

Anticancer Activity of Pristimerin in Epidermal Growth Factor Receptor 2-Positive SKBR3 Human Breast Cancer Cells

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Pristimerin is a naturally occurring triterpenoid that causes cytotoxicity in several cancer cell lines. However, the mechanism of action for the cytotoxic effect of pristimerin has not been unexplored. The purpose of this study was to investigate the effect of pristimerin on cytotoxicity using the epidermal growth factor receptor 2 (HER2)-positive SKBR3 human breast cancer cell line. Pristimerin inhibited proliferation in dose- and time-dependent manners in cells. We found it to be effective for suppressing HER2 protein and mRNA expression. Fatty acid synthase (FASN) expression and FASN activity were downregulated by pristimerin. Adding of exogenous palmitate, the end product of *de novo* fatty acid synthesis, reduced the proliferation activity of pristimerin. The changes in HER2 and FASN expression induced by pristimerin altered the levels of Akt and mitogen-activated protein kinase (MAPK) phosphorylation (Erk1/2, p38, and c-Jun N-terminal kinase (JNK)). Pristimerin lowered the levels of phosphorylated mammalian target of rapamycin (mTOR) and its downstream targets such as phosphoprotein 70 ribosomal protein S6 kinase and 4E binding protein1. Pristimerin inhibited migration and invasion of cells, and co-treatment with the mTOR inhibitor rapamycin additionally suppressed these activities. Pristimerin-induced apoptosis was evaluated using Western blotting for caspase-3, -8, -9, and poly (ADP-ribose) polymerase expression and flow cytometric analysis for propidium iodide labeling. These results suggest that pristimerin is a novel HER2-downregulated compound that is able to decrease fatty acid synthase and modulate the Akt, MAPK, and mTOR signaling pathways to influence metastasis and apoptosis. Pristimerin may be further evaluated as a chemotherapeutic agent for HER2-positive breast cancers.

Key words epidermal growth factor receptor 2; breast cancer; pristimerin

Natural compounds have become more important in anti-cancer drug development as they are more tolerable to the human body.¹⁾ Pristimerin, a naturally occurring quinonemethide triterpenoid compound, is a traditional medicine derived from the Celastraceae and Hippocrateaceae families and has long been used as an anti-inflammatory, antioxidant, antimalarial, and insecticidal agent.^{2,3)} Some of the plants containing pristimerin, such as *Maytenus chuchuhuasca* and *Maytenus laevis*, have been used traditionally to treat arthritis and skin cancer in South America.⁴⁾ Pristimerin has promising clinical potential as both a therapeutic and chemopreventive agent for various cancers.⁵⁾ For example, pristimerin induces apoptotic cell death in MDA-MB-231 breast cancer cells in a caspase-dependent manner, as well as in human acute myeloid leukemia.^{6,7)} Pristimerin has several mechanisms of inducing cell death, including potent proteasomal inhibition,⁸⁾ suppression of nuclear factor- κ B (NF- κ B) activity and cyclin D1 expression,⁹⁾ and reactive oxygen species-dependent c-Jun N-terminal kinase (JNK) and poly (ADP-ribose) polymerase-1 (PARP-1) activation.¹⁰⁾ However, the molecular target of apoptosis induction by pristimerin remains largely unknown.

One of the most common molecular alterations associated with the malignant phenotype of breast cancer is overexpression of epidermal growth factor receptor-2 (also known as HER2).¹¹⁾ The HER2 receptor (185 kDa) is the protein product of the *HER2* proto-oncogene and a member of the epidermal

growth factor receptor family of transmembrane tyrosine kinases. HER2 overexpression can transform normal mammary epidermal cells and is amplified in 25–30% of breast cancers; it has also been associated with an aggressive form of the disease characterized by significantly shortened survival time.¹²⁾ Aberrant up-regulation of HER2 is found in many cancer types and promotes cell survival, tumor growth, metastasis, and angiogenesis.^{13,14)} HER2 dimerizes with members of the epidermal growth factor receptor (EGFR) family (EGFR, HER3, and HER4) to induce downstream signaling cascades, including phosphoinositol 3-kinase (PI3K)/Akt and Ras/mitogen-activated protein kinase (MAPK), which control cell growth, motility, cell invasiveness, and differentiation.^{15,16)} The humanized monoclonal antibody trastuzumab (Herceptin), which binds the HER2 extracellular region, is approved for treatment of HER2-positive breast cancer.¹⁷⁾ Nevertheless, only a fraction of patients with HER2-positive disease respond to trastuzumab in metastatic settings,¹⁸⁾ implying that many advanced cancers are trastuzumab-resistant. The antitumor mechanism of trastuzumab action remains unclear.

The mammalian target of rapamycin (mTOR) signaling is already active in HER2-positive breast cancers.¹⁹⁾ mTOR is a serine/threonine kinase and a link between oncogenic PI3K/Akt signaling and critical downstream pathways that drive cancer cell growth and survival.²⁰⁾ mTOR kinase exists in two complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTOR interacts with regulatory associated protein of mTOR (raptor), PRAS40, and mLST8 to form mTORC1, the sensitive target of rapamycin that phosphorylates

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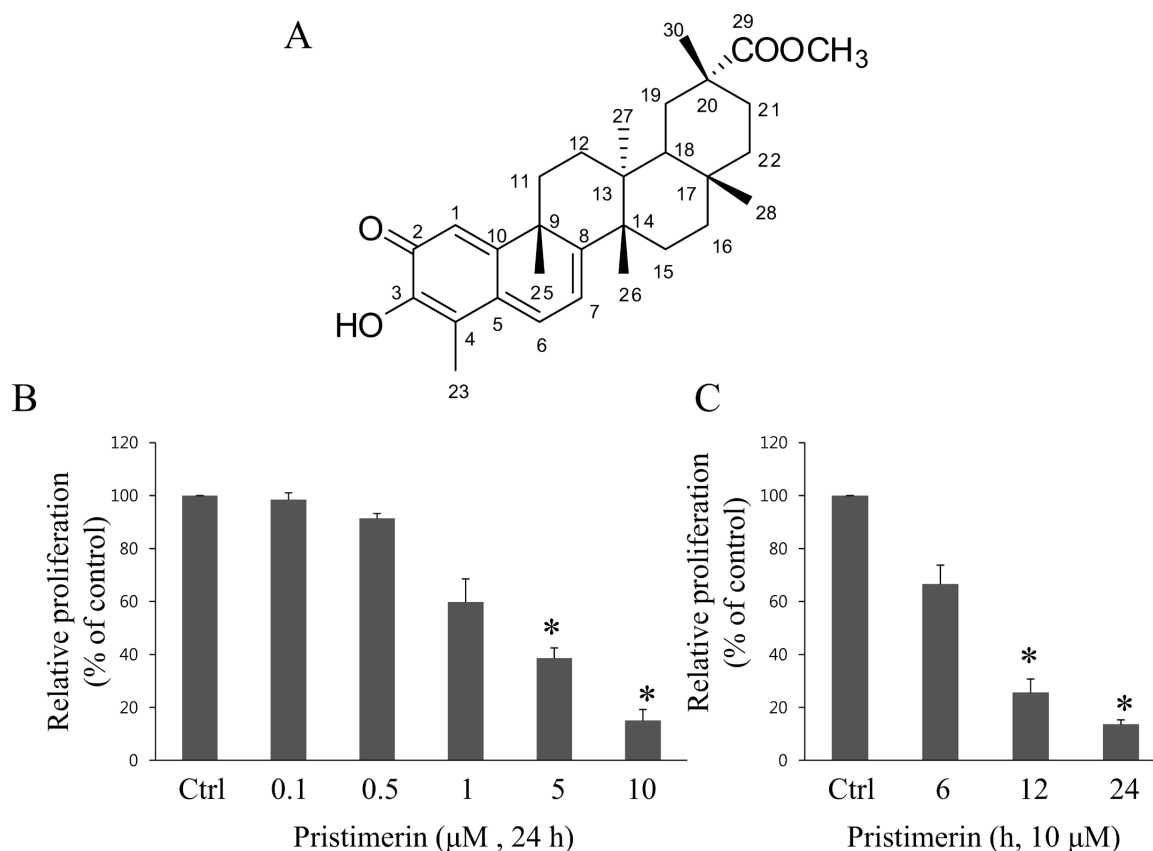


Fig. 1. Effect of Pristimerin on Cell Proliferation of SKBR3 Human Breast Cancer Cells Expressing High Levels of Epidermal Growth Factor Receptor 2 (HER2)

The structure of pristimerin (A). Cells were treated with various pristimerin concentrations (B) and for times (C) of up to 24 h. Cytotoxic effects were determined by CCK-8 assay. The number of viable cells after treatment with pristimerin is expressed as a percentage of the vehicle-only (0.1% DMSO) control. Values indicate the mean \pm standard deviation (S.D.) in triplicate tests. * $p < 0.01$ vs. the vehicle-treated control group.

downstream targets of phosphoprotein 70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1). It also controls cap-dependent protein translation, or rapamycin-insensitive companion of mTOR (rictor), mSIN1, PRR5/Protor, and mLST8 to form mTORC2, which is sensitive to acute rapamycin treatment and phosphorylates Akt at Ser473.²¹⁾ mTOR phosphorylates downstream mediators leading to the regulation of cell cycle progression, cell growth, and angiogenesis. mTOR signaling increases in most common human cancers, and activation of mTOR-dependent protein translation correlates with malignant progression, adverse prognosis, and resistance to both chemotherapy and targeted therapy such as trastuzumab.²²⁾ Clinical trials using rapamycin analogues, such as temsirolimus and everolimus, which are registered for treating advanced cancer, have validated the importance of mTOR inhibition as an anti-cancer treatment strategy.²³⁾ These mechanisms indicate that mTOR inhibiting agent in HER2-positive cancers could have a potent anticancer effect.

In this study, we investigated the effect of pristimerin on cytotoxicity using a HER2-positive SKBR3 human breast cancer cell line.

MATERIALS AND METHODS

Materials McCoy's 5A and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from Thermo Scientific

HyClone (Logan, UT, U.S.A.). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, U.S.A.). Antibodies to P-HER2, HER2, fatty acid synthase (FASN), P-Erk1/2, P-p38, p38, P-JNK, JNK, P-mTOR, mTOR, P-p70S6K, p70S6K, P-4EBP1, 4EBP1, caspase-3, caspase-8, caspase-9, PARP were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.), and Erk1/2, β -actin from Santa Cruz (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Transduction Lab (Lexington, KY, U.S.A.). Super Signal[®] West Pico Chemiluminescent substrate was purchased from PIERCE (Rockford, IL, U.S.A.). Cell Counting Kit-8 was from Dojindo Laboratories (Kumamoto, Japan). LY294002, PD98059, SB20356, SP600125, z-VAD-fmk, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Plant Material The roots of the plant *Salacia cochinchinensis* were collected in Nghe An Province, Vietnam in March 2009, and authenticated by Bs Ngo Van Trai, Department of Medicinal Plant Resource of the Vietnam National Institute of Medicinal, Hanoi, Vietnam. A voucher specimen was deposited at the Herbarium of the Department of Medicinal Plant Resource and the Department of Phytochemistry.

Isolation of Pristimerin Dry roots (4 kg) of *S. cochinchinensis* were powdered and extracted with methanol at room temperature for 48 h and the solvent was evaporated under reduced pressure to get a dry extract (methanol extract, 79 g).

This extract was suspended in water and partitioned with *n*-hexane and the organic solvent was concentrated to yield a hexane fraction (24g). A part of this fraction (10g) was chromatographed over a silica gel column and eluted with *n*-hexane–ethyl acetate (10:0, 9:1, 8:2→1:9, 0:10). Pristimerin (2g) was crystallized out from the selected fraction (eluted with *n*-hexane–ethyl acetate, 8:2, 7:3) with eluted solvent and then in *n*-hexane–dichloromethane (2.5:7.5). The purity of pristimerin was determined as 95% by high-performance liquid chromatography. The structure of pristimerin is illustrated in Fig. 1A.

Cell Culture Human breast carcinoma SKBR3 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.). Cells were grown in McCoy's 5A media supplemented with 10% (v/v) FBS, penicillin (100 U/mL)/streptomycin (100 µg/mL) at 37°C in a humidified CO₂ (5%)–controlled incubator.

Western Blotting Analysis Cells were incubated with pristimerin for 24h, and washed twice in cold phosphate buffered saline (PBS). Cells were lysed with lysis buffer (10mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 1% TritonX-100, 0.5% NP-40, 1mM propidium iodide (PI), 1mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF)) and placed on ice for 1h with occasional vortexing. Centrifugation followed at 10000rpm for 10min and each cell lysates (50 µg) were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 5% skim milk in PBS containing 0.05% Tween-20 for 1h at 25°C, then incubated with primary antibodies, followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG and visualized with enhanced chemiluminescence.

Cell Proliferation Assay Cells were seeded at 5×10³ cells/mL in 96-well microplates and allowed to attach for 24h. Pristimerin was added to the medium at various concentrations up to 50 µM and different duration. After treatment, cell cytotoxicity and/or proliferation was assessed by Cell Counting Kit-8 (CCK-8). Briefly, highly water-soluble tetrazolium salt, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt], produced an orange colored water-soluble product, formazan. The amount of formazan dye generated by dehydrogenases in cells was directly proportional to the number of living cells. CCK-8 (10 µL) was added to each well and incubated for 3h at 37°C, then cell proliferation and cytotoxicity were assessed by measuring the absorbance at 450nm using microplate reader. Three replicated wells were used for each experimental condition.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) After vehicle and drug treatment, cells were subjected to total RNA isolation by using RNAeasy mini kit. RNA concentrations were determined by measuring absorption at 260nm in a spectrophotometer. Aliquots of 1 µg of total RNA from each sample were reverse transcribed to cDNA using an AccuPower CycleScript RT PreMix (dT20) according to manufacturer's instructions from Bioneer Inc. (Daejeon, Korea). PCR primers used in this study included; HER2: 5'-AGC CGC GAG CAC CCA AGT and 5'-TTG GTG GGC AGG TAG GTG AGT T; β -actin: 5'-AAA CTG GAA CGG TGA AGG TG-3' and 5'-CTC AAG TTG

GGG GAC AAA AA-3'. HER2 and β -actin cDNAs were amplified with 25 cycles to ensure that the PCR reactions were performed in the linear range using the following PCR profile: 98°C for 30s, 57°C for 1min, and 72°C for 1min. The PCR products were separated on a 2% agarose gel and detected by ethidium bromide staining.

Fatty Acid Synthesis Activity The preparation, storage and use of fatty acid synthase from chicken liver were performed as described previously.²⁴⁾ Ammonium sulfate precipitations, gel filtration and anion exchange chromatography were used for purification. FASN has >95% purity as estimated from SDS-PAGE with Coomassie blue staining. FASN was incubated with reaction buffer (200mM K-P buffer, pH 7.0, 2.5mM DTT, 2.0mM EDTA and 1mg/mL bovine serum albumin (BSA)) at 37°C for 1h, and added 0.25mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.4mM malonyl-CoA, 0.06mM acetyl-CoA, and 0.02 µCi [³H]acetyl-CoA, then the reaction was terminated by 60% HClO₄. Fatty acids were extracted with 1mL of hexane and incorporation of radioactivity into the fatty acids was assessed by scintillation counting.

Flow Cytometric Analysis Cells with 10⁵ cells/mL were suspended in 300 µL PBS and 700 µL ethanol was added. Cells were incubated at 4°C for 1h and washed with PBS, and suspended in 250 µL of 1.12% sodium citrate buffer (pH 8.4) together with 0.125 units of RNase. Incubation was continued at 37°C for 30min. The cellular DNA was stained by applying 250 µL of PI (50 µg/mL) for 30min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on the BD FACSCanto™II flow cytometer using FACSCanto™II System Software for the percentage of apoptotic cells.

Wound Migration Assay Cells were seeded in 6-well plates and incubated for 18h in serum free McCoy's 5A. The cellular monolayer was wounded with a sterile 10 µL-pipette tip and washed with serum free McCoy's 5A to remove detached cells from the plates. The cells were incubated in the presence or absence of pristimerin for 48h in McCoy's 5A containing 10% FBS. The medium was replaced with PBS, and the cells were photographed using a phase-contrast microscope.

Transwell Invasion Assay Cell invasion assay was carried out with BD Biocoat™ Transwell Invasion Chambers with inserts. Each insert is equipped with 6.4mm in diameter, 8 µm pore size PET porous membrane which has been coated with Matrigel Matrix (BD Bioscience, Bedford, U.S.A.). Cells (2.5×10⁴) suspended in 300 µL of serum free McCoy's 5A with or without pristimerin were added to the upper chamber; 500 µL of McCoy's 5A containing 10% FBS were added to the lower chamber of 24-well plates. After incubation for 24h, non-invading cells were removed from the upper surface of the membrane by scrubbing, and invading cells on the lower surface of the membrane were stained hematoxylin. Membranes were then removed and invading cells were counted randomly by light microscopy. Each assay was performed in triplicate and repeated at least 3 times. Due to variation in the number of migrated cells from different experiment, the results are normalized to control cells and the relative invasion is expressed as mean±S.D. of migrating cells relative to control cells.

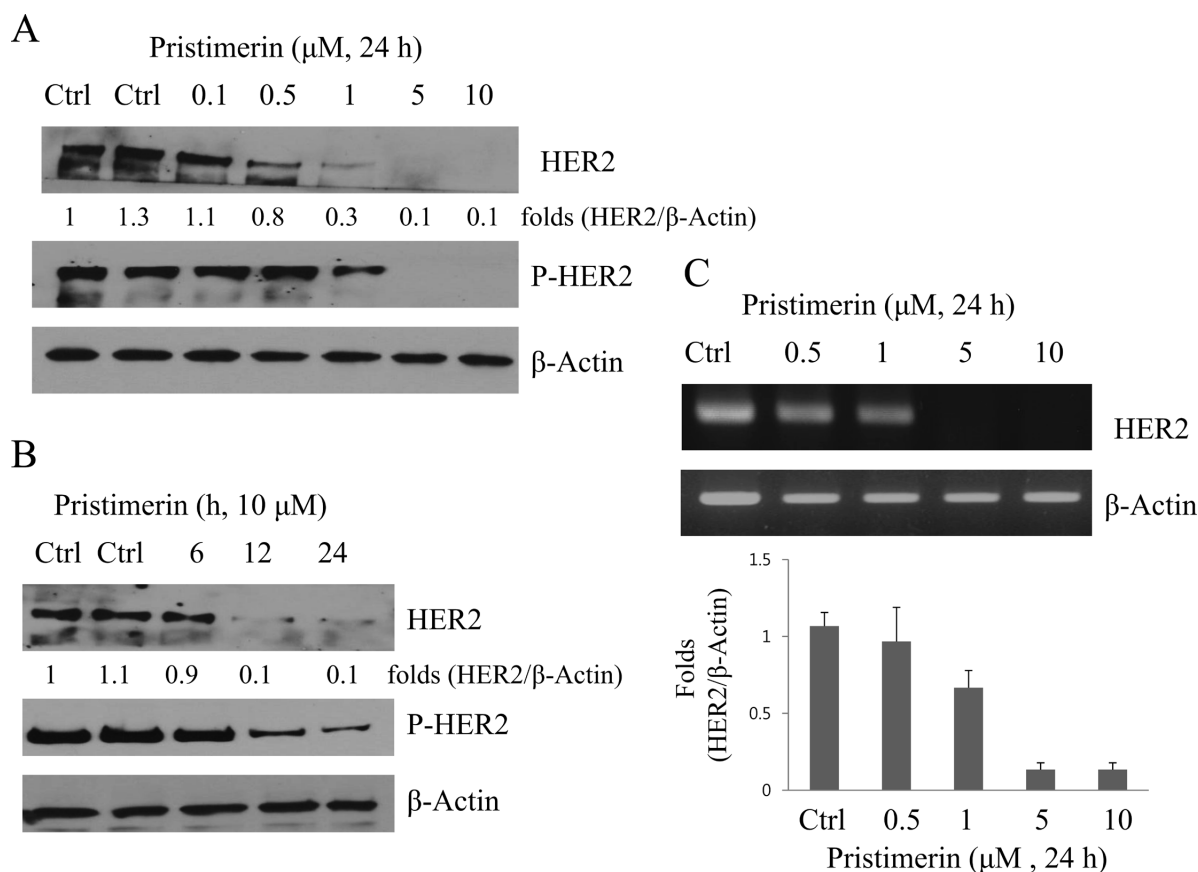


Fig. 2. Pristimerin Decreases Epidermal Growth Factor Receptor 2 (HER2) Expression in SKBR3 Human Breast Cancer Cells

Cells were treated with various concentrations (A) and times (B) of pristimerin and the vehicle control by DMSO for 24 h. Phosphorylated and total HER2 protein levels were analyzed by Western blotting. The values below the figures represent the change in protein expression normalized against β -actin. Three independent experiments were performed. (C) RNA expression by RT-PCR was assessed for the *HER2* gene in SKBR3 cells after treatment with 0.5, 1, 5, 10 μ M pristimerin for 24 h. PCR products were stained with ethidium bromide on an agarose gel (upper panel) and relative intensities are shown (lower panel). Histogram shows the average results of three independent experiments.

RESULTS

Pristimerin Inhibits the Proliferation of SKBR3 Cells

New treatment strategies are needed for patients with advanced HER2-positive breast cancers due to very limited therapeutic options available for fighting this disease. Overexpression of HER2 in SKBR3 human breast cancer cells is due to endogenous gene amplification. We assessed the effect of pristimerin on the proliferation of SKBR3 cells using the CCK-8 assay. Cells were treated with 0, 0.1, 0.5, 1, 5, and 10 μ M of pristimerin for 6, 12, and 24 h. Treatment with pristimerin significantly inhibited the proliferation of cells in dose- and time-dependent manners (Figs. 1B,C).

Pristimerin Downregulates HER2 Expression We found that pristimerin inhibits growth in SKBR3 cells. To further investigate whether the inhibitory proliferation was caused by the regulation of HER2 in HER2-positive cells, both HER2 protein and its phosphorylation levels were determined by Western blotting. Pristimerin dose- and time-dependently downregulated HER2 and HER2 phosphorylation levels in SKBR3 cells (Figs. 2A,B). To determine the mechanism of HER2 downregulation by pristimerin, we analyzed HER2 mRNA levels by RT-PCR. As a result, a decrease in HER2 mRNA levels was observed after pristimerin treatment (Fig. 2C). We conclude that pristimerin decreased HER2 at both mRNA and protein level.

Pristimerin Suppresses FASN Expression HER2 regulates FASN expression through PI3K/Akt signal transduction pathways, revealing a molecular connection between HER2 and FASN.²⁵⁾ To investigate their inhibitory activity on FASN protein level in HER2-positive cells, we treated SKBR3 cells with pristimerin (0.1, 0.5, 1, 5, 10 μ M) for 24 h, and FASN protein levels were analyzed by Western blotting. Similar to HER2 downregulation, pristimerin was effective for suppressing FASN expression (Fig. 3A). We confirmed that pristimerin altered FASN activity in SKBR3 cells, indicating that FASN activity was inhibited by pristimerin (Fig. 3B). To confirm that the anti-proliferation effect induced by pristimerin was related to FASN inhibition, cells were treated with pristimerin (10 μ M) for 24 h in the presence of palmitate (75 μ M). Adding exogenous palmitate reduced the proliferation activity of pristimerin (Fig. 3C). The changes in HER2 and FASN expression induced by pristimerin were confirmed by the levels of Akt and MAPK phosphorylation (Erk, p38, JNK). Pristimerin-induced tyrosine phosphorylation of Akt, Erk1/2, p38, and JNK was inhibited in SKBR3 cells, whereas total expression of Akt, Erk1/2, p38, JNK was unchanged (Fig. 3D). Furthermore FASN and HER2 expression was also synergistically inhibited by the PI3K inhibitor (LY294002), Erk inhibitor (PD98059), JNK inhibitor (SP600125), and p38 inhibitor (SB20356) (Fig. 3E). These data indicate that pristimerin is involved in the suppression of FASN physiological function in HER2-positive

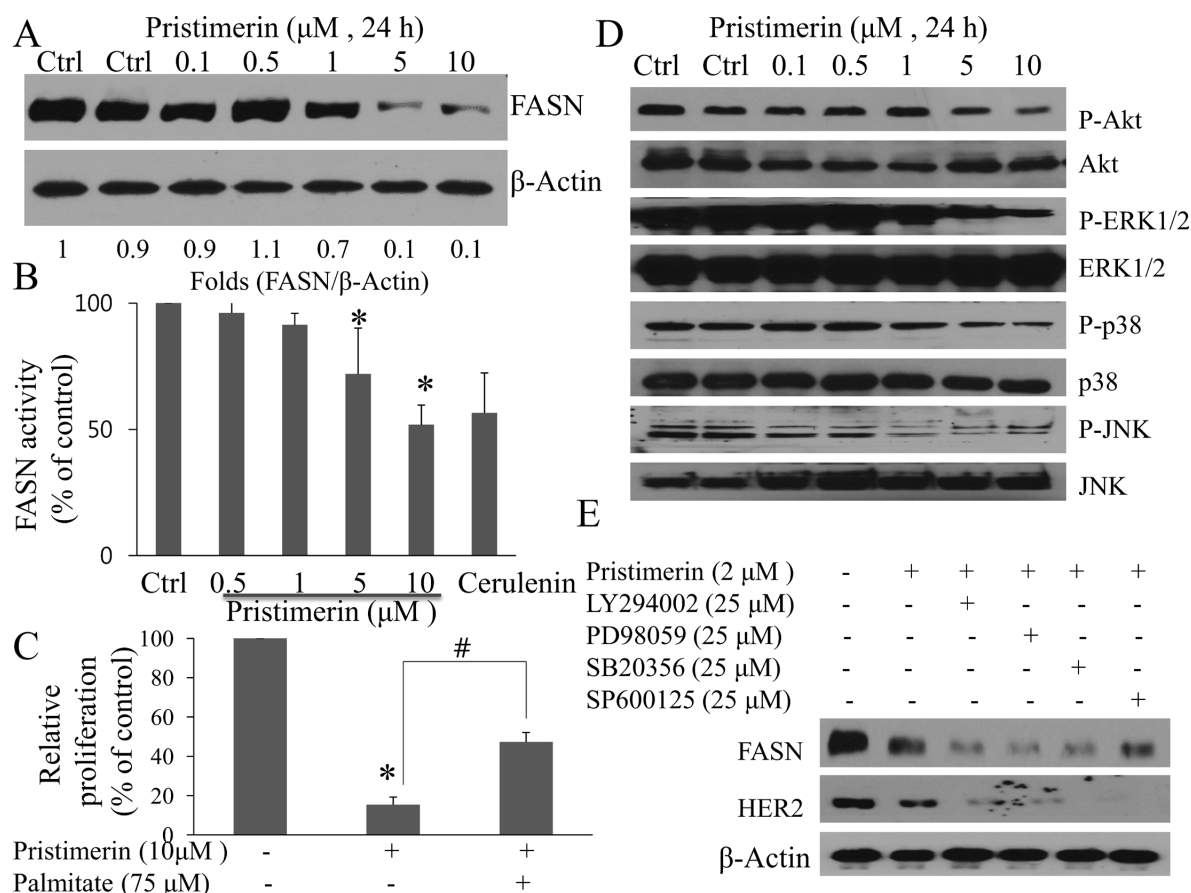


Fig. 3. Pristimerin Suppressed Fatty Acid Synthase (FASN) Expression and Activity and Inhibited the Phosphorylation Level of Akt and Mitogen-Activated Protein Kinases (MAPKs)

(A) Cells were treated with various concentrations (0.1, 0.5, 1, 5, $10\mu\text{M}$) of pristimerin and the DMSO vehicle control for 24h, and FASN protein expression level was analyzed by Western blotting. The values below the figures represent the change in protein expression normalized against β -actin. Three independent experiments were performed. (B) FASN activity was measured in cells. Cells were treated for 24h with pristimerin (0.5, 1, $10\mu\text{M}$) and incubated with [^3H]acetyl-CoA ($2\mu\text{Ci}$) for 4h, then cellular lipids were extracted and quantified relative to the vehicle-treated control. Cerulenin ($50\mu\text{M}$) was used as a positive control. Each data point represents the mean \pm standard deviation (S.D.) of three independent experiments. * $p < 0.05$ vs. vehicle-treated control. (C) Cells were exposed to vehicle or pristimerin ($10\mu\text{M}$) in the presence or absence of palmitate ($75\mu\text{M}$). After 24h, the cell proliferation rate was determined by the CCK-8 assay. Each data point represents the mean \pm S.D. of three independent experiments. * $p < 0.05$ vs. vehicle-treated control. # $p < 0.05$ vs. pristimerin-treated cells (D) Cell lysates from SKBR3 cells were subjected to Western blotting for Erk1/2, p38, JNK, phospho-Erk1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), and β -actin. Three independent experiments were performed. (E) Cells were exposed to PI3K inhibitor (LY294002), Erk inhibitor (PD98059), JNK inhibitor (SP600125), and p38 inhibitor (SB20356). After pristimerin ($2\mu\text{M}$) treatment for 24h, the HER2 and FASN protein expression levels were analyzed by Western blotting. Three independent experiments were performed.

SKBR3 cells, suggesting an interaction between HER2 and FASN.

Pristimerin Downregulates the mTOR/p70S6K/4EBP1 Pathway and Inhibits Metastasis Because of the pristimerin-induced connection between HER2 and FASN in SKBR3 cells, we focused on further signaling pathways regulated by HER2 and FASN downregulation. HER2 overexpression in breast cancer is often associated with aberrant activation of the mTOR pathway.^{14,26} To investigate the molecular changes in SKBR3 cells after pristimerin treatment, we analyzed the expression and phosphorylation of proteins relevant to mTOR signaling. The results showed that treatment with pristimerin dose-dependently lowered the levels of phosphorylated mTOR compared to those in vehicle treated cells (Fig. 4A). Additionally, pristimerin inhibited activity of mTOR downstream targets, such as p70S6K and 4EBP1 (Fig. 4A). Clinical trials using rapamycin analogues, such as temsirolimus and everolimus, registered for treatment of advanced renal cancer, have validated the importance of inhibiting mTOR as an anti-cancer treatment strategy.²³ To further investigate the combined effect of pristimerin and rapamycin in SKBR3 cells, we

performed Western blotting to measure activation of mTOR. Cells were treated for 24h with $10\mu\text{M}$ pristimerin, $2.7\mu\text{M}$ rapamycin, or a combination of both drugs. When both drugs were used together, a considerable reduction was noted in phospho-p70S6K and phospho-4EBP1 compared to the single drugs (Fig. 4B). Taken together, these results suggest that pristimerin inhibited mTOR/p70S6K/4EBP1 pathway in SKBR3 cells. Because HER2 enhances metastasis and angiogenesis by activating the mTOR/p70S6K pathway in human breast cancer cells,¹⁴ we examined the effect of pristimerin on SKBR3 cell migration in a wound migration assay. The migration distance between the leading edge and the wound line was compared between pristimerin-treated and untreated cells. Cell motility was controlled by $5\mu\text{M}$ pristimerin, and rapamycin additionally increased this activity (Fig. 4C). Next, cells were treated with or without pristimerin for 48h to determine whether pristimerin also inhibits cell invasion. Treatment of cells with $5\mu\text{M}$ pristimerin decreased invasive activity. Additionally, the pristimerin-induced decrease in Matrigel invasion decreased more following rapamycin treatment (Fig. 4D). Collectively, these results show that pristimerin-induced mTOR inhibition

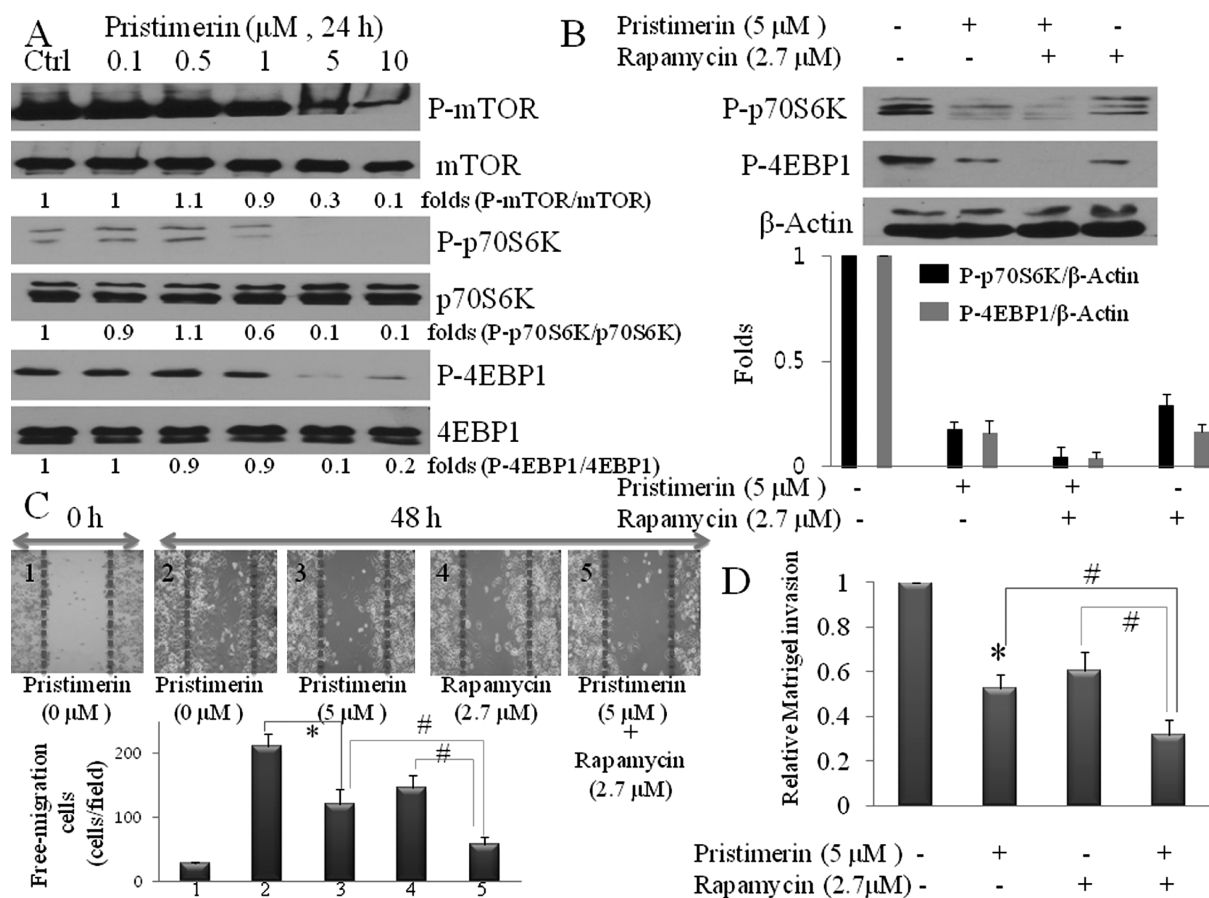


Fig. 4. Pristimerin Downregulates the mTOR/p70S6K/4EBP1 Pathways and Inhibits Migration and Invasion Activities

(A) Cells were treated with various concentrations of pristimerin or the DMSO vehicle control for 24h, and phosphorylated and total mTOR, p70S6K, 4EBP-1 protein levels were analyzed by Western blotting. Three independent experiments were performed. (B) Cells treated with rapamycin ($2.7 \mu\text{M}$) were blotted with antibodies against phospho-p70S6K and phospho-4EBP1. The values below the figures represent the change in protein expression normalized against β -actin. Three independent experiments were performed. (C) Cells were grown to confluence, scratched with a pipette tip and photographed immediately after scratching (0h). After a 48h incubation, the plates were observed with a phase-contrast microscope. Three independent experiments were performed. The cell migration into the wound area was counted based on the dash line as time zero. Quantitative assessment of the mean number of cells in the denuded zone is expressed as the mean \pm standard deviation (S.D.) of three independent experiments. * $p < 0.05$ compared with vehicle-treated control. # $p < 0.05$ compared with pristimerin- or rapamycin-treated cells. (D) Cells were plated with pristimerin ($5 \mu\text{M}$) in the upper part of a Matrigel-coated transwell chamber. After a 24h incubation, invasive cells that migrated to the lower surface of the membrane were stained with trypan blue and observed using phase-contrast microscopy. Values indicate the mean \pm standard deviation (S.D.) in triplicate tests. * $p < 0.05$ compared with untreated cells. # $p < 0.05$ compared with pristimerin- or rapamycin-treated cells.

reduced migration and invasion of SKBR3 cells.

Pristimerin Promotes Caspase-Dependent Apoptotic Cell Death Several studies have suggested that inhibiting mTOR could contribute to cell apoptosis.^{27–29} We examined whether cytotoxic effects were due to an increase in apoptotic cell death. The percentage of apoptotic cells in the sub- G_1 phase of pristimerin-treated cells was measured by flow cytometry. Compared with the vehicle-treated control, pristimerin dramatically increased the number of apoptotic cells (Fig. 5A). To investigate the biochemical features that lead to apoptosis induced by pristimerin, we examined the effects of pristimerin on caspase-3, -8, and -9, and PARP activation. Cells were treated with pristimerin ($10 \mu\text{M}$) for 24h, and samples were collected and blotted with antibodies that recognize the inactive forms of the caspases. Following treatment with $1 \mu\text{M}$, reductions of the inactive forms of PARP and caspases-3, -8, and -9 could already be observed, and the bands disappeared completely after treatment with $10 \mu\text{M}$ pristimerin, indicating their full activation (Fig. 5B). Pretreatment of cells for 1h with $100 \mu\text{M}$ z-VAD-fmk, a pan-caspase inhibitor, eliminated apoptotic cells (Fig. 5C) and PARP cleavage (Fig. 5D). Taken

together, caspase-dependent apoptotic cell death increased following pristimerin treatment.

DISCUSSION

We examined the effect of pristimerin ((20 α)-3-hydroxy-2-oxo-24-nor-friedelan-1(10),3,5,7-tetraen-carboxylic acid-(29)-methylester, $\text{C}_{30}\text{H}_{40}\text{O}_4$) on the cytotoxic mechanism of HER2-positive SKBR3 human breast cancer cells. Pristimerin is a potent anti-tumor agent in a wide range of different human cancers, including breast cancer, prostate cancer, cervical cancer, and leukemia.^{6,8,10,30} However, the mechanism involved in the cytotoxic effect of pristimerin in cancer cells has not been fully explored. The nontumorigenic human mammary epithelial cell line MCF-10A was less sensitive to pristimerin in a previous study,⁶ and we also investigated the cytotoxicity of pristimerin using NIH-3T3 fibroblasts, resulting in non-toxicity at concentrations up to $50 \mu\text{M}$ (data not shown). These results suggest that pristimerin has therapeutic potential in cancers. In a previous study, pristimerin was highly toxic to cell lines with IC_{50} values ranging from $0.55 \mu\text{M}$ (MDA-MB-435)

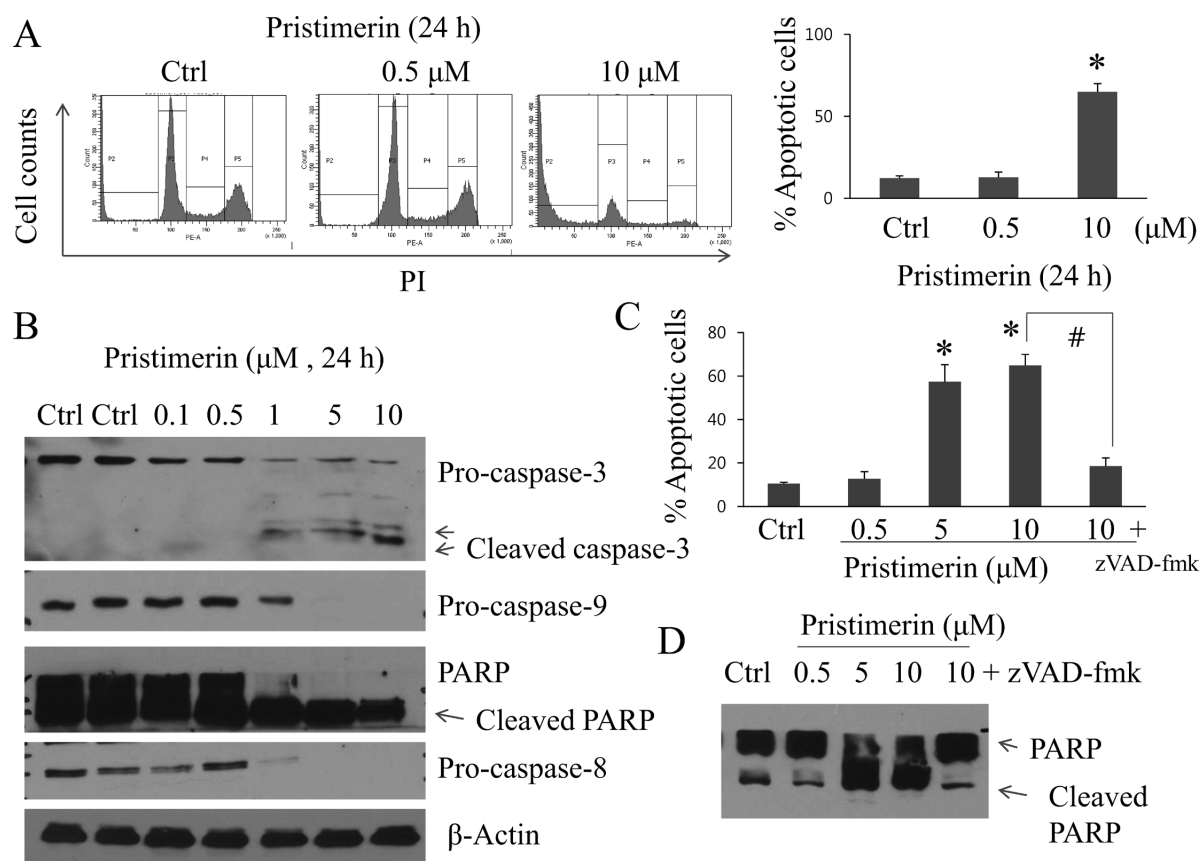


Fig. 5. Pristimerin Activates Caspase-3, Caspase-8, and Caspase-9 Activation as well as Poly(ADP-ribose) Polymerase (PARP) Cleavage

(A) Cells were treated with pristimerin (0.5 or 10 μ M) for 24 h, and harvested cells were fixed with 70% ethanol, and stained with 50 μ g/mL propidium iodide (PI). The cell cycle was then examined using FACS. Values indicate the mean \pm standard deviation (S.D.) in triplicate tests. * p < 0.05 vs. vehicle-treated control. (B) Cells were treated with pristimerin for 24 h, and Western blotting showed the kinetics of PARP, as well as caspase-3, -8 and -9 processing. Three independent experiments were performed. (C) Cells pre-treated with 100 μ M z-VAD-fmk were incubated with 50 μ M PA for different time intervals, stained with propidium iodide (PI) (50 μ g/mL) and the sub-G₁ peak was measured by FACS. Results represent the mean of three different experiments. Values indicate the mean \pm standard deviation (S.D.) in triplicate tests. * p < 0.05 vs. vehicle-treated control. # p < 0.05 vs. pristimerin-treated cells. (D) Z-VAD-fmk (100 μ M) was pre-treated for 2 h and pristimerin (0.5, 5, 10 μ M) was added to the cells, and PARP cleavage was measured by Western blot analysis. Three independent experiments were performed.

to 3.2 μ M (K-562).³⁰ In addition, pristimerin inhibits cell growth of the A-594 (lung), MCF-7 (breast), HepG2 (hepatocarcinoma), and Hep3B (liver) cancer cell lines with IC₅₀ values of 0.42–0.61 μ M.⁶ This present study also showed similar data with those of others, showing an IC₅₀ value 2.4 μ M after 24 h in the SKBR3 human breast cancer cell line (Fig. 1). The SKBR3 cell line overexpresses the *HER2/erbB2* oncogene product. HER2 overexpression has been correlated with poor prognosis, enhanced metastatic potential, increased chemoresistance, and the regulation of a variety of vital functions, such as cell growth, differentiation, and apoptosis, of human cancers.³¹ In this study, we demonstrated for the first time, that pristimerin inhibited HER2 expression in HER2-positive SKBR3 cells (Figs. 2A,B). Pristimerin also reduced HER2 mRNA levels (Fig. 2C), suggesting that pristimerin downregulates HER2 expression by diminishing *HER2* gene transcription. Together with the anti-proliferative activity of pristimerin in Fig. 1, these results suggest that the anticancer activity of pristimerin on HER2-positive SKBR3 cells is mediated by depleting HER2 from cancer cells. To test whether the effects of pristimerin decreased HER2 expression and cell growth, we evaluated the activities using another HER2-positive gastric cancer cell line (NCI-N87). As a result, HER2 expression and cell growth were downregulated in NCI-N87 cells following pristimerin treatment (data not shown) and showed a growth

inhibitory effect (IC₅₀ of approximately 7 μ M after 24 h). Although pristimerin has broad cytotoxicity in various cancer cell lines, these data show that downregulation of *HER2* gene expression by pristimerin may lead to cell toxicity.

FASN is a homodimeric multienzymatic protein of 250–270 kDa that is divided into seven functional domains assembled into two homodimers.³² Through a series of 32 reactions, FASN is able to synthesize long chain fatty acids, mainly palmitate, using acetyl-CoA and malonyl-CoA as substrates and nicotinamide adenine dinucleotide phosphate as an electron donor.³³ FASN expression remains at low levels and its regulation is complex and highly dependent on nutritional status and on the hormonal profile in normal cells.³³ However, FASN expression and activity are a common phenotype in most human tumor cells, demonstrating that FASN plays an essential role in tumor growth and survival.³⁴ The pathways responsible for FASN overexpression in cancer cells are not yet well understood. Four different mechanisms have been proposed, including enhanced transcription, increased translation, greater stability of proteins, and gene amplification.³⁵ These mechanisms may be active at the same time and may prevent FASN from physiological regulation; thus, resulting in constitutive activation of the lipogenic pathway in tumors.²⁵ FASN is highly expressed in a large number of human cancers, including breast, colon, ovary, pancreas, and prostate

cancers.³⁶⁾ Several breast cancer studies have demonstrated an association between FASN expression and disease prognosis. Patients with stage I breast cancer and high levels of FASN show a four-fold increased risk of death from this pathology than patients with lower FASN protein levels.³⁷⁾ FASN expression has also been associated with HER2 expression in poor prognosis tumors, suggesting that FASN is a potential therapeutic target for treatment of HER2-positive breast cancer. In this study, we observed that pristimerin inhibited FASN expression and FASN activity dose-dependently (Figs. 3A,B), and further analysis indicated that the pristimerin-induced anti-proliferation effect was reduced by exogenous treatment with palmitate in HER2-positive SKBR3 cells (Fig. 3C). In previous reports, the addition of exogenous palmitate markedly suppresses the cytotoxic effects of natural FASN inhibitors such as luteolin and amentoflavone in breast cancer cell lines.^{38,39)} These results provide evidence that pristimerin may function as a HER2 targeting suppressor by inhibiting FASN in SKBR3 cells. At the molecular level, the FASN-related signaling cascade, such as the PI3K/Akt, MAPKs, and mTOR pathways, are modified by FASN inhibition.⁴⁰⁾ FASN blockade destabilizes lipid rafts, which trigger degradation of HER2 and impede membrane recruitment of downstream mediators such as Akt and Erk thereby causing downregulation of phospho-Akt and phospho-Erk.⁴¹⁾ Moreover, Kumar-Sinha *et al.* demonstrated that elevated FASN expression is driven by HER2 through PI3K/Akt signaling.²⁵⁾ The JNK pathway represents one sub-group of MAPK and plays a role in the induction of apoptosis, cell survival, and proliferation.⁴²⁾ JNK signaling plays a role in FASN expression and apoptosis.^{43,44)} It is important that HER2 and FASN expression in cancer cells occurs in most cases through the same signaling pathways (MAPK/Erk-1).⁴⁵⁾ In this study, Akt and MAPK phosphorylation (Erk, p38, JNK) was suppressed by pristimerin treatment in SKBR3 cells (Fig. 3D). Furthermore FASN and HER2 expression was also synergistically inhibited by the PI3K inhibitor (LY294002), Erk inhibitor (PD98059), JNK inhibitor (SP600125), and p38 inhibitor (SB20356) (Fig. 3E). These data support that HER2 regulates FASN expression through Akt-dependent and/or MAPKs-dependent mechanisms.

A clear association exists between mTOR activation and HER2 overexpression in breast cancers,²⁶⁾ and activation of the mTOR signaling pathway has been associated with poorer prognoses. The mTOR signaling pathway plays an important role in FASN upregulation in HER2-overexpressing cancer cells.^{46,47)} Many reports have suggested that the mTOR pathway plays a role in the susceptibilities of chemopreventive or chemotherapeutic agents to kill cancer cells.^{48,49)} Therefore, blocking the mTOR pathway provides a promising target for novel therapeutic strategies. In this study, we demonstrated that pristimerin inhibited mTOR phosphorylation and phosphorylation of its substrates p70S6K and 4EBP1 in HER2-positive SKBR3 cells (Fig. 4A). Moreover, the mTOR inhibitor rapamycin synergistically enhanced pristimerin activity (Fig. 4B). To test whether the pristimerin-induced downregulation of HER2 blocks mTOR signaling, we evaluated the activities using another HER2-positive gastric cancer cell line (NCI-N87), which revealed similar results to those of SKBR3 cells (data not shown). The mTOR pathway, which is aberrantly activated in the majority of invasive breast cancers, is involved in many cellular processes including invasion and

tumor angiogenesis.^{50,51)} In addition, HER2 overexpression has been demonstrated to increase invasive gene expression (matrix metalloproteinase [MMP]2 and MMP9) in mammary epithelial cells;⁵²⁾ HER2 increases angiogenesis and spontaneous metastasis of human breast cancer cells *via* activation of mTOR/p70S6K.¹⁴⁾ Although HER2 activates the mTOR pathway, the role of mTOR signaling in HER2-mediated cancer metastasis and angiogenesis remains unclear. We found that pristimerin inhibited migration and invasion activities through mTOR signaling (Figs. 4C,D). Taken together, these results suggest that targeting the mTOR pathway using pristimerin may be a potential adjuvant strategy to inhibit migration and invasion of highly metastatic cancers. Dual mTOR and HER2 blockade results in antitumor activities in HER2-resistant breast cancers.⁵³⁾ Therefore, direct inhibition of mTOR signaling is an attractive clinical strategy for this disease. Combination therapies in which both the mTOR pathway and key reactivated targets such as HER2 are inhibited together may result in improved efficacy.⁵³⁾

Apoptosis is a key mechanism by which anticancer drugs kill tumor cells, which is a cellular suicide program through the mitochondrial (intrinsic) or death receptor pathways (extrinsic).⁵⁴⁾ One mechanism is the mitochondrial pathway, which is activated by the release of proapoptotic factors (cytochrome *c*) from mitochondria.⁵⁵⁾ The released cytochrome *c* interacts with Apaf-1 and activates caspase-9, which, in turn, proteolytically activates downstream caspase-3, one of the principle proteases participating in the execution phase of apoptosis.⁶⁾ Activated caspase-3 cleaves many substrates including PARP, a DNA repair enzyme, and leads to inevitable cell death.⁶⁾ The other pathway, the death receptor pathway, involves Fas and other members of the tumor necrosis factor receptor family, which activate caspase-8.⁵⁶⁾ Caspase-8 activates downstream events by directly activating caspase-3, which then triggers the mitochondrial pathway.⁶⁾ Pristimerin directly induces release of cytochrome *c* from mitochondria and activates the caspase cascade.⁶⁾ Pristimerin induces apoptosis by targeting the proteasome in prostate cancer cells.⁸⁾ Pristimerin induces apoptosis in imatinib-resistant chronic myelogenous leukemia cells harboring the T315I mutation by blocking NF- κ B signaling and depleting Bcr-Abl.⁵⁷⁾ However, the mechanism for apoptosis by pristimerin has not been fully explored. In this study, we confirmed that pristimerin induces apoptotic cell death (Fig. 5A) and activates caspase-3, caspase-9, followed by PARP cleavage, which are modulators in the mitochondrial pathway (Fig. 5B). Inhibiting caspase activation by z-VAD-fmk prevented pristimerin-induced apoptosis (Figs. 5C,D). Interestingly, we found that pristimerin induced apoptosis *via* the cell death receptor pathway (extrinsic) by activating caspase-8 and caspase-3 (Fig. 5B). This is the first report that pristimerin induces apoptosis *via* the extrinsic apoptotic pathway. One supportive study showed that apigenin induces apoptosis *via* the extrinsic pathway in HER2-positive breast cancer cells⁵⁸⁾, however, we suggest that further investigation is needed to identify the molecular mechanism of pristimerin-induced apoptosis targeting HER2-positive cancers.

Taken together, pristimerin represents a novel HER2-targeted compound that is able to decrease fatty acid synthase and modulate Akt, MAPKs (Erk, JNK, p38), and mTOR signaling pathways to influence metastasis and apoptotic cell death. Our results suggest that pristimerin may be a potential agent

for cancer prevention and treatment of HER2-positive human cancers.

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