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ORIGINAL ARTICLE

Effect of a V-ATPase inhibitor, FR202126, in syngeneic mouse model of experimental bone metastasis

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Abstract
Purpose It has been demonstrated that vacuolar ATPase (V-ATPase) is involved in various aspects of bone metastasis. The aim of this study is to investigate the effect of the anti-bone resorptive activity of the V-ATPase inhibitor FR202126 on bone metastases in mice with metastatic breast cancer.
Method As a spontaneous model of breast cancer metastasis to bone, mouse breast cancer cells, 4T1, were injected into the mammary fat pad in immunocompetent syngeneic mice. The mice were orally treated with FR202126 for 29 days. Tumor volume was measured once a week. Thirty days after the injection of the cells, the bone mineral density (BMD) of the proximal tibia was measured using peripheral quantitative computed tomography. Histomorphometric analysis of the distal femurs and the proximal tibiae was performed. To elucidate the mechanism behind the anti-osteolytic effect of FR202126, 4T1 cells were treated directly in vitro with FR202126. Cell viability was measured, and cell invasion was assessed using matrigel.
Results Oral administration of FR202126 significantly increased BMD by reducing the eroded bone surface ratio. While FR202126 is known to potently inhibit osteoclast mediated bone resorption, it did not prevent invasion by cancer cells or their proliferation.
Conclusion The V-ATPase inhibitor FR202126 was found to be effective at ameliorating osteolysis induced by metastatic breast cancer, even when the cancer cells themselves are not significantly affected by it. These results suggest that the anti-bone resorptive effect of the V-ATPase inhibitor might be useful for treating bone metastases associated with breast cancer.

Keywords Vacuolar ATPase · Bone metastasis · Osteolysis · Breast cancer · Osteoclast

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Introduction

It is clinically recognized that bone metastasis occurs in most breast cancer patients and results in bone fracture, bone pain, and hypercalcemia [4]. The biological regulation of breast cancer metastasis to bone must be understood if symptoms arising from bone metastases are to be alleviated. Experimental evidence indicates that the progression of bone metastases consists of several steps in which cancer cells play essential roles. These studies also indicate that one of the three kinds of bone cells, osteoclasts, is required for this progression to occur. Each cell type works in conjunction with the other to develop bone metastases. It has been demonstrated that vacuolar ATPase (V-ATPase) is involved with functions in both cell types during this time.

There are three major classes of cation-translocating ATPases (P-, F-, and V-ATPases). V-ATPases can be distinguished from the P- and F-ATPases on the basis of ion specificity, structure, and inhibitor sensitivity [7, 25, 33]. V-ATPases were discovered as the proton pump of intracellular compartments. In addition, V-ATPases have also been identified in the plasma membrane of specialized cells, such as renal tubular

epithelial cells [9], osteoclasts [2], macrophages [34], ocular ciliary epithelium [36], and breast cancer cells [19, 31]. V-ATPases are essential not only for basic cellular processes, but also for the distinctive functions of specialized cells or tissues.

V-ATPase expressed in the plasma membranes of tumor cells have specialized functions in cell growth [3] and metastasis [31]. Therefore, treatment of breast cancer patients with V-ATPase inhibitor may prevent the developing bone metastasis. Moreover, using V-ATPase inhibitor to treat cancer cells that undergo autophagy to protect themselves from the effects of anti-cancer therapies may cause them to induce apoptosis [12]. In addition, V-ATPase may also induce multidrug resistant tumor cells to increase intracellular pH [35]. These findings suggest that V-ATPase inhibitors can sustain the therapeutic effects caused by other anti-tumor agents.

In the ruffled border membranes of osteoclasts, V-ATPases are highly expressed and work to acidify the resorption lacunae [2]. An acidic microenvironment is required beneath the ruffled border in order for bone resorption to occur. This is because pH plays an essential role in the destruction of two major components of bone. Collagen is degraded by cathepsin K, which achieves optimal activity at an acidic pH, and hydroxyapatite is solubilized by the acidic solution [1]. For this reason, osteoclast V-ATPase is considered to be necessary for bone resorption to occur. Indeed, it is known that in vitro bone resorption is inhibited when V-ATPase genes are suppressed by anti-sense RNA and DNA molecules [15] or small interfering RNA [11]. In in vivo studies, a one subunit knockout of V-ATPase was found to cause severe osteopetrosis due to the loss of osteoclast-mediated extracellular acidification [16]. It has been reported in previous studies that V-ATPase inhibitors significantly prevented in vitro and in vivo bone resorption [23, 24].

Stimulation of osteoclasts by factors produced by tumor cells, such as parathyroid hormone-related protein, interleukin-1, interleukin-6, and interleukin-11 [5, 13, 27], results in osteolysis. Conversely, enhanced osteoclastic bone resorption leads to the expansion of intramedullary space for tumor growth and the release of bone-derived growth factors that enhance cancer cell proliferation in bone metastases [20]. It is possible that the inhibition of bone resorption may directly and indirectly suppress osteolytic bone metastasis. In fact, the prevention of osteoclastogenesis by osteoprotegerin, also known as osteoclastogenesis inhibitory factor, has been shown to be associated with a significant reduction in the skeletal tumor burden in mice with metastatic breast cancer [18]. Bisphosphonates, chemical analogs of pyrophosphate, are known to inhibit osteoclastic bone resorption, although the mechanisms of action are not completely clear [21, 29]. Bisphosphonates inhibit tumor-induced osteolysis in patients with breast cancer [6] and animal models of breast cancer [37]. This suggests that bone resorption inhibitors are effective treatments for bone metastasis. In addition, the acidic microenvironment produced by osteoclasts may contribute significantly to the pain associated with bone cancer because it activates acid-sensitive nociceptors (transient receptor potential vanilloid subtype 1) [8].

In this study, the effect of a V-ATPase inhibitor on bone metastases in mice with metastatic breast cancer was shown for the first time. This study focused on anti-bone resorptive activity, which is one of the functions of V-ATPase, in order to elucidate the roles of individual factors in the improvement of the disease state.

Materials and methods

Animals

Five-week-old female BALB/c mice and pregnant female BALB/c mice (day 14 of the gestation period) were obtained from Nihon SLC (Hamamatsu, Japan). Animals were housed in our animal care facility and kept under standard conditions (room temperature: 23 ± 2°C, humidity: 55 ± 5%, a 12:12 light–dark cycle, free access to regular commercial diet and water). All experimental procedures were evaluated and approved by the animal ethics committee of Fujisawa Pharmaceutical Co. (now known as Astellas Pharma Inc., Tokyo, Japan).

Mouse model of experimental bone metastasis

The mouse breast cancer model developed by Yoneda et al. was used [37]. Mouse breast cancer cells from line 4T1 were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in minimum essential medium alpha modification (α-MEM) containing 10% fetal calf serum (FCS) and 4 mM glutamine at 37°C in a humidified atmosphere of 5% CO_2. After a 1-week acclimatization period, 6-week-old mice were randomized into five groups according to body weight ($n = 10$–11). Fresh medium was supplied to subconfluent 4T1 cells 24 h before injection. Cells (1×10^6 cells) were suspended in 0.1 ml phosphate buffered saline (PBS) and injected into the mammary fat pad of mice under pentobarbital anesthesia. One

group of mice was injected with PBS only (Normal). FR202126 (2,6-dichloro-*N*-[3-methyl-4-(3-methyl-2-oxo-1-imidazolidinyl)-8-quinolinyl]benzamide) suspended in 0.5% methylcellulose was orally administered by gavage at the rate of 5 ml/kg once a day for 29 days starting the day after the cells were injected. One group of mice injected with 4T1 cells (Control) and the mice in the Normal group received the vehicle. Body weights were measured once a week.

The longest and shortest tumor lengths were measured with vernier calipers once a week and the tumor volume was estimated using the following formula: (longest length) × (shortest length)^2/2.

Thirty-days after cell injection, the right tibia was removed from all mice and stored in 70% ethanol. Cross-sectional bone mineral density (BMD) was measured at three points from the edge of the proximal end (1.3, 1.6, and 1.9 mm) using peripheral quantitative computed tomography (pQCT) (XCT-960A, Stratec Medizintechnik, Birkenfeld, Germany). The following settings were used: voxel size = 0.148 mm, contour mode = 1, threshold = 145 mg/cm^3. Individual values are expressed as the mean of the BMDs measured at three points.

The left hind limbs were dissected and fixed in 10% formalin neutral buffer solution. After decalcification with 10% EDTA solution for 4 weeks, longitudinal paraffin sections 5 μm thick were prepared. All specimens were stained with hematoxylin-eosin. The bone eroded surface (with osteoclast-like cells) per bone surface was measured on the primary spongiosa of the metaphysis of both the distal femur and proximal tibia using commercial software (Image Pro Plus Version 4, Media Cybernetics, Silver Spring, MD, USA) and without knowledge of the type of treatment.

Oral pharmacokinetic studies

Female BALB/c mice, 8 weeks of age, were used for pharmacokinetic studies. Under light ether anesthesia, blood samples were collected from heart ventricle by heparinized syringes at 30, 60, 120, 240, and 360 min after oral administrations of FR202126. Plasma was separated and assayed by HPLC using the following equipment and analytical conditions. Plasma (0.1 ml) was mixed with 0.2 ml of methanol, 0.5 ml of 50 mM phosphate buffer (pH 10.0) and 4 ml of ethyl acetate and the mixture was shaken for 5 min. Then it was centrifuged (2,500 rpm, 5 min) and 3 ml of the organic phase were dried under N_2 gas. The residue was dissolved in 100 μl of methanol and 20 μl of the solution were injected onto the HPLC system (HPLC 600E system, Waters, Milford, MA, USA) equipped with a Develosil (Nomura Chemical, Seto, Japan) and ultraviolet light detector at 255 nm. The mobile phase was composed of CH_3CN (43%, v/v) and 10 mM phosphate buffer (pH 9.0) (57%, v/v). The flow rate was 1 ml/min, and the column temperature was 30°C.

Effect of FR202126 on the growth of breast cancer cells

4T1 Cells (6.5×10^3 cells per 100 μl) were seeded into 96-well tissue culture dishes and incubated for 4 days with various concentrations of FR202126. Cell growth was determined by measuring the fluorescence of an oxidized indicator (reading wavelength: 590 nm, excitation wavelength: 544 nm), which formed after the cells were treated with alamarBlue (BioSource International, Camarillo, CA, USA) for 4 h at 37°C.

Effect of FR202126 on invasion by breast cancer cells

Matrigel invasion chambers (BD Biosciences, Bedford, MA, USA) were used to evaluate in vitro cell invasion through extracellular matrix proteins. Conditioned media prepared from bone organ culture were used as the chemoattractant. Specifically, calvariae from neonatal BALB/c mice were excised and cultured in α-MEM (10% FCS, 4 mM glutamine) supplemented with 10 nM human parathyroid hormone (PTH) fragment (1–34) (Peptide Institute, Osaka, Japan), which induced bone resorption. The volume of medium was 1.5 ml per calvaria. Six days later, the concentration of Ca^{2+} in the media was measured using a commercial kit (Wako Pure Chemical Industries, Osaka, Japan), and was found to have increased due to PTH-stimulated bone resorption. Conditioned media (700 μl) was then placed in the wells of a companion plate, after which the matrigel inserts or control inserts were added. Next, 200 μl of 4T1 cells (5×10^4 cells) suspended in α-MEM (10% FCS, 4 mM glutamine) was immediately added to the chambers. At that time, the concentration of Ca^{2+} in the cell suspension was adjusted to be equal to that in the conditioned media. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h in the presence or absence of FR202126. The cells invading through the membrane insert were stained with Diff-Quik solution (International Reagents Corporation, Kobe, Japan) and counted under a microscope. The data is expressed as the percent of cells that invaded by passing through the matrigel matrix and membrane relative to the number that migrated through the control membrane. A similar experiment using slightly metastatic human breast cancer cells (MCF-7 cells) in Dulbecco's modified Eagle's Medium (rather than α-MEM) was conducted as a negative control.

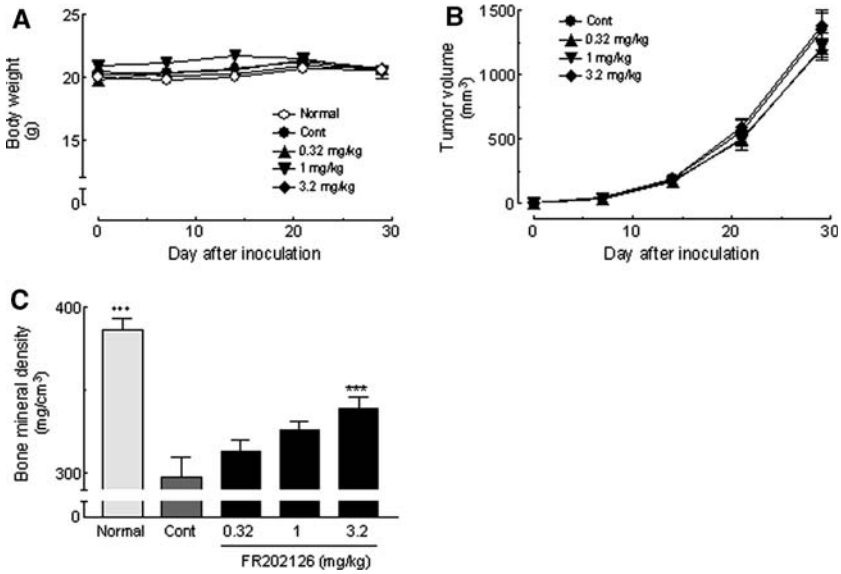

Fig. 1 Effect of FR202126 in mice inoculated with 4T1 cells. a Body weight change. Each value represents the mean ± SEM for 10–11 mice. b Tumor volumes in the mammary fat pad. Each value represents the mean ± SEM for 10–11 mice. c BMD of proximal tibia. Each value represents the mean ± SEM for 10–11 mice. A significant difference between the normal and control (*Cont*) groups was found $^{+++}P < 0.001$ (Student's *t*-test). A significant difference between the Cont and FR202126 treatment groups was also found $^{***}P < 0.001$ (*Dunnett*)

Statistical analysis

The results are expressed as the mean ± SEM. A one-way analysis of variance was performed, followed by Dunnett's test for multiple comparisons. The Student's *t*-test (two-tailed) for independent samples was applied to examine the significance of the differences between the means of the two measurements. Commercial software (GraphPad Prism Version 3, GraphPad Software, San Diego, CA, USA) was used to make the statistical comparisons. Differences were considered to be statistically significant at $P < 0.05$.

Results

Mouse model of experimental bone metastasis

Our preliminary experiments demonstrated that mice begin to die 30 days after tumor inoculation (data not shown). Therefore, the effect of FR202126 on osteolysis was estimated after 29 days of administration. FR202126 treatment did not affect body weight (Fig. 1a) or tumor volume (Fig. 1b). Lung and liver metastases were also observed in some of the mice, but the occurrence of visceral metastases did not appear to have been affected by treatment with FR202126.

At 30 days after cell injection, the hind limbs began to show clinical landmarks of bone metastasis. Breast cancer cells had metastasized into both ends of the shafts of the hind limb bones, and the trabecular bone decreased noticeably in mice injected with 4T1 cells compared with the Normal group (Fig. 2a, b). When viewed at higher magnification, a vigorous resorption of trabecular bone was apparent, and a large number of osteoclast-like multinuclear giant cells were present near the metastatic tumor (Fig. 2d). On the other hand, tumor cells were not observed in the resorption lacunae.

Administration of FR202126 partially restored trabecular bone loss, and metastatic skeletal lesions tended to become smaller (Fig. 2c). When examined at higher magnification, a reduced number of osteoclasts on the bone surface were noted, although tumor cells remained near the bone (Fig. 2e). This finding was reflected in the fact that the bone eroded surface ratio (with osteoclast-like cells) in the 3.2 mg/kg FR202126 group decreased significantly (Table 1).

There was a significant decrease in the BMD of the proximal tibia in mice injected with 4T1 cells. Oral administration of 3.2 mg/kg FR202126 significantly improved the BMD of the proximal tibia (Fig. 1c).

Pharmacokinetics in mice

Figure 3 shows pharmacokinetics of FR202126 in fed mice. C_{max} was achieved at 1 h after administration, and then the plasma concentration was gradually reduced. C_{max} and $AUC_{0-6\,h}$ value were 0.14 μg/ml and 0.301 μg h/ml for 3.2 mg/kg.

Effect of FR202126 on the growth of breast cancer cells

To elucidate the mechanism of FR202126's anti-osteolytic effect, 4T1 cells were treated with FR202126 directly in vitro. It has been demonstrated previously that treatment with 0.1 μM FR202126 caused almost complete inhibition of in vitro bone resorption induced

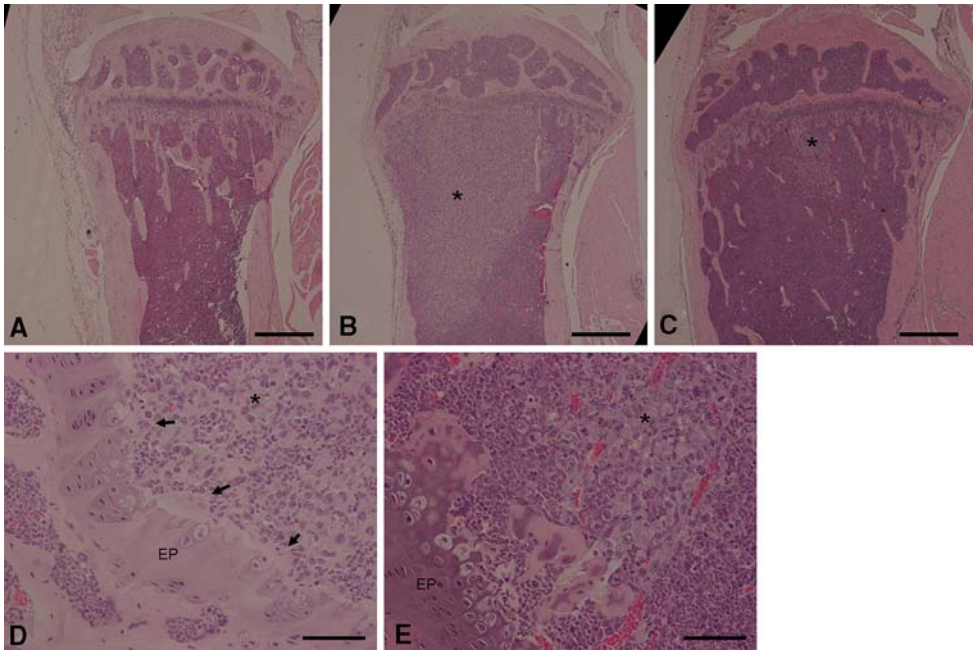

Fig. 2 Histopathology of the proximal tibiae of Normal mice (**a**), Control mice (**b**), and FR202126 (3.2 mg/kg)-treated mice (**c**). The scale bar indicates 500 μm. Hematoxylin-eosin staining. Higher magnification of the epiphysial plate area in the Control (**d**) and FR202126 (3.2 mg/kg)-treated groups (**e**). *Arrows* indicate activated osteoclasts. *Metastasized tumor. *EP* epiphysial plate. The *scale bar* indicates 50 μm. Hematoxylin-eosin staining

Table 1 Effect of FR202126 on bone eroded surface ratio

	Normal	Metastasis	
		Control	FR 3.2 mg/kg
ES/BS (%)	$4.0 \pm 0.4^{+++}$	10.7 ± 1.0	7.5 ± 0.8*

Values are expressed as the mean ± SEM for 10–11 mice. A significant difference between the Normal and Control was found $^{+++}$ $P < 0.001$ (Student's *t*-test). A significant difference between the control and 3.2 mg/kg FR202126 (FR 3.2 mg/kg) was found $^{*}P < 0.05$ (Student's *t*-test)

ES eroded surface, *BS* bone surface

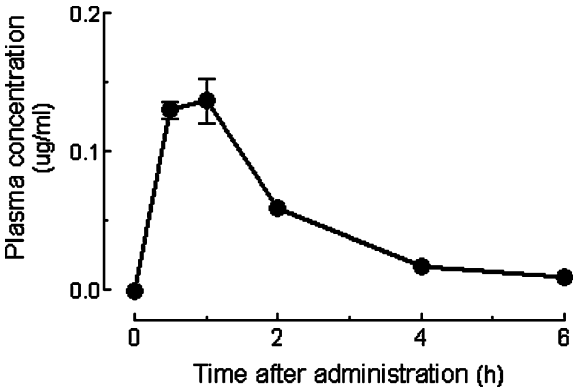

Fig. 3 Concentrations of FR202126 determined in the plasma of female BALB/c mice given orally at doses of 3.2 mg/kg. Each value represents the mean ± SEM for three mice

by interleukin-1, interleukin-6, or PTH [23]. For this reason, the effect of FR202126 on the growth of breast cancer cells was examined in vitro at concentrations up to 0.1 μM. Unlike bone resorption, the growth of 4T1 cells was not inhibited at these concentrations (Fig. 4). On the other hand, doxorubicin (1 μM), which was used as a positive control compound, inhibited the proliferation of 4T1 cells remarkably (Fig. 4).

Effect of FR202126 on the invasion of breast cancer cells

Compared to slightly metastatic breast cancer cells (MCF-7 cells), 4T1 cells were able to invade by passing through the matrix proteins (Fig. 5). This result reflects

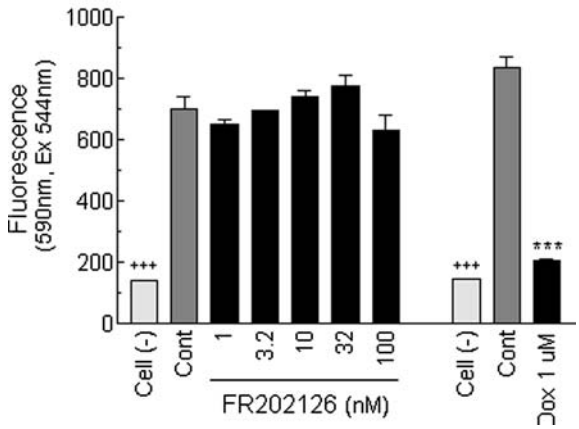

Fig. 4 Effect of FR202126 on the proliferation of 4T1 cells. Cells were cultured for 4 days in the presence or absence (*Cont*) of FR202126 or 1 μM doxorubicin (*Dox*). Medium alone was added to the dishes that contained no cells [Cell (-)]. The *bars* represent the mean ± SEM for four dishes. A significant difference between Cell (-) and Cont was found $^{+++}P < 0.001$ (Student's *t*-test). A significant difference between Cont and 1 μM Dox was also found $^{***}P < 0.001$ (Student's *t*-test)

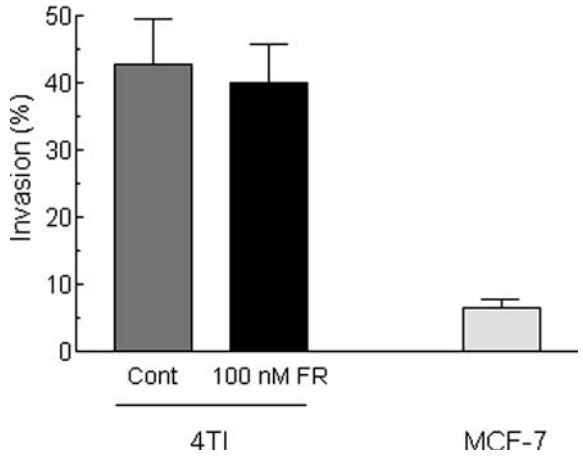

Fig. 5 Effect of FR202126 on the invasion of 4T1 cells through matrigel. Data are expressed as the percent invasion through the matrigel matrix and membrane relative to the migration through the control membrane using the mean of three dishes for each. A similar experiment was conducted using MCF-7 cells

the metastatic abilities of these cells. In order to evaluate the contribution of V-ATPase to the invasion of 4T1 cells, parallel experiments were performed with cells incubated with 0.1 μM FR202126. No significant effects occurred (Fig. 5), which suggests that V-ATPase is not involved in the invasion of 4T1 into bone.

Discussion

This study is the first to show that V-ATPase inhibitor is effective against osteolysis induced by metastatic breast cancer using a spontaneous model of bone metastasis. Since it has been shown that this model includes all the steps in bone metastasis and reflects the pathologic features well [37], it seemed suitable for examining the effects of FR202126. The severity of osteolysis has traditionally been estimated by examining radiographs of long bone and then assigning a score. However, this is the first study assessed using a quantitative method, pQCT, which is the method used in the clinical setting.

V-ATPase has been implicated in several steps of the osteolytic bone metastasis of breast cancer cells [31, 32]. V-ATPase is located in the ubiquitous intracellular acidic compartments such as endosomes, lysosomes, Golgi apparatus, and secretory vesicles. V-ATPase is also present on the plasma membranes of specialized cells, including osteoclasts [2] and breast cancer cells [19, 31], and works to extrude protons from the cytosol to the extracellular fluid. In the development of bone metastases, it is known that the key players include not only tumor cells, but osteoclasts as well [20, 38]. Therefore, in this experiment it is possible that the amelioration of osteolysis was brought about in both cells by the inhibition of V-ATPase.

It has been demonstrated that V-ATPase inhibitors have the potential to prevent bone resorption. It has been shown that V-ATPase inhibitors are highly effective in preventing proton extrusion from the cytoplasm of osteoclasts, while simultaneously inhibiting bone resorption induced by various stimulators [22, 23]. It has been clearly demonstrated that FR202126 exerts inhibitory effects on bone resorption in vitro with IC_{50} values of 2.6–20 nM. FR202126 has also been shown to reduce the hypercalcemia induced by retinoic acid in thyroparathyroidectomized-ovariectomized rats, which indicates an in vivo anti-resorptive effect [23]. In experiments on rats with periodontitis, treatment with FR202126 improved the condition of damaged alveolar bone by inhibiting bone resorption [23]. In the current study, histological examination revealed that the bone eroded surface (with osteoclast-like cells) decreased when 3.2 mg/kg doses of FR202126 were administered. This result agrees with those found in the previous studies.

On the other hand, in the present study, the effect of FR202126 on breast cancer cells does not contribute to the prevention of osteolysis. V-ATPase is present on the plasma membranes of several breast cancer cell lines and tumor cells derived from breast cancer patients and is thought to have specialized functions [19, 31]. In vitro studies using the V-ATPase inhibitor bafilomycin A_1 suggest that V-ATPase is involved in the growth [3] and invasive ability of tumor cells [31]. Accordingly, the inhibition of osteolysis by FR202126 might have been the result of its direct effect on 4T1 cells. However, the direct effects of FR202126 against 4T1 cells were not clearly observed in vitro, and the primary tumor volume was not changed by the administration of FR202126 in vivo. In addition, there are no reports about the presence of V-ATPase on the plasma membrane in 4T1 cells. V-ATPase could not be found in the plasma membrane of 4T1 cells immunohistochemically stained using anti-body against bovine V-ATPase subunit A (data not shown). Consequently, it seems likely that, in this experiment, the anti-osteolytic effect of FR202126 was not a result of the direct effect on tumor cells, but rather the inhibition of osteoclastic bone resorption. The decrease in the tumor area in the bone might be also have been caused indirectly by suppressing the release of bone-derived growth factors, which was induced by the inhibition of osteoclastic bone resorption.

Considering the result from previous experiment and the pharmacokinetic data, we estimated the

in vitro effect of FR202126 up to 0.1 μM. After oral administration of FR202126 at dose of 3.2 mg/kg in fed mice, the pharmacokinetic analysis showed that for the most part the plasma concentration of FR202126 was less than 0.05 μg/ml, which was comparable to 0.1 μM and the concentration at 6 h after administration was ~0.01 μg/ml. In addition, it has been demonstrated previously that 0.1 μM of FR202126 is sufficient enough to inhibit in vitro bone resorption [23]. It is thought that the effect at the higher concentrations of FR202126 would be hardly exerted in the mice treated with 3.2 mg/kg FR202126.

In this study, we found that administration of FR202126 reduced the number of osteoclasts. It may be due to interaction between osteoclasts and metastasized cancer cells. It has been demonstrated that cytokines secreted by tumor cells appears to stimulate osteoclast differentiation and bone resorption in a paracrine manner through cytokines receptor-immunopositive cells. Conversely, enhanced osteoclastic bone resorption leads to the expansion of intramedullary space for tumor growth and the release of bone-derived growth factors that enhance cancer cell proliferation in bone metastases [20]. Therefore, inhibition of bone resorption might break the correlation, resulting in a reduced number of osteoclasts. In addition, it is possible that FR202126 specifically promoted cell death of osteoclast by induction of cytosolic acidification. Our previous study has reported that exposure to V-ATPase inhibitor results in cytosolic acidification of osteoclast due to inhibition of the plasma membrane V-ATPase though the pHi of other cells did not be affected [22]. It has been shown that several apoptotic signaling pathways are affected by a cytosolic acidification, and consequently, low intracellular pH facilitates the occurrence of apoptosis [14]. Accordingly, cell death might be directly induced by FR202126.

Bisphosphonates are also potent inhibitors of bone resorption [21] that have been used to treat skeletal metastases [6]. However, discrepant clinical study results have been reported for the efficacy of bisphosphonates against skeletal metastases [26, 30]. In addition, resistance to bisphosphonates has been observed in hypercalcemic patients treated with bisphosphonates [17] and in those with Paget's disease treated with pamidronate [10]. Moreover, unlike FR202126 [23], bisphosphonates have a relatively slow onset of action (1–3 days) [28]. Therefore, V-ATPase inhibitors may be a better treatment option for patients with bone metastases.

The results of this study have shown that V-ATPase inhibitor is efficacious against osteolysis induced by metastatic breast cancer, 4T1. Moreover, it seems likely that the beneficial effect was produced by the anti-resorptive activity of FR202126 alone. Considering that FR202126 is expected to ameliorate the bone cancer-associated pain caused by activation of acid-sensitive nociceptors (transient receptor potential vanilloid subtype 1), [8] and have a direct effect on cancer cells that express V-ATPase in the plasma membranes, it would be interesting to investigate the effects of V-ATPase inhibitor in the future using other experimental models.

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