Full Paper

Effects of Chronic Administration of Zonisamide, an Antiepileptic Drug, on Bone Mineral Density and Their Prevention With Alfacalcidol in Growing Rats

Atsushi Takahashi¹, Kenji Onodera^{2,*}, Junzo Kamei³, Shinobu Sakurada⁴, Hisashi Shinoda⁵, Shuichi Miyazaki⁶, Takashi Saito¹ and Hideaki Mayanagi¹

¹Clinics of Dentistry for the Disabled, Tohoku University Dental Hospital, Sendai 980-8575, Japan

²Department of Dental Pharmacology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8525, Japan

³Department of Pathophysiology, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, Tokyo 142-8501, Japan

⁴Department of Physiology and Anatomy, Tohoku Pharmaceutical University, Sendai 981-8558, Japan

⁵Department of Pharmacology, Tohoku University School of Dentistry, Sendai 980-8575, Japan

⁶Immunology Laboratory, Diagnostics Department, Yamasa Corporation, Choshi 288-0056, Japan

Received November 13, 2002; Accepted February 7, 2003

Abstract. We investigated the effects of chronic administration of zonisamide, an antiepileptic agent, on bone metabolism in growing rats. Administration of zonisamide at a dose of 80 mg/kg per day, s.c. for 5 weeks significantly decreased bone mineral density (BMD) at the tibial metaphysis and the diaphysis. The percent rate of decrease in BMD at the tibial metaphysis and the tibial diaphysis was 9.2% and 5.0%, respectively. There was no significant difference between these groups in the growth of the rats. Treatment with zonisamide at a dose of 80 mg/kg increased serum pyridinoline level, a marker of bone resorption, while it does not affect the serum intact osteocalcin level, a marker of bone formation. Combined administration of alfacalcidol, an active vitamin D₃ metabolite, at a dose of 0.1 µg/kg per day with zonisamide prevented a decrease in BMD and showed an increase of serum pyridinoline levels. These results suggest that zonisamide may cause bone loss by accelerating bone resorption rather than inhibiting bone formation. Moreover, the bone loss induced by zonisamide could be prevented by combining zonisamide with alfacalcidol.

Keywords: zonisamide, drug-induced osteopenia, bone mineral density, alfacalcidol, serum pyridinoline

Introduction

Previously, we reported that repeated administration of phenytoin (20 mg/kg), which is the most commonly used drug for the therapy of patients with various types of seizures, for 5 weeks induces decreased bone mineral density (BMD) in all regions of bones tested such as the mandible head, tibial metaphysis, tibial diaphysis, femoral metaphysis, and femoral diaphysis (1). Combined administration of either alfacalcidol or vitamin K₂

with phenytoin for 5 weeks prevented the reduction of BMD induced by phenytoin (1, 2). Moreover, previous biochemical data indicated that the serum osteocalcin (OC), a marker of bone formation, was significantly decreased, but there were no significant differences in the levels of serum calcium, pyridinoline (PYD), 25-hydroxyvitamin D (25OHD), and parathyroid hormone (PTH) (1). These data and morphometric results indicated that phenytoin-induced osteopenia may be due to bone loss caused by inhibition of bone formation and/or by accelerating bone resorption rather than osteoid accumulation in rats (1, 2).

Clinical studies have shown that the chronic use of

*Corresponding author. FAX: +81-86-235-6664
E-mail: onoderak@md.okayama-u.ac.jp

antiepileptic agents induce mostly bone loss, which led to bone fractures and osteoporosis (3–5). In a series of our studies, we have been interested in the mechanisms of bone loss induced by antiepileptic agents, whether they are due to common chemical structures or something related to calcium and/or bone metabolism. Zonisamide, an atypical antiepileptic medication, has shown a broad spectrum of efficacy in the treatment of seizures including infantile spasms and myoclonic seizures (6). Zonisamide is used especially for patients with severe epilepsy such as West syndrome or Rennox-Gastaut syndrome. However, there is no data available about the effects of zonisamide on BMD, calcium, and bone metabolism. Therefore, we investigated the effects of chronic administration of zonisamide alone and in combination with alfacalcidol on bone metabolism by measurements of BMD using image analysis of soft X-ray microradiographs (1, 2, 7) and determination of serum bone markers in rats. Especially, since many types of epilepsy appear in childhood, we used young growing rats.

Materials and Methods

Animals

Male Wistar rats (SLC Co., Ltd., Shizuoka) weighing 70 ± 5 g were used. The animals were individually housed in wire-mesh cages ($170 \times 250 \times 370$ mm) in an air-conditioned room at constant temperature ($22 \pm 2^\circ\text{C}$) and humidity ($60 \pm 10\%$) on a 12 h/12 h light-dark cycle (light on 07:30). They were given laboratory chow (F2 of the following composition: 0.74 g Ca, 0.65 g P, 200 IU vitamin D₃ per 100 g; SLC Co., Ltd.) and deionized water ad libitum. The animals were treated carefully in compliance with the regulations of Tohoku University School of Medicine and Dentistry.

Experiment I

Grouping and drug treatments: Each group (10–11 animals) received drug-treatment once per day (between 17:30 and 18:30) for 5 successive weeks. Zonisamide (Dainippon Pharmaceutical Co. Ltd., Osaka) was suspended in 0.5% Tween-80 (Wako Pure Chemicals Industries, Ltd., Osaka) solution. Vehicle or zonisamide (50 and 80 mg/kg) was administered subcutaneous (s.c.) in a volume of 0.1 ml per 100 g body weight. Blood samples were collected on the 14th day and on the 35th day. These samples were centrifuged at 3,000 rpm for 15 min. Each serum sample was stored in the refrigerator until use. At the end of the experimental period, rats were sacrificed under intraperitoneal administration of sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA) and the tibiae were removed. BMD

at the tibial metaphysis and diaphysis were measured as described bellow.

Measurement of bone mineral density: Under pentobarbital anesthesia, rats were perfused with 4% paraformaldehyde (Wako Pure Chemicals Industries, Ltd.) in 0.1 M phosphate buffer (pH 7.4). After perfusion, the tibiae were dissected and soaked in 4% paraformaldehyde in 0.1 M phosphate buffer. After removing the adhesive soft tissues, the bones underwent soft X-ray microradiography with a soft X-ray apparatus (Type-Softex; Softex Co., Ltd., Tokyo). A step-wedge made of synthetic hydroxyapatite plates (Mitsubishi Kasei, Co., Ltd., Tokyo) of differing thickness was placed on the same radiographic films (Soft X-ray film, Type FR; Fuji Photofilm, Tokyo) to serve as a standard for measuring the BMD of each sample. Each BMD was measured at the metaphysis and diaphysis as shown in Fig. 1, essentially according to the previously described method (1, 2, 7). In brief, BMD was determined by analyzing the grey levels of the target area in microradiographs with an image analyzer (Aspect; Mitani Corp., Fukui). A standardized relation was established between the grey levels (0 to 255) and hydroxyapatite content/mm² by analyzing the image of the standard step-wedge. All images analyzed were fed to a video camera (TI-23A CCD; NEC Japan, Tokyo) at a magnification of $\times 20$.

Measurement of serum calcium: The levels of serum calcium were measured by spectrophotometry (228 type; Hitachi, Tokyo) for absorbance at 570 nm using a commercially available kit (Calcium-C Test Wako; Wako Pure Chemicals Industries, Ltd.).

Fig. 1. Diagram of the sample areas of tibiae used in the determination of bone mineral density. The enclosed parts of tibiae were measured using image analysis of soft X-ray microradiographs. Metaphysis, 3-mm width from the growth plate without cortical bone; Diaphysis, 3-mm width 15-mm away from the growth plate with cortical bone.

Experiment II

Each group (10–11 animals) received drug-treatment once per day (between 17:30 and 18:30) for 5 successive weeks. Zonisamide was suspended in 0.5% Tween-80 solution. The concentration of alfacalcidol was adjusted by dilution in ethanol. Zonisamide (80 mg/kg) was administered s.c. in a volume of 0.1 ml per 100 g body weight. $1\alpha(\text{OH})\text{vitaminD}_3$ (alfacalcidol; Chugai Pharmaceutical, Co., Ltd., Tokyo) at a dose of 0.1 $\mu\text{g/kg}$ was administered s.c. in a volume of 20 μl per 100 g body weight with a microsyringe. The tibiae were removed, and BMD at the tibial metaphysis and tibial diaphysis were measured by the method used in experiment I.

Measurement of serum bone markers: At the end of the experimental period, rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg. Blood samples were collected from the carotid arteries of the rats and then centrifuged at 3,000 rpm for 15 min. Each serum sample was stored in the refrigerator until use. The levels of serum calcium were again measured by the method described in experiment I. The activities of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) were measured by spectrophotometry (228 type, Hitachi) for absorbance at 405 nm using a commercially available kit (ALP B-Test Wako and ACP B-Test Wako, Wako Pure Chemicals Industries). The levels of serum OC, PYD, 25OHD, and PTH were determined using the Osteocalcin rat ELISA system[®] (Amersham Pharmacia Biotech K.K., Tokyo), Serum Pyd[®] (Metra Biosystems, Inc., Mountain View, CA, USA), 25-OH Vit D (Biomedica, Vienna, Austria), and Parathyroid hormone rat ELISA system[®] (Amersham Pharmacia Biotech K.K.), respectively. Each sample was analyzed in duplicate.

Statistics

In all experiments, statistical analyses were performed by analysis of variance followed by Duncan's multiple comparison test or Scheffe's multiple comparison test.

Results

Experiment I

Effect of zonisamide on growth curves in rats: At the beginning of the experimental period, the average body weights were 70 g in each group. At the end of the experiment, the average body weights were 235.0, 240.7, and 220.8 for the treatment with vehicle, zonisamide (50 mg/kg), and zonisamide (80 mg/kg), respectively. There were no significant differences in final body weight in the zonisamide-treated groups compared to the vehicle-treated group.

Effects of zonisamide on BMD: Effects of zonisamide

on BMD at the tibial metaphysis and diaphysis are shown in Fig. 2. After five weeks, the mean values of BMD in the metaphysis were 1622, 1599, and 1470 $\mu\text{gHA}/\text{mm}^2$ in the group treated with vehicle, zonisamide (50 mg/kg), and zonisamide (80 mg/kg), respectively. The mean values of BMD in the diaphysis were 1477, 1434, and 1407 $\mu\text{gHA}/\text{mm}^2$ in the group treated with vehicle, zonisamide (50 mg/kg), and zonisamide (80 mg/kg), respectively. The values of BMD of both tibial diaphysis and metaphysis were significantly decreased by the treatment with zonisamide (80 mg/kg) compared with that of vehicle ($P<0.05$). The percent rates of decrease in BMD of zonisamide at doses of 50 mg/kg and 80 mg/kg were 1.6% and 9.2% in the metaphysis and 2.8% and 5.0% in the diaphysis, respectively.

Effects of zonisamide on serum calcium: The levels of serum calcium are shown in Table 1. At 2 weeks and

Fig. 2. Effects of daily treatment with zonisamide for 5 successive weeks on bone mineral density (BMD) of tibial metaphysis (A) and diaphysis (B) in rats. Each column and bar represents the mean $\pm$ S.E.M., respectively. * $P<0.05$, compared with the vehicle-treated group; # $P<0.05$, compared with zonisamide (50 mg/kg)-treated group.

5 weeks after the beginning of the experiment, there were no significant differences in serum calcium levels among all groups.

Experiment II

Effect of zonisamide and/or alfacalcidol on growth curves in rats: At the beginning of the experimented period, the average body weights were 70 g in each group. At the end of the experiment, the average body weights were 240.0, 238.5, 242.0, and 241.4 g in the treatment with vehicle, zonisamide (80 mg/kg), zonisamide (80 mg/kg) + alfacalcidol (0.1 μ g/kg), and alfacalcidol (0.1 μ g/kg), respectively (Fig. 3). There was no significant differences in final body weight in the zonisamide- and/or alfacalcidol-treated groups com-

pared to the vehicle-treated group.

Effects of zonisamide and/or alfacalcidol on BMD: The BMD at the tibial metaphysis and tibial diaphysis are shown in Fig. 4. Zonisamide (80 mg/kg) induced a decrease of BMD at both areas. Combined administration of alfacalcidol (0.1 μ g/kg) with zonisamide (80 mg/kg) blocked the zonisamide-induced effect on BMD, and showed the same degree as the vehicle group in each area measured. There was no significant difference between the vehicle and zonisamide + alfacalcidol groups. Moreover, the mean values of BMD of the zonisamide + alfacalcidol group in each bone area were higher than those of the vehicle group.

Effect of zonisamide and/or alfacalcidol on serum biochemical markers: There was no significant differ-

Table 1. Effect of daily treatment with zonisamide for 5 successive weeks on the levels of serum calcium in experiment I

Group	Serum calcium (mg/dl)	
	14th day	35th day
Vehicle	11.86 $\pm$ 0.20	11.45 $\pm$ 0.33
Zonisamide (50 mg/kg)	11.03 $\pm$ 0.18	11.76 $\pm$ 0.50
Zonisamide (80 mg/kg)	11.35 $\pm$ 0.58	11.26 $\pm$ 0.65

Each result represents the mean $\pm$ S.E.M. of 10 animals. There was no significant difference among all groups.

Fig. 3. Effects of daily treatment with zonisamide and/or alfacalcidol for 5 weeks on rat growth curves. Values represent the means $\pm$ S.E.M. VEH, vehicle-treated group; Z80, zonisamide (80 mg/kg)-treated group; Z80 + ALFA, zonisamide (80 mg/kg) plus alfacalcidol (0.1 μ g/kg)-treated group; ALFA, alfacalcidol (0.1 μ g/kg)-treated group. At the beginning of the experimental period, the average body weight was 70 g in each group.

Fig. 4. Effects of daily treatment with zonisamide and/or alfacalcidol for 5 weeks on bone mineral density (BMD) of tibial metaphysis (A) and diaphysis (B) in rats. Each column and bar represent the mean $\pm$ S.E.M., respectively. VEH, vehicle-treated group; Z80, zonisamide (80 mg/kg)-treated group; Z80 + ALFA, zonisamide (80 mg/kg) plus alfacalcidol (0.1 μ g/kg)-treated group; ALFA, alfacalcidol (0.1 μ g/kg)-treated group. * P <0.05, compared with VEH; # P <0.05, compared with Z80.

Table 2. Effect of daily treatment with zonisamide and/or alfacalcidol for 5 successive weeks on the levels of the serum bone markers in experiment II

	Calcium (mg/dl)	TRAP (IU)	ALP (IU)	OC (ng/ml)	PYD (nmol/l)	25OHD (ng/ml)	PTH (pg/ml)
Vehicle (VEH)	11.2 ± 0.17	25.5 ± 0.72	277 ± 8.67	66.9 ± 3.01	2.79 ± 0.14	15.36 ± 0.97	11.83 ± 1.91
Zonisamide (80 mg/kg) (Z80)	11.6 ± 0.18	27.0 ± 0.99	234 ± 6.98*	62.3 ± 4.85	4.83 ± 0.70*	15.21 ± 1.32	9.42 ± 1.84
Alfacalcidol (0.1 µg/kg) (ALFA)	11.8 ± 0.13	24.8 ± 0.61	241 ± 9.64*	76.7 ± 4.19 [#]	2.91 ± 0.26 [#]	11.10 ± 0.67* [#]	3.14 ± 0.62* [#]
Z80 + ALFA	11.2 ± 0.21	27.7 ± 0.53	246 ± 6.12	77.2 ± 3.05 [#]	4.12 ± 0.27* [†]	11.24 ± 0.51* [#]	4.41 ± 0.95* [#]

Values represent the means ± S.E.M. of 10 animals. TRAP, the activity of tartrate-resistant acid phosphatase; ALP, the activity of alkaline phosphatase; OC, osteocalcin; PYD, pyridinoline; 25OHD, 25-hydroxyvitamin D; PTH, parathyroid hormone. **P*<0.05, compared with VEH; [#]*P*<0.05, compared with Z80; [†]*P*<0.05, compared with Z80 + ALFA.

ence in the activities of TRAP and serum calcium levels among each group as shown in Table 2. The levels of OC were higher in the zonisamide + alfacalcidol and alfacalcidol groups compared with the zonisamide group, while the levels of ALP were lower in the zonisamide and alfacalcidol groups compared with the vehicle group. The levels of serum PYD were higher in the zonisamide and zonisamide + alfacalcidol groups compared with the vehicle group. The levels of PTH and 25OHD were lower in the alfacalcidol and the zonisamide + alfacalcidol group compared with the vehicle group. There were no significant differences in the levels of PTH and 25OHD between the zonisamide and the vehicle group.

Discussion

In this study, we found that zonisamide (1,2-benzisoxazole-3-methanesulfonamide) induced bone loss like phenytoin (5,5-diphenyl-2,4-imidazolidinedione) does in growing rats, although there is no similarity of chemical structures between these substances.

With regard to calcium metabolism, clinical studies have shown that the use of antiepileptic agents was frequently associated with the development of hypocalcemia, and there is a finding that antiepileptic agents decrease intestinal transport of calcium (8, 9). However, the hypocalcemia which usually leads to bone fracture was not observed when the treatment with zonisamide at 80 mg/kg for 5 weeks significantly decreased the BMD in tibial bones measured in this experiment. This result is consistent with our previous finding that the administration of phenytoin at a dose of 20 mg/kg for 5 weeks induced decreased BMD without decreasing the level of serum calcium (2). Moreover, it is well recognized that the severity of drug-induced bone loss depends on several factors including calcium and vitamin D intake, sun-light exposure, physical exercise, doses and duration of antiepileptic agents, duration of therapy, and individual susceptibility (10). From these

results, it is conceivable that zonisamide may induce the decreased BMD before the level of serum calcium decreases.

In the present study, co-administration of alfacalcidol with zonisamide prevented bone loss. A lack of vitamin D is well known to lead to rickets and/or osteomalacia in animals (11). Antiepileptic agents induced liver microsomal P-450-containing oxidases, which subsequently leads to an increased rate of catabolism of vitamin D and its derivatives to inactive metabolites (12, 13). However, the present biochemical data indicated that the serum 25OHD levels are not decreased compared with that of the normal group. This finding indicated that zonisamide-induced bone loss does not participate in the abnormality of the vitamin D metabolism such as rickets or osteomalacia, although co-administration of alfacalcidol is effective to prevent the bone loss induced by zonisamide.

Furthermore, to clarify the bone abnormality induced by zonisamide, we determined several serum bone markers in this study. Chronic treatment with zonisamide significantly increased the serum PYD levels, a marker of bone resorption, while the serum OC levels, a marker of bone formation were not affected. This finding indicates that zonisamide may accelerate bone resorption rather than inhibit bone formation. For this mechanism, there was no significant difference in the level of PTH between the vehicle-treated and zonisamide-treated group, suggesting that bone loss induced by zonisamide was not mediated by the secretion of PTH. It is certain that alfacalcidol has the capacity of inhibiting bone resorption (14, 15). Chronic treatment of phenytoin significantly decreased the OC levels, while the serum PYD levels were not affected (1). This finding indicates that long-term phenytoin exposure may inhibit bone formation which, at least in part, contributed to bone loss in rats. It is also certain that alfacalcidol has the capacity of accelerating bone formation (14, 15).

Thus, the treatment with zonisamide and phenytoin

caused bone loss and combined administration of alfacalcidol prevented the bone loss induced by these drugs, although each causal mechanism is different from each other.

Acknowledgments

Parts of the data in this manuscript were presented at the 27th European Symposium on Calcified Tissue in Tampere, Finland, May, 2000. This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science, and Technology (No. 12671796 for K.O.). We thank Daiippon Pharmaceutical Co., Ltd. (Osaka) for generously supplying zonisamide.

References

- 1 Onodera K, Takahashi A, Shinoda H, Wakabayashi H, Kamei J and Mayanagi H: Phenytoin-induced bone loss and its prevention with alfacalcidol or calcitriol in growing rats. *Calcif Tissue Int* **69**, 109 – 116 (2001)
- 2 Onodera K, Takahashi A, Sakurada S and Okano Y: Effects of phenytoin and/or vitamin K2 (menatetrenone) on bone mineral density in the tibiae of growing rats. *Life Sci* **70**, 1533 – 1542 (2002)
- 3 Hunter J, Maxwell JD, Stewart DA, Parsons V and Williams R: Altered calcium metabolism in epileptic children on anti-convulsants. *Br Med J* **4**, 202 – 204 (1971)
- 4 Hahn TJ, Hendin BA, Scharp CR, Boisseau VC and Haddad JG: Serum 25-hydroxycalciferol levels and bone mass in children on chronic anticonvulsant therapy. *N Engl J Med* **292**, 550 – 554 (1975)
- 5 Richens A and Rowe DJF: Disturbance of calcium metabolism by anticonvulsant drugs. *Br Med J* **4**, 73 – 76 (1970)
- 6 Oomen KJ and Mathews S: Zonisamide: a new antiepileptic drug. *Clin Neuropharmacol* **22**, 192 – 200 (1999)
- 7 Shoji K, Horiuchi H and Shinoda H: Inhibitory effects of a bisphosphonate (risedronate) on experimental periodontitis in rats. *J Periodont Res* **30**, 277 – 284 (1993)
- 8 Caspary WF: Inhibition of intestinal calcium transport by diphenylhydantoin in rat duodenum. *Naunyn Schmiedeberg Arch Pharmacol* **274**, 146 – 153 (1972)
- 9 Koch H-U, Kraft D, Von Herrath D and Schaefer K: Influence of diphenylhydantoin and phenobarbital on intestinal calcium transport in the rat. *Epilepsia* **13**, 829 – 834 (1972)
- 10 Hahn TJ: Drug-induced disorders of vitamin D and mineral metabolism. *Clin Endocrinol Metab* **9**, 107 – 129 (1980)
- 11 Suda T, DeLuca HF and Tanaka Y: Biological activity of 25-hydroxycalciferol in rats. *J Nutr* **100**, 1049 – 1056 (1970)
- 12 Hahn TJ, Birge SJ, Scharp CR and Avioli V: Phenobarbital-induced alterations in vitamin D metabolism. *J Clin Invest* **51**, 741 – 748 (1972)
- 13 Silver J, Neale G and Thompson GR: Effect of phenobarbitone treatment on vitamin D metabolism in mammals. *Clin Sci Mol Med* **46**, 433 – 448 (1974)
- 14 Shiraishi A, Takeda S, Masaki T, Higuchi Y, Uchiyama Y, Kubodera N, Sato K, Ikeda K, Nakamura T, Matsumoto T and Ogata E: Alfacalcidol inhibits bone resorption and stimulates formation in an ovariectomized rat model of osteoporosis: distinct actions from estrogen. *J Bone Miner Res* **15**, 770 – 779 (2000)
- 15 Chen JT, Shiraki M, Hasumi K, Tanaka N, Katase K, Kato T, Hirai Y, Nakamura T and Ogata E: 1- α -Hydroxyvitamin D3 treatment decreases bone turnover and modulates calcium-regulation hormones in early postmenopausal women. *Bone* **20**, 557 – 562 (1997)