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Original Articles

Ovatodiolide sensitizes aggressive breast cancer cells to doxorubicin anticancer activity, eliminates their cancer stem cell-like phenotype, and reduces doxorubicin-associated toxicity

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ABSTRACT

Triple-negative breast cancer (TNBC) is chemotherapy–refractory and associated with poor clinical prognosis. Doxorubicin (Doxo), a class I anthracycline and first-line anticancer agent, effective against a wide spectrum of neoplasms including breast carcinoma, is associated with several cumulative dose-dependent adverse effects, including cardiomyopathy, typhilitis, and acute myelotoxicity. This study evaluated the usability of Ovatodiolide (Ova) in sensitizing TNBC cells to Doxo cytotoxicity, so as to reduce Doxo effective dose and consequently its adverse effects. TNBC cell lines MDA-MB-231 and HS578T were used. Pre-treatment of the TNBC cell with 10 μM Ova 24 h before Doxo administration increased Doxo anticancer effect (IC_{50} 4.4 μM), compared to simultaneous treatment with Doxo (IC_{50} 10.6 μM), or Doxo alone (IC_{50} 9.4 μM). Intracellular accumulation of Doxo was least in Ova pre-treated cells at all Doxo concentrations, when compared with Doxo or simultaneously treated cells. In comparison to the Doxo-only group, cell cycle analysis of MDA-MB-231 cells treated concurrently with 2.5 μM Ova and 1.25 μM Doxo showed decreased percentage of cells arrested at G0/G1; however, pre-treatment with the same concentration of Ova 24 h before Doxo showed greater tumor growth inhibition, with increased cell number in G_0/G_1 arrest (2.4-fold), lesser Doxo-induced apoptosis, and significantly reduced intracellular Doxo accumulation. Ova-sensitized TNBC cells also lost their cancer stem cell-like phenotype evidenced by significant dissolution and necrosis of formed mammospheres, as well as their terminal differentiation. Put together, Ovatodiolide treatment sensitizes TNBC cells to the anticancer activity of Doxorubicin and potentiates Doxorubicin-induced elimination of TNBC cancer stem cell-like phenotype.

Introduction

Breast carcinoma presents an enormous socio-economic and clinical challenge, being the second most diagnosed cancer for both sexes combined, the most prevalent female malignancy and the leading cause of female cancer mortality worldwide. Most recent data showed that global cancer mortality was 7.6 million with breast cancer accounting for over 6% of this mortality [1–3], and a yearly incidence of over one million newly diagnosed cases. Breast cancer, like many other polyetiologic human pathologies, is a product of cumulative genetic, epigenetic, somatic, and endocrine aberrations. The polyetiologism and complexity of breast cancer present a challenge for prevention and treatment. Currently, there are five breast cancer subtypes, among which the basal epithelial or so-called 'triple negative breast cancer' is the most aggressive, with worst prognosis and preference for younger women, especially of African American and Latina ancestry. TNBCs are highly invasive breast carcinomas devoid of estrogen (ER), progesterone (PR), and human epidermal growth factor receptors (HER2). TNBC is associated with high cell proliferation, early disease recurrence, and poor overall survival.

In spite of remarkable advances in anticancer therapy, systemic chemotherapy remains a vital part of breast cancer treatment. To date, the total elimination of TNBC remains elusive in clinical

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oncology practice, as a result of the intrinsic refractoriness of the highly metastatic TNBC to most known chemotherapeutic agents. To facilitate this process, an expanded cancer stem cell pool is essential. It is probable that this intrinsic chemoresistance is associated with TNBC constitutive heterogeneity; with a significant fraction of its tumor bulk exhibiting enhanced tumor-initiating capabilities, marked low chemosensitivity, conferred pseudo-immortality, and subsequently, post-therapy relapse. This cancer stem cell-like phenotype of the TNBC differentiates it from other subtypes of breast cancer and contributes to its enhanced drug resistance, dissemination to secondary site and disease recurrence.

Doxorubicin (Doxo), a non-selective class I anthracycline antibiotic, is a first-line anticancer agent, effective against a wide spectrum of neoplasms, including breast carcinoma, by inhibiting the action of DNA replication enzymes, intercalating with DNA double helix base pairs, interfering with topoisomerases I and II to prevent DNA unwinding and consequently inducing cell death. However, its administration is associated with several dose-dependent adverse effects. Thus, despite recent significant improvement in systemic chemotherapy-associated favorable clinical endpoints, adverse multiorgan effects of doxorubicin accumulation characterized by severe toxicity profile such as cardiotoxicity, myelosuppression, typhilitis, and other doxorubicin-induced pathologies remain limiting factors [4–7]. To minimize these cumulative-dose-dependent Doxo-induced toxicities, reducing Doxo effective therapeutic dose via combination with organ-protective agents has been investigated. The use of certain phytocompounds such as Berberine [8] and Resveratrol [9] in the combination doxorubicin has proved to be effective in limiting cardiotoxicity in mice and human MCF7 breast cancer cell line, respectively. However, none to our knowledge has been demonstrated to be effective against the incurable, chemotherapy– and radiotherapy–refractory hypermalignant TNBCs.

Ovatodiolide is a biologically active macrocyclic diterpenoid extracted from *Anisomeles indica* (L.) Kuntze (Labiatae) [10,11], a plant with broad traditional therapeutic usage, including antiviral [12], anti-inflammatory [11,13,14], analgesic and anti-hyperalgesia [15], treatment of gastrointestinal and hepatic disorders [16,17], hypotensive [18] and antiproliferative activities [19,20]; making Ova an ideal candidate for Doxo combination chemotherapy. Thus, this study aimed to investigate whether the phytocompound Ova interferes with the oncogenic signature of TNBC to effect sensitization of these highly malignant tumor cells to Doxo antiproliferative effects.

The ability of Ova to modulate Doxo was assessed in human TNBC cell lines MDA-MB-231 and HS578T noted for their cancer stem cell-like phenotype and activities, including high oncoaggression and refractoriness to therapy. The cells were treated with varying concentrations of Doxo and Ova simultaneously or sequentially, and the possible mechanisms of Doxo–Ova interaction, effect on cellular accumulation of Doxo, Doxo cytotoxicity, induced cell death were investigated, and we compared results from simultaneous and sequential treatments.

Materials and methods

Drugs and chemicals

Doxorubicin hydrochloride was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Stock solution of 1 mM dissolved in PBS was stored at –20 °C away from light. Ovatodiolide (99.7% purity) was generously provided by Professor Yew-Min Tzeng (Chaoyang University of Technology, Taichung, Taiwan). Ovatodiolide was dissolved in DMSO and further diluted in sterile culture medium immediately prior to use. Drug solutions were diluted to the desired concentration, before use, in RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM), for MDA-MB-231 and HS578T respectively. Gibco® RPMI 1640, DMEM, Trypsin/EDTA, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), sulforhodamine B (SRB) medium, Acetic acid and TRIS base were also purchased from Sigma Aldrich Co.

Cell culture

The human TNBC cell lines MDA-MB-231, HS578T and MCF-10A were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI1640 or DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), Penicillin (100 IU/ml) and Streptomycin (100 μg/ml), in humidified atmosphere with 5% CO_2 at 37 °C. Cells were passaged every 3 days and periodically checked for mycoplasma contamination.

Cytotoxicity assay

Cells were seeded in 96 well microtiter plates at 0.3×10^4 cells/well in appropriate supplemented medium, and incubated for 24 h in humidified atmosphere with 5% CO_2 at 37 °C. Cultured cells were then treated with varying concentrations of the drugs: 0–10 μM and 2.5–40 μM for Doxo and Ova respectively. Drug administration was done concurrently or sequentially. For concurrent treatment, both drugs were introduced simultaneously and incubated for 24 h, while for sequential treatment, cells were pre-treated with Ova overnight and then Doxo administered for 24 h. Cytotoxicity was evaluated using SRB assay as previously described by Vichai and Kirtikara [21]. Wells containing medium only with untreated cells served as control. All quantification was in triplicates and performed thrice. Optical density (OD) was measured at 495 nm wavelength, using SpectraMax microplate reader (Molecular devices, Kim Forest Enterprises Co., Ltd, Taiwan). Cancer cell viability/survival at specific mono or combo concentration was expressed as percentage of untreated control, and estimated as follows:

$$\text{Cell viability}\,(\%) = [\text{OD}_{\text{treated cells}}/\text{OD}_{\text{untreated control cells}}] \times 100$$

$$\text{Cytotoxic Index}\,(\text{CI}) = 1 - [\text{OD}_{\text{treated cells}}/\text{OD}_{\text{untreated control cells}}]$$

The half maximal inhibitory concentration (IC_{50}) of Doxo was determined using the $y = mX + b$ linear equation approach, where $y = 0.5$ (to determine 50% cytotoxicity or cell growth inhibition); m = gradient; X = Doxo concentration that induces 50% cytotoxic or cytocidal effect, and $b = y$-intercept.

Western blotting

Protein extracts were harvested by centrifugation, boiled with lysis buffer for 5 min, loaded and ran by SDS-PAGE, then transferred onto equilibrated PVDF membranes. After blocking with 5% skimmed milk in TBS–Tween-20 (TBST) for 1 h, membranes were incubated with antibodies BCL-2 and BAK (Abnova, Abnova Corporation, Taipei, Taiwan), STAT3, pSTAT3 and GAPDH (Santa Cruz, Santa Cruz Biotechnology Inc., Texas, USA) at 4 °C overnight. After washing with TBST, blots were probed for 1 h with a horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG secondary antibody as appropriate. Protein bands were visualized by UVP Biospectrum Imaging System (Vision Works LS 6.8, Level Biotechnology Inc. Taiwan) using ECL reagents (Thermo Scientific).

Intracellular Doxo accumulation assay

Doxo intracellular accumulation was assessed as previously described [22]. Cells were seeded at 0.1×10^5 in 96 well plates and cultured for 24 h at 37 °C in a 5% CO_2 incubator. Cells were washed and replenished with serum-free culture medium. Cells were then treated with Doxo and Ova concurrently for 2 h, or ova initially for 1 h and then Doxo for another hour, incubating in the 5% CO_2 incubator. Thereafter, cells were washed with PBS twice and lysed with 1% sodium dodecyl sulfate (SDS). We determined the amount of intracellular Doxo accumulated by measuring fluorescence intensity using spectrofluorometer (SpectraMax M3, Molecular Devices, Kim forest Enterprises Co., Ltd, Taiwan). Excitation and emission wavelengths (λ) used were 502 and 588 nm, respectively.

$$\text{Intracellular Doxo Accumulation} = \frac{\text{Doxo concentration}_{\text{ova-sensitized cells}} - \text{Doxo concentration}_{\text{doxo only cells}}}{\text{Doxo concentration}_{\text{doxo only cells}}}$$

Flow-cytometric cell cycle analysis

Cell cycle distribution was determined by collecting 1×10^6 cells after drug treatment. Cell fixation in 70% ethanol at 4 °C was done overnight, followed by PI (1 mg/ml) staining in the presence of 1% RNase A for 30 min. The percentages of the cells in sub-G_1, G_0/G_1, S and G_2/M phases were analyzed by flow-cytometry at excitation and emission of 488 nm and 630 nm, respectively.

In vitro *scratch assay*

Cells were seeded and cultured until about 98–100% confluence in appropriate complete cell growth media, then culture media were suctioned and cells were treated with Doxo alone, Ova and Doxo simultaneously, or Ova then Doxo sequentially. Following treatment, confluence cells were gently wounded along the well central axis

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