

Deguelin Restores Paclitaxel Sensitivity in Paclitaxel-Resistant Ovarian Cancer Cells via Inhibition of the EGFR Signaling Pathway

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Background: Ovarian cancer is one of women's malignancies with the highest mortality among gynecological cancers. Paclitaxel is used in first-line ovarian cancer chemotherapy. Research on paclitaxel-resistant ovarian cancer holds significant clinical importance.

Methods: Cell viability and flow cytometric assays were conducted at different time and concentration points of deguelin and paclitaxel treatment. Immunoblotting was performed to assess the activation status of key signaling molecules important for cell survival and proliferation following treatment with deguelin and paclitaxel. The fluo-3 acetoxymethyl assay for P-glycoprotein transport activity assay and cell viability assay in the presence of N-acetyl-L-cysteine were also conducted.

Results: Cell viability and flow cytometric assays demonstrated that deguelin resensitized paclitaxel in a dose- and time-dependent manner. Cotreatment with deguelin and paclitaxel inhibited EGFR and its downstream signaling molecules, including AKT, ERK, STAT3, and p38 MAPK, in SKOV3-TR cells. Interestingly, cotreatment with deguelin and paclitaxel suppressed the expression level of EGFR via the lysosomal degradation pathway. Cotreatment did not affect the expression and function of P-glycoprotein. N-acetyl-L-cysteine failed to restore cell cytotoxicity when used in combination with deguelin and paclitaxel in SKOV3-TR cells. The expression of BCL-2, MCL-1, and the phosphorylation of the S155 residue of BAD were downregulated. Moreover, inhibition of paclitaxel resistance by deguelin was also observed in HeyA8-MDR cells.

Conclusion: Our research showed that deguelin effectively suppresses paclitaxel resistance in SKOV3-TR ovarian cancer cells by downregulating the EGFR and its downstream signaling pathway and modulating the BCL-2 family proteins. Furthermore, deguelin exhibits inhibitory effects on paclitaxel resistance in HeyA8-MDR ovarian cancer cells, suggesting a potential mechanism for paclitaxel resensitization that may not be cell-specific. These findings suggest that deguelin holds promise as an anticancer therapeutic agent for overcoming chemoresistance in ovarian cancer.

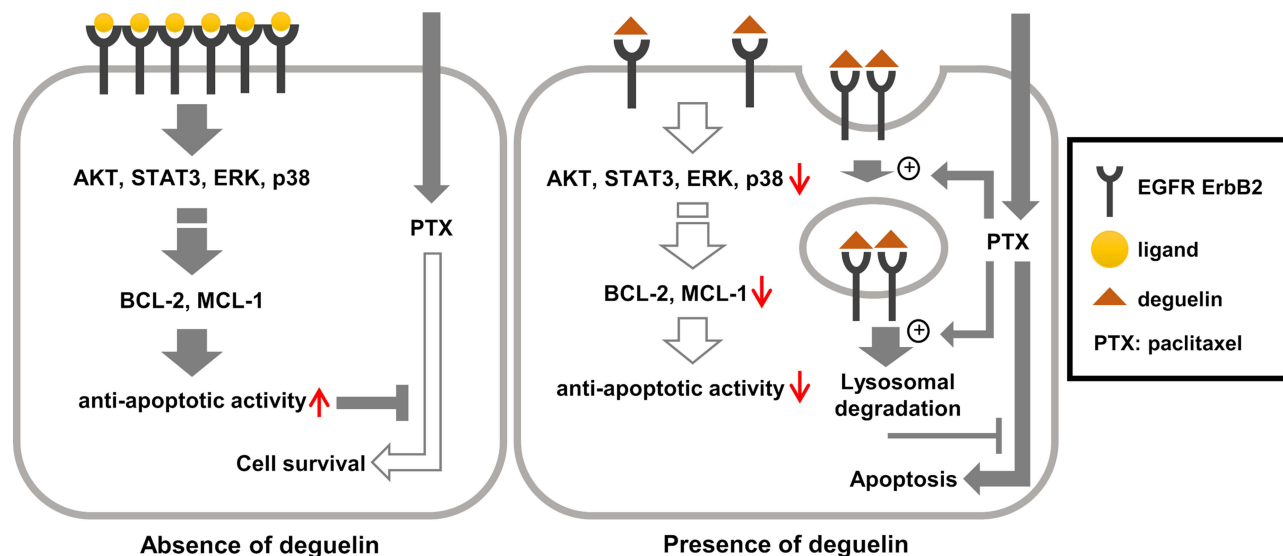
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Introduction

Ovarian cancer is one of the most lethal gynecological diseases, with a five-year survival rate of only 30%.¹ Developing biomarkers for early diagnosis and treatment of ovarian cancer is considered important to overcome ovarian cancer economically and cost-effectively.² Epithelial ovarian cancer, which accounts for approximately 90% of malignant ovarian cancer, presents as a heterogeneous gynecological disease that has been classified into six histotypes, including serous, mucinous, endometrioid, clear cell, seromucinous, and transitional cells (Brenner).^{3,4} Histopathological and molecular genetic studies have provided a subdivision of epithelial ovarian cancer into two groups: Type I ovarian carcinoma is defined as a relatively slow-growing and indolent cancer with a precursor lesion in the ovary, and type II carcinoma is a more aggressive and genetically unstable cancer that develops de novo from serous tubal intraepithelial carcinoma or ovarian epithelium.⁵ Type I carcinomas include low-grade serous, mucinous, endometrioid, clear cell, and Brenner carcinomas, while type II carcinomas include high-grade serous carcinoma, undifferentiated, and carcinosarcoma.⁶ Type I and type II ovarian carcinomas have different molecular genetic characterizations. High-grade

Graphical Abstract

Proposed mechanism of deguelin-mediated resensitization of paclitaxel in SKOV3-TR cells



serous ovarian carcinoma is the most common type of ovarian carcinoma (type II carcinoma), which is frequently mutated in *TP53* and *BRCA*, but not in genes that are predominantly mutated in type I carcinomas such as *KRAA*, *BRAF*, *ERBB2*, *PTEN*, *CTNBN1*, and *PIK3CA*.⁷ Paclitaxel has been employed as the initial chemotherapeutic option for these specific ovarian cancer types.⁸ Nonetheless, the emergence of chemoresistance remains a significant challenge to the effectiveness of ovarian cancer chemotherapy.⁹ While CA125 and HE4 measurements are currently approved diagnostic tools for ovarian cancer, they are insufficient for early detection.¹⁰ The discovery of suitable biomarkers for the development of new therapeutic agents and the diagnosis of chemosensitivity to paclitaxel to overcome chemoresistance have been considered important research areas aiming to improve ovarian cancer patient outcomes.

Paclitaxel, a natural product originally derived from *Taxus brevifolia*,¹¹ has been extensively used as an anticancer drug against several malignant cancers, including advanced ovarian and triple-negative breast carcinomas.¹² Previous studies have demonstrated that paclitaxel induces apoptotic cell death through different mechanisms or apoptotic pathways in multiple cell types.^{13,14} Paclitaxel stabilizes cellular microtubules and inhibits the formation of the mitotic spindle.¹⁵ Consequently, p53 is induced in cells and eventually activates an apoptotic pathway called mitotic catastrophe.¹⁶ In addition, it has been reported that there are various pathways by which paclitaxel can induce apoptosis through p53-independent mechanisms.¹⁷ Paclitaxel induces apoptosis by accumulating reactive oxygen species (ROS) and downregulating the ROS-scavenging enzyme superoxide dismutase (SOD) in canine mammary gland tumor cells.¹⁸ Also, paclitaxel induces apoptosis in breast cancer cells through the calcium efflux mechanism.¹⁹ Miller et al reported that paclitaxel-induced cell death requires the intrinsic apoptotic pathway, including BAK and MCL-1, in breast cancer cells.²⁰ Several reports have demonstrated that PI3K/AKT, MAPK, and STAT3 signaling, which are key cellular mediators for cell survival from various stress, are significantly involved in paclitaxel-induced apoptosis in various cells.^{21,22}

Epidermal growth factor receptors (EGFRs), members of the ErbB receptor family, consist of four members with a common structural backbone: EGFR (ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4).²³ EGFR contains a cytoplasmic tyrosine-kinase domain, a transmembrane domain, and an extracellular ligand-binding domain.²⁴ EGFR is able to form a variety of homo- or heterodimers, each combination modulating intracellular signaling while conferring diversity to different ligand affinities and receptor tyrosine kinase activity.²⁵ EGFR signaling plays critical roles in various cellular events, including proliferation, differentiation, development, and apoptosis.²⁶ The oncogenic

property of EGFR was first identified when the v-ErbB viral oncogene of the avian erythroblastosis virus was found to be a homolog of the human EGFR gene.²⁷ Many reports have indicated that EGFR is commonly upregulated in various cancers, including non-small-cell lung cancer, pancreatic cancer, glioblastoma, breast cancer, and head and neck cancer.^{28–33} Amplifications of the EGFR gene have been reported in more than half of glioblastoma multiforme (GBM), and it has been known that high levels of EGFR activity directly affect the malignancy of GBM.³⁴ In non-small cell lung cancer (NSCLC), EGFR overexpression or intracellular EGFR mutations represent about 43–89% of cases.²⁹ Also, it has been reported that 25% of NSCLC have mutations in the EGFR tyrosine kinase domain, correlating with increasing receptor expression in 75% of cases.³⁵ Epithelial ovarian cancer has also been linked to amplifications and overexpression of multiple EGFR family members.³⁶ Although anti-EGFR-targeted therapy has shown limited clinical activity in ovarian cancer to date,³⁷ several reports have suggested that the expression level of EGFR is a prognostic biomarker for ovarian cancer.^{38,39} However, the effect of EGFR signaling on paclitaxel resistance in ovarian cancer has not been reported yet.

EGFR can modulate its activity level through spatiotemporal control of the receptor induced by endocytic trafficking.⁴⁰ The regulation of EGFR expression occurs not only by the ubiquitination/proteasomal degradation pathway but also by the endocytosis/lysosomal degradation pathway.^{41–43} The strength of ligand-binding between the EGFR heterodimers could affect ligand dissociation efficiency in the endosome, which leads to lysosomal degradation of EGFR, affecting the stability of the EGFR family.⁴¹ In canonical ligand-dependent EGFR signaling, receptor endocytosis and recycling have been reported to be important for the temporal regulation of EGFR signaling.⁴⁴ Also, clathrin-dependent or non-clathrin endocytic pathways are utilized differently depending on the ligand concentration or cellular environment to maintain or weaken the EGFR signaling mechanism.⁴⁵ A number of studies suggest that the mechanisms of EGFR endocytosis and degradation, facilitated by small molecules, are stimulus-dependent and complex.^{46,47} Consequently, the exploration of mechanisms of EGFR degradation and the discovery of novel EGFR degraders are crucial for the development of new strategies to control EGFR-positive tumors.

Deguelin, a member of the rotenone family, is a compound isolated from *Derris trifoliata* Lour. or *Mundulea sericea* (*Leguminosae*).⁴⁸ Rotenone, a highly toxic deguelin-analogue, has traditionally served as an insecticide and a herbicide for an extended period of time,⁴⁹ and has been studied as an inducer of Parkinson's disease.^{50,51} Despite their structural similarities to rotenone, several studies have reported that deguelin exhibits distinct aspects of toxicity and molecular function compared to rotenone.^{52–56} Accumulating evidence has supported the significant anticancer potential of the natural product deguelin across various human cancer models, including lung cancer,⁵⁷ hepatocellular carcinoma,⁵⁸ colorectal cancer,⁵⁹ esophageal carcinoma,⁶⁰ metastatic melanoma,⁶¹ and breast cancer.⁶² Molecular mechanisms underlying deguelin-mediated anti-tumor effects have been elucidated, involving the inhibition of Aurora B kinase to delay cell cycle progression, modulation of metabolic pathways such as glycolysis suppression, attenuation of angiogenesis and metastasis, and activation of intrinsic apoptosis.^{63–65} However, the effect of deguelin on paclitaxel-resistant ovarian cancer cells was not clear. In the present study, we first investigated the inhibitory effect of deguelin on paclitaxel resistance in SKOV3-TR cells.

Materials and Methods

Cell Culture and Reagents

Ovarian cancer cell lines in this study (SKOV3, SKOV3-TR, and HeyA8-MDR) were provided by Dr. Anil K. Sood (University of Texas MD Anderson Cancer Center, TX, USA). SKOV3 cells were subcultured in RPMI 1640 medium (Biowest, Nuaille, France) supplemented with 10% fetal bovine serum (FBS) (Corning, NY, USA) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, USA). SKOV3-TR and HeyA8-MDR cells were grown in culture medium with 50 nM paclitaxel (Cayman Chemical Company, Ann Arbor, MI, USA) to sustain their paclitaxel resistance. All cells were cultured in a humidified incubator at 37 °C with 5% CO₂. Antibodies against AKT (#9272), phospho-AKT (Ser473) (#9271), ERK (#9102), phospho-ERK (Thr202/Tyr204) (#9101), p38 (#9212), phospho-p38 (Thr180/Tyr182) (#9211), STAT3 (#9139), phospho-STAT3 (Tyr705) (#9145), phospho-STAT3 (Ser727) (#94994), EGFR (#2232), phospho-EGFR (Tyr1068) (#2234), ERBB2 (#2165), phospho-ERBB2 (Tyr1248) (#2247), BCL-XL (#2764), cleaved caspase 3 (#9664), cleaved PARP (#5625), MCL-1 (#5453), BAX (#5023), survivin (#2803), phospho-BAD

(Ser115) (#9297), and MDR1/ABCB1 (#12693) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-BAD (sc-8044) and anti-Actin (sc-47778) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-BCL-2 antibody was obtained from ABclonal (Woburn, MA, USA). Anti-procaspase 3 antibody was from Abcam (Waltham, Boston, USA). Deguelin and paclitaxel were obtained from Cayman Chemical Company (MI, USA). Dimethyl sulfoxide (DMSO), N-acetyl-L-cysteine (NAC), Bafilomycin A1, MG132, and epidermal growth factor (EGF) were purchased from Sigma-Aldrich (Darmstadt, Germany). Gefitinib and erlotinib were from MedChemExpress (NJ, USA).

Cell Viability Assay Using Water-Soluble Tetrazolium (WST-1) Analysis and Crystal Violet Staining

WST-1 analysis was performed using EZ-Cytox (DoGenBio, Seoul, Republic of Korea) according to the manufacturer's protocol. SKOV3-TR (5×10^3) and HeyA8-MDR (7×10^3) cells were seeded on 96-well plates and further cultured for 24 h. Cells were treated with serially diluted paclitaxel (0–1000 nM) together with deguelin (0, 5, and 10 μ M), and further incubated for 48 h. The EZ-Cytox solution was diluted to 10% in the medium and subsequently added to each well. Cell viability was investigated by measuring the absorbance at 450 nm using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). SKOV3-TR (4×10^4) and HeyA8-MDR (5×10^4) cells were seeded in 24-well plates and further incubated for 24 h. Cells were treated with serially diluted paclitaxel (0, 25, 50, 100, 200, and 400 nM) together with deguelin (5 and 10 μ M). After 48 h of incubation, the cells were added to 500 μ L of 0.2% crystal violet solution (BioPure, Seoul, Republic of Korea) in each well and stained for 30 min.

Flow Cytometry for Apoptosis Analysis

Apoptosis induced by combined treatment with deguelin and paclitaxel was analyzed by the Annexin V-FITC/PI apoptosis staining/detection kit (Abcam, Cambridge, UK) following the manufacturer's protocols. SKOV3-TR (2×10^5) cells were treated with paclitaxel (200 nM), deguelin (10 μ M), and cotreated with paclitaxel and deguelin for 48 h. Cells were resuspended in 1X binding buffer and incubated with Annexin V-FITC and PI staining solution for 5 min in the dark. The quantity of apoptotic cells was analyzed using BD FACSCalibur (BD Bioscience, Mountain View, CA, USA).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNAs were extracted using a Ribo-EX reagent (GeneAll Biotechnology Co., Ltd., Seoul, Republic of Korea), and cDNAs were synthesized from 1 μ g of the total RNAs using oligo dT, 1.5 mM dNTP, 0.1 M DTT, 5X First Strand Buffer, and M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). The EvaGreen qPCR Mix Plus (ROX) master mix from Solis BioDyne (Tartu, Estonia) was utilized in the RT-qPCR. The StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) was employed for the analysis of relative quantifications of mRNA for the target gene. The expression levels of mRNA were normalized to the expression level of *glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)* housekeeping gene, an internally expressed reference gene. The primers used for the amplification of specific genes in this study were listed in [Supplementary Table 1](#).

Fluo-3/AM Assay (P-Glycoprotein Transport Assay)

SKOV3 (6×10^5) cells and SKOV3-TR (6×10^5) cells were incubated in culture medium for 24 h. Then, cells were treated with paclitaxel (200 nM), deguelin (10 μ M), and a combination of deguelin (10 μ M) and paclitaxel (200 nM), respectively. After treatment, cells were further incubated for 24 h. Following this, 4 μ M fluo-3 acetoxymethyl (AM) solution (Sigma Aldrich, St. Louis, USA) was added, and the cells were further incubated for 1 h at 37 °C. The cells were visualized by UV microscopy (Zeiss, Oberkochen, Germany).

Statistical Analysis

All statistical analyses were performed in triplicate. All data are presented as the mean \pm standard deviation (SD). Normally distributed data were evaluated using a one-way analysis of variance (ANOVA), as presented in the figure legends. For all results, values of * $p < 0.05$ and *** $p < 0.001$ were considered statistically significant.

Results

Deguelin Resensitized Paclitaxel in SKOV3-TR Ovarian Cancer Cells

We first validated the differential sensitivity of paclitaxel in SKOV3 and SKOV3-TR ovarian cancer cells. Cells were treated with serially diluted paclitaxel (0–1000 nM) for 48 h, and cell viability was examined using the WST-1 analysis (Figure 1A). As a result, SKOV3-TR cells showed cell viability of up to 80% or more even when treated with 1000 nM paclitaxel, while SKOV3 cells showed cell viability of less than 50% even at a concentration of 7.8125 nM paclitaxel. This indicates that SKOV3-TR cells are highly resistant to paclitaxel (Figure 1A). Next, we examined whether cell viability was affected by deguelin treatment alone in SKOV3-TR cells using the WST-1 analysis. SKOV3-TR cells were treated with deguelin at concentrations of 2.5, 5, 10, and 20 μ M for 48 h. As shown in Figure 1B, the WST-1 analysis showed no significant effect on cell viability by deguelin at concentrations of up to 10 μ M ($p > 0.1$), but 20% cytotoxicity was observed when treated with 20 μ M deguelin. We examined the effect of deguelin on paclitaxel resistance in SKOV3-TR cells. Cells were treated with serially diluted paclitaxel (0–500 nM) together with dose-different combinations (0, 5, and 10 μ M) of deguelin for 48 h. WST-1 analysis showed that deguelin effectively decreased cell viability in paclitaxel-treated SKOV3-TR cells in a dose-dependent manner (** $p < 0.001$) (Figure 1C). To determine whether the mechanism of deguelin-induced resensitization of paclitaxel was associated with apoptosis in SKOV3-TR cells, immunoblotting was performed to detect cleaved PARP. As shown in Figure 1D and E, the cleaved PARP proteins were effectively increased by deguelin in paclitaxel-treated SKOV3-TR cells in a time- and concentration-dependent manner, indicating that deguelin could restore the paclitaxel sensitivity in SKOV3-TR cells through the apoptotic pathway. Next, the effect of deguelin on restoring paclitaxel resistance in SKOV3-TR cells was confirmed by microscopic observation and FACS analysis. SKOV3-TR cells were treated with paclitaxel, deguelin, and the combination of paclitaxel and deguelin, respectively, for 48 h. Microscopic observations also showed that deguelin clearly resensitized the paclitaxel in SKOV3-TR cells (Figure 1F). As shown in Figure 1G, the level of apoptotic activity was analyzed by Annexin V-FITC and PI staining. The percentage of apoptotic cells was significantly increased in SKOV3-TR cells cotreated with paclitaxel and deguelin compared to SKOV3-TR cells treated with deguelin and paclitaxel alone (** $p < 0.001$). These results demonstrated that deguelin effectively restored paclitaxel sensitivity in SKOV3-TR ovarian cancer cells.

Deguelin Inhibited the Phosphorylation of AKT, STAT3, ERK, and p38 MAPK in Paclitaxel-Treated SKOV3-TR Cells

According to previous reports, the pivotal role of intracellular signaling molecules in affecting cell proliferation and survival has been identified, and their expression and phosphorylation levels are known to play an important role in the chemoresistance of various cancer cells.^{22,66–68} Therefore, we investigated the expression and phosphorylation levels of cellular signaling kinases involved in cell proliferation and survival, including AKT, STAT3, ERK, and p38 MAPK, through treatment with deguelin and paclitaxel in SKOV3-TR cells. Immunoblotting showed that the expression and phosphorylation of these molecules were not affected in SKOV3-TR cells treated with either paclitaxel or deguelin alone compared to control (Figure 2). However, the phosphorylation levels of AKT, STAT3, ERK, and p38 MAPK were inhibited in SKOV3-TR cells cotreated with deguelin and paclitaxel (Figure 2). These data revealed that deguelin can simultaneously inhibit AKT, STAT3, ERK, and p38 MAPK, crucial for cell proliferation and survival, and may also affect upstream signaling mechanisms common to their activation in paclitaxel-treated SKOV3-TR cells.

Deguelin Inhibited the Expression Level of EGFR in Paclitaxel-Treated SKOV3-TR Ovarian Cancer Cells via Enhancement of the Lysosomal Degradation Pathway

Recently, several studies have reported that deguelin can inhibit EGFR signaling and induce apoptosis in malignant cancer cells, including non-small cell lung cancer,⁶⁹ breast cancer,⁷⁰ and head and neck squamous cell carcinomas.⁷¹ Although apoptosis of SKOV3-TR ovarian cancer cells was not observed by treatment with deguelin alone in the above experimental results, we tested whether the resensitization of paclitaxel by deguelin was due to inhibition of EGFR signaling in SKOV3-TR cells. Immunoblotting showed that the expression of EGFR protein and its phosphorylation at the Tyr1068 residue were decreased by deguelin in paclitaxel-treated SKOV3-TR cells (Figure 3A). However, no significant differences in transcriptional expression were observed by RT-qPCR (Figure 3B). In addition, the expression

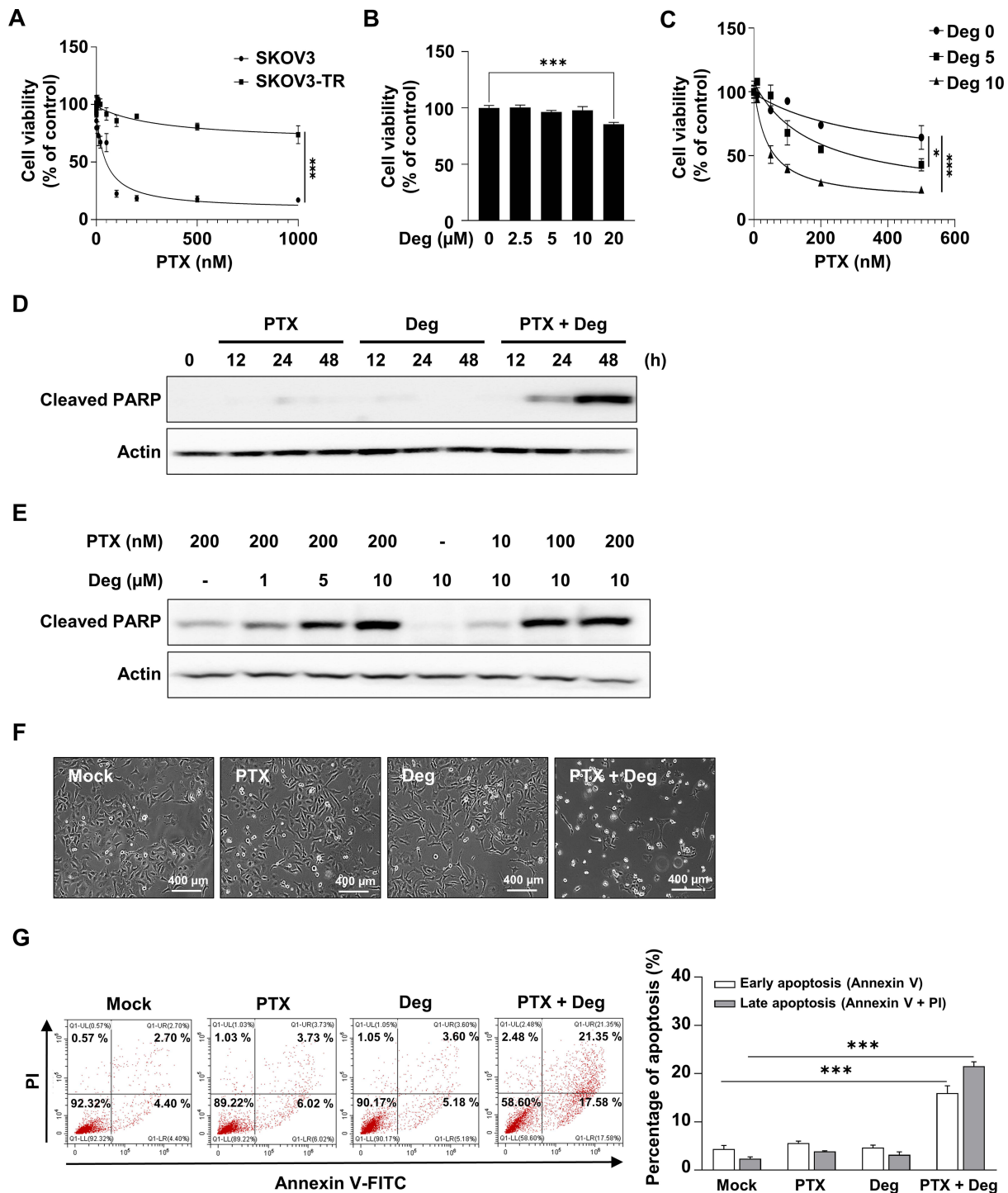


Figure 1 Cotreatment with deguelin and paclitaxel induced cell cytotoxicity in SKOV3-TR ovarian cancer cells. **(A)** SKOV3 (5×10^3) and SKOV3-TR (5×10^3) cells were seeded in 96-well plates and further incubated for 24 h. Cells were treated with serially diluted paclitaxel (0–1000 nM) for 48 h. **(B)** SKOV3-TR cells were treated with deguelin (0, 2.5, 5, 10, and 20 μ M) for 48 h. **(C)** SKOV3-TR (5×10^3) cells were seeded in a 96-well plate for 24 h, then treated with serially diluted paclitaxel (0–500 nM) together with dose-different combinations (0, 5, and 10 μ M) of deguelin. The cell viability was examined by WST-1 analysis. **(D)** SKOV3-TR (5×10^5) cells were seeded in 100 mm plates. After 24 h, cells were treated with DMSO (mock), paclitaxel (200 nM), deguelin (10 μ M), and cotreated with deguelin (10 μ M) and paclitaxel (200 nM) for 12 h, 24 h, and 48 h, respectively. **(E)** SKOV3-TR cells were treated with paclitaxel (200 nM) together with dose-different combinations (0, 1, 5, and 10 μ M) of deguelin, and treated with dose-different combinations (0, 10, 100, and 200 nM) of paclitaxel together with deguelin (10 μ M). At 48 h after treatment, cells were subjected to immunoblotting to examine the level of cleaved PARP. Actin was used as a loading control. **(F)** SKOV3-TR cells were treated with DMSO (mock), paclitaxel (200 nM), deguelin (10 μ M), and treated with a combination of paclitaxel (200 nM) and deguelin (10 μ M) for 48 h. The morphological changes of SKOV3-TR cells were observed by microscopy at 48 h. **(G)** SKOV3-TR (2×10^5) cells were seeded in 6-well plates and treated with paclitaxel (200 nM) and deguelin (10 μ M) for 48 h. Living, early apoptotic, and late apoptotic cells were determined using Annexin V-FITC and PI staining, followed by flow cytometry. The statistical experiments were repeated three times. * $p < 0.05$ and *** $p < 0.001$ analyzed by concentration were considered significant.

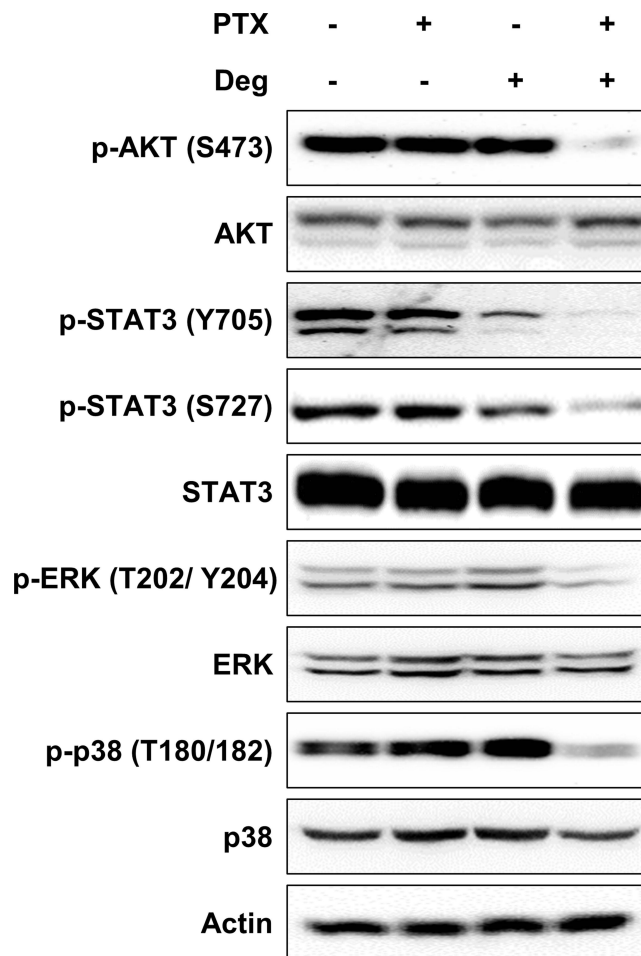


Figure 2 Inhibition of phosphorylation of AKT, STAT3, ERK, and p38 MAPK by cotreatment of deguelin and paclitaxel in SKOV3-TR ovarian cancer cells. SKOV3-TR cells were treated with DMSO (mock), paclitaxel (200 nM), deguelin (10 μ M), and cotreated with paclitaxel (200 nM) and deguelin (10 μ M), respectively. At 48 h after treatment, cells were harvested, and then the expression of AKT, STAT3, p38 MAPK, and p42/44 ERK and their phosphorylation status were examined by immunoblotting. Actin was used as a loading control.

and its phosphorylation at the Tyr1248 residue of ERBB2, one of the EGFR families, were also inhibited in SKOV3-TR cells cotreated with deguelin and paclitaxel in the same pattern of EGFR (Figure 3A and B). Also, we confirmed that the expressions of EGFR and ERBB2 in SKOV3-TR cells by cotreatment with deguelin and paclitaxel were inhibited in a concentration-dependent manner by deguelin and paclitaxel, respectively (Figure 3C). We next analyzed whether the decrease in EGFR expression by the cotreatment of deguelin and paclitaxel was caused by the ubiquitination/proteasomal degradation pathway or by the lysosomal degradation pathway. As shown in Figure 3D, immunoblotting showed that treatment with bafilomycin A1, which inhibits endosome acidification by inhibiting vacuolar H⁺-ATPase and thus prevents lysosomal degradation, restored the expression of EGFR in deguelin and paclitaxel-cotreated SKOV3-TR cells. In contrast, MG132, a proteasome inhibitor, did not affect the status of the expression of EGFR in deguelin and paclitaxel-treated SKOV3-TR cells (Figure 3D). Collectively, these results indicated that deguelin effectively inhibited the expression of EGFR by downregulating protein stability via the lysosomal degradation pathway in paclitaxel-treated SKOV3-TR cells.

Deguelin Did Not Affect the Expression and Function of P-Glycoprotein in SKOV3-TR Cells

It has been known that the most common mechanism of multidrug resistance in cancer is the overexpression of drug efflux transporters of the ATP binding cassette (ABC) family.⁷² P-glycoprotein (P-gp/MDR1) is a 170 kDa transmembrane protein and one of the members of the ABC family.⁷³ P-gp-mediated drug efflux has been widely recognized as a major driver of

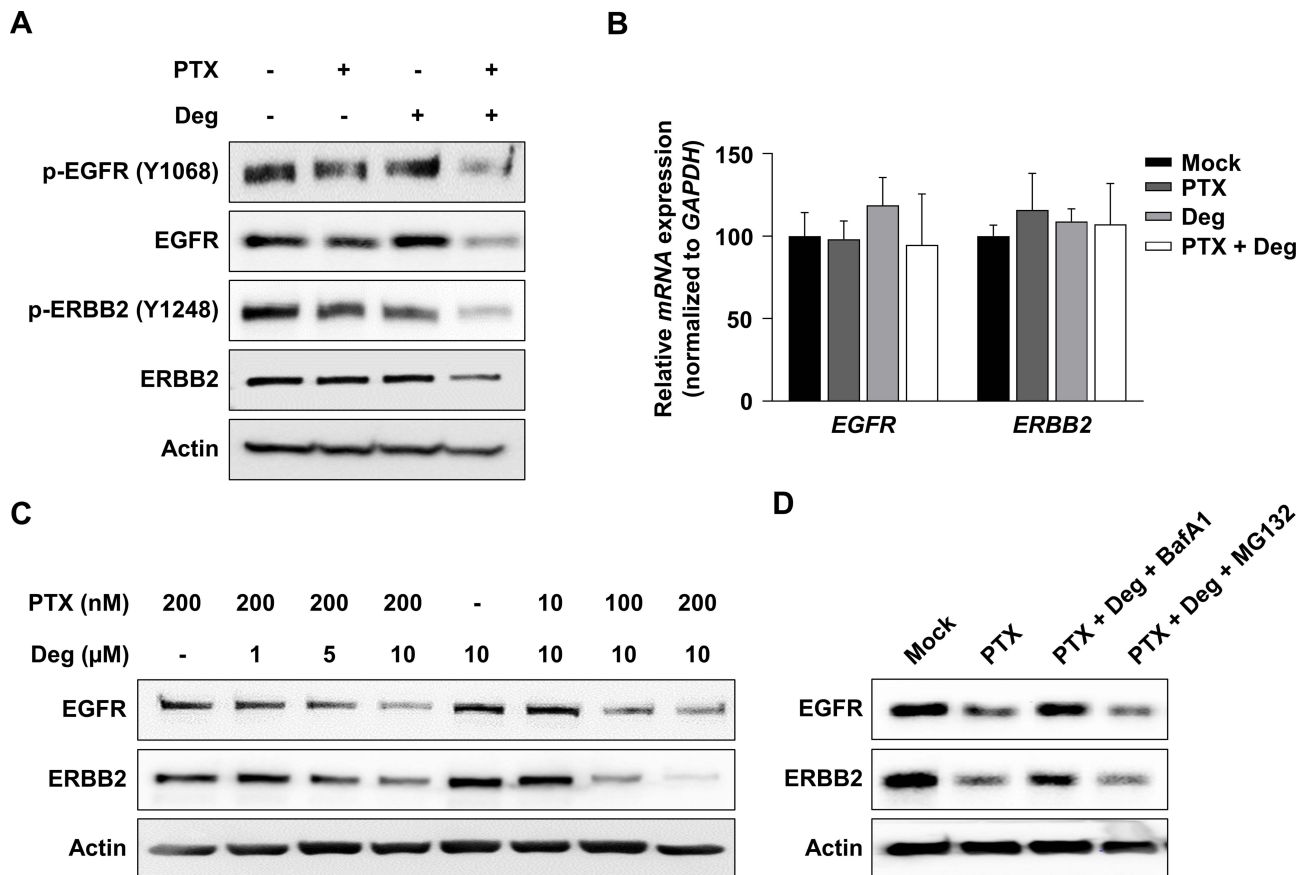


Figure 3 Downregulation of EGFR by cotreatment of deguelin and paclitaxel via the lysosomal degradation pathway in SKOV3-TR cells. **(A)** SKOV3-TR cells were treated with DMSO (mock), paclitaxel (200 nM), deguelin (10 μM), and cotreated with paclitaxel (200 nM) and deguelin (10 μM) as indicated for 48 h. The expression and phosphorylation levels of the EGFR family, including EGFR and ERBB2, were examined by immunoblotting. Actin was used as a loading control. **(B)** The transcription levels of *EGFR* and *ERBB2* were examined by RT-qPCR in the same treatment. The mRNA expression of *GAPDH* was used as a loading control. **(C)** SKOV3-TR cells were treated with paclitaxel (200 nM) together with dose-different combinations (0, 1, 5, and 10 μM) of deguelin and treated with dose-different combinations (0, 10, 100, and 200 nM) of paclitaxel together with deguelin (10 μM) for 48 h. And then the expression levels of EGFR and ERBB2 were examined by immunoblotting. **(D)** SKOV3-TR cells were cotreated with paclitaxel and deguelin. After 24 h of treatment, cells were additionally treated with DMSO, the lysosomal degradation inhibitor bafilomycin A1, and the proteasome inhibitor MG132 for 24 h. The expression levels of EGFR and ERBB2 were analyzed by immunoblotting. The statistical experiments were repeated three times.

resistance to anticancer agents including paclitaxel, cisplatin, and doxorubicin.^{74,75} We investigated whether deguelin may reduce paclitaxel resistance in SKOV3-TR cells by modulating the expression of P-gp or its function. Immunoblotting showed that P-gp was overexpressed in SKOV3-TR cells (Figure 4A), but the cotreatment of deguelin and paclitaxel did not affect the expression of P-gp in SKOV3-TR cells (Figure 4B). The cell-permeant dye fluo-3/AM is a fluorescent substrate for P-gp, and several reports have used it to measure the transport activity of P-gp.^{76,77} SKOV3-TR cells were treated with deguelin and paclitaxel alone or cotreated with deguelin and paclitaxel, and then further incubated with 4 μM fluo-3/AM for an additional 1 h. SKOV3-TR cells did not internalize the fluorescent dye compared to SKOV3 cells, as indicated by UV-microscopy, showing that fluo-3/AM was effectively excluded in SKOV3-TR cells (Figure 4C). However, the uptake of fluo-3/AM fluorescence was not increased either alone or cotreated with deguelin and paclitaxel in SKOV3-TR cells, indicating that deguelin did not affect the function of P-gp in SKOV3-TR cells (Figure 4C). These results revealed that cotreatment of deguelin with paclitaxel did not inhibit the expression of P-gp or substrate transport function in SKOV3-TR cells.

Deguelin Suppressed the Expression of BCL-2 and MCL-1 and the Phosphorylation of BAD in SKOV3-TR Cells

Next, we attempted to examine the mechanism by which deguelin inhibits paclitaxel resistance in SKOV3-TR cells. Several reports indicated that the mechanism of cell death caused by paclitaxel treatment may be due to excessive

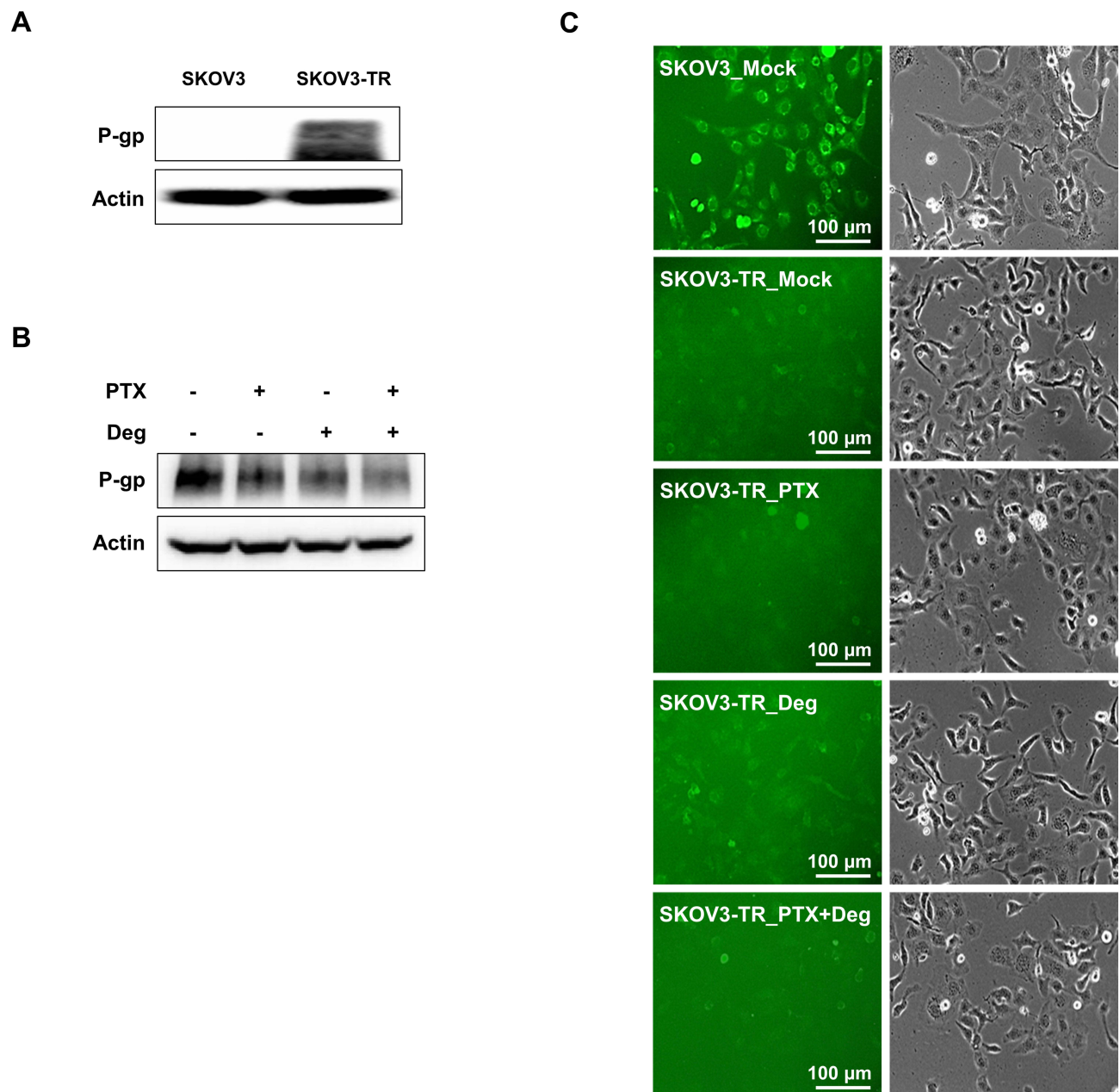


Figure 4 The expression of P-glycoprotein (P-gp) and its efflux function were not affected by deguelin or paclitaxel in SKOV3-TR cells. **(A)** The expression levels of P-gp in SKOV3 and SKOV3-TR cells were confirmed by immunoblotting. Actin was used as a loading control. **(B)** SKOV3-TR cells were treated with paclitaxel, deguelin, and cotreated with paclitaxel and deguelin as indicated. At 48 h posttreatment, the expressions of P-gp were examined by immunoblotting. **(C)** SKOV3-TR cells were treated with paclitaxel and deguelin as above. At 48 h of treatment, cells were additionally treated with the fluorogenic P-gp substrate fluo-3/AM for 1 h. Fluorescent SKOV3-TR cells were visualized by UV microscopy. SKOV3 cells were also treated with fluo-3/AM as a positive control.

induction of intracellular ROS.^{18,78} To determine whether ROS mediates the cell cytotoxicity in SKOV3-TR cells cotreated with deguelin and paclitaxel, we additionally treated NAC and then examined cell viability by WST-1 analysis. **Figure 5A** showed that NAC did not inhibit cell cytotoxicity induced by cotreatment with deguelin and paclitaxel in SKOV3-TR cells. Crystal violet staining also indicated that NAC did not restore cell viability in SKOV3-TR cells cotreated with deguelin and paclitaxel (**Figure 5B**). It has been reported that upon ligand recognition, EGFR directly recruits a class I phosphatidylinositol 3-kinase (PI3K), followed by activation of AKT.²² Moreover, AKT has been reported as a significant signaling effector for several pathways, including cell growth, modulation of metabolism, and inhibition of apoptosis.^{79,80} Since it has been reported that the PI3K/AKT pathway is important for multidrug resistance

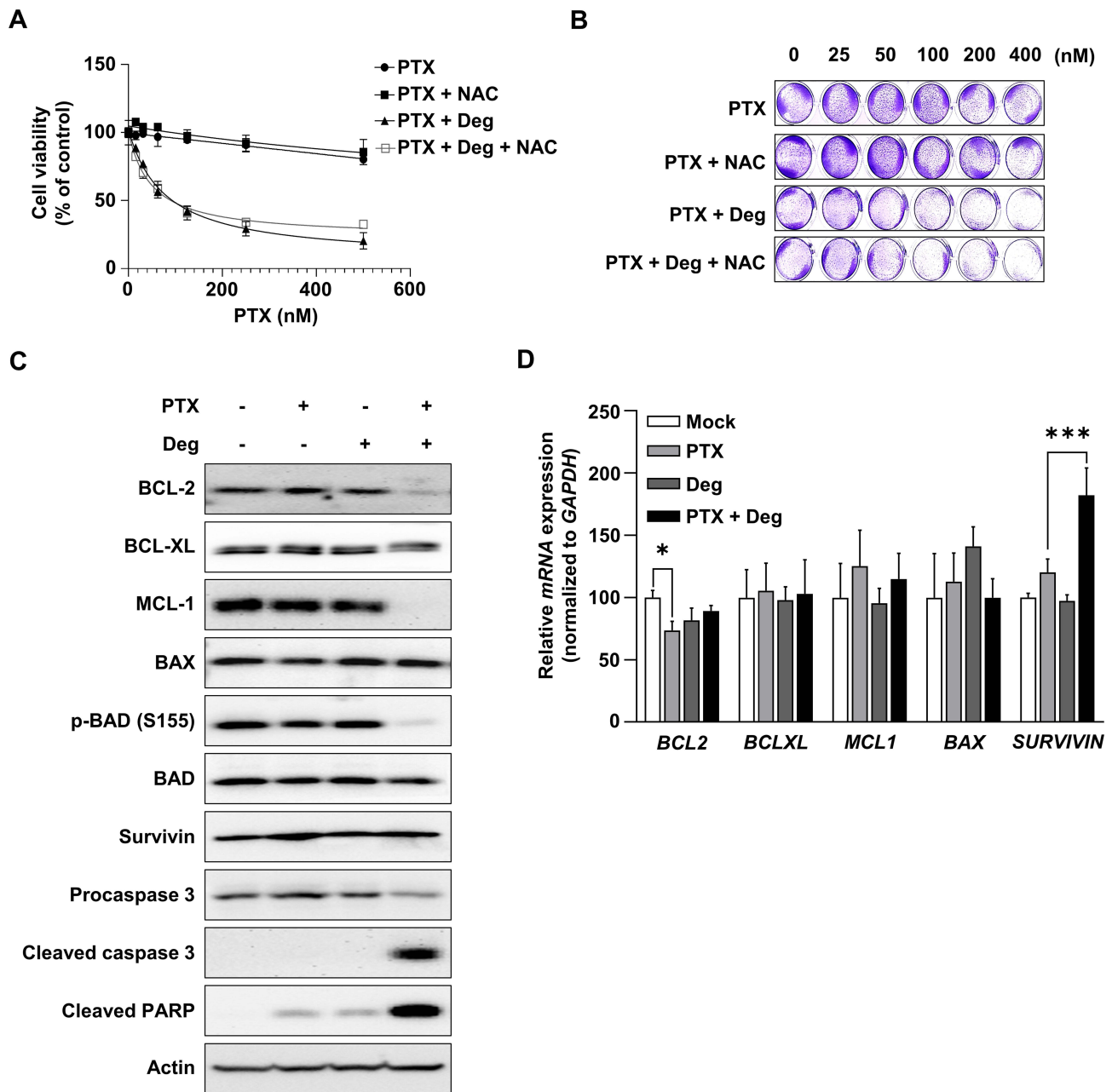


Figure 5 The expression levels of BCL-2 and MCL-1 were downregulated by cotreatment with deguelin and paclitaxel in SKOV3-TR cells. SKOV3-TR cells were seeded for 24 h and then treated with serially diluted paclitaxel (0–500 nM) together with deguelin (0 and 10 μ M). At 24 h of treatment, cells were additionally treated with 5 mM NAC for 24 h. The cell viability was examined by WST-1 analysis (A) and a crystal violet assay (B). (C) SKOV3-TR cells were treated with paclitaxel, deguelin, and cotreated with deguelin and paclitaxel as indicated. At 48 h of treatment, the expression levels of BCL-2 family proteins, including BCL-2, BCL-XL, MCL-1, BAX, BAD, phospho-BAD (S155), and survivin, were examined by immunoblotting. Also, procaspase 3, cleaved caspase 3, and cleaved PARP were analyzed as apoptosis indicators. Actin was used as a loading control. (D) The transcriptions of BCL-2 family genes were examined by RT-qPCR. The mRNA expression of GAPDH was used as a loading control. The statistical experiments were repeated three times. * $p < 0.05$ and *** $p < 0.001$ analyzed by concentration were considered significant.

processes in various cancer cells via the inhibition of apoptosis,⁸¹ we next examined whether deguelin may modulate BCL-2 family proteins. Immunoblotting indicated that the expression of BCL-2, MCL-1, and the phosphorylation of BAD at the S155 residue were downregulated by cotreatment of deguelin and paclitaxel in SKOV3-TR cells (Figure 5C). However, RT-qPCR showed the inhibition of the expression of BCL-2 and MCL-1 was not at the transcriptional level (Figure 5D). Our results indicated that deguelin downregulated the expression of BCL-2, MCL-1, and the phosphorylation of BAD at S155 through inhibition of the EGFR signaling pathway in SKOV3-TR cells.

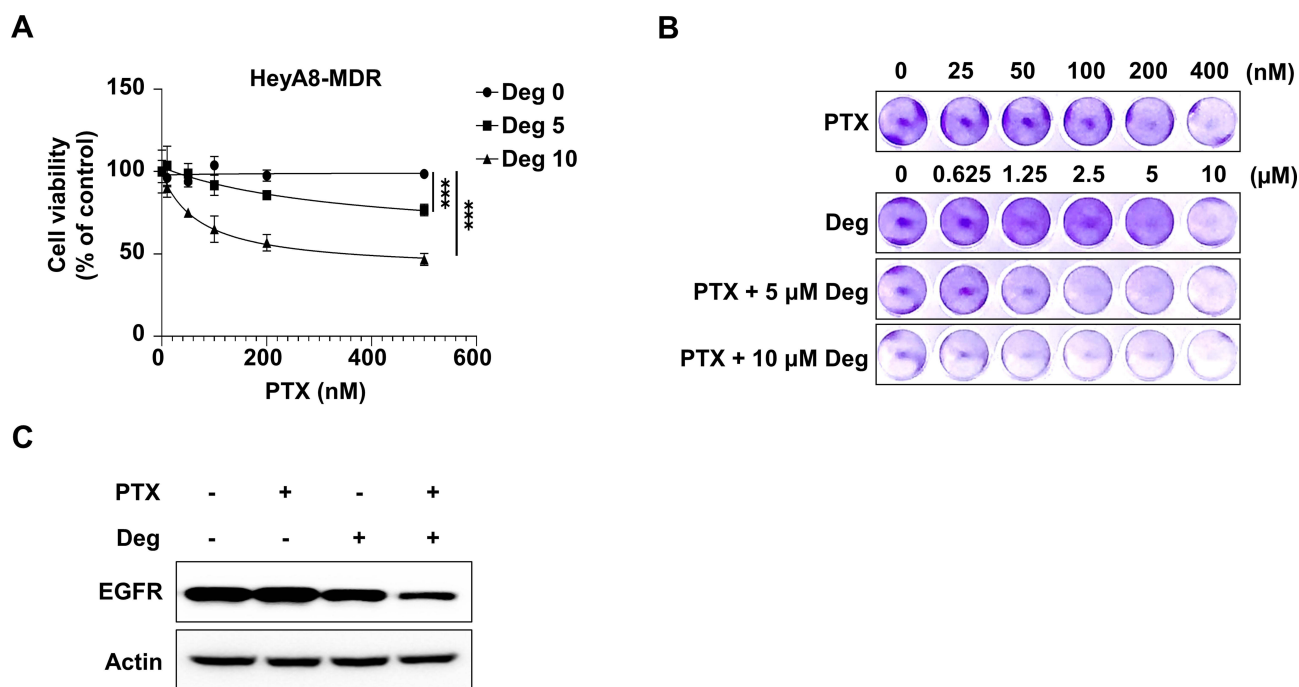


Figure 6 Cell cytotoxicity was induced by cotreatment of deguelin and paclitaxel in paclitaxel-resistant ovarian cancer HeyA8-MDR cells. **(A)** HeyA8-MDR (2×10^4) cells were seeded in a 96-well plate for 24 h, then treated with serially diluted paclitaxel (0–500 nM) together with dose-different combinations (0, 5, and 10 μ M) of deguelin. The cell viability was examined by WST-1 analysis. **(B)** HeyA8-MDR (1×10^5) cells were seeded in a 24-well plate for 24 h, then treated with paclitaxel (0, 25, 100, 200, and 400 nM) together with dose-different combinations (0, 5, and 10 μ M) of deguelin for 48 h. Then, cells were fixed and stained with a crystal violet solution. **(C)** HeyA8-MDR cells were treated with DMSO (mock), paclitaxel (200 nM), deguelin (10 μ M), and cotreated with deguelin (10 μ M) and paclitaxel (200 nM) as indicated for 48 h. The expression levels of EGFR were examined by immunoblotting. Actin was used as a loading control. The statistical experiments were repeated three times. *** $p < 0.001$ analyzed by concentration was considered significant.

Deguelin Resensitized Paclitaxel in Paclitaxel-Resistant HeyA8-MDR Ovarian Cancer Cells

To investigate whether the resensitization of paclitaxel by deguelin treatment was a cell-specific phenomenon in SKOV3-TR cells, we performed a cell viability assay using HeyA8-MDR in the same experiments as above. Notably, WST-1 analysis showed that deguelin inhibited cell viability in paclitaxel-treated HeyA8-MDR cells in a dose-dependent manner (Figure 6A). The crystal violet assay also showed that deguelin inhibited cell viability in paclitaxel-treated HeyA8-MDR cells (Figure 6B). Moreover, the expression of EGFR was downregulated by cotreatment with deguelin and paclitaxel in HeyA8-MDR cells (Figure 6C). These results indicated that restoration of paclitaxel sensitivity and inhibition of EGFR by cotreatment of deguelin and paclitaxel were not cell type-specific in SKOV3-TR cells.

Discussion

As our understanding of the molecular genetics of ovarian cancer has deepened, significant progress has been made in developing effective anticancer drugs. Recently, new therapeutic approaches for ovarian cancer patients have emerged as promising options. Among these, PARP inhibitors stand as a novel class of anticancer drugs with selective lethality in *BRCA1/2*-mutated breast and ovarian cancers lacking homologous recombination.⁸² In addition to direct lethality, PARP inhibitors can activate antitumor immunity, partially through a stimulator of interferon genes (STING)-dependent mechanism.⁸³ This effect is further enhanced by PD-1/PD-L1 blockade and occurred independently of *BRCA1/2* mutation status.⁸⁴ Moreover, some PARP inhibitors have demonstrated the ability to trap PARP1 and PARP2 on chromosomal DNA-damaging sites, resulting in the formation of PARP-DNA complexes.⁸⁵ This phenomenon, known as “PARP trapping”, interferes with the recruitment of DNA repair enzymes,⁸⁶ leading to mitotic catastrophe and subsequent cell death by aberrant DNA replication in the absence of proper DNA repair.⁸⁷ This synergistic effect is observed not only with PARP inhibitors but also with alkylating agents and platinum-based agents.⁸⁸ Despite numerous pharmacological

studies against various cancers having been reported and clinical applications of next-generation anticancer drugs represented by targeted therapy and immunotherapy having been developed, chemical anticancer drugs, including taxane and platinum-based chemicals, are still the first-line treatment for ovarian cancer patients to date.^{89,90} Recently, the most important research fields against ovarian cancer have been studied on the technology of early diagnosis, the overcoming strategies of chemoresistance, and research on the ovarian cancer-specific tumor microenvironment to reveal the reason why treatment options are limited, unlike other carcinomas. Proteomics has emerged as a promising technology with the potential to address the challenges in molecular research for early diagnosis and classification of tumors for precision medicine.⁹¹ In the context of ovarian cancer, proteomic analyses offer valuable insight not only into the molecular mechanisms underlying the disease but also into the adaptive responses of tumors to therapy. Through proteomic analysis of ovarian cancer, researchers can identify potential therapeutic targets that may help mitigate chemoresistance and improve ovarian cancer patient outcomes.⁹² We describe here a novel paclitaxel-resensitizing capacity of deguelin against paclitaxel-resistant ovarian cancer SKOV3-TR cells.

Deguelin is a member of the rotenoid family of compounds and utilized as an insecticide.⁴⁸ According to several studies, it is well known that rotenone is an inhibitor of mitochondrial NADH dehydrogenase, one of the major factors in energy metabolism, and causes cell toxicity by ATP depletion.^{93,94} In contrast, deguelin has been reported to selectively inhibit the growth of cancer cells by targeting oncogenic functions including cell cycle, apoptosis, and angiogenesis.^{48,54,95–97} For instance, it has been reported that treatment with deguelin induces apoptosis in lung cancer cells by downregulating AKT phosphorylation and stimulating ROS.⁹⁸ Li et al demonstrated that deguelin inhibits cell growth in NSCLC by downregulating hexokinase 2, a key player in glycolysis metabolism.⁵⁷ It has also been reported that deguelin induces cell death through the inhibition of AKT signaling and induction of the AMP-activated protein kinase (AMPK)-dependent autophagy pathway in Hep-2 head and neck cancer cells.⁷¹ Mehta et al reported that deguelin inhibits the cell growth of triple-negative breast cancer MDA-MB-231 cells by downregulating EGFR and c-Met, along with their downstream target proteins.⁹⁹ Recently, Wang et al reported a study investigating the enhancement of cell cytotoxicity in triple negative-breast cancer cells by loading deguelin and paclitaxel into a nano-micelles system.¹⁰⁰ Notably, our study is the first time that deguelin could affect paclitaxel resistance in ovarian cancer cells.

Figure 1B interestingly indicated that deguelin alone did not exert a significant effect on cell viability in SKOV3-TR cells. This raised the question of why the treatment of deguelin alone did not significantly affect the cell cytotoxicity (Figure 1B) as well as EGFR/AKT signaling (Figures 2 and 3A lane 3), and its downstream signaling (Figure 5C lane 3) in SKOV3-TR cells. Two hypotheses were considered to explain this phenomenon. Firstly, it is plausible that the sensitivity of deguelin might vary among different types of cancer cells. Second, the mechanism of action of deguelin on EGFR inhibition may be different. Gao et al indicated that deguelin directly interacts with EGFR, inhibiting its kinase activity in NSCLC cells in vitro and ex vivo.⁶⁹ We compared the activation levels of EGFR in SKOV3-TR cells treated with deguelin and the EGFR-specific small molecule inhibitors gefitinib and erlotinib. As a result, gefitinib and erlotinib completely inhibited the phosphorylation of EGFR at 24 h, but deguelin did not significantly affect the phosphorylation of EGFR in SKOV3-TR cells (Supplementary Figure 1A). In addition, we demonstrated that deguelin can inhibit the activation of EGFR by EGF treatment in a dose-dependent manner in SKOV3-TR cells (Supplementary Figure 1B). This mode of action of deguelin on EGFR inhibition led us to propose that the sensitivity of EGFR, which forms various homo- or heterodimers in different cells, may inevitably vary among different cell types. Further research is underway to analyze the detailed mechanism of action of deguelin in SKOV3 cells.

We also sought to understand why the degradation of EGFR (Figure 3A lane 4) and downregulation of its downstream signaling AKT and apoptotic regulators BCL-2 and MCL-1 (Figures 2 and 5C lane 4), occurred with cotreatment with deguelin and paclitaxel rather than with deguelin alone. It has been reported that paclitaxel might affect microtubule dynamics and upregulate endocytic trafficking in cells.¹⁰¹ Li et al reported that paclitaxel treatment shortened the endosomal trafficking of EGFR to the perinuclear area, delivering it rapidly into the peripheral lysosomes.¹⁰² Our findings showed that the downregulation of EGFR was not significant by the treatment with deguelin alone, but the effective degradation of EGFR by the cotreatment with deguelin and paclitaxel in SKOV3-TR cells suggested that paclitaxel may enhance the lysosomal degradation of EGFR in deguelin-treated SKOV3-TR cells. Consequently, we

concluded that deguelin restored paclitaxel sensitivity by promoting the lysosomal degradation of EGFR and inhibiting EGFR and its downstream signaling in SKOV3-TR cells.

Over the past decades, researchers have extensively investigated the molecular mechanisms of endocytic trafficking of EGFR and the role of the traffic in signal transduction by employing techniques such as fluorescence microscopy, receptor mutagenesis, and siRNA knockdown techniques.^{103–106} Accumulated evidence revealed that ligand-stimulated EGFR is recycled or degraded by receptor internalization through clathrin-mediated endocytosis (CME) with recruitment of clathrin adaptor proteins AP-2 and Grb2 or through clathrin-independent endocytosis (CIE) with the recruitment of E3 ligase c-Cbl.^{45,107} Under the physiological ligand environment, EGFR is rapidly recycled through the CME pathway; however, under the saturated ligand environment, EGFR is highly phosphorylated, then ubiquitinated by c-Cbl E3 ligase, and degraded through the CIE mechanism.⁴⁵ The EGFR signaling pathway exerts spatial and temporal regulation to modulate various cellular processes, including proliferation, survival, differentiation, and motility, in response to environmental cues such as ligand concentration and receptor distribution. Recent studies have demonstrated that small molecules can inhibit the proliferation of cancer cells by binding to EGFR and promoting its degradation.^{47,108} Xiao et al reported that proguanil enhanced EGFR endocytic degradation by receptor ubiquitination in a clathrin-independent manner in bladder cancer cells.⁴⁷ Yao et al reported that DPBA binds like an EGFR ligand, promotes EGFR degradation, and inhibits the proliferation of EGFR-positive NSCLC cells.⁴⁶ They reported that DPBA induced EGFR degradation via the lipid raft-dependent mechanism using flotillin-1 in A549 and H1975 lung cancer cell lines.⁴⁶ They also showed that DPBA does not induce degradation of EGFR via its ubiquitination. In this study, the expression level of EGFR was restored by bafilomycin A1, which inhibits lysosomal degradation, rather than the inhibitor of proteasomal degradation MG132 (Figure 3D), suggesting that deguelin can have an inhibitory mechanism of EGFR like that of DPBA, and further experiments to determine the detailed mechanism are in progress. We propose, for the first time, that deguelin promotes lysosomal degradation of EGFR, inhibits its downstream signaling, and can resensitize paclitaxel in paclitaxel-resistant ovarian cancer cells.

While many chemotherapeutic agents, including paclitaxel, are known to exert their effects through intrinsic apoptotic mechanisms, the chemoresistance mechanism of paclitaxel can vary.^{13,109–111} Paclitaxel binds to the N-terminal MT-loop domain of β -tubulin, affecting the dynamics of microtubule polymerization.¹¹² It has been reported that a point mutation in the paclitaxel-binding region of β -tubulin (T26A) leads to paclitaxel resistance in cells.¹¹³ Overexpression of the ABC transporter P-gp has also been reported in various chemoresistant cancer cells.^{114,115} Extracellular efflux of anticancer agents, including paclitaxel and cisplatin, has been implicated in playing an important role in the chemoresistance of ovarian cancer.¹¹⁶ In this study, we found that deguelin did not influence the expression and function of P-gp (Figure 4). These results suggest that deguelin exerts its effects by inhibiting cell survival signaling in SKOV3-TR, leading to apoptosis induced by paclitaxel. Despite observing a significant increase in the transcriptional levels of Survivin, which functions in cancer cell survival, this upregulation did not affect the combined treatment of deguelin and paclitaxel-induced cell cytotoxicity in SKOV3-TR cells (Figures 5C and 5D). On the other hand, the effectively inhibited protein expression of BCL-2 and MCL-1 in the cotreatment implied that the expression of BCL-2 and MCL-1 was down-regulated at the post-transcriptional level (Figures 5C and 5D).

AKT signaling is considered an attractive anticancer target for therapeutics. It has been known that AKT-mediated survival signals preserve various cancer cells from intrinsic and extrinsic stress.^{117–121} Several reports have examined the possibility that the inhibition of AKT activity can induce cell cytotoxicity by reactive oxygen species (ROS) through the suppression of the expression of ROS scavenging enzymes, including superoxide dismutase (SOD), inhibiting the FOXO signaling pathway in cancer cells.^{122–125} In particular, Xu et al reported that deguelin induces apoptosis through ROS by inhibiting AKT activity, and the cell cytotoxicity can be attenuated by the treatment of NAC in lung cancer cells.⁹⁸ Although our results showed that the phosphorylation of AKT was inhibited by cotreatment with deguelin and paclitaxel in SKOV3-TR cells, NAC did not affect cell cytotoxicity (Figures 5A and 5B), suggesting that apoptosis induced by cotreatment with deguelin and paclitaxel in SKOV3-TR ovarian cancer cells was not caused by ROS. Since we cannot rule out the possibility of diversity in the mechanism of action of deguelin, it cannot be asserted that cotreatment with deguelin and paclitaxel does not inhibit the AKT/FOXO signaling pathway or the activation of the ROS generation mechanism in SKOV3-TR cells. The PI3K/AKT pathway is frequently altered (<70%) in ovarian cancer and contributes to aggressiveness and chemoresistance in

ovarian cancer.¹²⁶ It has been reported that active PI3K signaling may increase cell survival by regulating DNA damage response (DDR).¹²⁷ Several studies have shown that active PI3K signaling is involved in DNA replication and cell cycle regulation, and thus, the inhibition of PI3K signaling increases replication stress and reduces the activity of the spindle assembly checkpoint protein Aurora kinase B, thereby causing mitotic catastrophe.^{84,127} It has also been reported that treatment with the PI3K/AKT inhibitors wortmannin and LY294002 can enhance paclitaxel-induced apoptosis in ovarian cancer cells.¹²⁸ These reports showed that the function of AKT plays an important role in cell survival in response to external stress, such as anticancer drugs, and suggested that paclitaxel-induced cytotoxicity can be effectively enhanced through the regulation of the AKT downstream pathway. It has been reported that AKT can upregulate BCL-2 expression through cAMP-response element-binding protein (CREB).¹²⁹ Moreover, studies have shown that the phosphorylation of BAD by AKT is involved in the paclitaxel resistance mechanism in human bladder carcinoma cells.¹³⁰ Gao et al reported that deguelin downregulated MCL-1 via the AKT/GSK3 β /FBW7 pathway in NSCLC cells.⁶⁹ We confirmed that the expressions of BCL-2 and MCL-1 were downregulated by cotreatment with deguelin and paclitaxel in SKOV3-TR cells (Figure 5C). Although downregulations of BCL-2, MCL-1, and phosphorylation of BAD were marginally reduced by treatment with deguelin alone, it showed that cell cytotoxicity can be restored by treatment with deguelin in paclitaxel-resistant ovarian cancer cells, as the same mechanism occurs in bladder and lung cancer cells. In Figure 2, we indicated that cotreatment with deguelin and paclitaxel also inhibited the phosphorylation of STAT3, ERK, and p38 MAPK, which are common downstream signaling molecules of EGFR. Several studies have reported that STAT3,¹³¹ ERK,¹³² and p38 MAPK¹³³ signaling pathways affect the proliferation of various cancer cells as well as chemoresistance, and we are analyzing the effects of these signaling pathways on proliferation and chemoresistance in ovarian cancer cells through additional studies.

Conclusion

The findings of this study underscore the potential of deguelin as an anticancer drug for the treatment of chemoresistant ovarian cancer. Our data showed that deguelin induced lysosomal degradation of EGFR, thereby inhibiting the cell survival signaling pathway. In addition, our results demonstrated that deguelin effectively resensitized the paclitaxel in SKOV3-TR cells by activating the intrinsic apoptosis pathway through downregulation of antiapoptotic factors, including BCL-2 and MCL-1. According to several studies showing the role of EGFR signaling in conferring chemoresistance to various anticancer drugs, including cisplatin in various carcinomas,^{26,134,135} deguelin holds promise for combating chemoresistance in EGFR-positive cancer cells.

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Author Contributions

All authors have played a crucial role in the development of this work, contributing significantly to its conception, study design, execution, data acquisition, analysis, and interpretation. Each author has actively participated in drafting, revising, and critically reviewing the article. Moreover, all authors have provided their final approval for the version to be published, unanimously selected the journal for submission, and committed to being accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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