

Cynomolgus monkeys were obtained from Simian Conservation Breeding and Research Center, Inc. (Philippines) and Scientific Primates Filipinas, Inc. (Philippines). Out of the purchased monkeys, six females (aged 5 to 7 years) were selected for use in this experiment based on health condition. The selected animals were orally given ONO-5334 (0.3, 3, and 30 mg/kg) or the vehicle (0.5%w/v methylcellulose) once daily for 7 consecutive days within a cross-over study with 14-day recovery period between treatments. Serum and urine samples were collected 2 days prior to the first administration day (Pre), on the first, third and seventh administration day; and 7 and 14 days after the last administration. Serum for measurement of CTX, osteocalcin and bone specific alkaline phosphatase (BAP) was collected 4 hours after administration during the dosing period, and at the same time each day during the pre-dosing and recovery periods. Twenty-four hour cumulated urine for measurement of CTX and creatinine was collected at each sampling day. Serum CTX was measured by Serum CrossLaps ELISA (Nordic Biosciences, Herlev, Denmark). Serum osteocalcin was measured by METRA Osteocalcin EIA kit (Quidel Corporation, San Diego, CA). Serum BAP was measured by Osteolinks BAP (Quidel Corporation, San Diego, CA). Urinary CTX was measured by β-CrossLaps ELISA (Nordic Biosciences, Herlev, Denmark), and urinary CTX data were normalized to creatinine concentration.

Table 1
Inhibitory effect of ONO-5334 against various proteases.

Proteases	Ki value (nM)[a]	Inhibition at 10 μM (%)[b]
Cysteine proteases		
Human cathepsin K	0.10	–
Rabbit cathepsin K	0.049	–
Rat cathepsin K	0.85	–
Human cathepsin S	0.83	–
Human cathepsin L	1.7	–
Human cathepsin B	32	–
Bovine cathepsin C	2500	–
Porcine calpain I	82	–
Porcine calpain II	69	–
Human caspase 3	–	2
Aspartic proteases		
Human cathepsin D	–	1
Human cathepsin E	–	1
Zinc proteases		
Human MMP-1	–	3
Human MMP-9	–	6
Serine proteases		
Human chymotrypsin	–	11
Human trypsin	–	1
Human elastase	–	3
Threonine protease		
Human proteasome	–	0

[a] Ki values are expressed as mean of 3 independent experiments.
[b] Enzyme assays were performed in duplicate at 10 μM.