Suppressive Effect of Norzoanthamine Hydrochloride on Experimental Osteoporosis in Ovariectomized Mice

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Norzoanthamine is an alkaloid isolated from a colonial zoanthid. We examined the effects of norzoanthamine hydrochloride on bone weight, strength and morphology in ovariectomized mice, a postmenopausal osteoporosis model. Norzoanthamine hydrochloride significantly suppressed the decrease in femoral weight and bone biomechanical parameters caused by ovariectomy without an increase in uterine weight. This means that norzoanthamine hydrochloride does not have an estrogen-like effect on reproductive organs. Morphological observations of longitudinally ground sections of the humeri showed that norzoanthamine hydrochloride administration (2 mg/kg/d, p.o,) completely suppressed the loss of trabecular bone. Furthermore, norzoanthamine hydrochloride thickened the cortical bone. Based on these results, norzoanthamine hydrochloride may act as both a suppressor of bone resorption and an enhancer of bone formation in vivo.

Key words norzoanthamine hydrochloride; ovariectomized mouse; bone weight; bone mechanical parameter; bone morphology

Osteoporosis is caused by an imbalance between bone resorption and bone formation, which results in bone loss and fractures after mineral flux. The frequency of fractures is significantly increased in osteoporosis, and hip fractures in senile patients is a very serious problem because it often limits the patients’ quality of life. Therefore, besides the prevention of bone mass loss, the maintenance of bone mechanical strength is a very important point for the evaluation of osteoporotic drugs.

The purpose of our research is the discovery of new candidate for anti-osteoporotic drugs. Simple and sensitive in vitro assay systems are essential to efficiently screen active compounds. However, although a variety of in vitro screening systems for osteoporotic drugs have been developed, the results of the in vitro experiments do not always match those of in vivo experiments. Furthermore, there is no in vitro experimental system to evaluate the mechanical bone strength or its morphology. Therefore, we have emphasized the importance of evaluating drugs using an osteoporosis model mice in vivo. This postmenopausal osteoporosis model using ovariectomized mice is useful for the evaluation of osteoporotic drugs, because several parameters are clearly decreased by the ovariectomy within 4 or 5 weeks after the operation. 1) The effects of a drug would be based on bone mass, biomechanical property and bone morphology in this model.

During the in vivo screening of osteoporotic drugs, we found that norzoanthamine hydrochloride showed a potent suppressive effect on osteoporosis in ovariectomized mice. Norzoanthamine (Fig. 1) is an alkaloid isolated from a colonial zoanthid, Zoanthus sp. 2) Recently, the absolute configuration of this compound has been completely determined. 3) The suppressive effects of norzoanthamine hydrochloride on osteoporosis in ovariectomized mice are described in this paper.

MATERIALS AND METHODS

Materials Norzoanthamine hydrochloride was prepared from Zoanthus sp. as previously described. 4) 17β-Estradiol was purchased from Wako Pure Chemical Industries (Japan). Alendronate sodium hydrate, a bisphosphonate, was purchased from Teijin (Japan).

Ovariectomized Mice 5) Four-week-old female ddY mice were obtained from Japan SLC, Inc. The mice were allowed free access to commercial chow (Labo MR stock, Nihon Nosan Kogyo, Kanagawa, Japan) and tap water, and were maintained at 25±0.5 °C. Ovariectomies and sham operations were carried out under ketamine hydrochloride anesthesia. The mice were divided into 5 groups: sham-operated, ovariectomized control and norzoanthamine hydrochloride-treated ovariectomized groups (0.08, 0.4, 2 mg/kg/d, p.o.). Each group consisted of 5 mice. The administration of the vehicle or norzoanthamine hydrochloride dissolved in sterilized water was started from the day after the operations and continued 5 d a week for 4 weeks. After 4 weeks’ administration, mouse body weight was measured, then the animals were sacrificed to retrieve femurs, humeri and uteri for measurements. The right femurs were used to measure length and dry weight (dried at 60 °C for 24 h), and the left femurs were used to test a biomechanical parameter: failure load. The right humeri were used to observe bone morphology. The experiments for 17β-estradiol and alendronate were carried out in the same way as described above. Alendronate was dissolved in sterilized water containing 0.17 mg/ml of citric acid.

Fig. 1. Chemical Structure of Norzoanthamine

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Fig. 2. Effect of Norzoanthamine Hydrochloride and 17β-Estradiol on Uterine Weight in Ovariectomized Mice

A: norzoanthamine hydrochloride (NZ) was orally administered for 4 weeks. B: 17β-estradiol was intra-peritoneally administered for 4 weeks. The uterine weights were measured 4 weeks after the operations. Each point is the mean ± S.E., n=5. ***, p<0.001, ****, p<0.0005 vs. ovariectomized group treated by vehicle using Student's t-test.

Fig. 3. Effect of Norzoanthamine Hydrochloride and 17β-Estradiol on the Femoral Weight in Ovariectomized Mice

A: norzoanthamine hydrochloride (NZ) was orally administered for 4 weeks. B: 17β-estradiol was intra-peritoneally administered for 4 weeks. The dry weight of the femur was measured 4 weeks after the operations. Each point is the mean ± S.E., n=5. **, p<0.01, ***, p<0.001, ****, p<0.0005 vs. ovariectomized group treated by vehicle using Student's t-test.

(0.2 ml/mouse/d).

**Measurement of a Bone Biomechanical Parameter**

Failure load measurement was performed using a bone strength tester (Model TK-252C, Muromachi Kikai Co., Ltd.). The femurs were tested in three-point bending until failure. The lower supports of the bending apparatus contacted the posterior surface of the femoral shaft 3.8 mm proximal and distal from the midpoint. The upper loading point was aligned with the anterior midpoint of the femoral shaft. The actuator was displaced at a rate of 5 mm/min and stopped after failure had occurred. The yield load, the failure load and the displacement at failure were recorded.

**Observation of Bone Morphology** The dried humeri were ground from the backside using a fine grindstone. The ground humeri were then washed with water, acetone and bleaching solution containing 0.5% sodium hypochlorite. The humeri were dried again and observed using a microscope. The thickness of the cortical bone was measured on a photograph of the ground bone.

**RESULTS**

**Effect of Norzoanthamine Hydrochloride on Body Weight and Uterine Weight** The ovariectomy did not make any difference in body weight in growing mice, and norzoanthamine hydrochloride and 17β-estradiol also did not affect the body weight in our experiments (data not shown). Although 17β-estradiol significantly and dose-dependently increased the uterine weight, which was significantly reduced by ovariectomy (Fig. 2B), norzoanthamine hydrochloride did not affect the uterine weight (Fig. 2A). Uterine atrophy was caused by the depletion of estrogen produced by the ovary and indicated that the ovary had been completely removed by the operation. Uterine hypertrophy by 17β-estradiol indicates a side effect on reproductive organs, but norzoanthamine hydrochloride did not show such estrogen-like side effect on the reproductive organs.

**Effect of Norzoanthamine Hydrochloride on Femoral Length and Femoral Weight** Although norzoanthamine hydrochloride did not affect the femoral length (data not shown), norzoanthamine hydrochloride significantly affected on femoral weight. The administration of norzoanthamine hydrochloride significantly suppressed bone weight loss by ovariectomy at the dose of 0.08–2.0 mg/kg/d, p.o. (Fig. 3A).

Also, 17β-estradiol did not affect femoral length and suppressed bone weight loss by ovariectomy at the dose of 0.08 mg/kg/d, i.p. (Fig. 3B). 17β-Estradiol did not suppress bone loss at the dose of 0.4 mg/kg/d. This could be explained by the down-regulation of the estrogen receptor.

**Effect of Norzoanthamine Hydrochloride on the Bio-
Fig. 4. Effect of Norzoanthamine Hydrochloride and 17β-Estradiol on the Failure Load in Ovariectomized Mice

A: norzoanthamine hydrochloride (NZ) was orally administered for 4 weeks. B: 17β-estradiol was intra-peritoneally administered for 4 weeks. The failure load was measured 4 weeks after the operations. Each point is the mean ± S.E., n = 5. * p < 0.05, ** p < 0.01, *** p < 0.0005 vs. ovariectomized group treated by vehicle using Student's t-test.

Fig. 5. Effect of Norzoanthamine Hydrochloride, 17β-Estradiol and Alendronate on Humeralis Morphology in Ovariectomized Mice

A: sham-operated mouse treated with vehicle, p.o. (BW: 30.5 g, UW: 128 mg). B: ovariectomized mouse treated with vehicle, p.o. (BW: 30.6 g, UW: 26.1 mg). C: ovariectomized mouse treated with 0.08 mg/kg/d norzoanthamine hydrochloride, p.o. (BW: 28.7 g, UW: 22.0 mg). D: ovariectomized mouse treated with 0.4 mg/kg/d norzoanthamine hydrochloride, p.o. (BW: 29.4 g, UW: 26.0 mg). E: ovariectomized mouse treated with 2.0 mg/kg/d norzoanthamine hydrochloride, p.o. (BW: 29.3 g, UW: 19.8 mg). F: ovariectomized mouse treated with 0.08 mg/kg/d 17β-estradiol, i.p. (BW: 30.8 g, UW: 57.4 mg). G: ovariectomized mouse treated with 1.0 mg/kg/d alendronate, i.p. (BW: 29.0 g, UW: 23.5 mg). Each mouse was treated for 4 weeks after the operations. BW: body weight, UW: uterine weight. Scale bar = 0.5 mm.

**mechanical Parameter** The failure load of the femur was measured using a bone strength tester. The failure load was recorded as the load at the point of failure.1,5,6) As for the failure load of the femur, the administration of norzoanthamine hydrochloride significantly suppressed the reduction caused by ovariectomy at the dose of 0.4 mg/kg/d, p.o. (Fig. 4A).

17β-Estradiol showed a similar effect to norzoanthamine hydrochloride at the dose of 0.016—0.4 mg/kg/d, i.p., in term
of failure load (Fig. 4B).

Effect of Norzoanthamine Hydrochloride on Bone Morphology The ovariectomy caused a decrease in the humeralis trabeculae (Fig. 5B). Norzoanthamine hydrochloride significantly suppressed the decrease in a dose-dependent manner (Figs. 5C, D, E). Although 17β-estradiol and alendronate also suppressed the decrease in trabeculae (Figs. 5F, G), the effect of norzoanthamine hydrochloride was apparently different from alendronate. In the ovariectomized mice treated with alendronate, the resorption of cancellous bone was almost suppressed, and the morphology was occupied by a primary spongiosa-like structure. The medullary cavity of the metaphysis significantly narrowed. On the other hand, in ovariectomized mice treated with norzoanthamine hydrochloride or 17β-estradiol, the primary spongiosa was not significantly increased and the morphology of the metaphysis nearly returned to normal.

Furthermore, norzoanthamine hydrochloride increased the thickness of the cortical bone in the humeralis diaphysis (Fig. 6A). Norzoanthamine hydrochloride was proven to have protective effects on both the trabecular and cortical bone in the humerus.

DISCUSSION

Norzoanthamine hydrochloride significantly suppressed bone weight loss and the failure load caused by ovariectomy through the increase in the trabecular and cortical bone. Furthermore, unlike 17β-estradiol, norzoanthamine hydrochloride did not affect uterine weight. Although norzoanthamine hydrochloride has a steroid-like structure and effects similar to 17β-estradiol on bone morphology in ovariectomized mice, it was not an agonist of estrogen, at least in the reproductive organs.

Although this method is not generally used, a microscopic observation of simply ground bone provided good information about bone morphology. The microscopic observation of ground bone presented information of the trabecular structure in three dimensions and also about the thickness of the cortical bone. Although this information can also be obtained using micro computed tomography, our method is very simple and useful for the primary evaluation of bone morphology.

Considering the morphology of the metaphysis, the effect of norzoanthamine hydrochloride was more similar to that of 17β-estradiol than alendronate, although norzoanthamine hydrochloride was effective at a higher dose than 17β-estradiol. Alendronate, a bisphosphonate, deposits calcium to the bone and inhibits bone resorption through the suppression of osteoclast activity. The effects of norzoanthamine hydrochloride were apparently different from those of alendronate. On the other hand, norzoanthamine hydrochloride had effects similar to 17β-estradiol on the trabecular bone. The morphology of the metaphysis treated with norzoanthamine hydrochloride or 17β-estradiol nearly returned to normal. 17β-Estradiol is known to regulate bone metabolism through binding to estrogen receptors. Because norzoanthamine hydrochloride did not have an estrogen-like effect on the reproductive organs, it would regulate bone metabolism similarly to estrogen through a process different from 17β-estradiol.

Norzoanthamine hydrochloride significantly increased the failure load decreased by ovariectomy. Because the failure load directly indicates cortical bone strength, norzoanthamine hydrochloride suppressed a decline in cortical bone strength. Other bone biomechanical parameters, yield energy and stiffness, were also measured in these experiments. Although these parameters showed a large standard error in providing a significant difference among each group, the administration of norzoanthamine hydrochloride and 17β-estradiol tended to increase these parameters of the femur (data not shown). Evaluation of the bone biomechanical property in the experimental osteoporosis model should predict the preventive effect on bone fractures. Since osteoporotic drugs are required to prevent bone fractures induced by osteoporosis, the evaluation of bone biomechanical properties is very important. Norzoanthamine hydrochloride can be expected to prevent bone fracture. Furthermore, norzoanthamine hydrochloride was effective using oral administration. Drugs for chronic diseases like osteoporosis must be effective by oral administration, and norzoanthamine hydrochloride meets this requirement.

It can be rationalized that the increase in trabecular and cortical bone by norzoanthamine hydrochloride caused the gains in bone weight and strength. However, the target molecule of norzoanthamine hydrochloride is not clear. Norzoanthamine hydrochloride was previously reported to be a suppressor of interleukin-6 production stimulated by the parathyroid hormone in preosteoblastic MC3T3-E1 cells in vitro \cite{IC50=4.5 μg/ml}. Interleukin-6 is known to be a stimulator of osteoclast formation. There is the possibility that norzoanthamine hydrochloride might act in vivo through the inhibition of interleukin-6 secretion and osteoclast formation. However, the suppression of interleukin-6 secretion has not been confirmed in vivo, and osteoclast formation in the presence of the osteoblasts in vitro was not inhibited by norzoanthamine hydrochloride (data not shown). Therefore, norzoanthamine hydrochloride may act in an unrelated manner on the suppression of interleukin-6 production in vivo. In any case, norzoanthamine hydrochloride is expected to work in a manner different from known anti-osteoporotic drugs and the study of its target molecule would be very interesting. Elucidation of the target molecule will provide new ideas for de-
veloping novel anti-osteoporotic reagents. Further detailed studies with norzontamine hydrochloride will be needed.

REFERENCES