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Tissue Distribution and Pharmacological Potential of SM-16896, a Novel Oestrogen-bisphosphonate Hybrid Compound*

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Abstract

Postmenopausal osteoporosis is caused mainly by a deficiency of oestrogen with rapid bone loss. To target oestrogen to the bone effectively, we have synthesized and evaluated the effects of a novel hybrid compound of oestrogen and bisphosphonate, SM-16896. The tissue distribution pattern and pharmacological potential are reported.

Although the affinity for calf uterine oestrogen receptor was very low (IC50: 73·3 μM; 1/25 000 of that of 17β-oestradiol (2·84 nM)), SM-16896 showed oestrogenic activity. SM-16896 (1 μM) induced a 4·5-fold transcriptional activity in rat osteosarcoma UMR-106 cells compared with vehicle-treated control, when we used the expression vector for human oestrogen receptor and a CAT reporter plasmid containing an oestrogen-responsive element. The distribution of SM-16896 after a subcutaneous administration to 7-week-old female rats was examined by radioluminography using ^{3}H-labelled SM-16896. At 30 min after the administration, significant radioactivity was detected in the bone. At 24 h after administration, a high level of radioactivity was detected in the bone, but in the uterus it was only at a background level. Daily subcutaneous administration of 0·5 mg kg^{-1} SM-16896 for 12 weeks (five times per week) to 13-week-old ovariectomized rats suppressed the ovariectomized-induced reduction in bone mineral density. A bone mineral density ratio of 120% was maintained compared with sham-operated rats, whereas a relatively low suppression of uterine weight was observed (about 50% loss compared with sham-operated rats). In the same experiment, the implantation of a 17β-oestradiol time-release pellet (0·25 mg/pellet/90 days) almost completely suppressed the reduction of both the bone mineral density and uterine tissue weight. It is likely that the effect of SM-16896 on bone was due to its oestrogenic activity, since 1·0 mg kg^{-1} SM-18108, the bisphosphonate moiety of this compound, had no effect on bone in 7-week-old ovariectomized rats.

The results suggest that SM-16896, a bisphosphonate-conjugated oestrogen, showed a preference profile in the uterus and bone due to its characteristic distribution pattern compared with the natural oestrogen analogue 17β-oestradiol. Thus, bisphosphonate-conjugated oestrogens have the potential to improve patient compliance in oestrogen therapy by minimizing adverse effects and reducing the frequency of medication.

Postmenopausal osteoporosis is caused mainly by a deficiency of oestrogen with rapid bone loss (Riggs 1991). Thus, for the treatment of postmenopausal osteoporosis, the administration of oestrogen (so-called hormone replacement therapy), is now widely used (Lindsay 1993; Lobo 1995; Maxim et al 1995). Though the benefits of oestrogen treatment are very clear, this hormone causes some undesired effects i.e. it affects the functions of reproductive organs and may sometimes cause an increase in the risk of cancer (Bergkvist et al 1989; Colditz et al 1995; Cummings 1991). For that

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reason, drugs without undesired effects are needed for the treatment of postmenopausal osteoporosis, and oestrogens that act selectively on bone are under development (Evans & Turner 1995).

To create such bone-selective oestrogens, two approaches can be taken. One approach is to make oestrogens that act specifically on bone irrespective of their distribution, with a very weak effect on reproductive tissues (Black et al 1994; Ke et al 1995). Examples are the so-called selective oestrogen-receptor modulators such as tamoxifen, raloxifene and drolroxifene (Evans & Turner 1995). These compounds function as oestrogen antagonists in reproductive organs, whereas they act as oestrogen agonists on bone and lipid metabolism. The second approach is to make oestrogens that distribute specifically in bone (Bauss et al 1996; Orme & Labroo 1995; Fujisaki et al 1998). Examples of these are hybrids made with tetracyclin, which is known to have high affinity for bone, but few details regarding these hybrid compounds have been reported (Orme & Labroo 1995).

To obtain a highly bone-selective oestrogen that distributes in the bone effectively, we have synthesized oestrogen-bisphosphonate hybrid compounds. Bisphosphonate was used because it is known to have high affinity for hydroxyapatite in the bone and was suggested to function as a good carrier of oestrogen to the bone (Bauss et al 1996; Fujisaki et al 1998). We found that one of these hybrid compounds, SM-16896, had strong affinity for the bone and exhibited a selective profile in the bone.

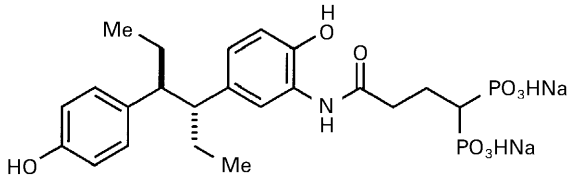
Materials and Methods

Chemicals

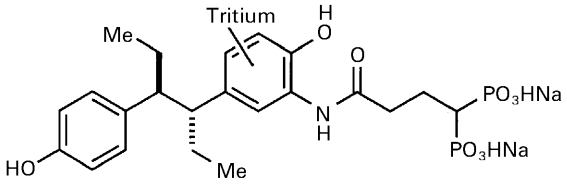
SM-16896 (erythro 3-(3-(4,4-diphosphonobutyryl-amino)-4-hydroxyphenyl)-4-(4-hydroxyphenyl) hexane disodium salt), SM-16897 (oestrogenic amino moiety), SM-18108 (bisphosphonate moiety) and etidronate were synthesized at the Sumitomo Pharamaceuticals Research Center (Figure 1). [Aminophenyl-G-^{3}H]SM-16896 was synthesized at Sumitomo Chemical Co., Ltd and was supplied as a solution in 80% ethanol. The radiochemical purity was greater than 97% on radio high-performance liquid chromatography (HPLC), and the specific activity was 1·39 TBq mmol^{-1}. 17β-Oestradiol and hexestrol were purchased from Sigma (St Louis, MO). 17β-Oestradiol pellets (0·25 mg/pellet/90 days and 0·05 mg/pellet/21 days) were purchased from Innovative Research of America (Sarasota, FL). ICI-164384 was a kind gift from Dr A. E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK).

Animals

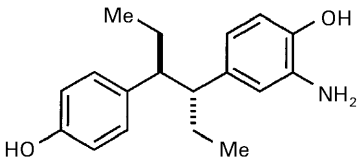
For the ovariectomized rat studies, female Wistar rats (Oriental Bioservice, Kyoto, Japan) were used. Rats were given a commercial diet (CE-2, Clea



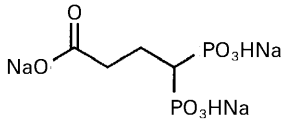
SM-16896



Tritium-labelled SM-16896
([amino phenyl-G-^{3}H]SM-16896)



SM-16897



SM-18108

Figure 1. Chemical structures of SM-16896, its oestrogenic amino moiety SM-16897 and the bisphosphonate moiety of SM-18108.

Japan, Tokyo, Japan) and tap water was freely available. The rats were housed in a temperature ($23 \pm 2^{\circ}C$) and humidity ($55 \pm 10\%$)-controlled environment with a 12-h light/dark cycle. Animals were allowed to acclimate for one week before the experiment.

For the whole body radioluminography, 7-week-old female Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) were used. The rats were given a commercial diet (CRF-1, Charles River Japan) and tap water was freely available. The housing conditions were the same as those of the Wistar rats.

Calf uterine oestrogen receptor binding assay

The procedures used were almost the same as those described by Borgna & Scali (1991). Samples of calf uterine cytosol were incubated at 4°C with various concentrations of ligand and 10 000 d min^{-1} [2,4,6,7-^{3}H]oestradiol (3·55 TBq $mmol^{-1}$, NEN, Boston, MA). After a 24-h incubation period, the cytosol was incubated with an equal volume of charcoal suspension (1% charcoal and 0·1% dextran T70 in 50 mM sodium phosphate buffer, pH 7·0) for 30 min at 4°C. The charcoal was pelleted and the radioactivity of the supernatant was measured.

Cell culture and chloramphenicol acetyl transferase (CAT) assay

The rat osteosarcoma cell line UMR-106 (obtained from Dainippon Pharmaceuticals, Osaka, Japan) was maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red supplemented with 5% foetal calf serum stripped with dextran-coated charcoal. The cells were transfected at 40–60% confluence in 9-cm Petri dishes with a total of 20 μg DNA by means of calcium phosphate co-precipitation. A Xenopus vitellogenin–oestrogen responsive element (ERE)-globin CAT reporter plasmid (1 μg/dish) was co-transfected with 0·5 μg of the expression vector for oestrogen receptor α (ERα) and 3 μg of the reference plasmid pCH110 (Amersham Pharmacia Biotech, Uppsala, Sweden). Bluescribe M13 + (Stratagene, La Jolla, CA) was used as the carrier to adjust the amount to 20 μg (Harada et al 1998). Tested compounds were added to the medium 1 h after transfection. After 24 h, the medium was replaced and the cells were incubated for an additional 24 h. The CAT assay was performed as described (Kato et al 1992; Harada et al 1998), after normalizing the transfection efficiency with β-galactosidase activity expressed by the reference plasmid pCH110 in UMR-106 cells (2·5 units).

Whole body radioluminography

Appropriate portions of the [^{3}H]SM-16896 solution and an aqueous solution of unlabelled SM-16896 (5 mg mL^{-1} water) were added to a glass tube. The mixture was concentrated to dryness in a vacuum. The residue was dissolved in isotonic saline to give a solution of 0·5 mg mL^{-1}. Seven-week-old female Sprague–Dawley rats received a single subcutaneous administration at a dose of 0·5 mg mL^{-1} kg^{-1}.

At 30 min or 24 h after this administration, rats were killed by ether anaesthesia, and the carcasses were placed in a dry ice/acetone bath. Each frozen carcass was embedded in a 5% carboxymethylcellulose solution at −25°C. Sections (30 μm) were collected on adhesive tape (Salotape RI-70, Hisamitsu Pharmaceutical Co., Tokyo, Japan) in a PMV 450MP cryomicrotome (LKB Co., Uppsala, Sweden) maintained at −25°C. After drying at −25°C, the sections were contacted with imaging plates (BAS-TR2040, Fuji Film, Tokyo, Japan) for seven days, and the radioluminograms were prepared by a Bio-imaging Analyzer (BAS-2000, Fuji Film). The relative levels of radioactivity were identified by colour imaging, red indicating high radioactivity and blue low.

In-vitro metabolism

A mixture containing the rat liver S9 fraction (0·1 mL, Oriental Yeast, Tokyo, Japan), β-NADPH (1·1 mg, Oriental Yeast) and 0·5 M phosphate buffer (pH 7·4; 0·14 mL) was pre-incubated at 37°C for 2 min. To start the reaction an aqueous solution of [^{3}H]SM-16896 (1 mg mL^{-1}, 10 μL) was added to the mixture. After incubation for either 30 min, 1 or 2 h, the reaction was stopped by adding ethanol (0·25 mL), and the mixture was then centrifuged (1800 *g*, 4°C, 10 min) to obtain the supernatant. For the incubation of the zero-time sample, ethanol was added to the pre-incubated mixture before adding the substrate. The recovery of the radioactive component to the supernatant was calculated by measuring the radioactivity in the supernatant (20 μL) with a liquid scintillation counter (2500TR, Packard, Downers Grove, IL). A 20-μL sample of the supernatant was analysed by an HPLC system consisting of a column (Cosmosil 5C18-AR, 6 × 150 mm, 5 μm, Nacalai Tesque, Kyoto, Japan) and a radiodetector (Radio-Chromatography

Detector FLO-ONE beta, Packard) with a mobile phase of 5 mM PIC-A (Waters, Milford, MA)/acetonitrile 60/40 (v/v) and a flow rate of 1·2 mL min^{-1}. Under this condition, SM-16896 and SM-16897 (Figure 1) were eluted in 7–8 and 12–13 min, respectively.

Ovariectomy study

Thirteen-week-old female Wistar rats were bilaterally ovariectomized via the ventral approach under ethyl ether anaesthesia. The next day, the rats were sorted into experimental groups of nine animals per group, and the compound administration was initiated. SM-16896 dissolved in phosphate-buffered saline (PBS) (0·25, 0·5, 1·0 mg kg^{-1}, volume: 1 mL kg^{-1} body weight) or vehicle (PBS, volume: 1 mL kg^{-1} body weight) was given subcutaneously five days a week for 12 weeks. For the oestradiol administration, one time-release pellet of 17β-oestradiol (0·25 mg/pellet/90 days) was implanted subcutaneously in each rat. After the 12-week treat-

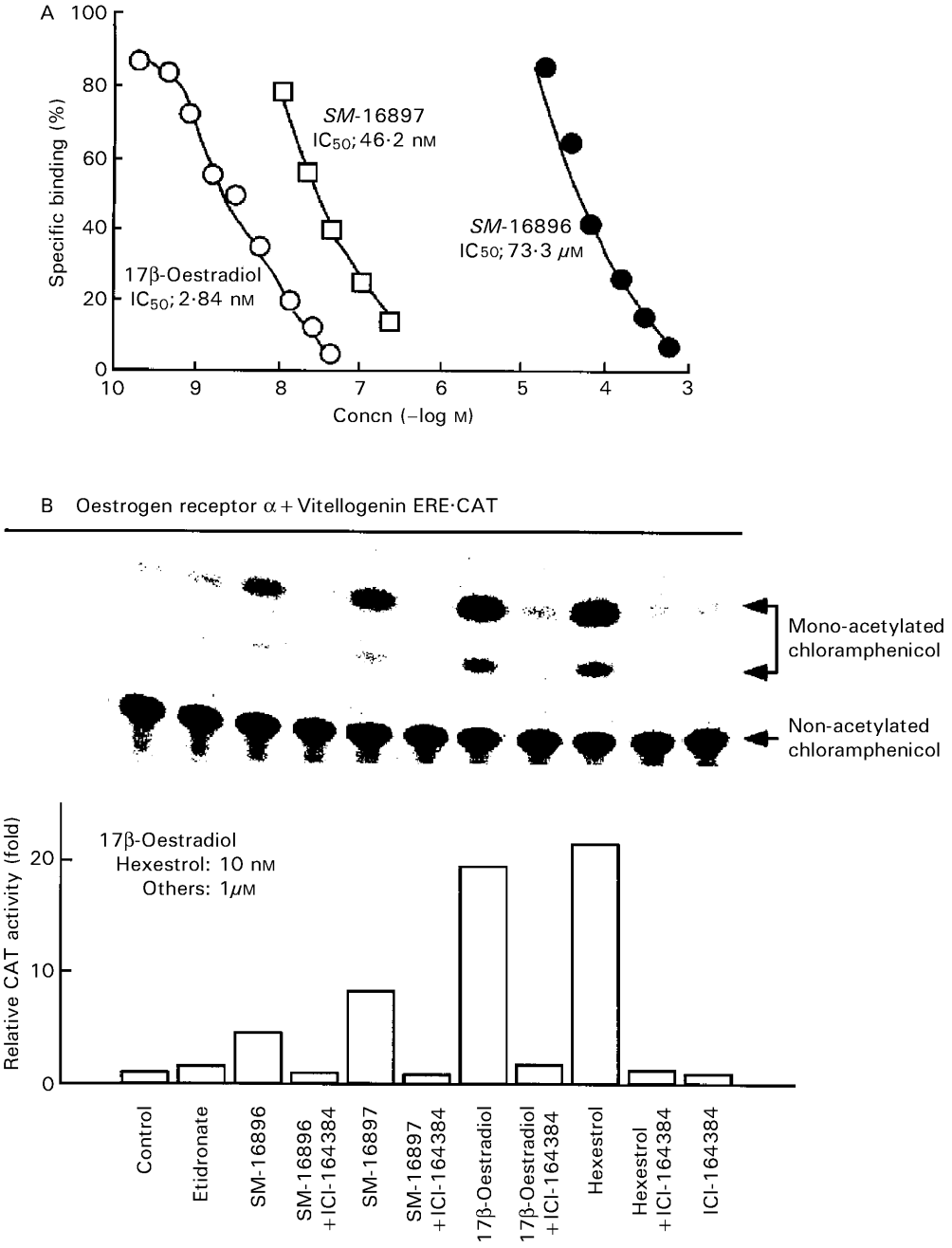


Figure 2. A. Relative binding affinity of SM-16896 for calf uterine oestrogen receptor. B. Transcriptional activity of SM-16896 upon Xenopus vitellogenin ERE in UMR-106 cells.

ment, the body weight and uterine tissue weight of each rat were determined at autopsy. Both tibiae from each rat were fixed with 70% ethanol, and the bone mineral density of the proximal tibiae was determined by Dual Energy X-ray Absorptiometry (DEXA) (DCS-600, Aloka, Tokyo, Japan). The bone mineral density was calculated from the bone mineral content and the area from the proximal end to the 7-mm distal part. Relative comparisons were made by calculating the percent protection using the following formula: preservation percentage = [(each group mean value− ovariectomized group mean value)/(sham-operated group mean value − ovariectomized group mean value)] × 100.

For the comparison of the pharmacological potential of SM-16896, SM-16897 and SM-18108, 7-week-old ovariectomized rats were used. The compound administration was carried out daily (five times/week) for three weeks. The doses were 0·3 mg kg^{-1} and 1·0 mg kg^{-1} for SM-16896 and SM-16897 and 1·0 mg kg^{-1} for SM-18108. Both the bone mineral density and the uterine tissue weight were examined as stated above.

Statistics

The results are expressed as the mean ± s.d. Differences between sham-operated (or intact) and ovariectomized rats were tested by Student's *t*-test. Analyses between groups (excluding the sham-operated group) were based on one-way analysis of variance. When significant differences were indicated by the analysis of variance, the results were compared using the Dunnett test or the Tukey multiple test. Probability values (P) less than 0·05 were considered significant.

Results

SM-16896 is a weak oestrogen agonist

SM-16896 has a hexestrol-like structure as an oestrogen and an etidronate-like structure as a bisphosphonate (Figure 1).

It is thought that most of the effects of oestrogen are exerted through its cognate receptor (Evans 1988; Kato et al 1992). Therefore, we examined whether SM-16896 binds oestrogen receptor. The affinity of SM-16896 for calf uterine oestrogen receptor was found to be very low (IC50 73·3 μM) compared with 17β-oestradiol (2·84 nM) and SM-16897 (46·2 nM), the oestrogen part of SM-16896. With a competitive radiometric binding assay using ^{3}H-labelled 17β-oestradiol, it was demonstrated that SM-16896 binds oestrogen receptor (Figure 2A), whereas SM-18108 (the bisphosphonate moiety of SM-16896) and etidronate, one of the bisphosphonates, do not (data not shown). The structure of the oestrogen receptor is highly conserved among species (Green et al 1986; Krust et al 1986; Koike et al 1987; White et al 1987), and because many chemical compounds have been evaluated for their binding affinity for the oestrogen receptor using calf uterus cytosol (Ouellet et al 1984; Kohle et al 1989), we used it in our experiments.

Oestrogenic compounds mainly exert their effects through the transcriptional regulation of the target genes (Evans 1988; Kato et al 1992), therefore we investigated the transcriptional activity of SM-16896 through a Xenopus vitellogenin ERE-CAT reporter plasmid when human oestrogen receptor α cDNA was overexpressed in the rat osteosarcoma cell line UMR-106 (Figure 2B). SM-16896 and SM-16897 enhanced the transcriptional activity by about 4·5- and 8·2-fold, respectively, compared with vehicle-treated control at 1 μM, and the activity was completely suppressed in the presence of ICI-164384, which is a pure anti-oestrogen (Wakeling 1995; Macgregor & Jordan 1998) (Figure 2B). 17β-Oestradiol and hexestrol, which are highly potent oestrogens, induced transcriptional activity by over 20-fold at the dose of 10 nM, and this activity was also completely suppressed in the presence of ICI-164384 (Figure 2B).

Distribution of SM-16896 in-vivo

To determine whether the selective distribution of SM-16896 occurs in-vivo, we synthesized the radiolabelled compound and investigated its tissue distribution pattern by radioluminography (Figures 3, 4 and 5).

At 30 min after administration, the highest levels of radioactivity were found in the subcutaneous tissue at the injected site and in the intestinal content (Figure 3). The levels of radioactivity in the liver, kidney and bone tissues (vertebra, femur and tibia) were higher than that in the blood. The bone tissue displayed an uneven distribution, i.e. the radioactivity was localized in the edges of the vertebral body and the metaphysis of the tibia. The radioactivity in the uterus was at a level similar to that in the blood.

At 24 h, while the radioactivity levels in most tissues including the subcutaneous tissue (injected site), liver and kidney were decreased, those in the intestinal content and bone tissues (vertebra, femur and tibia) were increased compared with those at 30 min (Figure 4). Regarding the distribution in the

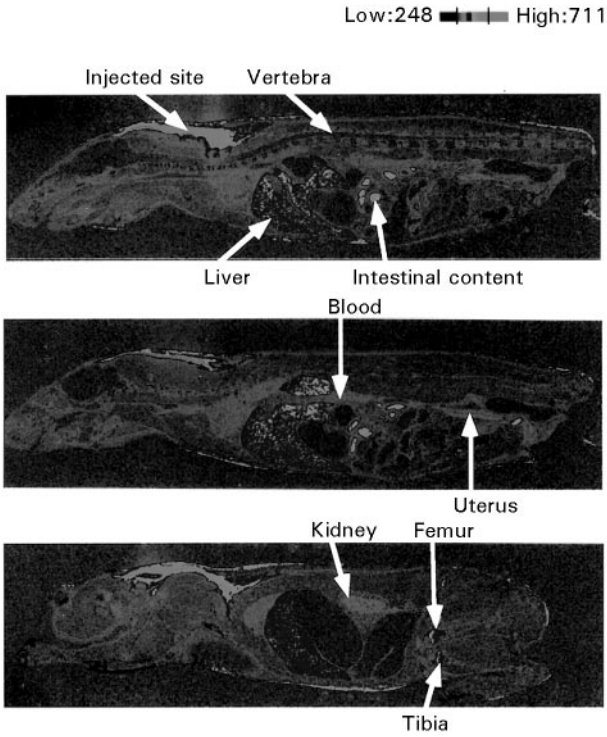


Figure 3. Radioluminograms of whole rats 30 min after a single subcutaneous administration of 0·5 mg kg^{-1} [aminophenyl-G-^{3}H]SM-16896 to a female rat. Numbers (248 and 711) at the gradation bar indicate the dynamic range (arbitrary unit).

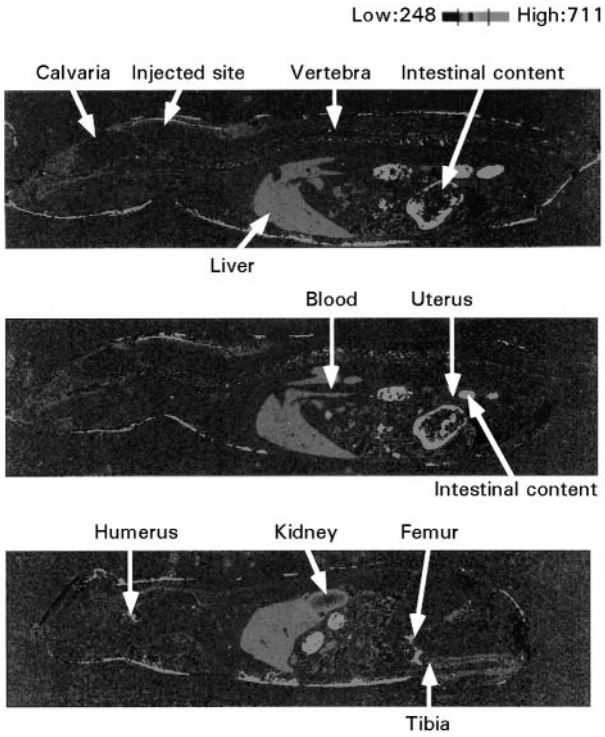


Figure 4. Radioluminograms of whole rats 24 h after a single subcutaneous administration of 0·5 mg kg^{-1} [aminophenyl-G-^{3}H]SM-16896 to a female rat. Numbers (248 and 711) at the gradation bar indicate the dynamic range (arbitrary unit).

tibia, the highest level was observed in the growth plate, and higher levels were seen in the epiphysis and metaphysis than the diaphysis (Figure 5), the pattern closely resembling that for bisphosphonates (Monkkonen et al 1987; Mochizuki et al 1995a; Masarachia et al 1996). The radioactivity in the uterus decreased to the background level (Figure 4).

To elucidate the metabolism of SM-16896 in-vivo, we examined the in-vitro stability of the compound using rat liver S9 fraction (Figure 6). The recovery of the radioactivity in the supernatant

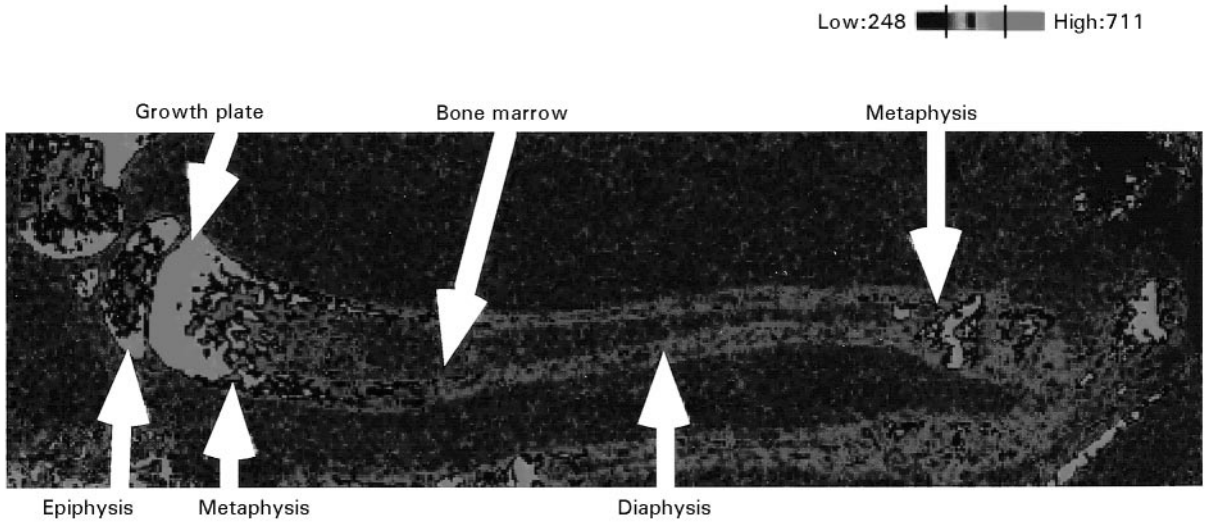


Figure 5. Radioluminogram of the tibia 24 h after a single subcutaneous administration of 0·5 mg kg^{-1} [aminophenyl-G-^{3}H]SM-16896 to a female rat. Numbers (248 and 711) at the gradation bar indicate the dynamic range (arbitrary unit).

was over 87%, and the recovery of the injected radioactivity from the HPLC column was almost 100% at all time points (data not shown). The HPLC analysis demonstrated that most of the radioactive compound remained unchanged for up to 2 h, suggesting that SM-16896 is stable in-vivo.

Effects of SM-16896 on ovariectomy-induced bone loss

The data given above strongly suggested that SM-16896 would have a highly bone-selective effect. Thus, we examined the effects of this compound on bone using 13-week-old ovariectomized rats given a 12-week subcutaneous administration. As an index of the main effect, the bone mineral density of proximal tibia was analysed, and the uterine tissue weight was analysed as an index of the side effects.

A body weight gain was observed in the ovariectomized rats, and this gain was prevented by the administration of 17β-oestradiol or SM-16896. The ovariectomy decreased the bone mineral density (to the level of 86·1%), and decreased the uterine tissue weight to 15·4% (Table 1). Taking the decreased amount of each parameter as 100% preservation, the administration of 17β-oestradiol suppressed the decrease of the bone mineral density to 104·2%, and the uterine tissue weight to 87·1% (Table 1). This effective preservation of both the bone mineral density and uterine tissue weight matches the findings of Ke et al (1995, 1997). The administration of SM-16896 produced a recovery of the bone mineral density and uterine tissue weight dose-dependently: 87·0% and 15·6% (0·25 mg kg^{-1}), 119·4% and 49·5% (0·5 mg kg^{-1}), 183·3% and 78·7% (1·0 mg kg^{-1}), respectively (Table 1). The administration of 0·5 or 1·0 mg kg^{-1} SM-16896 preserved the bone mineral density to levels above the sham-operated level.

For the comparison of the pharmacological potential among SM-16896, SM-16897 and SM-18108, the latter two compounds are the oestrogenic amino moiety and the bisphosphonate moiety of SM-16896, respectively. We evaluated these compounds by using 7-week-old ovariectomized rats (Tables 2 and 3). SM-16896 administered daily (five times/week) for three weeks prevented a decrease of the bone mineral density and uterine tissue weight in a dose-dependent manner with similar profile as stated above: 53·1% and 3·5% (0·3 mg kg^{-1}), 182·3% and 37·3% (1·0 mg kg^{-1}), respectively. SM-16897 showed effective preservation like that of 17β-oestradiol as stated above: 173·5% and 77·4% (0·3 mg kg^{-1}), 157·5% and 101·0% (1·0 mg kg^{-1}), respectively (Table 2). SM-18108 1·0 mg kg^{-1} had almost no effect on the preservation of the bone mineral density or the uterine tissue weight (Table 3).

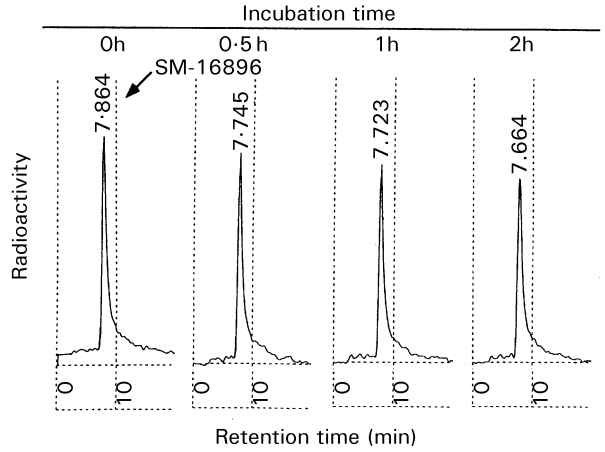


Figure 6. HPLC profiles of [aminophenyl-^{3}H]SM-16896 incubated in rat S9 liver enzyme mixture in the presence of NAPDH. Numbers nearby the peak top represent the retention time of the peaks. SM-16896 and SM-16897 (oestrogenic amino moiety, Figure 1) were eluted in 7–8 and 12–13 min, respectively.

Table 1. Effect of the 12-week treatment with SM-16896 (subcutaneously) or 17β-oestradiol on ovariectomy-induced bone loss.

Group	Dose (mg kg^{-1})	Body weight (g)	Bone mineral density of proximal tibia (mg cm^{-2})	Preservation (%)	Uterine tissue weight (mg)	Preservation (%)
Sham operated		347·0 ± 14·1	156·4 ± 9·6	100	726 ± 166	100
Ovariectomized		371·4 ± 17·9	134·8 ± 5·5[b]	0	112 ± 11[b]	0
17β-Oestradiol	0·25[a]	332·8 ± 22·1[c]	157·3 ± 18·2[c]	104·2	647 ± 180[c]	87·1
SM-16896	0·25	363·8 ± 17·5	153·6 ± 7·0[c]	87·0	208 ± 20	15·6
	0·5	350·1 ± 22·5	160·6 ± 8·6[c]	119·4	416 ± 129[c]	49·5
	1·0	341·0 ± 14·2[c]	174·4 ± 10·6[c]	183·3	595 ± 38[c]	78·7

Values are mean ± s.d. (n = 8 or 9). [a]mg/pellet/90 days. [b]$P < 0·01$ vs Sham-operated rats. [c]$P < 0·01$ vs ovariectomized rats. Preservation percentage = ((each group mean value − ovariectomized group mean value)/(sham-operated group mean value − ovariectomized group mean value)) × 100.

Table 2. Effects of 3-week subcutaneous treatment of SM-16896 and SM-16897 on ovariectomy-induced bone loss.

Group	Dose ($mg\,kg^{-1}$)	Bone mineral density of proximal tibia ($mg\,cm^{-2}$)	Preservation (%)	Uterine tissue weight (mg)	Preservation (%)
Sham operated		118·9 ± 4·0	100	604 ± 11	100
Ovariectomized		107·6 ± 3·6	0	121 ± 11	0
17β-Oestradiol	0·05[a]	133·7 ± 8·7	231	541 ± 33	87
SM-16896	0·3	113·6 ± 5·8	53·1	138 ± 13	3·5
	1·0	128·2 ± 5·9	182·3	301 ± 30	37·3
SM-16897	0·3	127·2 ± 8·1	173·5	495 ± 66	77·4
	1·0	125·4 ± 6·3	157·5	609 ± 63	101

Values are mean ± s.d. (n = 3–5). [a]mg/pellet/3 weeks. Preservation percentage is calculated as shown in Table 1.

Table 3. Effects of 3-week subcutaneous treatment of SM-18108 on ovariectomy-induced bone loss.

Group	Dose ($mg\,kg^{-1}$)	Bone mineral density of proximal tibia ($mg\,cm^{-2}$)	Preservation (%)	Uterine tissue weight (mg)	Preservation (%)
Sham operated		122·6 ± 5·7	100	527 ± 130	100
Ovariectomized		104·1 ± 3·3	0	123 ± 5	0
17β-Oestradiol	0·05[a]	136·2 ± 4·9	173·5	582 ± 68	113·6
SM-18108	1·0	105·3 ± 3·3	6·5	122 ± 13	< 0

Values are mean ± s.d. (n = 3–5). [a]mg/pellet/3 weeks. Preservation percentage is calculated as shown in Table 1.

Discussion

Oestrogen analogues are promising drugs for postmenopausal osteoporosis (Lindsay 1993; Lobo 1995; Maxim et al 1995), but because of their possible side effects such as increased risk of cancer (Bergkvist et al 1989; Cummings 1991; Colditz et al 1995), oestrogens which exert their oestrogenic effects selectively on bone are desired. To target the drugs to the bone effectively, tetracyclin (Orme & Labroo 1995) and bisphosphonate (Bauss et al 1996; Fujisaki et al 1998), which have high affinity for the bone mineral, are used as carriers. Bauss et al (1996) examined the effects of pro-drug type bisphosphonate-conjugated oestrogen but their pro-drug failed to reduce the systemic effects of oestrogen. Fujisaki et al (1998) also reported the usefulness of bisphosphonate-conjugated compounds, but the distribution pattern in-vivo and pharmacological potential including uterus tissue soon after administration have not yet been clarified. Those two studies suggested that pro-drug type bisphosphonate-conjugated oestrogens do not have a good profile and/or have to be investigated in detail to exert a bone selective effect. Thus, we have evaluated the activity of bisphosphonate-conjugated oestrogen, which displays the following characteristics. The bisphosphonate-conjugated compound itself shows oestrogenic activity without being cleaved. The conjugation is resistant to cleavage. The compound distributes in the bone selectively. The effect of the compound is bone selective even in daily administration.

After making conjugated compounds, we examined the binding activity of the compounds for oestrogen receptor, because bisphosphonate-oestrogen-conjugated compounds may not bind oestrogen receptor easily. Our conjugated compound, SM-16896, significantly bound calf oestrogen receptor, though its affinity was very low (approximately 1/25 000 of that of 17β-oestradiol, Figure 2A). In addition, although SM-16896 had very low affinity for oestrogen receptor, this compound clearly showed oestrogen agonistic activity similar to that of 17β-oestradiol in a transient transfection assay, since the effect of SM-16896 was completely suppressed in the presence of ICI-164384 (Figure 2B). There seemed to be some contradiction for the effective dose identified in the two in-vitro assays (receptor binding and transcriptional activity) for 17β-oestradiol and SM-16896. The IC50 for 17β-oestradiol in the binding assay was 2·84 nM and approximately 20-fold the transcriptional activity at 10 nM in the CAT assay. The IC50 for SM-16896 in the binding assay was 73·3 μM and approximately 4·5-fold the transcriptional activity at 1 μM in the CAT assay. This may be derived from the sensitivity of the assay system, because 17β-oestradiol, which showed nanomolar-

order IC50 values in the receptor binding assay, enhanced transcriptional activity from a dose of 10^{-10} M in the CAT assay system (data not shown).

The experiment using the radiolabelled compound to show bone-selective distribution showed that SM-16896 was bone-targeted rather than uterus-targeted (Figures 3 and 4). Strong radioactivity accumulated in both the liver and intestinal content after subcutaneous treatment, suggesting that the hepato-biliary system contributed to the systemic clearance of this compound. An accumulation of radioactivity in the bone was observed only at 30 min after the administration of radiolabelled compound (Figure 3), and the radioactivity in the bone increased with time, whereas it rapidly decreased in the uterus (Figure 4). This result was consistent with the recent finding that an intravenous injection of bisphosphonate–oestradiol-conjugated compound resulted in the compound remaining in the bone for a longer period compared with the similar use of unconjugated oestradiol (Fujisaki et al 1997). Moreover, the injected compound might not be cleaved. This was suggested by the results that ^{3}H was substituted in the oestrogen phenyl part of SM-16896 (Figure 1); thus, if it was cleaved, the cleaved compound might accumulate in the organs which have greater amounts of oestrogen receptor. No metabolites were detected by the HPLC analysis after the incubation of SM-16896 with the rat S9 enzyme mixture from liver, where much of this compound accumulated after subcutaneous administration (Figure 6). The distribution of SM-16896 in the uterus was much less than that of non-conjugated oestrogen compounds such as droloxifene, tamoxifen and oxo-hexestrol (Bergmann et al 1994; Tanaka et al 1994; Yang et al 1994). The distribution pattern of SM-16896 in the bone is quite similar to those of bisphosphonates (Monkkonen et al 1987; Mochizuki et al 1995a; Masarachia et al 1996).

In ovariectomized rats the distribution pattern of the administered compound might be different. This is because in uterus and adipose tissue, which are major target tissues of oestrogen, the oestrogen receptor content of the tissue is several fold that in ovary-intact rats (Eisenfeld & Axelrod 1966; Nishikawa et al 1993). But, as for SM-16896, there might not be a big difference between the distribution pattern in normal rats shown in this paper and that in ovariectomized rats, because SM-16896 does not show an oestrogen receptor-dependent distribution pattern (Figures 3 and 4). However, to predict the tissue distribution pattern of the administered compound in patients a detailed examination in ovariectomized animals needs to be carried out. This is because the whole body metabolism might change, as stated below.

Ovariectomy increases body weight and the administration of tamoxifen and raloxifene, as well as 17β-oestradiol, prevents this change (Turner et al 1987b; Evans et al 1996). This weight gain is caused by increased food consumption, and a predominant accumulation of fat, but cannot be completely prevented by pair-feeding to adjust the food intake of ovariectomized rats to the amount ingested by ovary-intact normal rats (Kalu 1984; Turner et al 1987a). Thus, the change of body weight suggests an alteration of whole body metabolism and this change is partly caused by the oestrogenic regulation of the central nervous system (Roy et al 1977). SM-16896 prevents the body weight increase caused by ovariectomy, as well as 17β-oestradiol, possibly under central regulation judging from its oestrogenic activity in-vitro. It is unlikely that the toxicity of SM-16896 caused body weight decrease, because the subcutaneous administration of SM-16896 to 3-week-old normal rats daily for one week at a dose of 1 or 10 mg kg^{-1} caused no prevention in body weight increase (data not shown). However, since repeated administration of these bisphosphonates may increase the compound concentration in bone tissue correlating with the frequency of administration (Mochizuki et al 1995b), the administration schedule will have to be carefully examined in the future to prevent the toxic effects of the compound.

As for the pharmacological potential, SM-16896 showed desirable and strong potential. In the experiment using the 12-week daily administration of SM-16896 to 13-week-old ovariectomized rats, SM-16896 prevented ovariectomized-induced bone loss in a dose-dependent manner (Table 1). The bone mineral density preservation was 119·4% at a dose of 0·5 mg kg^{-1}, and 183·3% at 1·0 mg kg^{-1}. This suggests that SM-16896 has potent effects on bone, and this preservation effect of the bone mineral density to levels above the sham-operated level was similar to the effect of CP-336156, a selective oestrogen-receptor modulator under development (Ke et al 1998). In addition to this strong activity, SM-16896 is highly bone-selective compared with 17β-oestradiol, since the recovery of the uterine tissue weight is predicted to be only 35% when full recovery (100%) of the bone mineral density is attained with SM-16896. This highly bone-selective effect is possibly exerted by the bone selective distribution of this compound because of its bisphosphonate moiety, since SM-16897, the oestrogenic amino moiety of SM-16896, showed almost no selective activity for bone (Table 2). In addition, the effect of SM-16896 on bone is

likely to be due to its oestrogenic activity, since SM-18108, the bisphosphonate moiety of this compound, had no effect on bone in 7-week-old ovariectomized rats at a dose of $1{\cdot}0\,mg\,kg^{-1}$ (Table 3). This is well correlated with the results of a similar bisphosphonate derivative substituted by the carboxyl group (Gil et al 1999).

In conclusion, we found that an oestrogen-bisphosphonate hybrid compound, SM-16896, was a potent bone-targeting oestrogen. This compound showed a strong ability to prevent bone loss and its effect on uterine growth was much weaker than that of 17β-oestradiol. The selective effect of SM-16896 on bone may be derived from its characteristic distribution pattern, though the binding affinity of the compound itself for oestrogen receptor is very low. Thus, using bisphosphonates for conjugating compounds is a promising method of effectively targeting oestrogens to the bone.

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