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Iejimalides A and B Inhibit Lysosomal Vacuolar H^+-ATPase (V-ATPase) Activity and Induce S-Phase Arrest and Apoptosis in MCF-7 Cells

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ABSTRACT

Iejimalides are novel macrolides that are cytostatic or cytotoxic against a wide range of cancer cells at low nanomolar concentrations. A recent study by our laboratory characterized the expression of genes and proteins that determine the downstream effects of iejimalide B. However, little is known about the cellular target(s) of iejimalide or downstream signaling that lead to cell-cycle arrest and/or apoptosis. Iejimalides have been shown to inhibit the activity of vacuolar H^+-ATPase (V-ATPase) in osteoclasts, but how this inhibition may lead to cell-cycle arrest and/or apoptosis in epithelial cells is not known. In this study, MCF-7 breast cancer cells were treated with iejimalide A or B and analyzed for changes in cell-cycle dynamics, apoptosis, lysosomal pH, cytoplasmic pH, mitochondrial membrane potential, and generation of reactive oxygen species. Both iejimalides A and B sequentially neutralize the pH of lysosomes, induce S-phase cell-cycle arrest, and trigger apoptosis in MCF-7 cells. Apoptosis occurs through a mechanism that involves oxidative stress and mitochondrial depolarization but not cytoplasmic acidification. These data confirm that iejimalides inhibit V-ATPase activity in the context of epithelial tumor cells, and that this inhibition may lead to a lysosome-initiated cell death process. J. Cell. Biochem. 109: 634–642, 2010.

KEY WORDS: MACROLIDE; BREAST CANCER; V-ATPase; LYSOSOME; MITOCHONDRIA; CELL CYCLE; REACTIVE OXYGEN SPECIES

Iejimalides constitute a small group of closely related marine macrolides that are composed of a 24-membered ring with two methoxy groups and an *N*-formyl-L-serine side chain [Nozawa et al., 2006]. The compounds are bioactive at nanomolar concentrations and have shown anti-cancer activity in vitro [Kobayashi et al., 1988; Kikuchi et al., 1991]. In the National Cancer Institute 60 tumor cell line screen iejimalide showed cytostatic and/or cytotoxic activity against a broad range of tumor cells, however, the mechanism of the anti-tumor activity of iejimalide is still unknown. There are two predominant chemical forms of iejimalide, A and B, that differ only by the presence of an additional methyl group on the macrolide ring of iejimalide B. A recent study of iejimalide B compared the differential cellular effects and underlying gene and protein expression levels between two prostate cancer cell lines, p53-wildtype LNCaP, and p53-null PC-3 [Wang et al., 2008]. In LNCaP cells, iejimalide B modulates the expression of many genes that regulate cell cycle and cell death, and induces G_0/G_1 cell-cycle arrest and apoptosis. In contrast, iejimalide B does not modulate the expression of these genes in PC-3 cells and induces S-phase arrest but not apoptosis, suggesting that the cellular response to

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iejimalides might be determined by the p53 status of the cell [Wang et al., 2008].

The total synthesis of iejimalide B has been described by two groups [Furstner et al., 2006; Schweitzer et al., 2007], opening the way for potential pharmacological development of this compound. However, to facilitate structure–function relationship studies on iejimalide, knowledge of the cellular target and mechanism of action is required. So far, osteoclast vacuolar H^+-ATPase (V-ATPase) is the only identified target of iejimalide action [Kazami et al., 2006]. In these cells the proton pump is localized on the plasma membrane and is responsible for the acidification of the extracellular compartment during bone resorption. In epithelial cells the V-ATPase is responsible for acidification of intracellular compartments including the Golgi, endosomes, lysosomes, and secretory vesicles and may play an important role in homeostatic regulation of cytosolic pH [Boron, 2004; Beyenbach and Wieczorek, 2006].

There is still much that is unknown about the effects iejimalide has on tumor cells. To further develop iejimalide as an anti-cancer compound, more information is needed on the cellular pathways involved in iejimalide-induced cell death and cell-cycle arrest. In the current study, p53-wildtype MCF-7 breast cancer cells were used to investigate a broad range of cellular activities affected by iejimalide to identify the pertinent targets and biological pathways involved in iejimalide-induced cell death. The cells were also treated with both iejimalides A and B to compare the activities of the related compounds. Both compounds rapidly neutralize the pH of lysosomes, induce S-phase cell-cycle arrest, and trigger apoptosis in MCF-7 cells through a mechanism that involves mitochondrial depolarization and oxidative stress but not cytoplasmic acidification or inhibition of mitochondrial ATP synthase. These results shed further light on the potential use of these compounds for use in cancer chemotherapy.

MATERIALS AND METHODS

CULTURE OF MCF-7 BREAST CANCER CELLS

MCF-7 cells were grown in αMEM medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Except when otherwise specified, MCF-7 cells were routinely plated at a density of 1×10^6 cells per 150-cm^2 dish and incubated at 37°C in 95% air/5% CO_2 in a humidified cell culture incubator 24 h before initiating treatments.

CRYSTAL VIOLET ASSAY TO DETERMINE DOSE RESPONSE OF MCF-7 BREAST CANCER CELLS TO IEJIMALIDES

MCF-7 cells were seeded at a density of 20,000 cells/well in 24-well plates and treated for 24–72 h with the indicated concentrations of iejimalide A or B. An equal volume of DMSO served as a vehicle control. Cells were fixed with 2% glutaraldehyde in phosphate-buffered saline (PBS), washed with PBS, stained with 0.1% crystal violet, destained in deionized water, and air dried. The stain retained by the cells was solubilized in 0.2% Triton X-100, transferred to a 96-well optical plate, and the optical density at 590 nm was determined.

CELL-CYCLE ANALYSIS OF MCF-7 BREAST CANCER CELLS TREATED WITH IEJIMALIDES

MCF-7 cells were treated with the indicated concentrations of iejimalide A or B for 48 h and harvested by trypsinization. Medium and PBS washes were collected and combined with trypsinized cells to include both adherent and non-adherent cells in the analysis. Harvested cells were fixed in ice-cold 90% ethanol and stained with propidium iodide in the presence of RNase. Cells were analyzed by flow cytometry at 620 nm on a Beckman Coulter Cytomics FC 500 and MXP software. A minimum of 10,000 events were analyzed for each experimental condition.

CELL DEATH ANALYSIS OF MCF-7 BREAST CANCER CELLS TREATED WITH IEJIMALIDES

MCF-7 cells were seeded and harvested as for cell-cycle analysis. Cells were treated with the indicated concentration of iejimalide A or B for 72 h; harvested cells were fixed in 2% formaldehyde in PBS on ice and permeabilized with 70% ethanol in PBS at −20°C. Cells were treated with Br-dUTP, cobalt chloride, and terminal deoxynucleotide transferase (TdT) and incubated with FITC-conjugated anti-BrdU monoclonal antibody (Phoenix Flow Systems, San Diego, CA) and propidium iodide. Cells were analyzed by bivariate flow cytometry at 620 and 525 nm on a Beckman Coulter Cytomics FC 500 and MXP software. A minimum of 10,000 events were analyzed for each experimental condition.

LYSOSOMAL pH ANALYSIS OF MCF-7 BREAST CANCER CELLS TREATED WITH IEJIMALIDES

MCF-7 cells were grown on coverslips in six-well plates and loaded with 100 nM LysoTracker Red (Invitrogen, Carlsbad, CA) and treated with 100 nM iejimalide A or B for 3 h. Coverslips were washed with PBS, fixed for 10 min with 1% formaldehyde, washed again with PBS, and mounted on slides for fluorescence or DIC imaging using a Leica DMRXA2 confocal microscope and Simple PCI software (Compix Media, Irvine, CA).

MEASUREMENT OF CYTOPLASMIC pH IN MCF-7 BREAST CANCER CELLS TREATED WITH IEJIMALIDE B

MCF-7 cells were treated for 24 h with 50 nM iejimalide B, collected by scraping, and washed in Hank's balanced salt solution (HBSS; with 25 mM HEPES, without calcium, magnesium, bicarbonate, and phenol red). Cells were loaded with 10 μM SNARF-AM (Invitrogen) diluted in HBSS for 10 min at 37°C, resuspended in HBSS and kept on ice. Iejimalide B was added to all buffers used for processing iejimalide B-treated samples to maintain inhibitory effects until analysis. Calibrator cells were prepared by treating control cells with 10 μM nigericin in the presence of high-K^+ buffers with pH of 6.2–7.4. Cells were analyzed by flow cytometry at 675 and 575 nm on a Beckman Coulter Cytomics FC 500 and MXP software, and the ratio of the intensities at each wavelength was plotted. The pH of each sample was calculated using Excel software by comparing the ratio value of the sample to a linear standard curve based on the calibrator cells.

MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL IN MCF-7 BREAST CANCER CELLS TREATED WITH IEJIMALIDES

MCF-7 cells were seeded and harvested as for cell-cycle analysis. Cells were treated for 18–30 h with 50 nM iejimalide A or B. Harvested cells were washed once with 0.2% BSA in PBS and loaded with 1 μM tetramethylrhodamine ethyl ester (TMRE) in 130 mM KCl in PBS for 15 min at 37°C in the dark. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), which abrogates the mitochondrial proton gradient, was used as a positive control. Cells were washed in PBS and analyzed by flow cytometry at 620 nm on a Beckman Coulter Cytomics FC 500 and MXP software.

MEASUREMENT OF MITOCHONDRIAL RESPIRATION IN RAT HEART MITOCHONDRIA TREATED WITH IEJIMALIDE B

Mitochondria were isolated from normal rat hearts as described [McKee et al., 1990]. Mitochondrial respiration was measured with a Clark type oxygen electrode connected to a Yellow Springs Model 53 oxygen monitor (Yellow Springs Instruments, Yellow Springs, OH) as previously described. Briefly, 3.7 ml of respiration buffer (100 mM KCl, 50 mM MOPS, 10 mM glutamate, 5 mM KH_2PO_4, 2.5 mM malate, 1 mM EGTA, pH 7.4) was added to a rapidly stirred chamber and equilibrated with room air at 30°C. The chamber was sealed with the oxygen electrode, mitochondria were added (equivalent to 1 mg protein), and rates of respiration recorded with a chart recorder. State 3 rates of respiration were initiated by the addition of 0.6 μmol ADP. After 2.5 min 0.3 μM iejimalide B was added, followed by addition of a second aliquot of 0.6 μmol ADP. After a further 2 min, oligomycin was added followed by a third aliquot of 0.6 μmol ADP. The rate of respiration was calculated from the changes in slope of the dissolved oxygen versus time curve.

MEASUREMENT OF REACTIVE OXYGEN SPECIES IN MCF-7 BREAST CANCER CELLS TREATED WITH IEJIMALIDES

MCF-7 cells were seeded and harvested as for cell-cycle analysis. Cells were treated for 18–42 h with 50 nM iejimalide A or B. Harvested cells were washed once with 0.2% BSA in PBS and loaded with hydroethidine (HE) for 15 min at 37°C in the dark. 60 μM hydrogen peroxide was used as a positive control. Cells were diluted with 0.2% BSA in PBS and analyzed by flow cytometry at 620 nm on a Beckman Coulter Cytomics FC 500 and MXP software.

STATISTICAL ANALYSIS

Flow cytometric analysis of cells stained with HE or TMRE was repeated twice. All other experiments were repeated at least three times. Statistical significance was determined by a P-value of <0.05 using one-way analysis of variance (ANOVA) with Bonferonni's post-test for cell number assays and using an unpaired t-test for measurement of intracellular pH. Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Instat software (Intuitive Software for Science, San Diego, CA).

RESULTS

IEJIMALIDES ARE CYTOTOXIC IN MCF-7 BREAST CANCER CELLS

In crystal violet assays measuring adherent cell density, MCF-7 cells were sensitive to low nanomolar concentrations of both iejimalides A and B (Fig. 1). A statistically significant decrease in cell number compared to untreated control is observed at 48 and 72 h for concentrations of 10 nM indicative of a cytostatic effect. At doses of 30 nM and above, there is a significant decrease in cell number compared with the initial 24 h time point, suggesting that both iejimalides A and B are cytotoxic in MCF-7 cells.

IEJIMALIDES INDUCE S-PHASE CELL-CYCLE ARREST AND APOPTOSIS IN MCF-7 BREAST CANCER CELLS

Analysis of cell-cycle dynamics demonstrates that by 72 h both iejimalides A and B induce a decrease in the G_0/G_1 and G_2/M-phase populations, accompanied by an increase in the S-phase population (Fig. 2). A sub-G_0 peak is also observed, suggesting that apoptosis is initiated by 72 h. Enzymatic labeling of DNA cleavage with bromodeoxyuridine (BrdU) confirms that both iejimalides A and B induce cell death by 72 h in a dose-dependent manner (Fig. 3).

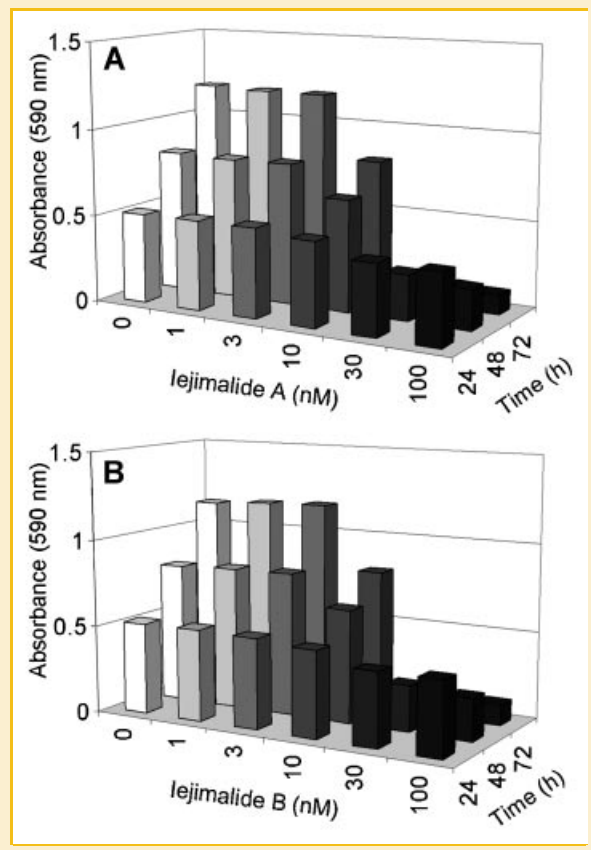

Fig. 1. Effects of iejimalides A and B on MCF-7 cell number. Cells were treated with increasing doses of iejimalide A (A) or iejimalide B (B) for 24–72 h as described in the Materials and Methods Section. Crystal violet staining reveals the relative number of cells remaining after treatment. The results shown are representative of three independent experiments. Error bars have been omitted for clarity.

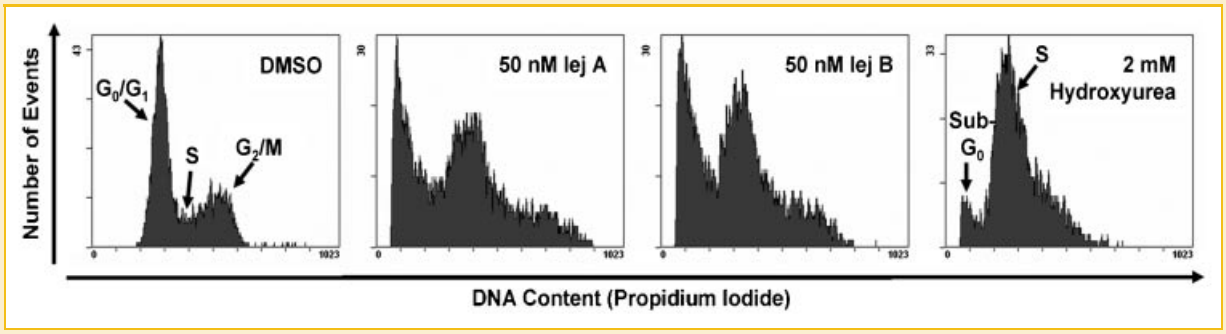

Fig. 2. Effects of iejimalides A and B on MCF-7 cell-cycle kinetics. Cells were treated for 72 h with 50 nM iejimalide A or B. The cells were stained with propidium iodide to measure DNA content and analyzed by flow cytometry as described in the Materials and Methods Section. DMSO served as a vehicle control, and hydroxyurea served as a positive control for S-phase arrest.

MCF-7 cells appear to be more sensitive to 10 nM iejimalide A than iejimalide B, although the predominant effect of this dose of iejimalide is on cell-cycle arrest. Taken together, these data suggest that iejimalides initially induce S-phase arrest, which is followed by apoptosis.

IEJIMALIDES INHIBIT ACIDIFICATION OF LYSOSOMES IN MCF-7 BREAST CANCER CELLS WITHOUT ALTERING CYTOPLASMIC pH

The proton gradient across the lysosomal membrane is maintained by the V-ATPase complex. To test the ability of iejimalides to inhibit lysosomal V-ATPase, MCF-7 cells were loaded with the pH-dependent lysosomal marker LysoTracker Red and treated with either 100 nM iejimalide A or B for 3 h. Compared to control cells, there is a significant decrease in pH-dependent lysosomal fluorescence in the iejimalide-treated cells after only 3 h (Fig. 4A), indicating that the two compounds rapidly block the accumulation of protons in the lysosomes, with essentially equal potency. These compounds also appear to induce rapid disruption of the lysosomal proton gradient in other epithelial cell lines, including the LNCaP and PC-3 prostate cancer cells (data not shown).

To determine whether there is significant acidification of the cytoplasm concomitant with the disruption of the lysosomal proton gradient, MCF-7 cells were treated with 50 nM iejimalide B for 24 h, and changes in cytoplasmic pH were monitored using the fluorescent pH indicator SNARF-1-AM. Treated cells show only

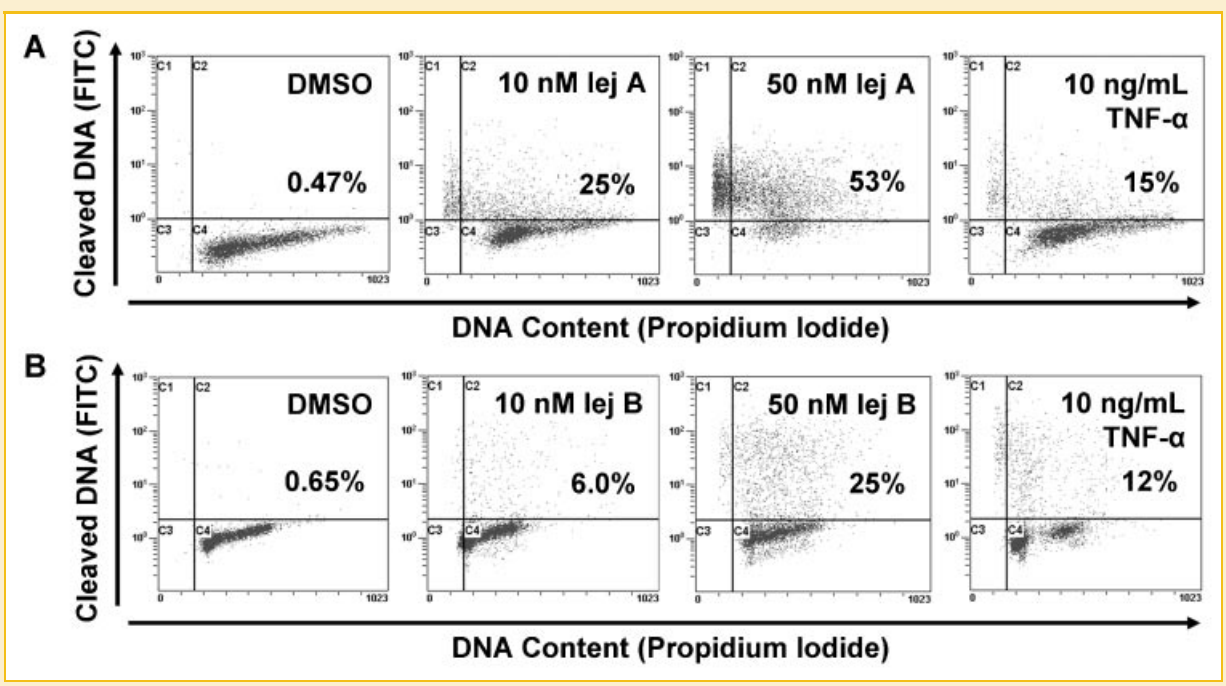

Fig. 3. Induction of apoptosis by iejimalides A and B in MCF-7 cells. Cells were treated for 72 h with 10 or 50 nM iejimalide A or B and labeled for DNA fragmentation using the Apo-BrdU method and counter-stained with propidium iodide as described in the Materials and Methods Section. Apoptotic events (upper right-hand quadrant) were recorded in populations of cells treated with iejimalide A (A) or iejimalide B (B), and the effect was dose dependent.

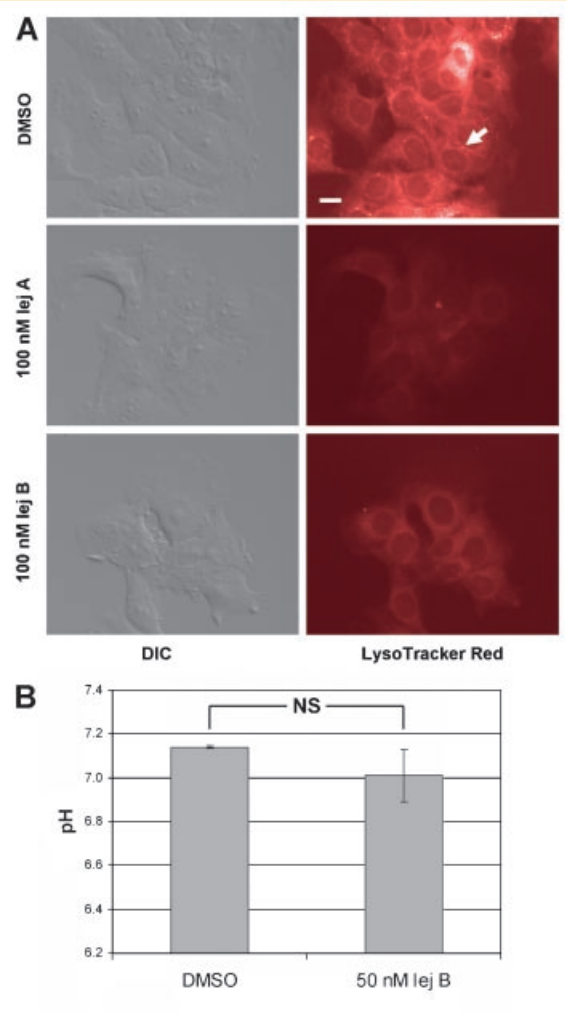

Fig. 4. Effects of iejimalides A and B on lysosomal and cytoplasmic pH in MCF-7 cells. A: Effects of iejimalides A and B on lysosomal acidification. Cells were stained with LysoTracker® Red, treated with DMSO or 100 nM iejimalide A or B for 3 h and imaged using DIC or fluorescent microscopy as described in the Materials and Methods Section. Arrow indicates punctuate lysosomal staining (scale bar: 10 μm). B: Effect of iejimalide B on cytoplasmic pH. Cells were treated for 24 h with DMSO or 50 nM iejimalide B and loaded with SNARF-1-AM to measure cytoplasmic pH as described in the Materials and Methods Section. There is no significant difference between control and treated cells. Results represent the mean (±SEM) of four independent experiments.

a slight decrease in pH compared to control cells (7.0 ± 0.1 vs. 7.1 ± 0.01) (Fig. 4B) that is neither statistically significant nor biologically relevant. Time points from 5 min to 24 h were tested with similar results (data not shown).

IEJIMALIDES CAUSE DELAYED MITOCHONDRIAL DEPOLARIZATION BUT DO NOT AFFECT MITOCHONDRIAL F-ATPASE ACTIVITY

Release and activation of several lysosomal proteases, including serine and cysteine proteases have been reported to induce cell death by promoting mitochondrial permeability transition through Bax activation. Measurement of the proton gradient of the mitochondrial inner membrane of MCF-7 cells treated with 50 nM iejimalide A or B demonstrates that there is a time dependent loss of membrane potential as evidenced by the appearance of a histogram peak with reduced fluorescence compared with the control population that is first evident at 24 h of treatment (Fig. 5A). To establish that this change in mitochondrial membrane potential is not due to a direct effect of iejimalides on the mitochondrial F-ATPase (ATP synthase), which has extensive structural homology to the lysosomal V-ATPase, the effect of iejimalide B on respiration of rat heart mitochondria was measured. As shown in Figure 5B, even very high doses of iejimalide B (0.3 μM) do not inhibit the oxygen consumption induced by the addition of ADP (529 natoms O_2/min/mg protein vs. 509 natoms O_2/min/mg protein in control vs. treated mitochondria). In contrast, oligomycin, a well-characterized inhibitor of the mitochondrial F-ATPase, blocks mitochondrial respiration in the same samples as effectively as depleting the endogenous ADP (83.4 natoms O_2/min/mg protein vs. 86.5 natoms O_2/min/mg protein). These results demonstrate that the effects of iejimalide B are not due to its direct interaction with the mitochondrial F-ATPase.

IEJIMALIDES CAUSE A GRADUAL INCREASE IN INTRACELLULAR LEVELS OF REACTIVE OXYGEN SPECIES

Loss of lysosomal and mitochondrial membrane potential has been shown to lead to an increase in the level of reactive oxygen species (ROS) that can be monitored by flow cytometry, measuring the changes in fluorescence induced by the conversion of HE to ethidium. Iejimalides A and B induce an increase in ROS levels that is first detectable at 24 h of treatment but increases gradually over time to approximately two times the level in control cells (Fig. 6).

DISCUSSION

Structurally iejimalides A and B are very similar, differing by a single methyl group on the macrolactone ring. The two compounds have been reported to have different activity profiles in the NCI 60 cancer cell line screen, raising the possibility that structure–function studies may help to elucidate the cellular target of the macrolides and define an appropriate pharmacophore for future drug development. Iejimalide B appears to be the more active compound in the NCI 60 cancer cell screen, and has previously been shown to induce cell-cycle arrest in $p53^{-/-}$ cells and cell death in $p53^{+/+}$ cell lines [Wang et al., 2008]. In the current study the two compounds have very similar effects on cell number, cell-cycle kinetics, and cell death, as well as lysosomal and mitochondrial membranes in MCF-7 cells. Both compounds induce dose- and time-dependent S-phase arrest and apoptosis in MCF-7 cells. Previous studies in p53 null or mutated cell lines (PC-3 prostate cancer cells and CHO-A8 Chinese hamster ovary cells, respectively) have demonstrated that iejimalide B induces S-phase arrest [Wang et al., 2008 and unpublished data], while in the p53-wildtype LNCaP cell line it predominantly induces apoptosis in response to iejimalide B.

Previous research has shown that both iejimalides A and B inhibit the V-ATPase in the plasma membrane of osteoclast cells and in the vacuolar membrane of *Saccharomyces cerevisiae* [Kazami et al.,

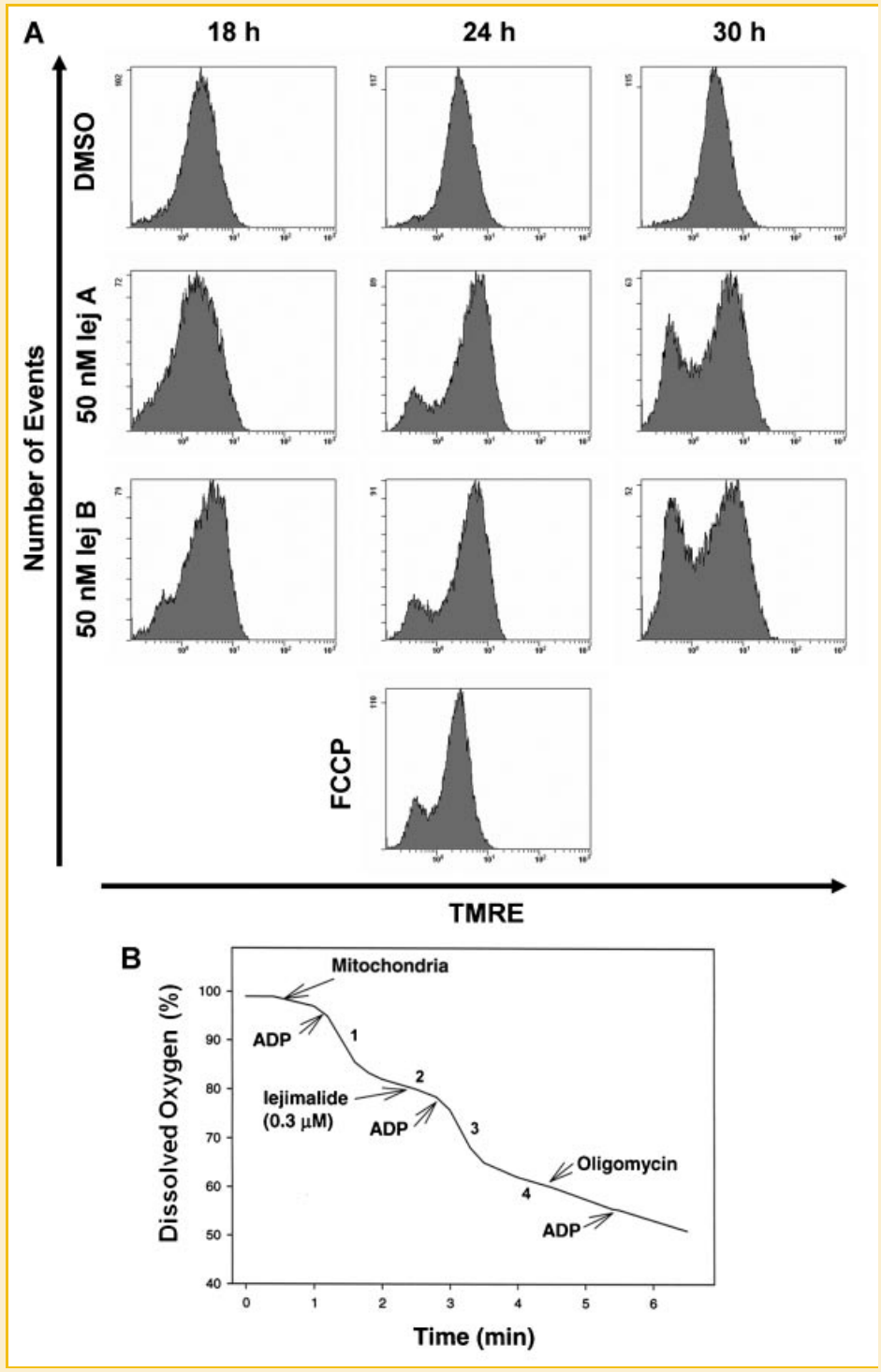

Fig. 5. Effects of iejimalides A and B on mitochondria. A: Cells were treated for 18–30 h with DMSO or 50 nM iejimalide A or B and loaded with TMRE to detect polarized mitochondria as described in the Materials and Methods Section. The proton ionophore FCCP was used as a positive control. Cells with decreased TMRE intensity indicate a loss of mitochondrial membrane potential. B: Effect of iejimalide B on mitochondrial respiration. Rat heart mitochondria were isolated and respiration measured as described in the Materials and Methods Section. Rate of oxygen consumption (in natoms O_2/min/mg) is (1) 529, (2) 86.5, (3) 509, and (4) 83.4. The data shown is from a representative experiment that was repeated on three independent preparations of mitochondria with equivalent results.

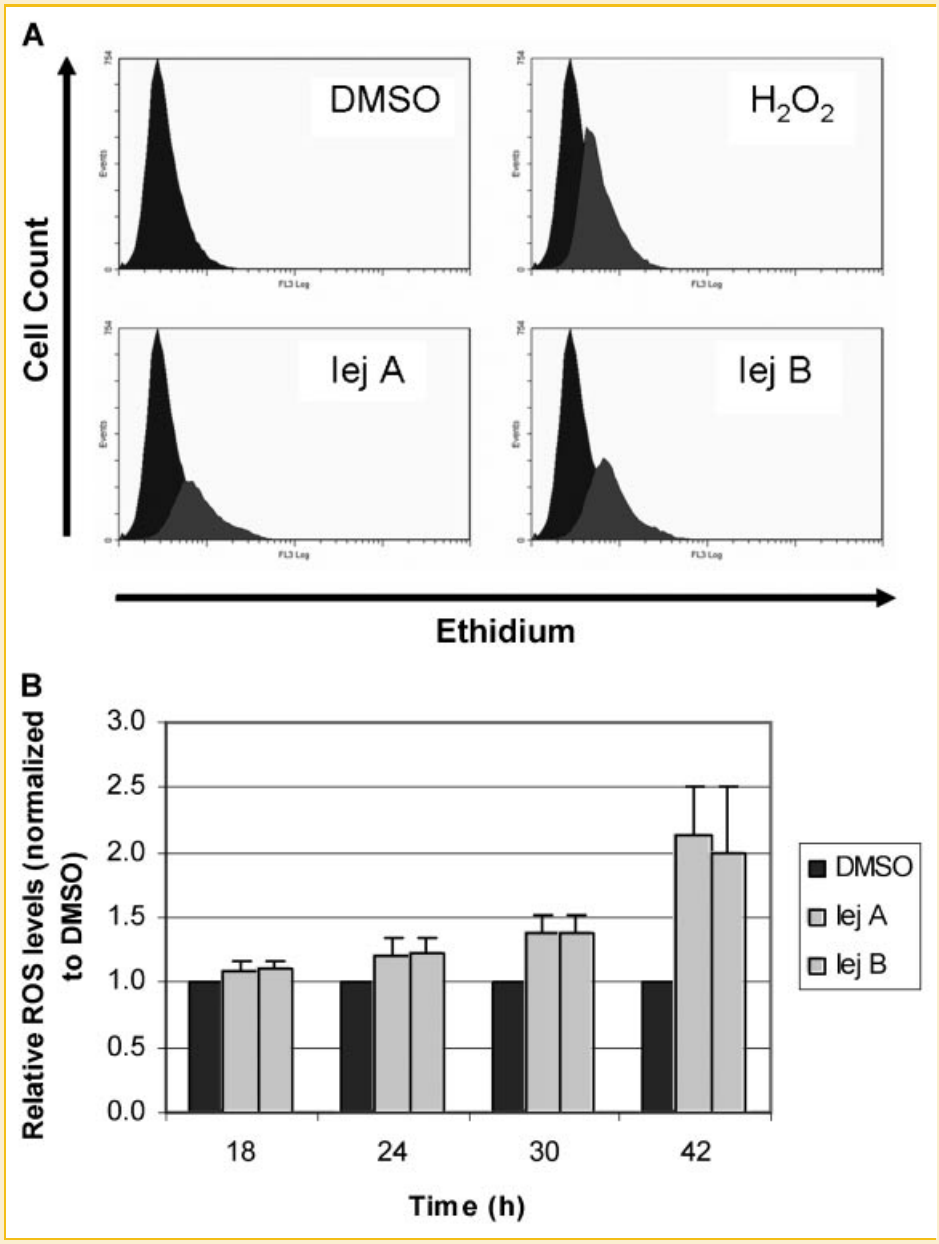

Fig. 6. Effects of iejimalides A and B on accumulation of ROS in MCF-7 cells. Cells treated for 18–42 h with 50 nM iejimalide A or B were loaded with HE to detect ROS using flow cytometry as described in the Materials and Methods Section. Hydrogen peroxide was used as a positive control. Cells with increased ethidium fluorescence indicate an increase in ROS. A: Histogram overlay of ROS in MCF-7 cells treated for 42 h. B: Relative ROS levels, measured by displacement on the *X*-axis and normalized to DMSO-treated control cells.

2006]. In MCF-7 cells the earliest detectable cellular response to iejimalide A or B is the loss of lysosomal proton gradient which is maintained by the V-ATPase, however, the decrease in cytoplasmic pH is modest and does not approach pH 6.5, the level needed to induce apoptosis [Counis and Torriglia, 2006]. This may be explained by the presence of several proton pumps, such as the sodium–hydrogen exchangers (NHEs), present in the plasma membrane that maintain pH homeostasis within the cell [Beltran et al., 2008]. In MKN-1 gastric cancer cells, another V-ATPase inhibitor, bafilomycin A_1, induces the loss of lysosomal membrane integrity, selectively releasing cathepsin D into the cytoplasm [Nakashima et al., 2003]. Lysosomal cysteine proteases, particularly cathepsin B, have been shown to activate pro-apoptotic proteins such as Bid while inactivating other, anti-apoptotic Bcl-2 family members [Stoka et al., 2001; Blomgran et al., 2007; Turk and Stoka, 2007]. This raises the possibility that in addition to blocking the acidification of lysosomes, iejimalides may induce the release of lysosomal proteases leading to apoptosis.

A significant sub-population of MCF-7 cells lose their mitochondrial proton gradient 24 h after treatment with iejimalide A or B, significantly later than the loss of the lysosomal proton gradient

(Fig. 5A). Since iejimalide B does not reduce the rate of oxygen consumption in isolated rat heart mitochondria, it is unlikely that iejimalides induce mitochondrial membrane permeability transition through direct effects on the mitochondrial F-ATPase. In this respect the iejimalides appear to be different from the well-characterized V-ATPase inhibitor bafilomycin A_1, which has also been shown to act as a potassium ionophore that can rapidly depolarize mitochondria and reduce mitochondrial respiration within several seconds after addition to isolated mitochondria [Teplova et al., 2007].

Studies similar to ours have been conducted on the structurally very similar macrolide archazolid. Like iejimalide, this compound is growth inhibitory in a number of mammalian cell lines at low nanomolar concentrations and neutralizes lysosomal pH as indicated by LysoTracker staining. In addition, it inhibits the V-ATPase holoenzyme in a biochemical assay. However, archazolid fails to inhibit the F-ATPase or P-ATPase enzymes even at micromolar concentrations [Huss et al., 2005]. In our studies, iejimalide also failed to inhibit the F-ATPase activity in rat heart mitochondria or to acidify the cytoplasm—suggesting that the activity of P-ATPase and other pH homeostatic proton-exchangers was not inhibited.

Yeast mutants deficient in V-ATPase activity have been shown to have elevated levels of ROS and to be more sensitive to oxidants [Milgrom et al., 2007], and several V-ATPase inhibitors, including bafilomycin A_1, have been shown to induce oxidative stress in cells. ROS levels in MCF-7 cells treated with iejimalide increase especially after 30 h of treatment. This is significantly longer than the time required to inhibit V-ATPase activity (3 h or less), and apparently after the onset of mitochondrial membrane transition. Assuming that biologically relevant levels of ROS are not below the lower limit of detection of our system, it is significant that ROS accumulate after mitochondrial depolarization, suggesting that the increase in ROS is related to the mitochondrial membrane permeability transition pore, which releases ROS-producing enzymes into the cytoplasm. This increase in ROS levels may lead to direct effects on DNA replication by causing DNA damage via oxidative stress. Single-strand breaks caused by ROS that are encountered by replicative machinery during S-phase may lead to double-strand breaks, which would then be detected by the DNA repair proteins ATM and/or ATR and begin a signaling cascade through the checkpoint kinases Chk1 and Chk2 and through p53, initially leading to S-phase arrest and subsequently apoptosis. Induction of S-phase arrest followed by apoptosis in MCF-7 cells suggests that iejimalides may stimulate an intra-S-phase checkpoint mechanism, possibly through indirect effects on Brca2 or Nek11 activation, which have been shown to arrest cells during DNA synthesis [Noguchi et al., 2002; Eastman, 2004].

Iejimalides A and B induce a variety of cellular responses over a relatively long period of time (Fig. 7). Within 3 h the lysosomal V-ATPase activity is inhibited; by 24 h there is a loss of mitochondrial membrane potential, after which ROS begin to accumulate. By 48–72 h dramatic effects are observed on cell cycle and cell death. These data demonstrate that iejimalides are potent

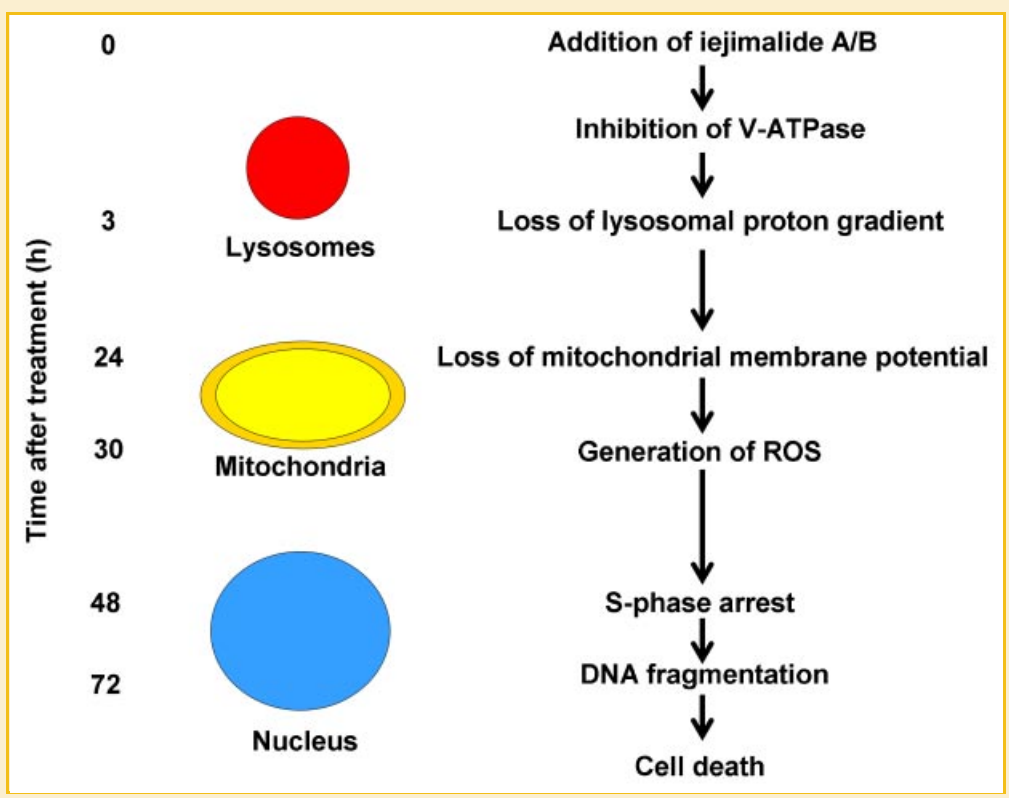

Fig. 7. Time course of iejimalide-induced cellular effects in MCF-7 cells.

inhibitors of V-ATPase activity in MCF-7 breast cancer cells, leading to the induction of S-phase arrest and cell death through the sequential activation of several cellular responses, initiated by lysosomal signaling.

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REFERENCES

Beltran AR, Ramirez MA, Carraro-Lacroix LR, Hiraki Y, Reboucas NA, Malnic G. 2008. NHE1, NHE2, and NHE4 contribute to regulation of cell pH in T84 colon cancer cells. Pflugers Arch 455:799–810.

Beyenbach KW, Wieczorek H. 2006. The V-type H+ ATPase: Molecular structure and function, physiological roles and regulation. J Exp Biol 209: 577–589.

Blomgran R, Zheng L, Stendahl O. 2007. Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. J Leukoc Biol 81:1213–1223.

Boron WF. 2004. Regulation of intracellular pH. Advan Physiol Educ 28:160–179.

Counis MF, Torriglia A. 2006. Acid DNases and their interest among apoptotic endonucleases. Biochimie 88:1851–1858.

Eastman A. 2004. Cell cycle checkpoints and their impact on anticancer therapeutic strategies. J Cell Biochem 91:223–231.

Furstner A, Nevado C, Tremblay M, Chevrier C, Teply F, Aissa C, Waser M. 2006. Total synthesis of iejimalide B. Angew Chem Int Ed Engl 45:5837–5842.

Huss M, Sasse F, Kunze B, Jansen R, Steinmetz H, Ingenhorst G, Zeeck A, Wieczorek H. 2005. Archazolid and apicularen: Novel specific V-ATPase inhibitors. BMC Biochem 6:13.

Kazami S, Muroi M, Kawatani M, Kubota T, Usui T, Kobayashi J, Osada H. 2006. Iejimalides show anti-osteoclast activity via V-ATPase inhibition. Biosci Biotechnol Biochem 70:1364–1370.

Kikuchi Y, Ishibashi M, Sasaki T, Kobayashi Ji. 1991. Iejimalides C and D, new antineoplastic 24-membered macrolide sulfates from the Okinawan Marine Tunicate *Eudistoma* cf *Rigida*. Tetrahedron Lett 32:797–798.

Kobayashi J, Cheng J, Ohta T, Nakamura H, Nozoe S, Hirata Y, Ohizumi Y, Sasaki T. 1988. Iejimalides A and B, novel 24-membered macrolides with potent antileukemic activity from the Okinawa Tunicate *Eudistoma* cf *rigida*. J Org Chem 53:6147–6150.

McKee EE, Grier BL, Thompson GS, McCourt JD. 1990. Isolation and incubation conditions to study heart mitochondrial protein synthesis. Am J Physiol 258:E492–E502.

Milgrom E, Diab H, Middleton F, Kane PM. 2007. Loss of vacuolar proton-translocating ATPase activity in yeast results in chronic oxidative stress. J Biol Chem 282:7125–7136.

Nakashima S, Hiraku Y, Tada-Oikawa S, Hishita T, Gabazza EC, Tamaki S, Imoto I, Adachi Y, Kawanishi S. 2003. Vacuolar H+-ATPase inhibitor induces apoptosis via lysosomal dysfunction in the human gastric cancer cell line MKN-1. J Biochem (Tokyo) 134:359–364.

Noguchi K, Fukazawa H, Murakami Y, Uehara Y. 2002. Nek11, a new member of the NIMA family of kinases, involved in DNA replication and genotoxic stress responses. J Biol Chem 277:39655–39665.

Nozawa K, Tsuda M, Ishiyama H, Sasaki T, Tsuruo T, Kobayashi J. 2006. Absolute stereochemistry and antitumor activity of iejimalides. Bioorg Med Chem 14:1063–1067.

Schweitzer D, Kane JJ, Strand D, McHenry P, Tenniswood M, Helquist P. 2007. Total synthesis of iejimalide B. An application of the Shiina macro-lactonization. Org Lett 9:4619–4622.

Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ, Ellerby LM, Bredesen D, Freeze H, Abrahamson M, Bromme D, Krajewski S, Reed JC, Yin XM, Turk V, Salvesen GS. 2001. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. J Biol Chem 276:3149–3157.

Teplova VV, Tonshin AA, Grigoriev PA, Saris NE, Salkinoja-Salonen MS. 2007. Bafilomycin A1 is a potassium ionophore that impairs mitochondrial functions. J Bioenerg Biomembr 39:321–329.

Turk B, Stoka V. 2007. Protease signalling in cell death: Caspases versus cysteine cathepsins. FEBS Lett 581:2761–2767.

Wang W, McHenry P, Jeffrey R, Schweitzer D, Helquist P, Tenniswood M. 2008. Effects of iejimalide B, a marine macrolide, on growth and apoptosis in prostate cancer cell lines. J Cell Biochem 105:998–1007.