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Resveratrol enhances chemosensitivity of doxorubicin in multidrug-resistant human breast cancer cells *via* increased cellular influx of doxorubicin

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ABSTRACT

Background: Multidrug resistance is a major problem in the treatment of breast cancer, and a number of studies have attempted to find an efficient strategy with which to overcome it. In this study, we investigate the synergistic anticancer effects of resveratrol (RSV) and doxorubicin (Dox) against human breast cancer cell lines.
Methods: The synergistic effects of RSV on chemosensitivity were examined in Dox-resistant breast cancer (MCF-7/adr) and MDA-MB-231 cells. *In vivo* experiments were performed using a nude mouse xenograft model to investigate the combined sensitization effect of RSV and Dox.
Results and conclusion: RSV markedly enhanced Dox-induced cytotoxicity in MCF-7/adr and MDA-MB-231 cells. Treatment with a combination of RSV and Dox significantly increased the cellular accumulation of Dox by down-regulating the expression levels of ATP-binding cassette (ABC) transporter genes, MDR1, and MRP1. Further *in vivo* experiments in the xenograft model revealed that treatment with a combination of RSV and Dox significantly inhibited tumor volume by 60%, relative to the control group.
General significance: These results suggest that treatment with a combination of RSV and Dox would be a helpful strategy for increasing the efficacy of Dox by promoting an intracellular accumulation of Dox and decreasing multi-drug resistance in human breast cancer cells.

1. Introduction

Breast cancer is the second most common cancer in women and, worldwide, it is the leading cause of cancer-related death in women. Anthracycline drugs are commonly used as chemotherapeutic treatments for malignant breast cancer. Doxorubicin (Dox) is a typical anthracycline drug, and it is widely used for the treatment of breast cancer in patients who are endocrine resistant or for those whose cancer has metastasized [1,2]. Although the exact mechanisms underlying its chemotherapeutic activity are not fully understood, Dox primarily induces apoptotic cell death in cancer cells [3–5]. When Dox is given as a single-agent treatment, response rates are typically 40% to 60%, and they can be as high as 80% [6]. Despite the successful treatment of primary cancer, Dox-resistance is a major clinical problem and an important cause of treatment failure [7]. Several mechanisms have been suggested to mediate resistance to Dox in breast cancer cells. One of these suggested mechanisms is the overexpression of multi-drug resistance (MDR) proteins and other plasma membrane multidrug transporters, such as P-glycoprotein (P-gp) [8]. Others are the failure of cancer cells to undergo apoptosis, which is caused by alterations in the cellular signaling pathways, and a chemo-resistant phenotype [9–12]. Thus, targeting MDR is a promising approach to reducing the need for additional chemotherapeutic drugs.

Naturally occurring dietary compounds possessing chemopreventive properties exhibit a potent sensitization effect on cancer cells, rendering them susceptible to apoptosis induced by a variety of anticancer drugs [13–15]. Among them, it has been noted that resveratrol (RSV) displays a wide spectrum of effects against various tumor cell lines [16–18]. RSV also enhances the growth inhibition activities of chemotherapeutic drugs, without affecting normal cells. This suggests that it may function by modulating and interacting with a broad range of cellular targets that are associated with regulating the proteins involved in apoptosis, cell-cycle arrest, or anti-oxidant properties [19–24]. However, the effects

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethylsulfoxide; Dox, Doxorubicin; RSV, Resveratrol; MCF-7/adr, Doxorubicin-resistant MCF-7; ABC transporter, ATP-binding cassette transporter; MDR, Multi-drug resistance; MRP, Multi-drug resistance associated protein; P-gp, P-glycoprotein; RT-PCR, Reverse transcription polymerase chain reaction; TUNEL, Terminal deoxynuclotidyl transferase dUTP nick end labeling

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of RSV on Dox-resistant breast cancer cells have not been clearly demonstrated.

We hypothesized that treatment with a combination of RSV and Dox would enhance the efficacy of the drug by increasing its accumulation in the target cells. Combining RSV with Dox is a novel strategy that has the potential for improving the anticancer activity of Dox while protecting against its dose-limiting toxicity.

2. Materials and methods

2.1. Chemicals and reagents

Doxorubicin (50 mg/25 mL, Boryung Pharmacy, Gyeonggi-do, Korea) was kindly provided by the National Cancer Center in Korea (Ilsan, Korea). RSV was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture medium and supplements, including antibiotics and fetal bovine serum (FBS), were obtained from GIBCO (NY, USA). Primers for reverse transcriptase-polymerase chain reaction (RT-PCR) were synthesized by Takara PCR Thermal Cycler Dice (Shiga, Japan). Antibodies against MDR1, MRP1, MRP2, BCRP, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell lines and culture conditions

Dox-resistant human breast cancer MCF-7 (MCF-7/adr) cells were kindly provided by Professor Keon Wook Kang of Seoul National University (Seoul, Korea). MCF-7/adr cells were derived from MCF-7 cells by continuous treatment with 0.3 μM Dox. Human breast cancer cells (MDA-MB-231) were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained as monolayers at 37 °C in an atmosphere containing 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells grown to 80% confluence were subcultured to fresh medium, and the culture was allowed to grow for an additional 24 h before being treated with drugs. Immediately before treatment, RSV was dissolved in dimethylsulfoxide (DMSO).

2.3. Cell viability and cell growth assay

Cell viability was determined by the microculture tetrazolium (MTT) assay. MCF-7/adr and MDA-MB-231 cells were cultured in 96-well plates for 24 h and then treated with different concentration of RSV and Dox for 24 h to 72 h. At the end of the treatment period, 15 μL of the 5 mg/mL MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffered saline, PBS) was added to each well. The plates were incubated again for 4 h at 37 °C in the dark. The supernatants were aspirated, and the formazan crystals were dissolved in 100 μL of DMSO at 37 °C for 10 min with gentle agitation. The absorbance of each well was measured at 540 nm with a VersaMax™ Microplate Reader (Molecular Devices, CA, USA). Morphological alterations were observed under TS-100 inverted microscope (Nikon, Tokyo, Japan). For recovery assays, MCF-7/adr and MDA-MB-231 cells were seeded in 6 well plates and allowed to attach for several hours. Cells were incubated with Dox or RSV, either alone or in combination for 24 h. After exposure, cells were replaced in fresh medium and incubated for 24 or 48 h in order to evaluate cell recovery. The level of cell growth was determined by using a hemocytometer to count the number of cells.

2.4. Intracellular influx of Dox

A laser scanning confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany) was used to measure the intracellular accumulation of Dox. Cells were seeded in culture dishes and incubated overnight to allow for attachment. After a 24 h incubation, cells were treated with 1 μM Dox alone or in combination with 50 μM RSV for 2, 6, and 12 h. Subsequently, the culture media was removed and the cells were washed 3 times with PBS. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, and then washed 3 more times with PBS. Intracellular fluorescent signals were visualized under the FV10i confocal laser-scanning microscopy (Olympus, Tokyo, Japan). The intrinsic fluorescence of Dox was excited with an argon laser at 488 nm, and the emission was collected through a 550 nm long-pass filter.

The accumulation of Dox in cells was evaluated by flow cytometry. The MCF-7/adr and MDA-MB-231 cells were cultured in 24-well plates and then treated for 24 h with 1 μM Dox alone or in combination with 50 μM RSV. The extracellular drugs were then removed and the cells were placed in the incubator with fresh media. Next, cells were treated with trypsin for 10 min at 37 °C to detach the cells from the dish surface. The cell suspension was centrifuged and resuspended in PBS. A minimum of 50,000 events from each sample was analyzed in order to generate histograms for the fluorescence intensity. Flow cytometry analyses were performed using an Accuri C6 flow cytometer (BD Biosciences), and the data were analyzed using Accuri C6 software.

2.5. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Trizol Reagent (Invitrogen Corporation, Carlsbad, CA) based on the manufacturer's protocol, and cDNA was synthesized using reverse transcription. The synthesized cDNAs were further amplified by PCR with the primers listed below. Briefly, the cDNA (2 μL) was amplified in a 25-μL reaction mixture containing 10× PCR buffer (2.5 μL), 50 mM $MgCl_2$ (0.75 μL), 10 mM dNTP mixture (0.5 μL), and 20 μM each of sense (0.25 μL) and antisense (0.25 μL) primers. The reaction was initiated at 94 °C for 5 min, and PCR was performed using a variable number of the following amplification cycles: denaturation at 94 °C for 45 s, annealing at 56–66 °C for 45 s, and extension at 72 °C for 45 s. The number of PCR cycles was estimated in a preliminary study and optimized in the exponential phase of PCR. A final cycle of extension at 72 °C for 5 min was also included. A 20 μL aliquot of each PCR product was separated by 2% agarose gel electrophoresis and visualized using ethidium bromide.

The resulting cDNA was amplified by PCR with the following primers: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; 110 bp) 5′-ATCGA CCACTACCTGGGCAA-3′ (sense) and 5′-AG GATAACGCAGGCGATGT-3′ (antisense); multidrug resistant protein (*MDR*) *1* (158 bp) 5′-CCCATCAT TGCAATAGCAGG-3′ (sense) and 5′-GTTCAAACTTCT GCTCCTGA-3′ (antisense); multiple drug resistance protein (*MRP*) *1* (155 bp) 5′-ATCA AGACCGCTGTCATTGG-3′ (sense) and 5′-TCTCGTTCCTACTGAACGTC-3′ (antisense); *MRP2* (78 bp) 5′-ACAGAGGCTGGTGG CAACC-3′ (sense) and 5′-ACCATTACCTTGTCACTGTC-3′ (antisense); and breast cancer resistance protein (*BCRP*; 172 bp) 5′-TGCCCAGGACTCAATGCAAC-3′ (sense) and 5′-ACAATTTCAGGTAGGC AATT-3′ (antisense).

2.6. Western blotting

Cell lysates were prepared using a lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, phenylmethylsulphonyl fluoride 100 μg/mL, aprotinin 2 μg/mL, pepstatin 1 μg/mL, and leupeptin 10 μg/mL), and placed on ice for 30 min. The suspension was collected after centrifugation at 15,000 g for 15 min at 4 °C. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, USA), using bovine serum albumin as a standard. Equal amounts of lysates were separated by SDS-polyacrylamide gels (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes in buffer containing 25 mM Tris-HCl pH 8.5, 192 mM glycine, 20% methanol. Membranes were blocked with 5% skim milk and probed sequentially with specific primary antibody (dilution range, 1:1000) followed by HRP-conjugated secondary antibody for protein detection. The blots

were developed using enhanced chemiluminescence (Millipore Corporation, MA, USA).

2.7. P-gp ATPase activity assay

The P-gp-Glo™ Assay Systems (Promega, WI, USA) was used to perform P-gp ATPase assays. A P-gp ATPase assay detects the effect of compounds on recombinant human P-gp in the cell membrane fraction. The effect of RSV on the P-gp ATPase activity was measured according to the manufacturer's instructions. Briefly, ATP was first incubated with P-gp membranes and MgATP, then the P-gp ATPase reaction was stopped, and the remaining ATP was detected as a luciferase-generated luminescent signal. Basal P-gp ATPase activities were investigated by detecting activity in the presence or absence of sodium orthovanadate. Verapamil- or Dox-stimulated P-gp ATPase activity was measured in the presence of 50 μM of each drug. Luminescence was read on a plate-reading luminometer (GloMax®-Multi Detection System, Promega, WI, USA). The converted drug-stimulated ATPase activity (nmol ATP consumed/μg P-gp/min) was determined by comparing the luminescence of the samples.

2.8. In vivo anti-tumor activity in a xenograft model

Six-week-old female BALB/c athymic nude mice (SLC, Inc., Hamamatsu, Shizuoka, Japan) were housed under controlled temperature (22 ± 2 °C) and lighting (12 h light/dark cycle) conditions in filtered-air laminar-flow cabinet, and manipulated using aseptic procedures. The institutional animal care committee of Pusan National University approved the experimental procedure. MCF-7/adr cells (5×10^5 cells in 0.1 mL) suspended in serum-free medium containing 50% Matrigel were inoculated subcutaneously through 25-gauge needles into the right flank of each nude mouse. Prior to drug administration, mice were randomized to 3 groups (n = 5): Group 1 Control; Group 2 Dox (4 mg/kg); Group 3 RSV (20 mg/kg) and Dox (4 mg/kg). Dox was diluted with PBS, and given intraperitoneally (i.p.) once a week. RSV (dissolved in 5% ethanol followed by dilution with corn oil) was orally administered for 4 consecutive weeks. Tumor diameters were determined every 3 days with a caliper, and the tumor volume was calculated using a standard formula: tumor size (mm^3) = width^2 × length × 0.52. Body weights were recorded before dosing. At the end of the treatment period, the mice were sacrificed by carbon dioxide asphyxiation.

2.9. Immunohistochemistry

Paraffin-embedded tumor sections were used to examine the expression of MDR1, MRP1, and MRP2. Deparaffinization was achieved with xylene, followed by a descending series of ethanol concentrations. Antigen retrieval was carried out in microwave-heated citrate buffer for 20 min. The endogenous peroxidases were depleted with 0.3% H_2O_2 for 10 min at room temperature. The sections were blocked with 10% normal horse serum for 1 h and then incubated overnight at 4 °C with the specific primary antibody. After washing with PBS 3 times, secondary antibodies were biotinylated and visualized with the avidine-biotinylated enzyme complex (ABC) technique using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and subsequent 3,3′-diaminobenzidine (DAB) staining (Vector Laboratories, Burlingame, CA, USA). The nuclei were counterstained with hematoxylin. The immunoreactions were viewed under an optical microscope (Axiovert200, Zeiss, Jena, Thuringen, Germany). Apoptosis was assessed by TUNEL staining using an ApopTag fluorescein *in situ* apoptosis detection kit (Millipore Corporation, MA, USA) according to the manufacturer's procedure. Images were observed using confocal laser scanning microscopy (FV10i, Olympus).

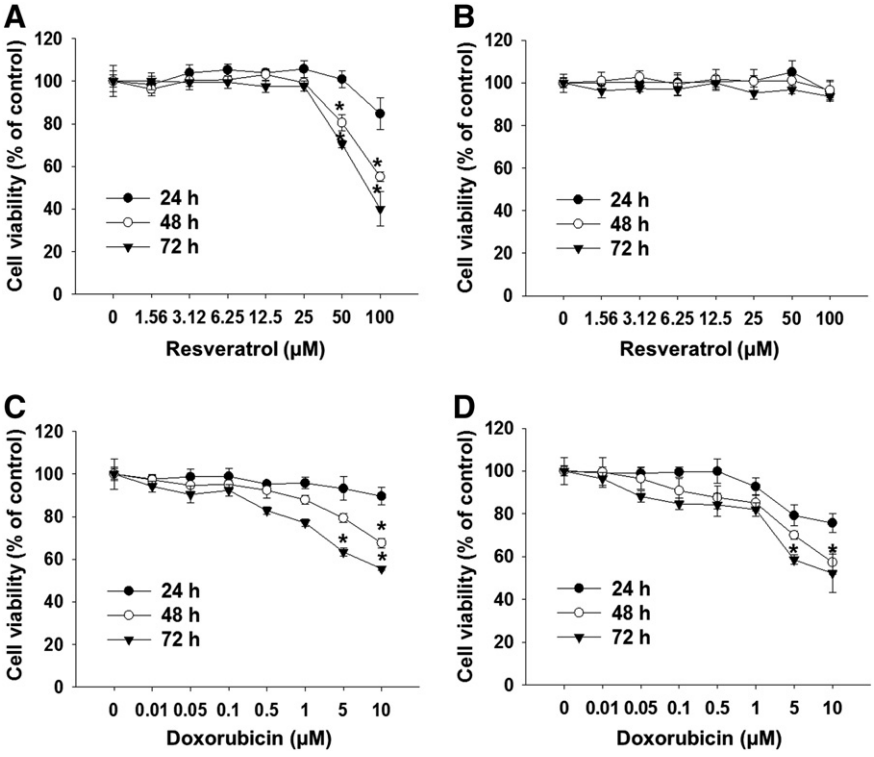

Fig. 1. Cell viability of resveratrol and doxorubicin on human breast cancer cells. (A) MCF-7/adr and (B) MDA-MB-231 cells were treated with RSV at various concentrations (0–100 μM) for indicated times, and the cell viability was measured using the MTT assay. (C) MCF-7/adr and (D) MDA-MB-231 cells were treated with Dox at various concentrations (0–10 μM) for indicated times, and the cell viability was measured using the MTT assay. Cell viability (%) was expressed as a percentage compared to the untreated control cells. The data are expressed as the mean ± SEM of 3 independent experiments. *$p < 0.05$, as determined by ANOVA test compared to the vehicle control.

2.10. Statistical analysis

All values are expressed as the mean ± the standard error of the mean (SEM). Statistical analysis was performed by using *SigmaStat* (SPSS Inc., IL, USA). The significance of differences was determined using one-way analysis of variance (ANOVA) followed by Dunn's test. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Resveratrol potentiates doxorubicin-induced cytotoxicity of breast cancer cells

To measure the cytotoxicity of RSV and Dox, human breast cancer cell lines MCF-7/adr and MDA-MB-231 were cultured either with various

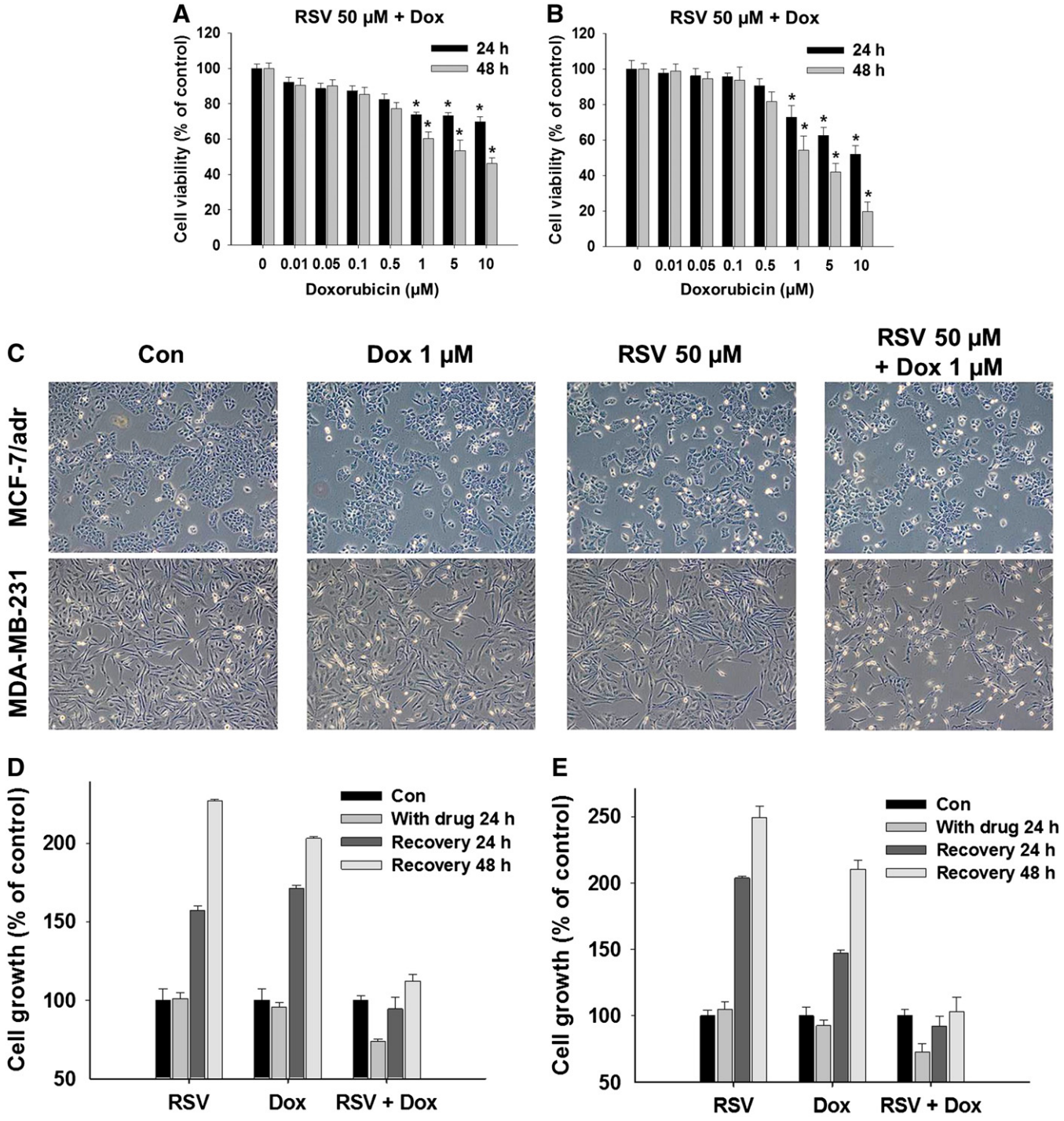

Fig. 2. Resveratrol enhances chemosensitivity of doxorubicin in human breast cancer cells. (A) MCF-7/adr and (B) MDA-MB-231 cells were treated with different concentrations of Dox (0.01–10 μM) and RSV (50 μM) for 24 h and 48 h, respectively. Cell viability was measured using the MTT assay. Cell viability (%) was expressed as a percentage compared to the untreated control cells. The data are expressed as the mean ± SEM of 3 independent experiments. *$p<0.05$, as determined by ANOVA test compared to the vehicle control. (C) Morphological features of human breast cancer cell lines after drug treatment. The MCF-7/adr and MDA-MB-231 cells were treated with 1 μM Dox or with 50 μM RSV, either alone or in combination. After 24 h incubation, cells were photographed at 100×. (D) MCF-7/adr and (E) MDA-MB-231 cells were treated with 1 μM Dox or with 50 μM RSV, either alone or in combination for 24 h. After treatment, media were replaced with fresh media without drugs, and the cells were allowed to recover for 24 or 48 h. The bar diagrams represent the level of cell growth at each time point.

concentrations of RSV (1.56 μM-100 μM) or Dox (0.01–10 μM) for 24 to 72 h. Cell viability was evaluated by MTT assay. As shown in Fig. 1A, RSV (100 μM) produced a remarkable reduction in the viability of MCF-7/adr cells at 24 h. By contrast, RSV showed no cytotoxic effect in MDA-MB-231 cells (Fig. 1B). After 24 h treatment, 50 μM RSV had induced no cytotoxicity in either cell line. From the above results, a 50 μM concentration of RSV was selected for use in further experiments, and we note that this concentration has been used elsewhere [25–29]. As shown in Fig. 1C and D, there was no significant inhibition of cell viability with 1 μM Dox. However, there was a difference in the

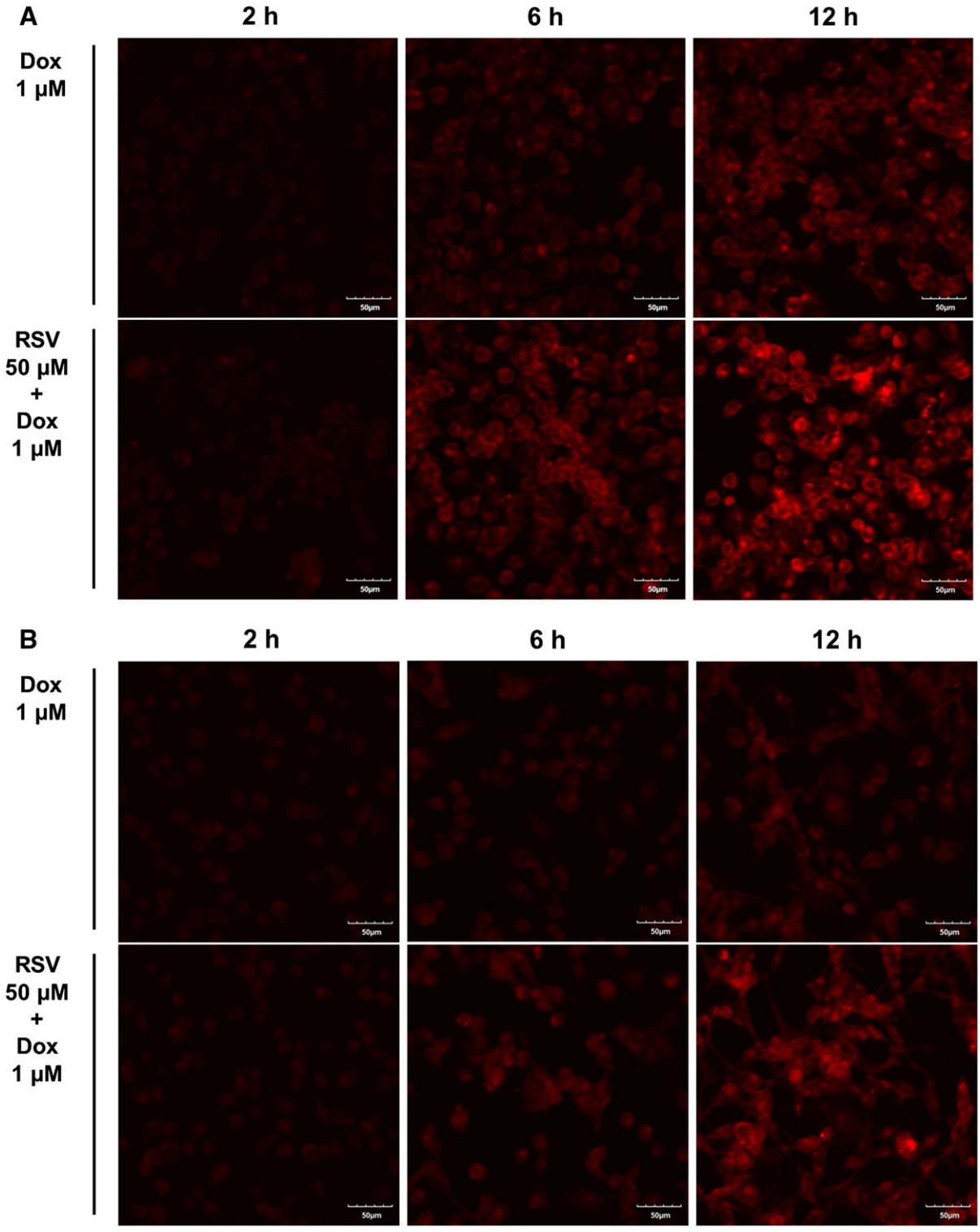

Fig. 3. Resveratrol increased the intracellular accumulation of doxorubicin in MCF-7/adr and MDA-MB-231 cells. Cells were treated with 1 μM Dox alone or in combination with 50 μM RSV for up to 12 h. Representative confocal images of (A) MCF-7/adr and (B) MDA-MB-231 cells, comparing the distribution of red fluorescence for Dox treatment alone or in combination with RSV. Treatment with RSV enhanced the intracellular accumulation of Dox in either cell line, whereas in the absence of RSV, Dox appeared to be exclusively located within the cytoplasm. (C) Left: Amount of Dox uptake in each cell line was measured by detecting Dox auto-fluorescence. The histogram shows the relative fluorescence intensity of cells. Right: Fluorescence intensities were measured using flow cytometer, and bar graphs represent the relative units ± SEM. $^{*}p < 0.05$, as determined by ANOVA test.

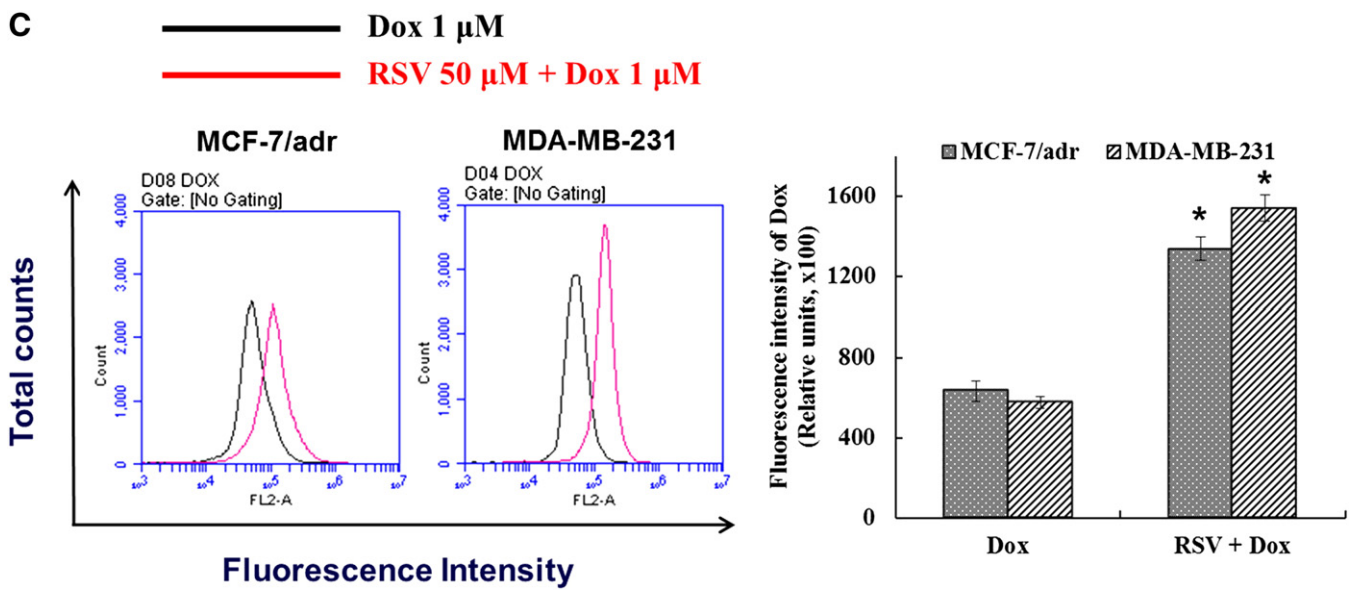

Fig. 3 (*continued*).

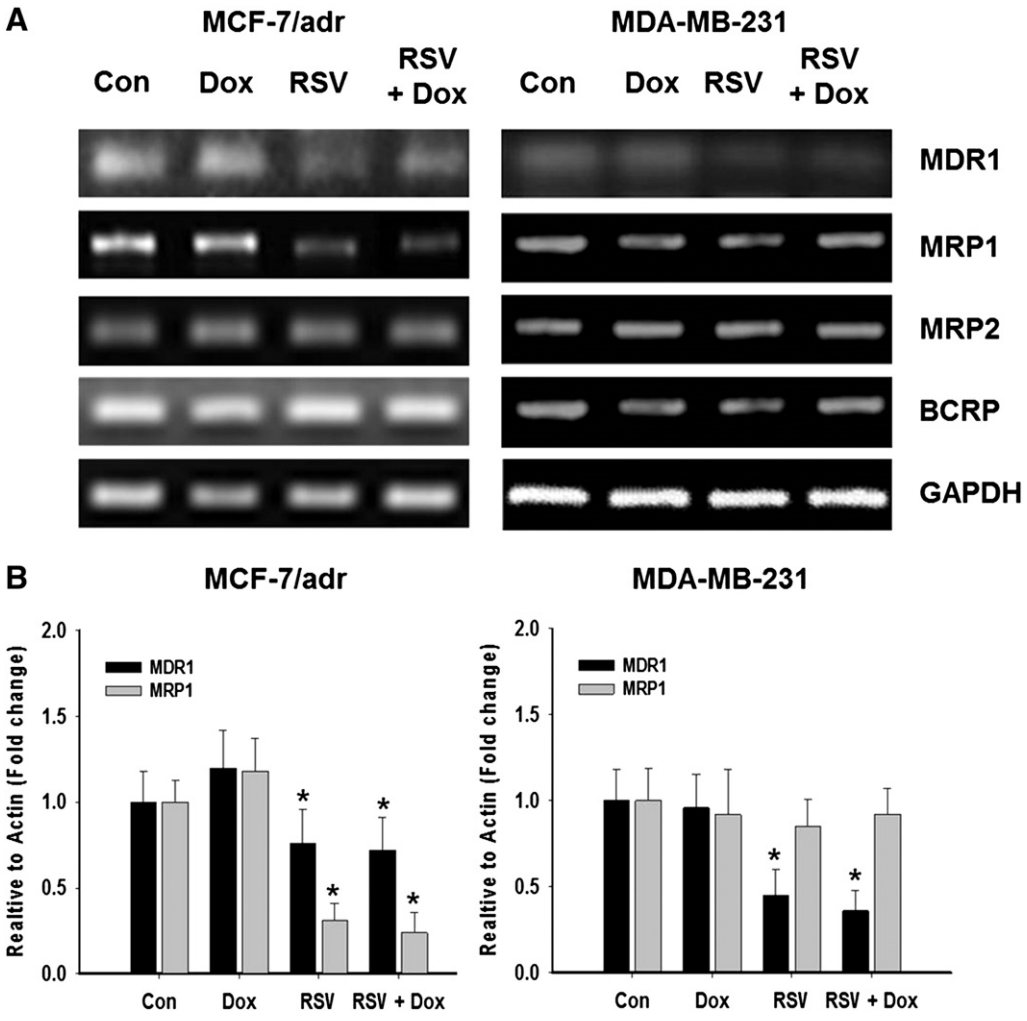

Fig. 4. Effects of resveratrol and doxorubicin on the mRNA expression levels of ABC transporter related genes in MCF-7/adr and MDA-MB-231 cells. Cells were treated with 1 μM Dox or with 50 μM RSV, either alone or in combination. (A) After 24 h treatment, mRNA expression was evaluated by reverse-transcription PCR in each cell line. *GAPDH* was used as an internal control. (B) The mRNA expression levels of *MDR1* and *MRP1* were quantified by densitometry analysis with the control value set at 100%. Values correspond to the mean ± SEM of three independent experiments. $^{*}p < 0.05$, as determined by ANOVA test compared to the vehicle-treated control.

susceptibility of the cell lines to Dox alone. The MDA-MB-231 cells were more sensitive to Dox, whereas the MCF-7/adr cells were relatively resistant to Dox levels of up to 10 μM.

To determine the synergistic effects of RSV against Dox-induced cytotoxicity, MCF-7/adr and MDA-MB-231 cells were treated with a combination of RSV (50 μM) and Dox. RSV markedly enhanced Dox-mediated cytotoxicity in MCF-7/adr and MDA-MB-231 cells. The growth-inhibitory effects of the combination treatment were synergistic and more pronounced with increasing concentrations of Dox. Treatment with RSV markedly enhanced the cytotoxicity of Dox in MCF-7/adr and MDA-MB-231 cells by up to 25% and 30%, respectively (Fig. 2A and B).

To investigate morphological changes due to the drug treatment, the MCF-7/adr and MDA-MB-231 cells were treated with 1 μM Dox or with 50 μM RSV, either alone or in combination. There were no significant changes in the cellular condition or shape in either cell line after treatment with RSV or with Dox alone. We observed that when cells were treated with a combination of RSV and Dox, they underwent morphological alterations resembling that of cell death (Fig. 2C). Next, we performed a recovery assay to determine whether the Dox treatment in combination with RSV had merely arrested the cells or if there was an element promoting cell death. During the overall recovery time, the level of cell growth was markedly decreased in the combination treatment group, while a complete recovery was observed in other groups when compared to the control cells (Fig. 2D and E). These results suggest that RSV markedly enhanced Dox-induced cytotoxicity in human breast cancer cell lines.

3.2. Resveratrol enhances intracellular accumulation of doxorubicin

To investigate the potential mechanism by which RSV sensitizes Dox-induced cytotoxicity, we examined the effect of RSV on the intracellular accumulation of Dox using a fluorescence microscope. Measurement of the fluorescence intensity of intracellular Dox has been used to determine cellular uptake [30,31]. The intracellular Dox amount is in direct proportion to its fluorescence, as Dox itself is fluorescent. In the absence of RSV, the intracellular levels of Dox were low in either cell line. However, treatment with 50 μM RSV significantly increased the intracellular accumulation of Dox in a time-dependent manner (Fig. 3A and B). Furthermore, Dox uptake analysis by flow cytometry (FACS) indicated that MCF-7/adr and MDA-MB-231 cell lines accumulated increased amounts of Dox in the presence of RSV, as the fluorescence intensity was higher in the combination treatment group relative to the group that was treated with Dox alone (Fig. 3C).

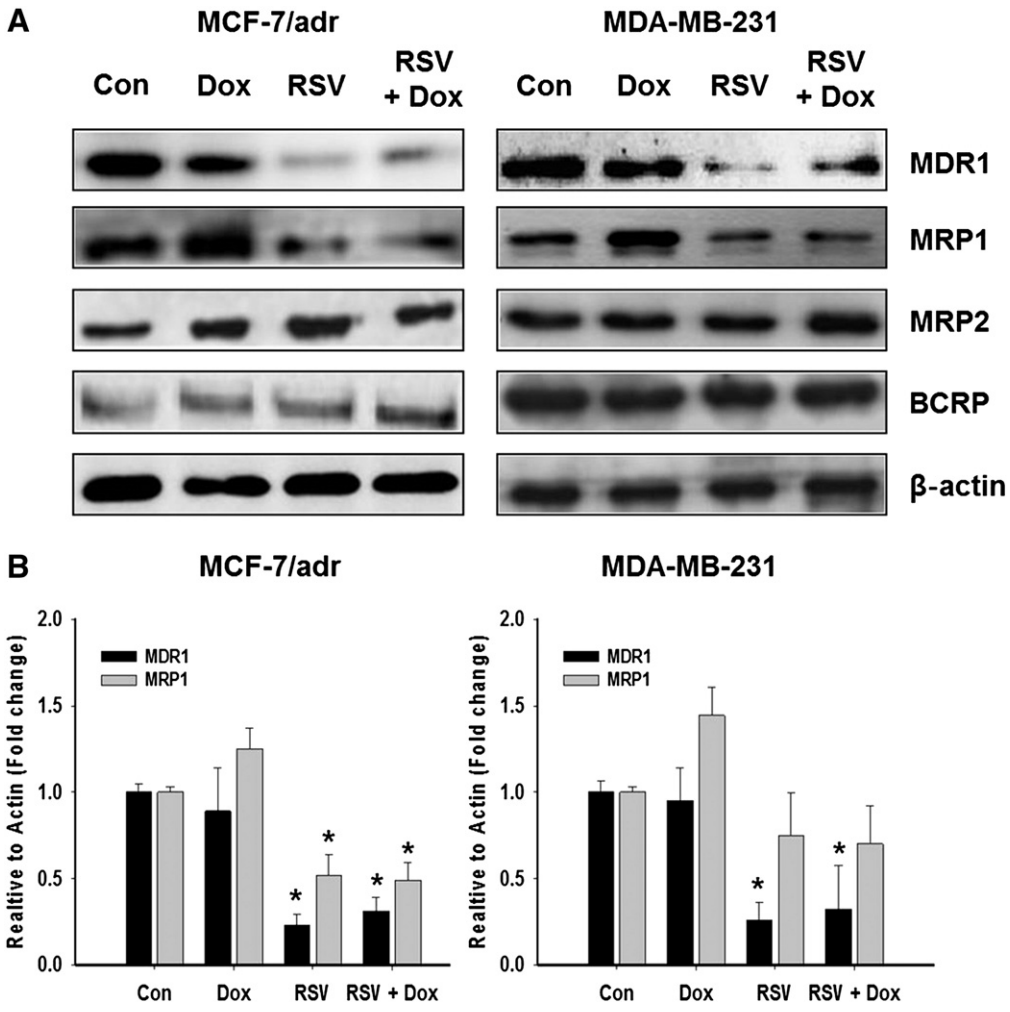

Fig. 5. Effects of resveratrol and doxorubicin on the expression levels of ABC transporter-related proteins in MCF-7/adr and MDA-MB-231 cells. Cells were treated with 1 μM Dox or with 50 μM RSV, either alone or in combination. (A) After 24 h treatment, protein levels in cell lysates were analyzed by Western blot. A β-actin was used as an internal control. (B) The protein expression levels of MDR1 and MRP1 were quantified by densitometry analysis with the control value set at 100%. Values correspond to the mean ± SEM of three independent experiments. *$p < 0.05$, as determined by ANOVA test compared to the vehicle-treated control.

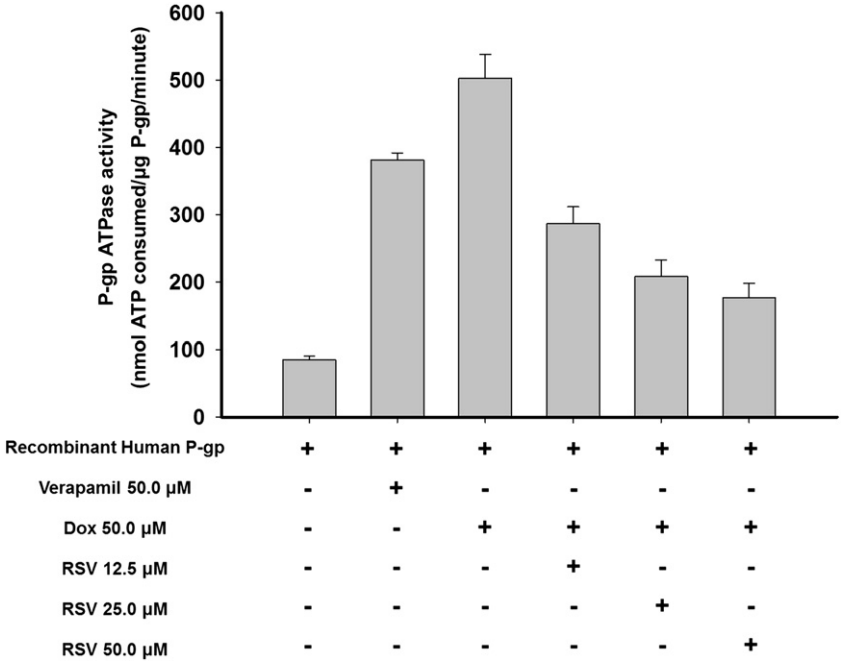

Fig. 6. Effect of resveratrol on the P-gp ATPase activity in human P-gp-expressing membranes. RSV was tested at a range of concentrations (12.5–50 μM) for its capacity to inhibit doxorubicin-stimulated P-gp ATPase activity. Membranes were treated with Verapamil, Dox, or RSV, as indicated. The differences in sample luminescence were used to calculate ATPase activity, which was expressed as namomoles of ATP consumed in reaction. Each bar represents the mean ± SEM of P-gp ATPase activity.

3.3. Effect of resveratrol on ABC multidrug transporters

In order to determine whether RSV mediated reversal effects on drug transporters, the cellular RNAs were isolated from the cell lines, and the mRNA expression of each of the selected genes was analyzed by RT-PCR. Treatment with a combination of RSV and Dox down-regulated the MDR1 mRNA level in both cell lines. We also observed a detectable decrease in the level of MRP1 in MCF-7/adr cells, while the expression level of GAPDH was unchanged (Fig. 4A and B). By contrast, the mRNA levels of MRP1 and MRP2 did not show any significant changes in MDA-MB-231 cells after combination treatment. These results indicated that RSV could overcome the Dox-resistant status of cancer cells *via* down-regulation of MDR1.

The expression levels of MDR1, MRPs, and BCRP proteins were confirmed again by Western blot analysis. After the combination treatment with RSV and Dox, MDR1 and MRP1 expressions were significantly decreased in both cell lines, as expected. However, there was no change in the expression of MRP2 or BCRP (Fig. 5A and B). These results suggest that treatment with RSV increases the intracellular accumulation of Dox *via* down-regulating MDR1 and MRP1 expression in MCF-7/adr and MDA-MB-231 cells.

To determine whether RSV is able to reverse drug resistance, drug-stimulated P-glycoprotein (P-gp) ATPase activity was estimated using the P-gp-Glo assay systems. The function of P-gp is connected to ATP hydrolysis by the ATPase, and its activity is influenced by P-gp substrates or modulators. As shown in Fig. 6, Verapamil and Dox are substrates that stimulate P-gp ATPase activity. However, treatment with RSV significantly inhibited the P-gp ATPase activity in a concentration-dependent manner, indicating that RSV acts as an inhibitor of P-gp ATPase.

3.4. Effect of resveratrol on the growth of MCF-7/adr cell tumors in nude mice

To evaluate the effect of RSV on tumor growth, athymic nude mice inoculated with MCF-7/adr cells were treated with Dox (4 mg/kg) alone or in combination with RSV (20 mg/kg). When these two drugs were combined, RSV was administered every day for 4 consecutive weeks. Treatment of nude mice with Dox (4 mg/kg) did not significantly inhibit tumor growth compared to the vehicle-treated control group. However, treatment with RSV (20 mg/kg) significantly inhibited tumor volume by up to 61.5% and reduced tumor weight by up to 54.7%, compared to the vehicle-treated control group (Fig. 7A and B).

The synergistic effects of RSV in the *in vivo* model were examined in paraffin-embedded tumor sections. Immunohistochemical staining was performed using specific antibodies against MDR1, MRP1, or MRP2. Dox treatment in combination with RSV significantly decreased the percentage of MDR1- or MRP1-positive tumor epithelial cells (Fig. 7C). These results support that inhibition of MCF-7/adr cells tumor growth contributes to the *in vivo* anticancer activity of RSV and Dox. Next, apoptotic cell death was examined by a TUNEL assay. The labeling indices of TUNEL positive cells were significantly increased in the combination group compared to those in the vehicle-treated control group (Fig. 7C and D).

4. Discussion

Among a variety of chemotherapeutic agents, Dox is commonly used as a primary and first-line treatment for patients with breast cancer [32,33]. Dox incorporates into the DNA of cancer cells and prevents cell replication by inhibiting protein synthesis [34]. However, Dox can cause many unwanted effects in the patients. Moreover, chemotherapeutic drugs often fail to overcome cancers due to the development of drug resistance, which blocks their activity. Therefore, increasing the sensitivity to Dox is an attractive goal for improving the clinical management of breast cancers.

Among natural compounds with beneficial effects on human health, RSV has attracted considerable interest due to its sensitizing effect on anticancer drugs. Previous studies have demonstrated that RSV exerts anticancer activity by inducing cell cycle arrest or inducing apoptosis in human cancer cells of different origins [35–37].

In the present study, we described a novel mechanism of RSV by which it regulates ABC transporter expression, thus resulting in the accumulation of Dox in human breast cancer cells. It was observed that Dox and RSV show a synergistic effect in inhibiting the proliferation of MCF-

7/adr and MDA-MB-231 cells. Based on the cytotoxicity results, effects on ATP-binding cassette (ABC) transporters were examined to clarify the exact mechanism of the Dox accumulative effect following RSV treatment. Treatment of cancer cells with anticancer drugs, such as Dox, frequently results in severe multi-drug resistance (MDR) due to the high expression of ABC transporters. Regulation of ABC transporter expression is considerably complex, and selective down-regulation of these transporter genes is a promising approach to anticancer therapeutics [38]. In particular, MDR1 is well known to play a crucial role in multidrug resistance and is over-expressed in a variety of cancers [39]. The role of MDR1 in protecting cells from cytotoxic effects induced by chemotherapeutic agents has been demonstrated in several cellular systems, including MCF-7/adr cells [40]. Furthermore, MRP1 is also one of the most widely studied members of the ABC transporter superfamily [41]. Since both MDR1 and MRP1 contribute extensively to multidrug resistance in MCF-7/adr and MDA-MB-231 cells, a simultaneous reversal of both would be clinically important [42,43]. Here, we demonstrated that RSV treatment decreased the expression levels of MDR1 and MRP1 in breast cancer cell lines. This is also supported by the P-gp ATPase activity demonstrating RSV decrease Dox-stimulated P-gp ATPase activity in a concentration dependent manner.

Based on our *in vitro* study, the antitumor effect of RSV was also demonstrated in a MCF-7/adr cell xenograft model. In general, the animal dose should not be extrapolated to a human equivalent dose (HED) by a simple conversion. Here, we used a body-surface-area normalization method to determine the dosage of RSV to be administered. Based on the reference dose [44], the HED was calculated as follows: animal dose (mg/kg) multiplied by (animal Km factor/Human Km factor). In this study, RSV was administered by oral gavage with 20 mg/kg, which equates to a 1.6 mg/kg for humans. Additionally, the dosage used in this study was based on several reports by other researchers [45–47]. Importantly, Dox administration with RSV significantly reduced tumor volume by approximately 50%, compared to the untreated control group. We also found that the expression levels of MDR1 and MRP1 were decreased in the tumors that were treated with RSV and Dox together. Other studies have shown that RSV or analogs have a reversal effect on multidrug resistance, and this is followed by potentiating cytotoxic properties of

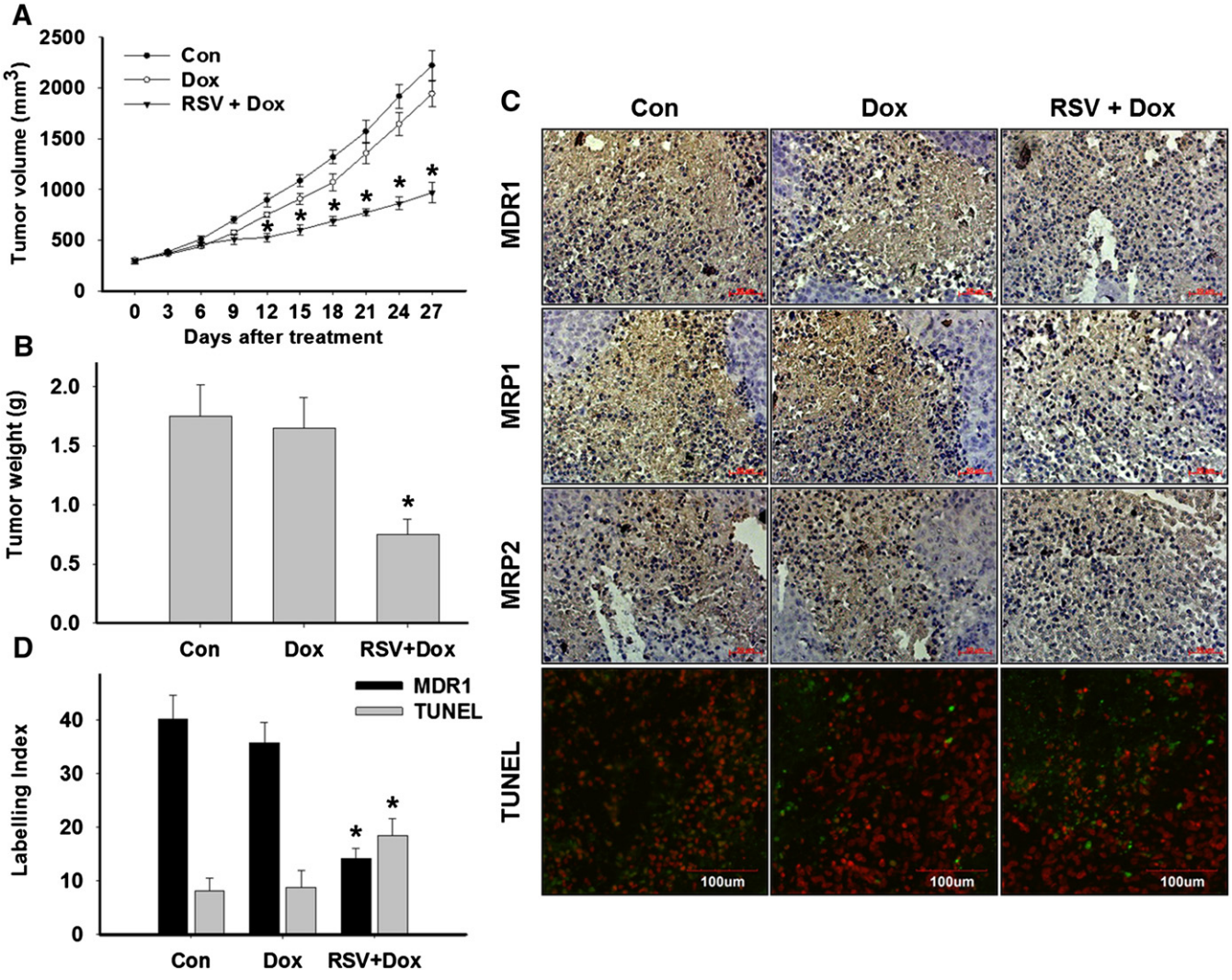

Fig. 7. Effect of resveratrol and doxorubicin on the growth of established MCF-7/adr tumors in nude mice. Each mouse was inoculated subcutaneously in the right flank with 5×10^5 MCF-7/adr cells in a total volume of 0.1 mL serum-free medium containing 50% Matrigel. Mice with established tumor volumes ($185.3 + 25.6\,mm^3$) were randomized into three groups. Dox (i.p.) and RSV (p.o.) were given to the tumor-bearing mice. There was no statistically significant difference in changes in the body weight of mice between treatment groups, and no signs of other toxicity effects were observed during the period. (A) Mean tumor volumes for each treatment group (as a function of day of treatment and tumor burdens of each treatment) are indicated. Results are represented as the mean ± SEM of 5 animals per group. $^*p < 0.05$, as determined by ANOVA test, compared to the control. (B) Each bar represents the mean ± SEM of tumor weight of 5 animals per group. $^*p < 0.05$, as determined by ANOVA test, compared to the control. (C) The tumors were fixed in formalin and embedded in paraffin. Representative images of immunohistochemical staining for MDR1, MRP1, and MRP2 from the control and drug-treated groups. (D) Changes in labeling indices of MDR1 (black bar) and TUNEL (gray bar) positive cells in 10 random fields at 400× magnification. Error bars show SEM (n = 5 at each group). $^*p < 0.05$, as determined by ANOVA test compared to the control.

chemotherapeutic drugs [48–50]. However, the precise mechanism of RSV on ABC transporters has not been determined. Several pathways have been shown to mediate the expression of ABC transporters, including NF-κB, cyclooxygenases-2, CYP3A4, reactive oxygen species, the mitogen-activated protein kinase pathway, phosphoinositide 3-kinase, and protein kinase C [51–56]. That is, it is possible that RSV is associated with some of these elements in relation to drug transport. Further research will be needed to better understand why RSV inhibits multidrug resistance in breast cancers.

In conclusion, we showed an increase in Dox-induced anticancer activity after treatment with non-cytotoxic concentrations of RSV. Furthermore, the RSV-induced intracellular accumulation of Dox was mediated by the inhibition of MDR1 and MRP1, a novel mechanism for its anticancer effect. Taken together, these results suggested that RSV can increase the efficacy of Dox and help in overcoming drug resistance in Dox-resistant human breast cancer cells.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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