Triterpenoid pristimerin synergizes with taxol to induce cervical cancer cell death through reactive oxygen species-mediated mitochondrial dysfunction

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A combined treatment with conventional chemotherapies can enhance the effectiveness of chemotherapeutic agents against cancers. Here, we have shown that the naturally occurring triterpenoids synergistically enhance the response of cervical cancer cells to taxol. Of the triterpenoid compounds, pristimerin enhanced the anticancer effect of taxol with the highest efficiency by combination. Pristimerin synergizes with taxol to inhibit clonogenic survival and tumor growth in nude mice, and to enhance cell death in cervical cancer cells. A combined treatment with taxol and pristimerin induced cervical cancer cell death by increasing intracellular reactive oxygen species levels, upregulation of death receptor death receptor 5 (DR5), activation of Bax, and dissipation of mitochondrial membrane potential. Treatment with *N*-acetyl-L-cysteine, a thiol-containing antioxidant completely blocked combined treatment-induced Bax translocation as well as DR5 upregulation. Moreover, inhibition of Jun N-terminal kinase/c-Jun pathway attenuated cell death by blocking DR5 upregulation and Bax activation. These results indicate that the triterpenoid, pristimerin, synergistically enhances taxol response of cervical cancer cells through DR5 expression and Bax activation. Furthermore, the reactive oxygen species-dependent activation of the Jun N-terminal kinase/c-Jun pathway is required for the DR5 upregulation and Bax activation. The molecular mechanism revealed by this study may aid in the design of future combination cancer therapies against cells with intrinsically reduced sensitivity to taxol. *Anti-Cancer Drugs* 22:763–773 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Taxol shows great efficacy against malignant tumors especially in cervical, breast, and ovarian cancer [1,2]. Taxol acts by binding to tubulin and by promoting its assembly into microtubules. This disturbs the dynamic instability of microtubules, thus preventing completion of mitosis and inducing mitotic arrest, ultimately leading to cell death [3–5]. However, the clinical use of taxol is often limited by acquisition of anticancer drug resistance [6–8]. Moreover, its toxicity to normal cells also limits clinical application of taxol. For these reasons, combined treatment is often used to enhance the effectiveness of chemotherapy and to avoid the cancer cells from growing resistant to a single agent [9]. As a novel combination of anticancer treatments could synergistically induce cancer cell death, each single anticancer agent could be used at a reduced dose when combined with each other, overcoming high apoptotic thresholds of cancer cells.

Recently, certain natural and synthetic triterpenoids have shown its effect as both therapeutic and chemopreventive agents against cancer. One such compound is pristimerin, a naturally occurring quinonemethide triterpenoid compound [10–12]. Pristimerin induces apoptotic cell death in human cancer cells, including breast and lung cancer [13,14], acute myeloid leukemia [15], chronic myelogenous leukemia cells [16], multiple myeloma [11], and prostate cancer [17]. Although many reports have emphasized the anticancer activities of pristimerin, the role of pristimerin as a potential modulator of drug sensitivity when combined with other anticancer drug is largely unstudied.

In this study, we show that pristimerin synergistically enhances taxol response of cervical cancer cells when combined with each other at reduced doses, whereas taxol or pristimerin alone cannot induce cancer cell death.

All supplementary data are available directly from the authors.

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Furthermore, we elucidate the mechanisms by which combined treatment with taxol and pristimerin increases intracellular reactive oxygen species (ROS) and consequently upregulates death receptor 5 (DR5) and triggers mitochondrial cell death pathways, finally leading to cell death in the cervical cancer cell line. The molecular mechanisms that we elucidate in this study may provide insights that aid in the design of future combination cancer therapies against cells that are intrinsically less sensitive to taxol treatment.

Materials and methods

Materials

Taxol and caspase inhibitors were purchased from Sigma (St Louis, Missouri, USA). Pristimerin was prepared as described in Byun *et al.* [13] and was dissolved in dimethylsulfoxide. Antibodies specific for polyclonal anti-apoptosis-inducing factor (AIF), anti-cytochrome *c*, anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-Bid, anti-phosphorylated extracellular receptor kinase (ERK), anti-DR4, anti-DR5, anti-Fas, anti-tumor necrosis factor receptor (TNFR), anti-α-tubulin, anti-Bcl-2, anti-p-Bcl-2, anti-Bcl-xl, anti-Mcl-1, and anti-HSP60 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Antibodies specific for polyclonal anticleaved caspase-3, anti-phosphorylated Jun N-terminal kinase (JNK), anti-JNK1, anti-p-p38, anti-p38, anti-ERK2, anti-p-c-Jun, and anti-poly (ADP-ribose) polymerase were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Monoclonal anti-Bax and anti-Bak antibodies were from Pharmingen (San Diego, California, USA). ERK inhibitor (U0126), p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580), and JNK inhibitor (SP600125) were purchased from Calbiochem (San Diego, California, USA). Flag-S70A-Bcl-2 was cloned to a retroviral vector, MFG.

Cell culture and transfection

Human cervical carcinoma cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and nonessential amino acids. The media were supplemented with penicillin (100 units/ml) and streptomycin (100 ug/ml), and cells were incubated at 37°C in 5% CO_2. Cells were transfected with specific small interfering RNA (siRNA) duplexes purchased from Ambion (Austin, Texas, USA), using the lipofectamine reagent (Invitrogen, California, USA) according to the manufacturer's recommendations. MFG and MFG-S70A-Bcl-2 retrovirus were produced in 293 T cells as described by Byun *et al.* [18].

Clonogenic survival assay

Cells were plated in triplicate in 60-mm culture dishes at a density of 500 colonies per dish. After 10 to 14 days of incubation, the culture medium was decanted and the colonies were fixed with a mixture of 75% methanol and 25% acetic acid. Colonies were stained with 0.4% trypan blue dye and the number of colonies containing more than 5 mm cells was counted.

Tumor xenografts on nude mice

All animal procedures and care were approved by the Institutional Animal Care and Usage Committee of Hanyang University. HeLa cells (5×10^6) were injected subcutaneously to the right flank of athymic Balb/c female nude mice (5 weeks of age; Charles River Laboratories, KBT Oriental, Charles River Grade, Tosu, Saga, Japan). Taxol (10 nmol/l, 100 μl) or pristimerin (0.5 μmol/l, 100 μl) or a combination of taxol (10 nmol/l, 100 μl) and pristimerin (0.5 μmol/l, 100 μl) was injected directly into the center of xenograft tumor with a 30-gauge needle when the tumor volume reached 200 mm^3 in diameter (around 5 days after HeLa cell injection). Drugs were administered three times at a 3-day interval. Tumor size was measured with a caliper (calculated volume = shortest diameter^2 × longest diameter/2) at a 3-day interval.

Quantification of cell death

Cell death was analyzed by propidium iodide staining by means of the dye entering the cells. For the assessment of cell death, the cells were plated in a 60-mm dish with a cell density of 2×10^5 cells per dish and combined treatment with taxol and pristimerin the next day. After 24 h, cells were harvested and washed in phosphate buffer solution (PBS). Propidium iodide-positive cells were quantified using a FACScan flow cytometer fitted with CellQuestPro software (Becton Dickinson, BD Biosciences, Seoul, Korea).

Measurement of mitochondrial membrane potential and reactive oxygen species generation

In brief, cells were incubated in 3,3-dihexyloxacarboxyanine iodide [$DiOC_6(3)$; 40 nmol/l] and 2,7-dichlorodihydrofluorescein diacetate (10 μmol/l; Molecular Probes, Eugene, Oregon, USA) at 37°C for 15 min and washed with cold PBS three times. Retained $DiOC_6(3)$ and dichlorodihydrofluorescein were analyzed by a flow cytometer fitted with CellQuestPro software (Becton Dickinson).

Preparation of cytosolic and mitochondrial fractions

Cells were washed with ice-cold PBS, left on ice for 10 min, and then resuspended in isotonic homogenization buffer [sucrose (250 mmol/l), KCl (10 mmol/l), $MgCl_2$ (1.5 mmol/l), Na-EDTA (1 mmol/l), Na-ethylene glycol tetra-acetic acid (EGTA; 1 mmol/l), dithiothreitol (1 mmol/l), phenylmethylsulfonylfluoride (0.1 mmol/l), and Tris–HCl (10 mmol/l); pH7.4] 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 800 *g* for 10 min and 14 000 *g* for 30 min, respectively, from the supernatant.

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For cytosolic fractionation, after 10 strokes with a loose homogenizer, the collected supernatant was spun down at 800 *g* for 10 min and 14 000 *g* for 30 min.

Cross-linking of Bax and Bak proteins

Cells were permeabilized at room temperature with 0.015–0.02% digitonin for 1–2 min in isotonic buffer A [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mmol/l), NaCl (150 mmol/l), $MgCl_2$ (1.5 mmol/l), and EGTA (1 mmol/l), pH 7.4] containing protease inhibitors. The permeabilized cells were shifted to 4°C, scraped, and collected into centrifuge tubes. The supernatants (digitonin/cytosol) were collected after centrifugation at 15 000 *g* for 10 min at 4°C. The pellet was further extracted with ice-cold lysis buffer [2% 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate (CHAPS) in buffer A containing protease inhibitors] for 60 min at 4°C to obtain the membrane fraction. Cells permeabilized with digitonin or membranes extracted with CHAPS were incubated with a cross-linker (dissuccinimidyl suberate with linker lengths of 11.4 Å) on a head-to-head rocker for 30 min at room temperature. After quenching the unreacted cross-linkers with 1/10 volume of Tris–HCl (2 mol/l; pH 7.4), cells or extracts were incubated for another 30 min at room temperature with rocking. After cross-linking, membranes were extracted with 2% CHAPS in buffer A and mixed with a nondenaturing loading buffer before SDS-polyacrylamide gel electrophoresis.

Analysis of death receptor expression

In brief, after fixation (0.25% paraformaldehyde, 5 min) and washing, cells were incubated for 30 min in the presence of digitonin (100 μg/ml) with anti-DR4 (H-130, Santa Cruz Biotechnology), anti-DR5 (N-19, Santa Cruz Biotechnology), anti-Fas (C236, Santa Cruz Biotechnology), and anti-TNFR (55R-170, Santa Cruz Biotechnology), respectively. After incubation with fluorescein isothiocyanate-conjugated secondary antibodies for 30 min, cells were analyzed using a flow cytometer.

Statistical analysis

All experimental data are reported as mean and the error bars represent the experimental standard error. Statistical analysis was performed by the nonparametric Student *t* test.

Results

Naturally occurring triterpenoids synergistically enhance taxol response of human cervical cancer cells

To examine whether naturally occurring triterpenoids enhance taxol-induced cell death in human cervical cancer cells, we treated HeLa cells with the triterpenoids, celastrol, iguesterin, tingenone, 22β-hydroxytingenone, 6β-acetonyl-22β-hydroxytingenone or pristimerin (200 nmol/l each) together with taxol (300, 500, or 700 pmol/l) and analyzed clonogenic survival by the colony-forming assay. As shown in Fig. 1a, all six natural triterpenoid compounds clearly enhanced the response of cervical cancer cells to taxol. Moreover, triterpenoids (0.5 μmol/l each), in combination with taxol (10 nmol/l), synergistically enhanced cell death. However, treatment with either triterpenoid (0.5 μmol/l) or taxol (10 nmol/l) alone did not cause significant cell death (Fig. 1a). Of the six triterpenoid compounds, pristimerin induced the highest level of cell death in combination with taxol; almost 50% of HeLa cells underwent cell death within 24 h after the combined treatment. Moreover, combined treatment with taxol and pristimerin markedly inhibited clonogenic survival, and synergistically enhanced cell death in three different human cervical cancer cell lines (HeLa, CaSki, and SiHa; Fig. 1b). The combined treatment with taxol and pristimerin also clearly suppressed tumor growth in nude mice (Fig. 1c). These findings suggest that naturally occurring triterpenoids synergistically enhance the response of human cervical cancer cells to taxol.

Upregulation of death receptor 5 contributes to enhanced cell death after combination treatment

To determine whether DRs are involved in the combination treatment-induced apoptotic cell death, alteration in DRs expression was analyzed by flow cytometry after treatment of HeLa cells with pristimerin or taxol alone, or a combination of them (Fig. 2). Single treatment with taxol (10 nmol/l) or pristimerin (0.5 μmol/l) did not induce the expression of DRs, TNFR, DR4, DR5, and Fas. However, the combined treatment significantly increased the expression of DR5, whereas other DRs (TNFR, DR4, and Fas) were unchanged in their expression levels of proteins (Fig. 2a and b, left). Moreover, siRNA-mediated DR5 downregulation effectively attenuated apoptotic cell death (Fig. 2b, right), caspase-8 activation, Bid cleavage (Fig. 2c), and Bax oligomerization (Fig. 2d) caused by the combined treatment. Taken together, these results suggest that the combined treatment with taxol and pristimerin induces the expression of DR5 that could mediate the cell death pathway.

Activation of Bax is involved in mitochondrial cell death induced by combined treatment

To determine whether the increase of DR5 caused by the combined treatment with taxol and pristimerin is linked to mitochondrial pathway to induce cell death, we first examined changes in mitochondrial membrane potential using the carbocyanine dye $DiOC_6(3)$ and the release of proapoptotic molecules from the mitochondria in HeLa cells after treatment with pristimerin or taxol alone, or combination of them. Although single treatment with pristimerin or taxol had no effect, the combined treatment induced a significant dissipation of mitochondrial membrane potential and promoted subsequent cytosolic redistribution of cytochrome *c* and AIF (Fig. 3a), indicating that cell death is accompanied by mitochondrial dysfunction. As proapoptotic Bcl-2 family members, especially Bax and Bak, are crucial to the mitochondrial cell death pathway [19], we next analyzed the activation of Bax and Bak after the combined

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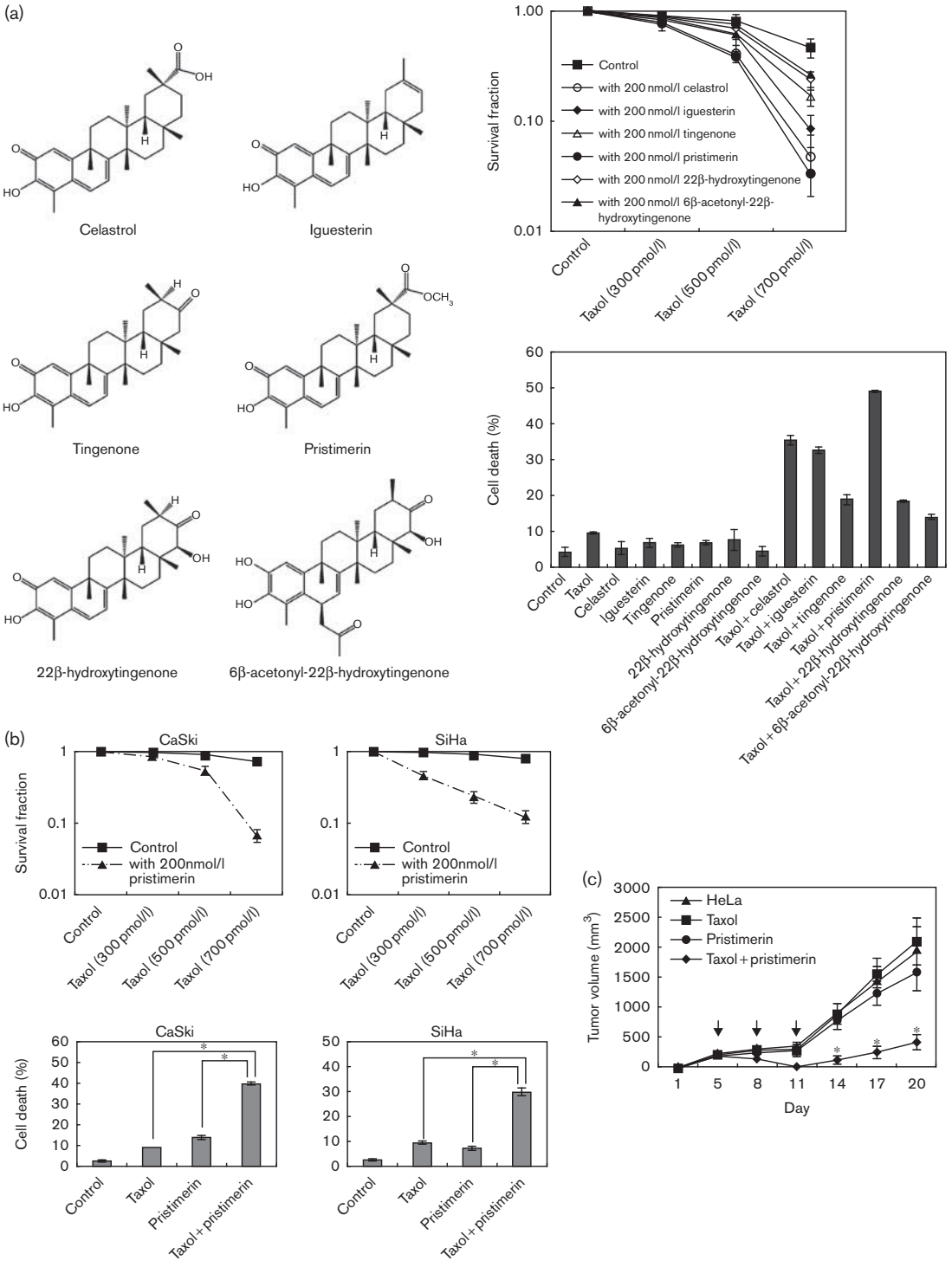
Fig. 1



Naturally occurring triterpenoids synergistically enhance taxol response of cervical cancer cells. (a) The chemical structures of triterpenoids (left) and the effect of either taxol or tripernoid alone or the combination of taxol and tripernoid on cell survival (upper right) and cell death (lower right) in HeLa cells. Cell survival was analyzed by counting colony formation after staining with 0.5% crystal violet, and cell death was measured as the percentage of propidium iodide-positive cells using flow cytometry. Error bar represents mean ± standard deviation (SD) of triplicate samples. (b) The effect of taxol in combination with pristimerin on cell survival and cell death in other human cervical cancer cell lines (CaSki, SiHa). *$P<0.01$. Error bar represents mean ± SD of triplicate samples. (c) Effect of either taxol or pristimerin alone or the combination of taxol and pristimerin on tumor growth in athymic nude mice (each group, $n=3$) after subcutaneous injection of HeLa cells (5×10^6 cells). Drugs were injected directly to the center of tumors three times at a 3-day interval as indicated with arrows. Tumor volumes were measured at a 3-day interval. Significantly different from pristimerin; *$P<0.01$. Error bar represents mean ± SD of triplicate samples.

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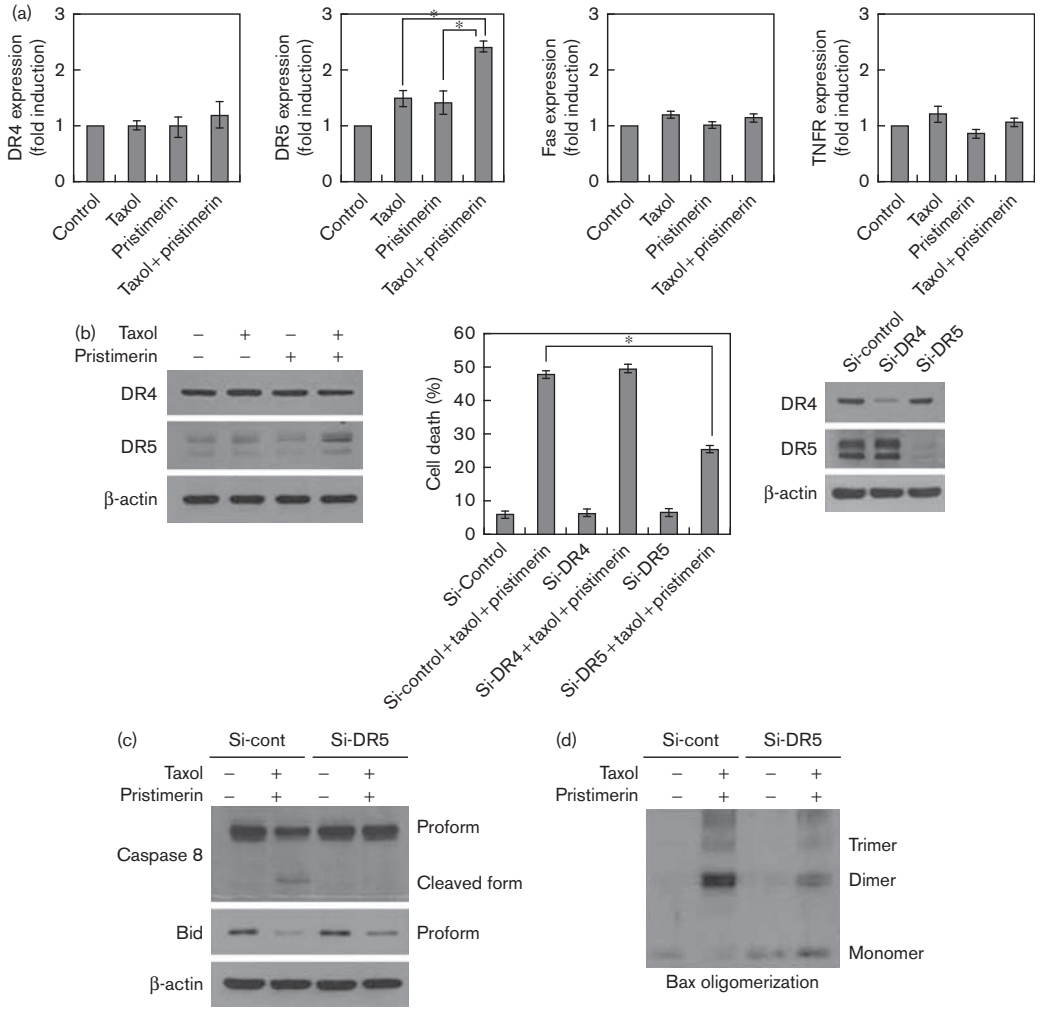
Fig. 2



Upregulation of death receptor 5 (DR5) contributes to enhanced cell death after combined treatment. (a) Flow cytometry analysis reveals upregulation of DR5 expression in HeLa cells treated with taxol (10 nmol/l) in combination with pristimerin (0.5 μmol/l), whereas the levels of DR4, DR5, Fas, and tumor necrosis factor receptor (TNFR) expression were not altered in flow cytometry analysis. However, single treatment with taxol or pristimerin did not upregulate the level of DR5 expression. $*P<0.01$. Error bar represents mean ± standard deviation (SD) of triplicate samples. (b) Western blot analysis indicates upregulation of DR5 but not DR4 in HeLa cells treated with the combination of taxol and pristimerin (left). Small interfering RNA (siRNA)-mediated DR5 downregulation attenuates cell death induced by the combined treatment (right). Cell death was determined by the percentage of propidium iodide-positive cells using flow cytometry. β-actin was used as a loading control. (c) siRNA-mediated DR5 downregulation inhibited caspase-8 activation and Bid cleavage induced by the combined treatment with taxol and pristimerin. (d) siRNA-mediated DR5 downregulation inhibited Bax oligomerization induced by the combined treatment with taxol and pristimerin. $*P<0.01$. Error bar represents mean ± SD of triplicate samples.

treatment. To this end, we extracted membrane fractions (mitochondria enriched) and allowed chemical cross-linking with disuccinimidyl suberate. Western blot analysis of cross-linked proteins revealed that the combined treatment induced oligomerization of Bax, but not Bak (Fig. 3b, left), suggesting that the activation of Bax is involved in the mitochondrial cell death. Consistent with the results, Bax was relocalized from the cytosol to mitochondria (Fig. 3b, right). Moreover, targeted suppression of Bax by siRNA significantly attenuated mitochondrial membrane potential loss (Fig. 3c) and cell death (Fig. 3d). We also observed that Bcl-2 phosphorylation at Ser 70 was increased accompanying the decrease of Bcl-2 in response to the combined treatment, whereas the levels of Bcl-xL or Mcl-1 were not altered in HeLa cells (Fig. 3e). Consistently, when a mutant form of Bcl-2 (Flag-S70A-Bcl-2), in which Ser70 of Bcl-2 is replaced by Ala, was expressed in HeLa cells by retrovirus-mediated gene transfer before treatment, the protein level of Flag-S70A-Bcl-2 was not altered by the combined treatment (Fig 3f). Moreover, overexpression of the mutant form of Bcl-2 effectively attenuated the combined treatment-induced apoptotic cell death (Fig. 3g). Taken together, these results suggest that DR5 upregulation by the combined treatment induces a loss of mitochondrial membrane potential by the activation of Bak and Bcl-2.

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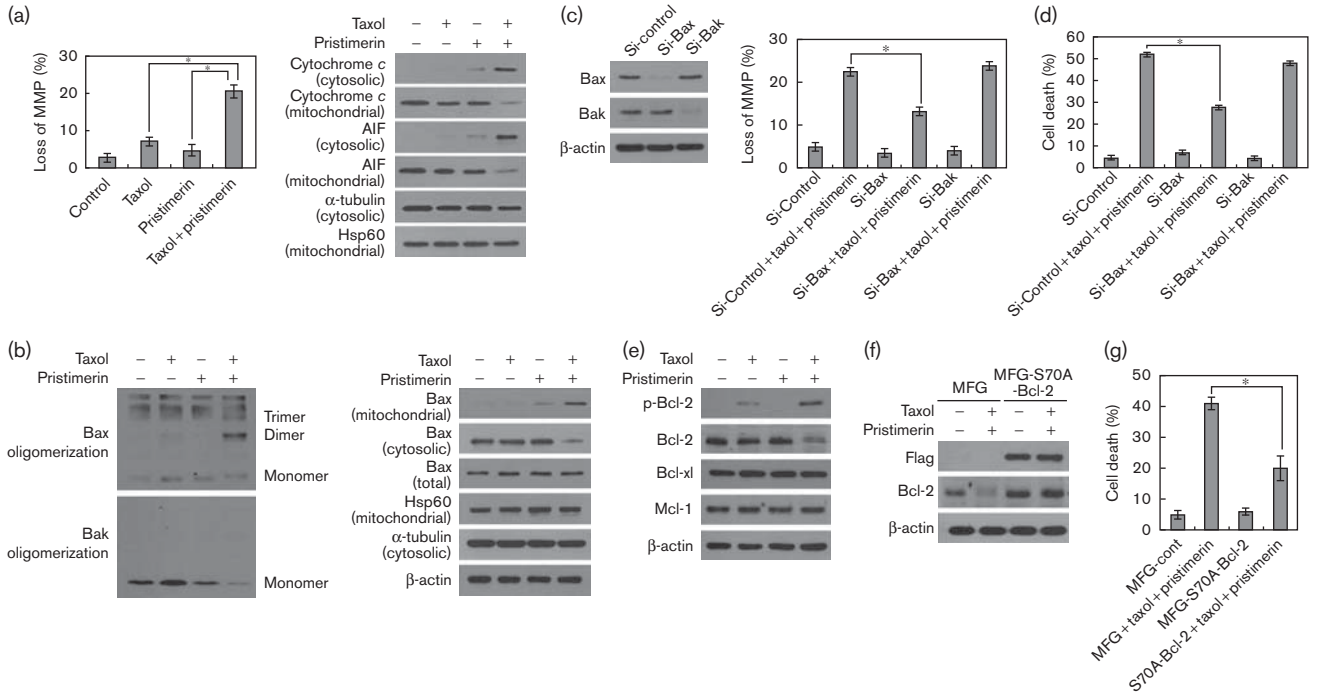
Fig. 3



Combined treatment with taxol and pristimerin induces Bax-mediated mitochondrial cell death. (a) Mitochondrial membrane potential of the cells was determined by retention of 3,3′dihexylocarbocyanine iodide [DiOC(3)]. The amount of retained DiOC(3) was measured by flow cytometry. Combined treatment with taxol (0.5 μmol/l) and pristimerin (10 nmol/l) induced mitochondrial membrane potential loss (left) and the release of cytochrome *c* and apoptosis-inducing factor (AIF) to cytosol (right) in HeLa cells. Alpha-tubulin and HSP60 were used as cytosolic and mitochondrial marker proteins, respectively. **P*<0.01. Error bar represents mean ± standard deviation (SD) of triplicate samples. (b) Oligomerization of Bax was detected in mitochondria-enriched membrane fractions of HeLa cells treated with the combination of taxol and pristimerin, whereas oligomerization of Bak was not (left). Bax was relocalized from the cytosol to mitochondria (right). Alpha-tubulin and HSP60 were used as cytosolic and mitochondrial marker proteins, respectively. (c and d) Small interfering RNA (siRNA)-mediated downregulation of Bax-attenuated mitochondrial membrane potential loss (c) and cell death (d) caused by combined treatment of taxol and pristimerin, whereas siRNA targeted to Bak had no effect on mitochondrial membrane potential and cell death. **P*<0.01. Error bar represents mean ± standard deviation (SD) of triplicate samples. (e) Increase in Bcl-2 phosphorylation at Ser 70, accompanies the decrease in Bcl-2, whereas the levels of Bcl-xl and Mcl-1 were not altered in HeLa cells treated with the combination of taxol and pristimerin. (f) The expression of Flag-S70A-Bcl-2, the point mutant form of Bcl-2, in which Ser 70 is replaced by Ala, was not decreased by the combined treatment with taxol and pristimerin, compared with control vector, MFG. (g) Combined treatment-induced cell death was attenuated in HeLa cells expressing Flag-S70A-Bcl-2. β-actin was used as a loading control. **P*<0.01. Error bar represents mean ± SD of triplicate samples. MMP, matrix metalloproteinase.

Intracellular reactive oxygen species generation in mitochondria is required for the cell death pathway caused by combined treatment

Oxidative damage has been shown to play an important role in the anticancer effect of chemotherapeutic drugs [20–22]. As the combined treatment with taxol and pristimerin caused cell death through mitochondria pathway and the mitochondria is a major source of ROS, we next examined changes in intracellular ROS levels after the combined treatment. As shown in Fig. 4a, the combined treatment led to an approximately three-fold increase in mean DCF fluorescence, indicating an

Fig. 4

Intracellular reactive oxygen species (ROS) generation is required for the activation of mitochondrial cell death pathway. (a) Dichlorodihydro-fluorescein (DCF) fluorescence visualization in fluorescence microscopy (left) and flow cytometry analysis (right) indicate the increase in ROS levels in HeLa cells treated with the combination of taxol and pristimerin. The ROS scavenger, *N*-acetyl-L-cysteine (NAC) treatment (10 mmol/l) for 24 h attenuated the effects of the combination treatment of taxol and pristimerin by abolishing ROS, recovering cell survival, and attenuating cell death. **P*<0.01. Error bar represents mean ± standard deviation (SD) of triplicate samples. (b) Combined treatment with taxol and pristimerin in the presence of NAC-attenuated mitochondrial membrane potential loss (left) and inhibited Bax oligomerization, relocalization from cytosol to mitochondria, and the release of cytochrome *c* and apoptosis-inducing factor (AIF) to cytosol (right), compared with only combination treatment. **P*<0.01. Error bar represents mean ± standard deviation (SD) of triplicate samples. (c–e) NAC treatment also inhibited Bcl-2 phosphorylation at Ser 70 (c), the expression of death receptor 5 (DR5; d), caspase-3, caspase-8, and caspase-9 activation, Bid cleavage, and anti-poly (ADP-ribose) polymerase (PARP) cleavage (e), all of which are induced by combined treatment with taxol and pristimerin. β-Actin was used as a loading control. **P*<0.01. Error bar represents mean ± SD of triplicate samples. DRS, death receptors; MMP, matrix metalloproteinase.

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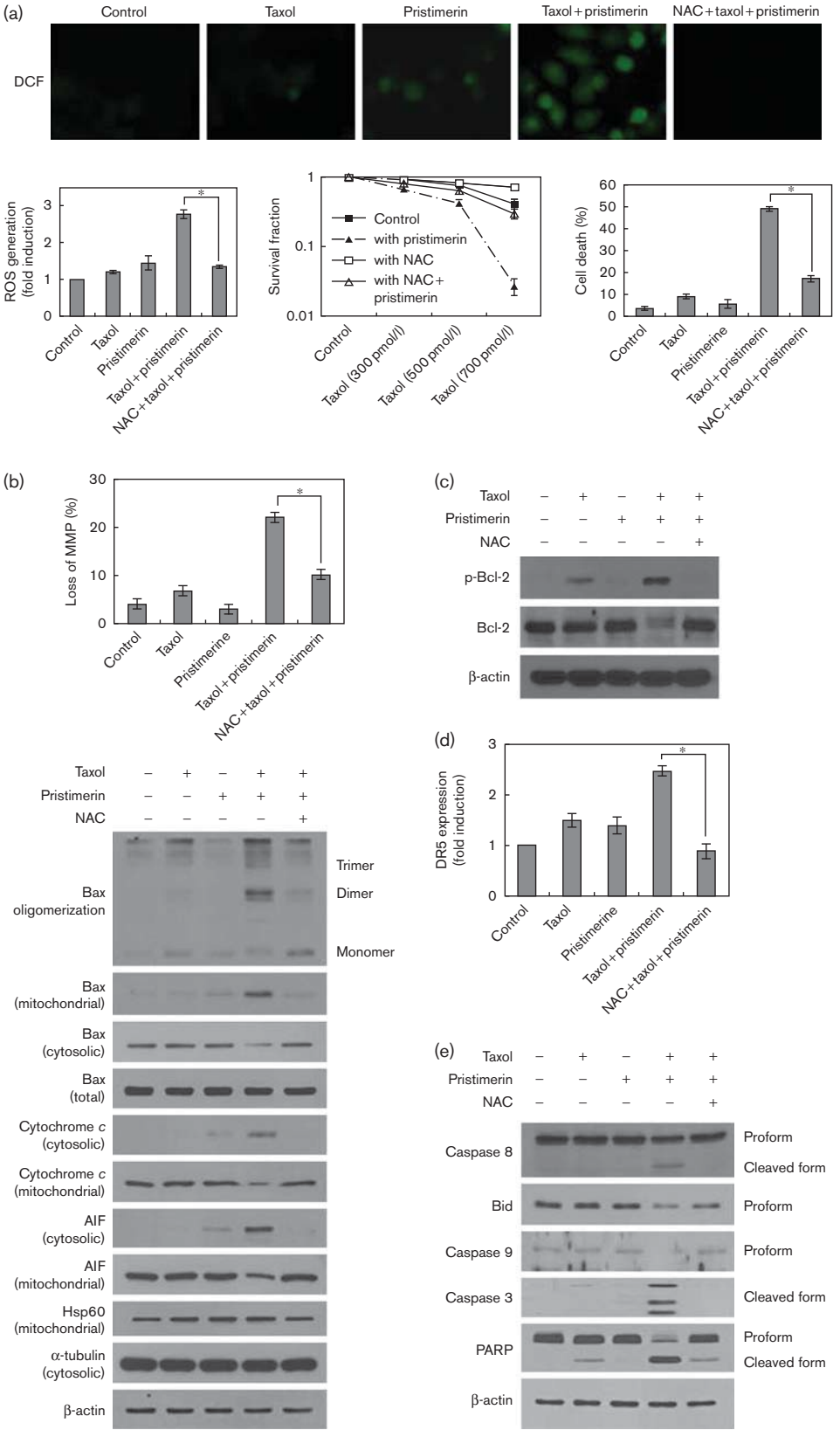
Fig. 4



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increase in intracellular ROS; however, treatment with either reagent alone did not increase significant ROS levels at the concentration we used. In addition, combined treatment of all six triterpenoids with taxol induced intracellular ROS generation (Fig. S1A).

To further establish a link between elevation of intracellular ROS and cell death, we incubated cells with the antioxidant NAC before the addition of taxol and pristimerin. NAC (10 mmol/l) treatment for 24 h markedly reversed the inhibition of clonogenic survival and attenuated cell death caused by the combined cell death (Fig. 4a). Moreover, inhibition of ROS with NAC treatment completely prevented the oligomerization and mitochondrial redistribution of Bax, the mitochondrial membrane potential loss, and the release of AIF and cytochrome *c* caused by combined taxol and pristimerin treatment (Fig. 4b). Inhibition of ROS also suppressed the phosphorylation of Bcl-2 accompanying the downregulation of Bcl-2 (Fig. 4c). Furthermore, pretreatment with NAC completely blocked the combined treatment-induced DR5 upregulation (Fig. 4d) and subsequent caspase-3, caspase-8, and caspase-9 activation and Bid cleavage (Fig. 4e), indicating that combined treatment with taxol and pristimerin increases the intracellular ROS level before the expression of DR5 to trigger cell death pathway.

To further determine whether the mitochondria contributed to an increase in the intracellular ROS level caused by the combined treatment, we measured fluorescence of MitoSOX Red, the mitochondrial superoxide indicator. Combined treatment with taxol and pristimerin increased the fluorescence of MitoSOX Red (Fig. S1B), indicating that the mitochondria contribute to the overall elevation in ROS. The stippled linear pattern of fluorescence in HeLa cells loaded with MitoSOX Red is consistent with mitochondrial localization of ROS. Mitochondrial contribution to the increase of intracellular ROS was further confirmed by using dihydroethidium after the combined treatment (Fig. S1C). Taken together, these results suggest that combined treatment with taxol and pristimerin induces an increase of intracellular ROS through mitochondria, which further induces the expression of DR5, and finally leads to cell death.

Jun N-terminal kinase 1 is an important mediator of death recptor 5 upregulation and Bax activation

We next attempted to examine a signaling pathway that links between intracellular ROS and the expression of DR5. As MAPKs have been implicated in regulating the cell death response to various stimuli [23], we tested whether the MAPK signaling pathway is involved in the combination treatment-induced cell death. To this end, the level of phosphorylation of JNK, p38, and ERK was analyzed after treatment of HeLa cells with a combination of taxol and pristimerin. Immunoblotting analysis revealed an increase of the phosphorylated form of JNK, whereas the level of phosphorylated form of p38 and ERK was not altered (Fig. 5a), indicating that JNK was activated by the combined treatment with taxol and pristimerin. Moreover, pretreatment with the JNK-specific inhibitor SP600125 (10 μmol/l) or siRNA targeting of JNK-attenuated cell death caused by combined treatment with taxol and pristimerin, whereas a p38 MAPK inhibitor SB203580 (10 μmol/l) and an MEK inhibitor U0126 (10 μmol/l) or siRNA treatment targeting to p38 or ERK2 had no effect (Fig. 5b). In addition, inhibition of JNK by pretreatment with SP600125 or by siRNA targeting of JNK-attenuated mitochondrial membrane potential loss (Fig. 5c) as well as oligomerization and mitochondrial translocation of Bax (Fig. 5d), whereas intracellular ROS level was not altered (data not shown). These results implicate JNK as a mediator of the mitochondrial cell death caused by a combined treatment with taxol and pristimerin. However, pretreatment with NAC before the combined treatment with taxol and pristimerin blocked the activation of JNK (Fig. 5f), indicating that the activation of JNK depends on intracellular ROS.

We next examined whether JNK is involved in the combination treatment-induced upregulation of DR5 and subsequent activation of the apoptotic cell death cascade. To this end, HeLa cells were treated with the JNK-specific inhibitor, SP600125, or siRNA targeting of JNK1 before combined treatment with taxol and pristimerin, and 24 h later the expression level of DR5 was analyzed by flow cytometry. The expression of DR5 was not upregulated by the combined treatment when cells are pretreated with the JNK-specific inhibitor, SP600125, or siRNA targeting of JNK1. Moreover, caspase-3, caspase-8, and caspase-9 activation and Bid cleavage were abrogated by pretreatment with SP600125 (Fig. 5g).

The combined treatment of HeLa cells activated c-Jun, as evidenced by a marked increase in phosphorylated c-Jun (Fig. S2A). Moreover, siRNA targeting of c-Jun completely inhibited the combination treatment-induced DR5 expression (Fig. S2B), caspase-8 activation, Bid cleavage (Fig. S2C), and Bax activation (Fig. S2D). However, siRNA targeting of c-Jun did not alter Bcl-2 phosphorylation and downregulation (data not shown). Taken together, these results suggest that JNK signaling links between intracellular ROS and the expression of DR5, acting as a critical upstream mediator of DR5 upregulation and Bax activation in response to combined treatment with taxol and pristimerin.

Discussion

The anticancer activities of pristimerin have been emphasized in terms of its therapeutic and chemopreventive potential in cancer [12]. However, a role of pristimerin as a modulator of chemotherapeutic drug sensitivity has been largely unstudied. Here, we examined

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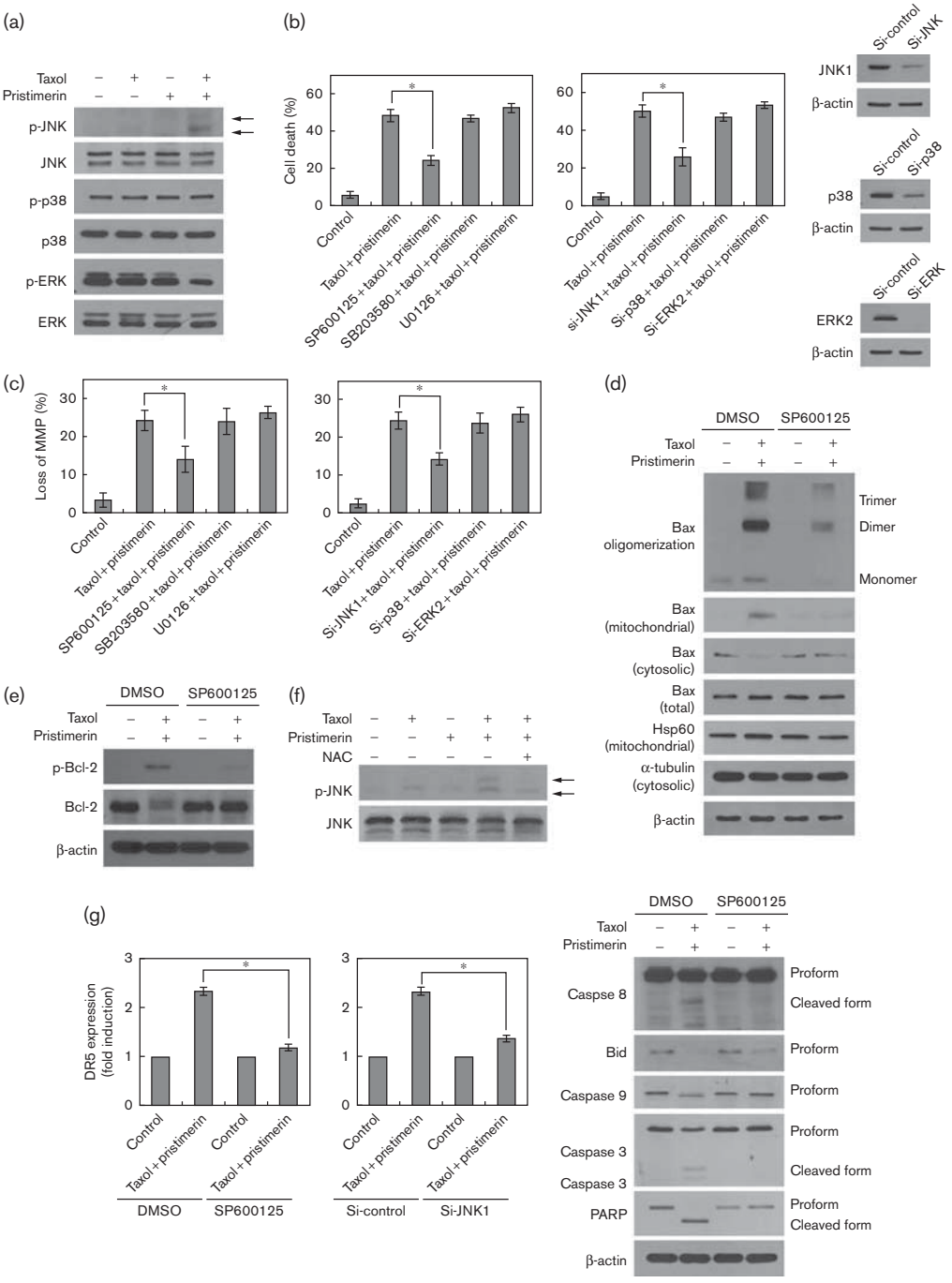
Fig. 5



Jun N-terminal kinase (JNK1) is an important mediator of death receptor 5 (DR5) upregulation and Bax activation. (a) The phosphorylated form of JNK was increased in HeLa cells treated with the combination of taxol and pristimerin, whereas the phosphorylated form of p38 and extracellular receptor kinase (ERK) was not. (b and c) Pretreatment with the JNK-specific inhibitor, SP600125, or siRNA targeted to JNK-attenuated cell death (b) and mitochondrial membrane potential loss (c) induced by the combination treatment, whereas a p38 MAPK inhibitor, SB203580 or a MEK inhibitor, U0126, or siRNA targeted to p38 and MEK had no effect. *$P<0.01$. Error bar represents mean ± standard deviation (SD) of triplicate samples. (d and e) Pretreatment with the JNK-specific inhibitor, SP600125, inhibited Bax oligomerization, mitochondrial translocation (d), and Bcl-2 phosphorylation (e) caused by the combined treatment with taxol and pristimerin. Alpha-tubulin and HSP60 were used as cytosolic and mitochondrial marker proteins, respectively. (f) Pretreatment with *N*-acetyl-L-cysteine (NAC) before the combined treatment with taxol and pristimerin blocked the activation of JNK. (g) Pretreatment with the JNK-specific inhibitor, SP600125, or siRNA targeted to JNK suppressed the increase in DR5 expression induced by combined treatment with taxol and pristimerin. Moreover, combined treatment with taxol and pristimerin in the presence of the JNK-specific inhibitor, SP600125, attenuated the activity of caspase-3, caspase-8, and caspase-9, Bid cleavage, and Poly (ADP-ribose) polymerase (PARP) cleavage caused by the combined treatment with taxol and pristimerin. Beta-actin was used as a loading control. *$P<0.01$. Error bar represents mean ± SD of triplicate samples. DMSO, dimethylsulfoxide; MMP, matrix metalloproteinase.

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whether pristimerin enhances taxol response in cervical cancer cells. We found that combined treatment with taxol (10 nmol/l) and pristimerin (0.5 μmol/l) synergistically enhances cell death in cervical cancer cell lines and inhibits tumor growth in xenograft mice. Moreover, we elucidated the mechanisms by which combined treatment with taxol and pristimerin increases the intracellular ROS level and consequently upregulates DR5 and finally triggers mitochondrial cell death pathways in cervical cancer cell lines. In previous studies, the expression of DR4 and DR5 in some human cancer cells is also enhanced by treatment with chemotherapeutic agents, interferon, hyperthermia, and ionizing radiation [24,25]. The upregulation of DR4 and DR5 expression is known to be important in sensitizing tumor cells to the stimuli-induced apoptosis [26–28]. In this study, pristimerin in combination with taxol also induced selective expression of DR5 in cervical cancer cells, and siRNA targeting of DR5 effectively suppressed the combined treatment-induced Bax activation and subsequent mitochondrial cell death.

We also found that JNK1 signaling is involved in these mitochondrial cell death pathways, mediating between intracellular ROS and upregulation of DR5 expression. By using JNK-specific inhibitor or JNK-targeting siRNA, we provide evidence that the JNK/c-Jun pathway plays a critical role in the upregulation of DR5 and subsequently caspase-3, caspase-8, and caspase-9 activation, Bid cleavage, and Bax activation in response to the combined treatment with taxol and pristimerin. These results are consistent with previous studies in several cell types that JNK activates caspases and may also target other factors that have been implicated in the regulation of apoptotic cell death, including Bax and Bak [23,29].

Recently, several studies have put forward the hypothesis that antiapoptotic function of Bcl-2 depends on its phosphorylation status [30–32]. Consistent with these findings, we observed a marked phosphorylation of Bcl-2 and subsequent downregulation of Bcl-2 after the combined treatment. Moreover, we found that Bcl-2 phosphorylation is closely associated with JNK activation, as its inhibition leads to suppression of Bcl-2 phosphorylation, indicating that phosphorylation and subsequent downregulation of Bcl-2 might be associated with the enhancement of mitochondrial cell death induced by the combined treatment with taxol and pristimerin.

Recent studies have provided evidence for a role of ROS as inducers of MAPK activation during apoptotic cell death [20,21,33], supporting our results that ROS are essential for the activation of JNK and subsequent mitochondrial cell death caused by the combined treatment with taxol and pristimerin. Consistent with this, NAC completely blocked combination treatment-induced JNK activation, Bax relocalization, Bcl-2 downregulation, and mitochondrial cell death. One mechanism has been proposed to account for JNK pathway activation by ROS. In nonstressed cells, apoptosis signal-regulating kinase 1 (ASK1) is known to associate with reduced thioredoxin [34]. Oxidation of thioredoxin by ROS releases ASK-1 and leads to JNK activation, possibly through dimerization of ASK-1 [34,35]. In this study, we found no evidence for ASK-1 activation in response to the combined treatment. Moreover, siRNA targeted to ASK-1 did not affect JNK activation or cell death (data not shown), indicating that ROS-dependent JNK activation by the combined treatment is independent of ASK-1. This observation is consistent with the ASK-1-independent activation of MAPK by ROS [35]. Thus, the precise mechanisms by which combined treatment with taxol and pristimerin induces ROS-dependent activation of JNK remain to be elucidated.

Pristimerin has been shown to act as proteasome inhibitor both *in vitro* and *in vivo* [11,17]. Proteasome inhibition by pristimerin led to the accumulation of ubiquitinated proteins and proteasome target proteins Bax, p27, and IκB-α, and consequently blocked the NF-κB pathway and induced cell death. Moreover, proteasome inhibition with chemical inhibitors has shown to induce significant oxidative stress [36–39]. Proteasome inhibitors can also induce JNK activation and subsequent mitochondrial cell death [40–42]. In this study, we also showed that pristimerin in combination with taxol-induced JNK activation and subsequent mitochondrial cell death in a ROS-dependent manner. However, treatment with either pristimerin or taxol alone did not cause significant ROS generation, JNK activation, and cell death, suggesting that the combined treatment-induced ROS-dependent cell death may not be caused by proteosome inhibition activity of pristimerin. This discrepancy may be due to differential sensitivity to pristimerin between cell lines or use of lower dose of pristimerin (0.5 μmol/l) from previous studies [11,13,17]. Thus, it is unclear whether the cell death caused by the combined treatment with taxol and pristimerins directly due to ROS generation or by proteasome inhibition. The exact mechanism by which pristimerin raises intracellular ROS level and subsequent mitochondrial cell death remains to be elucidated.

In summary, we demonstrated in this study that combined treatment with taxol (10 nmol/l) and pristimerin (0.5 μmol/l) synergistically enhances cell death in cervical cancer cell lines. Moreover, we elucidated the mechanisms by which combined treatment with taxol and pristimerin upregulates DR5 by activation of the JNK/c-Jun pathway in a ROS-dependent manner, and subsequently activates Bax, finally leading to mitochondrial cell death. Elucidating the molecular mechanisms by which naturally occurring products modulate conventional chemotherapeutic drug sensitivity would contribute to our understanding of cell death events and the development of cancer treatment technologies.

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Conflicts of interest

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