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

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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+/plus.bold -ATPases (V-ATPases). However IEJL-C and -D, which are sulfonylated 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 850kDa. 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 vitro4,5 and in vivo.6) 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 terminus9,10) (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 antosteoporotic compounds. Further investigation revealed that the molecular target of IEJL-A and -B is V-ATPase and that IEJLs-A and -B show antosteoporotic 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.
Here, we investigated the effects of IEJL-C, a sulfonylated 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. 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. The reaction mixture (150 μL) contained 5 mM Na₂ATP, 5 mM MgCl₂, 10 mM NH₄Cl, 5 mM NaN₃, 0.1 mM Na₂VO₄, 25 mM Mes-Tris (pH 6.9) and vacuolar membrane vesicles (10 μg 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 μL of 0.6 M perchloric acid followed by placement on ice. To measure the released inorganic phosphate, 300 μL 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. 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 μM 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 (3×10³ cells/well) in 96 well plates were incubated overnight. Then, cells were treated with various concentrations of each compound. After 48 h incubation, 10 μL 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 yrr1::HIS3 yrr1::TRP1 vma3::natNT2 leu2-prRS305-VMA3 or leu2-prRS305-vma3-T32I, respectively) 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 prewarmed YPDA plates (1% yeast extract, 2% polypeptide, 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. In vitro actin polymerization assay was performed using fluorescence spectroscopy as described previously.

RESULTS

IEJL-C Inhibited V-ATPase Activity in Vivo and in Cells We previously reported that IEJL-A (1) and -B (2) exerted antitumor and antiestopertor activity via inhibition of vacuolar H⁺-ATPases (V-ATPases). 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.
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.\textsuperscript{12} Yeast V-ATPases activities were inhibited by bafilomycin \( \Lambda_1 \) (Baf\( \Lambda_1 \)), a potent V-ATPase inhibitor, with an IC\( _{50} \) value of 43.1 nM, as previously reported (Fig. 2A). Compound 3 also inhibited the activity of V-ATPases in vitro, with an IC\( _{50} \) 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 \( \gamma \) subunit in the \( V_0 \) domain of V-ATPase.\textsuperscript{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 Baf\( \Lambda_1 \), compounds 2, 3, and latrunculin A (Lat\( \Lambda \)). On the contrary, the sensitivity of yeast cells harboring wild-type \( VMA3 \) gene was inhibited by Baf\( \Lambda_1 \), compounds 2, 3, and Lat\( \Lambda \). The low pH of the intracellular acidic organelles is known to be maintained by V-ATPases, and this effect of IEJLs on in vitro actin polymerization using pyrenyld 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 Baf\( \Lambda_1 \), 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 Baf\( \Lambda_1 \) and 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.

Table 1. IC\( _{50} \) Values on Growth of HeLa Cell

| Compound | IC\( _{50} \) (nM) |
|----------|------------------|
| 2 (IEJL-B) | 2.7 |
| 3 (IEJL-C) | 107.8 |
| Baf\( \Lambda_1 \) | 9.8 |
| Lat\( \Lambda \) | 105.0 |

HeLa cells (3 \( \times \) 10\(^4\) 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 Lat\( \Lambda \) (B, G, L), 10 nM Baf\( \Lambda_1 \) (C, H, M), 30 nM IEJL-B (2, D, I, N) and 1000 nM IEJL-C (3, E, J, O) for 2 h (A–E) or 24 h (F–O). Actin filaments was stained with rhodamine–phalloidin (A–J), and DNA was stained with Hoechst 33258 (K–O). Bar, 50 \( \mu \)m.
in the cells treated with BaFα₁, 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[1,10] 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, respectively)[12], 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 I). 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, BaFα₁ (Fig. 3H). These results suggest 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 A 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 subunit 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 BaFα₁ 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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