Pristimerin induces caspase-dependent apoptosis in MDA-MB-231 cells via direct effects on mitochondria

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Abstract

Pristimerin, a naturally occurring triterpenoid, has been shown to cause cytotoxicity in several cancer cell lines. However, the mechanism for the cytotoxic effect of pristimerin was never explored. In the present study, human breast cancer MDA-MB-231 cells treated with pristimerin (1 and 3 μmol/L) showed rapid induction of apoptosis, as indicated by caspase activation, DNA fragmentation, and morphologic changes. Pretreatment of a pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) completely prevented pristimerin-induced apoptosis. Treatment of tumor cells with pristimerin resulted in a rapid release of cytochrome c from mitochondria, which preceded caspase activation and the decrease of mitochondrial membrane potential. In addition, neither benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone nor permeability transition pore inhibitor cyclosporin A markedly prevented pristimerin-induced mitochondrial cytochrome c release. Pristimerin did not significantly alter the protein level of Bcl-2 family members (Bcl-2, Bcl-X_L, and Bax), nor did it induce Bax translocation. Moreover, Bcl-2 overexpression fails to prevent pristimerin-induced apoptosis. The generation of reactive oxygen species in MDA-MB-231 cells was also not affected by pristimerin. In a cell-free system, pristimerin induced cytochrome c release from isolated mitochondria. Taken together, these results suggested that pristimerin is a novel mitochondria-targeted compound and may be further evaluated as a chemotherapeutic agent for human cancer. [Mol Cancer Ther 2005;4(8):1277–85]

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Introduction

Activation of apoptosis pathways is a key mechanism by which anticancer drugs kill tumor cells (1, 2). It is known that anticancer drugs can induce apoptosis signaling through two major pathways. One is the mitochondrial (intrinsic) pathway which is activated by the release of proapoptotic factors from mitochondria, such as cytochrome c (3–5). Released cytochrome c interacts with Apaf-1 and activates caspase-9 that in turn proteolytically activates downstream caspase-3, one of the principle protease participating in the execution phase of apoptosis. Activated caspase-3 cleaves a lot of substrates, including poly(ADP-ribose)polymerase (PARP), a DNA repair enzyme, and leads to inevitable cell death. The other apoptosis pathway is the death receptor (extrinsic) pathway involving Fas and other members of the tumor necrosis factor receptor family that activate caspase-8 (6, 7). Caspase-8 can activate downstream events either by directly activating caspase-3 or by cleaving Bid, which then in turn trigger the mitochondrial pathway.

Many compounds that are used in cancer chemotherapy are derived from plant sources, such as *Vinca* alkaloids, paclitaxel, camptothecin, and etoposide. In the course of our continuing search for cytotoxic plant components, 21 compounds have been isolated from *Reissantia buchananii* (Celastraceae), and have been tested for their cytotoxicity. Among them, pristimerin showed the most potent activity against nine tumor cancer cell lines, including vincristine-resistant oropharyngeal cancer KB cells and taxol-resistant ovarian cancer PTX10 cells (8). Pristimerin is a quinonemethide triterpenoid compound which has been found in various species belonging to Celastraceae and Hippocrateaceae. Some of the plants, such as *Maytenus chuchuhuasca* and *Maytenus laevis*, have been used traditionally in the treatment of arthritis and skin cancer in South America (9, 10). It is known that pristimerin exhibits antimicrobial, antiinflammatory, antiperoxidation, and antitumor effects (8, 10–12). Pristimerin has been reported to be effective in preventing inflammatory responses in several animal models (13). In addition, pristimerin inhibited the induction of inducible nitric oxide synthase in macrophages by suppressing nuclear factor κB activation, an effect which may be responsible for its antiinflammatory activity (14). However, the mechanism for the antitumor activity of pristimerin was never explored. In the present study, we show that pristimerin induces caspase-dependent apoptosis in the human breast cancer cell line MDA-MB-231. Our results also suggest that the mechanism of action of pristimerin includes direct induction of the release of cytochrome c from mitochondria and activation of the caspase cascade.

Materials and Methods

Materials

RPMI 1640, fetal bovine serum, and streptomycin/penicillin G were obtained from Life Technologies (Gaithersburg, MD). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, propidium iodide, Hoechst 33342, cyclosporin A, and N-acetylcysteine were obtained from Sigma Chemical Co. (St. Louis, MO). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) was from Calbiochem (San Diego, CA). 2',7'-Dichlorodihydrofluorescein diacetate and rhodamine 123 were obtained from Molecular Probes (Eugene, OR). Anti-caspase-3 monoclonal antibody (mAb), anti-PARP mAb, anti-cytochrome c mAb, anti-Bcl-2 mAb, anti-Bcl-X_L mAb, and anti-Bax mAb were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). All other chemicals were obtained from Sigma Chemical.

Pristimerin (Fig. 1A) was isolated from *R. buchananii* as described previously (8). The DMSO stock solution was kept at −20°C and freshly diluted to the desired concentration with cultured medium immediately before use (the final concentration of DMSO in culture medium was 0.2%).

Cell Culture

The human breast cancer cell lines (MCF-7 and MDA-MB-231), the human liver cancer cell lines (HepG2 and Hep3B), the human lung cancer cell line (A549), and the human mammary epithelial cell line (MCF-10A) were from American Type Culture Collection (Rockville, MD). All cell lines, except MCF-10A cells, were propagated in RPMI 1640 supplement with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. MCF-10A cells were cultured in a 1:1 mixture of DMEM/Ham's F-12 medium containing 10 μg/mL bovine insulin, 20 ng/mL epidermal growth factor, 100 ng/mL cholera enterotoxin, 0.5 μg/mL hydrocortisone, and 10% fetal bovine serum.

In the Bcl-2 overexpression study, transient transfections were carried out using LipofectAMINE 2000 reagent (Life Technologies). MDA-MB-231 cells were cultured to 80% confluence in 6 × 35 mm plates. Cells were transfected either with 2 μg of pUSE-empty vector or same vector containing a cDNA encoding human Bcl-2 (generously provided by Professor C.M. Teng) with 5 μL LipofectAMINE 2000 in serum-free Opti-MEM (Life Technologies) at 37°C for 4 hours. Then the cells were cultured in regular medium for 24 hours before appropriate treatment.

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 5,000 to 10,000 cells/well in 96-well tissue culture plates. On day 2, cells were treated with pristimerin for various time periods. After drug 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 IC_{50} is the concentration of agent that reduced the cell viability by 50% under the experimental conditions.

Detection of Apoptosis by Flow Cytometry

Apoptotic cells were detected by flow cytometry using propidium iodide staining. Briefly, after incubation with
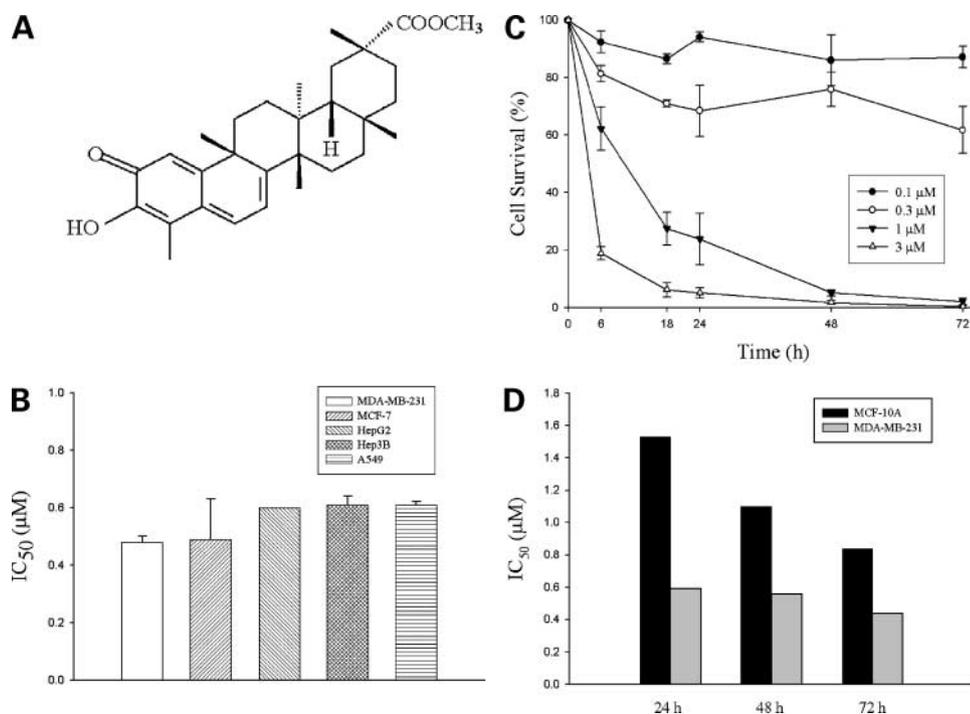
Figure 1. **A,** the chemical structure of pristimerin. **B,** effect of pristimerin on the viability of various cancer cell lines. Cells were treated with pristimerin for 72 h and cell viability was determined by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The IC_{50} is the concentration of agent that reduced the cell numbers by 50% under the experimental conditions ($n = 4$). **C,** concentration- and time-dependent inhibition of cell viability of MDA-MB-231 cells by pristimerin ($n = 4$). **D,** effect of pristimerin on viability of MCF-10A cells and MDA-MB-231 cells. Results represent the means from two independent experiments.

pristimerin, floating cells in the spent medium and adherent cells were collected by combining the spent medium and trypsin-treated samples, and cells were harvested by centrifugation (3,000 × g for 5 minutes), washed with PBS and fixed in ice-cold 75% ethanol at −20°C overnight. Cells were washed and resuspended in PBS containing 25 μg/mL RNase and 0.5% Triton X-100. Samples were then incubated with 50 μg/mL propidium iodide at 37°C for 30 minutes and analyzed in a flow cytometer (Beckman Coulter, Miami, FL).

Nuclear Staining with Hoechst 33342

After treatment, cells were harvested and washed with ice-cold PBS, fixed in 4% paraformaldehyde at 4°C for 30 minutes. The cells were then incubated in nuclear fluorochrome Hoechst 33342 at a final concentration of 5 μg/mL at room temperature for 30 minutes. Nuclear morphology was then examined with a Zeiss fluorescent microscope.

Western Blot Assay

Cells were lysed with lysis buffer containing 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 5,000 units/mL aprotinin, 20 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium orthovanadate, 50 mmol/L NaF, 5% glycerol, 10 mmol/L Tris-HCl (pH 7.4), and 2% SDS. Protein concentrations were determined using a protein assay kit (Bio-Rad, Richmond, CA). Equal amounts of protein were separated by SDS-PAGE (7.5% for PARP, 12% for caspase-3, and Bcl-2 family proteins, 15% for cytochrome c), and electroblotted onto nitrocellulose membrane, which was then blocked with 5% nonfat milk in 0.1% Tween 20-PBS overnight at 4°C. The membrane was immunoblotted with primary antibodies against caspase-3, PARP, Bcl-X$_L$, Bcl-2, Bax, and cytochrome c. After washing with Tween 20-PBS, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. After washing with Tween 20-PBS, protein bands on the membrane were visualized by an enhanced chemiluminescence Western blotting detection system (Western Lightning, Perkin-Elmer, Norwalk, CT).

Translocation of Bax to Mitochondria

Cells treated with pristimerin were harvested, resuspended in buffer A [250 mmol/L sucrose, 20 mmol/L HEPES-KOH, 10 mmol/L KCl, 1.5 mmol/L Na-EGTA, 1.5 mmol/L Na-EDTA, 1 mmol/L MgCl$_2$, 1 mmol/L DTT, and cocktail of protease inhibitors (pH 7.4)], and incubated on ice for 30 minutes. After the cells were disrupted by 50 strokes with a glass Dounce homogenizer/B-type pestle, homogenates were centrifuged at 800 × g for 10 minutes at 4°C. Supernatants were further centrifuged at 15,000 × g for 30 minutes at 4°C, and the subsequent supernatants were stored as cytosolic fractions at −20°C. The pellet was washed once with buffer A and finally lysed with buffer B [50 mmol/L HEPES, 1% Nonidet P-40, 10% glycerol, 1 mmol/L EDTA, 2 mmol/L DTT, cocktail of protease inhibitors (pH 7.4)]. The lysates were then centrifuged at 15,000 × g for 30 minutes at 4°C, and the supernatants were stored as mitochondrial fractions at −20°C. The protein

concentrations of cytosolic or mitochondrial fractions were determined using a protein assay kit (Bio-Rad). Aliquot of cytosolic or mitochondrial fractions were used for Western blot assay of Bax.

Cytochrome *c* Release from Mitochondria in Drug-Treated Cells or from Isolated Mitochondria

After treatment of pristimerin for indicated periods, MDA-MB-231 cells were harvested, resuspended in buffer A and disrupted with a glass Dounce homogenizer/B-type pestle. The resultant homogenate was centrifuged at 15,000 × g for 30 minutes at 4°C, and the subsequent supernatants were used for Western blot assay of cytochrome c. For detection of cytochrome c release from isolated mitochondria, cell homogenates obtained from untreated MDA-MB-231 cells were centrifuged at 800 × g for 10 minutes at 4°C. Supernatants were further centrifuged at 15,000 × g for 15 minutes at 4°C. The resultant mitochondrial pellet was washed once with buffer A and finally resuspended in assay buffer [250 mmol/L sucrose, 2 mmol/L KH$_2$PO$_4$, 5 mmol/L sodium succinate, 2 mmol/L EGTA, 10 mmol/L HEPES-KOH (pH 7.5)]. The suspension of isolated mitochondria was incubated with pristimerin at 37°C for various periods and then centrifuged at 15,000 × g for 15 minutes at 4°C. The cytochrome c content of the supernatant was measured by Western blot assay.

Measurement of Mitochondrial Membrane Potential

Changes of mitochondrial membrane potential ($\Delta\Psi_m$) were monitored by determination of the rhodamine 123 fluorescence. Rhodamine 123 is a cationic lipophilic fluorochrome whose distribution to the mitochondria matrix correlates with the $\Delta\Psi_m$ (15). After treatment, cells were loaded with 10 μmol/L rhodamine 123 and incubated at 37°C for 30 minutes in the dark. Cells were then harvested, washed, and resuspended in PBS and analyzed immediately using flow cytometry with the excitation and emission wavelengths of 488 and 525 nm, respectively.

Measurement of Generation of Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) accumulation was monitored using 2′,7′-dichlorodihydrofluorescein diacetate, which is converted to highly fluorescent dichlorofluorescein in the presence of intracellular ROS (16). At the end of the treatments, cells were loaded with 20 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate and incubated at 37°C for 30 minutes in the dark. Cells were then harvested, washed and resuspended in PBS and analyzed immediately using flow cytometry with the excitation and emission wavelengths of 490 and 530 nm, respectively.

Results

Effects of Pristimerin on the Viability of Various Tumor Cell Lines

As shown in Fig. 1B, pristimerin inhibited the cell viability of A549, MCF-7, MDA-MB-231, HepG2, and Hep3B cells, with IC$_{50}$ values ranging from 0.42 to 0.61 μmol/L after a 72-hour treatment. These results are consistent with those of the previous study in which

pristimerin displayed broad-spectrum inhibition of nine human tumor cell lines (8). The effects of pristimerin on cell viability occurred very rapidly. After a 6-hour treatment, 1 and 3 µmol/L of pristimerin decreased cell viability in MDA-MB-231 cells by 38% and 81%, respectively (Fig. 1C).

To examine the selectivity of pristimerin toward tumor cells, we assessed the effect of pristimerin in a non-tumorigenic human mammary epithelial cell line MCF-10A. As shown in Fig. 1D, MCF-10A cells exhibited two to three times higher resistance to pristimerin than MDA-MB-231 cells, especially at early time points (24 hours). This result suggests that pristimerin may have potential selectivity toward tumor cells.
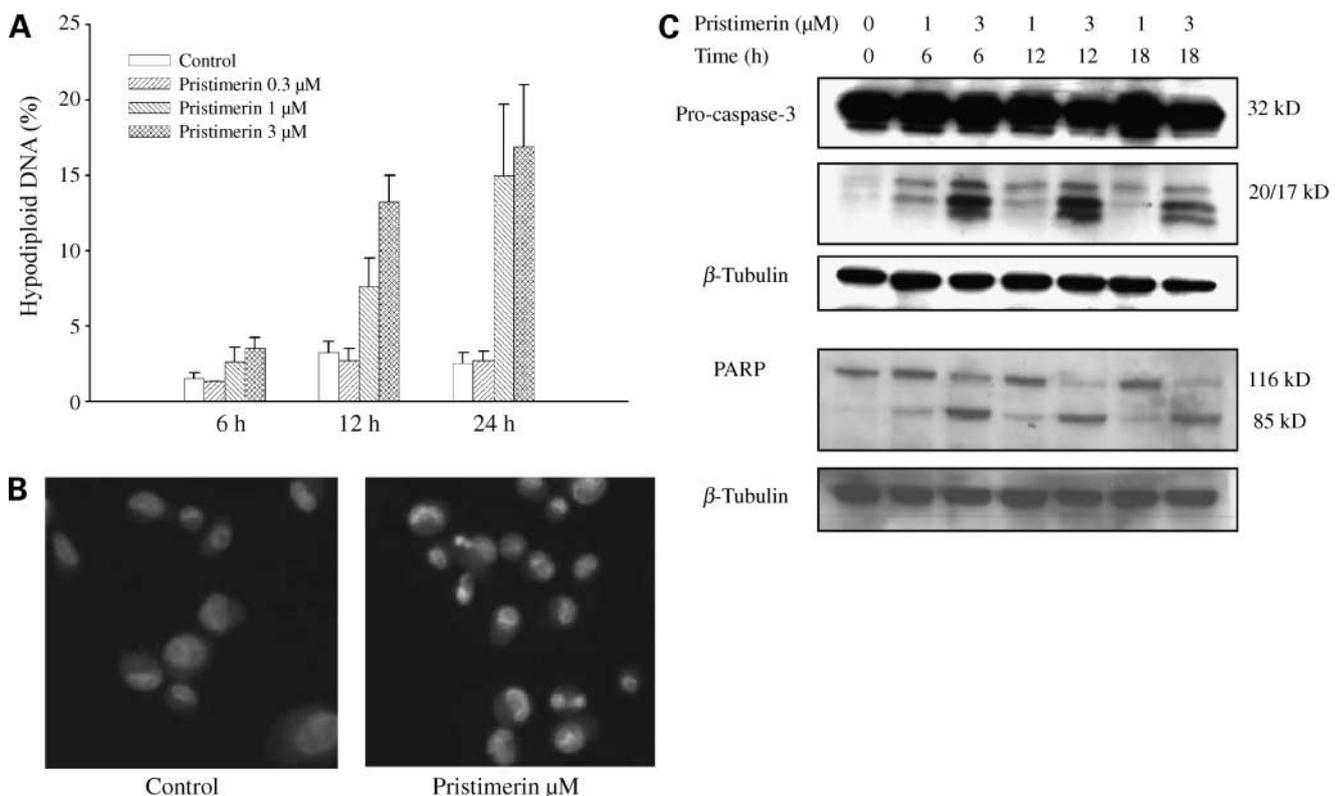
Pristimerin Induces Apoptosis in MDA-MB-231 Cells

We next focused on MDA-MB-231 cells, which are p53-mutant and highly metastatic breast cancer cells, to investigate whether pristimerin decreases cell viability through the induction of apoptosis. As shown in Fig. 2A, pristimerin concentration- and time-dependently induced an increase in the proportion of sub-G_1 population, suggesting that the cells underwent DNA fragmentation which is a biochemical hallmark of apoptosis. Moreover,

pristimerin-treated cells displayed typical morphologic features of apoptotic cells, with shrinkage and apoptotic bodies as well as fragmented nuclei visualized by fluorescence microscopy after DNA staining with Hoechst 33342 (Fig. 2B). Taken together, these results suggest that pristimerin is able to induce apoptosis in MDA-MB-231 cells.

Pristimerin-Induced Apoptosis Is Caspase-Dependent

To investigate whether pristimerin-induced apoptosis is mediated by caspases, the proteolytic activation of caspase-3, which plays a central role in the caspase cascade, was examined. As shown in Fig. 2C, pristimerin (1 and 3 µmol/L) induced cleavage of procaspase-3 to the active form (p20/p17) in a time- and concentration-dependent manner. The cleavage products were detectable as early as 6 hours after cells exposure to pristimerin. The activation of caspase-3 is further confirmed by detecting the degradation of PARP, which is a DNA repair enzyme and undergo cleavage by caspase-3 during apoptosis. In pristimerin-treated cells (3 µmol/L), the cleavage of PARP occurred at 6 hours and was completed at 12 hours. To address the significance of caspase activation in pristimerin-induced apoptosis, we used a broad-range caspase inhibitor,

Figure 2. Induction of apoptosis by pristimerin in MDA-MB-231 cells. **A,** MDA-MB-231 cells were treated with different concentrations of pristimerin for the indicated periods. The harvested cells were fixed and stained with propidium iodide, followed by flow cytometric analysis. The percentages of cells with hypodiploid DNA content (sub-G_1) represent fractions undergoing apoptotic DNA fragmentation. *Columns*, mean; *bars*, ± SE ($n = 3–4$). **B,** to observe the chromatin condensation, cells were treated with DMSO (control) or pristimerin (3 µmol/L) for 12 h. The harvested cells were fixed and stained with Hoechst 33342, and then the nuclear morphology was examined with a fluorescent microscope. **C,** pristimerin induces caspase-3 activation and PARP cleavage. MDA-MB-231 cells were treated with pristimerin (1 or 3 µmol/L) for the indicated periods. Immunoblotting analysis was done to detect the processing of procaspase-3 to the p20/p17 cleavage products and the processing of PARP (116 kDa) to the 85 kDa fragment. Results are representative of three independent experiments.

z-VAD-fmk. As shown in Fig. 3, pretreatment of cells with z-VAD-fmk (50 µmol/L) almost completely prevented pristimerin-induced apoptosis as measured by the appearance of sub-G_1 peak (Fig. 3A) and Hoechst staining (Fig. 3B); in addition, the cleavage of PARP was also totally inhibited (Fig. 3C), indicating that caspase activity had been abolished. These results suggest that activation of caspase cascade is essential for pristimerin-induced apoptosis in MDA-MB-231 cells.

Pristimerin Induces Cytochrome *c* Release in MDA-MB-231 Cells

A key step in intrinsic apoptotic pathway is the release of cytochrome *c* from mitochondria to activate Apaf-1, thus turns on the caspase cascade. When MDA-MB-231 cells were treated with pristimerin, cytochrome *c* was detectable in the cytosolic fractions as early as 0.5 hours after treatment and peaked at 3 hours (Fig. 4A), thereafter, cytochrome *c* levels remained elevated up to 18 hours (data not shown). Pretreatment of cells with either z-VAD-fmk (50 µmol/L) or the permeability transition pore inhibitor cyclosporin A (1 µmol/L) had only little effect on the release of cytochrome *c* caused by pristimerin (Fig. 4B). Furthermore, cyclosporin A did not prevent pristimerin-induced apoptosis in MDA-MB-231 cells (data not shown).

### Effect of Pristimerin on the Mitochondrial Membrane Potential

The release of cytochrome *c* from mitochondria is usually preceded or accompanied with a reduction in the $\Delta\Psi_m$. To address whether pristimerin-induced cytochrome *c* release is associated with the change of $\Delta\Psi_m$, MDA-MB-231 cells were treated with pristimerin and were stained with rhodamine 123 to assess the $\Delta\Psi_m$. Treatment of MDA-MB-231 cells with pristimerin for up to 1 hour did not produce any significant drop in the $\Delta\Psi_m$. A slight decrease of $\Delta\Psi_m$ became detectable only after 3 hours of treatment and a remarkable loss of $\Delta\Psi_m$ was observed after 6 hours of treatment. Neither z-VAD-fmk nor cyclosporin A markedly affected the change of $\Delta\Psi_m$ caused by pristimerin (Fig. 4C).

Effect of Pristimerin on ROS Generation

It has been reported that the production of ROS is involved in the induction of apoptosis by various cytotoxic agents (17). To determine whether oxidative stress was associated with pristimerin-induced apoptosis, we examined the intracellular generation of ROS by using 2′,7′-dichlorodihydrofluorescein diacetate fluorescence. Treatment of MDA-MB-231 cells with pristimerin for up to 6 hours (Fig. 4D) or 12 hours (data not shown) did not produce any significant increases in the generation of ROS. Furthermore, pretreatment of cells with an antioxidant *N*-acetylcysteine did not prevent pristimerin-induced cytochrome *c* release (data not shown). These results indicate that ROS production was not directly associated with pristimerin-induced apoptosis in MDA-MB-231 cells.

Effect of Pristimerin on Antiapoptotic Proteins and Proapoptotic Proteins

Because of the important role of Bcl-2 family proteins in the regulation of apoptosis, we examined the effects of
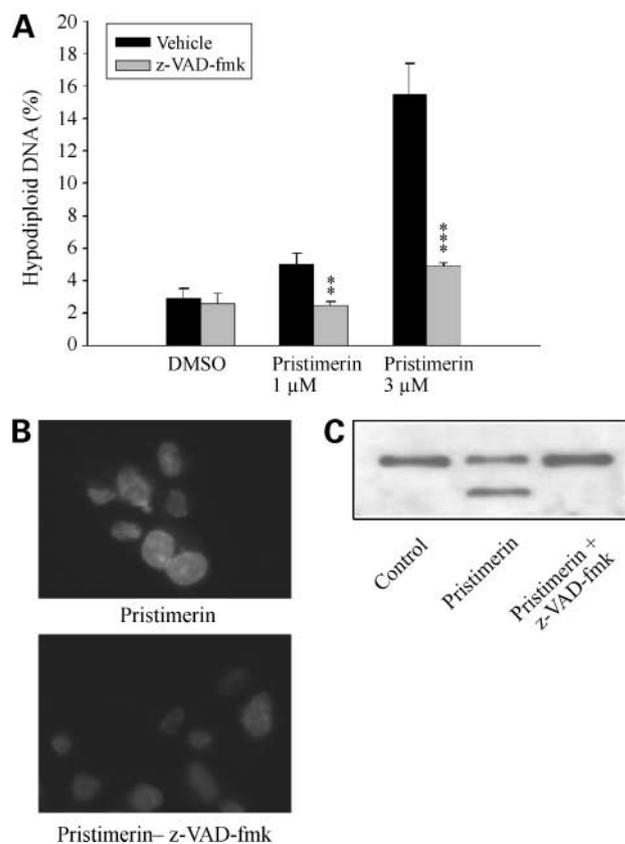

Figure 3. Inhibition of pristimerin-induced apoptosis by the caspase inhibitor z-VAD-fmk. MDA-MB-231 cells pretreated with or without z-VAD-fmk (50 µmol/L, 1 h) were further incubated with DMSO (control) or pristimerin (3 µmol/L) for another 12 h. **A,** apoptosis was assessed by sub-G_1 DNA contents; *columns,* mean; *bars,* $\pm$ SE ($n = 3$); **, $P < 0.01$; ***, $P < 0.001$ as compared with the respective controls. **B,** apoptosis assessed by Hoechst 33342 staining. **C,** cleavage of PARP was examined by immunoblotting analysis.

pristimerin on the expression of antiapoptotic proteins (Bcl-2 and Bcl-X_L) and the proapoptotic proteins (Bax) in MDA-MB-231 cells. As shown in Fig. 5A, pristimerin did not significantly affect the expression levels of Bcl-2 family proteins during apoptosis. We next examined if pristimerin induced translocation of Bax to mitochondria. As shown in Fig. 5B, after treatment of MDA-MB-231 cells with pristimerin for up to 6 hours, we did not observe a significant change of Bax levels in both cytosolic fractions and mitochondrial fractions. This indicates that Bax did not translocate to mitochondria during pristimerin-induced cytochrome *c* release.

To examine the effect of Bcl-2 overexpression on pristimerin-induced apoptosis, MDA-MB-231 cells overexpressing Bcl-2 (Fig. 5C, *top*) were exposed with pristimerin. Figure 5C (*bottom*) shows that Bcl-2 overexpression failed to prevent apoptotic cell death caused by pristimerin.

Pristimerin Induces Cytochrome *c* Release from Isolated Mitochondria

To examine whether pristimerin had a direct effect on mitochondria, we incubated pristimerin with isolated
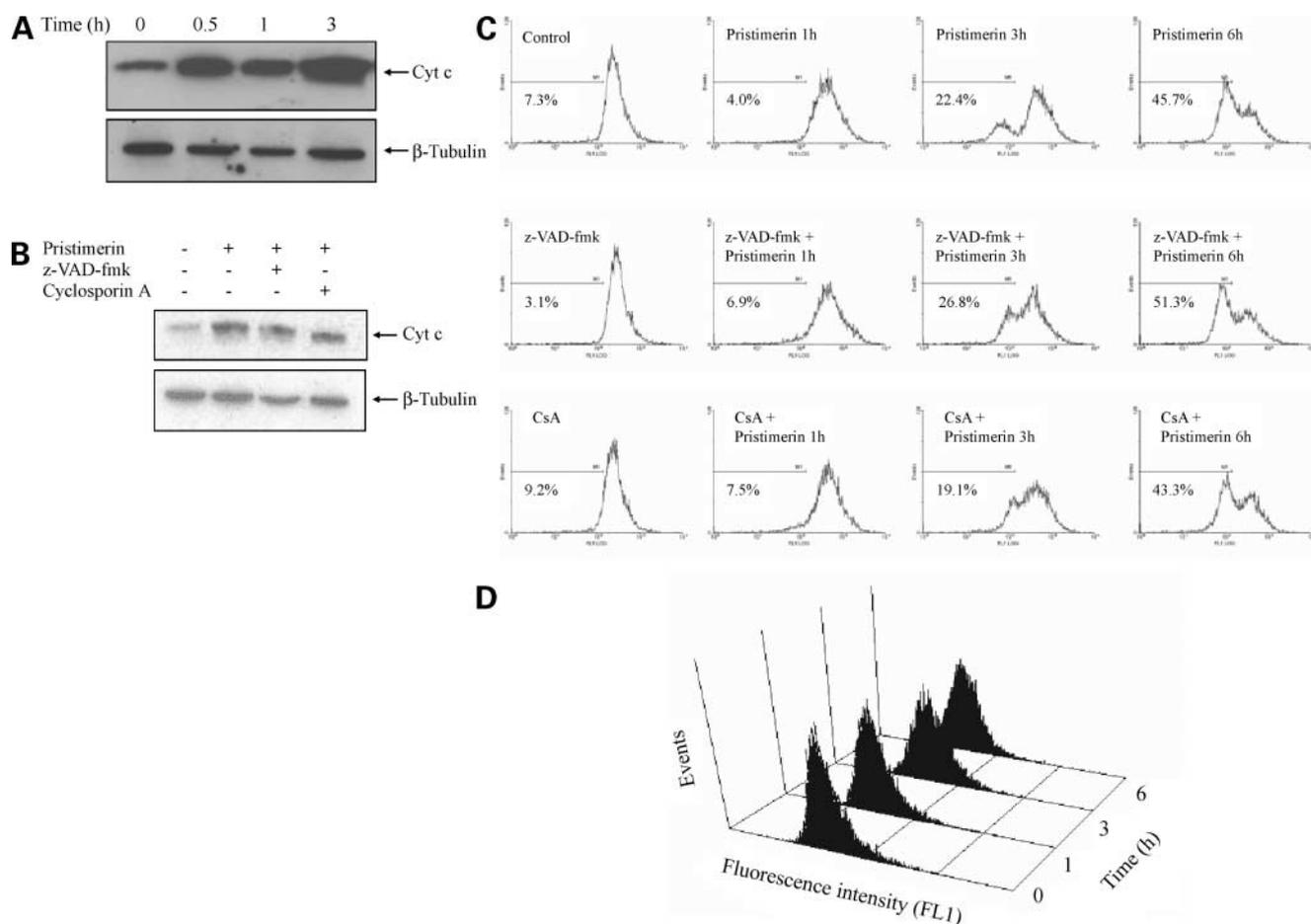
Figure 4. Effects of pristimerin on mitochondrial cytochrome *c* release, mitochondrial membrane potential and ROS formation. **A**, time course of pristimerin-induced cytochrome *c* release in intact cells. MDA-MB-231 cells were treated with pristimerin (3 μmol/L) for the indicated periods and cytochrome *c* in the cytosolic fraction was detected by immunoblotting analysis. **B**, effects of z-VAD-fmk and cyclosporin A on pristimerin-induced cytochrome *c* release. MDA-MB-231 cells were treated with pristimerin (3 μmol/L) for 3 h in the presence of z-VAD-fmk (50 μmol/L) or cyclosporin A (1 μmol/L) and then cytochrome *c* in the cytosolic fraction was detected. **C**, effect of pristimerin on mitochondrial membrane potential. MDA-MB-231 cells were treated with pristimerin (3 μmol/L) for the indicated periods in the absence or presence of z-VAD-fmk (50 μmol/L) or cyclosporin A (1 μmol/L). Cells were then harvested, stained with rhodamine 123 to determine mitochondrial membrane potential and analyzed by flow cytometry. Results are representative of three independent experiments. **D**, effect of pristimerin on the generation of ROS. MDA-MB-231 cells were treated with pristimerin (3 μmol/L) for the indicated periods. Cells were then harvested, stained with 2′,7′-dichlorodihydrofluorescein diacetate to determine ROS generation and analyzed by flow cytometry. Results are representative of two to three independent experiments.

mitochondrial fractions from MDA-MB-231 cells. As shown in Fig. 6A, pristimerin induced cytochrome *c* release in a time-dependent manner, which was consistent with the results obtained in the intact cells. Similar results were also observed in pristimerin-treated mitochondria that were isolated from MCF-7 cells (Fig. 6B). These results suggest that pristimerin can trigger cytochrome *c* release through a direct effect on mitochondria.

Discussion

In the present and previous work, we have shown that pristimerin, a triterpenoid compound from plants, is a potent and broad-spectrum antitumor agent, with activity against a wide range of different human cancers. In contrast, the nontumorigenic human mammary epithelial cell line MCF-10A is less sensitive to pristimerin, although significant cytotoxicity is also detectable after prolonged treatment. These results imply that pristimerin may have therapeutic potential in cancer patients if the lower doses can be achieved clinically. Besides, there is no correlation between cell sensitivity to pristimerin and p53 status, because the IC_{50} values of pristimerin for Hep3B cells and MDA-MB-231 cells, which carry mutant p53 are similar to those for HepG2 cells and MCF-7 cells, which have wild-type p53 (18, 19). We also showed that pristimerin induced rapid apoptosis in MDA-MB-231 breast cancer cells as judged by cell morphology, chromatin condensation, and DNA fragmentation. Pristimerin activated caspase-3, the major effector caspase, and was followed by the cleavage of PARP. Inhibition of caspase activation by z-VAD-fmk prevented pristimerin-induced
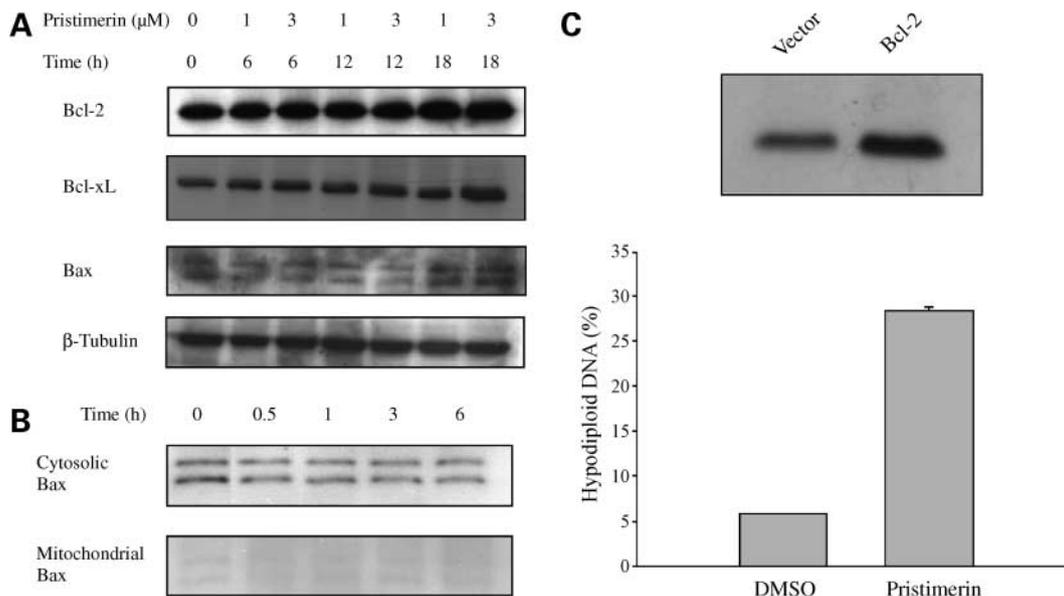
Figure 5. **A**, effect of pristimerin on the levels of Bcl-2 family proteins. MDA-MB-231 cells were treated with pristimerin (1 and 3 μmol/L) for the indicated periods. Bcl-2, Bcl-X$_L$, and Bax in the cell lysates were detected by immunoblotting assay. **B**, effect of pristimerin on Bax translocation to mitochondria. MDA-MB-231 cells were treated with pristimerin (3 μmol/L) for the indicated periods. Bax in cytosolic fractions and mitochondrial fractions were detected by immunoblotting assay. Results are representative of two to three independent experiments. **C**, effect of Bcl-2 overexpression on pristimerin-induced apoptosis. Western blots demonstrating the expression level of Bcl-2 protein in MDA-MB-231 transfected with vector control or Bcl-2 (*top*). MDA-MB-231 cells overexpressing Bcl-2 were treated with DMSO (control) or pristimerin (3 μmol/L) for 12 h (*bottom*). Apoptosis was assessed by sub-G$_1$ DNA contents. *Columns*, mean; *bars*, ± SE (*n* = 2).

apoptosis. Taken together, these results suggest that pristimerin is able to decrease the viability of MDA-MB-231 cells through induction of caspase-dependent apoptosis.

Mitochondria have been shown to play a central role in the apoptotic process, because both the intrinsic pathway and the extrinsic pathway can converge at the mitochondrial level and trigger mitochondrial membrane permeabilization (20, 21). After apoptotic-stimulated mitochondrial membrane permeabilization, cytochrome *c* and other proa-poptotic proteins, such as apoptosis-inducing factor, SMAC/Diablo and EndoG release into the cytosol. Released cytochrome *c* subsequently triggers the activation of cas-pases, substrate cleavage, and cell death. In the present study, MDA-MB-231 cells treated with pristimerin showed rapid cytochrome *c* release from mitochondria, indicating induc-tion of mitochondrial membrane permeabilization. Our data suggest that pristimerin acts by triggering the intrinsic apoptosis pathway rather than the extrinsic pathway. First, pristimerin triggered a rapid release of cytochrome *c* from mitochondria within 0.5 hours, which preceded the activation of caspase-3 and the cleavage of PARP. Second, z-VAD-fmk did not significantly inhibit pristimerin-induced cytochrome *c* release indicating that the mitochondrial effect of pristimerin is not caspase-dependent. Because cytochrome *c* release in death receptor–mediated apoptosis is second-ary to caspase activation (22), these results suggest that the extrinsic apoptotic pathway is probably not involved in pristimerin-induced apoptosis in MDA-MB-231 cells.

Although the precise mechanism that mediates the release of cytochrome *c* from the mitochondria during apoptosis is unclear, a high-conductance channel called the permeability transition pore has been suggested to play an important role, because cytochrome *c* release is usually preceded or accompanied by a drop in $\Delta\Psi_m$ (23, 24). The permeability transition pore is thought to consist of the voltage-dependent anion channel, localized to the outer mitochondrial membrane, the adenine nucleotide
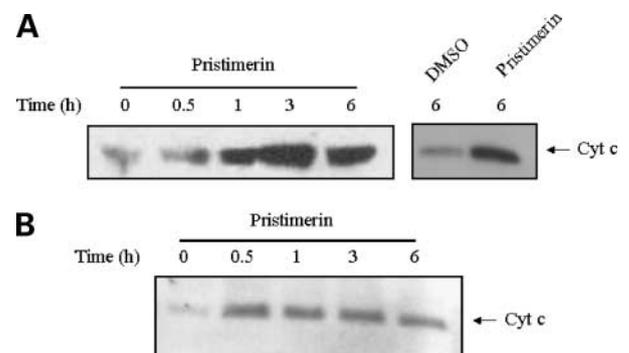
Figure 6. Pristimerin induces cytochrome *c* release from isolated mitochondria. Mitochondrial fractions were isolated from (**A**) MDA-MB-231 cells or (**B**) MCF-7 cells and then incubated with DMSO or pristimerin (3 μmol/L) for the indicated periods. Mitochondria were then pelleted by centrifugation, and cytochrome *c* in the resulting supernatants was detected by immunoblotting analysis. Results are representative of two to three independent experiments.

translocase in the inner mitochondrial membrane, the mitochondrial benzodiazepine receptor, and cyclophilin D. As the permeability transition pore opens, small molecular weight solutes and water enter, leading to a rapid loss in $\Delta\Psi_m$, mitochondrial matrix swelling and rupturing of the outer mitochondrial membrane. Subsequently, cytochrome c and other proapoptotic factors release into the cytosol. However, there are also data indicating that in some cases, the release of cytochrome c during apoptosis can occur before any drop in $\Delta\Psi_m$, suggesting that the permeability transition pore is not involved (25, 26). For example, it has been shown that the proapoptotic proteins Bid and Bax cause cytochrome c release through outer mitochondrial membrane, but preserve $\Delta\Psi_m$ and the integrity of inner mitochondrial membrane (27, 28). In the present study, cytochrome c is first observed in the cytosol after 0.5 hours treatment of MDA-MB-231 cells with pristimerin, which is 2.5 hours prior to any detectable drop in $\Delta\Psi_m$. This finding suggests that the inner mitochondrial membrane is intact even after pristimerin-induced cytochrome c release. Although further treatment with pristimerin for up to 6 hours led to large loss of $\Delta\Psi_m$, this may be a consequence of sustained cytochrome c loss, because cytochrome c depletion can impair the generation of $\Delta\Psi_m$ (29). Moreover, the permeability transition pore inhibitor cyclosporin A only slightly affected pristimerin-induced cytochrome c release and did not prevent pristimerin-induced apoptosis. These results indicate that pristimerin is able to trigger mitochondrial cytochrome c release and subsequent apoptosis through a permeability transition pore opening–independent mechanism.

Bcl-2, Bcl-X_L, and Bax have been implicated as major regulators in the control of mitochondrial cytochrome c release (30, 31). Bcl-2 and Bcl-XL bind to the outer membrane of mitochondria and block cytochrome c efflux. In contrast, upon apoptosis induction, Bax translocates from the cytosol to the mitochondria where it enhances cytochrome c release through the outer membrane of mitochondria. Many anticancer agents or apoptotic stimuli can trigger cytochrome c release through either down-regulation of Bcl-2/Bcl-X_L and/or up-regulation of Bax. However, we show here that pristimerin neither significantly altered the protein level of these three Bcl-2 family members nor induced Bax translocation. Moreover, Bcl-2 overexpression fails to prevent pristimerin-induced apoptosis. Therefore, pristimerin can induce apoptotic cell death irrespective of Bcl-2 family protein levels and does not require translocation of Bax to the mitochondria.

Pristimerin failed to alter Bcl-2 family protein levels and did not enhance the generation of ROS, which is another important factor for induction of mitochondrial membrane permeabilization during apoptosis in MDA-MB-231. Moreover, z-VAD-fmk failed to prevent pristimerin-induced mitochondrial dysfunctions, indicating that these effects are independent on caspases. We therefore asked whether pristimerin has a direct effect on mitochondria. Indeed, in a cell-free system, pristimerin is also able to induce cytochrome c release from isolated mitochondrial fractions. This result clearly indicates that pristimerin can induce mitochondrial membrane permeabilization by a direct action on mitochondria. A number of cytotoxic agents have been reported to have such an effect, including BH3 mimetics (BaxBH3-domain-derived peptides, chelerythrine), adenine nucleotide translocase ligands (MT-21), benzodiazepine receptor ligands (PK11195, Ro5-4864), cationic ampholytes (FTY720), and steroid analogues (betulinic acid, avicins; refs. 32–39). Agents acting directly on mitochondria have been regarded as promising anticancer drugs. Because mitochondrial membrane permeabilization is the ''point of no return'' in the apoptotic pathway, the mitochondria-targeted agents might be able to overcome resistance to apoptosis because of mutations in premitochondrial signal-transducing machinery, such as p53 and PTEN (40, 41). In contrast to pristimerin, most, if not all, agents acting directly on mitochondria trigger mitochondrial membrane permeabilization preceded or accompanied by a drop in $\Delta\Psi_m$, and pretreatment of the permeability transition pore inhibitor cyclosporin A or bongkrekic acid prevents mitochondrial dysfunctions and apoptosis caused by these agents. Therefore, pristimerin seems to exert effects on mitochondria by a novel manner which is distinct from other mitochondria-targeted agents. Although the precise molecular target for pristimerin in mitochondria remains unidentified, the steroid-like core structure of pristimerin may be critical for its mitochondrial effects. Agents possessing a steroid-like core have the potential to insert into biological membranes, leading to perturbations in the lipid composition of these membranes and alterations in the membrane permeability. The diverse structural characteristics of steroid-like agents allow them to act on biological membranes with different affinity. It has been reported that digitonin, a triterpenoid sponin, preferentially partitions into cholesterol-rich outer mitochondrial membranes but not in inner mitochondrial membranes (42, 43), and leads to permeability transition pore–independent cytochrome c release from mitochondria (44). In contrast, the triterpenoids betulinic acid and 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid disrupt the inner mitochondrial membrane, as evidenced by the collapse in $\Delta\Psi_m$ (38, 45). It is unclear whether the absence of a carboxyl group in pristimerin or digitonin makes the difference. Nevertheless, pristimerin probably exerts a digitonin-like effect on mitochondria, and further study of this issue is needed.

In conclusion, pristimerin represents a novel mitochondria-targeted compound which is able to induce caspase-dependent apoptosis in human cancer cells irrespective of p53 status and Bcl-2 levels. Based on these results, we suggest that pristimerin may be a candidate for further evaluation as a chemotherapeutic agent for human cancer.

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