A NOVEL INHIBITOR OF VACUOLAR ATPase, FR202126, PREVENTS ALVEOLAR BONE DESTRUCTION IN EXPERIMENTAL PERIODONTITIS IN RATS

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ABSTRACT — An acidic microenvironment formed by vacuolar ATPase (V-ATPase) expressed in plasma membranes of osteoclasts is thought to be indispensable for bone resorption. This study examined the efficacy of a novel V-ATPase inhibitor, FR202126, in reducing alveolar bone loss caused by experimental periodontitis in rats. FR202126 inhibited H+ transport in plasma membrane vesicles of murine osteoclasts, whereas FR202126 exerted no effect on H+ transport of mitochondrial ATPase or gastric H+,K+-ATPase, indicating that FR202126 is a specific inhibitor of V-ATPase. As expected from the mechanism, FR202126 remarkably inhibited in vitro bone resorption whatever bone resorptive factors were added. Moreover, FR202126 was also able to exert an inhibitory effect on in vivo bone resorption. Experimental periodontitis was induced by ligature wire tied around the contact between the first and second maxillary molars. Insertion of ligature wire for 7 days induced alveolar bone destruction by activating osteoclasts. Oral administration of FR202126 (u.i.d.) significantly prevented alveolar bone loss in experimental periodontitis which may offer a new approach to treatment of periodontal disease.

KEY WORDS: Periodontitis, Vacuolar ATPase, Alveolar bone loss, Osteoclast

INTRODUCTION

The primary cause of periodontitis is accumulation of persistent bacterial plaque, which results in inflammation of the gums. Inflammation finally induces excessive resorption of alveolar bone, which supports tooth roots. Therefore inhibition of alveolar bone destruction is expected to directly lead to blockage of tooth loss. Nonetheless, direct inhibitors of bone resorption have never been therapeutically used.

Matrix metalloproteinases (MMPs), which are optimally active at neutral pH, have been thought to promote degrade of alveolar bone in periodontitis. MMPs are secreted by polymorphonuclear leukocytes, macrophages and fibroblasts, which are activated by inflammatory cytokines. On the other hand, every bone, including alveolar bone, is directly resorbed by activated osteoclasts which attach to the bone surface and form an acidic microenvironment. This acidic microenvironment is required for bone resorption because hydroxyapatite is solubilized in acidic solution and a collagen-digested protease, cathepsin K, is optimally active at acidic pH (Aibe et al., 1996). Accordingly, from the point of view of osteoclast function, disturbance of acid environment is expected to lead to blockage of alveolar bone resorption.

V-ATPase is one of three major classes of ATP-driven cation pumps (V-, P- and F-ATPase). V-ATPases are highly expressed in plasma membrane of osteoclasts and function to acidify the resorption lacunae (Blair et al., 1989). Suppression of gene expression of V-ATPases by antisense RNA and DNA molecules inhibits bone resorption (Laitala and Vaananen, 1994). One subunit knockout of V-ATPase caused severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification (Li et al., 1999). Consequently, plasma membrane V-ATPase of osteoclasts (osteoclast V-ATPase) is thought to be a good molecular target for reducing bone loss.

Bafilomycin A1, the first V-ATPase inhibitor

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described (Bowman et al., 1988), remarkably inhibits bone resorption in vitro (Sundquist et al., 1990). However, bafilomycin A₁ cannot be administered even in animal experiments, because it exhibits in vivo severe toxic effect (Keeling et al., 1998). In addition, it is not clear whether the toxic effect of bafilomycin A₁ is derived from the inhibition of V-ATPase. Recently, we obtained a novel potent V-ATPase inhibitor, FR202126. This study showed for the first time the effect of the V-ATPase inhibitor on alveolar bone destruction in a periodontal model using FR202126. In this study, oral administration of FR202126 significantly prevented alveolar bone loss in experimental periodontitis, which suggests that acidic microenvironment plays an important role in periodontal alveolar bone destruction as well and may offer a new approach to the treatment of periodontal disease.

**MATERIALS AND METHODS**

**Animals**

Fourteen days pregnant female Wistar rats and 9-week-old female Wistar rats were obtained from Clea Japan (Tokyo, Japan). Eight-week-old male ddY mice, 14 days pregnant female ddY mice and 13-week-old Japanese White rabbits were obtained from Nihon SLC (Hamamatsu, Japan). Animals were housed in our animal care facility and kept under standard conditions (room temperature of 23 ± 2°C, humidity of 55 ± 5%, 12 : 12 light–dark cycle, free access to normal commercial diet and water). All experimental procedures have been subjected to evaluation and were approved by the animal ethics committee of Astellas Pharma Inc..

**H⁺-transport assay**

Mouse osteoclasts were formed in the coculture of spleen cells obtained from ddY mice and ST2 cells (murine bone marrow stromal cells) (Riken, Tsukuba, Japan) according to Niikura et al. (2004). Plasma membrane vesicles of murine osteoclasts were isolated (Koizumi et al., 1976). Mitoplasts (inner membrane vesicles of mitochondria) were obtained from ddY mouse liver according to Wlodawer et al. (1966). Rabbit gastric microsomes were prepared according to Tomoi et al. (1988). H⁺ transport by various membrane vesicles was assayed with a dual wavelength spectrophotometer (UV-3000, Shimadzu, Kyoto, Japan) by measuring uptake of acridine orange (Niikura et al., 2004).

**Assay for in vitro effect of bone resorption**

Calvariae from neonatal ddY mice or Wistar rats were excised and cultured in 2 ml DMEM (10% FBS) supplemented with 1 ng/ml human IL-1β (Genzyme–Technne, Cambridge, MA, USA) in the presence of FR202126. Six days later, the concentration of Ca²⁺ in the media was measured by commercial kit (Wako Pure Chemical Industries, Osaka, Japan) according to the methylene blue method.

In addition, murine calvariae were treated with 100 ng/ml human IL-6 plus 500 ng/ml human IL-6 soluble receptor (R&D Systems, Minneapolis, MN, USA) or 10 nM human parathyroid hormone (PTH) (Peptide Institute, Osaka, Japan). The effects of FR202126 in these stimulated cultures were also estimated.

**Assay for in vivo effect of bone resorption**

The method was previously described (Niikura et al., 2005). Ten-week-old female Wistar rats were ovarioctomized (OVX) and thyroparathyroidectomized (TPTX), which caused hypocalcemia. These rats were administered 6 mg/kg of all-trans retinoic acid (RA) (Sigma, St. Louis, MO, USA) subcutaneously for 3 days. Rats were fasted overnight after the last RA administration. On the day of the assessment, blood was taken by retroorbital puncture prior to administration of FR202126 and at indicated time, and then serum Ca²⁺ was measured by the methylene blue method. FR202126 was suspended in 0.5% methylcellulose and orally administered at a volume of 5 ml/kg (body weight).

**Oral pharmacokinetic studies**

Male and female Wistar rats, 10 weeks of age, were used for pharmacokinetic studies. Prior to drug administration, femoral arterial catheters for blood sample withdrawal were implanted. Blood samples were collected by heparinized syringes at 15, 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 (2500 rpm, 5 min) and 3 ml of the organic phase were dried under N₂ 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$_3$CN (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.

Serum concentration of FR202126 in the TPTX-OVX rats was also monitored.

**Periodontal model**

Ten-week-old male Wistar rats were randomized according to body weight into 4 groups. Experimental periodontitis was induced by ligature wire (diameter: 0.25 mm) (Tomy International, Tokyo, Japan) tied around the contact between the first (M1) and second (M2) maxillary molars (Konishi et al., 2000). One group of rats was sham operated (Sham), FR202126 suspended in 0.5% methylcellulose was orally administered once a day at a volume of 5 ml/kg from the day of surgery. One group of operated rats (Cont) and sham group rats received vehicle. Seven days after tying, the maxillas were dissected and fixed in 10% formalin neutral buffer solution. After decalcification with 10% EDTA solution for 4 weeks, 5 μm-thick mesio-distal longitudinal paraffin sections, including interdental alveolar bone between M1 and M2, were prepared. All specimens were stained with hematoxylin-eosin. Two sections per side, which were cut through the center of the distal buccal root of M1 and the center of the mesial buccal root of M2, and the center of the distal lingual root of M1 and the center of the mesial lingual root of M2, were chosen for histomorphological analysis (Fig. 5 A). The remaining area of alveolar bone was measured within the field, which was enclosed by the cemento-enamel junction (CEJ) of M1, the CEJ of M2, the apical end of the distal root of M1, and the apical end of the mesial root of M2. The ratio of the area of alveolar bone to the interdental area within the above-mentioned field was also calculated. The distance between the alveolar bone crest and the CEJ taken from the two molars was measured. All measurements were performed using commercial software (Image Pro Plus Ver 4, Media Cybernetics, Silver Spring, MD, USA) without knowledge of the treatment allocation.

**Statistical analysis**

Results are expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance followed by Dunnett test (Dunnett, 1955) for multiple comparisons. Student’s t test (Gosset, 1908) (two-tailed) for independent samples was applied to examine the significance of the differences between the means of the two measurements. A 50% inhibitory concentration (IC$_{50}$) was obtained by fitting sigmoidal curve to data. For statistical comparisons and calculation of IC$_{50}$, commercial software (GraphPad Prism Ver 4, GraphPad Software, San Diego, CA, USA) was used. Differences were considered to be statistically significant at p<0.05.

**RESULTS**

**Inhibition of H$^+$ transport Activity**

FR202126, 2,6-dichloro-N-[3-methyl-4-(3-methyl-2-oxo-1-imidazolidinyl)-8-quinolinyl]benzamidine, (Fig. 1) inhibited H$^+$ transport in plasma membrane vesicles of murine osteoclasts (IC$_{50}$ 99 nM), whereas it exerted no effect on H$^+$ transport of mitochondrial ATPase (F-ATPase) or gastric H$^+$.K$^+$-ATPase (P-ATPase) up to 10 μM (Fig. 2). Therefore, FR202126 is thought to be a specific inhibitor of V-ATPase.

**Effect on Bone Resorption in vitro**

Next we investigated whether inhibition of osteoclast V-ATPase by FR202126 consequently can cause blockade of bone resorption. IL-1 and IL-6 were detected in gingival crevicular fluid from patients with chronic periodontitis (Reinhardt et al., 1993). In addition, it has been reported that both cytokines induce osteoclast formation and exhibit potent bone resoring activity through separate mechanisms (Suda et al., 1999).

![Fig. 1. Chemical structure of FR202126.](image-url)
Mouse calvariae treated with each of two stimulators, IL-1 or IL-6, released more calcium into the culture media than did the control. FR202126 could significantly inhibit each bone resorption induced by these two stimulators (IC50, 20 nM and 2.6 nM, respectively) (Fig. 3). In addition, FR202126 also exerted a similar inhibitory effect on PTH-induced bone resorption (IC50, 9.6 nM) (Fig. 3). FR202126 also inhibited bone resorption induced by IL-1 in rat calvariae with an IC50 of 12 nM, suggesting that FR202126 can inhibit rat as well as mouse osteoclast V-ATPase.

**Effect on in vivo Bone Resorption**

It has been known that excess vitamin A directly leads to stimulation of osteoclastic bone resorption in vitro (Teti et al., 1987) and administration of a large amount of vitamin A increases serum Ca2+ in rats (Trechel et al., 1987). Therefore, we used hypercalcemic rats induced by administration of excess RA as an *in vivo* model to investigate the effect on bone resorption.

TPTX-OVX caused hypocalcemia with serum Ca2+ concentrations around 50 mg/l. The increase in serum Ca2+ (around 90 mg/l) was observed in TPTX-OVX rats given RA.

FR202126 promptly reduced hypercalcemia induced by RA in TPTX-OVX rats in a dose-dependent manner, reaching a plateau at a dose of 1 mg/kg (Fig. 4). The results indicate that FR202126 is also effective in inhibiting *in vivo* bone resorption.

**Effect on Alveolar Bone Loss in Periodontal Rats**

Insertion of ligature wire induced alveolar bone destruction (Photo 1 A, B). On the surface of alveolar bone were large numbers of vigorous osteoclasts with many resorption lacunae (Photo 1 C). All three parameters (Fig. 5 A) used in the histomorphometric analysis also demonstrated alveolar bone loss around ligated teeth in Cont rats (Fig. 5 B-D).

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**IC50 (nM)**

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<th>V-ATPase (osteoclast)</th>
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<th>P-ATPase (stomach)</th>
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**Fig. 2.** Effect of FR202126 on ATP-dependent H+ transport activity of V-ATPase, F-ATPase and P-ATPase. H+ transport was assayed by monitoring absorption change of acridine orange at 492-540 nm (an index of the amount of acridine orange in free solution) in the presence of FR202126. The reaction was initiated by adding ATP (final concentration 1 mM). The initial rate of change in the absorbance of acridine orange was used for calculation of IC50 value. FR202126 did not inhibit H+ transport activity of F-ATPase and P-ATPase up to 10 μM. Each value represents the average of triplicated determinations with SEM shown by vertical bars.

**IC50 (nM)**

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<th>IL-1</th>
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**Fig. 3.** Effect of FR202126 on bone resorption of mouse calvariae. Values are expressed as percentage of inhibition, which was calculated by taking the difference between the mean medium Ca2+ concentration in non-stimulated wells and stimulated wells as 100%. The increases in the mean medium Ca2+ concentration were 16.8 mg/l for hIL-1β, 6.6 mg/l for IL-6 and 18.4 mg/l for hPTH. Values are the mean ± SEM for 5 dishes. Significantly different between non-stimulated wells (○, △, ○) and each of the three different stimulated wells (***p<0.01 (IL-6), **p<0.001 (IL-1β and PTH)) (Student’s t test). *p<0.05, **p<0.01 vs. stimulator control (Dunnett).
A Novel V-ATPase inhibitor, FR202126, prevents alveolar bone loss.

Using pharmacokinetic analysis with oral dosing, it was shown that plasma concentration of FR202126 was affected by feeding and sex. FR202126 was administered to TPTX-OVX female rats under fasting condition whereas periodontal male rats were fed. Maximum concentration (Cmax) and area under the concentration-time (0-6 hr) curve (AUC0-6 hr) value of 3.2 mg/kg in TPTX-OVX rats were almost 10-fold higher than those of 3.2 mg/kg in male rats under feeding condition (AUC0-6 hr : 0.66 vs. 0.049 µg . hr/ml, Cmax : 0.16 vs. 0.017 µg/ml ). Thus, FR202126 was given to periodontal male rats, which were under feeding condition, up to 10 mg/kg/day because 1 mg/kg of FR202126 exerted maximum effect on TPTX-OVX model (Fig. 4).

The administration of FR202126 restored the decrease in the alveolar bone area and the ratio of alveolar bone area in a dose-dependent manner (Fig. 5 B, C). Increase in the distance between top of the alveolar bone and CEJ was significantly inhibited by the oral administration of FR202126 (3.2, 10 mg/kg) (Fig. 5 D). Femoral bone mineral density was increased by the treatment with FR202126 (data not shown). No significant weight or behavior changes were noted in any of the animals during the administration with FR202126 (data not shown).

DISCUSSION

Periodontal diseases result from mixed serious

Photo 1. Histopathology of the region between the first and second molars of rats subjected to periodontitis. Photographs of Sham group (A) and Cont group (B). Arrows indicate CEJ. M1 : the first molar, M2 : the second molar, * : alveolar bone. The scale bar indicates 1mm. Hematoxylin-eosin stain. (C) Higher magnification in Cont group. Arrows indicate activated osteoclasts. *: alveolar bone. The scale bar indicates 100 µm. Hematoxylin-eosin stain.
bacterial infections that finally destroy alveolar bone, which holds the teeth, and lead to tooth loss. Therefore, from a therapeutical point of view it must be important to suppress the progressive destruction of alveolar bone in periodontal patients. Bacterial infection results in periapical osteoclastic bone resorption by directly or indirectly stimulating production of inflammatory cytokines. In our experimental model, insertion of ligature wire induced an acute inflammatory response and caused vertical and horizontal alveolar bone resorption by activated osteoclasts, which is pathologically similar to periodontal patients. Though several other experimental periodontal rat models have been reported, we adopted the ligature wire insertion model because it has been shown that alveolar bone destruction progresses rapidly and the amount of destruction is large enough to estimate the effect of compounds (Konishi et al., 2000).

Several studies have reported that administration of bisphosphonates, which are known to inhibit bone

![Histological analysis of alveolar bone. The illustration (A) shows the methodology for morphological evaluation. Histomorphological parameters, the remaining area of alveolar bone (B), the ratio of the area of alveolar bone to the interdental area (C) and the distance between the alveolar bone crest and the CEJ (D), are measured. Data are means ± SEM (n=30, 31, 10 and 18 for Sham, Cont, 3.2 mg/kg and 10 mg/kg, respectively). ***p<0.001 vs. Cont (Student’s t test). *p<0.05, **p<0.01 vs. Cont (Dunnett).](image-url)
A novel V-ATPase inhibitor, FR202126, prevents alveolar bone loss.

Resorption, is effective in preventing alveolar bone loss in experimental periodontitis (Brunsvold et al., 1992, Shoji et al., 1995, Konishi et al., 2000). However, bisphosphonates have some weaknesses. First, because they have a slow onset of action, it may be difficult to follow progressive bone loss. Next, after taking them, they easily deposit in bone and remain for a long time. These effects lead to modulation of proliferation and differentiation of periodontal ligament cells (Lekic et al., 1997). Bone morphogenetic protein has started to be clinically applied for reconstruction of the bone defects caused by periodontitis. However, bisphosphonate inhibits maturation of ectopic bone induced by bone morphogenetic protein (Gong et al., 2003) due to continuous blockage of bone resorption. Consequently, it is important to develop a new approach for the treatment of periodontal alveolar bone loss.

There has been no study on the effect of V-ATPase inhibitors in periodontitis. We have demonstrated that a novel V-ATPase inhibitor, FR167356, exerted an inhibitory effect on in vitro bone resorption (Niikura et al., 2004; Niikura et al., 2005). To obtain more potent V-ATPase inhibitors, chemically modified compounds were screened by monitoring H+ transport activity of chicken osteoclast microsomes, which were inhibited by bafilomycin A1 but not by oligomycin (F-ATPase inhibitor) or orthovanadate (P-ATPase inhibitor), suggesting that H+ transport in the osteoclast microsomes was driven by V-ATPase (data not shown). FR202126 is one of the successors and displayed 1.7-fold higher potency against murine osteoclast V-ATPase. Thus, we undertook to investigate the role of V-ATPase on the alveolar bone destruction using FR202126.

In our experiments FR202126 could prevent in vitro bone resorption induced by IL-1 and IL-6. In addition, FR202126 could also inhibit bone resorption induced by PTH. PTH and IL-6 exert their activities via osteoblasts/stromal cells surface receptors whereas IL-1 directly acts on osteoclasts in addition to osteoblasts/stromal cells. Moreover, their signal transduction at the cellular level are different from each other (Suda et al., 1999). Nonetheless, FR202126 could exert a similar inhibitory effect, suggesting that V-ATPase is involved in a final indispensable step of bone resorption and that V-ATPase inhibitors can cope with every bone resorative factor. This characteristic of FR202126 is thought to be suitable for the treatment of periodontitis because several kinds of cells are associated with the differentiation, activation and survival of osteoclasts and a number of factors are working in periodontitis.

We have studied 2-week toxicity tests of FR202126 in rat (data not shown). In the experiment, rats were orally administered once a day for 2 weeks. FR202126 did not affect any plasma biochemical parameters measured up to 10 mg/kg. However, histomorphological analysis revealed that the femoral trabecular bone was increased. In addition, the femoral bone density in periodontal models was increased in parallel with the inhibition of alveolar bone destruction (data not shown). The results of bone density would be expected but FR202126 will have to be administered topically for clinical application in periodontitis.

In conclusion, the pharmacological studies of FR202126 showed it has specificity for osteoclast V-ATPase inhibition. Since V-ATPase is involved in a final step of bone resorption, FR202126 is expected to affect only osteoclast activity. In this regard, FR202126 is thought to be superior to bisphosphonates. FR202126 significantly inhibited alveolar bone loss in experimental periodontitis, which suggests that an acidic microenvironment plays an important role in periodontal alveolar bone destruction as well and may offer a new approach to treatment of periodontal disease.

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