Deoxyrhapontigenin, a Natural Stilbene Derivative Isolated From *Rheum undulatum* L. Induces Endoplasmic Reticulum Stress–Mediated Apoptosis in Human Breast Cancer Cells

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**Abstract**

Although current chemotherapeutic agents are active at the beginning of therapy, the most common risk is the development of resistance during later stages in almost all cancer types including breast cancer. Hence, investigation of novel drugs is still a priority goal for cancer treatment. The objective of the present study is to investigate the anticancer effect of a derivative of stilbene, deoxyrhapontigenin (DR) isolated from *Rheum undulatum* L. root extracts against the chemoresistant MCF-7/adr and its parental MCF-7 human breast cancer cells. The morphological images indicate that DR induces an extensive cytoplasmic vacuolation in breast cancer cells. Mechanistic investigations revealed that DR treatment causes endoplasmic reticulum (ER) dilation and upregulated the expression of ER stress markers GRP78, IRE1α, eIF2α, CHOP, JNK, and p38. Subsequently, we also identified that DR increases the levels of apoptotic fragment of PARP (89 kDa) in breast cancer cells. Blocking the expression of one of the components of the ER stress–mediated apoptosis pathway, CHOP using siRNA significantly decreased DR-induced apoptotic cleavage of PARP. In summary, the present study suggests that the induction of ER stress–mediated apoptosis by DR may account for its cytotoxic effects in human breast cancer cells.

**Keywords**

*Rheum undulatum* L., deoxyrhapontigenin, breast cancer, chemoresistant, ER stress, apoptosis

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**Introduction**

Currently, breast cancer continues to be the second leading cause for cancer-related deaths among women worldwide. It is one of the most difficult types of cancer to treat because of its heterogeneity, poor response and resistance to therapy, as well as tumor relapse even after surgical treatment.¹ In the year 2012, approximately 1.7 million new breast cancer cases were diagnosed worldwide, which represents about 12% of all new cancer cases and 25% of all cancers among women. Among them, 24% were within the Asia-Pacific region with the greatest number in China (46%), Japan (14%), and Indonesia (12%). Notably, in the region of Eastern Asia, the incidence of breast cancer in South Korea was almost comparable to Japan (both 52 per 100 000).²

For many decades, natural products have served us well in combating various diseases including cancer. Plants and microbes remain the main source for these successful compounds because of their fewer side effects and greater therapeutic efficiency.³ Doxorubicin is an anthracycline antibiotic derived from bacterial species recognized as an essential component of many treatment regimens for solid and blood tumors. Furthermore, it is broadly considered as the most active single agent available for breast cancer treatment. However, the development of resistance and its intrinsic cardiotoxicity leads to an unsuccessful outcome in many patients.³ Hence finding a novel drug for breast cancer treatment is highly desirable. Extensive research...
over the past several decades has led to identification of numerous plant-derived metabolites with diverse chemical structures such as flavonoids, stilbenes, terpenes, and alkaloids, and they have been proposed as cancer chemopreventive agents. The present study aimed to investigate the cytotoxic potential of a naturally occurring stilbene derivative, deoxyrhapontigenin (DR), isolated from root extracts of *Rheum undulatum* L. (Polygonaceae) against human breast cancer using the doxorubicin resistant, MCF-7/adr and its parental, MCF-7 breast cancer cell lines.

### Materials and Methods

#### Plant Material

The roots of *R. undulatum* L. were collected from Kyungdong market, Seoul, Korea. The plant material was authenticated by the corresponding author (Y.K.K). A voucher specimen (RhuD2P1) was stored at the herbarium of the College of Forest Science, Kookmin University, Seoul, Korea. The collected roots were washed with water, cut into small pieces, dried, and milled to get powder. Then the powder (4.5 kg) was extracted with methanol 3 times, for 5 hours each at 45°C. The extracts were combined, filtered, and concentrated under reduced pressure at 45°C to 50°C (crude yield: 1.22 kg).

#### Isolation and Purification of Compounds

The crude extracts were sequentially fractionated with dichloromethane, ethyl acetate and followed by aqueous solvents. When the dichloromethane fraction was concentrated to dryness, the yield was 288.83 g. Around 60 g of crude extract from dichloromethane fraction was subjected to column chromatography using silica gel column. The column was eluted in sequence by increasing the percentage of ethyl acetate from 25% to 100% with hexane to give D1, D2, D3, and D4 fractions. The D2 fraction was concentrated (24.16 g), eluted in sequence by increasing the ratio of methanol from 2 to 100 with dichloromethane. By following thin layer chromatography, the fractions containing the same compound was spooled and dried. The compound was subjected to purification (purity >98%) by high-performance liquid chromatography (HPLC) using X-Terra RP column, No. 186000456 (2.1 × 150 mm, 5 μm) and isocratic elution was performed with methanol and distilled water (HPLC grade) (1:1) with 0.1% formic acid (flow rate 0.2 mL/min; injection volume 5 μL; retention time 7-8 minutes) and then crystallization was done. The crystals (melting point 175°C to 178°C) were characterized by nuclear magnetic resonance (Bruker Ascend 400MHz NMR), and mass spectroscopy using HPLC–mass spectrometry with water micromass ZQ detector. The resulting compound was characterized and identified as deoxyrhapontigenin (DR).

#### Drugs and Inhibitors

A stock solution of DR was prepared in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. Staurosporine, actinomycin-D (Act-D), and bafilomycin A1 (BafA1) stock solutions were also prepared in concentrations of 1 mM in DMSO. Cycloheximide (CHX) was prepared at 2 mM concentration in ethanol. 3-Methyladenine (3-MA) was prepared in cell culture media at 10 mM concentration. All stock solutions were stored at −20°C and diluted to the required concentration as and when needed.

#### Cells and Treatment

Human breast cancer cell line MCF-7 was procured from Korean cell line bank (KCLB). Chemo-resistant (Doxorubicin/adriamycin) cell line, MCF-7/adr was graciously given by Professor Jae-Hong Kim, School of Life Sciences and Biotechnology, Korea University, 5-1 Anamdong, Sungbuk-gu, Seoul, Korea. Cancer cells were maintained either in RPMI1640 (MCF-7) or Dulbecco’s modified Eagle medium (DMEM) (MCF-7/adr) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and glutamine (2 mM). For experimental conditions, cells were treated with DR diluted in RPMI1640 or DMEM without FBS and antibiotics for up to 24 hours.

#### Cytotoxicity Assay

The colorimetric MTT cell viability assay was performed to determine the cytotoxic effects of DR on human breast cancer cells. Briefly, cells (1 × 10^4 cells/well) were seeded in 96-well plates and incubated for attachment overnight. After treatment for 24 hours with DR, MTT solution was added to a final concentration of 0.5 mg/mL and incubated for additional 4 hours at 37°C. Then the medium was removed and formazan crystals were solubilized in DMSO after which absorbance was measured at 570 nm. To assess the effects of autophagic (3-MA, BafA1), and paraptotic (CHX, Act-D) inhibitors on DR-induced breast cancer cell death, cells were treated with inhibitors 1 hour prior to treatment with DR for 24 hours. Then, MTT assay was performed to determine the percentage level of cell viability.

#### Detection of Acidic Vacuoles

In order to identify the formation of acidic vacuoles during autophagy, cells were stained with acridine orange (AO) at a final concentration of 1 μg/mL for 30 minutes in the dark after a 12-hour treatment with DR. Images were then captured under a fluorescent microscope. AO is a weak base that can accumulate in acidic cellular compartments and emits bright red fluorescence. The intensity of red
fluorescence is proportional to the volume of cellular acidic compartments.12

Characterization of Cellular Morphological Changes

To monitor the effect of DR on cellular morphological changes, cells were treated with DR for 12 hours, and images were captured under a phase-contrast microscope. To examine the morphology of endoplasmic reticulum (ER), cells were visualized by staining with specific fluorescent tracker dye according to the instructions of the manufacturer with slight modification. Briefly, after treatment with DR, cells were incubated with ER-Tracker Blue-White DPX for 30 minutes at 37°C. Cells were then washed with phosphate buffered saline (PBS) and visualized under a fluorescence microscope. Furthermore, to characterize the morphology of ER, cells were cultured on sterile poly-l-lysine coated glass cover slips for approximately 50% to 60% confluent condition in DMEM. For staining of ER membrane protein calnexin, cells were fixed using 4% paraformaldehyde and permeabilized with 0.2% Triton-X 100 in PBS for 10 minutes followed by PBS washes. After blocking with 2% bovine serum albumin (BSA), cells were incubated with rabbit anti-calnexin antibody (SC-11397) in T-TBS (100 mM Tris-HCl, pH 7.5; 150 mM NaCl and 0.05% Tween-20) containing 2% BSA for overnight at 4°C. After T-TBS washes, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG for 1 hour in the dark. The cover slips were washed with T-TBS, mounted with a drop of mounting medium and sealed with nail polish to prevent drying and movement under fluorescence microscope.13

Immunoblotting

Cells were seeded into 100 mm dish and allowed to grow up to 70% to 80% confluence and were subjected to treatment with DR for 0 to 24 hours. Cells were harvested at different time intervals by scraping, washed with TBS twice, and cell pellets were stored at −80°C. Cell pellets were lysed in RIPA buffer (CST, 9806S) supplemented with proteases and phosphatases inhibitors (Roche Diagnostics) and clarified by centrifugation at 12,000 rpm for 15 minutes at 4°C. Equal amount of proteins were loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, electrophoresed and Western transferred to membranes using Trans-blot turbo system (Bio-Rad). The membranes were blocked with 5% milk or 5% BSA in TBS with 0.1% Tween-20 for 1 hour at room temperature. The membranes were then incubated with appropriate primary antibodies (anti-LC3, Sigma- L8918; anti-p62, CST-5114; anti-GRP78, SC-1050; anti-phospho elF2α, CST-3597; anti-elF2α, CST-5324; anti-phospho IRE1α, abcam-ab48187; anti-JNK, CST-9252; anti-phospho JNK, CST-9251; anti-p38, CST-9211; anti-phospho p38, CST-9212; anti-CHOP, SC-56107; anti-cleaved PARP, CST-9541; anti-RIp1, 610458, BD Transduction Laboratories; anti-GAPDH, SC-25778) overnight at 4°C. Following washes in TBS containing 0.1% Tween-20, the membranes were incubated with appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies for 2 hours at room temperature. Finally, the membranes were washed thrice with TBS containing 0.1% Tween-20, and once with TBS alone. Subsequently protein expression was visualized by enhanced chemiluminescent (ECL) detection method.

siRNA Transfection

Cells were transfected according to Invitrogen protocols with some modifications. Cells were seeded into 100 mm dish and allowed to grow up to 30% to 50% confluency. Then, cells were washed once with Opti-MEM medium (Invitrogen) and transfected with siRNA-CHOP (sc-35437) or control siRNA (sc-37007). Briefly, for each dish, 10 μL of Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) was diluted with 40 μL of Opti-MEM medium (Invitrogen) in an eppendorf tube and was incubated at room temperature for 10 minutes. In another eppendorf tube, 20 μL of 10 μM siRNA stock was diluted with 440 μL of Opti-MEM medium (Invitrogen). Diluted Transfection Reagent was gently mixed with diluted siRNA solution, and incubated at room temperature for 20 minutes. This mixed siRNA solution and 2 mL of Opti-MEM medium (Invitrogen) was added to each dish containing cells and incubated at 37°C and 5% CO2. After 4 hours, 2.5 mL of Opti-MEM medium and 400 μL of FBS was added and incubated at 37°C and 5% CO2. The final concentration of siRNA was 40 nM. Next day, cells were split into 6-well plates and allowed to adhere overnight. Then cells were treated with DR and used for further experiments.

Statistical Analysis

Data are presented as the mean ± SD of 3 or 6 values in each group. Data were analyzed using SPSS 16.0 student software (SPSS Inc, Chicago, IL). One-way analysis of variance followed by post hoc testing with a least significance difference test was used to obtain P values to determine statistically significant (P < .05) differences between controls and treated groups.

Results

DR-Mediated Breast Cancer Cell Death Is Independent of Autophagy

The effect of DR on the viability of MCF-7, and doxorubicin resistant MCF-7/adr human breast cancer cells was
studied by MTT assay after a 24-hour treatment of different concentrations of DR. The results indicate that DR significantly reduced the viability of both the breast cancer cell lines (Figure 1A). To determine the mode of cell death induced by DR, we examined the morphological changes under microscopy. As shown in Figure 1B, DR induced the accumulation of cytoplasmic vacuoles, indicating DR-induced cell death assumed to be associated with autophagy. Thus, we first examined the existence of acidic autophagic vacuoles by acridine orange staining assay. Data revealed that DR did not induce the accumulation of acidic autophagic vacuoles in breast cancer cells (Figure 1C). However, the levels of autophagosomes marker, microtubule-associated protein light chain 3 (LC-3II) was increased in breast cancer cells treated with DR (Figure 1D). Thus, to further explore the effect of DR on autophagic flux, we also measured the levels of p62, a ubiquitin-binding protein involved in lysosomal or proteasome dependent degradation of proteins. During autophagic flux, p62 incorporates into the autophagosome by direct interaction with LC-3II and degrades when autophagosomes fuse with lysosomes. Inhibition of fusion of autophagosomes with lysosomes lead to increased levels of p62. As shown in Figure 1D, the increased levels of p62 as well as LC-3II indicate that the autophagic process remained incomplete in breast cancer cells treated with DR. Moreover, we found that the autophagy inhibitors, 3-MA and BafA1 failed to suppress the DR induced cytoplasmic vacuolation and cell death in breast cancer cells (Figure 1E and F). Taken together, these data revealed that DR-induced cytoplasmic vacuolation mediated cell death in human breast cancer cells is independent of autophagy.

**DR-Induced Breast Cancer Cell Death Is Also Independent of Paraptosis**

There are increasing evidences of paraptosis, a nonapoptotic cell death mechanism characterized by extensive cytoplasmic vacuolization. Notably, in our study, the insensitivity of DR-induced cytoplasmic vacuoles to the autophagic inhibitors indicated that DR might be inducing paraptosis in breast cancer cells. Since paraptosis requires transcription and translation, we investigated the effects of Act-D (transcription inhibitor) and CHX (translation inhibitor) on DR-induced cytoplasmic vacuolation and cell death. At first, we analyzed the effects of Act-D and CHX on DR-induced cell death by MTT assay. Different concentrations of DR were used to treat cells after a 1 h pretreatment with Act-D or CHX. The results showed that the percentage of cell viability was almost the same as that of the cells treated with DR alone (Figure 2A). Next, we investigated the effects of these paraptotic inhibitors on DR-induced cytoplasmic vacuolation, and we found out that act-D, as well as CHX, did not influence the DR-induced cytoplasmic vacuolation in breast cancer cells (Figure 2B). Collectively, DR-induced cytoplasmic vacuolation and cell death does not require cellular transcription and translational processes and also revealed the involvement of paraptotic independent cell death mechanism in DR treated breast cancer cells.

**DR-Induced Cytoplasmic Vacuoles Originated From Endoplasmic Reticulum**

We investigated whether the cytoplasmic vacuoles in DR-treated breast cancer cells originated from endoplasmic reticulum by staining with ER tracker dye or fluorescence staining of ER membrane protein, calnexin. The fluorescence images confirmed the involvement of ER in the vacuolization of DR-treated breast cancer cells (Figure 3A and B).

**ER Stress Pathway Is Activated in DR-Treated Human Breast Cancer Cells**

As cytoplasmic vacuoles originated from the ER and their formation was insensitive to both the autophagic and paraptotic inhibitors in DR-treated breast cancer cells, we suspected that ER stress might be responsible for the induction of breast cancer cell death. To address this issue, cells were treated with DR for 24 hours and then immunoblotting was performed to monitor the ER stress markers. As shown in Figure 3C, DR treatment increased the activation of ER stress markers GRP78, eIF2α, IRE1α, CHOP, JNK, and p38 in human breast cancer cells. These observations exemplified the contribution of ER stress in DR-induced breast cancer cell death.

**DR Induces ER Stress–Mediated Apoptotic Cell Death in Human Breast Cancer Cells**

Previously, it was thought that prolonged ER stress triggers cell death either through apoptosis or autophagy pathways. But recent findings demonstrated that ER stress can also induce necroptosis cell death. Since DR-induced cell death was not associated with autophagy, we determined the expression of apoptotic and necrotic markers. Like staurosporine, a representative proapoptotic alkaloid, DR also induced apoptosis in human breast cancer cells evidenced from proteolytic processing of PARP (Figure 4A). On the other hand, DR did not increase the expression of necroptotic marker protein RIP1 in human breast cancer cells (Figure 4B). Furthermore, suppression of the expression of CHOP, which is one of the pro-apoptotic transcription factors in the ER stress-mediated downstream pathways, significantly reduced the PARP cleavage induced by DR.
Figure 1. Deoxyrhapontigenin (DR) induces nonautophagic cell death in human breast cancer cells. (A) DR induces cell death in human breast cancer cells. MCF-7 and chemoresistant MCF-7/adr cell viability was determined by MTT colorimetric assay method after a 24-hour treatment with different concentrations of DR (25-200 µM). Data are from 3 independent experiments done in triplicates ± SD. *Significantly different from untreated control ($P < .05$). (B) Observation of the effect of DR on the morphology of human breast cancer cells. Cells were treated with DR for 12 hours and then visualized under phase contrast microscope. Image magnification, 400×. (C) Acridine orange (AO) staining was used to observe the formation of autophagic vacuoles in breast cancer cells. Under AO staining, both the cytoplasm and nucleus fluoresce green, whereas the acidic compartments like autophagosomes fluoresce orange to bright red. Image magnification, 400×. (D) Whole cell lysates were subjected to Western blot for analysis of autophagic markers, LC3 and p62. Images are representative of 3 independent experiments. (E) Assay of the effect of autophagic inhibitors on DR-induced cytoplasmic vacuoles in human breast cancer cells. Both MCF-7 and MCF-7/adr cells were pretreated with 3-MA (2 mM) for 1 hour followed by DR (IC$_{50}$) treatment for 12 hours, visualized, and images were captured under phase contrast microscope. Magnification, 200×. (F) Effect of autophagic inhibitors on DR-induced breast cancer cell death. Cells were pretreated with 3-MA or BafA1 1 h before DR (50, 100 and 150 µM) treatment for 24 hours. Cell viability was determined by MTT assay. *Significantly different from untreated control ($P < .05$).
Collectively, the results indicated that DR induces ER stress-mediated apoptosis in drug-resistant MCF-7/adr as well as in its parental MCF-7 cells.

**Discussion**

Natural stilbenes are important group of phytochemicals that attract great attention due to their wide range of pharmacological properties. Resveratrol is a natural stilbene derivative, which plays a potentially important role in many disorders. The research on this phenolic compound started through observations of the “French paradox,” which describes improved cardiovascular outcomes despite a high-fat diet in French people. Later resveratrol has been extensively studied and those findings demonstrated that it has several pharmacological properties including anticancer activity. Deoxyrhapontigenin (DR), a natural analog of resveratrol has already been reported to have antidiabetic and anti-inflammatory activities. However, the effect of DR on human cancer cell lines has not been explored so far. Hence to explore the effect of DR on cancer cells, we isolated DR from one of the bioactive fractions of *R. undulatum* L. root extracts and studied against human breast cancer cells.

Increased drug resistance is one the major obstacles in cancer treatment including breast cancer. There are various factors contributing to the drug resistance namely, tumor cell heterogeneity, drug efflux, genetic or epigenetic alterations. Autophagy is activated as a protective mechanism in cancer cells during chemotherapy. Autophagy is a normal cellular process by which cellular macromolecules and organelles are sequestered within the double membrane vesicles and then reach lysosomes for degradation and recycling to maintain cell survival under starvation and various metabolic stress conditions. Generally, autophagy in cancer cells plays a vital role in overcoming metabolic stresses such as nutrient deprivation, hypoxia, and absence of growth.
Figure 3. Deoxyrhapontigenin (DR) induces endoplasmic reticulum (ER) stress–mediated cell death in human breast cancer cells. (A) DR-induced cytoplasmic vacuoles originated from ER. Cells were treated with DR for 12 hours followed by stained with ER-Tracker Blue-White DPX for 30 minutes, and after removal of excess dye, cells were observed under fluorescence microscope. Image magnification, 400×. (B) Observation of ER morphology by immunofluorescence staining of ER membrane protein calnexin. Images were captured under fluorescent microscope. Image magnification, 200×. (C) To analyze the expression of ER stress markers, whole cell lysates were prepared after DR treatment and equal amount of proteins were subjected to immunoblotting of GRP78, elf2α, phospho-elf2α, phospho-IRE1α, CHOP, phosphor-JNK, JNK, phosphor-p38, and p38 proteins. Blots are representative of 3 independent experiments.

Recent evidence has shown that either genetic or pharmacological inhibition of autophagy in cancer cells enhances the therapeutic efficiency. Hence, autophagy has been considered as one of the key targets in cancer chemotherapeutic intervention. In our study, the increased autophagosome markers, LC-3II and p62 levels clearly indicated the blockade of autophagic flux by DR in human breast cancer cells.
Venkatesan et al

NP51

Figure 4. Deoxyrhapontigenin (DR) induces endoplasmic reticulum (ER) stress–mediated apoptosis in human breast cancer cells. (A) Immunoblotting of apoptosis marker, cleaved PARP (89 kDa). (B) Analysis of necroptosis marker protein. Cells were treated with DR for 0 to 24 hours and whole cell lysates were subjected to Western blotting of RIP1 protein. Blots are representative of 3 independent experiments. (C) Effect of CHOP on DR-induced apoptosis. Cells were transfected with CHOP-SiRNA or control-SiRNA using Opti-MEM medium (Invitrogen) and Lipofectamine RNAiMAX Transfection Reagent (Invitrogen), according to the manufacturer’s protocol with some modifications. Transfection efficiency was confirmed by immunoblotting of CHOP protein. Transfected cells were treated with DR for 24 hours and whole cell lysates were subjected to Western blotting of PARP (89 kDa). Blots are representative of 3 independent experiments.

The ER is an important intracellular organelle mainly responsible for protein folding and translocation. Many cellular conditions can alter ER function. As a consequence, unfolded and or misfolded proteins accumulate and develop stress in the ER lumen. Under physiological conditions, ER stress induces an adaptive response called unfolded protein response (UPR) to reestablish the functional homeostasis within the ER. However, this adaptive mechanism turns into a toxic signal when UPR is insufficient. There are reports stating that ER stress is usually maintained at higher levels in cancer cells as compared with normal cells due to persistent increased metabolic rate. This gives an opportunity for therapeutic intervention that specifically targets the already engaged ER stress in cancer cells. Recently, Papandreou et al have reported that plant stilbenes are potent inducers of ER stress. Here we found that deoxyrhapontigenin, a natural stilbene derivative induced breast cancer cell death via triggering ER stress–mediated apoptosis.

Conclusion

In summary, this study supports the fact that activation of endoplasmic reticulum stress–mediated apoptosis pathway represents the mechanism of action of deoxyrhapontigenin-induced cytotoxic effects in human breast cancer cells.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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