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Effects of Iejimalide B, a Marine Macrolide, on Growth and Apoptosis in Prostate Cancer Cell Lines

Wei-Lin Winnie Wang,[1,2,3] Peter McHenry,[1] Randy Jeffrey,[1] Dirk Schweitzer,[4] Paul Helquist,[4] and Martin Tenniswood[1,2*]

[1]*Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556*

[2]*Gen*NY*Sis Center of Excellence in Cancer Genomics, Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, Rensselaer, New York 12144*

[3]*Department of Biochemistry, Queen's University, Kingston, ON, Canada K7L 3N6*

[4]*Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556*

ABSTRACT

Iejimalide B, a marine macrolide, causes growth inhibition in a variety of cancer cell lines at nanomolar concentrations. We have investigated the effects of Iejimalide B on cell cycle kinetics and apoptosis in the $p53^{+}/AR^{+}$ LNCaP and $p53^{-}/AR^{-}$ PC-3 prostate cancer cell lines. Iejimalide B, has a dose and time dependent effect on cell number (as measured by crystal violet assay) in both cell lines. In LNCaP cells Iejimalide B induces a dose dependent G_0/G_1 arrest and apoptosis at 48 h (as measured by Apo-BrdU staining). In contrast, Iejimalide B initially induces G_0/G_1 arrest followed by S phase arrest but does not induce apoptosis in PC-3 cells. qPCR and Western analysis suggests that Iejimalide B modulates the steady state level of many gene products associated with cell cycle (including cyclins D, E, and B and $p21^{waf1/cip1}$) and cell death (including survivin, p21B and BNIP3L) in LNCaP cells. In PC-3 cells Iejimalide B induces the expression of $p21^{waf1/cip1}$, down regulates the expression of cyclin A, and does not modulate the expression of the genes associated with cell death. Comparison of the effects of Iejimalide B on the two cell lines suggests that Iejimalide B induces cell cycle arrest by two different mechanisms and that the induction of apoptosis in LNCaP cells is p53-dependent. J. Cell. Biochem. 105: 998–1007, 2008.

KEY WORDS: CELL DEATH; CELL CYCLE; MARINE MACROLIDE; CYCLINS

The limitations of current cancer chemotherapies have led to the exploration for novel treatments and anticancer agents. Nearly 60% of all drugs, and over 75% of cancer drugs, introduced between 1981 and 2006 were first identified as natural products [Newmann and Cragg, 2007]. Anti-cancer agents derived from terrestrial plants, including taxol, vinblastine, vincristine, etoposide, and camptothecin, have demonstrated in vitro efficacy for a variety of cancers and are now in clinical use. Many other compounds are in clinical development [Cragg and Newmann, 2005]. New technologies in deep sea diving and collection have made marine organisms attractive sources for bioactive compounds with anti-inflammatory, anti-viral, antibiotic, immunosuppressive, or anti-tumor activities [Amador et al., 2003; Sipkema et al., 2005]. Natural products isolated from marine organisms are often produced by symbiotic bacteria, fungi, or cyanobacteria [König et al., 2006]. Such microorganisms produce compounds for defense that are foreign to terrestrial systems and can therefore be exploited for novel cytotoxic drugs. High-throughput screening systems, such as the National Cancer Institute NCI 60 tumor cell line screen, have increased speed of the identification of potential anti-cancer compounds from marine sources [Amador et al., 2003]. Iejimalides are one such class of natural products. Iejimalides are macrocyclic lactones, or macrolides, composed of a 24-member ring, with two methoxy groups, and an *N*-formyl-L-serine side chain [Nozawa et al., 2006]. Iejimalides were first extracted from the tunicate *Eudistoma* cf. *rigida* and were named after the island where the tunicates were found, Ie Jima, Japan [Kobayashi et al., 1988]. The compounds are bioactive and have shown anti-tumor activity in vitro [Kobayashi et al., 1988; Kikuchi et al., 1991]. In the NCI 60 tumor cell line screen Iejimalides showed cytostatic and/or cytotoxic activity against a broad range of tumor cells at nanomolar concentrations. However, the mechanism of the anti-tumor activity of Iejimalides is still not known. Two

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*Correspondence to: Martin Tenniswood, Gen*NY*Sis Center of Excellence in Cancer Genomics, State University of New York at Albany, 1 Discovery Drive, Rensselaer, NY 12144. E-mail: mtenniswood@albany.edu

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groups have recently described distinct synthetic routes to the complete synthesis of Iejimalide B, making it possible to develop new therapeutics for the treatment of a variety of cancers [Fürstner et al., 2007; Schweitzer et al., 2007].

To characterize the mechanism of action of Iejimalide B, we compared the effects of Iejimalide B in $p53^+$, androgen receptor positive (AR^+), LNCaP and $p53^-/AR^-$, PC-3 prostate cancer cell lines. These two cell lines were chosen since they represent models of early stage, hormone responsive prostate cancer (LNCaP) and late stage hormone refractory disease (PC3). The data presented here demonstrate that Iejimalide B induces cell cycle arrest and cell death in the $p53^+$ cell line in a dose- and time-dependent manner. In the $p53^-$ cell line Iejimalide B induces S phase arrest but not cell death. In each of these cell lines, these biological processes are accompanied by distinct changes in gene expression, suggesting that Iejimalide B exerts some, or all of its effects, through transcriptional regulation. In LNCaP cells many of the genes that are affected by Iejimalide B regulate cell cycle and cell death (as characterized by Gene Ontology), and are p53 target genes. A different spectrum of genes is induced in PC-3 cells, suggesting that Iejimalide B induces both p53 dependent and independent responses in prostate cancer cell lines.

MATERIALS AND METHODS

CELL CULTURE

The LNCaP and PC-3 human prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). PC-3 cells were cultured in Ham's F-12K (Kaign's modified) medium (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Cells were passaged regularly every 4 or 5 days with 2.5× Trypsin-EDTA (Invitrogen). LNCaP cells were cultured in RPMI 1640 medium (Invitrogen) with 10% FBS (Atlas, Fort Collins, CO). Penicllin and streptomycin was added to all media to final concentrations of 100 Units/ml and 100 μg/ml, respectively. Cells were passaged every 4 or 5 days with 1× Trypsin-EDTA. Both cell lines were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2.

CRYSTAL VIOLET ASSAY

Cells were plated in 24-well Costar plates (Corning Inc., Corning, NY) at densities of 2×10^4 or 4×10^4 cells/well for PC-3 and LNCaP, cells respectively. At 24 h intervals cells were fixed with 2% glutaraldehyde in phosphate buffer saline (PBS) for 20 min and air dried, and stained with 0.1% crystal violet in water for 30 min and washed extensively with water. Crystal violet was re-dissolved with 0.2% Triton X-100 and the absorbance was measured by Wallac Victor^2 1420 Multilabel Counter at 590 nm.

FLOW CYTOMETRY

Cells were plated at 1×10^6 cells per dish in tissue culture flasks, and treated with 30 or 50 nM Iejimalide B (the optimal doses determined from the crystal violet assay). Cells treated with vehicle (DMSO) served as the negative control. Cells were harvested by trypsinization, permeabilized with 90% ethanol and incubated with 50 μg/ml propidium iodide and 0.015 U/ml RNase (Roche Applied Science, Indianapolis, IN) in PBS for 20 min at room temperature. Samples were kept on ice and analyzed within 3 h on a Beckman Coulter Cytomics FC 500MPL (Coulter, Miami, FL). Results were analyzed by Wincycle (Phoenix Flow Systems, San Diego, CA).

Apoptosis was detected by flow cytometry using Apo-BrdU staining. Cells were harvested by trypsinization and fixed with 4% formaldehyde in PBS and permeabilized by 70% ethanol. Samples were enzymatically labeled with bromodeoxyuridine triphosphate in TdT reaction buffer (Br-dUTP, 2.5 mM cobalt chloride, and terminal transferase 24,000 U) for 1 h at 37°C to label the 3′ OH ends of fragmented DNA (Roche Diagnostics). DNA strand breaks were detected using the Apo-BrdU detection kit according to the manufacturer's directions (Phoenix Flow Systems). Cells were counter-stained with 50 μg/ml propidium iodide and at least 10,000 cells were analyzed on Beckman Coulter Cytomics FC 500MPL within 3 h, and modeled using Multiplus AV software (Phoenix Flow Systems).

Q-PCR ANALYSIS

LNCaP, and PC-3 cells were plated at 1×10^6 cell/dish and treated with control vehicle, or 30 or 50 nM Iejimalide B prior to harvesting at 24, 48, and 72 h by trypsinization. Total mRNA was extracted using the RNeasy Midi kit (Qiagen, Valencia, CA). Reverse transcription PCR reactions were performed with 1.5 μg RNA samples using Taqman® Reverse Transcription Reagents (Cat # N8080234, Applied Biosystems, Foster City, CA). The reaction mixture was incubated for 10 min at 25°C, 1 h at 37°C and 5 min at 95°C and kept at 4°C until further analysis. Q-PCR SYBR Green probes for each gene were designed using Primer Express™ 1.5 (Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA) (see Table I Supplemental Data for sequences). SYBR Green reactions were performed using the SYBR® Green PCR Master Mix (Applied Biosystems). SYBR Green reactions were performed using an ABI Prism™ 7700 Sequence Detector (Applied Biosystems) and incubated for 2 min at 50°C, 10 min at 95°C, 15 s at 95°C and 1 min at 60°C for 50 cycles. Relative expression levels of each gene in real time were analyzed using the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001] and presented as ratio to the expression of the housekeeping gene GAPDH. Each sample was replicated twice from three independent sets of RNA preparations. Results are tabulated as mean ± SE analyzed by an established mathematic model [Fu et al., 2005]. Differences greater than twofold were considered to be biologically relevant and means were considered statistically significant (*) at $P < 0.05$.

SUBCELLULAR FRACTIONATION

Cells were scraped and pelleted by centrifugation at 1,500× rpm for 3 min at 4°C. Pellets were resuspended in ice-cold wash buffer (25 mM Tris, pH 7.5, 250 mM sucrose, 2.5 mM $MgCl_2$, 10 mM benzamidine, 10 mM NaF, 1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin and 1 mM PMSF), pelleted at 1,500× rpm for 3 min at 4°C and resuspended in Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM sodium vanadate, 1 mM DTT, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 2.5 μg/ml pepstatin, 1 mM PMSF, 10 mM benzamidine and 20 mM NaF). Pellets were

resuspended with three volumes of Buffer A and lysed with a Dounce homogenizer. Homogenates were centrifuged twice at 1,500× rpm for 6 min at 4°C and the nuclear pellets were resuspended in Buffer A, sonicated for 20 s, and stored at -20°C. The supernatants were ultracentrifuged at 55,000× rpm (100,000*g*) for 1 h at 4°C. The resulting supernatant containing cytosolic fraction was designated S100 and stored at −20°C. Protein concentrations were determined by the Micro BCA protein assay (Pierce, Rockford, IL).

WESTERN BLOT ANALYSIS

Nuclear fraction (30 μg), and cytosolic fraction S100 (30 μg) isolated as described above were solubilized in loading buffer containing 5% β-mercaptoethanol, separated by SDS–PAGE, and transferred to nitrocellulose. Equal loading and transfer of proteins were confirmed by Ponceau-S staining (BDH, Dorset, England). Protein derived from total cell lysate, nuclear and S100 extracts were immunoblotted with anti-cyclin D1 (DCS6) mouse monoclonal (1:1,000); anti-$p21^{Waf1/Cip1}$ (DCS60) mouse monoclonal (1:1000) from Cell Signaling Technology; anti-p53 (clone BP53-12) mouse monoclonal (1:500), anti-cyclin B1 mixed mouse monoclonal (1:1,000), anti-cyclin E (clone HE12) mouse monoclonal (1:1,000), from Upstate (Biotech Lake Placid, NY); anti-survivin rabbit polyclonal (1:2,000) from R&D Systems (Minneapolis, MN); anti-androgen receptor mouse monoclonal (1:1,000) from BD Pharmingen (San Diego, CA); anti-GAPDH mouse monoclonal (1:1000) from Biogenesis (Kingston, NH); anti-lamin A/C (N-18) goat polyclonal (1:100) from Santa Cruz Biotech (Santa Cruz, CA), diluted in blocking solution (5% BSA in 0.05% PBS-Tween 20). Specific antibody binding was detected by goat anti-mouse from Bio-Rad Lab (Hercules, CA), goat-anti rabbit from Bio-Rad Lab or donkey anti-goat from Pierce, IgG antibody conjugated with horseradish peroxidase diluted 1:5,000 in 5% skim milk in 0.05% PBS-Tween 20 and autoradiographed with enhanced chemeriluminescence (Pierce). The band intensities on all gels were measured and analyzed with the Kodak 1D imaging software. Blots were stripped with Western Re-Probe™ Buffer (Geno Technology, St. Louis, MO). Changes in protein levels were determined relative to the appropriate loading control (lamin A/C for nuclear proteins; GAPDH for cytoplasmic proteins).

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to assess statistical significance between means. Differences between means were considered significant when $P < 0.05$ for mRNA analyses or $P < 0.1$ for protein analyses using the Bonferroni post-test. All statistical analyses were performed with the GraphPad Instat software (Intuitive Software for Science, San Diego, CA).

RESULTS

Iejimalide B exerts dose- and time-dependent effects on both LNCaP and PC-3 cell lines (Fig. 1, panels A,B). In LNCaP cells, 30 nM Iejimalide B inhibits the time dependent increase in cell number, whereas 50 and 100 nM induce a substantial decrease in cell number. In contrast, the compound blocks the increase in cell number observed in untreated PC-3 cells at doses of 30 nM or greater, but does not cause a decrease in cell number, even at the highest dose tested (100 nM). Cell cycle analysis (Table I) demonstrates that LNCaP cells treated with 30 or 50 nM Iejimalide B showed an increased G_0/G_1 population, (79% and 73.9% compared to 66.2% in the untreated control cells), with a concomitant decrease in G_2/M relative to untreated cells. The S phase population remained relatively unchanged after treatment with 30 or 50 nM Iejimalide B for 48 h. In addition, these cells show a dose-dependent increase in

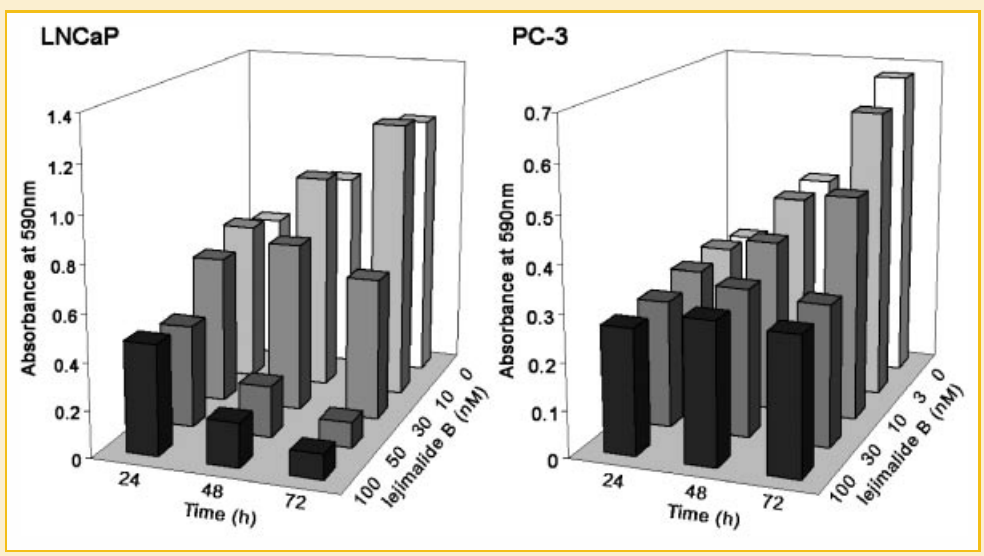

Fig. 1. Dose and time-dependent effect of Iejimalide B on PC-3 and LNCaP cells. Cells were treated with control vehicle or increasing doses of Iejimalide B (10–100 nM) for periods up to 72 h. Cell number was measured by crystal violet assay. Means and standard deviation of three independent replicates were calculated using Excel. Statistical was performed using one way ANOVA (error bars not shown for clarity).

TABLE I. Cell Cycle Kinetics of LNCaP Cells Treated With Iejimalide B for 48 h

Treatment		Percentage of cell cycle			
Time (h)	Compound	Sub-G_0	G_0/G_1	S	G_2/M
48	Vehicle control	0	66.2	18.5	15.2
	Iejimalide B (30 nM)	8.9	79.0	15.0	6.0
	Iejimalide B (50 nM)	21.5	73.9	21.1	5.0
	TNFα (10 ng/ml)	9.3	74.3	14.5	11.2

LNCaP cells were plated in RPMI-1640 media supplemented with 10% FBS and grow for 24 h prior to treatment. Cells were treated with either 30 and 50 nM Iejimalide B and 10 ng/ml TNFα, the positive control of this experiment. Cells were fixed and analyzed by flow cytometry as described in Materials and Methods Section.

the number of sub-G_0 (apoptotic events) in response to Iejimalide B (8.9% and 21.5%, respectively) that is as effective as 10 ng/ml TNFα, which is known to induce cell death in this cell line. In contrast, in PC-3 cells Iejimalide B initially induces G_0/G_1 arrest at 24 h, but at later times the blockade is predominantly in S phase (Table II). The time dependent increase in the proportion of the cells demonstrating G_0/G_1 arrest in untreated PC-3 cells is indicative of the induction of cell cycle arrest due to contact inhibition. At doses of 30 nM or greater, Iejimalide B is as effective as hydroxyurea which is known to induce S phase arrest by inhibiting ribonucleotide reductase (Yarbro, 1992). In PC-3 cells, Iejimalide B does not increase the number of sub-G_0 events, even at longer times of incubation (Table II) and higher doses (not shown), indicating that the compound does not induce apoptosis in this cell line. Analysis of DNA fragmentation using apo-BrdU staining, confirms that 30 or 50 nM Iejimalide B induces apoptosis in LNCaP cells, but neither dose induces apoptosis in PC-3 cells after 72 h (Fig. 2).

We have surveyed the effects of Iejimalide B on 55 genes associated by the Gene Ontology Consortium (www.geneontology.org) with the biological processes of cell cycle (GO:0007049) or apoptosis (GO:0006915) and their children (more refined functional subcategories of these biological processes in GO), particularly DNA replication (GO:0006260) and anti-apoptosis (GO:0006916).

TABLE II. Cell Cycle Kinetics of PC-3 Cells Treated With 30 nM Iejimalide B

Treatment		Percentage of cell cycle			
Time (h)	Compound	Sub-G_0	G_0/G_1	S	G_2/M
24	Vehicle control	0	36.5	26.2	37.3
	Iejimalide B (30 nM)	0	44.7	38.8	16.5
	Hydroxyurea (2 mM)	0	47.3	38.9	13.7
48	Vehicle control	0	48.8	27.1	24.1
	Iejimalide B (30 nM)	0	49.2	38.0	12.7
	Hydroxyurea (2 mM)	0	45.9	50.3	3.8
72	Vehicle control	0	60.6	19.9	19.5
	Iejimalide B (30 nM)	0	39.6	45.5	14.8
	Hydroxyurea (2 mM)	0	56.3	42.9	0.7

PC-3 cells were plated in Ham's F-12K media supplemented with 10% FBS and grow for 24 h before treatment. Cells were treated with 30 nM Iejimalide B or 2 mM hydroxy-urea for up to 72 h. Cells treated with hydroxyurea served as the positive control for this experiment. Cells were fixed and analyzed by flow cytometry as described in Materials and Methods Section.

Changes in the steady state level mRNA of selected genes induced by 30 and 50 nM Iejimalide B in both cell lines were monitored at 24, 48, and 72 h using Q-PCR. Of the 55 transcripts selected for analysis, 40 show greater than twofold change in one or both cell lines (Supplemental Data, Table II). The majority of the transcripts that are significantly modulated by Iejimalide B are associated with G_1/S or G_2/M transition or apoptosis. To establish that the changes in mRNA levels monitored by RT-PCR are reflected in changes at the protein level, a select group of genes and gene products, associated with androgen regulation, cell cycle and cell death were monitored in parallel.

Iejimalide B induces a dose dependent decrease in the steady state level of the androgen receptor (AR) mRNA in LNCaP cells reaching a level of 5.8 ± 0.3-fold lower by 72 h (Fig. 3). The decrease in AR mRNA induced by 50 nM Iejimalide B is mirrored by a significant decrease in both the nuclear and cytoplasmic levels of the AR, which is first evident at 48 h. Neither AR mRNA nor AR protein was detected in PC-3 cells (data not shown).

Iejimalide B induces an early and statistically significant decrease in the levels of cyclin D1 mRNA in LNCaP cells by 24 h (-3.1 ± 0.5-fold), but has no statistically significant effect in PC-3 cells (Fig. 4). There is a corresponding dose dependent decrease in the protein level of cyclin D1 in LNCaP cells that is quite profound by 48 h. In PC-3 there is a slight but significant decrease in the protein levels of cyclin D1 by 72 h which does not appear to correlate with changes in the cyclin D1 mRNA levels. Similarly Iejimalide B induces a dose dependent early decrease in the steady state mRNA levels of cyclin E in LNCaP cells that reaches significance at 48 h (-2.7 ± 0.4-fold), but has no detectable effect on the cyclin E mRNA and protein levels in PC-3 cells (Fig. 5). In LNCaP cells the decrease in cyclin E protein levels correlate to these changes in the steady state RNA levels, and is very pronounced by 72 h. The most dramatic effect of Iejimalide B on the major cyclin genes appears to be exerted on cyclin B1 mRNA which is down regulated by more than 20-fold in LNCaP cells by 72 h, and by more than 6-fold in PC-3 cells. The decrease in cyclin B1 protein levels in both cell lines parallels the changes in mRNA levels (Fig. 6). The decrease in cyclin B1 mRNA levels is also paralleled the changes in cyclin B2 mRNA levels (Table II, Supplemental Data). These data demonstrate that Iejimalide B induces cell cycle arrest through the substantial down regulation of several cyclins in LNCaP cells and to a lesser extent in PC-3 cells.

In addition to these changes Iejimalide B also induces a substantial increase in the mRNA levels of the cyclin kinase inhibitor (CKI) p21 in both LNCaP and PC-3 cells. However, the effects on the mRNA are not reflected in the changes in the level of the protein. Iejimalide B has a more dramatic effect on the p21 protein levels in PC-3 cells than LNCaP cells. In PC-3 cells, p21 protein levels are rapidly increased in response to Iejimalide B, but then decrease significantly by 72 h (Fig. 7).

Other genes associated with G_1/S transition (GO:0000082) including cdk2 which is significantly down regulated by 50 nM Iejimalide B after 48 and 72 h of treatment in LNCaP cells. Cyclin A, which along with cyclin E, heterodimerize with cdk2 are also significantly down regulated by Iejimalide B (-17.7 ± 1.4 6-fold) as early as 24 h in LNCaP cells. Cyclin A is also down regulated in PC-3

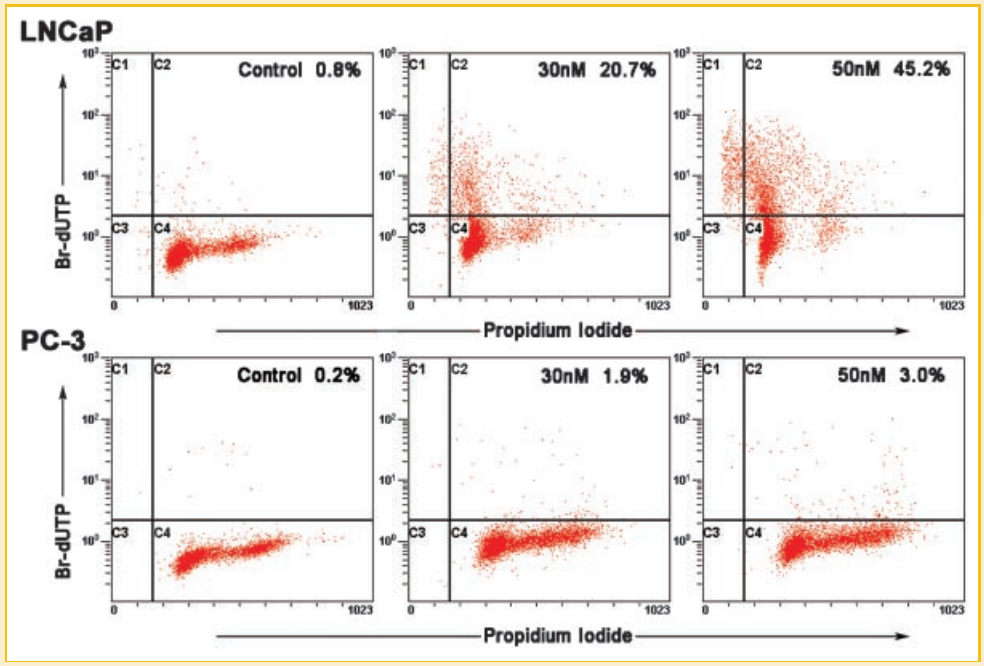

Fig. 2. Induction of DNA fragmentation by Iejimalide B in PC-3 and LNCaP cells. Cells were treated with control vehicle, 30 and 50 nM Iejimalide B for 72 h. Cell death was measured by apo-BrdU staining and detected by flow cytometry. Percentage of cell death for both the control and treated is indicated as %(C1/C2)/total events in upper right quadrant of each graph.

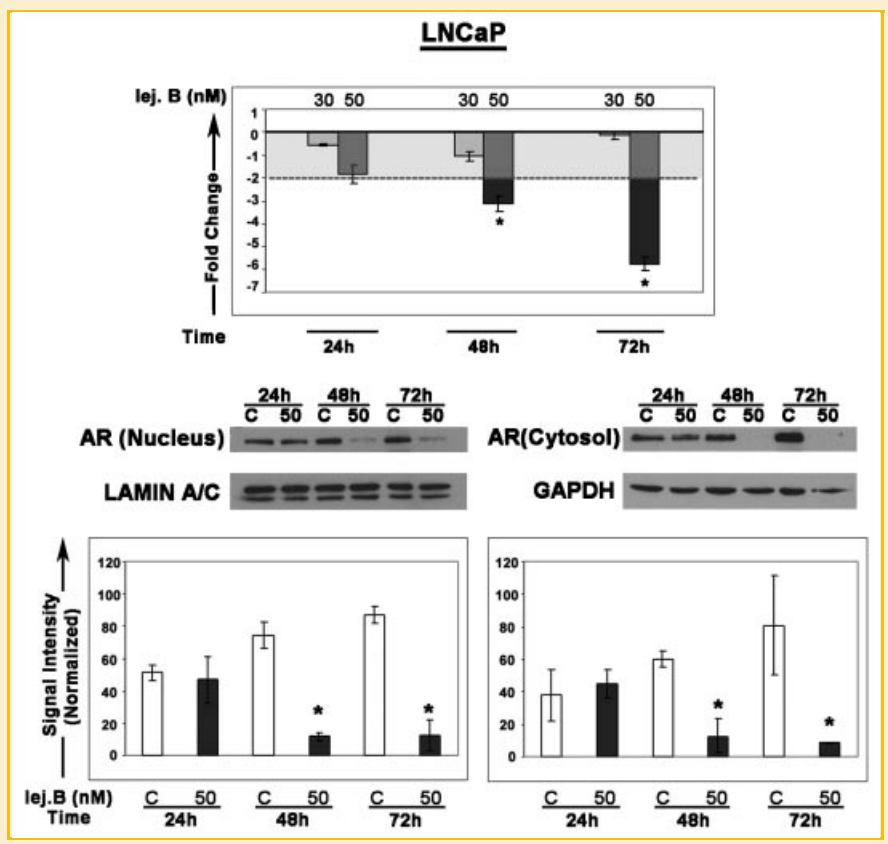

Fig. 3. Effect of Iejimalide B on AR mRNA and protein levels in LNCaP cells. Q-PCR (upper panel) and Western blot (bottom panel) analyses for androgen receptor (AR) in LNCaP cells incubated with control vehicle, 30 or 50 nM Iejimalide B. Statistical significance ($P < 0.05$) is indicated by asterisks; while changes less than twofold with respect to control group are shaded. Protein expression was normalized to lamin A/C for nuclear proteins or GAPDH for cytoplasmic proteins. Significant changes in protein level relative to the control group ($P < 0.1$) are indicated by asterisks.

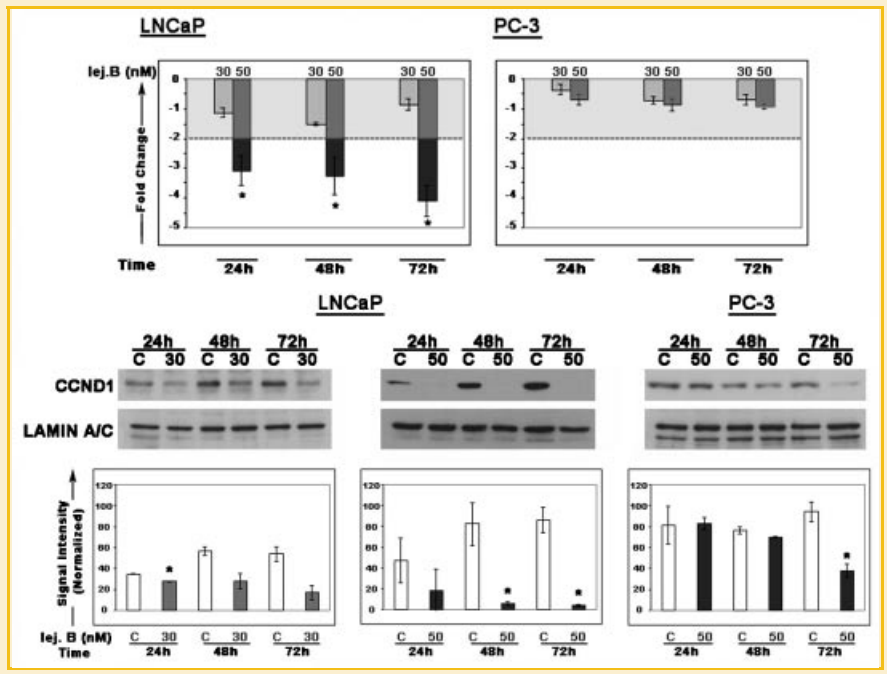

Fig. 4. Effects of Iejimalide B on cyclin D1 mRNA and protein levels in LNCaP and PC-3 cells. Q-PCR (upper panel) and Western blot (bottom panel) analysis for cyclin D1 in LNCaP and PC-3 cells treated with control vehicle, 30 or 50 nM Iejimalide B. Statistical significance ($P < 0.05$) is indicated by asterisks while changes less than twofold with respect to control group are shaded. Protein expression was normalized to lamin A/C. Significant changes in protein level relative to the control group ($P < 0.1$) are indicated by asterisks.

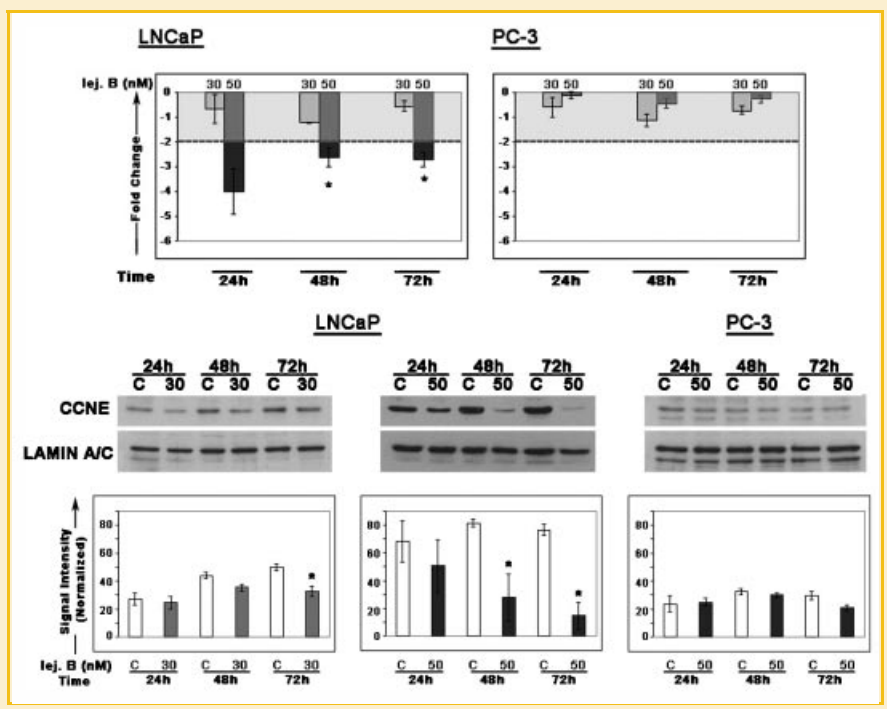

Fig. 5. Effects of Iejimalide B on cyclin E1 mRNA and protein levels in LNCaP and PC-3 cells. Q-PCR (upper panel) and Western blot (bottom panel) analysis for cyclin E1 in LNCaP and PC-3 cells treated with control vehicle, 30 or 50 nM Iejimalide B. Statistical significance ($P < 0.05$) is indicated by asterisks; while changes less than twofold with respect to control group are shaded. Protein expression was normalized to lamin A/C. Significant changes in protein level relative to the control group ($P < 0.1$) are indicated by asterisks.

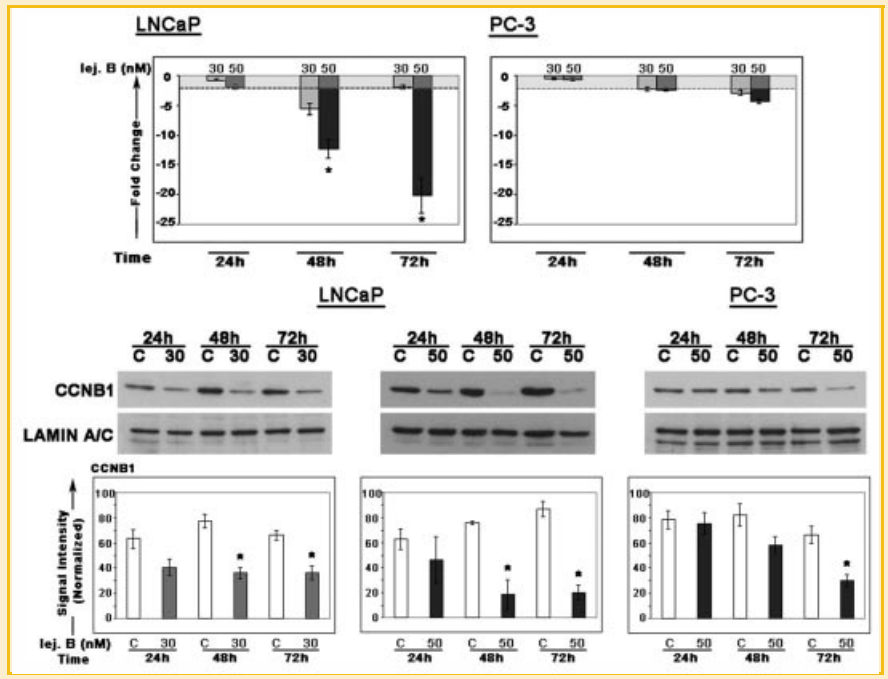

Fig. 6. Effects of Iejimalide B on cyclin B1 mRNA and protein levels in LNCaP and PC-3 cells. Q-PCR (upper panel) and Western blot (bottom panel) analysis for cyclin B1 in LNCaP and PC-3 cells treated with control vehicle, 30 or 50 nM Iejimalide B. Statistical significance ($P < 0.05$) is indicated by asterisks; while changes less than twofold with respect to control group are shaded. Protein expression was normalized to lamin A/C. Significant changes in protein level relative to the control group ($P < 0.1$) are indicated by asterisks.

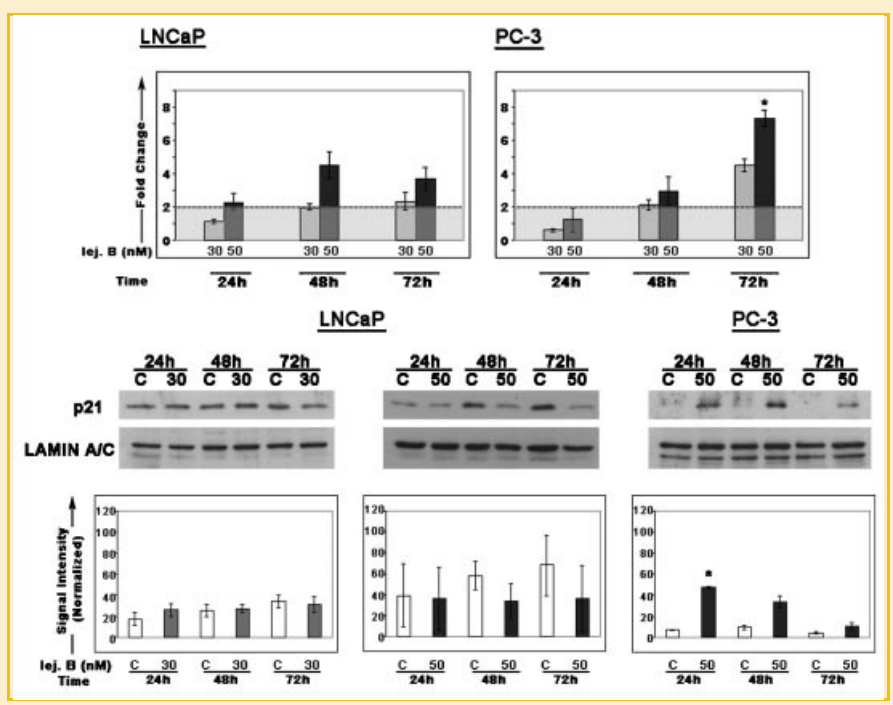

Fig. 7. Effects of Iejimalide B on p21 mRNA and protein levels in LNCaP and PC-3 cells. Q-PCR (upper panel) and Western blot (bottom panel) analysis for p21 in LNCaP and PC-3 cells treated with control vehicle, 30 or 50 nM Iejimalide B. Statistical significance ($P < 0.05$) is indicated by asterisks; while changes less than twofold with respect to control group are shaded. Protein expression was normalized to lamin A/C. Significant changes in protein level relative to the control group ($P < 0.1$) are indicated by asterisks.

cells, although it is more gradual and less dramatic, decreasing 6-fold by 72 h.

In addition to these genes directly associated with cell cycle progression, Iejimalide B also modulates the transcript levels of several transcripts such as MCM-7 and cdc45 associated with licensing of DNA replication and the formation of pre-replicative complexes in both LNCaP and to a lesser extent PC-3 cells (see Table II, Supplemental Data).

Iejimalide B also modulates the expression of a number of genes associated with G_2/M transition (GO:0000086). In LNCaP cells, concurrently with the decreases in cyclin B, the steady state levels of cdc2 (cdk1), cdc20 and polo like kinase-1 (PLK1) which are associated with G_2/M transition or centrosome assembly (GO:0005813) are also significantly downregulated 15–30-fold in LNCaP cells by 50 nM Iejimalide B but not at the lower dose of 30 nM or in PC-3 cells (see Table II, Supplemental Data).

In LNCaP cells, Iejimalide B also modulates the expression of several genes associated with apoptosis, including the anti-apoptotic protein, survivin, which is substantially down regulated at the mRNA level (-12.5 ± 1.8-fold) by 48 h. This decrease in survivin mRNA is reflected in the substantial decrease in both the nuclear and cytoplasmic levels of survivin after 48 h of treatment in LNCaP cells. Iejimalide B also down regulates survivin mRNA expression in PC-3 cells, however the response is more muted (-2.2 ± 0.3-fold) and is delayed (reaching a nadir at 72 h) relative to LNCaP cells (Fig. 8).

Several genes associated with apoptosis (GO:0006915), are also modulated at the higher dose of Iejimalide B in LNCaP but not in PC-3 cells as early as 24 h after treatment including the transcripts for p21B and BNIP3L (Fig. 9). Since there are no antibodies specific for these pro-apoptotic proteins it is not possible to characterize the effect of Iejimalide B on the expression of the cognate proteins.

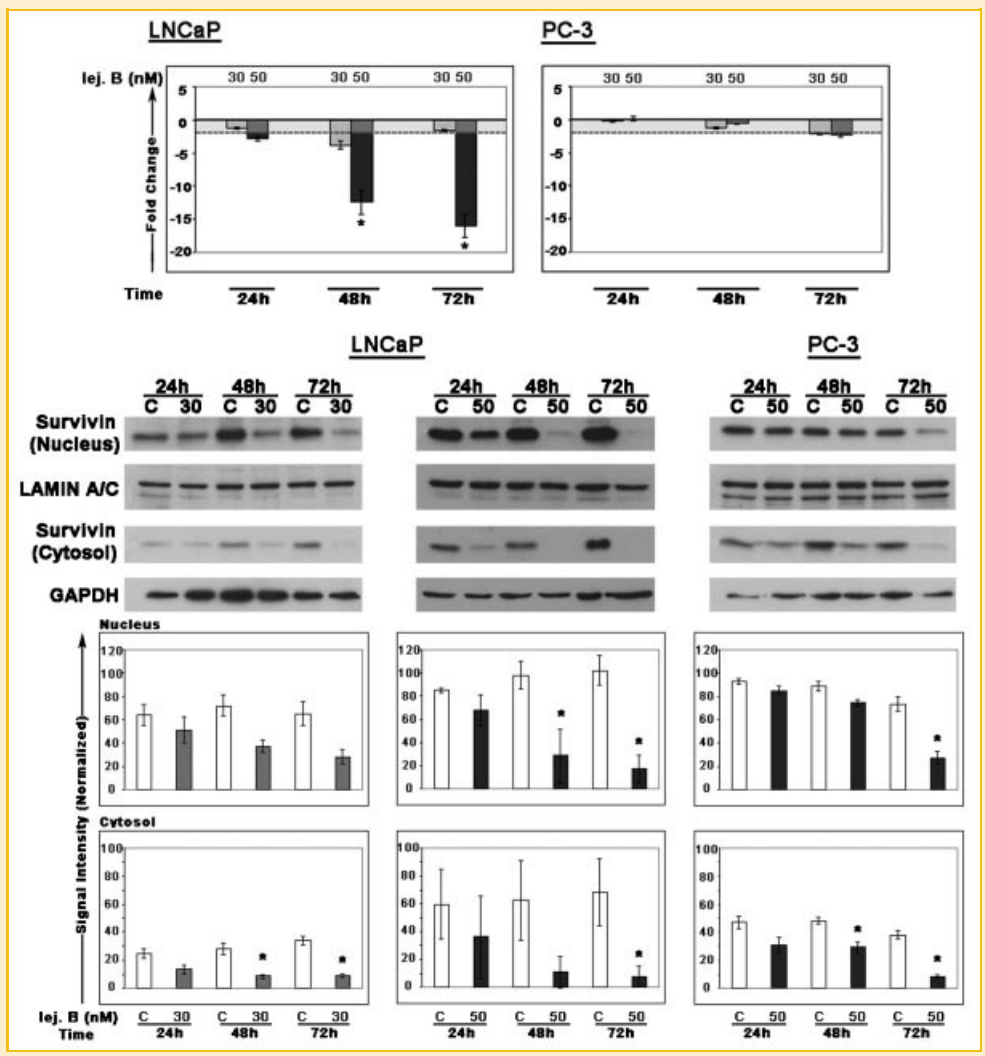

Fig. 8. Effects of Iejimalide B on Survivin mRNA and protein levels in LNCaP and PC-3 cells. Q-PCR (upper panel) and Western blot (bottom panel) analysis for survivin in LNCaP and PC-3 cells treated with control vehicle, 30 or 50 nM Iejimalide B. Statistical significance ($P < 0.05$) is indicated by asterisks; while changes less than twofold with respect to control group are shaded in grey area. Protein expression was normalized to lamin A/C for nuclear proteins or GAPDH for cytoplasmic proteins. Significant changes in protein level relative to the control group ($P < 0.1$) are indicated by asterisks.

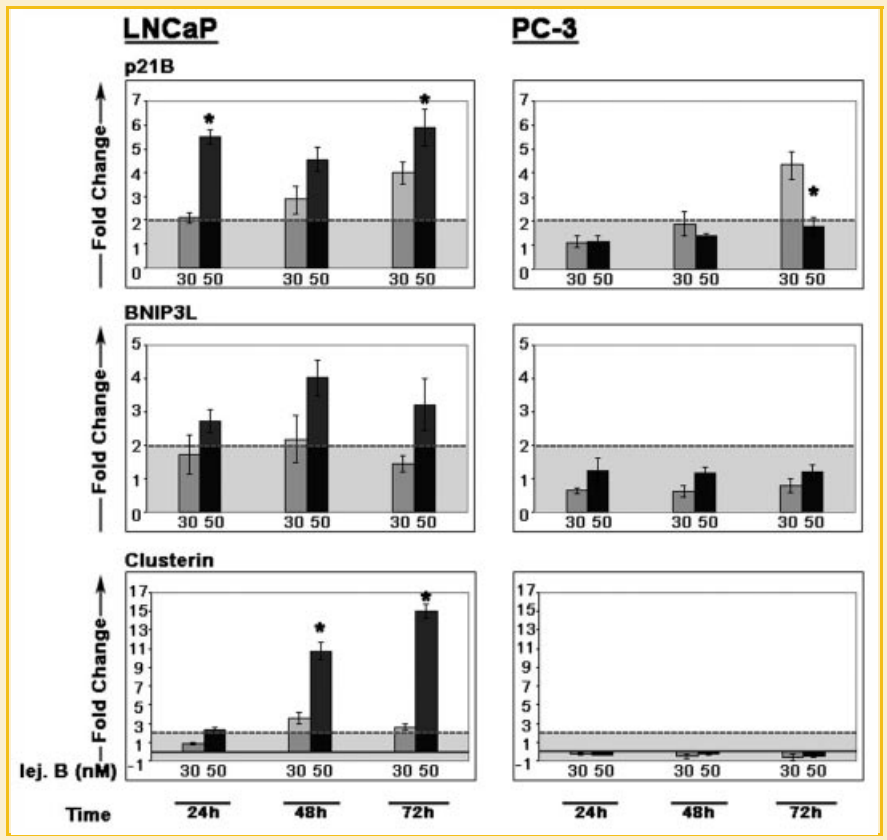

Fig. 9. Effects of Iejimalide B on p21B, BNIP3L and clusterin mRNA levels in LNCaP and PC-3 cells. Q-PCR analysis of p21B, BNIP3L and clusterin mRNA in LNCaP and PC-3 cells treated with control vehicle, 30 or 50 nM Iejimalide B. Statistical significance ($P < 0.05$) is indicated by asterisks; while changes less than twofold with respect to control group are shaded.

Clusterin expression is also dramatically upregulated (15-fold) in LNCaP cells in response to 50 nM Iejimalide B. Since the steady state levels of these transcripts do not change significantly in response to Iejimalide B in PC-3, it probably explains why the compound does not induce cell death in these cells.

DISCUSSION

In recent years the search for novel natural products that can be used for chemotherapy has been extended to a detailed inventory of compounds extracted from marine organisms [Mayer and Gustafson, 2006; Adrian, 2007]. A number of these compounds have been tested against a variety of cancer models in vitro and are active at the micro or nanomolar range in cell culture suggesting that they may be useful chemotherapeutic agents. Of these compounds, Aplidine, a cyclic depsipeptide isolated from *Aplidium albicans*, Bryostatin A, a macrocyclic lactone derived from *Bugula neritina* and Kalalahide F isolated from the mollusc *Elysia rufescen*, have been shown to induce apoptosis in breast and prostate cancer cell lines.

Iejimalide B is active in both LNCaP and PC-3 prostate cancer cell lines in the nanomolar range, but effects on the two cell lines are significantly different. The LNCaP and PC-3 cell lines differ in two important aspects: the expression of the androgen receptor and p53 status. In LNCaP cells there is a clear dose response to Iejimalide B: at doses below 30 nM iejimalide B induces cell cycle arrest in G_0/G_1, while at doses of 50 nM and above it induces cell death. This is most likely attributable to the rapid and prolonged induction of $p21^{waf1/cip1}$ and the co-ordinate down regulation of cyclin B and its heterodimeric partner cdc2/cdk1 in addition to the down regulation of the anti-apoptotic survivin [Ambrosini et al., 1997; Krajewska et al., 2003] and upregulation of the pro-apoptotic p21B and BNIP3L [Nozell and Chen, 2002; Zhang et al., 2003] in LNCaP cells. These genes are known to be p53 target genes [Roy et al., 2008], suggesting, but not conclusively establishing, that the induction of apoptosis by Iejimalide B is p53-mediated. We have also established that Iejimalide B induces apoptosis in MCF-7 breast cancer cells which also express wild type p53 (McHenry et al., unpublished data), suggesting that the response to Iejimalide B is not restricted to a single cell line.

The initial arrest of PC-3 cells in G_0/G_1 is likely caused by the early induction of p21. The decline in $p21^{waf-1/cip1}$ levels at later times allows the cells to transit into S phase. The S phase arrest in PC-3 seen at later times is probably explained by the prolonged down regulation of both Cyclin A and PCNA which would be predicted to cause the stalling of the DNA polymerase complex on the partially replicated template [Niculescu et al., 1998; Cazzalini

et al., 2003]. In PC-3 cells Iejimalide B does not induce significant levels of apoptosis even at very high doses. However Iejimlaide B clearly has significant effects on this and probably other cell lines that are not mediated by p53.

Aplidine has been shown to induce apoptosis through the activation of the epidermal growth factor receptor and its downstream effectors, Src, JNK and p38 MAP kinase [Cuadrado et al., 2003] while Bryostatin-1 appears to modulate apoptosis via a PKC-dependent pathway [Ali et al., 2003] or through a COX-2 mediated pathway [De Lorenzo et al., 2003]. The intracellular target of Iejimalide B in prostate and breast cancer cells has yet to be firmly identified. It has recently been reported that Iejimalide B inhibits the membrane associated vacuolar-ATPase (V-ATPase) in osteoclasts responsible for the acidification of the extracellular milieu [Kazami et al., 2006] in a manner similar to bafilomycin A1 [Schoonderwoert et al., 2000; Fernandes et al., 2006]. In non-osteoclast cells, such as prostate and breast cancer cells, the V-ATPase complex is primarily localized to the lysosomes, suggesting that Iejimalide B may initiate apoptotic and/or autophagic cell death through its interaction with lysosomes. The intracellular signaling pathways linking the inhibition of V-ATPase to the modulation of p53-dependent and independent gene expression in prostate and breast cancer cells remains to be elucidated. Nevertheless, given the data presented here, Iejimalide B appears to be a novel marine natural product that has considerable potential as a therapeutic for the treatment of early stage, solid tumors that continue to express wild type p53.

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