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Research Article

Synergistic Effect of Pomolic Acid Inducing Apoptosis on Doxorubicin-Resistant Human Gastric Carcinoma Cells

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Abstract

Doxorubicin resistance is one of chemotherapeutic failure in cancers. Pomolic Acid (PA), a pentacyclic triterpene inducing apoptotic cell death, has been suggested to overcome multi-drug resistance. However, the probability of PA as an agent to overcome doxorubicin resistance was not investigated. The present study investigated the synergistic effects of PA on doxorubicin-resistant human gastric carcinoma SNU-620-DOX cells. Co-treatment with PA and doxorubicin decreased the viability of SNU-620-DOX cells, compared with only PA- or doxorubicin-treated cells. Co-treatment with PA and doxorubicin induced the activation of caspase cascade, but this was not observed in SNU-620-DOX cells treated with only PA- or doxorubicin-treated cells. While the expression of anti-apoptotic protein Bcl-2 and Bcl-xL was not affected by the presence of PA. PA increased the loss of mitochondrial membrane potential in doxorubicin-treated SNU-620-DOX cells. In conclusion, our results indicated that co-treatment with PA and doxorubicin induced apoptosis in doxorubicin-resistant human gastric carcinoma cells, which is mediated by increasing the loss of mitochondrial membrane potential.

Keywords: Apoptosis; Doxorubicin-Resistant; Gastric Carcinoma Cells; SNU-620-DOX; Mitochondrial Membrane Potential ($\Delta\Psi m$)

Introduction

Multi-drug resistance is the major cause of chemotherapeutic failure in cancer. Multi-drug resistance exists against every effective anticancer drug and can develop by numerous mechanisms including decreased drug uptake, increased drug efflux, activation of detoxifying systems, activation of DNA repair mechanisms, and evasion of drug-induced apoptosis [1]. Doxorubicin, a naturally occurring anthracycline antibiotic, is an essential chemotherapy drug used to treat many types of cancer. Doxorubicin targets topoisomerase II by interfering with a catalytic cycle and inhibits the growth of cancer cells [2].

Pomolic Acid (PA) is a pentacyclic triterpene compound isolated from flowers of Osmathus fragrans var. aurantiacus Makino. PA has multiple effects including hepatoprotective, anti-inflammatory and anti-proliferative effects. PA inhibited d-galactosamine-induced cytotoxicity in primary cultured mouse hepatocytes and 12-O-tetradecanoylphrbol-13-acetate-induced inflammation in mice [3,4]. PA inhibited the growth of leukemia HL-60 cells and induced mitochondria-depen¬dent apoptotic cell death [5]. PA also showed anti-proliferative activities against human gastric adenocarcinoma (MK-1), Human Uterine Carcinoma (HeLa) and murine melanoma (B16F10) cells [6]. Recently, PA has been reported to suppress the invasiveness of breast cancer cells and HIF1α/VEGF-mediated angiogenesis [7,8]. PA-induced apoptosis was

mediated by activating the caspase cascade through the loss of the mitochondrial transmembrane potential (ΔΨm), indepen¬dently of anti-apoptotic Bcl-2 expression in leukemia cells [5,9]. PA has been suggested to overcome multi-drug resistance mediated by the overexpression of anti-apoptotic Bcl-2 proteins [9]. PA induced apoptosis in cells from chronic myeloid leukemia patients with clinical resistance to interferon and/or imatinib [10]. However, the effect of PA on multi-drug resistant cancer cells has not been fully investigated. To the best of our knowledge, the effect of PA on doxorubicin-resistant cancer cells has not been examined. In this study, we investigated the apoptotic effects of PA on doxorubicin-resistant human gastric carcinoma SNU-620-DOX cells [11].

Materials and Methods

Chemicals and Cell Culture

PA isolated from flowers of Osmathus fragrans var. aurantiacus Makino [12] was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and diluted in Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Scientific Hyclone, South Logan, UT) with 10% Fetal Bovine Serum (FBS; Thermo Scientific Hyclone) for use. Human gastric carcinoma SNU-620 cells and SNU-620-DOX cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). SNU-620 cells were maintained in RPMI-1640 with 10% FBS. SNU-620-Dox cells were maintained in RPMI-1640 with 10% FBS and 300 ng/ml doxorubicin (Sigma-Aldrich). Cells were cultured in a humidified incubator with 5% CO2 at 37˚C.

Cell Proliferation Assay

SNU-620 cells and SNU-620-DOX cells were seeded onto 12-well tissue culture plates at a density of 2×105 cells per well. The cells were treated with culture media containing different concentrations of doxorubicin and/or PA for 4 days. The cells were harvested by trypsinization. The cell suspensions were mixed with a trypan blue solution and viable cells were counted using a hemocytometer.

Protein Extraction and Western Blot Analysis

After seeding onto 6-well plates at a density of 4×105 cells per well, SNU-620 cells and SNU-620-DOX cells were treated with different concentrations of doxorubicin and/or PA for 4 days, then lysed with radioimmunoprecipitation assay buffer (Thermo Fisher Scientific Inc., Rockford, IL) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were determined using an RC/DC Bio-Rad assay kit (Bio-Rad, Hercules, CA). Protein samples were separated using electrophoresis on 8-15% polyacrylamide-sodium dodecyl sulfate gel. The electrophoresized proteins on the gel were transferred onto Polyvinlidene Fluoride (PVDF) membranes (PALL Life Science, Port Washington, NY). The membranes were blocked with 3% skimmed milk (BD Biosciences, San Jose, CA), incubated with a rabbit polyclonal anti-human caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-human cleaved caspase-8 (Cell Signaling Technology Inc. Danvers, MA), rabbit polyclonal anti-human caspase-9 (Cell Signaling Technology Inc.), mouse monoclonal anti-human Bcl-2 (Santa Cruz Biotechnology), mouse monoclonal anti-human Bcl-xL (Santa Cruz Biotechnology) or mouse monoclonal anti-human actin (Cell signaling Technology Inc.), then probed with a horseradish peroxidase-conjugated goat polyclonal anti-rabbit or anti-mouse immunoglobulin G antibody (Cell Signaling Technology Inc.). Enhanced chemiluminescent western blotting detection reagent (Pierce, Rockford, IL) was applied to allow detection of the protein bands.

Mitochondrial Membrane Potential (ΔΨm) Assay

SNU-620-DOX cells incubated for 4 day in culture media containing 300 ng/ml doxorubicin and/or 30 μM PA were treated with 10 μg/ml 5,5′,6,6′-tetrachloro-1,1′ 3,3′-tetraethylbenzimidazsoly-carbocyanine iodide (JC-1; Thermo Fisher Scientific Inc.) cationic dye for 30 min and collected by trypsinization. The fluorescence intensities of JC-1 retained by 10,000 cells per sample were measured on a flow cytometer with 530 nm (FL-1, green) and 590 nm (FL-2, red) channels.

Statistical Analysis

All data are represented as mean ± Standard Deviation (SD). Student's t-test was used for comparison between non-treated control and treated data groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

Doxorubicin Resistance of SNU-620-DOX Cells

To estimate the doxorubicin resistance of SNU-620-DOX cells, SNU-620 cells and SNU-620-DOX cells were treated with different concentrations (0, 100, 300, 600 ng/ml) of doxorubicin for 4 days, and cell densities were measured after trypsinization (Figure 1). The densities of SNU-620 cells treated with 100, 300 and 600 ng/ml doxorubicin decreased to 13.2, 7.9 and 3.9% of that for untreated control cells, respectively. Whereas the densities of SNU-620-DOX cells decreased to 83.5, 65.8 and 25.3% of that for untreated control cells, respectively. This result indicates that SNU-620-DOX cells exhibit doxorubicin resistance.

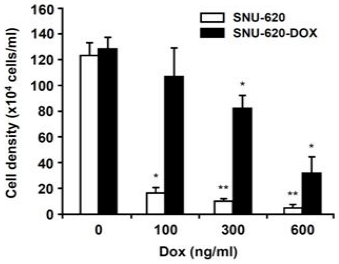

Figure 1: Effect of doxorubicin on the proliferation of gastric carcinoma SNU-620 and SNU-620-DOX cells. SNU-620 and SNU-620-DOX cells were treated with different concentrations (0, 100, 300, 600 ng/ml) of doxorubicin. Following 4 days of incubation, the cells were trypsinized and counted using a hemocytometer. The cell densities obtained from three independent experiments are represented as a bar diagram. Each experiment was performed in triplicate. Data are presented as mean ± Standard Deviation (SD) of three independent experiments (*p < 0.05, **p < 0.01). Dox, doxorubicin.

To further confirm the resistance of SNU-620-DOX cells against doxorubicin, the activation of caspase cascade were determined in SNU-620 cells and SNU-620-DOX cells treated with 100 and 300 ng/ml doxorubicin for 4 days. Western blot analysis showed that doxorubicin treatment resulted in cleavage of caspase-3 (band of 17 kDa), -8 (bands of 41 and 43 kDa), and -9 (bands of 25 and 35 kDa) in SNU-620 cells (Figure 2a). In contrast, the cleavage of caspase-3, -8, and -9 was not observed in doxorubicin-treated SNU-620-DOX cells. The levels of anti-apoptotic protein Bcl-2 and Bcl-xL were also determined in SNU-620 cells and SNU-620 -DOX cells treated with 100 and 300 ng/ml doxorubicin (Figure 2b). Bcl-2 and Bcl-xL decreased in doxorubicin-treated SNU-620 cells, but not in doxorubicin-treated SNU-620-DOX cells. The expression of Bcl-2 and Bcl-xL in SNU-620-DOX cells seemed instead to increase upon doxorubicin treatment. These results suggest that doxorubicin does not induce apoptosis in SNU-620-DOX cells, in used concentration.

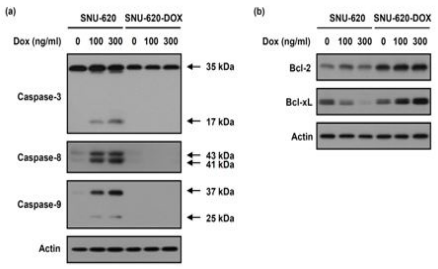

Figure 2: Effect of doxorubicin on caspase activation cascade and anti-apoptotic protein, Bcl-2 and Bcl-xL. Protein extracts were prepared from SNU-620 and SNU-620-DOX cells treated with different concentrations (0, 100, 300 g/ml) of doxorubicin. The cleavage of caspase-3, -8, and -9 (a) and the amounts of Bcl-2 and Bcl-xL (b) were determined using western blot analysis. Actin was used as a control.

To examine the effect of PA on SNU-620-DOX cells, cells were treated with different concentrations (0, 7.5, 15, 30 μM) of PA in the presence and absence of 300 ng/ml doxorubicin for 4 days and cell densities were measured after trypainization (Figure 3). The cell densities of SNU-620-DOX cells treated with 7.5, 15 and 30 μM PA in the absence of doxorubicin decreased by 18.3, 26.8 and 41.5%, respectively. Whereas, the cell densities of SNU-620-DOX cells treated with 7.5, 15 and 30 μM PA in the presence of 300 ng/ml doxorubicin decreased by 41.2, 55.9 and 73.5%, respectively. This result indicates that PA inhibits the proliferation of SNU-620-DOX cells. This also suggests that doxorubicin resistance of SNU-620-DOX cells can be overcome by the co-treatment of PA and doxorubicin.

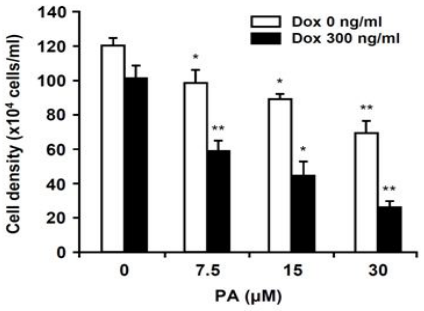

Figure 3: Effect of PA on the proliferation of SNU-620-DOX cells. SNU-620-DOX cells were treated with different concentrations (0, 7.5, 15, 30 μM) of PA in the presence and absence of 300 ng/ml doxorubicin. Following 4 days of incubation, the cells were trypsinized and counted using a hemocytometer. The cell densities obtained from three independent experiments are represented as a bar diagram. Each experiment was performed in triplicate. Data are presented as mean ± Standard Deviation (SD) of three independent experiments (*p < 0.05, **p < 0.01). PA, pomolic acid.

Effect of Co-Treatment with PA and Doxorubicin on the Activation of Caspase Cascade in SNU-620-DOX Cells

To determine the effect of PA on the activation of caspase cascade in SNU-620-DOX cells, cells were treated with different concentrations (0, 7.5, 15, 30 μM) of PA in the presence and absence of 300 ng/ml doxorubicin for 4 days and the cleavages of caspase-3, -8, and -9 were determined by western blot analysis (Figure 4a). Treatment of PA in the presence of 300 ng/ml doxorubicin increased the cleavages of caspase-3, -8, and -9, in concentration-dependent manner. While the cleavages of caspases were not observed in SNU-620-DOX cells treated with PA in the absence of doxorubicin. This result indicates that co-treatment with PA and doxorubicin induces the activation of caspase cascade in SNU-620-DOX cells. Furthermore, SNU-620-DOX cells were treated with different concentrations (0, 7.5, 15 and 30 μM) of PA in the presence and absence of 300 ng/ml doxorubicin and the levels of Bcl-2 and Bcl-xL were determined by western blot analysis

(Figure 4b). The treatment of PA slightly increased the levels of Bcl-2. The level of Bcl-xL was constant in all samples.

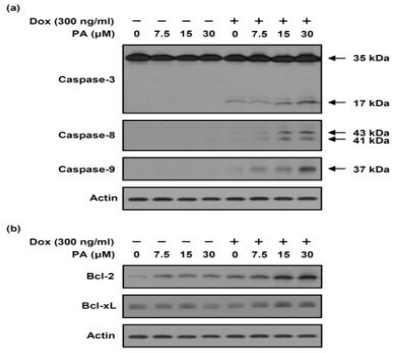

Figure 4: Effect of PA on caspase activation cascade and anti-apoptotic protein, Bcl-2 and Bcl-xL. Protein extracts were prepared from SNU-620-DOX cells treated with different concentrations (0, 7.5, 15, 30 μM) of PA in the presence and absence of 300 ng/ml doxorubicin. The cleavage of caspase-3, -8, and -9 (a) and the amounts of Bcl-2 and Bcl-xL (b) were determined using western blot analysis. Actin was used as a control.

Effect of PA on Mitochondrial Membrane Potential (ΔΨm)

To determine the effect of PA and doxorubicin on mitochondrial membrane potential (ΔΨm), SNU-620-DOX cells were treated with 30 μM PA and/or 300 ng/ml doxorubicin for 4 days and labeled with JC-1. The fluorescence intensity of JC-1 monomer (green fluorescence) and aggregate (red fluorescence) was measured using flow cytometry (Figure 5). PA increased the intensity of JC-1 monomer. The JC-1 monomer intensity was increased by the co-treatment with PA and doxorubicin in SNU-620-DOX cells, compared with single treatment of either PA or doxorubicin. This result indicates that PA reduces mitochondrial membrane potential and co-treatment with PA and doxorubicin enhances the loss of mitochondrial membrane potential.

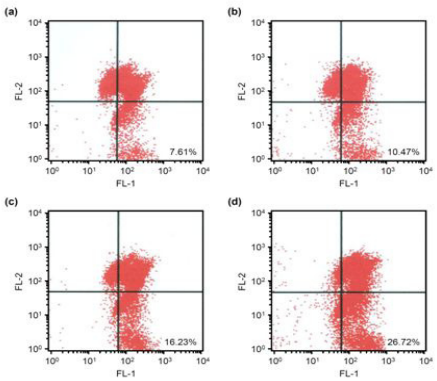

Figure 5 : Effect of PA on mitochondrial membrane potential of SNU-620-DOX cells. SND-620-DOX cells were treated with 300 ng/ml doxorubicin and/or 30 μM PA for 4 days. Flow cytometry was performed to determine the loss of mitochondrial membrane potential (ΔΨm) after staining with JC-1 cationic dye (FL-1, 530 nM green; FL-2, 590 nm red). The % of cells in down-right region were presented in the diagram.

(a) Control cells, (b) Doxorubicin-treated cells, (c) PA-treated cells, (d) Doxorubicin- and PA-treated cells.

Discussion

Human gastric cancer is the most prevalent form of cancer in East Asia, including Korea and Japan. The doxorubicin-resistant SNU-620-DOX cells were obtained from human gastric carcinoma SNU-620 cells [11,13]. Doxorubicin has been reported to reduce the survival rates of human gastric cancer cells less than 50% in ranges from 0.3 to 3 μM [14]. SNU-620-DOX cells had a high survival ability following treatment with 300 ng/ml doxorubicin, corresponding to 0.55 μM (Figure 1). This indicates that SNU-620 -DOX cells are resistant to doxorubicin.

Cells contain two main apoptotic pathways: the extrinsic pathway and the intrinsic or mitochondrial pathway [15]. Caspases, a family of cysteine proteases, are the central regulators of apoptosis. Initiator caspases (including caspase-2, -8, -9, -10, -11, and -12) are closely coupled to pro-apoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (including caspase-3, -6, and -7), which in turn execute apoptosis by cleaving cellular proteins following specific Asp residues [16]. Bcl-2 family proteins regulate the intrinsic pathway of apoptosis through the loss of mitochondrial membrane potential [17,18]. The current results demonstrated that treatment of SNU-620 cells with various concentrations of doxorubicin resulted in the reduction of procaspase-3 and increased cleaved capase-3, -8 and -9, but these results were not observed in SUN-620-DOX cells (Figure 2). Furthermore, the level of anti-apoptotic proteins was higher in doxorubicin-treated SUN-620-DOX cells. This implies that the doxorubicin resistance of SNU-620-DOX cells may be mediated by the overexpression of anti-apoptotic proteins.

The viability of doxorubicin-resistant SNU-620-DOX cells was decreased by co-treatment with PA and doxorubicin (Figure 3). Previous studies indicated that PA may be a potential agent to overcome drug resistance [9,10] and that PA induces apoptosis in human ovarian carcinoma cells via the mitochondria-mediated intrinsic pathway [12]. PA induced the activation of caspase-3, -8 and -9 in doxorubicin-treated SUN-620-Dox cells (Figure 4a), but the expressions of anti-apoptotic proteins (Bcl-2 and Bcl-xL) was not affected by PA treatment (Figure 4b). Treatment of PA reduced the mitochondrial membrane potential (Figure 5). These suggest that PA can partially overcome the doxorubicin resistance and induces apoptosis in SNU-620-DOX cells by a mitochondria-mediated intrinsic pathway, which is mediated by the loss of the mitochondrial membrane potential in anti-apoptotic protein-independent manner. To the best of our knowledge, this is the first report showing the

apoptotic effects of PA in doxorubicin-resistant gastric carcinoma cells.

Conclusion

Our results demonstrated for the first time PA-induced apoptotic effects in human carcinoma SNU-620-DOX cells showing doxorubicin resistance. Our finding indicates that co-treatment of PA and doxorubicin increases apoptosis in doxorubicin-resistant human gastric carcinoma cells, which is mediated by increasing the loss of mitochondrial membrane potential.

Acknowledgements

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