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ORIGINAL ARTICLE

Novel bisphosphonate compound FYB-931 preferentially inhibits aortic calcification in vitamin D3-treated rats

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Abstract

In patients with chronic kidney disease (CKD) or those undergoing hemodialysis, pathological calcific deposition known as ectopic calcification occurs in soft tissue, resulting in a life-threatening disorder. A potent and effective inhibitor of ectopic calcification is eagerly expected. In the current study, the effects of FYB-931, a novel bisphosphonate compound synthesized for the prevention of ectopic calcification, were compared with those of etidronate using both in vitro and in vivo models. In vitro, FYB-931 inhibited calcification of human aortic smooth muscle cells induced by high phosphate medium in a concentration-dependent manner, and the effect was slightly more potent than that of etidronate. In vivo, rats were administered with three subcutaneous injections of vitamin D3 to induce vascular calcification, and were given FYB-931 (1.5, 5, or 10 mg/kg) or etidronate (9, 30, or 60 mg/kg) orally once daily for 14 days. The increased aortic phosphorus content as an index of vascular calcification was inhibited by both FYB-931 and etidronate in a dose-dependent manner; however, FYB-931 was 10 times more potent than etidronate. FYB-931 inhibited serum tartrate-resistant acid phosphatase (TRACP) activity as a bone resorption marker 5.2 times more potently than etidronate. FYB-931, but not etidronate, significantly decreased serum phosphorus levels. The preferential inhibition of aortic calcification by FYB-931 suggested that possible additional effect including a decline in serum phosphorus may lead to an advantage in terms of its efficacy.

Keywords Bisphosphonate · Rat · Calcification · Bone resorption marker · Serum phosphorus

Introduction

Chronic kidney disease–mineral and bone disorder (CKD–MBD) adversely affects bone turnover and mineralization. These alterations occur in early stages of CKD and are accompanied by the development of cardiovascular calcifications [1]. The pathologic calcific deposition observed in soft tissues, including vasculature of the heart and kidney, is called as ectopic calcification and leads to serious complications [2, 3]. Vascular calcification particularly causes thrombosis, arteriosclerosis, and myocardial infarction. Valvular calcification, most frequently observed in the heart tissue, causes aortic stenosis and furthermore, heart failure and death [4]. Calcification in the renal tissue is the deposition of calcium phosphate crystals in the renal papilla which leads to kidney stone formation, and the growth of kidney stone is one of the causal factors of renal dysfunction [5].

It was traditionally believed that vascular calcification was the result of passive precipitation of calcium and phosphate, which were present in the blood at high concentrations. It now appears that vascular calcification is a consequence of tightly regulated processes that culminate in organized extracellular matrix deposition by osteoblast-like cells [6, 7]. Apoptosis, differentiation of vascular smooth muscle cells into osteoblast-like cells, elastin degeneration and degradation, and vessel wall remodeling are involved in these processes.

Although many patients with CKD or those undergoing dialysis are treated with drugs for hyperphosphatemia, such as lanthanum carbonate, relatively large number of patients still have ectopic calcification, indicating that novel and potent anticalcifying drugs are required. Etidronate, a bisphosphonate compound, has an ameliorating effect on

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osteoporosis because of inhibition of bone resorption. At higher doses, it can be used to treat heterotopic ossification caused by the inhibition of calcium phosphate mineralization. It has been reported that etidronate significantly suppresses aortic calcification in patients on hemodialysis [8–10]. Furthermore, etidronate is reported to decrease the intima-medial thickness of the carotid artery in osteopenic patients with type-2 diabetes mellitus [11]. These findings indicate that etidronate has the potential to prevent the progression of vascular calcification. However, high dose of etidronate sometimes causes osteomalacia that increases the risk of bone fracture. It is likely that bisphosphonate, which has a lower risk of causing osteomalacia than etidronate, is an attractive anticalcifying agent.

In rats or mice, bone resorption and vascular calcification seem to be closely correlated as evidenced by Bucay et al. [12] who showed that mice with deficiency of osteoprotegerin, a secreted protein that normally inhibits osteoclast activity, caused both loss of bone calcium and the calcification of arteries. In addition, three bisphosphonates inhibited calcification induced by both warfarin and vitamin D3 in rats at doses showing bone resorption inhibition [13]. It seems that bone resorption inhibition in rats or mice could inhibit calcification due to impairment of releasing calcium and phosphate from the bone. However, clinical trials of newer bisphosphonates that show potent inhibition of bone resorption demonstrated an equivocal effect [14, 15], suggesting that the inhibition of bone resorption alone is insufficient to inhibit calcification in humans. A preferable feature of bisphosphonate compound seems to exert an additional effect apart from being a calcification inhibitor because lowering of the dose based on an effect other than bone resorption inhibition may reduce the risk of osteomalacia which would be a common feature of bisphosphonate at high doses. Based on such a concept, a search for potent anticalcifying bisphosphonates in view of the dissociation of inhibition between calcification and bone resorption was performed, and this has led to the discovery of FYB-931, a novel compound. FYB-931 belongs to 1st generation of bisphosphonate without nitrogen in its molecule, but has more potent bone resorption inhibitory activity than etidronate. In the current study, the effects of FYB-931 on calcification were compared with those of etidronate using cultured human arterial smooth muscle cells and a vitamin D3-induced aortic calcification model of rats.

Materials and methods

Reagents

FYB-931 was synthesized by Research Laboratories 1 in Fuji Yakuhin Co., Ltd. (Saitama, Japan) (Fig. 1). Etidronate

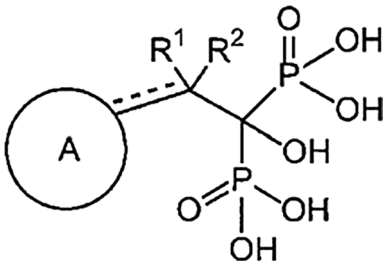

Fig. 1 General structure of FYB-931

was procured from LKT Laboratories Inc (St. Paul, MN, USA). Cholecalciferol (vitamin D3) was procured from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Additionally, all reagents were purchased from Kishida Chemical Co., Ltd. (Tokyo, Japan).

Cell culture and calcification study

Human aortic smooth muscle cells (HASMC) purchased from Kurabo industries Ltd. were cultured in a 24-well plate at 37 °C and 5% CO_2. The medium was changed every 2–3 days. HASMC were cultured in calcific medium containing 3 mmol/L sodium phosphate (pH 7.4) [16] for 7 days in the presence of FYB-931 or etidronate (0, 1, 2, or 4 µmol/L). The cultured cells were observed using a stereomicroscope. HASMC were washed twice with Hanks balanced salt solution and decalcified using 0.5 N hydrochloric acid for 24 h. Calcium content of the solutions as an index of the amount of calcium phosphate deposited in extracellular matrix was determined using the QuantiChrom™ Calcium Assay Kit (Bio Assay Systems, Hayward, CA, USA). The decalcified cells were solubilized using 0.1 N NaOH containing 0.1% sodium dodecyl sulfate and protein content of the samples was determined using the Takara BCA Protein Assay kit (Takara Bio, Inc. Shiga, Japan). Calcium content of the cells was expressed as µg/mg protein.

Vitamin D3-induced aortic calcification in rats

Animals and diets

All applicable institutional and/or national guidelines for the care and use of animals were followed. Eleven-week-old male Wistar/ST rats were purchased from Japan SLC Inc. (Shizuoka, Japan). Rats were given 7 days to acclimatize to the animal house conditions before experimentation. Rats were fed a CE-2 pelleted diet (CLEA Japan Inc., Tokyo, Japan) and were provided tap water ad libitum. Rats were maintained in sterilized cages (2 or 3 per cage) at a temperature of 22 ± 4 °C and relative humidity of 60 ± 20% under a 12-h light/dark cycle.

Treatment

Experiments for FYB-931 and etidronate were performed separately but in the exact same manner. In each experiment, except vitamin D3-untreated normal group (4 per group), rats were divided into groups (6 per group) matched by body weight, and were administered three subcutaneous injections of 125,000 IU/kg vitamin D3 at 0, 24, and 48 h to induce calcification. Each group was orally administered either vehicle, FYB-931 (1.5, 5, or 10 mg/kg), or etidronate (9, 30, or 60 mg/kg) once daily for 14 days. Rats were fasted from 4 h before to 2 h after oral administration because the oral absorption of bisphosphonate is reduced by feeding. After 9 days of initiating drug administration, blood was collected from a jugular vein under isoflurane anesthesia to measure tartrate-resistant acid phosphatase (TRACP) activity as an index of bone resorption. After the final administration, rats were killed by collecting blood via abdominal aorta; further, thoracic and abdominal aortas from aortic arch to bifurcation of the common iliac artery were isolated. Serum was obtained to measure the levels of phosphorus, calcium, and FGF23.

Measurement of calcification in aorta

Thoracic and abdominal aortas were isolated by removing connective tissues, washed with saline and homogenized in 1 N hydrochloric acid. After centrifugation (4 °C, 15,000 rpm, 15 min), supernatants were collected for the determination of phosphorus content. Phosphorus content measured using Phospha-C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as an index of calcification.

Bone metabolic markers in intact rats

Animals and diets

Five-week-old male SD rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The other methods employed were the same as those described in the section Vitamin D3-induced aortic calcification in rats.

Treatment

Rats were divided into groups (4 or 5 per group) and matched by body weight. They were orally administered either vehicle, FYB-931 (3, 10, 30, 100, 300, or 600 mg/kg), or etidronate (10, 30, 100, 300, or 600 mg/kg) once daily for 14 days. The rats were fasted before and after oral administration as described above. After the final administration, rats were killed by collecting blood via abdominal aorta to obtain serum for the measurement of TRACP activity and bone alkaline phosphatase (BAP) activity.

Biochemical analysis

Blood samples were kept for approximately 1 h at room temperature. Serum was separated by centrifuging at a speed of 5000 rpm at 4 °C for 10 min. Serum levels of calcium, phosphorus, FGF23, and TRACP activity were measured using Calcium-E test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), Phospha-C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), FGF-23 ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan), and TRACP & ALP assay kit (Takara Bio, Inc., Shiga, Japan), respectively. TRACP activity, an index of bone resorption, was expressed as the concentration of p-nitrophenol produced in the reaction. BAP activity was measured using the BAP ELISA kit (Kamiya Biomedical Company, WA, USA).

Statistical analysis

Values were expressed as mean or mean ± standard error. Dunnett's multiple comparison test was used to assess the difference in multiple groups after one-way ANOVA. P value less than 0.05 for control was considered statistically significant. The percent inhibition of calcium content of cultured cells, aortic calcification or TRACP was calculated using the mean value of each group according to the following equation:

$$\text{percent inhibition (\%)} = (1 - \text{mean value of each group} / \text{mean value of control group}) \times 100.$$

An ED_{50} and ID_{30}, a dose required for inhibition of aortic calcification by 50% and that required for inhibition of TRACP activity by 30%, respectively, with their 95% confidence interval were calculated using the probit method (statlight 2000, Yukms Co Ltd).

Results

Effects of FYB-931 and etidronate in cultured human aortic smooth muscle cells

Representative microphotographs are shown in Fig. 2. Culture of HASMC in the presence of 3 mmol/L of phosphate resulted in the deposition of calcium (Fig. 2b), which was

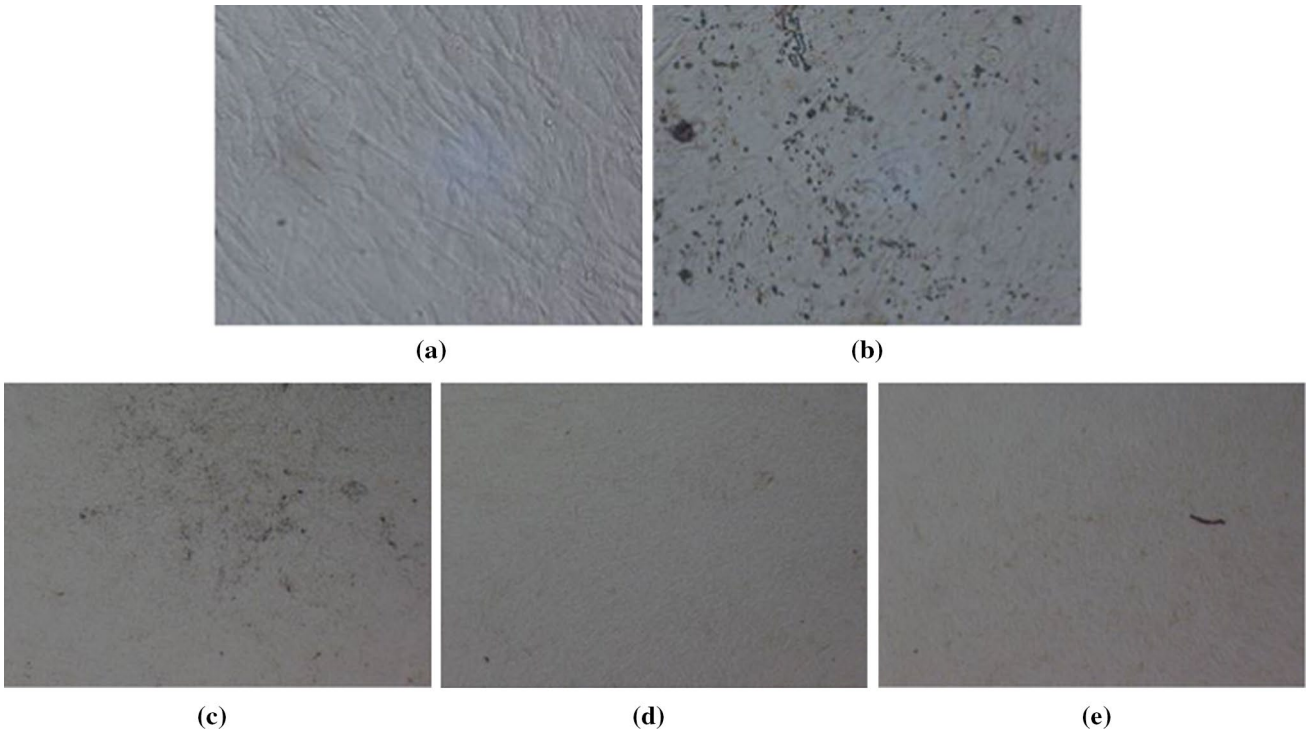

Fig. 2 Microphotographs of cultured human aortic smooth muscle cells in the normal or calcific medium and effects of FYB-931. Cells were cultured for 7 days in the normal (**a**) or calcific medium (**b**–**e**) containing 3 mmol/L phosphate. FYB-931 was added to the calcific medium at concentrations of 1 µmol/L (**c**), 2 µmol/L (**d**) or 4 µmol/L (**e**). The magnification of the stereomicroscope was set to 200 times (**a**, **b**) and 100 times (**c**–**e**). Black spots indicated calcium phosphate deposits (**b**, **c**)

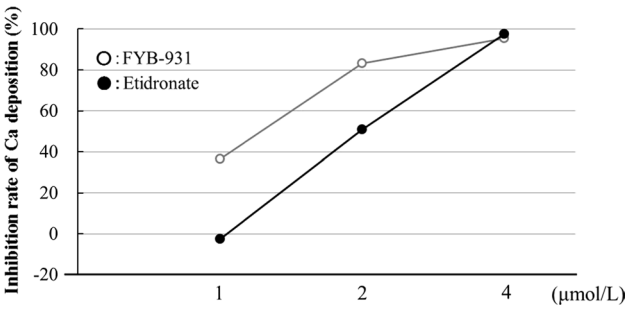

Fig. 3 Effects of FYB-931 and etidronate on calcification of cultured human aortic smooth muscle cells in the hyperphosphatemic condition. Cells were cultured for 7 days in the calcific medium containing 3 mmol/L phosphate with or without FYB-931 or etidronate. After decalcification, cells were solubilized to determine protein content. Calcium content of the cells was expressed as µg/mg protein, and inhibition rate by the compounds were determined. Results were expressed as mean of two separate experiments

inhibited by FYB-931 at 1 µmol/L (Fig. 2c), 2 µmol/L (Fig. 2d), or 4 µmol/L (Fig. 2e). Both FYB-931 and etidronate exhibited concentration-dependent inhibition of calcium deposition; however, FYB-931 was slightly more potent than etidronate (Fig. 3).

Effects of FYB-931 and etidronate on aortic calcification in vitamin D3-treated rats

Vitamin D3-treated rats showed a drastic increase in the phosphorus content in the aorta as compared with the normal group (Fig. 4a). Consecutive administration of FYB-931 dose-dependently decreased the phosphorus content in the aorta with a significant difference at doses of 5 and 10 mg/kg (Fig. 4a). ED_{50} value of FYB-931 for decreasing aortic phosphorus content was 2.5 mg/kg (Table 1). Moreover, etidronate demonstrated a preventive effect on aortic calcification with a significant difference at a dose of 60 mg/kg (Fig. 4a) with ED_{50} value being 26.6 mg/kg (Table 1).

Effects of FYB-931 and etidronate on biochemical parameters in vitamin D3-treated rats

Vitamin D3-treated rats did not exhibit an increase in serum phosphorus level but showed hypercalcemia when compared with the normal group (Fig. 4b, c). FYB-931 dose-dependently reduced the levels of serum phosphorus (Fig. 4b), calcium (Fig. 4c), and calcium-phosphorus product (Fig. 4d). A significant difference in these parameters was observed at doses of 5 and 10 mg/kg of FYB-931 when compared with the control group. Etidronate significantly decreased serum

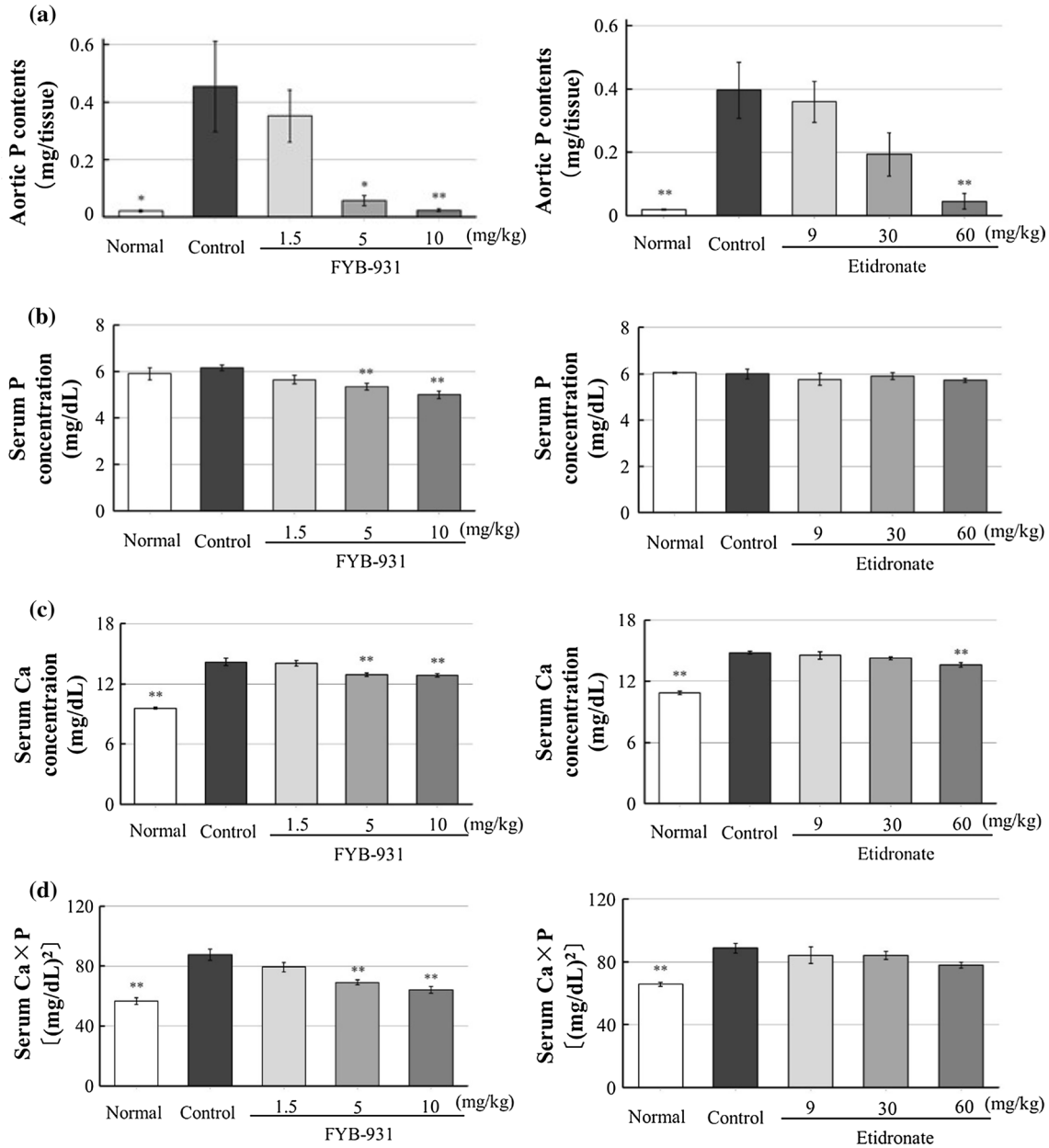

Fig. 4 Effects of FYB-931 and etidronate on aortic calcification and serum biochemical parameters in vitamin D3-treated rats. Rats given 3 subcutaneous injections of vitamin D3 were orally administered FYB-931 or etidronate for 14 consecutive days. On the next day of final treatment, rats were killed to measure the aortic phosphorus (**a**) as an index of calcification and serum phosphorus (**b**), calcium (**c**), and calcium-phosphorus product (**d**). The data were presented as mean ± standard error ($n = 4$ or 6). $^{**}p < 0.01$, $^{*}p < 0.05$: significantly different from the control (Dunnett's multiple comparison test)

calcium level at the dose of 60 mg/kg (Fig. 4c), whereas serum phosphorus and calcium-phosphorus product were not altered by etidronate (Fig. 4b, d).

Effects of FYB-931 and etidronate on bone resorption marker in vitamin D3-treated rats

Vitamin D3-treated rats exhibited an increase in TRACP activity as compared with the normal group (Fig. 5a). FYB-931 dose-dependently decreased TRACP activity with a significant difference as compared with the control group when administered at doses of 5 and 10 mg/kg (Fig. 5a). Etidronate also decreased TRACP activity in a dose-dependent manner with a significant difference at all doses tested (Fig. 5a). ID_{30} values of FYB-931 and etidronate on TRACP activity were 6.0 and 31.0 mg/kg, respectively (Table 1), indicating that FYB-931 more potently inhibited TRACP activity than etidronate by 5.2 times.

Table 1 Inhibitory effects of FYB-931 and etidronate on aortic calcification and serum TRACP activities in vitamin D3-treated rats

Compounds	Dose (mg/kg)	Aorta phosphorus content		Serum TRACP activity	
		Inhibition rate (%)	ED_{50} value (mg/kg)	Inhibition rate (%)	ID_{30} value (mg/kg)
FYB-931	1.5	22.5	2.5	12.4	6.0
	5	87.4		25.7	
	10	94.6		39.7	
Etidronate	9	9.2	26.6	21.5	31.0
	30	51.1		28.6	
	60	88.8		36.1	

Rats given 3 subcutaneous injections of vitamin D3 were orally administered FYB-931 or etidronate for 14 consecutive days. Aorta phosphorus content and TRACP activities were measured using aorta removed after 14 days and serum obtained after 9 days of initiating drug administration, respectively

Effects of FYB-931 and etidronate on serum FGF23 concentration in vitamin D3-treated rats

Vitamin D3-treated rats demonstrated an extreme increase in serum FGF23 concentration compared with the normal group (Fig. 5b). FYB-931 at 5 and 10 mg/kg remarkably and significantly decreased FGF23 concentration at both doses (Fig. 5b). Treatment of etidronate at 30 and 60 mg/kg resulted in a dose-dependent and significant decrease in FGF23 concentration (Fig. 5b).

Effects of FYB-931 and etidronate on bone metabolic markers in intact rats

As shown in Fig. 6a, the administration of FYB-931 and etidronate inhibited TRACP activity at doses of 100 mg/kg and more, and 300 mg/kg and more, respectively, indicating that the inhibitory effect of FYB-931 was more potent than that of etidronate, as shown in vitamin D3-treated rats. On the other hand, treatment of both compounds did not inhibit BAP activity; rather a trend of increase up to 30 mg/kg of FYB-931 and 100 mg/kg of etidronate was observed (Fig. 6b).

Discussion

In patients with diabetes and CKD, vascular calcification is found to be strongly associated with mortality in cardiovascular disease [1]. Thus far, a variety of therapies have been examined with the goal of preventing or retarding vascular calcification. Recent research indicates that SNF472 [17] and TNAP inhibitor [18] are possible candidates. The current study focuses on bisphosphonates, synthetic analogs

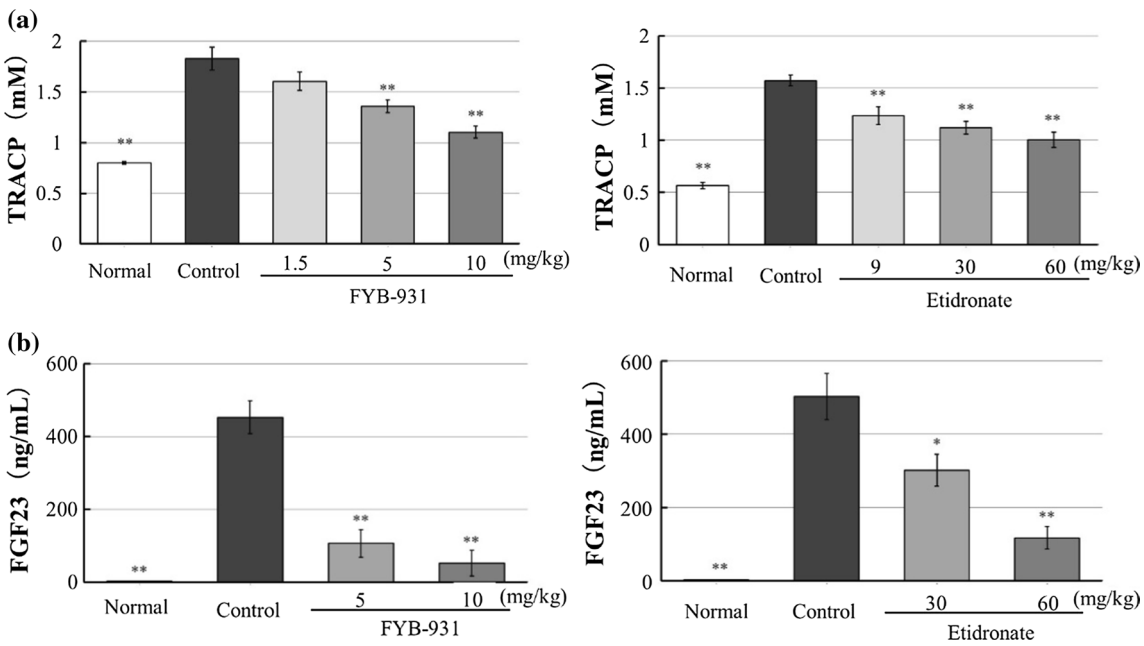

Fig. 5 Effects of FYB-931 and etidronate on serum TRACP activities, and FGF23 concentrations in vitamin D3-treated rats. Rats given 3 subcutaneous injections of vitamin D3 were orally administered FYB-931 or etidronate for 14 consecutive days. TRACP activities (**a**) and FGF23 concentrations (**b**) were measured using serum obtained after 9 days and 14 days of initiating drug administration, respectively. The data were presented as mean ± standard error ($n = 4$ or 6). $^{**}p < 0.01$, $^{*}p < 0.05$: significantly different from the control (Dunnett's multiple comparison test)

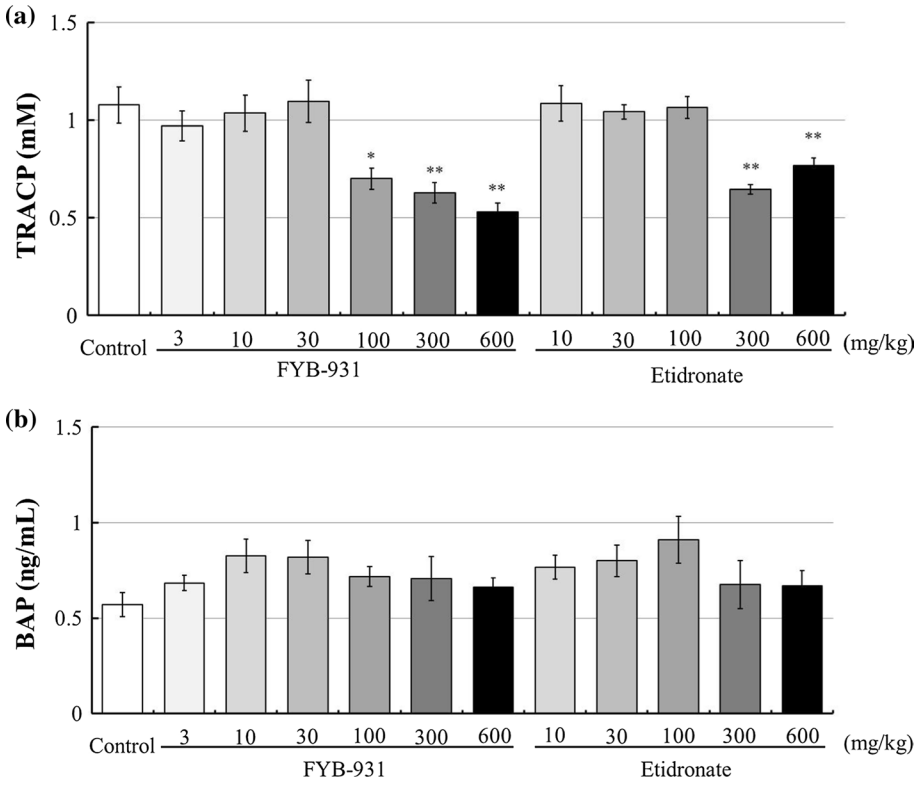

Fig. 6 Effects of FYB-931 and etidronate on bone metabolic markers in intact rats. Rats were orally administered FYB-931 or etidronate for 14 consecutive days. TRACP (**a**) and BAP (**b**) activities were measured using serum obtained after 14 days of initiating drug administration. The data were presented as mean ± standard error ($n = 4$ or 5). $^{**}p < 0.01$, $^{*}p < 0.05$: significantly different from the control (Dunnett's multiple comparison test)

of pyrophosphate, among various candidates of anticalcifying agents, because of their potent and direct effect. Taking into consideration the reports regarding structure–activity relationship of bisphosphonate compounds by Marma et al. [19], diverse compounds were synthesized. Based on the insufficient clinical efficacy of new generation bisphosphonate, a search was performed for bisphosphonate in view of the dissociation of inhibition between calcification and bone resorption. This led to the discovery of a novel compound, FYB-931.

We used rats treated with vitamin D3 as a model of ectopic calcification. Although the model may not be most suitable in view of the pathogenesis of CKD, similar persistent increase in serum calcium is observed in some CKD patients accompanied by second hyperparathyroidism (SHPT). The model shows the advantage in view of less variation of calcification degree than others such as adenine-induced nephropathy. In rats, treatment with high doses of vitamin D3 activates osteoclasts through RANKL/RANK system and increases bone resorption, which leads to hypercalcemia [20] and resultant calcification in soft tissues such as the aorta and kidneys. In the current study, experiments were performed by administering lower doses of vitamin D3 in a manner similar to that reported by Fleisch et al. [21] in contrast to other studies [22–24] in which a large amount of vitamin D3 was used. Consequently, a clear dose–response by FYB-931 and etidronate was obtained without animal deaths.

In this vitamin D3-treated model, FYB-931 was more potent than etidronate in inhibiting aortic calcification. Inhibition of calcification is considered to be mediated by inhibition of both the precipitation and growth of hydroxyapatite crystals. When artery calcification was induced by both warfarin and vitamin D3, three bisphosphonate compounds prevented the calcification at comparable doses that inhibit bone resorption [13], suggesting the predominant role of bone resorption in the calcification process in rats. In the current study, the ID_{30} value of FYB-931 against TRACP activity was 5.2 times lower than that of etidronate, whereas ED_{50} value of FYB-931 on aortic calcification inhibition was about 10 times lower than that of etidronate. These results indicate a difference of inhibition between calcification and bone resorption, in contrast to the results of known bisphosphonates [13], suggesting that FYB-931 has an additional effect independent of that by etidronate. Moreover, it seems that the inhibition of bone resorption by FYB-931 is more potent than that of etidronate, leading to a possible contribution to produce its anticalcifying effect. Furthermore, FYB-931, up to extremely high dose, did not show apparent inhibition of BAP activity, suggesting some therapeutic margin between efficacy and bone formation inhibition. These characteristics may lead to the preference of FYB-931 over etidronate in terms of efficacy at lower doses with less risk of adverse effects, such as osteomalacia.

In vitamin D3-treated rats, a remarkable increase in serum FGF23 was observed. Because renal impairment

was not remarkable as evidenced by slight increase in BUN (data not shown) or no change in serum phosphorus in this model, the increase in FGF23 seems unlikely to be mediated by decreased renal function but due to direct regulation by 1α,25-dihydroxyvitamin D [25]. However, increase in serum phosphorus can be masked by the phospho-diuretic effect of FGF23 and/or decrease in dietary phosphorus intake of animals due to deterioration of general condition of rats in the later period of the experiment. The exact mechanism of how FYB-931 decreased serum FGF23 levels in vitamin D3-treated rats in this experiment remains to be clarified.

In the in vitro study, culture of HASMC in high phosphate medium for 7 days resulted in the deposition of calcium, which was inhibited by FYB-931 and etidronate in a concentration-dependent manner. Regarding the inhibitory mechanism of calcification by bisphosphonate, the physicochemical property for inhibiting calcium phosphate aggregation [7, 26] and biological effect of inhibiting osteogenic differentiation of vascular smooth muscle cells [27–29] are documented. It is reasonable to consider that the superior inhibitory effect of FYB-931 over etidronate on calcification observed in the in vitro study along with more potent bone resorption inhibition than etidronate as shown by TRACP activity and the resultant decrease in calcium-phosphorus product in vivo may contribute to the efficacy of this compound in the vitamin D3 model. Further detailed studies are required to clarify the precise mechanism of anticalcifying effect and the decrease in serum phosphorus by FYB-931.

In conclusion, we consider the advantages of FYB-931 over etidronate are that FYB-931 could prevent aortic calcification more effectively and could inhibit bone resorption with a lesser risk of adverse effects such as osteomalacia. In addition to the inhibition of bone resorption, FYB-931 seems to possess different effects than etidronate, such as causing a decline in the serum levels of phosphorus and FGF23, which could contribute to its calcification inhibition.

Limitations of the study

We did not investigate bone morphology such as bone mass as a result of bone resorption inhibition induced by test compounds. Instead, TRACP, an enzyme secreted from osteoclasts was measured as a biomarker of bone resorption [30] at 9 days after initiating vitamin D3 treatment. Treatment of rats with vitamin D3 caused gradual decrease in locomotor activity and body weight loss. Under these conditions, we observed decrease in TRACP activity; the value at 0, 9 and 14 days after initiating vitamin D3 treatment was 0.474, 1.573 and 0.982 mM, respectively. To assess the effects of compounds on TRACP activity accurately, measurement was performed before the animal condition worsened.

Compliance with ethical standards

Conflict of interest Dr. Takashi Shigematsu serves as a consultant for Fuji Yakuhin. The manuscript does not contain clinical studies or patient data.

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