British Journal of Pharmacology (2004) 142, 558–566
www.nature.com/bjp

A novel inhibitor of vacuolar ATPase, FR167356, which can discriminate between osteoclast vacuolar ATPase and lysosomal vacuolar ATPase

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1 Vacuolar ATPase (V-ATPase) has been proposed as a drug target in lytic bone diseases. Studies of bafilomycin derivatives suggest that the key issue regarding the therapeutic usefulness of V-ATPase inhibitors is selective inhibition of osteoclast V-ATPase. Previous efforts to develop therapeutic inhibitors of osteoclast V-ATPase have been frustrated by a lack of synthetically tractable and biologically selective leads. Therefore, we tried to find novel potent and specific V-ATPase inhibitors, which have new structural features and inhibition selectivity, from random screening using osteoclast microsomes. Finally, a novel V-ATPase inhibitor, FR167356, was obtained through chemical modification of a parental hit compound.

2 FR167356 inhibited not only H^+ transport activity of osteoclast V-ATPase but also H^+ extrusion from cytoplasm of osteoclasts, which depends on the V-ATPase activity. As expected, FR167356 remarkably inhibited bone resorption *in vitro*.

3 FR167356 also showed inhibitory effects on other V-ATPases, renal brush border V-ATPase, macrophage microsome V-ATPase and lysosomal V-ATPase. However, FR167356 was approximately seven-fold less potent in inhibiting lysosomal V-ATPase compared to osteoclast V-ATPase. Moreover, LDL metabolism in cells, which depends on acidification of lysosome, was blocked merely at higher concentration than bone resorption, suggesting that FR167356 inhibits V-ATPase of osteoclast ruffled border membrane still more selectively than lysosome at the cellular level.

4 These results from the experiments seem to indicate that osteoclast V-ATPase may be different from lysosomal V-ATPase in respect of their structure.

5 FR167356 had a novel chemical structural feature as well as inhibitory characteristics distinctly different from any previously known V-ATPase inhibitor family. Therefore, FR167356 is thought to be a useful tool for estimating the essential characteristics of V-ATPase inhibitors for drug development.

British Journal of Pharmacology (2004) **142,** 558–566. doi:10.1038/sj.bjp.0705812

Keywords: V-ATPase; osteoclast; bone resorption; lysosome; bafilomycin A1; FR167356

Abbreviations: BCECF, 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein; LDL, low-density lipoprotein; M-CSF, macrophage colony-stimulating factor; PTH, parathyroid hormone; sRANKL, soluble receptor activator of NF-κB ligand; V-ATPase, vacuolar ATPase

Introduction

Bone mass is maintained under a delicate balance between bone formation and bone resorption. Increase of bone resorption causes several lytic bone diseases such as osteoporosis, hypercalcemia, rheumatoid arthritis, tumor metastasis into bone, periodontitis and Paget's disease. Therefore, bone resorption inhibitors are valuable for treating these diseases. Osteoclasts, the multinucleated large cells that are derived from precursor cells of the monocytic/macrophage lineage, perform bone resorption under regulation by various hormones and cytokines. Bone resorption means that crystalline hydroxyapatite is dissolved and organic bone matrix rich in collagen fiber is degraded. Breakdown of both bone components requires the bone surface acidic microenvironment beneath the ruffled border, special plasma membrane of osteoclasts formed in face of bone, because hydroxyapatite is solubilized in acidic solution and collagen-digested protease, cathepsin K, is optimally active at acidic pH. Accordingly, disturbance of acid environment provided by osteoclasts is expected to lead to blockage of bone resorption.

Vacuolar ATPases (V-ATPases) are ATP-dependent proton pumps, which are highly expressed in the ruffled border membranes and function to acidify the resorption lacunae (Blair *et al*., 1989). In addition, suppression of gene expression of V-ATPases by antisense RNA and DNA molecules inhibits bone resorption (Laitala & Vaananen, 1994). Consequently, plasma membrane V-ATPase of osteoclasts (osteoclast V-ATPase) is thought to be a good molecular target for reducing osteoclast activity.

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Advance online publication: 17 May 2004

V-ATPase is one of three major classes of ATP driven cation pumps (V-, P- and F-ATPase). These three ATPases show the different sensitivity to inhibitors (Forgac, 1989). Bafilomycin A1 is a first potent and specific inhibitor of V-ATPase. Bafilomycin A1 inhibits V-ATPases at nanomolar concentration, while P-ATPases are affected only at micromolar concentration and F-ATPase is not inhibited at all (Bowman *et al*., 1988). V-ATPase is not only localized in the plasma membranes of osteoclasts but also the plasma membranes of renal intercalated cells and macrophages. Moreover, V-ATPases are also present on ubiquitous intracellular acidic compartment such as lysosomes, endosomes, the Golgi apparatus and secretory vesicles, and responsible for their acidification (Nishi & Forgac, 2002). Since bafilomycin A1 equally inhibits all of these V-ATPase activities leading to systemically undesirable alteration of cellular physiology when administered to animals, the characteristics of bafilomycin A1 is not suited for therapeutic application. In order to obtain safe V-ATPase inhibitors for the treatment of lytic bone diseases, we must discover the compounds that affect the activities of neither P- nor F-ATPase, and moreover, display selectivity with respect to inhibition of V-ATPases.

V-ATPases consist of a complex of many subunits. Recently, there is growing evidence for the presence of subunit diversity among V-ATPases and it is reported that they are expressed differently in organelles and tissues (Nishi & Forgac, 2002; Sun-Wada *et al*., 2003; Toyomura *et al*., 2003; Ueda *et al*., 2003). The existence of osteoclast-specific isoform is controversial (Chatterjee *et al*., 1992; van Hille *et al*., 1993; 1995; Li *et al*., 1996; Scott & Chapman, 1998). However, recent report showed that the function of osteoclast V-ATPase can be depressed without influence on V-ATPase function in other cells. a3 subunit knockout mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification, whereas acidification of lysosomes and the proton transport in kidney microsomes are retained normally in these mice (Frattini *et al*., 2000). This fact suggests that it is possible to selectively inhibit osteoclast V-ATPase *in vivo*. However, it is not clear whether it can be achieved pharmacologically by agents. Recently, it was reported that bafilomycin A1 derivatives inhibited selectively osteoclast V-ATPase although this tissue selectivity was assessed in comparison to just one more tissue (adrenal gland or brain). However, chemical modification of bafilomycin is limited by its high complexity and low chemical stability. Accordingly, we tried to find novel potent and specific V-ATPase inhibitors, which have new chemical structures, by random screening using chicken osteoclast microsomes. Consequently, we obtained a novel specific V-ATPase inhibitor, FR167356. In this paper, pharmacological studies of FR167356 showed FR167356 had the inhibition profile distinctly different from any previously known V-ATPase inhibitor family.

Method

Animals

Female 1-day-old chickens were obtained from Sankyo Lab Service (Tokyo, Japan). Female Wistar,14 days pregnant rats, 8-week-old male Wistar rats and low Ca^{2+} (0.07%) diet for chicken were obtained from Clea Japan (Tokyo, Japan). Male ddY mice, 8 weeks old, and 13-week-old Japanese White rabbits were obtained from Nihon SLC (Hamamatsu, Japan). Male ICR mice, 8 weeks old, were obtained from Charles River Japan (Yokohama, Japan). Animals were housed in our animal care facility and kept under standard conditions (room temperature of $23 \pm 2^{\circ}C$, humidity of $55 \pm 5\%$, 12 : 12 light–dark cycle, free access to normal commercial diet (except chickens) and water). All experimental procedures have been subjected to evaluation and were approved by animal ethics committee of the Fujisawa Pharmaceutical Co.

Membrane preparation for H^+-transport assay

We used tibiae of chickens fed low Ca^{2+} diet to obtain sufficient amounts of pure osteoclasts for random screening. Chicken osteoclasts were isolated from tibiae of 1-month-old chicks fed low Ca^{2+} diet (0.07%) for 1 month (Hunter *et al*., 1991) and their microsomes were prepared (Blair *et al*., 1989). Mouse osteoclasts were formed in the coculture of spleen cells obtained from ddY mice and ST2 cells (Riken, Tsukuba, Japan), murine bone marrow stromal cells, in the presence of 10 nM 1,25-dihydroxyvitamin D_3 and 100 nM dexamethasone (Udagawa *et al*., 1989). After 1 week coculture, ST2 cells were eliminated by the treatment of 0.2% dispase. Remaining cells, which were mainly osteoclasts, were collected by cell scraper. Plasma membrane vesicles of murine osteoclasts were isolated (Koizumi *et al*., 1976). Lysosomes were isolated as tritosomes from ddY mice and Wistar rat liver. Then, membrane ghosts were prepared by osmotic lysis of tritosomes (Arai *et al*., 1993). Macrophages were harvested by peritoneal lavage from ddY mice injected with thioglycolate and their microsomes were fractionated. Murine (ddY mouse) and rat (Wistar rat) renal brush border membrane vesicles were isolated from renal cortical homogenate by Mg^{2+}-aggregation method (Biber *et al*., 1981). Mitoplasts (inner membrane vesicle 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 assay

H^+ transport by various membrane vesicles was assayed with a dual wavelength spectrophotometer (UV-3000, Shimadzu, Kyoto, Japan) by measuring uptake of acridine orange, in a reaction medium containing 10 μM acridine orange, 150 mM KCl, 2 mM $MgCl_2$, 10 mM bis-tris-propane and 1 μM valinomycin, pH 7.0. (Blair *et al*., 1989). A reaction buffer for measuring of lysosomal V-ATPase activity was 10 μM acridine orange, 40 mM Bicine-TMAH, 0.1 M KCl, 0.2 M Sucrose, 1 μM valinomycin and 1 mM $MgCl_2$, pH 8.5 (Moriyama *et al*., 1984). For measurement of V-ATPase activity, additionally 5 $\mu g\,ml^{-1}$ oligomycin was added. The reaction was initiated by adding ATP (final concentration 1 mM).

Measurement of ATPase activity

ATPase activity of gastric H^+,K^+-ATPase was determined by measuring the release of inorganic phosphate (Tomoi *et al*., 1988). Macrophage V-ATPase was purified by immunoprecipitation using rabbit anti-bovine V-ATPase (first antibody) and mouse anti-rabbit IgG (second antibody). V-ATPase activity bound to protein A-bearing beads (pansorbin cells)

was assayed (Gluck & Caldwell, 1987). The liberated phosphate was estimated by the malachite green method.

Measurement of acid extrusion

Previously glass bottom dishes (Asahi Technoglass, Funabashi, Japan) were coated with vitronectin by a 4 h incubation in 5 μg ml^{-1} bovine vitronectin at 37°C. Unfractionated bone cell suspension was prepared from tibiae and femora of 11- to 13-day-old ICR mice (Takada *et al*., 1992) and seeded on to the glass bottom dishes. The cells were incubated at 37°C in a CO_2 incubator (5% CO_2, 95% air) for 3 h. Then the glass bottom dishes were gently washed and added α-MEM supplemented with 10% FCS plus 10 nM 1,25-dihydroxyvitamin D_3. The medium was changed every 2 days. After the incubation for 6- and 8-days, the pH_i of cells was measured fluorometrically using the pH-sensitive probe, BCECF. Cells were bathed in a recording buffer (145 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1 mM Na_2SO_4, 1.8 mM $CaCl_2$, 30 mM HEPES, 1 mM $MgCl_2$, 10 mM glucose, pH7.4) containing 40 mM NH_4Cl for 15 min. After cytoplasmic acid loading accomplished by preincubating in 40 mM NH_4Cl for 15 min, the pH_i recovery rate was monitored (Fernandez & Malnic, 1998) in the presence of FR167356 plus 100 μM dimethylamiloride (Na^+/H^+-exchanger inhibitor) using the High speed [Ca^{2+}] analyzing system (ARGUS/HiSCA Ver1.7, Hamamatsu Photonics, Hamamatsu, Japan). Multinucleated cells were determined to be osteoclasts.

Calvaria organ culture

Calvariae from neonatal Wistar rats were excised and cultured in 2 ml DMEM (10% FCS) supplemented with 10 nM hPTH fragment (1–34) in the presence of FR167356. After 6 days, the concentration of Ca^{2+} in the media was measured by the commercial kit (Wako Pure Chemical Industries) according to methylxylenol blue method.

Pit formation

Mouse bone marrow cells were obtained from tibiae of 9-week-old ddY mice. Mouse bone marrow cells were cultured on collagen gel-coated dishes in the presence of sRANKL (100 ng ml^{-1}) and mM-CSF (25 ng ml^{-1}) for 10 days. Then, cells were retrieved from dishes by treatment with collagenase. The cells, which consisted of mainly osteoclasts, were put on ivory slices and cultured in α-MEM containing 10% FCS in the presence of sRANKL (100 ng ml^{-1}) and FR167356. After 24 h, the ivory slices were stained with acid hematoxylin and pit area at six fields (29.73 mm^2) a slice was measured by image analyzer (LUZEX 3U, Nikon, Tokyo, Japan) (Udagawa *et al*., 1999).

Assay for cholesteryl ester formation

Murine peritoneal resident macrophages were isolated by peritoneal lavage from ddY mice and incubated with DMEM (10% FCS) for 20 h. Then the medium was changed to DMEM (10% FCS) containing [^{14}C]-oleate-albumin, lipoprotein and FR167356. The cells were incubated for 4 h and then washed, and extracted with hexane/isopropanol. The extract was assayed for cholesteryl esters using thin-layer chromatography (Goldstein *et al*., 1983).

LDL metabolism

Human hepatocyte cell line, Hep G2 (ATCC, Manassas, VA, U.S.A.) was grown in monolayers in MEM (10% FCS). Then cells were switched to MEM (10% fetal calf lipoprotein-deficient serum) and incubated for 2 h in MEM (10% fetal calf lipoprotein-deficient serum) containing [^{125}I]-LDL and FR167356, either in the absence or presence of excess unlabeled LDL. After incubation, specific binding, uptake and degradation of LDL were assayed according to Sparrow *et al*. (1989).

Materials

Bafilomycin A1 and anti bovine V-ATPase A subunit antibody (rabbit IgG) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Pansorbin cells were obtained from Calbiochem (La Jolla, CA, U.S.A.). Anti-rabbit IgG (mouse) was obtained from OEM Concepts (Toms River, NJ, U.S.A.). Acetoxymethyl ester of 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) was obtained from Molecular Probe (Eugene, OR, U.S.A.). 1,25-dihydroxyvitamin D_3 was obtained from Biomol Res. Lab. (Plymouth Meeting, PA, U.S.A.). Bovine vitronectin was purchased from Koken (Tokyo, Japan). Recombinant human soluble RANKL (sRANKL) was purchased from PeproTech EC (London, England). Dispase was purchased from Godo Shusei (Tokyo, Japan). Human PTH (1–34) fragment was purchased from Peptide Institute (Osaka, Japan). Triton WR-1339 was purchased from Nacalai tesque (Kyoto, Japan). Collagen gel solutions (Cellmatrix, type I-A) were obtained from Nitta Gelatin (Osaka, Japan). [^{14}C]-oleic acid and [^{125}I] were obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). Fetal calf serum (FCS) was obtained from Moregate (Bulimba, Australia). Tissue culture wares were obtained from Corning (Elmira, NY, U.S.A.) and Becton Dickinson (San Jose, CA, U.S.A.). All other reagents were purchased from Sigma (St Louis, MO, U.S.A.).

Data analysis

Results are expressed as mean ± s.e.m. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. 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. A 50% inhibitory concentration was obtained by fitting sigmoidal curve to data. When multiple experiments were repeated, we took an average from IC_{50} value of each experiment. For statistical comparisons, calculation of 50% inhibitory concentration and kinetics analysis, commercial Software (GraphPad Prism Ver 4, GraphPad Software, San Diego, CA, U.S.A.) was used. Differences were considered to be statistically significant at $P<0.05$.

Results

H^+ transport

To discover novel inhibitors of osteoclast V-ATPase, we performed random screening by monitoring H^+ transport activity of chicken osteoclast microsomes, which were inhibited by bafilomycin A1 and *N*-ethylmaleimide but not by oligomycin (F-ATPase inhibitor) or orthovanadate (P-ATPase inhibitor) (data not shown), suggesting that H^+ transport in the osteoclast microsomes were driven by V-ATPase. FR167356 (Figure 1), 2,6-dichloro-*N*-[3-(1-hydroxy-1-methylethyl)-2-methyl-7-benzofuranyl]benzamide, was one of compounds which were obtained from chemical modifications of hit compounds. FR167356 inhibited H^+ transport in plasma membrane vesicles of murine osteoclasts (IC_{50} 170 nM) and chicken osteoclast microsomes (IC_{50} 220 nM), whereas FR167356 exerted no effect on H^+ transport of mitochondrial ATPase (F-ATPase) or gastric H^+,K^+-ATPase (P-ATPase) (Figure 2). Therefore FR167356 is thought to be a specific inhibitor of V-ATPase. Next we investigated the effect of FR167356 on H^+ transport by other V-ATPases, which are derived from macrophage microsomes, renal brush border membranes and lysosomal membranes. FR167356 showed inhibitory effects on H^+ transport activities of other V-ATPases (Table 1). The values of the inhibition (IC_{50}) are 220 nM (murine macrophage), 370 nM (murine kidney) and 310 nM (rat kidney), respectively, which are almost similar to that determined for the inhibition of osteoclast V-ATPase. However, FR167356 displayed lesser potency (IC_{50} 1200 nM) against murine and rat lysosomal V-ATPase, which raises the possibility that there is structural difference between lysosomal V-ATPase and osteoclast V-ATPase.

Inhibition kinetics

We determined the inhibition kinetics of FR167356 with respect to ATP. H^+ transport as a function of ATP concentration was measured in the presence and absence of FR167356 (Figure 3a). A concentration of 100 nM FR167356

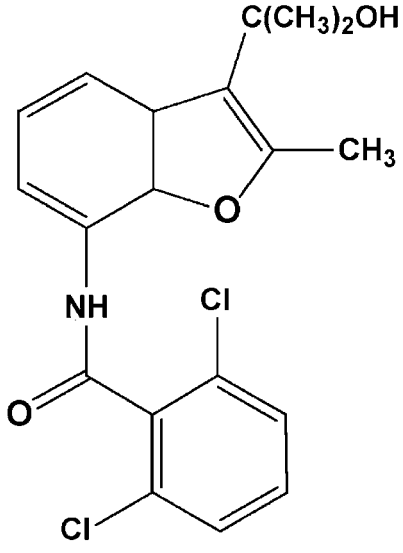

Figure 1 Chemical structure of FR167356.

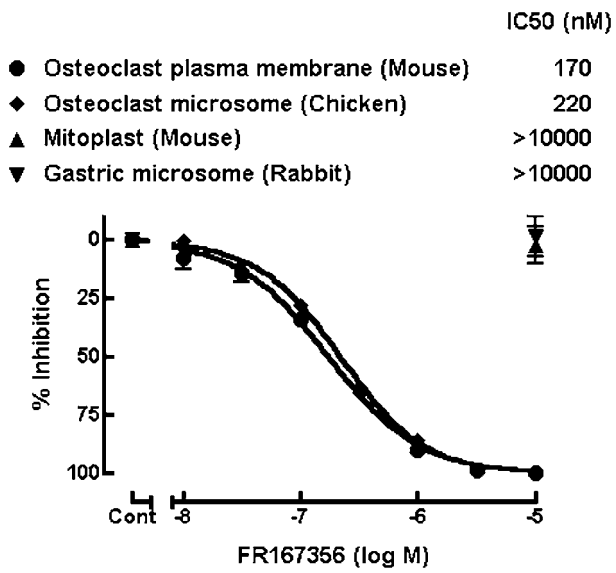

Figure 2 Effect of FR167356 on ATP-dependent H^+ transport activity of V-ATPase, F-ATPase and P-ATPase. H^+ transport was assayed by monitoring absorption change of acridine orange at 492–540 nm (an index of the amount of acridine orange in free solution). The initial rate of change in the absorbance of acridine orange was used for calculation of IC_{50} value. V-ATPase (murine osteoclast plasma membrane vesicles and chicken osteoclast microsomes) was assayed in the presence of 5 μg ml^{-1} oligomycin. FR167356 did not inhibited H^+ transport activity of F-ATPase (murine liver mitoplast) and P-ATPase (rabbit gastric microsomes) up to 10 μM. Each value represents the average of triplicated determinations. The bars represent s.e.m.

Table 1 Inhibition of ATP-dependent H^+ transport activity of V-ATPase by FR167356

	IC_{50} (nM)					
Mouse osteoclast	Chicken osteoclast	Mouse Mφ	Mouse kidney	Rat kidney	Mouse tritosome	Rat tritosome
170	220	220	370	310	1200	1200

Mφ, macrophage.
Values shown are averages from two independent experiments.

reduced the V_{max} of H^+ pumping of the macrophage microsomes from 0.00307 to 0.00178 $A_{492-540\,nm}$ min^{-1}, without remarkably altering the K_m values (424 μM *versus* 364 μM ATP for control and 100 nM FR167356, respectively). The double reciprocal transformation (Figure 3b) reveals changes in both slope and the *y*-intercept of the resulting lines. Thus, the inhibition by FR167356 is of the noncompetitive type.

ATPase activity

V-ATPases translocate protons across membranes utilizing the energy of ATP hydrolysis. V-ATPases are composed of two functional domains, V1 and V0. V1, composed of eight subunits, is responsible for ATP hydrolysis. V0 domain contains five different types of subunits and is mainly

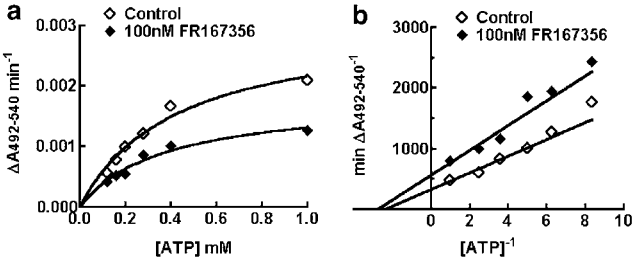

Figure 3 Noncompetitive inhibition of H^+ transport by FR167356. (a) An ATP-dependent activation curves of H^+ transport in the absence and presence of 100 nM FR167356. The continuous lines are derived by fitting the data points to a Michaelis–Menten-type kinetics. (b), Lineweaver–Burk plot of the reciprocal rate of H^+ transport *versus* the reciprocal of the different concentrations of ATP.

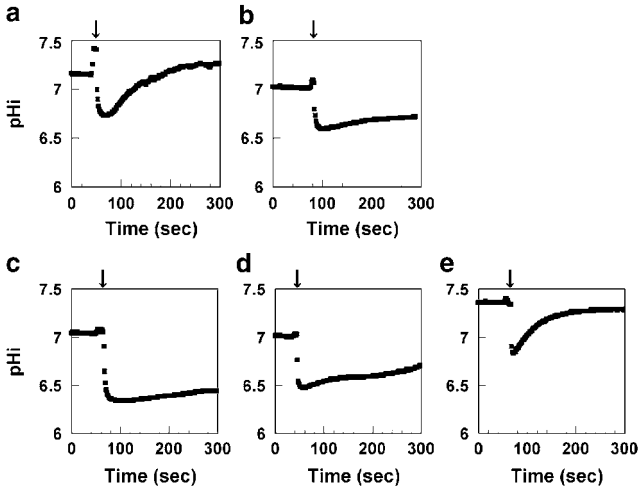

Figure 4 Effect of FR167356 on pH_i recovery of acid-loaded osteoclasts. pH_i was measured fluorometrically using the probe BCECF. Cells were acid-loaded by the NH_4^+ prepulse method. Following the removal of NH_4Cl (arrow), pH_i decreases acutely due to rapid efflux of NH_3. Then pH_i recovery in a recording buffer containing 100 μM dimethylamiloride (a) was inhibited by additional presence of 200 nM bafilomycin A1 (b). Effect of adding FR167356 (10 μM (c), 1 μM (d), 100 nM (e)) in the presence of dimethylamiloride was investigated. Traces are representative of 5–11 experiments. The initial rate of pH_i recovery was obtained by fitting tangent line and used for calculation of IC_{50} value.

responsible for proton translocation. We investigated whether FR167356 affected ATPase activity of V-ATPase. For measurement of ATPase activity, we used macrophage V-ATPase purified by immunoprecipitation because we could obtain sufficient amounts of uniform cells. FR167356 inhibited ATPase activity of macrophage microsome V-ATPase with an IC_{50} of 350 nM. Therefore, FR167356 may directly act on V-ATPase and inhibition of H^+ transport by FR167356 seems to be attributed to the inhibition of ATPase activity. On the other hand, FR167356 did not affect ATPase activity of H^+,K^+-ATPase up to 10 μM.

H^+ extrusion in acid-loaded osteoclasts

To examine the effect of FR167356 on V-ATPase activity at the cellular level, recovery from cytoplasmic acid loading was studied in murine osteoclasts. After 15 min exposure to 40 mM NH_4Cl, NH_4Cl removal caused a rapid acidification of pH_i as a result of NH_3 efflux. Then the pH_i recovered to basal level in spite of the presence of 100 μM dimethylamiloride (inhibitor of Na^+/H^+-exchanger) (Figure 4a), whereas the pH_i recovery in contaminating fibroblastic cells was not observed under the same experimental condition (data not shown). To confirm that V-ATPase residing in the plasma membrane of osteoclasts contributed to the dimethylamiloride-insensitive pH_i recovery, the effect of bafilomycin A1 was examined. When osteoclasts were treated with bafilomycin A1 in the presence of dimethylamiloride, pH_i recovery was inhibited (Figure 4b). It provided evidence that the dimethylamiloride-insensitive pH_i recovery of acid-loaded osteoclasts was mediated by V-ATPase. FR167356 also inhibited pH_i recovery with an IC_{50} of 220 nM, suggesting that FR167356 acted on V-ATPase residing in the plasma membrane of osteoclast at the cellular level (Figure 4c–e).

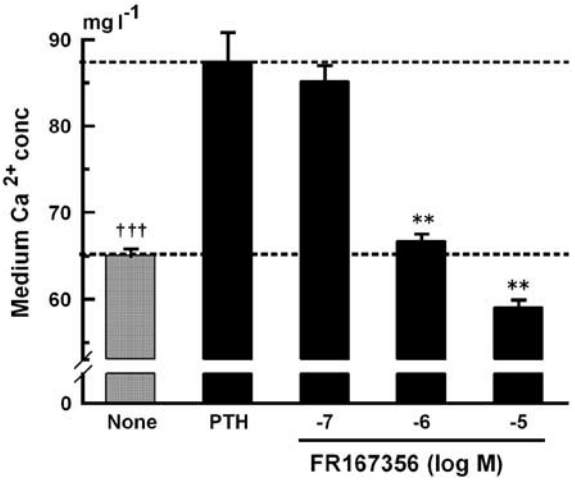

Figure 5 Effect of FR167356 on PTH-induced bone resorption of rat calvariae. Calvariae were incubated with FR167356 in the presence of 10 nM hPTH. In nontreated dishes (None), only distilled water was added. The bars represent the mean ± s.e.m. for five dishes. The figure is representative of two independent experiments. Significantly different between None and PTH alone (PTH): †††$P<0.001$ (Student's *t*-test). Significantly different between treatment with FR167356 and PTH: **$P<0.01$ (Dunnett).

Effect on bone resorption in vitro

Next, we investigated whether inhibition of osteoclast V-ATPase by FR167356 consequently can cause blockade of bone resorption or not. Calvariae treated with parathyroid hormone (PTH) released more calcium into the culture media than did the control. An amount of 1 μM FR167356 inhibited almost completely the bone resorption induced by PTH (Figure 5). Its dose response (IC_{50} 360 nM) appears to be similar to that observed in the effect on H^+ extrusion (Figure 4c–e). Calvariae contain not only osteoclasts but also osteoblasts, bone marrow cells and fibroblasts. Therefore, it was not clear which cells were target of FR167356. Pit assay using purified murine osteoclasts showed that adding 1 μM FR167356 remarkably decreased the number and area of resorption lacunae. Figure 6 shows that pit area was inhibited

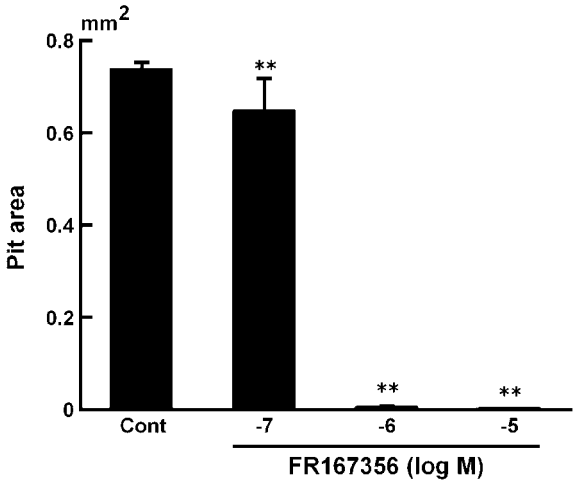

Figure 6 Formation of resorption pits by isolated osteoclasts in the presence of FR167356. The osteoclast preparation obtained from bone marrow cultures treated with mM-CSF (10 ng ml^{-1}) and hsRANKL (100 ng ml^{-1}) was cultured on ivory slices in the presence of FR167356 and hsRANKL (100 ng ml^{-1}). After culture for 24 h, ivory slices were stained with acid hematoxylin and pit area at six fields (29.73 mm^2) a slice was measured by image analyzer. Data are expressed as mean ± s.e.m. of triplicate cultures. Significantly different between treatment with FR167356 and Control (Cont): $^{**}P<0.01$ (Dunnett).

by FR167356 in a dose-dependent manner (IC_{50} 190 nM). It suggests that FR167356 directly acted on osteoclasts. The number of cells per slice did not change regardless of the presence of FR167356.

Lysosomal function

FR167356 inhibited lysosomal V-ATPase activity. However, FR167356 displayed lower inhibitory effect on lysosomal V-ATPase than osteoclast V-ATPase (Table 1). We examined the effect of FR167356 on lysosomal V-ATPase activity in living culture cells. Measurement of LDL metabolism was selected for the examination of the effect of FR167356 on lysosomal biological function, which depends on lysosomal pH. LDL took up by cells is hydrolyzed in lysosome, apolipoprotein being digested and free cholesterol being liberated. Then free cholesterol is effluxed into the cytoplasm, where much of it is re-esterified. As mentioned above, lysosome is an important organelle where the first step in LDL metabolism occurs, and moreover such functions operate under acidic pH. Based on these background, the effects of FR167356 on cholesteryl ester accumulation and LDL degradation were analyzed, both of which were inhibited by bafilomycin A1 (data not shown) (Naganuma *et al*., 1992). In all, 58% inhibition of accumulation of cholesteryl ester in macrophages was observed only at 10 μM FR167356 (IC_{50} 7800 nM) (Figure 7). Degradation of internalized LDL was blocked by 66% in human hepatocytes treated with 10 μM (IC_{50} 5200 nM) (Figure 8).

These results suggest that lysosomal V-ATPase (Figures 7 and 8) is less sensitive to FR167356 than V-ATPase of osteoclast ruffled border membrane (Figures 4–6) at the cellular level.

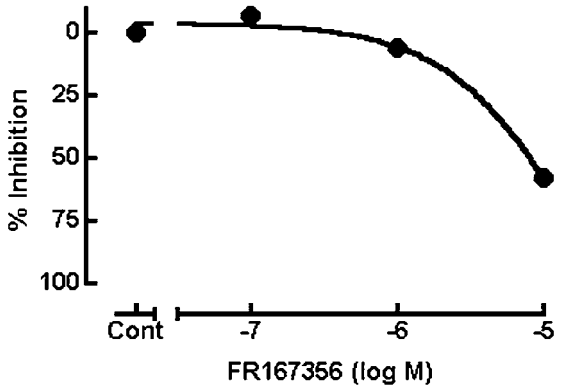

Figure 7 Effect of FR167356 on cholesteryl ester accumulation in macrophages. After culture for 20 h, murine macrophages received [^{14}C]-oleate in complex with albumin and the indicated concentrations of FR167356 in the presence of acetylated LDL. After incubation for 4 h, cholesteryl esters were determined. The value for control experiment was 347.7 PSL μg^{-1} (protein). Each value represents the average of duplicate determinations.

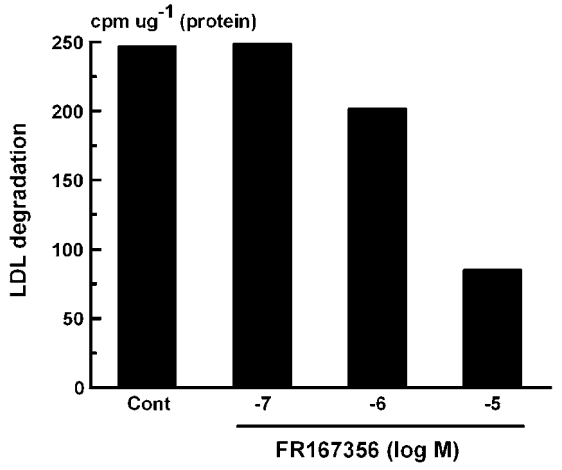

Figure 8 Effect of FR167356 on the degradation of [^{125}I]-LDL in hepatocyte Hep G2. Cells received 3 μg [^{125}I]-LDL and FR167356 in the presence or absence of 120 μg unlabeled LDL. After incubation for 2 h, specific values for degraded [^{125}I]-LDL were determined. Each value represents the average of duplicate determinations. The figure is representative of two independent experiments.

Discussion

Bone resorption process requires maintaining of acidic condition formed by osteoclast V-ATPase. Bafilomycin A1, the first specific V-ATPase inhibitor described (Bowman *et al*., 1988), remarkably inhibits bone resorption *in vitro* (Sundquist *et al*., 1990), which indicates that osteoclast V-ATPase plays a central role in the process of bone resorption. Inhibitors of osteoclast V-ATPase are expected to become therapeutic agents for treatment of several bone diseases that are caused by increase of bone resorption. The key issue regarding the therapeutic usefulness of V-ATPase inhibitors is their selectivity. V-ATPases are present not only in osteoclasts but also all eukaryotic cells where they are involved in many important cellular processes. Nonspecific inhibitor of V-ATPase can be expected to cause a multitude of effects, most of which are undesirable. Indeed, bafilomycin A1 is not used

therapeutically, because it exhibits *in vitro* and *in vivo* toxic effect (Keeling *et al*., 1997). Recent studies indicated that isoforms of several subunits of V-ATPase are present and they are expressed differently in organelles and tissues (Nishi & Forgac, 2002; Sun-Wada *et al*., 2003; Toyomura *et al*., 2003; Ueda *et al*., 2003). Although the presence of osteoclast-specific isoform remains to be elucidated, it may be possible that only osteoclast V-ATPase activity can be depressed without influence on other cells and functions of acidic cellular compartments (Frattini *et al*., 2000). However, it is not clear whether chemical compounds can pharmacologically achieve it.

Several tissue-selective V-ATPase inhibitors were reported. Salicylihalamide A had the selectivity for mammalian *versus* fungal V-ATPase, although there was not selectivity among tested human V-ATPases (kidney, liver and osteoclast) (Boyd *et al*., 2001). H362/48 was approximately six-fold less potent against brain V-ATPase as opposed to bone V-ATPase (Keeling *et al*., 1998). SB242784 inhibited osteoclast V-ATPase at 1000-fold lower concentration than V-ATPases in other evaluated tissues (liver, kidney and brain) (Visentin *et al*., 2000). However, in these experiments the inhibitory activity was determined by measuring bafilomycin-sensitive ATPase activity of tissue membranes without the purification steps. As variable amount of Mg^+-dependent ATPase activities were contaminated in these assays, these V-ATPase activities were calculated as difference of the $\pm$bafilomycin A1 treatment. Accordingly, percentage of inhibition by tested compounds completely depended on the inhibition by bafilomycin treatment (control value). Moreover bafilomycin-sensitive ATPase activity occupied only a small proportion of total Mg^+-dependent ATPase activities, which allows percentage of inhibition to fluctuate easily. Additionally, if tested compounds inhibited other Mg^+-dependent ATPase activities contaminating in these assays than V-ATPase activity, the inhibition of Mg^+-dependent ATPase could not be excluded from total inhibition by the compounds. After all, the IC_{50} value seems to be variable and not accurate in these assays.

There are some reports described about tissue selective V-ATPase inhibitors using H^+ transport assay. Vanadate, which is known as a P-ATPase inhibitor, could inhibit specifically osteoclast H^+ pump among other V-ATPases (Chatterjee *et al*., 1992). Tiludronate also had a significant degree of selectivity for osteoclast V-ATPase relative to kidney V-ATPase (David *et al*., 1996). However, these results of two compounds were not repeatable by other laboratories (Blair *et al*., 1989; Keeling *et al*., 1997). Therefore, it seems that only bafilomycin A1 derivatives had certainly selectivity. Gagliardi *et al*. (1998) reported that two of derivatives were three- or six-fold less potent against adrenal gland as opposed to bone and oppositely two of derivatives were five- or 50-fold less potent against bone. Other bafilomycin A1 derivative, (2Z,4E)-5-(5,6-dichloro-2-indolyl)-2-methoxy-*N*-[4-(2,2,6,6-tetramethyl)piperidinyl]-2,4-pentadienamide, was reported to be seven-fold more potent in inhibiting bone V-ATPase compared to brain V-ATPase (Mattsson *et al*., 2000).

Since chemical modification of bafilomycin is limited by its high complexity and low chemical stability, we tried to obtain novel potent and specific V-ATPase inhibitors, which have new structural features, from random screening using osteoclast microsomes. The structure of a hit compound was imidazopyridine and subsequently good structure–activity relationships were observed in chemical modification. Consequently, FR167356 was synthesized through replacement of imidazopyridine of a parental hit compound by benzofuran. FR167356 has potent inhibitory activity on V-ATPase and simple structure. Therefore, FR167356 derivatives seem to be more suitable for study of selective V-ATPase inhibitor.

FR167356 is the first V-ATPase inhibitor that can discriminate between osteoclast plasma membrane V-ATPase and lysosomal V-ATPase. In addition, FR167356 is the first compound that could distinguish between V-ATPases at cellular level. At cellular level, FR167356 also showed the selectivity between lysosomal function (LDL metabolism), which depends on lysosomal V-ATPase and osteoclast V-ATPase activities (H^+ extrusion and bone resorption). Lysosomes play many critical roles in biological functions, including digestion of exogenous or endogenous macromolecules, receptor recycling, processing of bioactive peptide substances and antigen presentation, with acidic environment. Therefore, the inhibitory profile of V-ATPase by FR167356, distinction between inhibition of osteoclast V-ATPase and inhibition of lysosomal V-ATPase, is noteworthy. The risk of undesirable alteration of cellular physiology may be decreased when FR167356 is administered to animals compared with other V-ATPase inhibitors.

The inhibitory selectivity of FR167356 suggests that there are differences in isoform expression between osteoclast and lysosomal V-ATPase. The presence of osteoclast-specific isoform is controversial. There is a report that described the presence of osteoclast-specific isoform in A subunit (van Hille *et al*., 1993), but the specificity was denied after that (van Hille *et al*., 1995). Recently, a3 was reported to be an osteoclast-specific isoform in a subunit (Li *et al*., 1996). a3 subunit-deficient mice exhibited severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification, whereas acidification of lysosomes was retained normally in these mice (Frattini *et al*., 2000). However, a3 was also reported to be ubiquitously expressed in man, and is found in all tissues studied using the polymerase chain reaction (Scott & Chapman, 1998). Toyomura *et al*. (2003) reported that a3 isoform was transported from the lysosome to the plasma membrane during osteoclast differentiation. Accordingly, inhibition profile of FR167356 may predict the presence of novel isoforms, which may differentiate osteoclast V-ATPase from lysosomal V-ATPase.

Osteoclasts contain not only plasma membrane V-ATPase but also lysosomal V-ATPase. It is very interesting to compare the effect of FR167356 on plasma membrane V-ATPase with intracellular lysosomal V-ATPase, both of which osteoclasts contain. However, it remains to be elucidated because it is difficult to isolate pure lysosomes from osteoclasts.

At first, we aimed to obtain the compound that inhibits nothing but osteoclast V-ATPase. However, FR167356 inhibited macrophage V-ATPase and kidney V-ATPase with a similar potency to inhibition of osteoclast V-ATPase. In macrophage, mechanism of pH_i regulation includes not only V-ATPase but also Na^+/H^+ exchanger and HCO^{3-}/Cl^- exchanger (Swallow *et al*., 1989). We actually observed that in acid-loaded macrophages H^+ extrusion did not affect by FR167356 but dimethylamiloride (Na^+/H^+ exchanger inhibitor) (data not shown). Kidney secretes H^+ for maintenance of acid–base balance. Multiple mechanisms (Na^+/H^+ exchanger, H^+,K^+-ATPase) involved for $H+$ secretion with the

exception of V-ATPase (Wang *et al*., 2002). Accordingly, the inhibitory effects of FR167356 on macrophage V-ATPase and kidney V-ATPase may not emerge *in vivo*.

Since bafilomycin A1 exerts cell death in parallel with inhibition of V-ATPase (Xu *et al*., 2003), we have never been able to judge whether both cell death and inhibition of H^+ secretion contribute to inhibition of bone resorption *in vitro*. As the number of cells per slice did not change regardless of the presence of FR167356 in pit formation assay, FR167356 clearly demonstrated that inhibition of H^+ secretion by V-ATPase inhibitors solely mediated inhibition of osteoclastic bone resorption.

In conclusion, the pharmacological studies of a novel V-ATPase inhibitor, FR167356, showed FR167356 had selectivity for osteoclast V-ATPase relative to lysosomal V-ATPase, suggesting that novel isoforms differentiate osteoclast V-ATPase from lysosomal V-ATPase. As FR167356 is available for oral administration, FR167356 may be a useful tool for indicating the direction of improvement for therapeutic use of V-ATPase inhibitor.

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(Received January 29, 2004
Revised March 23, 2004
Accepted March 26, 2004)