Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 457 (2002) 51–56
www.elsevier.com/locate/ejphar

Incadronate inhibits osteoporosis in ovariectomized rats

Kyoko Teramura*, Shinji Fukushima, Takaya Iwai, Kazutoshi Nozaki, Satoshi Kokubo, Koichiro Takahashi

Pharmacology Department, Clinical Pharmacology Research Laboratories, Institute for Drug Development Research, Yamanouchi Pharmaceutical Co., Ltd., 1-8, Azusawa 1-chome, Itabashi, Tokyo 174-8511, Japan

Received 23 October 2002; accepted 29 October 2002

Abstract

Incadronate is a highly effective inhibitor of stimulated bone resorption as demonstrated in a hypercalcemia model in rats, bone metastasis models in mice and rats, and an osteoporosis model in dogs. In this study, the effect of incadronate on osteoporosis in ovariectomized rats was examined. Incadronate dose-dependently inhibited decreases in second lumbar vertebrae bone mineral density (BMD) following oral administration for 4 or 12 weeks. Significant inhibition was observed at doses of more than 0.3 mg/kg. Incadronate dose-dependently inhibited the loss of distal femur metaphyseal compressive strength following 12 weeks of oral administration, and this was significant at a 3 mg/kg daily dose. Incadronate also dose-dependently inhibited the increases in urinary deoxypyridinoline levels after 4-or 12-week oral administrations. While incadronate had no effect on serum osteocalcin levels after 4 weeks of oral administration, it did dose-dependently reduce levels after 12 weeks of oral administration. These results suggested that incadronate may be a useful drug for osteoporosis due to stimulated bone resorption.

Keywords: Bisphosphonate; Osteoporosis; Ovariectomy; Bone mineral density; Deoxypyridinoline

1. Introduction

Cessation of ovarian function is a major cause of postmenopausal osteoporosis (Riggs et al., 1986; Dempster and Lindsay, 1993). Ovariectomized rats and dogs have been used extensively in osteoporosis models. In 1996, the effects of incadronate on ovariectomized dogs (Motoie et al., 1996) were reported. This study examined the effect of incadronate not only on bone mineral density (BMD) but also on bone strength using bone formation and resorption markers in ovariectomized rats as an osteoporosis model.

Metabolic markers, reflecting specific stages of bone catabolism and anabolism have been used to detect bone loss in early stages of morbidity (Robey, 1989; Delmas, 1993). These markers for bone formation include the protein osteocalcin (Price et al., 1981) and the enzyme alkaline phosphatase (Price et al., 1980) produced by osteoblasts. To monitor bone resorption, urinary collagen deoxypyridinoline crosslinks, which are produced by the catabolism of collagen, have been assayed (Black et al., 1988; Uebelhart et al., 1990). We used serum osteocalcin levels as a bone formation marker and urinary deoxypyridinoline levels as a bone resorption marker to clarify the effect of the bisphosphonate incadronate following 4- and 12-week oral administrations on bone turnover in ovariectomized rats.

Bisphosphonates are analogues of pyrophosphates, possessing a P–C–P bond. This bond exhibits high affinity for hydroxyapatite crystals and inhibits bone resorption. It has been reported that some of the bisphosphonates cause functional disorders in osteoclasts, and possibly osteoclast apoptosis, thereby, preventing bone resorption (Hughes et al., 1995; Selander et al., 1996).

Etidronate, a first generation bisphosphonate, demonstrated delayed bone mineralization after long-term administration as a major side effect. In contrast, new third generation bisphosphonates have no bone mineralization, but potently inhibit bone resorption. When we compared the effects of incadronate, etidronate and alendronate on BMD in ovariectomized rats, it was revealed that incadronate is 240-fold

* Corresponding author. Present address: Molecular Medicine Laboratories, Institute for Drug Discovery Research/Yamanouchi Pharmaceutical Co., Ltd., 21, Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan. Tel.: +81-298-63-6446; fax: +81-298-52-5391.
E-mail address: teramurk@yamanouchi.co.jp (K. Teramura).

0014-2999/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0014-2999(02)02659-6

more potent than etidronate, and 4-fold more potent than alendronate (Iwai et al., in preparation).

Incadronate is a novel bisphosphonate which potently inhibits bone resorption (Takeuchi et al., 1993; Abe et al., 1990; Kawamuki et al., 1990; Kudo et al., 1990; Fujimoto et al., 1990). This compound shows great potential for the treatment of many diseases caused by elevated bone resorption, such as hypercalcemia (Takahashi et al., 1998) and osteoporosis (Motoie et al., 1995, 1996, 1997).

2. Materials and methods

Incadronate disodium monohydrate was synthesized by Yamanouchi Pharmaceutical (Fig. 1) (Takeuchi et al., 1993). Mature female Sprague–Dawley rats (12 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). During the experimented period, rats had free access to tap water and commercially available standard solid food containing 1.18% calcium and 2.5 IU/g of vitamin D_3 (CE-2; CLEA Japan, Tokyo, Japan). After 8 days acclimatization, they were anesthetized using diethylether and their ovaries were removed. Sham-operation was performed in the same manner but only exposing the ovaries. From the day after ovariectomy, incadronate (0.03, 0.1, 0.3, 1 and 3 mg/kg) was orally administered six times a week for either 4 or 12 weeks. After the final administration, urine (0–24 h) was collected and then urinary deoxypyridinoline levels were measured using high performance liquid chromatography at Teijin Bio Laboratories (Tokyo, Japan). Rats were anesthetized and sacrificed by withdrawing blood from the abdominal aorta using vacuum sampling tubes VT070B (Terumo, Tokyo, Japan) through a laparotomic incision. Serum samples were prepared by centrifuging ($1000 \times g$ for 10 min) their blood, and serum osteocalcin levels were determined using a radioimmunoassay (Biomedical Technologies, MA, USA) at Teijin Bio Laboratories. Serum intact parathyroid hormone levels were determined using an immunoradiometric assay (Nichols Institute, CA, USA) at Teijin Bio Laboratories and serum $1,25(OH)_2D_3$ levels were determined using a radioreceptor assay at Teijin Bio Laboratories. Immediately after collecting urine and blood from rats, the second lumbar vertebrae, the fifth lumbar vertebrae and the femurs were removed. BMD of the second lumbar vertebrae were measured by dual energy X-ray absorptiometry using a bone densitometer QDR-2000 (Hologic; Waltham, MA, USA). The distal epiphysis of each femur was removed and a 7-mm portion was cut from the edge of the bone. The corpus of the fifth lumbar vertebrae was removed and a 5.5-mm length was cut off the edge of each bone in parallel. The strength of the distal femoral metaphysis under compression was measured by compressing the bone anterior-to-posterior in a bone strength tester (TK-252C; Muromachi Kikai Tokyo, Japan). The intra-and interassay variations (CV value) were about 0.69% and 0.76% for BMD by dual energy X-ray absorptiometry, 3.8% and 6.3% for urinary deoxypyridinoline levels, 2.6% and 3.9% for serum osteocalcin levels, 3.7% and 5.5% for serum intact parathyroid hormone levels, and 6.9% and 11.3% for serum

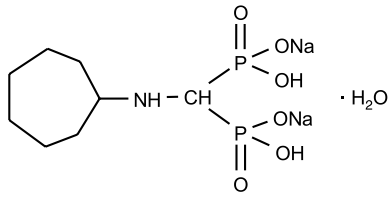

Fig. 1. Chemical structure of incadronate disodium monohydrate.

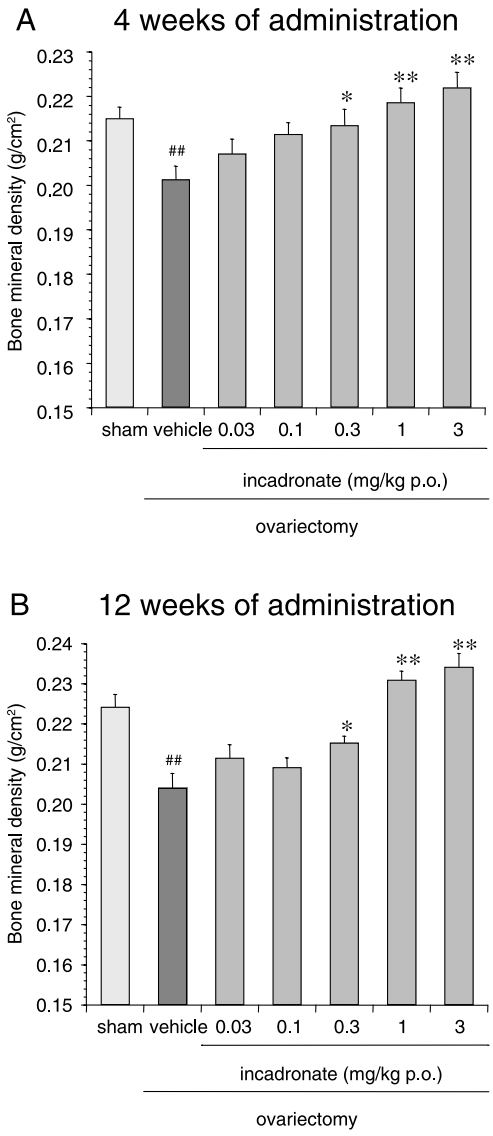

Fig. 2. Effect of incadronate on BMD in ovariectomized rats. Each column and bar represents the mean ± S.E.M. (A: $n=6-7$, B: $n=9-10$). # indicates statistical significance from the sham group (##: $P<0.01$, Student's t-test); * indicates statistical significance from the vehicle group (*: $P<0.05$, **: $P<0.01$, Dunnett's multiple range test). BMD levels of second lumbar vertebrae following repeated oral administration for 4 (A) and 12 (B) weeks were measured by dual energy X-ray absorptiometry. Significant inhibition was observed at doses of more than 0.3 mg/kg.

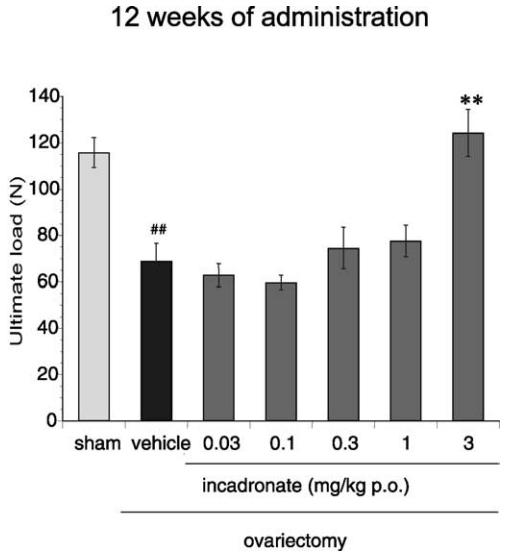

Fig. 3. Effect of incadronate on distal metaphyseal compressive strength of the femur in ovariectomized rats. Each column and bar represents the mean ± S.E.M. ($n = 9–10$). # indicates statistical significance from the sham group (##: $P < 0.01$, Student's t-test). * indicates statistical significance from the vehicle group (**: $P < 0.01$, Dunnett's multiple range test). The strength of femurs was measured by compressing the bone anterior-to-posterior. Incadronate inhibited the reduction in the strength. At a dose of 3 mg/kg, the inhibition was significant.

$1,25(OH)_2D_3$ levels. Measurements are expressed as means ± S.E.M. Comparison between two groups was performed using Student's t-test and comparison among multiple groups was performed using Dunnett's multiple range tests. A P value less than 0.05 was considered statistically significant.

3. Results

Ovariectomy resulted in significantly decreased BMD of the second lumbar vertebrae in rats, with reductions of 6.4% and 9.0% at 4 and 12 weeks, respectively, after ovariectomy compared to non-ovariectomized sham-operated animals. Oral administration of incadronate halted, and at higher doses reversed, the decreases in BMD. At doses of 0.3 mg/kg or more for 4 or 12 weeks, this effect was statistically significant (Fig. 2A,B). After 12 weeks, the compressive strength of the distal femoral metaphyses declined by 40% in ovariectomized rats compared to sham-operated rats. Incadronate inhibited this decrease significantly at a dose of 3 mg/kg (Fig. 3). Ovariectomy decreased the compressive strength of the fifth lumbar vertebrae by 6.5%, but this was not statistically significant. Oral administration of incadro-

Table 1
Effects on fifth lumbar vertebrae compressive strength in ovariectomized rats

Compound	Dose	Ultimate load (N)
Sham-operated	–	371.3 ± 11.4
Vehicle	0	347.1 ± 20.3
Incadronate (mg/kg p.o.)	0.03	339.8 ± 19.8
	0.1	356.8 ± 23.1
	0.3	352.2 ± 18.4
	1	373.7 ± 19.0
	3	380.4 ± 22.7

Ovariectomy decreased the compressive strength of the fifth lumbar vertebrae, but not significantly. Oral administration of incadronate showed some tendency to dose-dependently inhibit the decrease in compressive strength. At doses of 1 and 3 mg/kg, measured strength was at or above the level of the sham-operated group.

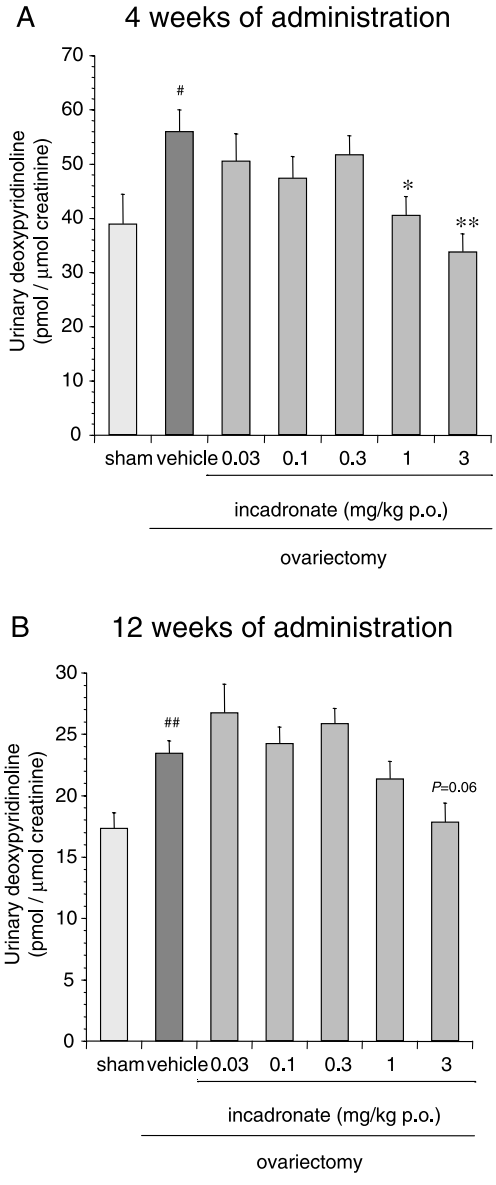

Fig. 4. Effect of incadronate on urinary deoxypyridinoline levels in ovariectomized rats. Each column and bar represents the mean ± S.E.M. (A: $n = 6–7$, B: $n = 9–10$). # indicates statistical significance from the sham group (#: $P < 0.05$, ##: $P < 0.01$, Student's t-test). * indicates statistical significance from the vehicle group (*: $P < 0.05$, **: $P < 0.01$, Dunnett's multiple range test). P value indicates statistical probability from vehicle group (Dunnett's multiple range test). Urinary deoxypyridinoline levels were measured by HPLC. Incadronate dose-dependently inhibited the increase in urinary deoxypyridinoline levels after repeated oral administration for 4-(A) and 12 weeks (B).

nate showed some tendency to dose-dependently inhibit the decrease in compressive strength, and at doses of 1 and 3 mg/kg, the compressive strength was equal to or higher than the sham-operated group level (Table 1). Urinary deoxypyridinoline levels were significantly elevated by 44% and 35% at 4 and 12 weeks after ovariectomy, and significantly elevated serum osteocalcin levels by 26% and 27% at 4 and 12 weeks, respectively, after ovariectomy, compared with levels in sham-operated animals. Incadronate dose-dependently inhibited these increases in urinary deoxypyridinoline levels after 4 or 12 weeks of administration and

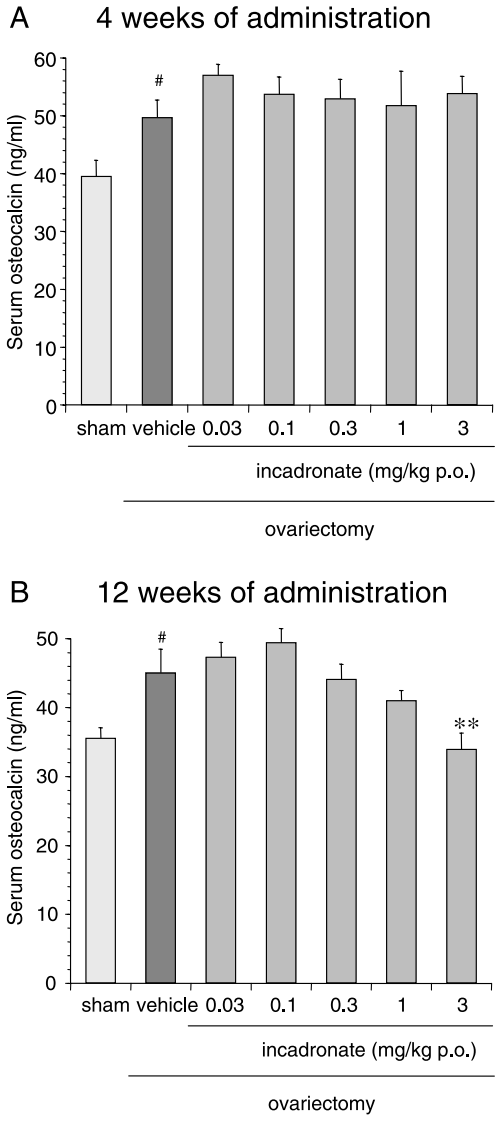

Fig. 5. Effect of incadronate on serum osteocalcin levels in ovariectomized rats. Each column and bar represents the mean ± S.E.M. (A: $n=6–7$, B: $n=9–10$). # indicates statistical significance from the sham group (#: $P<0.05$, Student's t-test). * indicates statistical significance from the vehicle group (**: $P<0.01$, Dunnett's multiple range test). Serum osteocalcin levels were determined by radioimmunoassay. While incadronate had no effect on serum osteocalcin levels after repeated oral administration for 4 weeks (A), it dose-dependently reduced serum osteocalcin levels after administration for 12 weeks (B).

Table 2
Effects on serum parathyroid hormone (PTH) and serum $1,25(OH)_2D_3$ in ovariectomized rats

Compound	Dose	Serum PTH (pg/ml)		Serum $1,25(OH)_2D_3$ (pg/ml)	
		4 weeks	12 weeks	4 weeks	12 weeks
Sham-operated	–	14.1 ± 2.0	12.9 ± 1.0	23.0 ± 3.6	12.0 ± 2.4
Vehicle	0	19.7 ± 4.9	14.5 ± 0.9	29.0 ± 3.5	11.5 ± 1.9
Incadronate	0.03	11.6 ± 0.9	16.0 ± 1.6	26.0 ± 4.1	7.5 ± 1.2
(mg/kg p.o.)	0.1	14.5 ± 2.2	16.3 ± 1.4	19.6 ± 3.2	7.5 ± 0.4
	0.3	12.0 ± 0.8	14.4 ± 1.2	21.9 ± 3.2	8.2 ± 1.1
	1	13.0 ± 0.4	17.0 ± 1.5	24.1 ± 3.6	11.6 ± 1.9
	3	10.5 ± 0.7	16.6 ± 1.3	23.0 ± 3.6	12.0 ± 1.5

No significant difference was observed in the serum levels of PTH and $1,25(OH)_2D_3$ among non-ovariectomized sham-operated rats, ovariectomized rats or ovariectomized–incadronate administered rats. However, the $1,25(OH)_2D_3$ levels in each group after 12 weeks of incadronate administration decreased to approximately half or less of the values determined after 4 weeks of administration.

reduced levels to normal at a dose of 3 mg/kg (Fig. 4A,B). Incadronate, however, had no significant effect on serum osteocalcin levels after 4 weeks, but showed inhibitory effect after 12 weeks of administration of a 3-mg/kg dose (Fig. 5A,B). No statistically significant alteration was observed in the serum levels of intact parathyroid hormone (Table 2), either in non-ovariectomized sham-operated rats, ovariectomized rats or ovariectomized–incadronate administered rats after both 4 and 12 weeks of incadronate administration. No significant difference was observed in the serum levels of $1,25(OH)_2D_3$ among non-ovariectomized sham-operated rats, ovariectomized rats or ovariectomized–incadronate administered rats, however, the levels in each group after 12 weeks of incadronate administration decreased to approximately half or less of the values determined after 4 weeks of administration (Table 2).

4. Discussion

Incadronate significantly inhibited declines in BMD and bone strength, suggesting that the drug may be effective in treating osteoporosis. In addition to distal femoral metaphyseal compressive strength, the compressive strength of fifth lumbar vertebrae was also tested as an alternative index.

Generally, it is known that there are differences in manifestation of a drug's effect on lumbar vertebrae vs. long bone (Yeh et al., 1994). In the present study, incadronate at a dose of 3 mg/kg showed statistically significant inhibition for the decrease in femur metaphysis bone strength. Although not statistically significant, incadronate showed inhibition for the decrease in the fifth lumbar vertebrae compressive strength in a dose-dependent manner. At doses of 1 and 3 mg/kg, where there was full inhibition of up to the levels as the sham-operation and there was similar tendency observed in the BMD of the second lumbar vertebrae (Fig. 2), indicating that the bone strength of the

fifth lumbar vertebrae (Table 1) and BMD of the second vertebrae (Fig. 2) are closely related to each other. Regarding the fifth lumbar vertebrae studies, no significant difference were obtained between ovariectomized rats and sham-operated rats, probably because quite a small availability of sample amount, unfortunately generated large data variation in the present studies.

Long-term experiments were not done. Other studies have confirmed the increase in both BMD and bone strength with no side-effect related to bone quality after 2 years of administration in normal rats (Motoie et al., 1997) and 18 months of administration in a beagle dog osteoporosis model (Motoie et al., 1995).

Incadronate potently inhibited the increase in urinary deoxypyridinoline levels, a bone resorption marker, following oral administration for both 4 and 12 weeks. However, incadronate had no effect on elevated serum osteocalcin levels, a bone formation marker, after 4 weeks of administration, but significantly inhibited this increase after 12 weeks of administration at a dose of 3 mg/kg. These results suggest bone formation may be uncoupled from bone resorption during the first 4 weeks of oral administration, and during longer periods of incadronate administration bone formation was reduced to original normal levels. The inhibition of bone formation by incadronate might be attributed mainly to inhibition of bone resorption after restoration of the balance between bone resorption and bone formation. In clinical practice, there is a general agreement that bisphosphonate derivatives first reduce increased deoxypyridinoline levels, then inhibit elevations in serum osteocalcin levels with some time lag (Christenson, 1997). These observations are consistent with our findings. Alkaline phosphatase, which is also a bone formation marker, was measured in this study but no significant difference between sham-operated rats and ovariectomized rats was detected (data not shown).

No change was observed in the serum levels of intact parathyroid hormone, either in non-ovariectomized sham-operated rats, ovariectomized rats or ovariectomized–incadronate administered rats both after 4 and 12 weeks of incadronate administration (Table 2). No significant difference was observed in the serum levels of $1,25(OH)_2D_3$ among non-ovariectomized sham-operated rats, ovariectomized rats or ovariectomized–incadronate administered rats, however, the levels in each group after 12 weeks of incadronate administration decreased to approximately half or less of the values determined after 4 weeks of administration (Table 2). Incadronate concentrations in bone increased dose-dependently at doses of between 0.3 and 3 mg/kg (data not shown).

These results suggest that parathyroid hormone and $1,25(OH)_2D_3$ may not be affected by ovariectomy. We speculate that the effects of incadronate seen in this study may depend on bone incadronate concentrations. This possibility is supported by a report that the uptake of incadronate into bone is site-dependent (Usui et al., 1995).

In conclusion, in the long run incadronate inhibits bone turnover (osteoclast and osteoblast activity), showing marked efficacy in treating osteoporosis-like disorders caused by ovariectomy in rats. Considering these results together with those obtained from several other models previously reported (Motoie et al., 1996; Takahashi et al., 1998; Tanaka et al., 1995), incadronate promises to be a highly effective treatment for hypercalcemia, bone metastasis and osteoporosis, commonly due to stimulated bone resorption.

Acknowledgements

The authors thank Drs. Toshiyuki Tanaka, Hisashi Ida, Saburo Higuchi and Shinji Usuda for helpful comments and discussions. We are grateful to Messrs. Mikio Suzuki and Yoshihito Gomi for technical assistance.

References

Abe, T., Flanagan, A.M., Chambers, T.J., 1990. Comparative studies of actions of bisphosphonates on bone resorption in vitro. J. Bone Miner. Res. 5 (Suppl. 2), S86.

Black, D., Duncan, A., Robins, S.P., 1988. Quantitative analysis of the pyridinium crosslinks of collagen in urine using ion-paired reversed-phase high performance liquid chromatography. Anal. Biochem. 169, 197–203.

Christenson, R.H., 1997. Biochemical markers of bone metabolism: an overview. Clin. Biochem. 30, 573–593.

Delmas, P.D., 1993. Biochemical markers of bone turnover. J. Bone Miner. Res. 8, s549–s555.

Dempster, D.W., Lindsay, R., 1993. Pathogenesis of osteoporosis. Lancet 341, 797–805.

Fujimoto, R., Nii, A., Okazaki, A., Miki, H., Kawashima, H., 1990. Effect of disodium dihydrogen (cycloheptylamino) methylene bisphosphonate monohydrate (YM175) on the bone formation and resorption in rats and dogs: histological examination. J. Bone Miner. Res. 5 (Suppl. 2), S157.

Hughes, D.E., Wright, K.R., Uy, H.L., Sasaki, A., Yoneda, T., Roodman, G.D., Mundy, G.R., Boyce, B.F., 1995. Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. J. Bone Miner. Res. 10, 1478–1487.

Kawamuki, K., Abe, T., Kudo, M., O'uchi, N., Motoie, H., Isomura, Y., Takeuchi, M., Kawashima, H., Murase, K., 1990. Pharmacological actions of the novel bisphosphonate YM175. Recent Prog. Osteoporos. Res. 5, 132–137.

Kudo, M., Abe, T., Kawamuki, K., Yamaoka, E., Isomura, Y., Takeuchi, H., Kawashima, H., 1990. Effect of YM175 on experimental hypercalcemia and tumor-induced osteolysis in rats. J. Bone Miner. Res. 5 (Suppl. 2), S166.

Motoie, H., Nakamura, T., O'uchi, N., Nishikawa, H., Kanoh, H., Abe, T., Kawashima, H., 1995. Effects of the bisphosphonate YM175 on bone mineral density, strength, structure, and turnover in ovariectomized beagles on concomitant dietary calcium restriction. J. Bone Miner. Res. 10, 910–920.

Motoie, H., Kanoh, H., Ogata, S., Kawamuki, K., Shikama, H., Fujikura, T., 1996. Prevention of bone loss by bisphosphonate YM175 in ovariectomized dogs with dietary calcium restriction. Jpn. J. Pharmacol. 71, 239–246.

Motoie, H., Okazaki, A., Kanoh, H., Shikama, H., Fujikura, T., 1997. Increase of bone mass and mechanical strength in rats after treatment

with a novel bisphosphonate YM175, for 2 years. Pharmacol. Toxicol. 81, 42–47.

Price, P.A., Parthemore, J.G., Deftos, L.J., Nishimoto, S.K., 1980. New biochemical marker for bone metabolism. J. Clin. Invest. 66, 878–883.

Price, P.A., Williamson, M.K., Lothringer, J.W., 1981. Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. J. Biol. Chem. 256, 12760–12766.

Riggs, B.L. , Melton III, I.J. 1986. Involutional osteoporosis. N. Engl. J. Med. 314, 1676–1686.

Robey, P.G., 1989. The biochemistry of bone. Metabolic bone disease, part I. Endocrinol. Metab. Clin. N. Am. 18, 859–902.

Selander, K.S., Monkkonen, J., Karhukorpi, E.K., Harkonen, P., Hannuniemi, R., Vaananen, H.K., 1996. Characteristics of clodronate-induced apoptosis in osteoclasts and macrophages. Mol. Pharmacol. 50, 1127–1138.

Takahashi, K., Shirahata, A., Fukushima, S., Kokubo, S., Teramura, K., Usuda, S., 1998. Effects of YM175, a new-generation bisphosphonate, on hypercalcemia induced by tumor-derived bone resorbing factors in rats. Jpn. J. Pharmacol. 76, 155–163.

Takeuchi, M., Sakamoto, S., Yoshida, M., Abe, T., Isomura, Y., 1993. Studies on novel bone resorption inhibitors: Part I. Synthesis and pharmacological activities of aminomethylene bisphosphonate derivatives. Chem. Pharm. Bull. 41, 688–693 (Tokyo).

Tanaka, S., Hiraga, T., Ozawa, Y., Teramura, K., Motoie, H., Tanaka, T., Yamamoto, M., 1995. YM175 inhibits tumor-induced osteolysis in nude mice with bone metastases. J. Bone Miner. Res. 10 (Suppl. 1), 262.

Uebelhart, D., Gineyts, E., Chapuy, M.C., Delmas, P.D., 1990. Urinary excretion of pyridinium crosslinks. A new marker of bone resorption in metabolic bone disease. Bone Miner. 8, 87–96.

Usui, T., Watanabe, T., Higuchi, S., 1995. Pharmacokinetics of YM175, a new bisphosphonate, in rats and dogs. Drug Metab. Dispos. 23, 1214–1219.

Yeh, J.K., Aloia, J.F., Chen, M., 1994. Growth hormone administration potentiates the effect of treadmill exercise on long bone formation but not on the vertebrae in middle-aged rats. Calcif. Tissue Int. 54, 38–43.