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Research article

Effect of sun ginseng potentiation on epirubicin and paclitaxel-induced apoptosis in human cervical cancer cells

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ABSTRACT

Background: Sun ginseng (SG), a specific formulation of quality-controlled red ginseng, contains approximately equal amounts of three major ginsenosides (Rk1, Rg3, and Rg5), which reportedly has antitumor-promoting activities in animal models.

Methods: MTT assay was used to assess whether SG can potentiate the anticancer activity of epirubicin or paclitaxel in human cervical adenocarcinoma HeLa cells, human colon cancer SW111C cells, and SW480 cells; apoptosis status was analyzed by annexin V-FITC and PI and analyzed by flow cytometry; and apoptosis pathway was studied by analysis of caspase-3, -8, and -9 activation, mitochondrial accumulation of Bax and Bak, and cytochrome c release.

Results: SG remarkably enhances cancer cell death induced by epirubicin or paclitaxel in human cervical adenocarcinoma HeLa cells, human colon cancer SW111C cells, and SW480 cells. Results of the mechanism study highlighted the cooperation between SG and epirubicin or paclitaxel in activating caspase-3 and -9 but not caspase-8. Moreover, SG significantly increased the mitochondrial accumulation of both Bax and Bak triggered by epirubicin or paclitaxel as well as the subsequent release of cytochrome c in the targeted cells.

Conclusion: SG significantly potentiated the anticancer activities of epirubicin and paclitaxel in a synergistic manner. These effects were associated with the increased mitochondrial accumulation of both Bax and Bak that led to an enhanced cytochrome c release, caspase-9/-3 activation, and apoptosis. Treating cancer cells by combining epirubicin and paclitaxel with SG may prove to be a novel strategy for enhancing the efficacy of the two drug types.

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1. Introduction

Cancer is one of the most fatal diseases that poses a threat to human health worldwide [1]. A deviant regulation of apoptosis is required for cancer initiation, development, and metastasis [2]. Recent anticancer treatment, including chemotherapy, immunotherapy, radiation, and cytokines, primarily induce apoptosis in targeted cancer cells [3]. Apoptosis, a programmed cell death, is initiated through two main pathways: the exogenous pathway, which is characterized by death receptor activation; and the endogenous pathway, which is characterized by mitochondrial

destruction [4]. The tumor necrosis factor receptor superfamily triggers the membrane receptor aggregation and then recruits Fas associated death domain (FADD) and caspase-8 by binding of its specific ligand. Upon recruitment, caspase-8 becomes activated and initiates apoptosis through the direct cleavage of the downstream effector caspases, particularly caspase-3 and -7. In the mitochondrial pathway, apoptogenic factors, such as cytochrome c, second mitochondria-derived activator of caspases (Smac), or apoptosis-inducing factor (AIF), are released into the cytosol from the mitochondria. Cytochrome c triggers the activation of caspase-9 by forming the cytochrome c/apoptotic protease-activating factor

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(Apaf-1)/caspase-9-containing apoptosome complex. Meanwhile, Smac promotes the activation of caspase by invalidating the inhibitory effects of the inhibitors of apoptosis (IAP) family [5–7]. Combination treatments prove to be advantageous in treating malignancies that still partially respond to a single treatment [8]. Drugs have long been combined to treat diseases and reduce suffering; this long-standing history of drug combinations is clearly depicted in traditional Chinese medicines [9].

Panax ginseng has been long used for several thousand years in the Orient as a tonic, prophylactic, and restorative agent [10]. Sun ginseng (SG), a new type of ginseng that is processed by heating at specific pressures, contains approximately equal amounts of three major ginsenosides (Rk1, Rg3, and Rg5). SG reportedly serves several functions, including radical scavenging and antitumor-promoting activities [11–13]. Ginsenoside Rg3, one of the most studied of the three major ginsenosides, is reported to inhibit the proliferation, migration, and invasion in many common cancers, such as liver, prostate, colorectal, and ovarian, and has been widely used in cancer treatment [14–17]. Rg3 can induce apoptosis and cell cycle arrest in different cancer cells via different pathways such as downregulating hypoxia inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) [18–21]. Rk1 was investigated to inhibit telomerase activity and cell growth and induce apoptosis through activation of caspase-8 and -3 via ERK pathway, whereas another article demonstrated that Rk1 could induce G1 arrest and autophagy [22,23]. Rg5 blocks the cell cycle at the G1/S transition phase by increasing p21^{Cip}/WAF1 and decreasing cyclin E and CDK2 [24].

Epirubicin is a third-generation anthracycline that treats a broad spectrum of cancers, including cervical, breast, lung (especially small cell lung cancer), ovarian, stomach, colon, and bladder, and malignant lymphoma [25,26]. Similar to widely used anticancer drugs, epirubicin exhibits some adverse effects on blood, the stomach, and the heart; these effects largely depend on the applied doses [27]. Paclitaxel is another important anticancer drug that is widely used as a chemotherapeutic agent for treating ovarian, breast, lung, colorectal, bladder, prostate, and gastric cancer, melanoma, and lymphoma [28–30]. Paclitaxel, which is an inhibitor of microtubule degradation, induces cell cycle arrest at the G2/M phase [31,32] and ultimately apoptosis [33,34]. This drug also has significant adverse effects, such as hypersensitivity, neutropenia syndrome, neurotoxicity, heart rhythm disorders, and intracellular toxicity [35–37]. Therefore, developing adjuvant agents to potentiate the anticancer activities of epirubicin and paclitaxel and to minimize their adverse effects is significant.

In the current study, SG significantly potentiated the anticancer activities of epirubicin and paclitaxel in a synergistic manner. These effects were associated with the increased mitochondrial accumulation of both Bax and Bak that led to an enhanced cytochrome c release, caspase-9/-3 activation, and apoptosis.

2. Materials and methods

2.1. Materials

SG was provided by Dr. Jeong Hill Park, College of Pharmacy, Seoul National University, Seoul, Korea. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Epirubicin was acquired from Pfizer (Wuxi, China). Newborn calf serum and Dulbecco modified Eagle's medium (DMEM) were purchased from Gibco (Life Technologies, Grand Island, NY, USA). Caspase substrates Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC were purchased from Calbiochem (La Jolla, CA, USA). The

Mitochondria Isolation Kit was purchased from Pierce (Rockford, IL, USA). Annexin V-FITC Apoptosis Detection Kit was purchased from KeyGEN Biotech (Nanjing, China). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Billerica, MA, USA). Antibodies against cytochrome c, poly (adenosine diphosphate-ribose) polymerase (PARP), Bak, Bax, α -tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against caspase-8, -9, and cytochrome c oxidase II (Cox II) were purchased from Cell Signaling Technology (Beverly, MA, USA). Clarity Western ECL Substrate Kit was purchased from Bio-Rad (Hercules, CA, USA).

2.2. Cell culture

HeLa, SW111C, and SW480 cells were grown in DMEM supplemented with 10% (by volume) heat-inactivated newborn calf serum, 100 μ g/mL of streptomycin 100 U/mL of penicillin, at 37°C in a humidified atmosphere with 5% CO₂.

2.3. Analysis of SG methanol extract

The SG methanol extract was analyzed as a previous report described [38]. Briefly, SG was dissolved in MeOH (3 mg/mL), and filtered with 0.45 μ m Millipore filter, and the solution was analyzed with a Waters 2695 liquid chromatograph (Waters Corporation, Milford, MA, USA) fitted with Knauer C-18, reverse-phase column (Knauer, Berlin, Germany; 5 μ m, ϕ 250 mm \times 3 mm) utilizing the solvent gradient system. The mobile phase consisted of acetonitrile water (Solvent A) and water (Solvent B) and the flow rate was 0.6 mL/min. The detector was a Waters 2996 PDA Detector (Waters Corporation). The gradient elution was used as follows: 0–20 min, 20% A; 20–31 min, linear gradient from 20–32% A; 31–40 min, linear gradient from 32–43% A; 40–70 min, linear gradient from 43–100% A; and 70 min, 100% A.

2.4. MTT assay

Exponentially growing cells were seeded into a 96-well plate at 0.8×10^4 cells/well in triplicate. After incubation for 20 h, cells were treated with increasing concentrations of SG, epirubicin, or paclitaxel for 48 h. At 44 h posttreatment, 20 μ L of MTT (5 mg/mL) was added to each well and incubated for 4 h. Then 150 μ L of DMSO was added to every well to solubilize the formazan crystals formed by viable cells, and the color intensity was measured at 550 nm with an enzyme-linked immunosorbent assay plate reader (TECAN, Männedorf, Switzerland).

2.5. Apoptosis assay

HeLa cells were cultured for 20 h and then treated with 80 μ g/mL SG with 0.5 μ g/mL epirubicin or 10nM paclitaxel alone or combined for 24 h. HeLa cells were harvested, washed with ice-cold phosphate buffered saline (PBS), and stained with annexin V/PI reagent as described previously [3]. The percentage of annexin V (+) cells was determined by flow cytometry (Becton Dickinson FACS Calibur Cytometer, San Jose, CA, USA). The percentage of annexin V (+) cells indicates the frequency of total apoptotic cells.

2.6. Cell-free caspase activity assay

As described [39], HeLa were treated and harvested. 50 μ g whole-cell lysates were incubated with 200nM Ac-LEHD-AFC (for caspase-9), Ac-IETD-AFC (for caspase-8), and Ac-DEVD-AFC (for caspase-3) in a reaction buffer containing 20mM

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 10mM dithiothreitol (DTT), 10% sucrose, 100mM NaCl, and 0.1% 3-((3-Cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS) at 37°C for 1 h. The reaction was monitored by fluorescence excitation at 405 nm and emission at 505 nm.

2.7. Preparation of mitochondrial and cytosolic protein extracts

HeLa cells were treated and harvested. Mitochondria and cytosolic protein extracts were prepared using a Mitochondria Isolation Kit (Pierce) according to the manufacturer's instructions. Isolated mitochondria were solubilized in a lysis buffer containing 20mM Tris-HCl (pH 7.5), 1% NP-40, 150mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2mM MgCl₂, 1mM ethylene glycol tetraacetic acid (EGTA), 50mM β-glycerol phosphate, 25mM NaF, 1mM DTT, 1mM Na₃VO₄ with 2 mg/mL leupeptin, 2 mg/mL pepstatin A, 2 mg/mL antipain, and 1mM phenylmethylsulfonyl fluoride (PMSF). The mitochondrial proteins were then subjected to immunoblotting analysis using antibodies against Bax and Bak. The cytosolic proteins were subjected to immunoblotting analysis using antibody against cytochrome c.

2.8. Immunoblotting analysis

The treated cells were washed with ice-cold PBS and solubilized in a lysis buffer containing 20mM Tris with a pH of 7.5, 2mM MgCl₂, 1mM DTT, 0.5% Triton X-100, 1mM EGTA, 25mM NaF, 1mM Na₃VO₄, 50mM β-glycerol phosphate, 2 mg/mL leupeptin, 2 mg/mL pepstatin A, 2 mg/mL antipain, and 1mM PMSF. After incubating on ice for 1 h, the insoluble materials were removed by centrifugation at 14,000 × g for 15 min. 50 μg of protein from each sample was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by electrotransfer onto a PVDF membrane (Millipore). The membrane was blocked with 5% nonfat milk in PBS with 0.1% Tween 20 and probed with the antibodies. The blots were washed and incubated with a horseradish peroxidase-coupled antimouse immunoglobulin G (IgG) or an antirabbit IgG antibody (Pierce) followed by detection with an electrogenerated chemiluminescence (ECL) revelation system (Bio-Rad).

2.9. Statistical analysis

All values are performed in triplicate and expressed as mean ± standard deviation with Microsoft Office 2013 and imaged with Sigmaplot 10 (Systat Software Inc, San Jose, CA, USA). A Student *t* test was used for quantitative analysis, and the significant difference is shown as * *p* < 0.05, ** *p* < 0.01, and *** *p* < 0.001.

3. Results and Discussion

3.1. Analysis of SG extract

To determine the types of ginsenoside in SG, we analyzed MeOH extract of SG by an analytical high-performance liquid chromatography. As shown in Fig. 1, the amount of four main ginsenosides in the total ginsenosides were 20(S)-Rg3 (11.33%), 20(R)-Rg3 (6.88%), Rk1 (16.72%), and Rg5 (11.97%).

3.2. Cytotoxic effect of SG on cancer cells

As shown in Fig. 1, the amount of ginsenoside Rg3, Rg5, and Rk1 reached 50% of total ginsenosides in SG. A number of studies showed that (20S) ginsenoside Rg3, Rg5, and Rk1 inhibit cell viability in various human cancer cells. We then examined whether SG features cytotoxic activity in human cancer cells in human cervical adenocarcinoma HeLa cells, human colon cancer SW111C cells, and SW480 cells through an MTT assay. Fig. 2 illustrates that SG exhibited a moderate cytotoxicity against the HeLa, SW111C, and SW480 cells with IC₅₀ values of 94 μg/mL, 78 μg/mL, and 224 μg/mL, respectively.

3.3. SG enhances the efficacy of anticancer drugs epirubicin and paclitaxel

To examine whether SG enhances the efficacy of clinical anticancer drugs, we determined the half maximal inhibitory concentration (IC₅₀) values of epirubicin and paclitaxel, either alone or with a subtoxic concentration of SG, in the three cancer cell lines (HeLa, SW111C, and SW480). Fig. 3 and Table 1 depict that the IC₅₀ values markedly decreased with the addition of SG to epirubicin and paclitaxel. The IC₅₀ value of epirubicin in the HeLa cells was 1.05 μg/mL, which decreased to 0.15 μg/mL with the addition of 80 μg/mL SG. This result indicates that a subtoxic concentration of SG significantly increases the cytotoxic efficacy of epirubicin. SG exhibited similar potentiating activities on paclitaxel in all three cancer cell lines.

3.4. SG helps induce strong apoptotic cell death in HeLa cells

To examine whether the role of SG in the cytotoxic effect of epirubicin and paclitaxel was caused by the enhanced apoptosis, we assessed the resulting apoptosis in the HeLa cells after separate treatments with epirubicin and paclitaxel alone and after the treatment with the combination of SG and the two drugs. The stage of apoptosis was determined through annexin-V analysis. As shown

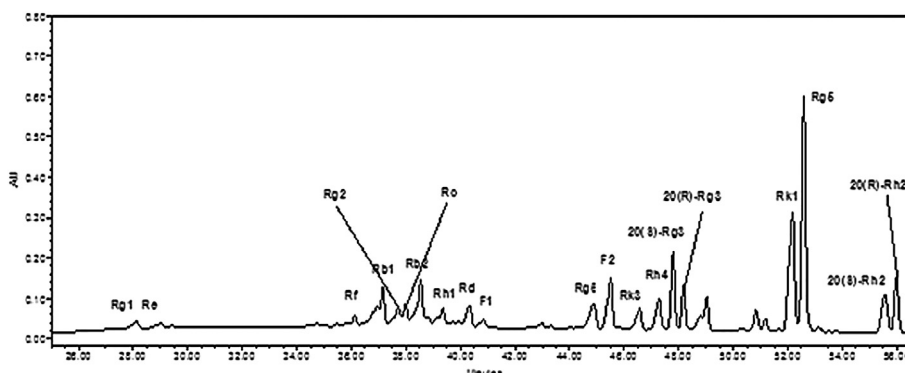


Fig. 1. High-performance liquid chromatography chromatogram of sun ginseng (SG) MeOH extract.

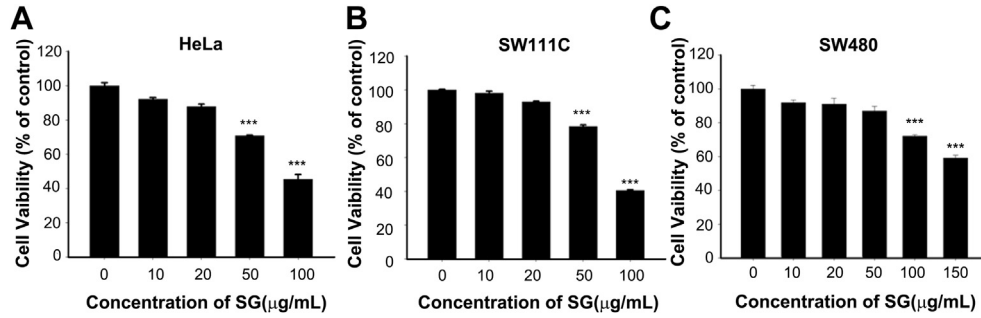


Fig. 2. Dose-dependent effects of sun ginseng (SG) on the cell viability of different human cancer cells. (A) Human cervical adenocarcinoma HeLa cells. (B and C) Human colon cancer, SW111C, and SW480. These cells were treated with various concentrations of SG for 48 h. Cell viability was defined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The results represent three independent experiments performed in triplicate. The values from each treatment are expressed as a percentage relative to the control (100%). Each point represents the mean \pm standard deviation. A Student *t* test was used for quantitative analysis, and the significant difference is shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with those not treated.

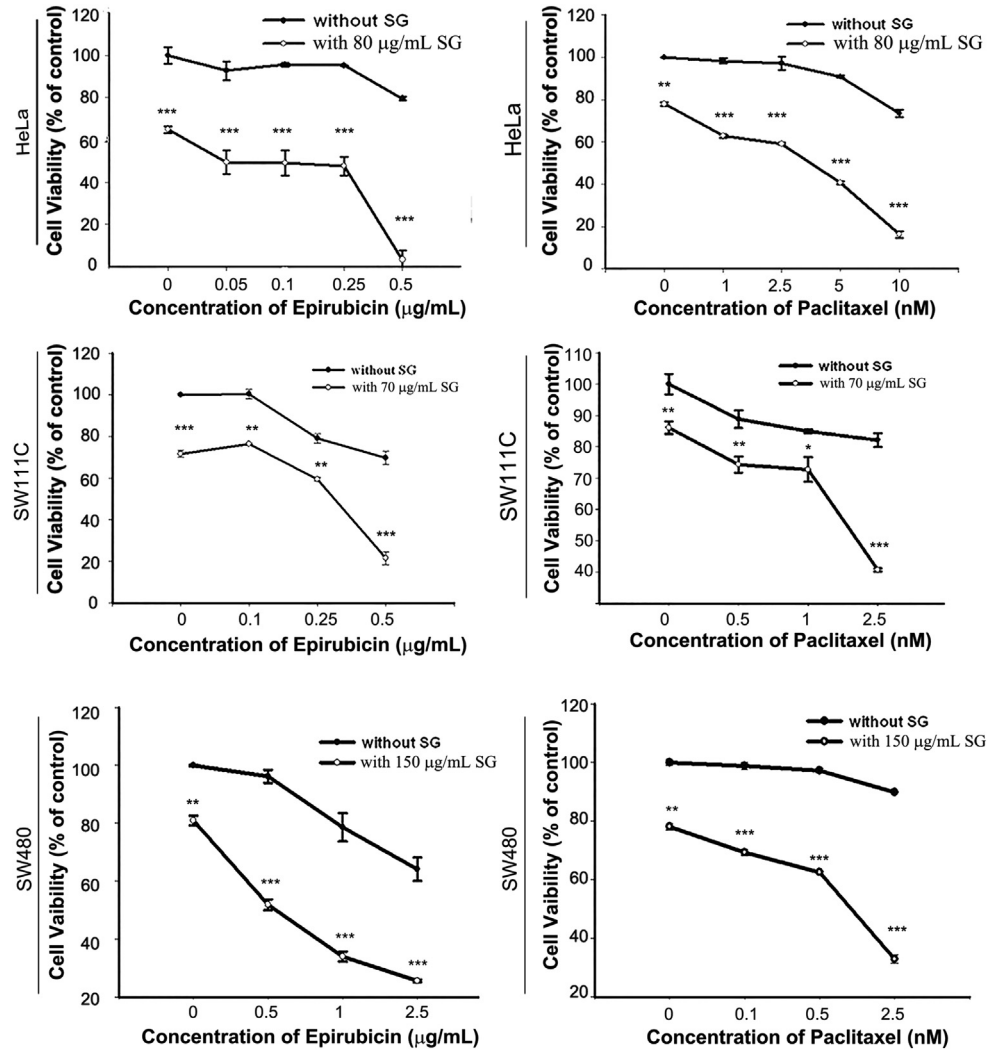


Fig. 3. Sun ginseng (SG) enhances the antiproliferative activities of epirubicin and paclitaxel. HeLa, SW111C, and SW480 cells were treated with different concentrations of epirubicin or paclitaxel with or without different concentrations of SG for 48 h. Cell viability was estimated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and expressed as the percentage of viable cells in the treated and controlled samples. Data are presented as mean \pm standard deviation of experiments performed in triplicate. A Student *t* test was used for quantitative analysis, and the significant difference is shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Table 1

The resulting half maximal inhibitory concentration values from the 48-h drug treatment with or without sun ginseng (SG) in three cell lines

	Epirubicin ($\mu\text{g}/\text{mL}$)	SG + Epirubicin ($\mu\text{g}/\text{mL}$)	Paclitaxel (nM)	SG + Paclitaxel (nM)
HeLa	1.05	0.15	12.57	1.9
SW111C	1.84	0.30	>100	1.92
SW480	3.32	0.53	>100	0.99

in Fig. 4A and C, the percentage of apoptotic cells was considerably higher in the cotreated cells than in the epirubicin- and paclitaxel-treated cells. To determine the activation of caspase in the cells, we detected the PARP cleavage through immunoblotting analysis. Fig. 4B and D show that PARP was cleaved to yield an 85-kD fragment in the drug-treated cells and that the amount of the cleaved 85-kD fragment was more significant in the co-treated cells than in the epirubicin- and paclitaxel-treated cells. On the basis of these results, we suggest that SG enhances the anticancer activities of epirubicin and paclitaxel through caspase-associated apoptosis.

3.5. SG enhances apoptosis via the mitochondrial pathway

To elucidate the initiation event of apoptosis, we inspected the activation kinetics of the two initiator caspases, namely, caspase-8 and -9, and the effector caspases, caspase-3/-7. As shown in Fig. 5, the activities of caspase-9 and -3/-7 greatly increased in the cotreated cells than in the epirubicin- and paclitaxel-treated cells. By contrast, the activity of caspase-8 did not show any change in all cells. We then determined the cleavage of caspase-9 and -8. Specifically, we examined the proteolytic activation of these caspases through immunoblotting analysis. Apparent cleavage was observed in caspase-9 but not in caspase-8. The amounts of the active form of

the cleaved caspase-9 were higher in the cotreated cells than in the epirubicin- and paclitaxel-treated cells. The data suggest that epirubicin and paclitaxel-induced apoptosis might be potentiated by SG via the intrinsic apoptosis pathway in HeLa cells.

3.6. Apoptosis of SG-enhanced HeLa cells mediated through cytochrome C release

The release of mitochondrial cytochrome c is the crucial event in caspase-9 activation [40]. The family members of the Bcl-2 family, namely, Bax and Bak, serve as an essential gateway for the release of cytochrome c [5,41]. Fig. 6 shows that cytochrome c appeared in cytosol after the cell was treated with the two drugs; the levels of this protein in the cytosol were much higher in the SG and epirubicin or paclitaxel-treated HeLa cells than in the epirubicin- and paclitaxel-treated cells. Similarly, the levels of Bax and Bak in the mitochondria were markedly increased in the epirubicin- and paclitaxel-treated cells, but this increase was more significant in the cotreated groups (Fig. 7). Moreover, the increase of Bax and Bak in the mitochondria upon drug treatment conformed well to the release of the enhanced cytochrome c in the apoptotic cells. However, no evident changes were observed in Bax or Bak in the whole-cell lysates. These results imply that the increased regulation of the released cytochrome c that was observed in the co-treated HeLa cells resulted from the enhanced translocation of Bax and Bak proteins.

The induction of apoptosis in cancer cells is a staple killing mechanism for most antitumor therapies [2]. The cotreatment of anticancer reagents has been shown to be advantageous in malignancies that still partially respond to epirubicin or paclitaxel treatment because they may help amplify weak death signals. In this study, SG markedly potentiated epirubicin- or paclitaxel-induced cancer cell death possibly because of the increase in the

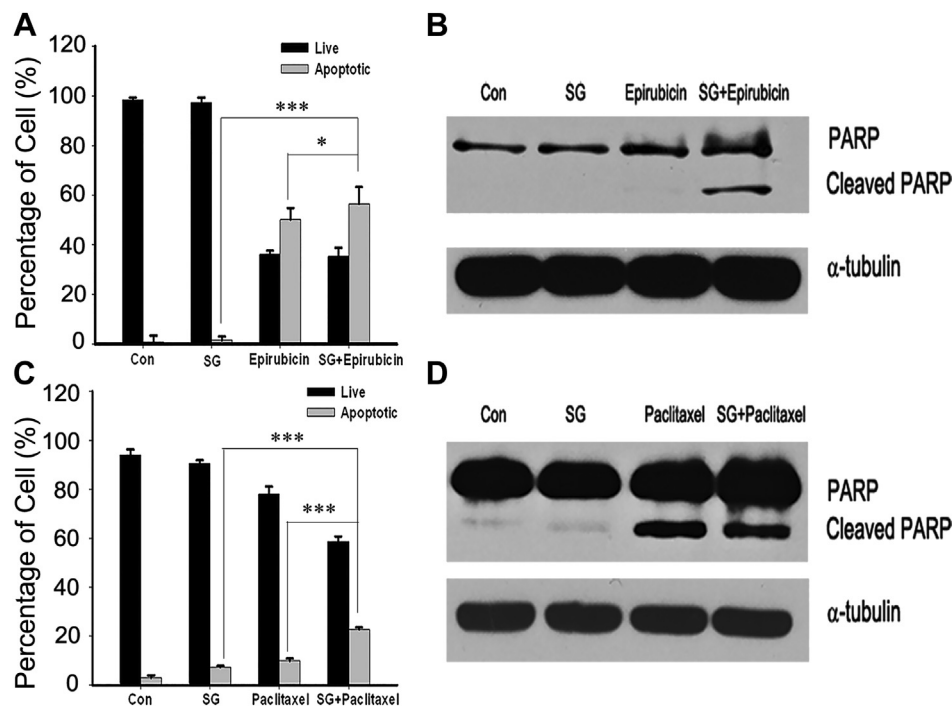


Fig. 4. Apoptosis was synergistically induced by sun ginseng (SG) and epirubicin or paclitaxel in HeLa cells. (A and B) HeLa cells were treated with 0.5 $\mu\text{g}/\text{mL}$ epirubicin alone and with the combination of 0.5 $\mu\text{g}/\text{mL}$ epirubicin and 80 $\mu\text{g}/\text{mL}$ SG for 24 h. (C and D) HeLa cells were treated with 10nM paclitaxel alone and with the combination of 10nM paclitaxel and 80 $\mu\text{g}/\text{mL}$ SG for 24 h. (A and C) Cells were stained with annexin V-FITC and PI and analyzed by flow cytometry. Data are presented as mean \pm standard deviation of experiments performed in triplicate. (B and D) The equal amounts of cell extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by immunoblotting using specific antibodies against poly (adenosine diphosphate-ribose) polymerase and α -tubulin.

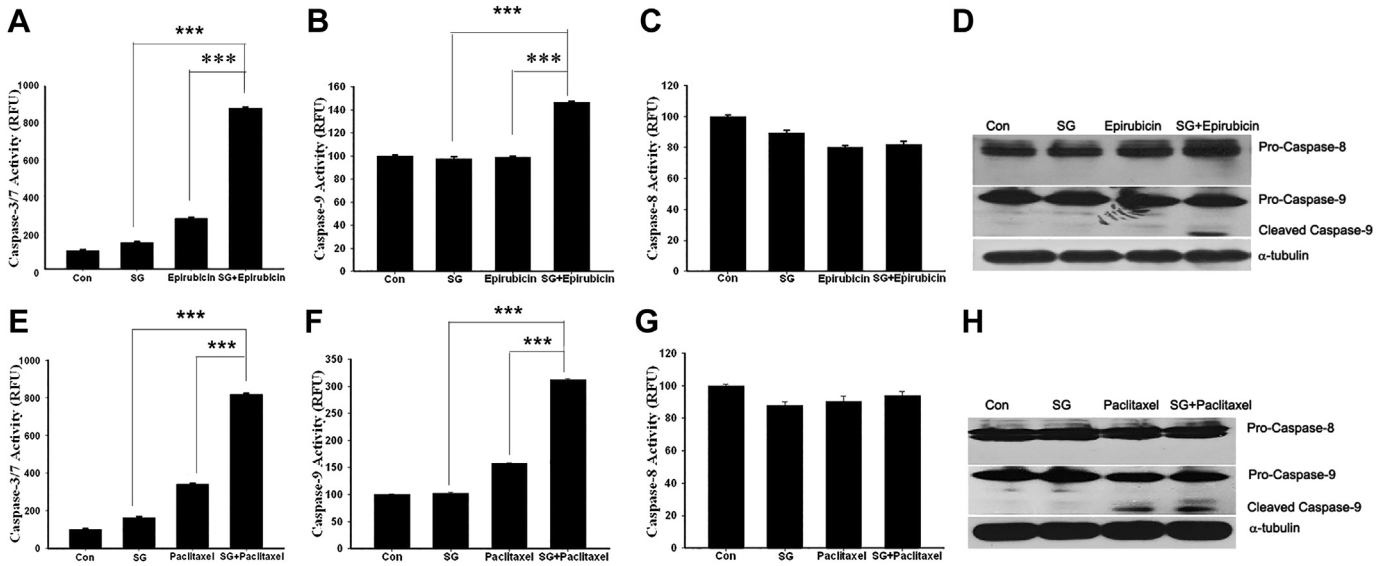


Fig. 5. Sun ginseng (SG) and epirubicin or paclitaxel synergistically acted to induce the activation of caspase-9 and -3 in HeLa cells. (A–D) HeLa cells were treated with 0.5 $\mu\text{g}/\text{mL}$ epirubicin alone and with the combination of 0.5 $\mu\text{g}/\text{mL}$ epirubicin and 80 $\mu\text{g}/\text{mL}$ SG for 24 h. (E–H) HeLa cells were treated with 10nM paclitaxel alone and with the combination of 10nM paclitaxel and 80 $\mu\text{g}/\text{mL}$ SG for 24 h. (A–C, E–G) Cell-free caspases-3, -9, and -8 activities were analyzed by using specific substrates (Ac-DEVD-AFC, Ac-LEHD-AFC, and Ac-IETD-AFC, respectively). Data are presented as mean \pm standard deviation of experiments performed in triplicate. A Student *t* test was used for quantitative analysis, and the significant difference is shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (D, H) Cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by immunoblotting using specific antibodies against caspase-8, caspase-9, and α -tubulin.

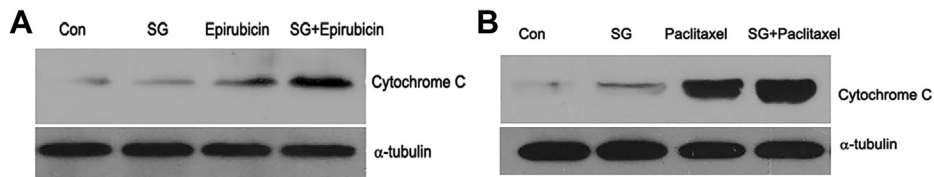


Fig. 6. Sun ginseng (SG) and epirubicin or paclitaxel synergistically induced the mitochondrial membrane depolarization and cytochrome c release in HeLa cells. (A) HeLa cells were treated with 0.5 $\mu\text{g}/\text{mL}$ epirubicin alone and with the combination of 0.5 $\mu\text{g}/\text{mL}$ epirubicin and 80 $\mu\text{g}/\text{mL}$ SG for 24 h. (B) HeLa cells were treated with 10nM paclitaxel alone and with the combination of 10nM paclitaxel and 80 $\mu\text{g}/\text{mL}$ SG for 24 h. The equal amounts of cytosol fractions from the treated or untreated HeLa cells were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (15%) and analyzed by immunoblotting using specific antibodies against cytochrome c and α -tubulin. Representative results from one of the three independent experiments are shown.

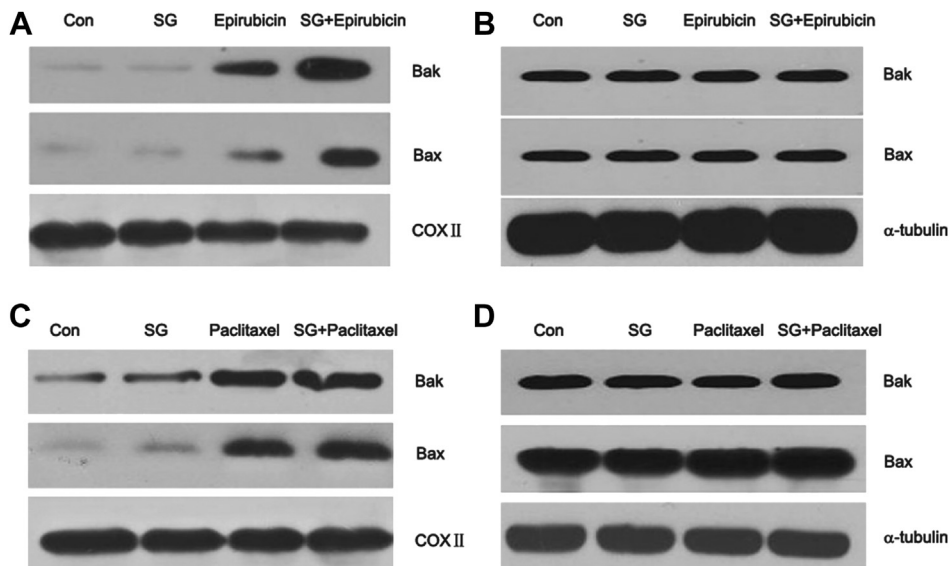


Fig. 7. Bak and Bax were translocated to the mitochondria in the sun ginseng (SG) and epirubicin or paclitaxel co-treated HeLa cells. (A and B) HeLa cells were treated with 0.5 $\mu\text{g}/\text{mL}$ epirubicin alone and with the combination of 0.5 $\mu\text{g}/\text{mL}$ epirubicin and 80 $\mu\text{g}/\text{mL}$ SG for 24 h. (C and D) HeLa cells were treated with 10nM paclitaxel alone and with the combination of 10nM paclitaxel and 80 $\mu\text{g}/\text{mL}$ SG for 24 h. The equal amounts of the mitochondrial fraction of proteins (A and C) and the total cell lysates (B and D) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (15%) and analyzed by immunoblotting using antibodies against Bax, Bak, COXII, and α -tubulin. COXII and α -tubulin acted as loading controls. Representative results from one of the three independent experiments are shown.

release of cytochrome c and the activation of caspases-9 and -3. Thus, cotreating cancer cells with SG and clinical drugs could be a novel strategy for enhancing the efficacy of current chemotherapies. The development of SG as a new adjuvant drug for cancer therapy also shows great potential.

Conflicts of interest

All authors declare no conflicts of interest.

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