Pharmacokinetic Advantage of an Intranasal Preparation of a Novel Anti-osteoporosis Drug, L-Asp-Hexapeptide-Conjugated Estradiol

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We examined the usefulness of intranasal (i.n.) administration of a novel osteotropic prodrug of estradiol, estradiol-17β-succinate-(L-aspartate)$_6$ ($E_2 \cdot 17D_6$), for selective drug delivery to bone. $E_2 \cdot 17D_6$ alone or with 5% 2,6-di-*O*-methyl-β-cyclodextrin (DMβCD), 5% β-cyclodextrin (βCD), or 10% hydroxypropyl cellulose (HPC) as an absorption enhancer was administered to ovariectomized (OVX) mice *via* the i.n. route. The oral and nasal bioavailability after *p.o.* or i.n. administration of $E_2 \cdot 17D_6$ (3.7 μmol/kg) in mice amounted to 9.9 and 23.0% of the dose, respectively. The values of nasal bioavailability of $E_2 \cdot 17D_6$ administered with DMβCD, βCD, and HPC were 74.9, 55.8, and 49.1%, respectively. The plasma concentration of $E_2 \cdot 17D_6$ after i.n. administration of $E_2 \cdot 17D_6$-DMβCD decreased rapidly to the endogenous level by 6 h, but the concentration in the bone was about 200 times higher than that in plasma, and decreased slowly over a period of about a week. When E_2 (total dose 4.4 μmol/kg, i.n., every 3rd day) was administered to OVX mice for 35 d, bone mineral density (BMD), liver weight, and uterus weight increased, whereas $E_2 \cdot 17D_6$-DMβCD (total dose 0.44 to 8.8 μmol/kg, i.n., every 7th day) increased only BMD in a dose-dependent manner. In conclusion, intranasally administered $E_2 \cdot 17D_6$-DMβCD has a potent antiosteoporotic effect without side effects, and has potential to provide an improved quality of life for patients with osteoporosis.

Key words estradiol; aspartic acid oligopeptide; intranasal administration; drug delivery system; osteoporosis; ovariectomized (OVX) mice

Estrogen replacement therapy for osteoporosis is an effective treatment to prevent the reduction of bone mineral density (BMD) in postmenopausal women.[1] However, the development of osteoporosis therapies that are more efficient and selective to bone without the adverse reactions of estrogens, such as intrauterine hemorrhage, endometrial and breast cancers, and fatty liver, are needed. We attracted that non-collagenous proteins, *i.e.* osteopontin and bone sialoprotein in bone matrix contain repeating sequences of acidic amino acids,[2,3] and revealed some repetitive acidic amino acid sequences can act as a carrier for selective drug delivery to bone.[4,5] Based on that finding, we have developed novel osteotropic pro-drugs, estradiol conjugated with 17β-succinate-(L-aspartate)$_6$ ($E_2 \cdot 17D_6$)[5] and 3β-succinate-(L-aspartate)$_6$ ($E_2 \cdot 3D_6$),[6] which showed selective, prolonged action on bone without significant side effects after an intravenous (i.v.) administration to ovariectomized (OVX) animals.

When considering the clinical use of these oligopeptide-conjugated estradiol compounds, i.v. injection is not desirable because of the potential problem of poor compliance. After oral administration, it is unlikely these compounds unlikely permeate across the intestinal membrane because of their high hydrophilicity, and become a target of peptidases in the gastrointestinal tract. We therefore considered the feasibility of intranasal (i.n.) administration. Previous studies have attempted to improve the bioavailability of nasally administered peptides by using absorption enhancers.[7] Currently available absorption enhancers include acylcarnitine,[8] sodium tauro-24,25-dihydrofusidate,[9] cyclodextrin,[10] chitosan,[11] calcium carbonate,[12] hydroxypropyl cellulose,[13] and polymer.[14]

In this study, we examined the effects of some nasal absorption enhancers for $E_2 \cdot 17D_6$ on its bioavailability and evaluated the efficacy and side effects after i.n. administration of $E_2 \cdot 17D_6$ with an absorption enhancer to OVX mice.

MATERIALS AND METHODS

Materials Estradiol (E_2) and β-estradiol 17-hemisuccinate were purchased from Sigma Co. Ltd. (St. Louis, MO, U.S.A.). *N*,*O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA), β-cyclodextrin (βCD) and 2,6-di-*O*-methyl-β-cyclodextrin (DMβCD) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Hydroxypropyl cellulose (HPC), trifluoroacetic acid and dimethyl sulfoxide were purchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan).

Chemical Synthesis of Estradiol-17β-succinate-(L-aspartate)$_6$ We synthesized estradiol-17β-succinate-(L-aspartate)$_6$ ($E_2 \cdot 17D_6$) (Fig. 1) from β-estradiol 17-hemisuccinate and L-aspartate-hexapeptide by a routine solid-phase synthesis method. The compound was purified by reverse-phase HPLC with a YMC D-ODS-5 120 A column (2×25 cm, YMC Co., Ltd., Kyoto, Japan).

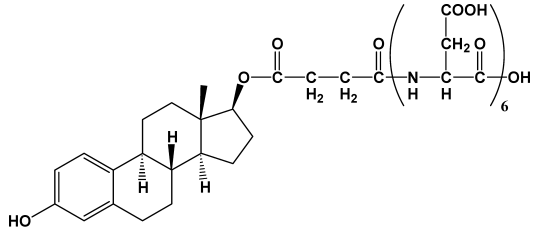

Fig. 1. Molecular Structure of Synthesized Estradiol-17β-succinate-(L-aspartate)$_6$ ($E_2 \cdot 17D_6$)

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Nasal Preparation of $E_2 \cdot 17D_6$ A preparation of $E_2 \cdot 17D_6$ for nasal delivery was prepared according to Iliescu *et al*.[15] βCD or DMβCD (25 μg) was added at a level of 5% (final concentration) to 500 μl of ethanol containing an $E_2 \cdot 17D_6$ (5 mg) and the mixture was stirred for 90 min at 60 °C, then for 24 h at room temperature. It was cooled in a refrigerator at 6 °C for 1 week, and evaporated to dryness under a stream of nitrogen gas at room temperature. The residue was dissolved in 500 μl of 1 M phosphate buffer (pH 7.4).

In addition, $E_2 \cdot 17D_6$ (5 mg) in 250 μl of 50% ethanol–1 M phosphate buffer (pH 7.4) was added to an equal volume of 1 M phosphate buffer (pH 7.4) containing HPC (10% to $E_2 \cdot 17D_6$, 500 μg). The mixture was stirred at 37 °C for 2 h and then used as a nasal preparation of $E_2 \cdot 17D_6$ with HPC.

Animal Experiments All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kanazawa University.

1) Drug Administration: We used female ddY mice (8 weeks old, Nippon SLC, Hamamatsu, Japan). E_2 solution in 30% ethanol or $E_2 \cdot 17D_6$ in saline was injected *via* the jugular vein at a dose of 3.7 μmol/kg in a volume of 50 μl or was orally (*p.o.*) administered in a volume of 200 μl. For i.n. administration, mice were surgically treated according to the procedure of Sinswat and Tengamnuay.[16] An incision was made at the neck to expose the trachea. A polyethylene tube was inserted about 0.5 cm into the trachea toward the lungs to maintain respiration during the experiment. The esophagus was also cannulated with a similar polyethylene tube, which was closed at the posterior nasal cavity. The nasopalatine was sealed with an adhesive agent to prevent drainage of the drug solution from the nasal cavity into the mouth. E_2 solution in 30% ethanol or $E_2 \cdot 17D_6$ without or with DMβCD, βCD or HPC in saline was intranasally administered *via* the cannula at a dose of 3.7 μmol/kg in a volume of 5 μl in mice. Blood samples were collected from the intraorbital venous plexus using heparinized capillary tubes at designated time intervals. The plasma was separated by centrifugation and stored at −30 °C until assay. Mice were sacrificed by decapitation, then bone (tibia and femur) was removed and stored at −30 °C until assay.

2) OVX Model: Female ddY mice (8 weeks old) were randomly divided into 7 groups of five mice each. Five mice of one group were sham-operated (Sham group) and all others were ovariectomized (OVX group) under pentobarbital anesthesia. During the experiments, the mice were pair-fed. Treatment was started 8 weeks after operation. The sham group and one OVX group (untreated-control group) were i.n. administered with the vehicle (1 M phosphate buffer) in a volume of 50 μl on every seventh day. One OVX group was i.n. administered E_2 at a dose of 0.37 μmol/kg on every third day (10 times) and the other four groups were i.n. administered $E_2 \cdot 17D_6$-DMβCD at doses of 0.11, 0.37, 1.1, and 2.2 μmol/kg on every seventh day (5 times). At 3 or 2 d after the last administration of E_2 or $E_2 \cdot 17D_6$-DMβCD, mice were sacrificed and the major organs were removed and weighed. The tibia and femur were removed and stored in ethanol. Bone mineral density (BMD) was measured by using a dual X ray absorptiometer (DCS-600R, Aloka Corp., Tokyo, Japan).

Analysis of E_2 and $E_2 \cdot 17D_6$ in Plasma and Bone For the measurement of E_2 in plasma, an aliquot of 10 μl of plasma was mixed with 1 ml of 1 M phosphate buffer (pH 7.0) and 3 ml of *n*-hexane. $E_2 \cdot 17D_6$ was measured as the equivalent concentration of E_2 after alkaline hydrolysis. Therefore, the concentration of $E_2 \cdot 17D_6$ was obtained by subtracting the measured E_2 value before alkaline hydrolysis from that after alkaline hydrolysis. To an aliquot of 10 μl of plasma was added 200 μl of 1 N NaOH. The mixture was incubated at 60 °C for 3 h, then neutralized with 200 μl of 1 N HCl, and 600 μl of 1 M phosphate buffer (pH 7.0) and 3 ml of *n*-hexane were added. For the extraction of E_2 in bone, the femur and tibia were crushed to pieces after they had been frozen with liquid nitrogen. An aliquot of about 0.2 g of bone sample was incubated with 200 μl of 10 N HCl at 60 °C for 2 h. The mixture was neutralized with 200 μl of 10 N NaOH, and 1 ml of 1 M phosphate buffer (pH 7.0) and 3 ml of *n*-hexane were added. For $E_2 \cdot 17D_6$, the bone sample was incubated with 200 μl of 10 N HCl at 60 °C for 2 h, then 400 μl of 10 N NaOH was added and incubation was continued at 37 °C for 3 h. The mixture was neutralized with 200 μl of 10 N HCl, and 1 ml of 1 M phosphate buffer (pH 7.0) and 3 ml of *n*-hexane were added.

The *n*-hexane extract was vigorously shaken for 1 min and centrifuged for 10 min at 3000×***g***. The supernatant organic phase was transferred to another glass tube and preconcentrated under a stream of nitrogen gas at room temperature. Then, 100 μl of 25% BSTFA in acetone was added to the residue, and an aliquot (1 μl) of the mixture was injected into the gas chromatography-mass spectrometry (GC-MS) system.

Measurement of E_2 by GC-MS The concentration of E_2 in plasma and bone was determined by GC-MS (Model GC-17 system Class 5000, Shimadzu, Kyoto, Japan), according to Dawling *et al*.[17] Analyses were carried out in the selected-ion monitoring mode, with monitoring at *m*/*z* 285, *m*/*z* 416 and *m*/*z* 417 for the derivative of E_2 with BSTFA. Chromatographic separation of derivatized E_2 was achieved with a 5% phenyl-methylpolysiloxane-crosslinked capillary column (DB-5; 30 m×0.32 mm I.D.; J & W Scientific Inc., U.S.A.) in a gas chromatograph equipped with a splitless injector. The oven temperature was set at 170 °C for 1 min and then programmed up to 310 °C at 15 °C/min. The final temperature was maintained for 10 min.

Data Analysis The plasma drug concentration was expressed as the equivalent concentration of E_2, after subtraction of the endogenous E_2 concentration before drug administration from obtained data. The pharmacokinetic parameters were estimated by means of model-independent moment analysis as described by Yamaoka *et al*.[18] The data were analyzed using Student's *t* test to compare the unpaired mean values of two sets of data. The number of determinations is noted in each table and figure. A value of $p<0.01$ or 0.05 was taken to indicate a significant difference between sets of data.

RESULTS

Disposition Pharmacokinetics of E_2 and $E_2 \cdot 17D_6$ after Administration *via* Various Routes Figure 2 shows the plasma concentration–time courses after a single i.v., *p.o.* or i.n. administration of E_2 (3.7 μmol/kg) in mice. The plasma

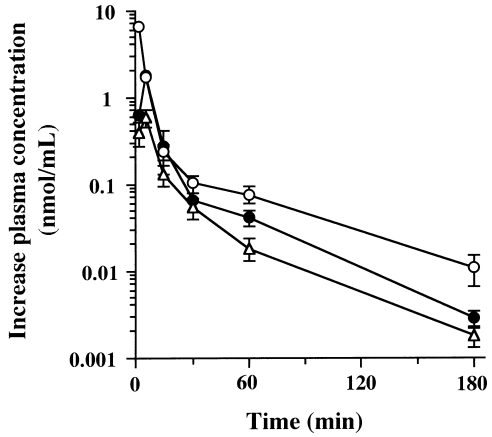

Fig. 2. Plasma Concentration–Time Courses of E_2 after an i.v. (○), *p.o.* (△) or i.n. (●) Administration of E_2 (3.7 μmol/kg) in Mice

Each point with bar represents the mean±S.E. of three mice.

Table 1. Pharmacokinetic Parameters after an i.v., *p.o.* or i.n. Administration of E_2 or $E_2 \cdot 17D_6$ (3.7 μmol/kg) in Mice

Parameter	E_2	$E_2 \cdot 17D_6$
i.v. administration		
AUC (nmol·min/ml)[a)]	45.7±2.9	60.2±3.1*
MRT (min)	14.4±3.1	11.5±2.0
Vd_{ss} (l/kg)	1.16±0.28	0.704±0.139
CL_{tot} (ml/min/kg)	80.6±5.2	61.2±3.0*
p.o. administration		
AUC (nmol·min/ml)[a)]	9.0±1.4	5.98±1.19*
Bioavailability (%)	19.7	9.93
i.n. administration		
AUC (nmol·min/ml)[a)]	21.1±2.3[#]	13.9±3.2*,[#]
Bioavailability (%)	46.2	23.1

Each value represents the mean±S.D. (n=3). *a*) The area under the blood concentration–time curve from zero to 3 h. * Significantly different from the value for the administration of E_2 at $p<0.05$. # Significantly different from the value for the *p.o.* administration at $p<0.05$.

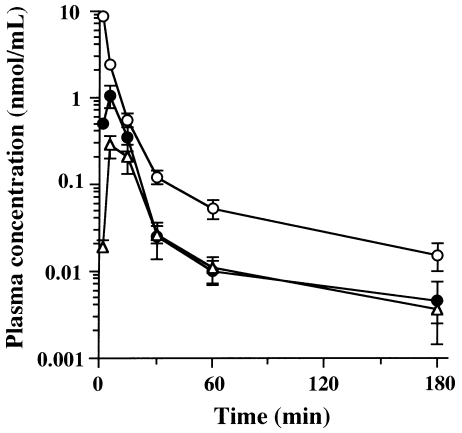

Fig. 3. Plasma Concentration–Time Courses of $E_2 \cdot 17D_6$ after an i.v. (○), *p.o.* (△) or i.n. (●) Administration of $E_2 \cdot 17D_6$ (3.7 μmol/kg) in Mice

Each point with bar represents the mean±S.E. of three mice.

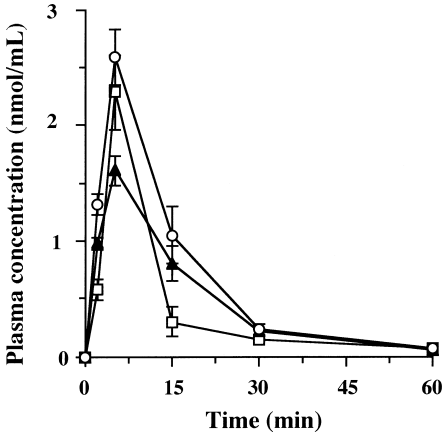

Fig. 4. Plasma Concentration–Time Courses of $E_2 \cdot 17D_6$ after an i.n. Administration of $E_2 \cdot 17D_6$-DMβCD (○), $E_2 \cdot 17D_6$-5%βCD (▲) or $E_2 \cdot 17D_6$-10%HPC (□) (3.7 μmol/kg) in Mice

Each point with bar represents the mean±S.E. of three mice.

concentration of E_2 after administration of E_2 is given as the observed concentration minus the endogenous concentration of E_2, because the plasma endogenous concentration of E_2, which varied widely among individuals (range, 42 to 489 pmol/ml), may be due to the sexual cycle. The behavior of E_2 was biphasic with a half-time for the elimination phase ($t_{1/2}$) of 45 min after i.v. administration. The plasma concentration decreased to approximately the endogenous level of E_2 by 6 h. The plasma concentration reached the maximum at 5 min, which was higher after i.n. administration than after *p.o.* administration, and then gradually decreased in parallel with the concentration after i.v. administration.

Figure 3 shows the plasma concentration–time course after a single i.v., *p.o.* or i.n. administration of $E_2 \cdot 17D_6$ (3.7 μmol/kg) in mice. The behavior of $E_2 \cdot 17D_6$ was biphasic, with a $t_{1/2}$ of 55 min after i.v. administration. After the *p.o.* or i.n. administration of $E_2 \cdot 17D_6$, the plasma concentration reached the maximum at 5 min, but was lower than the respective i.v. administration route of E_2 (Fig. 2). Table 1 shows the pharmacokinetic parameters of E_2 and $E_2 \cdot 17D_6$ after the i.v., *p.o.* or i.n. administration in mice. After i.v. administration, the value of the area under the plasma concentration–time curve from zero to infinity (*AUC*) of $E_2 \cdot 17D_6$ was significantly larger than that of E_2, and the total clearance (CL_{tot}) was significantly lower, but those of $E_2 \cdot 17D_6$ after *p.o.* and i.n. administrations were significantly smaller than those of E_2, and the bioavailability values were about half those of E_2. As predicted, the maximum concentration and the bioavailability value of $E_2 \cdot 17D_6$ after *p.o.* administration were the lowest among the administration routes.

Plasma Concentration–Time Courses of $E_2 \cdot 17D_6$ after the i.n. Administration of Various Preparations of $E_2 \cdot 17D_6$ Figure 4 shows the plasma concentration–time courses of $E_2 \cdot 17D_6$ after i.n. administration of $E_2 \cdot 17D_6$-DMβCD, $E_2 \cdot 17D_6$-βCD and $E_2 \cdot 17D_6$-HPC (3.7 μmol/kg). The plasma concentrations of $E_2 \cdot 17D_6$ rapidly increased after i.n. administration of these preparations and subsequently declined gradually. As shown in Table 2, the *AUC* value was largest for $E_2 \cdot 17D_6$-DMβCD, followed by $E_2 \cdot 17D_6$-βCD and $E_2 \cdot 17D_6$-HPC. The nasal BA value of $E_2 \cdot 17D_6$-DMβCD was the highest among these preparations and indicated satisfactory absorption efficacy.

Bone Concentration of $E_2 \cdot 17D_6$ after i.n. Administration of Various Preparations of $E_2 \cdot 17D_6$ Figure 5 shows the bone concentrations of E_2 or $E_2 \cdot 17D_6$ at 6 h after i.n. administration of E_2 or $E_2 \cdot 17D_6$ without or with various ab-

Table 2. Pharmacokinetic Parameters of $E_2 \cdot 17D_6$ after an i.n. Administration of $E_2 \cdot 17D_6$-DMβCD, $E_2 \cdot 17D_6$-βCD or $E_2 \cdot 17D_6$-HPC (3.7 μmol/kg) in Mice

Parameter	$E_2 \cdot 17D_6$-DMβCD	$E_2 \cdot 17D_6$-βCD	$E_2 \cdot 17D_6$-HPC
AUC (nmol min/ml)[a]	45.1±4.7	33.7±2.8	29.6±0.8
Bioavailability (%)	74.9	55.9	49.2

Each value represents the mean±S.D. (n=3). *a*) The area under the blood concentration–time curve from zero to 3 h.

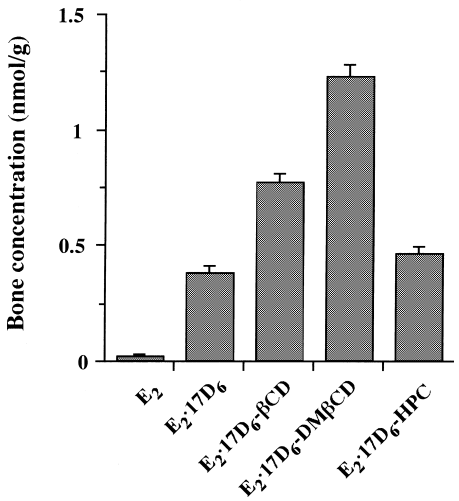

Fig. 5. Bone Concentrations of E_2 or $E_2 \cdot 17D_6$ at 6 h after an i.n. Administration of E_2, $E_2 \cdot 17D_6$ or $E_2 \cdot 17D_6$ with Various Absorption Enhancers (3.7 μmol/kg) in Mice

Each column with bar represents the mean±S.E. of three mice.

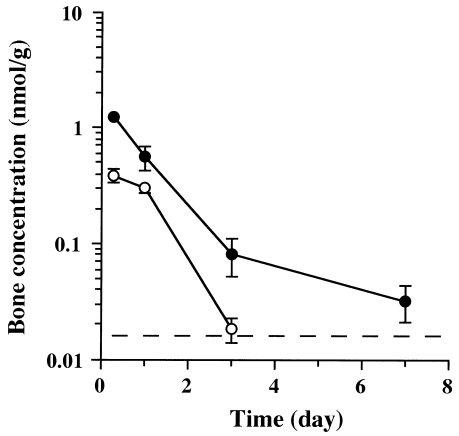

Fig. 6. Bone concentration–Ttime Courses of $E_2 \cdot 17D_6$ after an i.n. Administration of $E_2 \cdot 17D_6$ (○) or $E_2 \cdot 17D_6$-DMβCD (●) (3.7 μmol/kg) in Mice

Each point with bar represents the mean±S.E of three mice.

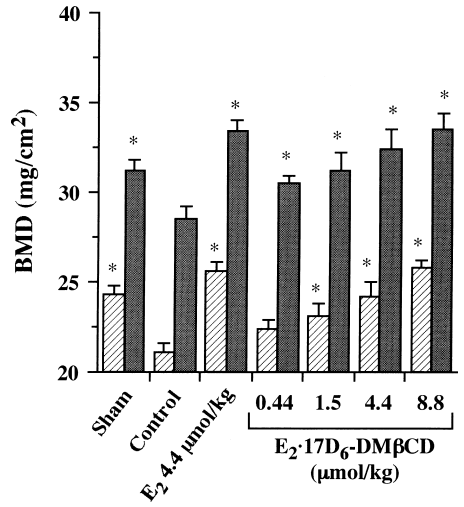

Fig. 7. Effects of E_2 and $E_2 \cdot 17D_6$-DMβCD on the Tibial (▨) and Femoral (■) BMD of OVX Mice

OVX mice were intranasally administered the indicated dose of E_2 (total dose, 4.4 μmol/kg, every 3rd day) or $E_2 \cdot 17D_6$-DMβCD (total dose, 0.44—8.8 μmol/kg, every 7th day) for 35 d. Each column with bar represents the mean±S.D. of six animals. * Significantly different from control mice at $p<0.01$.

sorption enhancers. The bone concentration was highest in the case of $E_2 \cdot 17D_6$-DMβCD, followed by $E_2 \cdot 17D_6$-βCD, $E_2 \cdot 17D_6$-HPC and $E_2 \cdot 17D_6$ alone, whereas the bone concentration of E_2 after administration of E_2 alone was very much lower, in the range of the endogenous concentration.

Figure 6 shows the bone concentration–time courses of $E_2 \cdot 17D_6$ after an i.n. administration of $E_2 \cdot 17D_6$ or $E_2 \cdot 17D_6$-DMβCD. The maximum concentration of $E_2 \cdot 17D_6$ in the bone after administration of $E_2 \cdot 17D_6$-DMβCD was about 3 times higher than that after administration of $E_2 \cdot 17D_6$. The bone concentration after $E_2 \cdot 17D_6$ administration gradually decreased to the endogenous level of E_2 after 3 d, whereas after $E_2 \cdot 17D_6$-DMβCD administration the bone concentration decreased more slowly and never reached the endogenous level of E_2 even until 7 d. The values of *AUC* (0 to 7 d) of $E_2 \cdot 17D_6$ after an i.n. administration of $E_2 \cdot 17D_6$ or $E_2 \cdot 17D_6$-DMβCD were 37.5±2.4 and 101±9 nmol min/ml (mean±S.D., n=3), respectively.

Pharmacology of $E_2 \cdot 17D_6$-DMβCD in OVX Mice

OVX mice were treated with E_2 (total dose, 4.4 μmol/kg) every 3rd day (12 times) or $E_2 \cdot 17D_6$-DMβCD (total dose, 0.44, 1.5, 4.4 or 8.8 μmol/kg) every 7th day (5 times) for 5 weeks, and the effects on the bone were evaluated.

Figure 7 shows the effect of i.n. administration of $E_2 \cdot 17D_6$-DMβCD on the tibial and femoral BMD of OVX mice. The tibial and femoral BMD of the OVX-control group were significantly decreased compared with that of the sham group, but the BMD of the E_2 treatment group recovered to the level of the sham group. The $E_2 \cdot 17D_6$-DMβCD treatment also restored BMD to a level of at least that of the sham group in a dose-dependent manner.

DISCUSSION

Although $E_2 \cdot 17D_6$ is useful for osteoporosis due to its bone selective action, there are some problems associated with its administration routes. The i.v. injection route is undesirable because of a lowering of the quality of life and poor compliance, while good bioavailability cannot be expected with *p.o.* administration because of the high hydrophilicity of the peptide moiety. Indeed, the bioavailability of $E_2 \cdot 17D_6$ after *p.o.* administration was less than that of E_2 (Fig. 2, Table 1). On the other hand, the bioavailability after i.n. ad-

ministration was significantly higher than after *p.o*. administration, though it was less than that of E_2. We then attempted to increase the efficacy of absorption of $E_2 \cdot 17D_6$ *via* nasal mucosa. We examined 5% βCD, 5% DMβCD[19—21] and 10% HPC[22]; these enhancers elevate drug permeability because they prolong the residence time of the drug in the nasal cavity by forming an inclusion complex with the drug. We found that the plasma concentration of $E_2 \cdot 17D_6$ after i.n. administration of $E_2 \cdot 17D_6$ with these absorption enhancers was clearly increased compared with the case of $E_2 \cdot 17D_6$ alone (Fig. 4). $E_2 \cdot 17D_6$-DMβCD gave the highest nasal bioavailability value, which was over about 3 times higher than that of $E_2 \cdot 17D_6$ alone (Tables 1, 2). Moreover, we confirmed that the bone concentration of $E_2 \cdot 17D_6$ became high in a bioavailability-dependent manner, at 6 h after i.n. administration of $E_2 \cdot 17D_6$-DMβCD it was about 50 times higher than that in the case of $E_2 \cdot 17D_6$ alone, and remained above the endogenous level of E_2 for about 1 week (Figs. 5, 6). These results indicate that $E_2 \cdot 17D_6$-DMβCD is pharmacokinetically useful as a nasal dosage form for drug delivery to bone.

It is well known that estrogen replacement therapy is an effective treatment in postmenopausal women to prevent the reduction of bone mineral density,[23] but prolonged therapy may increase the risk of fatty liver, endometritis, breast cancer, and uterine cancer.[24,25] Thus, we compared the efficacy and side effects of E_2 and $E_2 \cdot 17D_6$-DMβCD after i.n. administration in ovariectomized mice. The decreased BMD in tibial and femoral bone by OVX was increased in a dose-dependent manner by i.n. administration of $E_2 \cdot 17D_6$-DMβCD, and was as effective as E_2, despite being administered less frequently than E_2 (Fig. 7), and was without adverse effects, such as increases in uterine and liver cancer (data not shown). These results suggest that $E_2 \cdot 17D_6$-DMβCD had no side effects due to estrogen replacement therapy. Thus, DMβCD is considered to be effective at accelerating the absorption of $E_2 \cdot 17D_6$ across the nasal mucosa. It has been reported that β-cyclodextrin may occasionally induce nephrotoxicity owing to the formation of cholesterol–cyclodextrin complexes.[26,27] However, such adverse effects may be of limited significance, because the dosing interval of this preparation is longer than E_2.

In conclusion, we have demonstrated in this study that a unique prodrug of E_2 could be effectively delivered to the bone *via* the nasal mucous membrane. This drug may play an important role in the development of a potent and selective therapy for osteoporosis.

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