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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