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Reactive oxygen species-dependent activation of Bax and PARP-1 is required for mitochondrial cell death induced by triterpenoid pristimerin in human cervical cancer cells

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Running title: Molecular mechanism of pristimerin-induced cell death

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Abbreviations: MAPK, mitogen-activated protein kinase; JNK1, c-Jun N-terminal kinase; siRNA, small interfering RNA; PI, propidium iodide; PARP, poly (ADP-ribose) polymerase; $DiOC_6(3)$, 3,3'dihexylocarbocyanine iodide; DIQ 1,5-dihydroxyisoquinoline

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ABSTRACT

Naturally occurring triterpenoid compounds have long been used as anti-inflammatory, anti-malarial, and insecticidal agents. Recently, it has become evident that some of natural or synthetic triterpenoids have promising clinical potential as both a therapeutic and chemopreventive agent for cancer. However, the molecular basis for the antitumor activity of triterpenoid has yet to be defined. In this study, we show that pristimerin, a natural triterpenoid, induces mitochondrial cell death in human cervical cancer cells, and that reactive oxygen species (ROS)-dependent activation of both Bax and PARP-1 is critically required for the mitochondrial dysfunction. We also showed that c-Jun N-terminal kinase (JNK) is involved in ROS-dependent Bax activation. Treatment of pristimerin induced an increase in intracellular ROS, JNK activation, conformational change and mitochondrial redistribution of Bax, mitochondrial membrane potential loss, and cell death. The PARP-1 was also found to be activated by pristimerin treatment. An antioxidant, N-acetyl-L-cysteine (NAC), inhibited pristimerin-induced JNK activation, Bax relocalization, and PARP-1 activation as well as mitochondrial cell death. Moreover, inhibition of JNK clearly suppressed conformational change and mitochondrial translocation of Bax and subsequent mitochondrial cell death, but did not affect PARP-1 activation. Inhibition of PARP-1 with 1,5-dihydroxyisoquinoline (DIQ) or with siRNA of PARP-1 significantly attenuated pristimerin-induced mitochondrial membrane potential loss and cell death, but did not affect JNK activation and Bax relocalization. These results indicate that the natural triterpenoid pristimerin induces mitochondrial cell death through ROS-dependent activation of both Bax and PARP-1 in human cervical cancer cells, and that JNK is involved in ROS-dependent Bax activation.

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INTRODUCTION

Pristimerin, a naturally occurring quinonemethide triterpenoid compound, is a traditional medicine derived from the *Celastraceae and Hippocrateaceae* families which has long been used as anti-inflammatory, antioxidant, antimalarial and insecticidal agents (Brinker et al., 2007; Gao et al., 2007). Recently, it has been reported that pristimerin have promising clinical potential as both a therapeutic and chemopreventive agent for cancer (Salminen et al., 2008). Indeed, pristimerin induces apoptotic cell death in certain human cancer cells, including breast and lung cancer (Wu et al., 2005), human acute myeloid leukemia (Nagase et al., 2003). Pristimerin has been shown to have several mechanisms of cell death induction including proteosome inhibition (Yang et al., 2008), suppression of NF-kB activity and cyclin D1 expression (Tiedemann et al., 2008), and caspase activation (Wu et al., 2005; Yang et al., 2008). Although evidence has been accumulated that the mode of cell death by pristimerin is caspase-dependent apoptotic cell death, the involved mechanisms of action, especially cross-talk between signaling pathway and apoptotic cell death machinery, is largely unknown.

c-Jun N-terminal kinase (JNK) and p38 MAPK are weakly activated by growth stimuli, but respond strongly to stress signals, including tumor necrosis factor-α (TNF-α), ionizing and ultraviolet (UV) irradiation, hyperosmotic stress, and chemotherapeutic drugs (Li et al., 2005; Kim et al., 2008; Huang et al., 2004; Kang and Lee, 2008). Activation of these kinases is strongly associated with apoptotic cell death. Moreover, JNK and/or p38 MAPK-mediated cell death is often accompanied by regulating the redistribution of Bax from the cytoplasm to the mitochondria (Choi et al., 2006), and by modulating the phosphorylation status of Bcl-2 in response to various stimuli (Kim et al., 2008; Gustafsson and Gottlieb, 2007). These results are consistent with the fact that JNK and/or p38 MAP kinase acts at early step prior to dysfunction of mitochondria and caspase activation in several cell types (Choi et al., 2006; Kim et al., 2008; Gustafsson and Gottlieb, 2007; Seo et al., 2007).

In recent years, it has become apparent that reactive oxygen species (ROS) play an important role during apoptosis induction (Fleury et al., 2002). Many stimuli such as TNF-α, anticancer drugs, and chemopreventive agents, stimulate cells to produce intracellular ROS (Park et al., 2005; Kuwabara et al., 2008; Simizu et al., 1998). Intracellular production of ROS can directly lead to activation of the mitochondrial permeability transition and to loss of mitochondrial membrane potential (Chan et al., 2003). Several studies also provide evidence the role of ROS as the potential inducers of

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JNK or p38 MAPK activation during apoptotic cell death (Kim et al., 2008; Huang et al., 2004; Kang and Lee, 2008). In these studies, these kinases have been shown to activate caspases and may also target other factors that have been implicated in the regulation of cell death, including Bax, Bak, and Bcl-2 (Gustafsson and Gottlieb, 2007).

Poly (ADP-ribose) polymerase-1 (PARP-1) is the most abundant protein of the PARP family members, which is rapidly activated by DNA damage. Activation PARP-1 appears to facilitate DNA repair under moderate stress conditions (Chan et al., 2003), and utilizes NAD^+ to form poly (ADP-ribose) polymers on specific acceptor proteins. However, under certain conditions that cause extensive DNA damage such as excitotoxicity and ischemia, activation of PARP-1 is involved in cell death caused by NAD^+ depletion leading to ATP depletion (Ditsworth et al., 2007). Although PARP-1 activation-mediated cell death has been thought to be necrotic (Mathews and Berk, 2008), recent reports have demonstrated that PARP-1-mediated cell death also has many features in common with mitochondrial apoptotic cell death, such as mitochondrial release of cytochrome *c* and subsequent caspase activation (Xu et al., 2006), and nuclear translocation of AIF from mitochondria (Mathieu et al., 2008).

In this study, we investigate the molecular basis for the antitumor activity of pristimerin, and demonstrate that pristimerin induces mitochondrial cell death in human cervical cancer cells via ROS-dependent JNK and PARP-1 activations. These results indicate that both JNK and PARP-1 play critical roles in interconnection between increase in intracellular ROS and activation of mitochondrial cell death machinery during the triterpenoid pristimerin-induced apoptotic cell death.

MATERIALS AND METHODS

Materials. Antibodies specific for polyclonal anti-Bcl-2, -Bcl-xL, -AIF, -cytochrome *c*, -caspase-9, -p-ERK, -ERK1/2, -α-tubulin, and -HSP60 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). β-Actin was from Sigma. Antibodies specific for polyclonal anti-cleaved caspase-3, -p-JNK, -JNK, -p-p38, -p38, and -PARP were from Cell Signaling Technology (Beverly, MA). Monoclonal anti-Bax and -Bak antibodies were from Pharmingen (San Diego, CA). Polyclonal anti-PAR antibody was from Calbiochem (San Diego, CA). The specific PARP inhibitor 1,5-dihydroxyisoquinoline (DIQ) and the broad-spectrum caspase inhibitor z-VAD-fmk were from Sigma.

Purification of pristimerine. The stem root (5 kg) of *T.regelii* were air-dried,

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chopped and extracted three times with 95% MeOH (3 × 10 L) for 7 days at room temperature. The combined extract was concentrated, and the dark residue (453 g) was partitioned between water and chloroform (1 L : 1 L). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give a dark brown residue (310 g) which was chromatographed on a silica gel column. The resultant extract (310 g) was suspended in H_2O (2 × 1 L). The resultant extract diluted with H_2O has been partitioned with organic solvents ($CHCl_3$, BuOH) of the different polarities to afford $CHCl_3$ (162 g), BuOH (125 g), and H_2O (150 g) extracts, respectively. The $CHCl_3$ and BuOH extracts were subjected to column chromatography using silica gel with hexane-acetone gradient and hexane-EtOAc gradient. The $CHCl_3$ extract (162 g) was subjected to column chromatography (glass column 10 x 80 cm) over silica gel (1.5 kg; 70-230 mesh; Merk), eluted with gradient mixtures of hexane (4 L) and hexane-acetone, of increasing polarity (30/1 → 1/6), and finally with MeOH. Fifteen pooled fractions (Fr.1-Fr.30) were obtained after combining fractions with similar TLC profiles from this initial column chromatography. The column was eluted with solvents of increasing polarity (CHCl3-acetone) to give 30 fractions. Fraction 5 (14.3 g) was chromatographed on a silica gel column with hexane-acetone to give 45 fractions (5.1-5.30). The fractions from 5.11-5.18 and 5.21-32.27 were evaporated to give pristimerine (980 mg) [R*f* 0.53 (hexane/acetone= 4/1)]. The structures of pristimerine was confirmed by spectroscopic analysis and comparison with values previously reported. ^{1}H- and ^{13}C-NMR at 500 and 125 MHz data were obtained on a Bruker AM 500 spectrometer in $CDCl_3$, respectively. General procedures, product characterization data, and NMR spectra were available via Supplementary data.

Quantification of cell death. Cell death was investigated by both (i) propidium iodide staining detects cell death by means of the dye entering the cells, and (ii) Annexin-V labelling using a kit according to manufacturer's directions (Sigma-Aldrich). For the cell death assessment, the cells were plated in 60-mm dish with cell density of $2x10^5$cells per dish and treated with pristimerin the next day. At indicated time points, cells were harvested, washed in PBS. Annexin-V-positive early apoptotic cells or PI-positive cells were quantified using a FACScan flow cytometer fitted with CellQuestPro software (Becton Dickinson).

Cell Viability Assay. The inhibitory effect of pristimerin on the cell viability was measured by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric method. Cells were seeded at densities of 10,000 cells/well in 96-well tissue culture plates. On day 2, cells were treated with pristimerin for 72h. After drug

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treatment, attached cells were incubated with 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.5 mg/mL, 1 hour) and subsequently solubilized in DMSO. The absorbency at 550 nm was then measured using a microplate reader. The IC50 is the concentration of agent that reduced the cell viability by 50% under the experimental conditions.

Cell culture and transfection of siRNA. Human cervical carcinoma cells (HeLa, CaSki, and SiHa) and human breast cancer cells (SK-BR3, MCF7, MDA-MB-231) were obtained from the American Type Culture Collection (Manassas, VA). HeLa and CaSki cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, and SiHa and MCF7 cells were grown in modified Eagle's medium supplemented with 10% fetal bovine serum and nonessential amino acids. SK-BR3 and MCF7 cells were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum. All of the media were supplemented with 100 units/mL penicillin and 100 ug/mL streptomycin, and all of the cells were incubated at 37°C in 5% CO2. Cells were transfected with specific small interfering RNA duplexes purchased from Ambion (Austin, TX), using Lipofectamine reagent (Invitrogen, CA) according to the manufacturer's recommendations.

Measurement of mitochondrial membrane potential and ROS generation. Briefly, cells were incubated in 40 nM 3,3-dihexyloxacarboxyanine iodide [$DiOC_6(3)$] and 10 uM 2,7-dichlorodihydrofluorescein diacetate DCFH-DA (Molecular Probes, Eugene, OR) at 37˚C for 15 min and washed with cold PBS three times. Retained $DiOC_6(3)$ and DCF were analyzed by a flow cytometer.

Flow cytometric analysis of Bax and Bak activations. Upon induction of apoptosis, the proapoptotic Bax and Bak proteins undergo conformational changes which expose otherwise inaccessible N-terminal epitopes. At specific time points after pristimerin treatment, cells were detached using cell dissociation solution (Sigma). Cells were then fixed in paraformaldehyde (0.25%, 5 min), washed three times in PBS, and incubated for 30 min with a mouse monoclonal antibody against amino acids 12 to 24 of Bax (clone 6A7; PharMingen) or Bak (AM03TC100, Oncogene Research Products). Antibodies were diluted 1:50 in PBS containing digitonin (100 mg/ml). After three washes in PBS, cells were incubated with FITC-labeled anti-mouse antibody for 30 min, washed twice in PBS, and resuspended in PBS. The increases in accessibility of these epitopes can be monitored by flow cytometry.

Preparation of cytosolic and mitochondrial fractions. The cells were washed with ice-cold PBS, left on ice for 10 minutes, and then resuspended in isotonic

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homogenization buffer [250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L Na-EDTA, 1 mmol/L Na-EGTA, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonylfluoride, and 10 mmol/L Tris-HCl (pH 7.4)] containing a proteinase inhibitor mixture (Roche, Basel, Switzerland). After 80 strokes in a Dounce homogenizer, the unbroken cells were spun down at 30 *g* for 5 min. The mitochondria fractions were fractionated at 750 *g* for 10 minutes and 14,000 *g* for 20 minutes, respectively, from the supernatant. For cytosolic fractionation, 10 strokes with loose homogenizer, and collected supernatant after spun down at 750 *g* for 10 min and 14,000 *g* for 20 min.

Statistical analysis. Data was analyzed with unpaired two-tailed Student's *t*-test. Data were expressed as mean ±S.E.M derived from at least three independent experiments. Differences were considered significant at $p < 0.05$.

RESULTS

Pristimerin induces mitochondrial cell death in several human cancer cell lines.

To examine whether pristimerin induces cell death in human cervical cancer cells, three different human cervical cancer cell lines (HeLa, CaSki, and SiHa) were treated with 1 μM of pristimerin, and cell death was measured by flow-cytometric analysis with propidium iodide (PI) staining. Treatment of pristimerin effectively induced cell death in three types of cancer cells (Fig. 1A). The IC50 value ranged from 0.85 to 1.7 μM in HeLa, SiHa and CasKi cells after 72 hr of incubation with pristimerin (Fig. 1A). Moreover, there is a time- and dose-dependent increase of cell death in HeLa cells, reaching approximately 60% of cells after 36 hr of treatment with 1 μM of pristimerin (Fig. 1A). In addition, analysis of apoptotic cell death with Annexin-V staining also clearly revealed that Annexin-V-positive cell populations were markedly increased by prestimerin treatment (Fig. 1B). We next investigated whether caspase activities are required for induction of cell death by pristimerin. As shown in Fig. 1C, pristimerin induced activation of caspase-9 and caspase-3. Moreover, a broad-spectrum caspase inhibitor, z-VAD-fmk, completely attenuated the pristimerin-induced cell death (Fig. 1D). These findings suggest that pristimerin induces apoptotic cell death in human cancer cells in a caspase-dependent fashion.

Selective activation of Bax is required for the pristimerin-induced mitochondrial membrane potential loss and subsequent cell death.

To determine whether mitochondrial pathway is involved in the induction of cell

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death by pristimerin, we examined changes in mitochondrial membrane potential and release of proapoptotic molecules from the mitochondria by pristimerin treatment. As shown in Fig. 2A, pristimerin significantly induced dissipation of mitochondrial membrane potential and subsequent cytosolic redistribution of cytochrome *c* and AIF, indicating pristimerin-induced cell death is accompanied by mitochondrial dysfunction. Since it has been demonstrated that pro-apoptotic Bcl-2 family members, especially Bax and Bak, are crucial to the mitochondrial cell death pathways (Gustafsson and Gottlieb, 2007), we next analyzed activation of Bax and/or Bak after pristimerin treatment using flow cytometric analysis with activity-dependent antibodies (antibodies recognizing N-terminal epitopes) of Bax or Bak. Pristimerin induced a marked activation of Bax (Fig. 2B), but not Bak, and subsequently induced mitochondrial relocalization of Bax from cytosol (Fig. 2B). Moreover, small interfering RNA (siRNA) targeting of Bax clearly attenuated mitochondrial membrane potential loss (Fig. 2C) and cell death (Fig. 2D). These results indicate that selective activation of Bax plays a crucial role in pristimerin-induced mitochondrial cell death.

JNK1 acts as an important mediator of Bax activation and mitochondrial cell death in response to pristimerin treatment.

MAPK has been implicated in the regulation of apoptotic cell death in response to various stimuli (Li et al., 2005; Kim et al., 2008). Treatment of cells with pristimerin also resulted in a marked increase in the phosphorylated forms of all three MAPKs, indicating activation of these kinases in response to pristimerin (Fig. 3A). Moreover, as shown in Fig. 3B, pre-treatment of SP600125, a JNK-specific inhibitor, effectively blocked pristimerin-induced mitochondrial membrane potential loss and cell death, while SB203580, a p38 MAPK inhibitor, and PD98059, a MEK inhibitor, did not. Moreover, siRNA targeting of MAPK also revealed that si-JNK, but not si-ERK and si-p38 MAPK, inhibited mitochondrial membrane potential dissipation and cell death. Interestingly, inhibition of JNK with SP600125 treatment clearly attenuated conformational changes and mitochondrial relocalization of Bax (Fig. 3C). In addition, SP600125 clearly attenuated pristimerin-induced cytochrome *c* and AIF release form mitochondria, and caspase-9 and -3 activations (Fig. 3D). These results indicate that JNK1 acts as an important mediator of the Bax activation and subsequent mitochondrial dysfunction during pristimerin-induced cell death.

The induction of reactive oxygen species generation is critically required for the activation of mitochondrial cell death pathway by pristimerin.

Oxidative damage has been shown to plays an important role in anti-cancer effects of chemotherapeutic drugs (Park et al., 2005; Kuwabara et al., 2008; Simizu et al.,

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1998). Thus we subsequently examined changes in the intracellular reactive oxygen species (ROS) level in cells treated with pristimerin. As shown in Fig. 4A, treatment of pristimerin led to an approximately 3-fold increase of mean DCF fluorescence, indicating increase in intracellular ROS. To further determine a link between elevation of the intracellular ROS and mitochondrial cell death, cells were preincubated with antioxidant N-acetyl-L-cysteine (NAC) prior to pristimerin treatment. Treatment of pristimerin did not induce cell death in the presence of NAC (Fig. 4A). Moreover, NAC markedly inhibited pristimerin-induced mitochondrial membrane potential loss, AIF and cytochrome *c* release, and caspase activations (Fig. 4B). Inhibition of ROS with NAC treatment also completely attenuated pristimerin-induced activation of JNK (Fig. 4C) and conformational change and mitochondrial redistribution of Bax (Fig. 4D). These observations suggest that an increase in the intracellular ROS is critically required for pristimerin-induced cell death through JNK activation and subsequent Bax-mediated mitochondrial membrane potential loss.

PARP-1 activation is located downstream of the ROS signaling in pristimerin-induced mitochondrial cell death process.

Recently, it has been reported that ROS-mediated DNA damage triggers activation of the poly(ADP-ribose) polymerase-1 (PARP-1) and subsequent cell death (Hong et al., 2004). Thus we next examined whether PARP-1 is involved in pristimerin-induced mitochondrial cell death. Treatment of HeLa cells with pristimerin induced a marked activation of PARP-1 (Fig. 5A). Pretreatment of, 1,5-dihydroxyisoquinoline (DIQ), or siRNA targeting of PARP-1 significantly attenuated pristimerin-induced cell death (Fig. 5B). Moreover, as shown in Fig. 5C, antioxidant NAC completely blocked pristimerin-induced PARP-1 activation, whereas DIQ, a PARP-1 specific inhibitor, did not affect ROS production (Fig. 5C), indicating that PARP-1 is located downstream of the ROS signaling in pristimerin-induced cell death process. Treatment of DIQ also significantly blocked mitochondrial membrane potential loss and AIF and cytochrome *c* release from mitochondria (Fig. 5D). However, DIQ did not suppress pristimerin-induced JNK activation (Supplementary Fig. S1). Conversely, inhibition of JNK with SP600125 did not affect PARP-1 activation (Supplementary Fig. S1).

The activation of both JNK and PARP-1 are essentially required for the pristimerin-induced cell death.

To confirm activation of both JNK and PARP-1 is involved in pristimerin-induced mitochondrial cell death, HeLa cells were pretreated together with JNK specific inhibitor, SP600125, and PARP-1 specific inhibitor, DIQ, before treatment with pristimerin. As shown in Fig. 6A, the cells simultaneously treated with both inhibitors

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show more dramatic attenuation of the cell death than the cells treated with either reagent alone. Inhibitory effect of the simultaneous treatment was almost same degree as ROS inhibition by NAC. In addition, the combination of SP600125 and DIQ induces more dramatic inhibition of the loss of mitochondrial membrane potential, cytochrome c and AIF release, and caspase-9 and -3 activations than each reagent alone (Supplementary Fig. S2A, 2B and 2C), suggesting that activation of both JNK and PARP-1 are essentially required for the pristimerin-induced cell death. However, the combination treatment of inhibitors did not affect pristimerin-induced ROS production (Supplementary Fig. S2D), indicating that ROS is upstream effector for JNK and PARP-1 activation.

DISCUSSION

Pristimerin is a quinonemethide triterpenoid compound which has long been used as anti-inflammatory, antioxidant, antimalarial and insecticidal agents (Brinker et al., 2007; Gao et al., 2007). Although recently pristimerin has shown to induce apoptosis in certain cancer cells, the cell death mechanism by pristimerin is largely unknown. In this study, we investigate the molecular basis for the pristimerin-induced cell death in human cancer cells. We show that pristimerin induces mitochondria-mediated cell death, and that ROS-dependent activation of both JNK and PARP-1 are critically required for the mitochondrial dysfunction.

Many reports provide evidence that JNK can function as a proapoptotic kinase in response to a variety of stress signals (Kang and Lee, 2008; Kim et al., 2008). These reports suggested that JNK has been shown to activate caspases and may also target other factors that have been implicated in the regulation of apoptotic cell death, including Bax and Bak (Kim et al., 2008; Kang and Lee, 2008). Consistent with these findings, we also found that JNK is involved in pristimerin-induced Bax activation and subsequent mitochondrial cell death. Inhibition of JNK attenuated pristimerin-induced conformational change and mitochondrial translocation of Bax, but not Bak, as well as caspase activations. These results are correlated well with the fact that JNK acts at early step prior to dysfunction of mitochondria in several cell types (Kim et al., 2008; Kang and Lee, 2008).

Accumulation of intracellular ROS in response to diverse stimuli has shown to lead the mitochondrial membrane permeability transition and subsequent activation of cell death machinery (Fleury et al., 2002). Moreover, recent studies also provide evidence

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for the role of ROS as the potential inducers of MAPK activation during apoptotic cell death in response to a variety of different stimuli (Fleury et al., 2002; Park et al., 2005; Kuwabara et al., 2008). We further evidence that the ROS is essential for the JNK activation and subsequent mitochondrial cell death by pristimerin treatment. We showed that an antioxidant NAC completely attenuated pristimerin-induced JNK activation, Bax relocalization, and mitochondrial cell death. Recently several mechanisms have been proposed for JNK activation that involves ROS-dependent dissociation of regulatory factor that maintains the pathway in an active state. In non-stressed cells, apoptosis signal-regulating kinase 1 (ASK1) has known to be associated with reduced thioredoxin (Trx) (Hsieh and Papaconstantinou, 2006). The interaction between ASK1 and Trx was found to be highly dependent on the redox status of Trx**.** Oxidation of Trx by ROS releases ASK-1, and leads to JNK activation, possibly through dimerization of ASK-1 (Hsieh and Papaconstantinou, 2006; Torres and Forman, 2003). However, in this study, we failed to observe ASK-1 activation in response to pristimerin treatment. Moreover, siRNA targeting of ASK-1 did not have any effect on JNK activation and cell death (data not shown), indicating that pristimerin-induced JNK activation is ASK-1 independent. The precise mechanisms by which ROS-dependent activation of JNK in response to pristimerin treatment occurs remain to be elucidated.

PARP-1 is a nuclear protein involved in the cellular response to DNA damage that plays role in DNA damage signaling and repair (Ditsworth et al., 2007). Recent evidence indicate that excessive activation of PARP-1 depletes pools of intracellular NAD^+ and ATP, consequently leading to necrotic or apoptotic cell death (Ditsworth et al., 2007; Mathews and Berk, 2008). In this study, we provide further evidence that activation of PARP-1 is involved in pristimerin-induced mitochondrial dysfunction and cell death. We showed that inhibition of PARP-1 significantly attenuated mitochondrial membrane potential loss, cytochrome *c* and AIF release, and cell death by pristimerin treatment. It is possible that PARP-1 activation by pristimerin treatment in our study is caused by ROS-dependent DNA damage, because recent reports showed that ROS-mediated DNA damage in hydrogen peroxide injury or in neurodegenerative disease triggers PARP-1 activation and subsequent cell death (de Vries et al., 2008). As expected, we found that pristimerin induces significant degree of DNA damage as assessed by the phosphorylation level of histone H2AX (γ-H2AX) and its foci formation (data not shown), an established marker for double strand breakages in chromosomal DNA (Tu et al., 2005). Moreover, antioxidant NAC completely blocked pristimerin-induced DNA damage (data not shown) as well as PARP-1 activation, suggesting that PARP-1 activation by pristimerin treatment may be caused by ROS-dependent DNA

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damage.

Pristimerin has been shown to exert potent proteasome inhibition activity both in vitro and using proteasome extracts (Yang et al., 2008; Tiedemann et al., 2009). As noted above, proteasome inhibitors can induce significant oxidative stress (ROS); furthermore, cells exposed to proteasome inhibitors can be rescued by various antioxidants, including NAC (Ling et al., 2003; Minami et al., 2005; Llobet et al., 2008; Fribley et al., 2004). Moreover, proteasome inhibitors can also induce Bax and JNK activation (Yu et al., 2003; Dewson et al., 2003; Yang et al., 2004; Dai et al., 2003). In this study, in human cervical cancer cells, we also found that proteasome inhibitor induced ROS generation, JNK activation and subsequent mitochondrial cell death (data not shown). However, it is still unclear whether pristimerin-induced mitochondrial cell death is directly due to ROS generation or is occurring secondary to more direct proteasome inhibition. Therefore, the exact mechanisms by which pristimerin raises intracellular ROS level and subsequent mitochondrial cell death remain to be elucidated.

In summary, we demonstrated in this study that pristimerin induces mitochondrial cell death through ROS-dependent activation of both JNK1 and PARP-1 in human cancer cells; JNK1 activation is required for the activation of Bax and mitochondrial potential loss; PARP-1 is also involved in the mitochondrial membrane potential loss, but did not affect Bax activation (Fig. 6B). Elucidating the molecular mechanisms utilized by naturally occurring products to regulate cell death is critical for both our understanding of cell death events and the development of cancer therapeutic agents.

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FOOTNOTES

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J.B. and M.K. contributed equally to this work and should be considered co-first authors.

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LEGENDS FOR FIGURES

Fig. 1. Pristimerin induces mitochondrial cell death in human cancer cells.

(A) (left) Human cervical cancer cells (HeLa, Siha, CasKi) were treated with 1 μM pristimerin. After 24 hr, cell death was measured as the percentage of PI-positive cells using flow cytometric analysis. Results from three independent experiments are presented as means ± S.E.M. (middle) After 72 hr, (right) HeLa cells were treated with pristimerin at indicated dose and time. Cell death was measured as the percentage of PI-positive cells. (B) HeLa cells were treated with 1 μM pristimerin. After 24 hr, cell death was measured as the percentage of Annexin-V-positive cells using flow cytometric analysis. (C) Cells were harvested at the indicated times, and cell lysates were subjected to immunoblot analysis with indicated antibodies. β-actin was used as a loading control. (D) Cells were treated with 1 μM pristimerin in the presence or absence of 40 μM Z-VAD-fmk. After 24 hr, cell death was determined by the percentage of PI-positive cells. Significantly different from control; *P < 0.05.

Fig. 2. Selective activation of Bax is required for the pristimerin-induced induced mitochondrial membrane potential loss and subsequent cell death.

(A) (left) HeLa cells were treated with 1 μM pristimerin. Cells were harvested at the indicated times, and mitochondrial membrane potential of the cells were determined by retention of $DiOC_6(3)$ (40 μM) added during the last 30 min of the treatment. The amount of retained $DiOC_6(3)$ was measured by flow cytometry. Results from three independent experiments are presented as means ± S.E.M. Significantly different from control; *P < 0.01. (right) After 24 hr, cytosolic, mitochondrial, or nuclear fraction was prepared and subjected to immunoblot analysis with anti-cytochrome *c* and -AIF antibodies. α-tubulin, HSP60, and Histone H3 were used as cytosolic, mitochondria, and nuclear marker proteins, respectively. (B) (left) After 24 hr, activity related modulations of Bax and Bak were determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitope of Bax or Bak as described under "Materials and Methods". (right) After 24 hr, mitochondrial fractions were prepared and subjected to immunoblot analysis with anti-Bax antibody. HSP60 was used as a mitochondrial marker protein. (C) Cells were transfected with control or Bax siRNA and treated with 1 μM pristimerin for 24 hr. (left) Cell lysates were subjected to immunoblot analysis with anti-Bax antibody. β-actin was used as a loading control. (right) Mitochondrial membrane potential of the cells were determined by retention of $DiOC_6(3)$. The amount of retained $DiOC_6(3)$ was measured by flow cytometry.

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Significantly different from control; *P < 0.05. (D) HeLa cells transfected with control or Bax siRNA were treated with 1 μM pristimerin. After 24 hr, cell death was measured as the percentage of PI-positive cells using flow cytometric analysis. Significantly different from control; *P < 0.05.

Fig. 3. JNK1 acts as a critical mediator of pristimerin-induced Bax activation and mitochondrial cell death.

(A) HeLa cells were treated with 1 μM pristimerin for 24 hr. Cell lysates were subjected to immunoblot analysis with indicated antibodies. β-actin was used as a loading control. (B) Cells were pretreated with PD98059 (25 μM), SB203580 (10 μM), or SP600125 (10 μM), or transfected with ERK2, p38 MAPK, or JNK1 siRNA and then treated with pristimerin for 24 hr. (upper) Mitochondrial membrane potential of the cells were determined by retention of $DiOC_6(3)$. The amount of retained $DiOC_6(3)$ was measured by flow cytometry. Significantly different from control; *P < 0.05. (lower) Cell death was measured as the percentage of PI-positive cells using flow cytometric analysis. Significantly different from control; *P < 0.05. (C) Cells were treated with 1 μM pristimerin in a presence or absence of SP600125. (left) Activity related modulation of Bax was determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitopes of Bax as described under "Materials and Methods". Significantly different from control; *P < 0.01. (right) Mitochondrial and cytosolic fractions were subjected to immunoblot analysis with anti-Bax antibody. HSP60 and α-tubulin were used as mitochondrial and cytosolic maker protein, respectively. (D) After 24 hr, cytosolic, mitochondrial, or nuclear fraction was prepared and subjected to immunoblot analysis with anti-cytochrome *c* and -AIF antibodies. α-tubulin, HSP60, and Histone H3 were used as cytosolic, mitochondria, and nuclear marker proteins, respectively. Total cell lysates were subjected to immunoblot analysis with anti-caspase-3 and -9 antibodies. β-actin was used as a loading control.

Fig. 4. The induction of ROS generation is critically required for the activation of mitochondrial cell death pathway by pristimerin.

HeLa cells were treated with 1 μM pristimerin for 24 hr in the presence or absence of N-acetyl-L-cysteine (NAC). Cells were loaded with dichlorofluorescein-diacetate (DCFH-DA) for 30 min. (A) (left) The DCF fluorescence was visualized using fluorescence microscope, (right upper) and the amount of retained DCF was measured using flow cytometry as described in "Materials and Methods". (right lower) Cell death was measured as the percentage of PI-positive cells using flow cytometry. Significantly

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different from control; *P < 0.01. (B) (left) Mitochondrial membrane potential of cells was determined by retention of $DiOC_6(3)$ (40 μM) added during the last 30 min of the treatment. The amount of retained $DiOC_6(3)$ was measured by flow cytometry. Significantly different from control; *P < 0.01. (right) After 24 hr, cytosolic, mitochondrial, or nuclear fraction was prepared and subjected to immunoblot analysis with anti-cytochrome *c* and -AIF antibodies. α-tubulin, HSP60, and Histone H3 were used as cytosolic, mitochondria, and nuclear marker proteins, respectively. Total cell lysates were subjected to immunoblot analysis with anti-caspase-3, -9, and PARP-1 antibodies. *β*-actin was used as a loading control. (C) Total cell lysates were subjected to immunoblot analysis with specific antibodies as indicated. *β*-actin was used as a loading control. (D) (left) Activity related modulation of Bax was determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitopes of Bax as described under "Materials and Methods". Significantly different from control; *P < 0.01. (right) Mitochondrial and cytosolic fractions were subjected to immunoblot analysis with anti-Bax antibody. HSP60 and α-tubulin were used as mitochondrial and cytosolic maker proteins, respectively.

Fig. 5. Intracellular ROS induction is involved in PARP-1 activation in pristimerin-induced mitochondrial cell death process.

(A) HeLa cells were treated with 1 μM pristimerin at indicated times, and cell lysates were subjected to immunoblot analysis with anti-PAR antibody. *β*-actin was used as a loading control. (B) (left) Cells were pretreated with PARP-1 inhibitors, DIQ (50 μmol/L) or transfected with PARP-1 si-RNA(40 nM), and then treated with pristimerin for 24 hr. (left) Cell death was measured as the percentage of PI-positive cells using flow cytometric analysis. Significantly different from control; *P < 0.05. (right) Total cell lysates were subjected to immunoblot analysis with anti-PAR antibody. (C) (left) Cells were treated with 1 μM pristimerin in the presence or absence of NAC. After 24 hr, total cell lysates were subjected to immunoblot analysis with anti-PAR antibody. (right) HeLa cells were treated with 1 μM pristimerin for 24 hr in the presence or absence of DIQ (50 μmol/L). Cells were loaded with DCFH-DA for 30 min, and analyzed the retained DCF by flow cytometry. (D) (left) Cells were treated with 1 μM pristimerin in the presence or absent of DIQ. After 24 hr, mitochondrial membrane potential of the cells were determined by retention of $DiOC_6(3)$. The amount of retained $DiOC_6(3)$ was measured by flow cytometry. Significantly different from control; *P < 0.01. (right) Cytosolic and nuclar fractions were subjected to immunoblot analysis with anti-cytochrome *c* and -AIF antibody. α-tubulin and Histone H3 were used as cytosolic and

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nuclear marker proteins, respectively.

Fig. 6 The activation of both JNK and PARP-1 are essentially required for the pristimerin-induced mitochondrial cell death.

(A) HeLa cells were treated with 1 μM pristimerin in the presence of SP600125 (10 μM) and/or DIQ (50 μmol/L), or NAC (10 mM). After 24 hr, cell death was measured as the percentage of PI-positive cells using flow cytometric analysis. Significantly different from control; *P < 0.05, **P < 0.001. (B) A schematic model for the molecular basis of pristimerin-induced mitochondrial cell death, suggesting that ROS-dependent activation of both JNK and PARP-1 is essentially required for the pristimerin-induced mitochondrial cell death.

Fig. 1

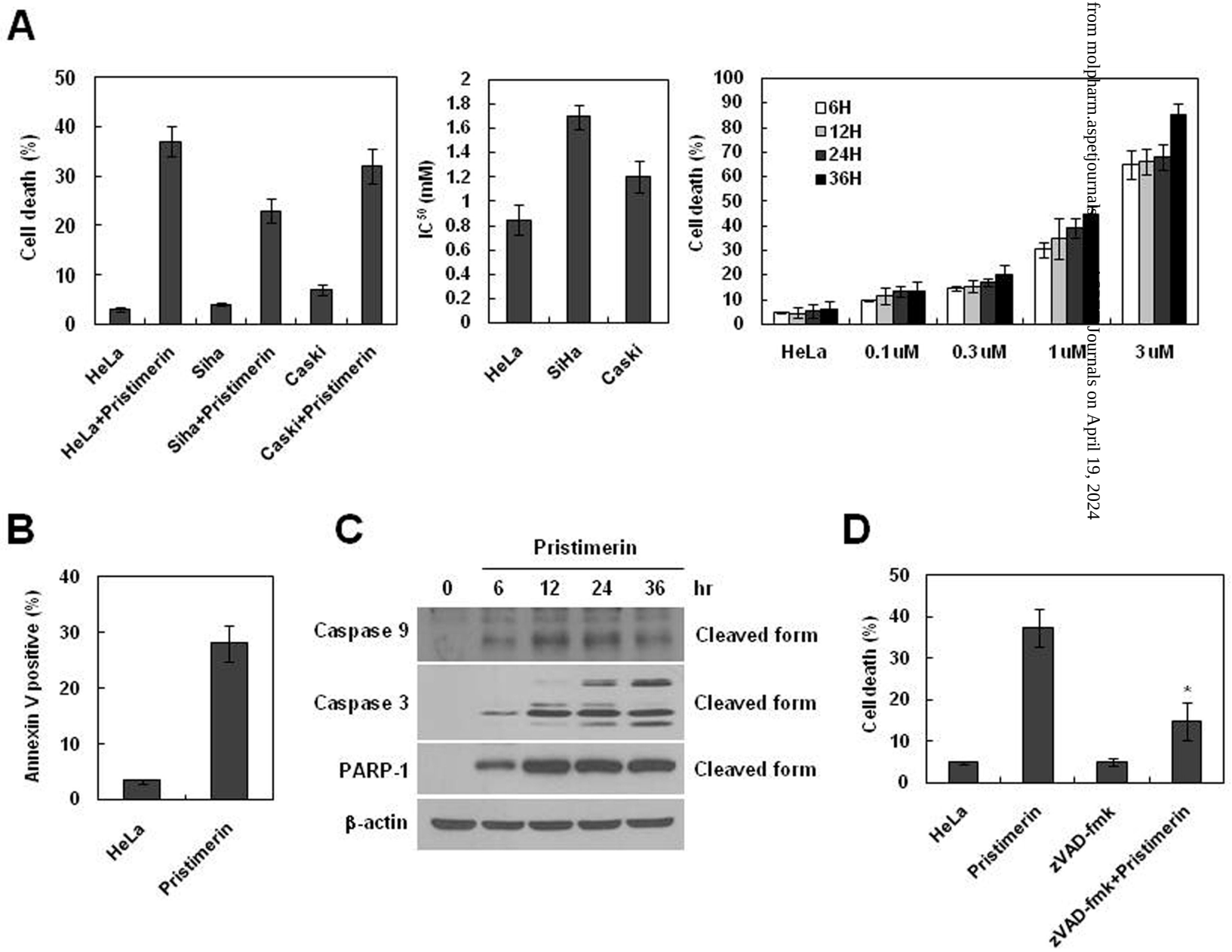
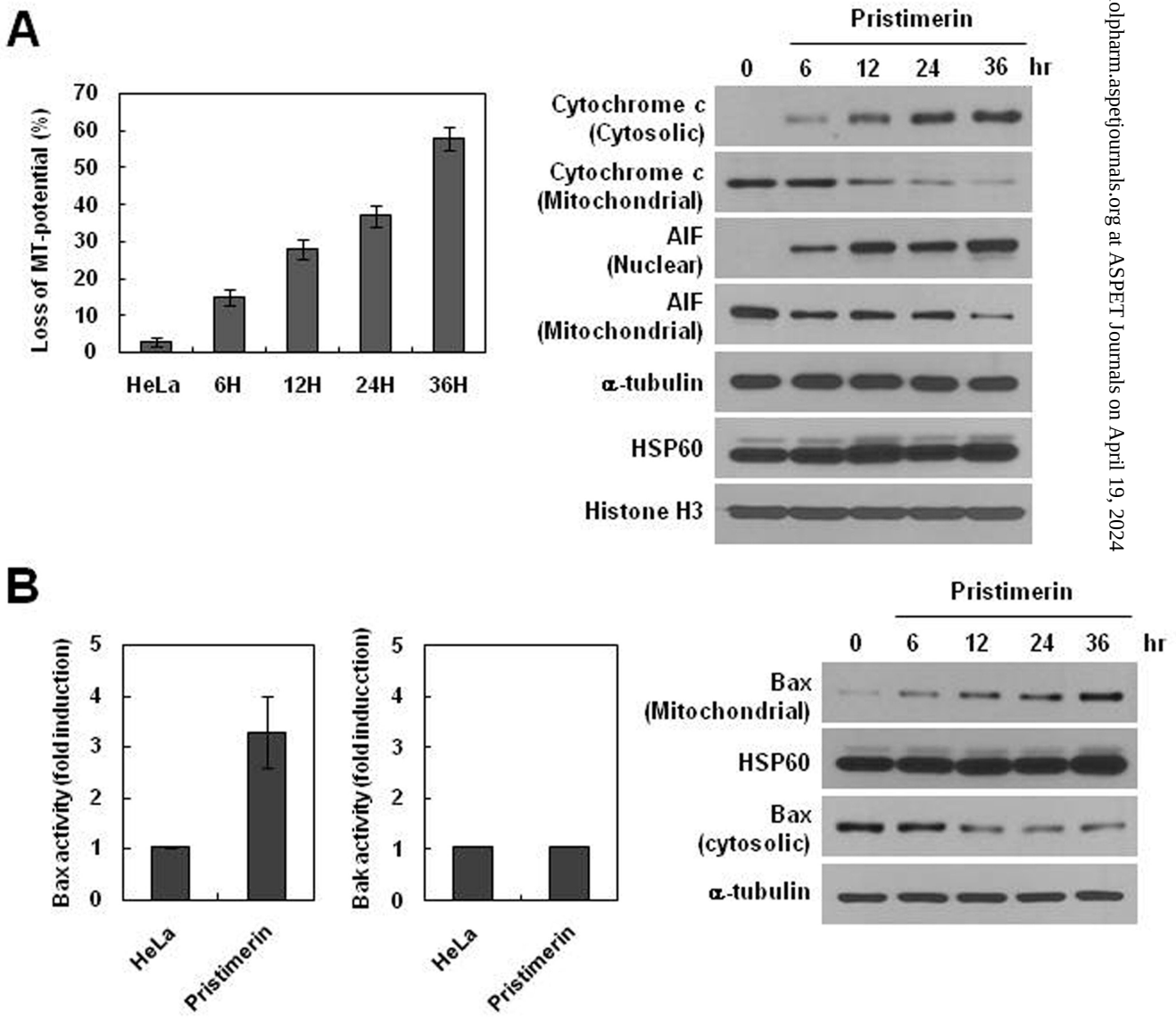


Fig. 2



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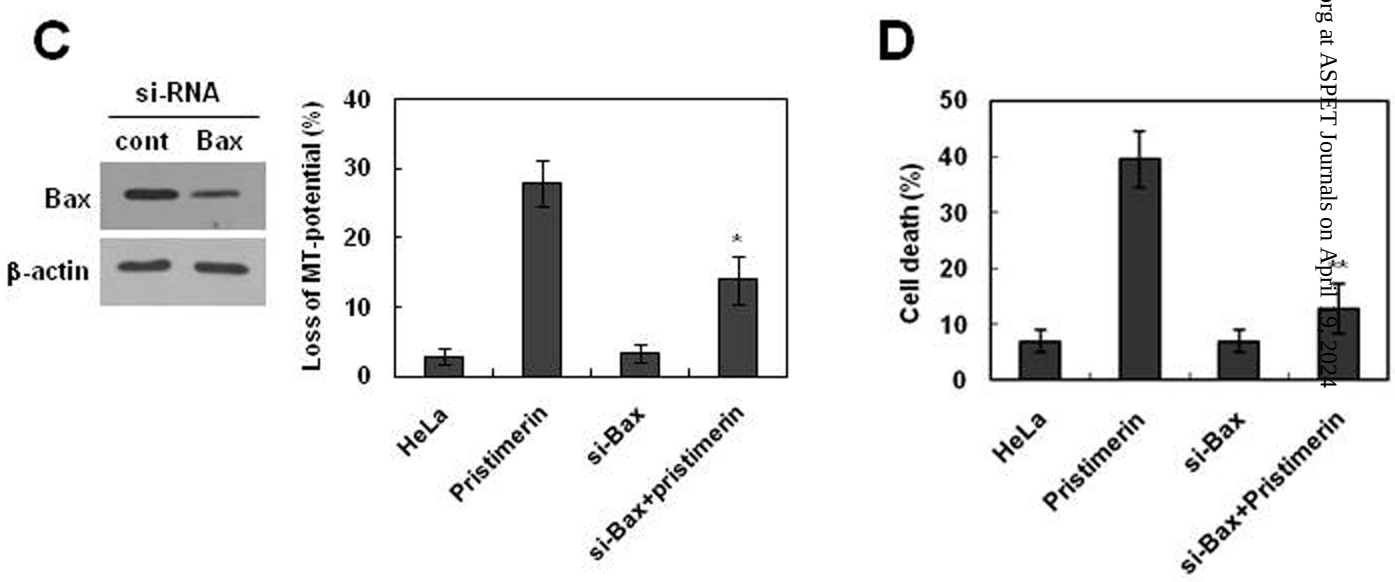

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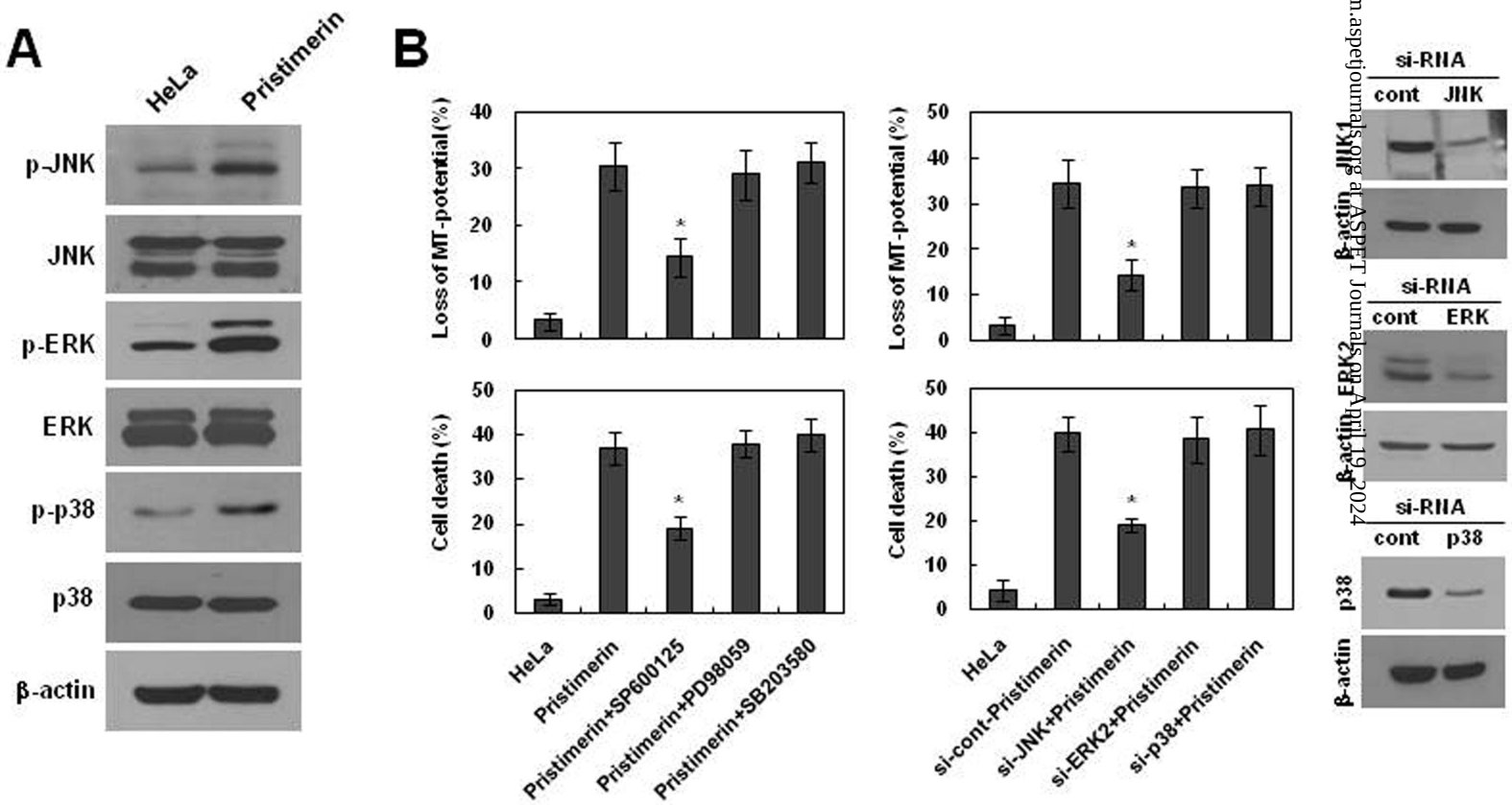

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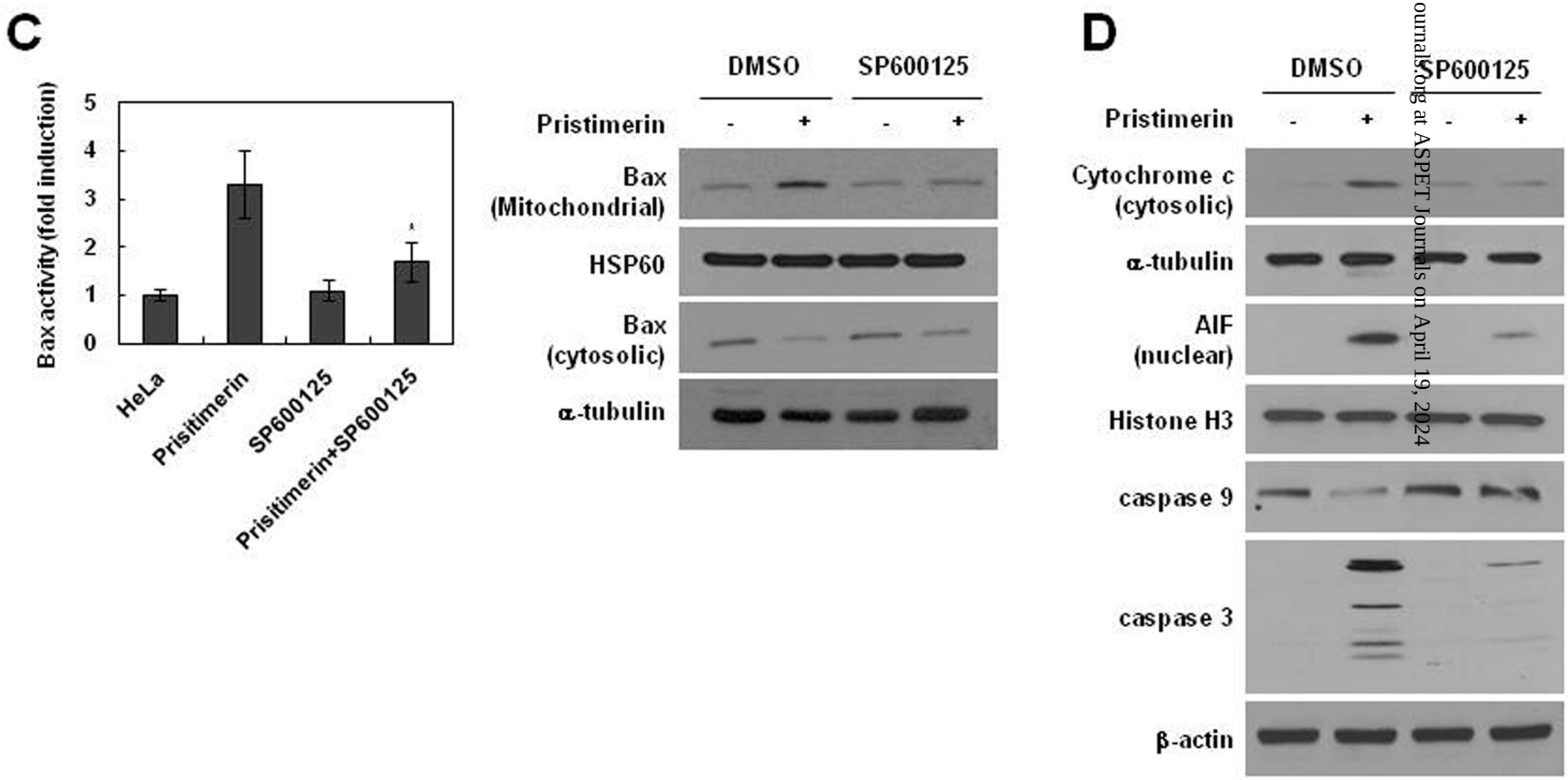
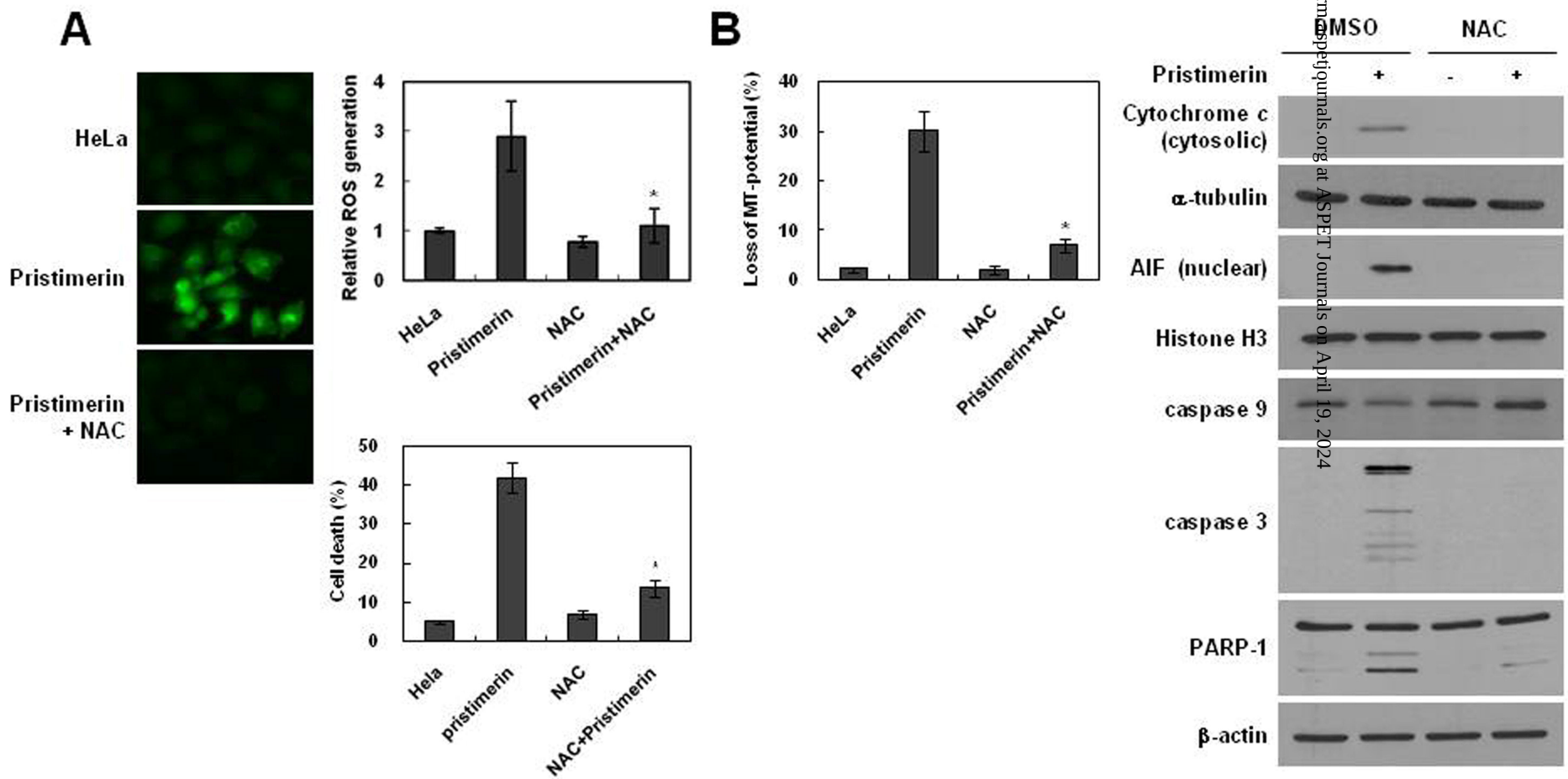


Fig. 4



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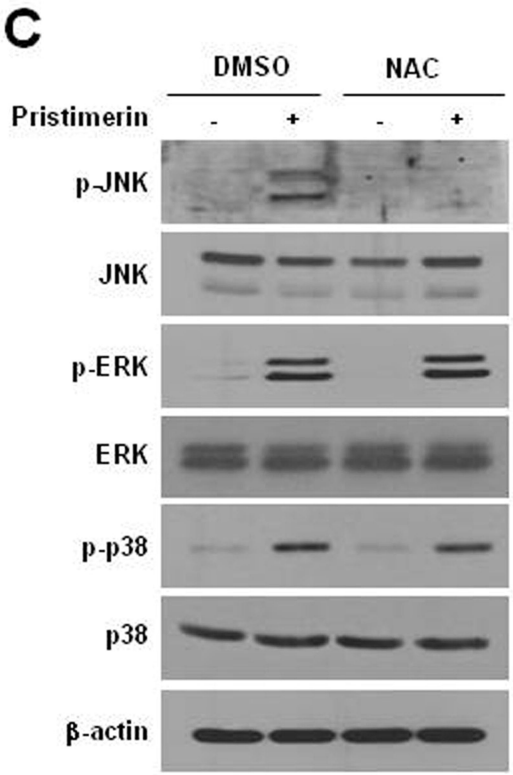
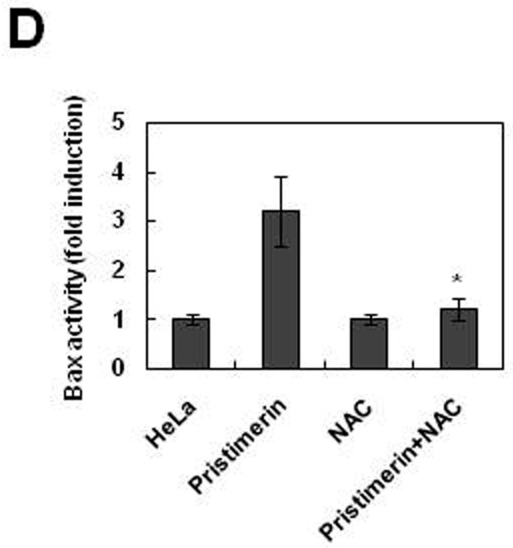




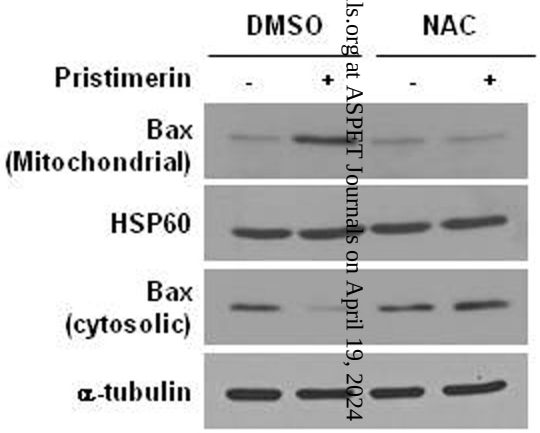
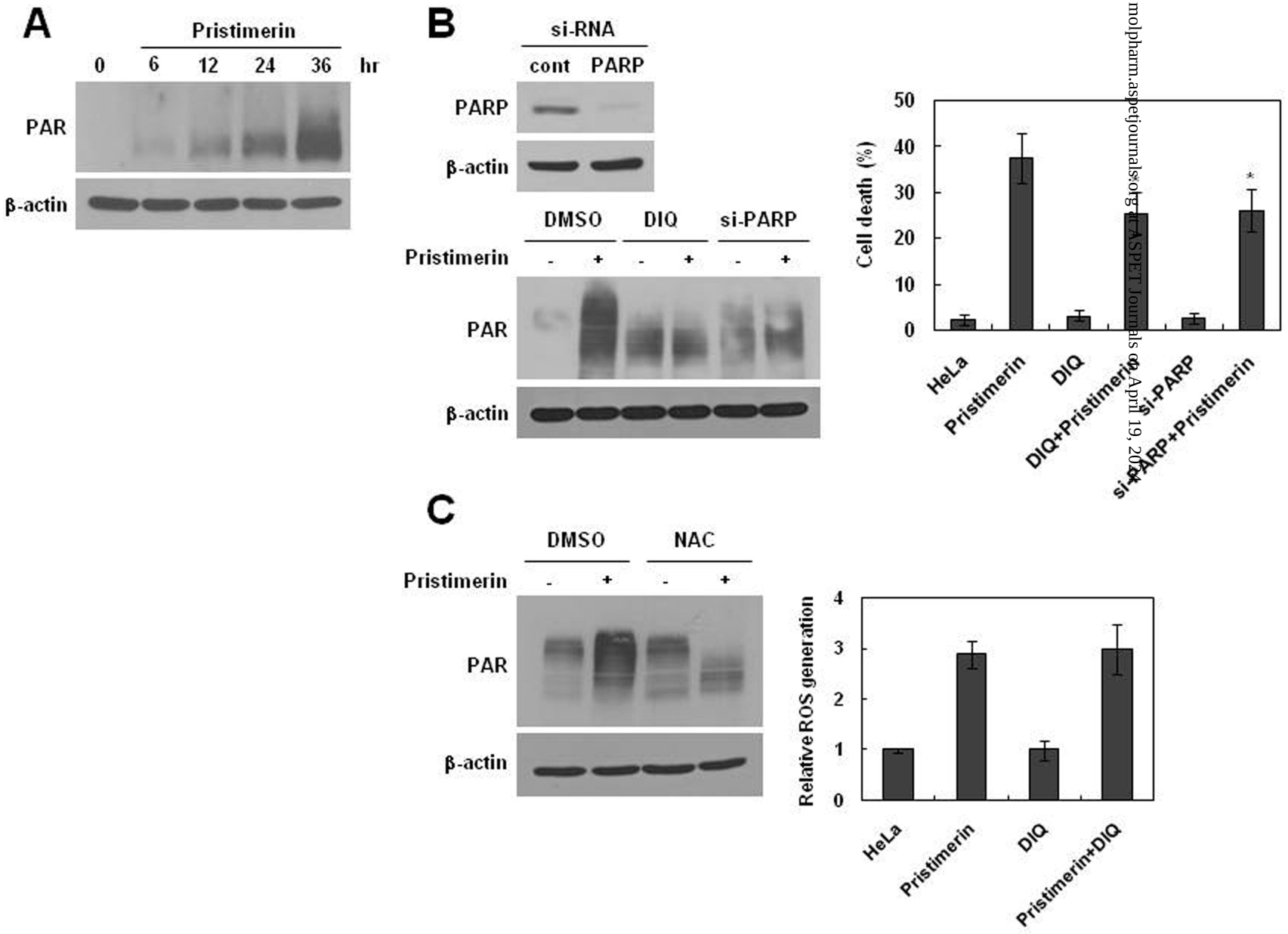


Fig. 5



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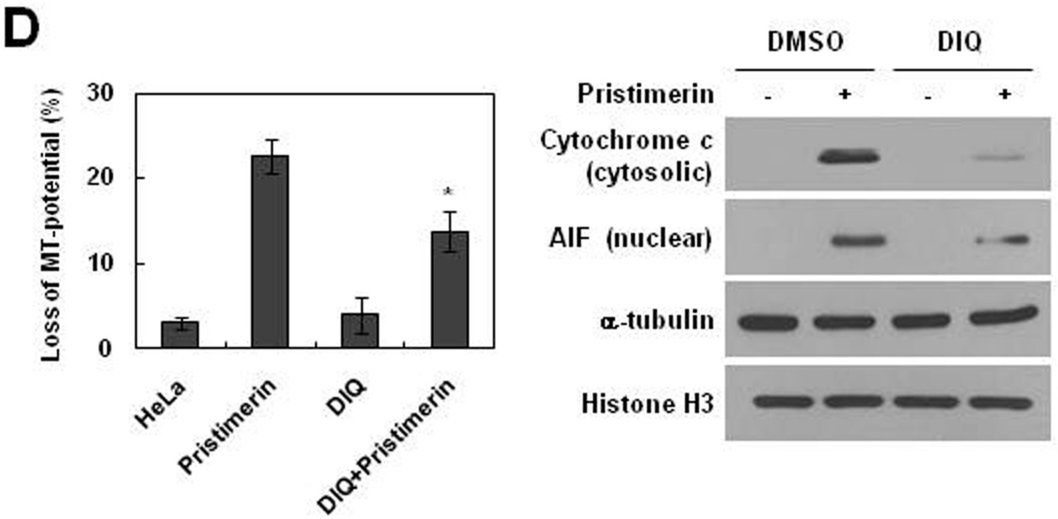

Fig. 6