Iejimalide C Is a Potent V-ATPase Inhibitor, and Induces Actin Disorganization

Sayaka Kazami,^a Masak Takaine,^b Hiroyasu Itoh,^a Takaaki Kubota,^c Jun'ichi Kobayashi,^c and Takeo Usui^{*b}

^aTsukuba Research Laboratory, Hamamatsu Photonics K.K.; Tokodai, Tsukuba 300–2635, Japan; ^bFaculty of Life and Environmental Sciences, University of Tsukuba; Tsukuba, Ibaraki 305–8572, Japan; and ^cGraduate School of Pharmaceutical Sciences, Hokkaido University; Sapporo, Hokkaido 060–0812, Japan.

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Iejimalides (IEJLs) A–D are 24-membered macrolides isolated from a tunicate *Eudistoma cf. rigida*, and exhibit potent cytotoxicity *in vitro* and antitumor activity *in vivo*. We previously reported that the molecular target of IEJL-A and -B was the vacuolar-type H⁺-ATPases (V-ATPases). However IEJL-C and -D, which are sulfonated IEJL-A and -B, respectively, show more potent antitumor activity, and their molecular targets remain to be discovered. Here, we report that IEJL-C is also a potent V-ATPase inhibitor by binding in a site similar to the bafilomycin-binding site. Two-hour treatment with IEJL-C resulted in the complete disappearance of acidic organelles in HeLa cells. Interestingly, after 24-h treatment, small actin aggregates were observed instead of actin fibers. The same actin reorganization was also observed in cells treated with another V-ATPase inhibitor, bafilomycin A₁. Because IEJLs did not inhibit actin polymerization *in vitro*, these results suggest that the primary target of IEJL-C, as well as IEJL-A and -B, is V-ATPase, and actin reorganizations are probably caused by the disruption of pH homeostasis *via* V-ATPase inhibition.

Key words antitumor; cytotoxicity; iejimalide C; V-ATPase; actin cytoskeleton

V-ATPases are among the most widely distributed ATP-driven proton pumps in nature, present in all eukaryotic cells and in various bacteria.^{1,2)} Within eukaryotic cells, the structure of these proton pumps is highly conserved from yeast to humans, as a multiple subunit complex with a molecular mass exceeding 850 kDa. The pumps contain at least 13 different subunits with various copy numbers, which are organized into two distinct domains, a peripheral V₁ domain that is the catalytic sector and a transmembrane V₀ domain that constitutes the proton channel. Recently, it has been reported that V-ATPases are expressed in the plasma membrane of malignant and metastatic tumor cells, and function in the maintenance of intracellular pH.³⁾ Furthermore, it has been found that V-ATPases are attractive therapeutic targets of tumor cells *in vitro*⁴⁾ and *in vivo*.⁵⁾ These reports strongly suggest that V-ATPase inhibitors are good candidates for anticancer agents. The mechanism by which such inhibitors interfere with pump function has been investigated in detail. The most potent inhibitors, bafilomycin A₁⁶⁾ and the closely related compound concanamycin A, inhibit V-ATPases at nanomolar concentrations and have become important tools for the detection and identification of V-ATPase activity. The site of bafilomycin inhibition has been located to subunit *c* in the V₀ proton channel.^{7,8)}

Iejimalides (IEJLs) A–D, isolated from the marine tunicate *Eudistoma cf. rigida*, are unique 24-membered macrolides having two methoxy groups, four diene units, and an *N*-formyl-L-serine terminus^{9,10)} (Fig. 1). Because these compounds exhibit potent cytotoxic activity *in vitro* and antitumor activity *in vivo*,¹¹⁾ their target molecules and antitumor mechanisms have been attractive subjects for investigation. During the screening of novel inhibitors of osteoclasts, we identified IEJL-A and -B as potent antiosteoporotic compounds. Further investigation revealed that the molecular target of IEJL-A and

-B is V-ATPase and that IEJLs-A and -B show antiosteoporotic activity *via* V-ATPase inhibition.¹²⁾ Recently, Fürstner *et al.* reported the total synthesis of IEJL-B¹³⁾ and IEJLs-A–D,¹³⁾ and their investigation of the biological activity of IEJL-B and its non-natural analogues was focused on the actin cytoskeleton. They found that IEJL-B induced severe changes of the actin cytoskeleton in NIH/3T3 cells. These results suggested that IEJLs phenotypically have at least two cellular targets in cells, V-ATPases and the actin cytoskeleton. However, it remains to be revealed whether IEJLs independently inhibit both V-ATPases and the actin cytoskeleton.

- 1: R¹ = R² = H: Iejimalide A
- 2: R¹ = CH₃, R² = H: Iejimalide B
- 3: R¹ = H, R² = SO₃Na: Iejimalide C
- 4: R¹ = CH₃, R² = SO₃Na: Iejimalide D

Bafilomycin A₁ (BafA₁)

Fig. 1. Structures of Iejimalide A–D (1–4) and Bafilomycin A₁

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*To whom correspondence should be addressed. e-mail: usui.takeo.kb@u.tsukuba.ac.jp

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Here, we investigated the effects of IEJL-C, a sulfonlated IEJL-A, on the V-ATPase and the actin cytoskeleton, and the relationships between the inhibition of V-ATPases and the inhibition of the actin cytoskeleton. Our results suggested that the primary target of IEJL-C is V-ATPase and that actin reorganizations are probably caused by the disruption of pH homeostasis in the cells *via* inhibition of V-ATPases.

MATERIALS AND METHODS

Materials IEJLs-A-D were isolated as described previously.^{9,10} Bafilomycin A₁ was the kind gift of Professor H. Ikeda (Kitasato University).

Vacuole Isolation and Measurement of V-ATPase Activities The isolation of yeast vacuoles and V-ATPase activity assay were performed as described previously.¹² The reaction mixture (150 μ L) contained 5 mM Na₂ATP, 5 mM MgCl₂, 10 mM NH₄Cl, 5 mM NaN₃, 0.1 mM Na₃VO₄, 25 mM Mes-Tris (pH 6.9) and vacuolar membrane vesicles (10 μ g of protein). Prior to the assay of ATPase activity, the reaction mixture (without ATP) was incubated with or without drugs at the indicated concentrations for 10 min on ice, and then the reaction was started by adding ATP. The V-ATPase reaction was run for 20 min at 37°C, and stopped by adding 150 μ L of 0.6 M perchloric acid followed by placement on ice. To measure the released inorganic phosphate, 300 μ L of malachite green solution containing 0.03% malachite green oxalate, 0.2% NaMoO₄, 0.05% Triton X-100, and 0.7 M HCl was added. After 40 min of incubation at room temperature, the absorbance at 650 nm was measured. Drugs were solved in dimethyl sulfoxide (DMSO) solution and control activities were determined in the presence of an equivalent amount of 0.1% DMSO, a concentration that did not inhibit the enzyme activity.

Staining of Intracellular Acidic Organelles Mammalian cells were stained with acridine orange as described previously.¹² In brief, human cervical carcinoma HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) in a humidified atmosphere containing 5% CO₂. Cells were seeded onto coverslips and incubated for 2 h with latrunculin A, bafilomycin A₁ or compounds **2** or **3** at the indicated concentrations. They were incubated for 15 min with 5 μ M acridine orange (Waldeck GmbH & Co., KG Division Chroma) and washed with phosphate-buffered saline (PBS). Acidic organelles were photographed using a cooled charge-coupled device (CCD) camera (Leica DFC 350 FX R2; Leica, Wetzlar, Germany).

Cell Growth Assay HeLa cells (3 $\times$ 10³ cells/well) in 96 well plates were incubated overnight. Then, cells were treated with various concentrations of each compound. After 48 h incubation, 10 μ L of WST-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to the culture. After 1 h incubation, the absorbance at 450 nm was measured with iMark microplate reader (BioRad Laboratories, Inc.)

Yeast Halo Assay Exponentially growing *vma3*-replaced yeast cell lines, SKY080 and SKY081 (*MATa his3 ura3 trp1 ade2 can1 pdr1::hisG pdr3::hisG ysr1::HIS3 yrr1::TRP1 vma3::natNT2 leu2-pRS305-VMA3* or *leu2-pRS305-vma3-T32I*, respectively)¹² were inoculated into 5 mL of 0.5% agar containing 0.004% sodium dodecyl sulfate (SDS) (cooled to 45°C), and the cell suspension was spread on the surface of

Fig. 2. IEJL-C Inhibited V-ATPase *in Situ* and *in Vitro*

(A), BafA₁ (line with closed circles) and IEJL-C (**3**, line with open circles) inhibited yeast V-ATPases *in vitro* in a dose-dependent manner. (B), *vma3-T32I* mutation of *c* subunit in *V₀* domain confers IEJL-C resistance in budding yeast. The sensitivities of wild-type *VMA3* and *vma3-T32I* mutant yeast against 30 nM LatA, 100 nM BafA₁, 100 nM of **2** and 300 nM of **3** were tested by halo assay. (C), IEJLs inhibited cellular acidification by interfering with V-ATPases. HeLa cells seeded onto coverslips were incubated for 2 h with 100 nM LatA, 10 nM BafA₁, 30 nM of **2** and 1000 nM of **3**, and then stained with acridine orange. Bar, 50 μ m.

prewarmed YPDA plates (1% yeast extract, 2% polypeptone, 2% glucose, 0.02% adenine, 1.5% agar). One micro liter of drug solutions were spotted on a plate containing each strain and the plates were cultured at 30°C for 2 d, and clear zones around the spots were photographed.

Actin Purification, and *in Vitro* Actin Polymerization Assay Actin was prepared from the acetone powder of rabbit skeletal muscle, according to the method of Spudich and Watt.¹⁴ *In vitro* actin polymerization assay was performed using fluorescence spectroscopy as described previously.¹⁵

RESULTS

IEJL-C Inhibited V-ATPase Activity *in Vitro* and in Cells We previously reported that IEJL-A (**1**) and -B (**2**) exerted antitumor and antiosteoporotic activity *via* inhibition of vacuolar H⁺-ATPases (V-ATPases).¹² It has also been reported that the sulfonlated IEJLs, IEJL-C (**3**) and -D (**4**), showed more potent antitumor activity compared with **1** and **2**.¹¹ To

determine whether **3** directly inhibits V-ATPases in the manner of **1** and **2**, we tested the effect of **3** on V-ATPases from the vacuole membrane of budding yeast as described previously.¹²⁾ Yeast V-ATPases activities were inhibited by bafilomycin A₁ (BafA₁), a potent V-ATPase inhibitor, with an IC₅₀ value of 43.1 nM, as previously reported (Fig. 2A). Compound **3** also inhibited the activity of V-ATPases *in vitro*, with an IC₅₀ value of 121.9 nM (Fig. 2A). These results show that IEJL-C, in addition to IEJL-A and -B, is a potent V-ATPase inhibitor.

It has been suggested that IEJL-A and -B bind a site similar to the bafilomycin-binding site by using budding yeast carrying the mutation in *VMA3*, which encodes the *c* subunit in the V_o domain of V-ATPase.¹²⁾ Therefore we next investigated the effects of IEJL-C on the growth of yeast cells carrying *vma3* mutation (Fig. 2B). The growth of cells containing the wild-type *VMA3* gene was inhibited by BafA₁, compounds **2**, **3**, and latrunculin A (LatA). On the contrary, the sensitivity of the *vma3-T32I* mutant against LatA was not changed but decreased against BafA₁, compounds **2** and **3**. These results suggest that IEJL-C bind a site similar to the bafilomycin-binding site like IEJL-A and -B.

Because compound **3** inhibited the activity of yeast V-ATPases *in vitro* and the growth of yeast harboring wild-type *VMA3* gene, we next investigated its effects on the acidification of intracellular acidic organelles in human cervical carcinoma HeLa cells.¹²⁾ The low pH of the intracellular acidic organelles is known to be maintained by V-ATPases, and this acidification is detected as an orange fluorescence when the cells are stained with acridine orange, a weak base used as a fluorescent reagent.¹⁶⁾ As shown in Fig. 2B, acidic organelles

in the control cells and in the cells treated for 2 h with 100 nM LatA, an actin polymerization inhibitor, were stained with acridine orange, but 10 nM BafA₁ and 30 nM of **2** caused a complete disappearance of fluorescence of acidic organelles (Fig. 2C). One thousand nano molar of **3** also caused a complete disappearance of fluorescence of acidic organelles, indicating that **3** also inhibited V-ATPases *in situ*. It should be mentioned that the concentrations of compound **3** for the disappearance of acidic organelle were much higher than that of **2**. The similar result was also seen in IC₅₀ values of compounds **2** and **3** on HeLa cell growth (Table 1).

The Cellular Actin Cytoskeleton Was Disrupted by Long-Term Treatment with IEJLs Recently, it was reported that **2** and synthetic analogues showed cytotoxicity *via* disorganization of the actin cytoskeleton,¹⁷⁾ suggesting that IEJLs phenotypically have at least two cellular targets in cells, V-ATPases and the actin cytoskeleton. We investigated the effect of IEJLs on *in vitro* actin polymerization using pyrenyl actin, but no inhibitory effects of **2** and **3** were observed (data not shown). Therefore, we next investigated the effects of a V-ATPase inhibitor on the actin cytoskeletons *in situ* by immunofluorescence microscopy (Figs. 3A–J). In the control cells, we observed both actin bundles running through the cell body and lamellipodia around the cells (Fig. 3A). After 2 h of treatment with 100 nM LatA, the actin fibers disappeared, and only a few disorganized aggregates were observed in the cells (Fig. 3B). In contrast, no changes in the actin bundles or lamellipodia were observed in the cells treated with 10 nM BafA₁, 30 nM of **2**, and 1000 nM of **3** (Figs. 3C–E). Under the same concentrations and treatment duration, IEJLs did inhibit V-ATPases (Fig. 2C), suggesting that the primary target of IEJLs is V-ATPases.

Although we did not observe any effects on the cellular actin cytoskeletons after 2 h of treatment with **2** and **3**, the different phenotypes were observed after 24 h of treatment (Figs. 3F–J). LatA-treated cells possessed two nuclei (91%; Fig. 3G), and the new actin fibers were formed, but a few disorganized actin aggregates were still present (Fig. 3G). On the other hand, the actin bundles and lamellipodia around cells disappeared completely, and disorganized actin aggregates emerged

Table 1. IC₅₀ Values on Growth of HeLa Cell

Compound	IC ₅₀ (nM)
2 (IEJL-B)	2.7
3 (IEJL-C)	107.8
BafA ₁	9.8
LatA	105.0

HeLa cells (3×10³ cells/well) in 96-well plate were treated with compounds for 48 h. The cell viability was determined by WST-8.

Fig. 3. Effects of IEJL-C on the Actin Cytoskeletons after Short- and Long-Term Incubation

HeLa cells seeded onto coverslips were incubated with 100 nM LatA (B, G, L), 10 nM BafA₁ (C, H, M), 30 nM IEJL-B (D, I, N) and 1000 nM IEJL-C (E, J, O) for 2 h (A–E) or 24 h (F–O). Actin filaments were stained with rhodamine-phalloidin (A–J), and DNA was stained with Hoechst 33258 (K–O). Bar, 50 μm.

in the cells treated with BafA₁, compounds **2** and **3** (Figs. 3H–J), although there were no effects on the microtubule network (data not shown) or nuclear morphology (Figs. 3K–O). These results suggest that the actin disorganization is a common phenotype induced by the loss of function of V-ATPases.

DISCUSSION

IEJL-C (**3**), a unique 24-membered macrolide (Fig. 1), was originally isolated as an agent exhibiting potent cytotoxic activity *in vitro*^{9,10} and antitumor activity *in vivo*.¹¹ Here, we showed that compound **3** is a potent V-ATPase inhibitor (Fig. 2A) by binding a site similar to the bafilomycin-binding site as IEJL-A and -B (Fig. 2B), and induced the complete disappearance of acidic organelles in HeLa cells within 2 h (Fig. 2C). Nonetheless the IC₅₀ value of compound **3** (121.9 nM) against *in vitro* V-ATPase activity is almost same with those of compounds **1** and **2** (71.1 and 95.0 nM, respectively¹²), much higher concentrations of **3** than **2** were required for the growth inhibition of budding yeast (300 and 100 nM, respectively, Fig. 2B), for the inhibition of cellular V-ATPase in HeLa (1000 and 30 nM, respectively, Fig. 2C) and for the inhibition of HeLa cell growth (107.8 and 2.7 nM, respectively, Table 1). Because compound **3** contains a sulfonyl moiety, these results suggest that a strong negative charge of sulfonyl moiety does not influence the inhibitory activity of the V-ATPase *in vitro* but decreases the cell permeability of compound **3**.

Interestingly, after 24 h of treatment of **3**, small actin aggregates were observed in the place of linear actin fibers, and the same actin reorganization was also observed in cells treated with another V-ATPase inhibitor, BafA₁ (Fig. 3H). These results suggested that the actin disorganization occurs in the cells lacking V-ATPase activity by an indirect mechanism. There have been several reports that V-ATPase subunits B and C are directly bound to actin filaments with high affinity,¹⁸ and thus there may be a V-ATPase-dependent actin regulatory mechanism. More importantly, it was reported that yeasts having mutation in V-ATPases subunits E induced actin delocalization,¹⁹ suggesting that V-ATPase-dependent acidification of organelles is required for the regulation of the actin cytoskeleton.

Irrespective to LatA inhibited the actin cytoskeleton, the cytotoxicity of LatA was weaker than that of V-ATPase inhibitors, such as BafA₁ and IEJL-B.

In this study, we did not investigate the inhibitory activity of compound **4** because it was difficult to purify the enough amounts of compound **4**. However, we obtained the almost same results in inhibition of V-ATPase activity *in vitro*, disappearance of acidic organelles and actin aggregate formation in HeLa cells, and yeast halo assay using the 1 : 1 mixture of sulfonyl IEJLs, compounds **3** and **4** (data not shown), suggesting that IEJL-D also inhibits V-ATPase activity by binding a site similar to the bafilomycin-binding site.

Taken together, our results suggested that the primary target of compound **3** is V-ATPase, and that the inhibition of V-ATPases causes the actin reorganizations in the cells.

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