

## RESEARCH ARTICLE

# Andrographolide Reverses Doxorubicin Resistance in Human Breast Cancer Stem Cells by Regulating Apoptotic Gene Expressions

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## Abstract

**BACKGROUND:** Breast cancer stem cells (BCSCs) have been identified as playing a crucial role in therapeutic resistance. This resistance can be attributed to the anti-apoptotic protein survivin and the antioxidant MnSOD high expression. To overcome the resistance to doxorubicin (DOX), this study proposed the utilization of andrographolide (ANDRO), the primary bioactive compound in *Andrographis paniculata* leaves. The objective was to examine the role of andrographolide in regulating survivin, caspase-9, and caspase-3 gene expressions to reverse doxorubicin resistance in human BCSCs.

**METHODS:** BCSCs were exposed to 0.1  $\mu$ M DOX every two days or 50  $\mu$ M rotenone (ROT) for 6 hours, subsequently supplemented with 0.3 mM ANDRO. Superoxide and peroxide levels were measured using DHE and DCFH-DA assay. The MnSOD, survivin, caspase-9, and caspase-3 mRNA expression levels were analyzed using qRT-PCR. Protein expressions were evaluated using

Western blotting assay. MnSOD activity was determined using xanthine oxidase inhibition assay. The apoptotic cells were determined using Annexin-V/PI staining.

**RESULTS:** This study indicated that the cytotoxic mechanisms of DOX, similar to ROT, in BCSCs were attributed to oxidative stress, as evidenced by an elevation in superoxide rather than peroxide levels, accompanied by a decrease in MnSOD activity. This study also highlighted that ANDRO reversed DOX resistance in BCSCs subjected to repeated DOX treatment by downregulating survivin and upregulating caspase-9 and caspase-3 mRNA expressions, thereby activating the intrinsic apoptotic pathway.

**CONCLUSION:** This study provides insights into the role of ANDRO in modulating the expression of apoptotic genes, such as survivin, caspase-9, and caspase-3, to overcome DOX resistance in BCSCs.

**KEYWORDS:** breast cancer, breast cancer stem cell, andrographolide, doxorubicin, oxidative stress, apoptosis

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## Introduction

Breast cancer continues to be the most frequently detected cancer and the primary reason for cancer-related fatalities

in women.(1,2) Despite the development of advanced treatments for breast cancer (3,4), the failure rate of breast cancer treatment remains high. This could be attributed to the existence of side populations displaying stemness characteristics akin to those of regular stem cells, known as

breast cancer stem cells (BCSCs).(5-7) Moreover, BCSCs have tumorigenicity which greatly contribute to breast cancer progression and therapy resistance. Nowadays, breast cancer research is focused on targeting BCSCs to prevent metastasis and disease recurrence.(8,9)

Doxorubicin (DOX) is a cytotoxic anthracycline agent that has been widely used as a broad-spectrum chemotherapy agent against several cancers, including breast cancer. Despite its well-known mechanism of action, which involves intercalating between the DNA base pairs and inhibiting DNA synthesis in cancer cells, DOX can also produce an overabundance of reactive oxygen species (ROS), resulting in oxidative damage and cancer cell death. Although DOX remains one of the most effective chemotherapeutic drugs, DOX resistance remains a major barrier to effective cancer treatment.(10,11) Previous study reported that the reduction of DOX sensitivity in BCSCs after repeated treatment is associated with the suppression of oxidative stress.(12) It has been also showed that oxidative stress induced by rotenone (ROT), an inhibitor of complex I mitochondrial electron transport chain, upregulates the synthesis of survivin, leading to a higher survival of BCSCs compared to non-BCSCs.(13) Survivin is an anti-apoptosis protein and is frequently upregulated in numerous types of cancers to suppress the activation of caspase-9 and caspase-3, thereby inhibiting apoptosis.(14-17)

The use of natural active compounds to overcome anticancer resistance has been widely reported, including andrographolide (ANDRO), which is the main bioactive compound in *Andrographis paniculata* leaves.(18,19) Recent studies have suggested that ANDRO has antioxidant, anticancer, and anti-inflammatory activities.(20) Previous *in silico* and *in vivo* studies have shown that ANDRO directly interacts with survivin to inhibit survivin phosphorylation, as well as with caspase-9 and caspase-3, which trigger intrinsic apoptosis.(21) Until now, the effectiveness of ANDRO in overcoming DOX resistance of survivin-overexpressed BCSCs via the modulation of oxidative stress remains unclear. This study aimed to examine the role of ANDRO in the regulation of survivin, caspase-9, and caspase-3 gene expressions to overcome DOX resistance in BCSCs associated with oxidative stress.

## Methods

### Cell Culture

Human BCSCs with a positive expression of aldehyde dehydrogenase (ALDH<sup>+</sup>), obtained from the pleural effusion

of a 79-year-old patient with metastatic breast cancer (estrogen receptor [ER]-positive, progesterone receptor [PgR]-positive, human epidermal growth factor receptor 2 [HER2]-negative), were generously provided by Professor Osamu Ohneda from the Laboratory of Regenerative Medicine and Stem Cell Biology at the Graduate School of Comprehensive Human Sciences, University of Tsukuba, Japan.(22) To maintain the stemness, ALDH<sup>+</sup> BCSCs were cultivated using a serum-free medium, specifically Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Invitrogen, Carlsbad, CA, USA) supplemented with 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) and 1% amphotericin B (Gibco) in a 37°C incubator with 5% CO<sub>2</sub> and 20% O<sub>2</sub>. It has been reported that subjecting ALDH<sup>+</sup> BCSCs to a 10% fetal bovine serum treatment led to the differentiation of these cells, resulting in significant alterations in their appearance, shifting from floating and sphere-like cells to adherent and epithelial-like cells.(23) Cell viability was assessed with trypan blue exclusion method by using Luna™ Automated Cell Counter (Logos Biosystems, Gyeonggi-do, South Korea).

### DOX and ROT Treatment

Both DOX (Sigma-Aldrich, St. Louis, MO, USA) and ROT (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and diluted in a DMEM/F12 medium without serum. A total of 1×10<sup>5</sup> cells per well were seeded in a 12-well plate. After overnight adaptation, BCSCs were treated with ROT of 50 μM for 6 hours or DOX of 0.1 μM for 2 days.(12,13) Afterwards, the cells were harvested and counted for total mRNA and protein extraction.

### Supplementation of ANDRO to DOX- and ROT-treated BCSCs

Cells were divided into three groups: control, DOX, and DOX+ANDRO cells. The DOX cells were treated with 0.1 μM DOX every two days starting from day 0 until day 20.(12) DOX+ANDRO cells were first treated like DOX cells until day 12, then treated with the combination of 0.1 μM DOX and 0.3 mM ANDRO every two days (from day 14 until day 20). Control cells were merely treated with 0.01% DMSO as vehicles using the same schedule as DOX and DOX+ANDRO cells. Cells were harvested and counted every two days starting from day 2 until day 22. To analyze the effect of ANDRO on ROT-treated BCSCs, BCSCs were first treated with 50 μM ROT for 6 hours, then supplemented with 0.3 mM ANDRO for 24 hours.

### Determination of Intracellular ROS Levels

To determine the intracellular ROS levels, a dihydroethidium (DHE) probe (Sigma-Aldrich) was utilized for superoxide, and a 2',7'-dichlorofluorescein-diacetate (DCFH-DA) probe (Sigma-Aldrich, St. Louis, Missouri, USA) was utilized for hydrogen peroxide as described in a previous study.(12) Briefly,  $2 \times 10^4$  cells were harvested and washed twice with sterile phosphate buffer saline (PBS). After centrifugation, cell pellets were re-suspended in 500  $\mu$ L PBS and exposed to either 20  $\mu$ M DHE or 20  $\mu$ M DCFH-DA for 30 minutes at 37°C in a dark environment. Subsequently, the fluorescence intensity was assessed using a fluorometer (Varioskan™ Flash Multimode Reader, ThermoFisher Scientific, Waltham, MA, USA) with excitation and emission wavelengths of 480 nm and 585 nm, respectively, for DHE and 485 nm and 530 nm, respectively, for DCFH-DA. Data were presented as the ratio of fluorescence intensity in treated cells to that in control.

### Measurement of Manganese-dependent Superoxide Dismutase (MnSOD) Activity

The xanthine oxidase inhibition assay was used to assess the MnSOD enzymatic activity (Randox Laboratories, Crumlin, UK), as described in a prior study.(12) The absorbance was measured using a spectrophotometer (Varioskan™ Flash Multimode Reader, ThermoFisher Scientific) at a wavelength of 505 nm. Data were presented as the ratio of MnSOD activity in treated cells to that in control.

### Analysis of mRNA Relative Expression Levels Using Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted using Tripure Isolation Reagent® (Roche Diagnostic, Basel, Switzerland) according to manufacturer's instructions. The concentration of total RNA was quantified by using spectrophotometry (Varioskan™ Flash Multimode Reader, ThermoFisher Scientific) with a wavelength of 260 nm and the purity of RNA was measured by comparing a wavelength at 260 and 280 nm. The qRT-PCR assay was performed in a one-step reaction using the SensiFAST™ SYBR® No-ROX kit (Bioline, London, UK) in 7500 Fast Real-Time PCR System (Applied Biosystem, Waltham, MA, USA) according to manufacturer's instructions.

The qRT-PCR was performed using the following primers: 18S rRNA Forward 5'-AAACGGCTACCACATCCAAG-3' and Reverse

5'-CCTCCAATGGATCCTCGTTA-3'; Survivin Forward 5'-GCCAGATGACGACCCCATAGAGGA-3' and Reverse 5'-TCGATGGGCACGGCGCACTTT-3'; Caspase-9 Forward 5'-TTGGTGATGTCGAGCAGAAA-3' and Reverse 5'-GGCAAAGTAGATATGGCGTC-3'; Caspase-3 Forward 5'-TGAGGCGGTTGTAGAAGAGTT-3' and Reverse 5'-CACACCTACCGATAACCAGAG-3'.(21) Levels of relative mRNA expression were normalized to control and presented as  $2^{-\Delta\Delta Ct}$  using Livak formula.(24)

### Analysis of Protein Expression Levels Using Western Blotting Assay

Western blotting assay was used to analyze the protein expression levels as previously described.(21) The nitrocellulose membranes were incubated with 1:4000 mouse anti- $\beta$ -actin (Cell Signaling Technology, Danvers, MA, USA), 1:250 rabbit anti-survivin (Elabscience, Houston, TX, USA), 1:4000 rabbit anti-MnSOD (Cell Signaling Technology), 1:250 rabbit anti-caspase-3 (Abcam, Cambridge, UK), or 1:500 rabbit anti-caspase-3 cleaved Asp175 (Genetex, Irvine, CA, USA) antibody for 24 hours at 4°C. Subsequently, the membranes were subjected to an incubation process with an HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 2 hours and visualized using Enhance Chemiluminescence (ECL) reagent (Biorad, Hercules, CA, USA) as the HRP substrate.

### Cell Apoptosis Assay Using Annexin V and Propidium Iodide (PI) Staining

Quantification of cell apoptosis was determined using flowcytometry assay with Annexin V-FITC Apoptosis Detection Kit (Abcam). The cells were harvested, washed using PBS, and then re-suspended in 500  $\mu$ L binding buffer solution. Cell suspension was incubated with 5  $\mu$ L Annexin V-FITC and 5  $\mu$ L PI for 5 minutes in a dark environment and subsequently analyzed using a flowcytometer (BD FACSVerser flowcytometry, BD Biosciences, Franklin Lakes, NJ, USA).

### Statistical Analysis

The data from three independent experiments, each with triplicates, were presented as the mean  $\pm$  standard deviation (SD). Significant differences were analyzed using analysis of variance (ANOVA), and subsequently, the Tukey's test was analyzed to demonstrate the differences among various groups.

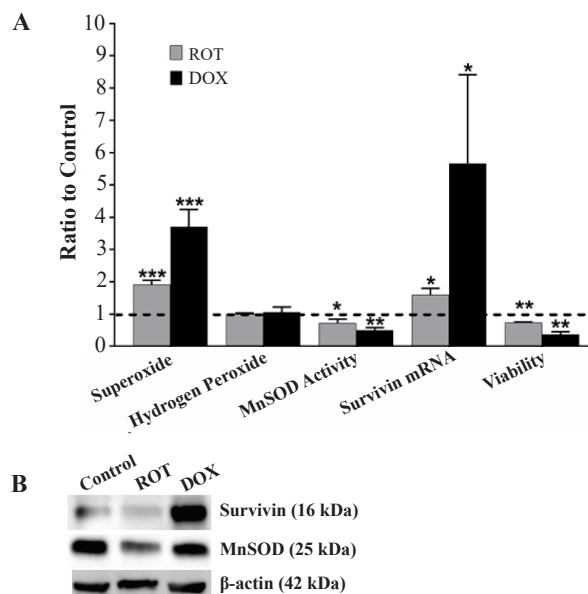
## Results

### DOX Increased Superoxide Level and Decreased MnSOD Activity in BCSCs

To evaluate oxidative stress modulation in DOX-treated BCSCs, the levels of superoxide and peroxide, as well as the MnSOD enzyme activity were analyzed. BCSCs were also treated with ROT to compare the analyses. Figure 1A showed a significant enhancement of superoxide level (~3.70-fold;  $p=0.0005$ ) and a significant suppression of MnSOD activity (~2.0-fold;  $p=0.0087$ ) in BCSCs after 2 days of DOX treatment compared to control. In contrast to superoxide, there was no significant differences in peroxide levels. This study also demonstrated that MnSOD protein expression was suppressed after 2 days of DOX treatment (Figure 1B). These results were consistent with those obtained from ROT treatment (Figure 1A, Figure 1B), indicating that DOX induces oxidative stress in a similar manner to ROT.

### ANDRO Reversed DOX Resistance in BCSCs Treated With Repeated DOX

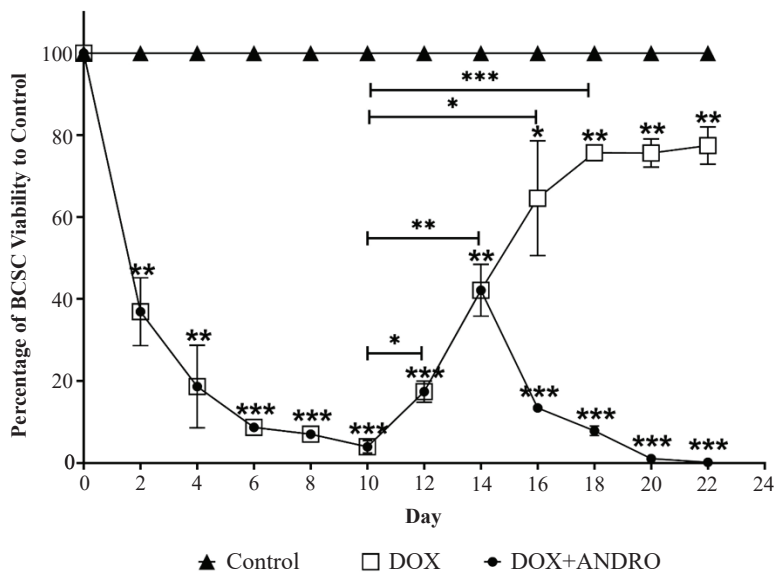
To evaluate the effect of ANDRO on DOX sensitivity of human BCSCs, BCSCs were first treated with 0.1  $\mu\text{M}$  DOX repeatedly every two days until the sensitivity of BCSCs to DOX was reduced. As shown in Figure 2, the viability of DOX-treated BCSCs gradually decreased until it reached 3.92% ( $p<0.001$ ) during the first ten days of DOX treatment. Subsequently, the viability of BCSCs started to increase, reaching 77.42% ( $p<0.01$ ) after 22 days of repeated DOX treatment, indicating a reduction in DOX sensitivity of BCSCs. On day 14, BCSCs were treated with the combination of 0.1  $\mu\text{M}$  DOX and 0.3 mM ANDRO every two days until day 20. The results demonstrated that ANDRO supplementation to repeated DOX treatment gradually resensitized BCSCs, with viability reaching 0.16% ( $p<0.001$ ) and a higher percentage of cells in early and late apoptosis (68.2%) compared to those in DOX (0.3%) and control (0%) cells on day 22 (Figure 3A - Figure 3C). Apoptosis analysis was also compared with that in ROT-treated BCSCs (Figure 3D - Figure 3F). The results of apoptosis in ROT-treated BCSCs were consistent with their reduced viability (Figure 1A). ANDRO supplementation at 0.3 mM increased the apoptosis of ROT-treated BCSCs (39%) compared to those without ANDRO supplementation (13.2%) and control (11%), confirming that ANDRO increased apoptosis in oxidative stress-induced BCSCs.



**Figure 1. Effects of DOX and ROT treatment on the ROS levels, MnSOD activity, survivin mRNA, and cell viability in human BCSCs.** Cells were treated with 0.1  $\mu\text{M}$  DOX for two days and 50  $\mu\text{M}$  ROT for 6 hours, respectively. A: Levels of superoxide, peroxide, MnSOD activity, survivin mRNA, and viability in DOX- or ROT-treated BCSCs were presented as ratio to the control treated with 0.01% DMSO as vehicle (mean  $\pm$  SD). The dashed line shows the value of the respective control. The significance differences between treated cells and controls were analyzed using ANOVA and Tukey's test and shown as \* $p<0.05$ , \*\* $p<0.01$ , and \*\*\* $p<0.001$ . B: Protein expressions of MnSOD and survivin in BCSCs were analysed using Western blotting assay.  $\beta$ -actin was used as the internal control.

### ANDRO Regulated the Gene Expressions Involved in Intrinsic Apoptosis of BCSCs

Figure 1A demonstrated that BCSC viability was suppressed into 36.92% ( $p=0.0042$ ) after 2 days of DOX-treatment, but the relative expression levels of their survivin mRNA were enhanced (5.66-fold;  $p=0.0208$ ). This data was supported by the increase of survivin expression at protein level (Figure 1B). Following repeated DOX treatment, the increase in survivin mRNA expression on day 16 and 18 was significantly higher 7.53-fold ( $p=0.026$ ) and 4.00-fold ( $p=0.0164$ ) than that on day 2, respectively (Figure 4). Consequently, BCSC viability on day 16 and 18 was increased 16.46-fold ( $p=0.0129$ ) and 19.29-fold ( $p=0.001$ ) compared to that on day 10, respectively (Figure 2). Additionally, this study found that after two-day of DOX treatment the expression level of caspase-9 mRNA in BCSCs significantly decreased 0.47-fold ( $p=0.0029$ ; Figure 5), while the caspase-3 mRNA level showed no significant change ( $p=0.2776$ ; Figure 6A) compared to their respective controls. In accordance

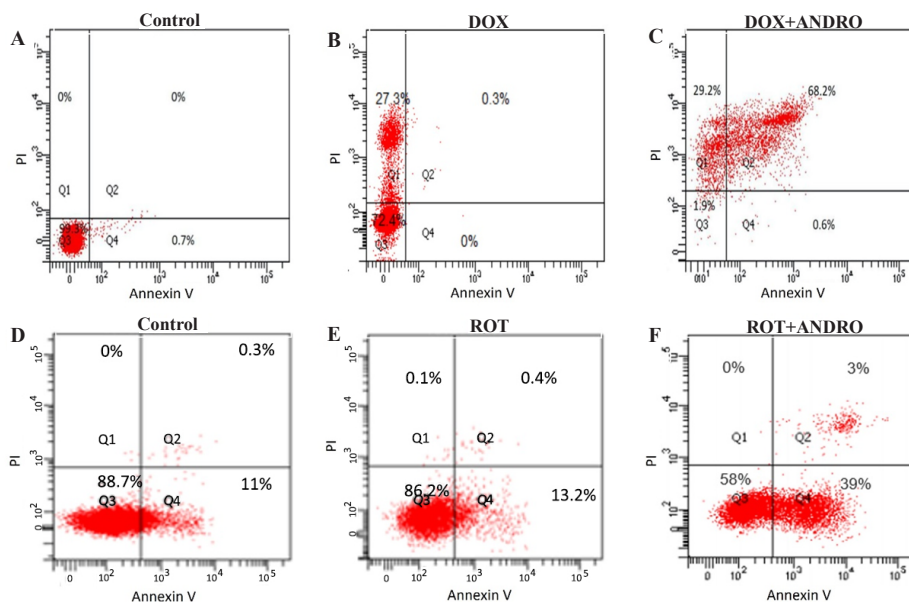


**Figure 2. ANDRO sensitized BCSCs treated with repeated DOX.** Cells were divided into three groups: control, DOX, and DOX+ANDRO cells. DOX and DOX+ANDRO cells were firstly exposed with 0.1 μM of DOX every two days until day 20. In addition to DOX treatment, DOX+ANDRO cells were supplemented with 0.3 mM ANDRO every two days starting from day 14 until day 20. Cells were harvested and counted every two days. Control cells were exposed with 0.01% DMSO as vehicle. BCSC viability was presented as the percentage to control (mean ± SD). The significance differences between treated cells and their respective control were analyzed using ANOVA and Tukey’s test and shown as \**p*<0.05, \*\**p*<0.01, and \*\*\**p*<0.001.

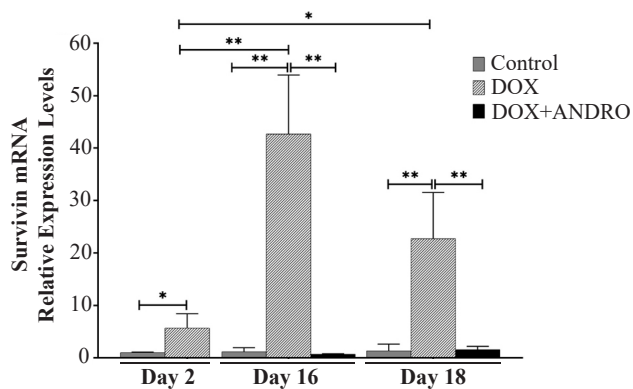
to the increased BCSC viability following repeated DOX treatment, the caspase-9 and caspase-3 mRNA expression levels on day 16 of DOX treatment decreased 1.41-fold (*p*=0.0326; Figure 5) and 1.36-fold (*p*=0.1262; Figure 6A) compared to those on day 2, respectively. Likewise, on day 18 the mRNA expressions of both pro-apoptotic proteins significantly reduced 1.45-fold (*p*=0.0190; Figure 5) and

2.63-fold (*p*=0.0186; Figure 6A) compared to those on day 2, respectively.

However, following two- and four-day of ANDRO supplementation to DOX-treated BCSCs, survivin mRNA relative expression levels were significantly reduced 64.76-fold (day 16; *p*=0.0015) and 14.66-fold (day 18; *p*=0.0071) compared to that without ANDRO supplementation,



**Figure 3. Supplementation of ANDRO increased apoptosis in DOX- and ROT-treated BCSCs.** DOX and DOX+ANDRO cells were repeatedly treated with 0.1 μM DOX every two days until day 20. In addition to DOX treatment, 0.3 mM ANDRO was supplemented to DOX+ANDRO cells every two days starting from day 14 until day 20. ROT and ROT+ANDRO cells were treated with 50 μM ROT for 6 hours. Then, ROT+ANDRO cells were supplemented with 0.3 mM ANDRO for 24 hours. Apoptosis assays were performed on day 22 of DOX and DOX+ANDRO treatment (A-C) or after 24-hour ROT and ROT+ANDRO treatment (D-F). Cells were double stained with Annexin V and PI for flowcytometry assay. A, D: Cells treated with 0.01% DMSO as controls; B: Cells treated with DOX; C: Cells treated with DOX+ANDRO; E: Cells treated with ROT; F: Cells treated with ROT+ANDRO.

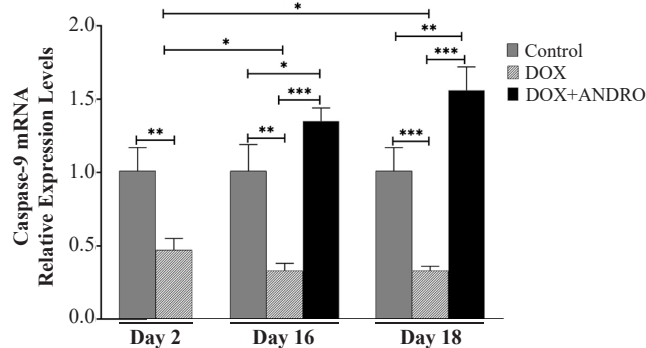


**Figure 4. ANDRO suppressed survivin expression in BCSCs treated with repeated DOX.** DOX and DOX+ANDRO cells were repeatedly treated with 0.1  $\mu\text{M}$  DOX every two days until day 20. In addition to DOX treatment, 0.3 mM ANDRO was supplemented to DOX+ANDRO cells every two days starting from day 14 until day 20. Control cells were treated with 0.01% DMSO as vehicle. The mRNA relative expression levels of survivin in BCSCs treated with DOX or DOX+ANDRO were determined on day 2, 16, and 18 using qRT-PCR assay and presented as  $2^{-\Delta\Delta Ct}$  using Livak formula. Data were presented as the mean  $\pm$  SD. The significance differences between treated cells and their respective control were analyzed using ANOVA and Tukey's test and shown as \*  $p < 0.05$  and \*\*  $p < 0.01$ .

respectively (Figure 4). In line with the reduction in survivin expression, the supplementation of ANDRO to DOX-treated BCSCs on day 14 increased caspase-9 mRNA expression levels by 4.04-fold (day 16;  $p < 0.001$ ) and 4.79-fold (day 18;  $p < 0.001$ ) compared to their respective DOX groups (Figure 5). Similarly, the supplementation of ANDRO to DOX-treated BCSCs significantly increased the expression levels of caspase-3 mRNA on day 16 (3.42-fold,  $p = 0.0005$ ) and day 18 (4.07-fold,  $p = 0.0006$ ) compared to their respective DOX groups (Figure 6A). Furthermore, the present study demonstrates that ANDRO increased the expression of both pro- and cleaved caspase-3 protein (Figure 6B). Altogether, these results indicate that ANDRO reversed DOX resistance in BCSCs by regulating the mRNA synthesis of anti- and pro-apoptotic proteins.

## Discussion

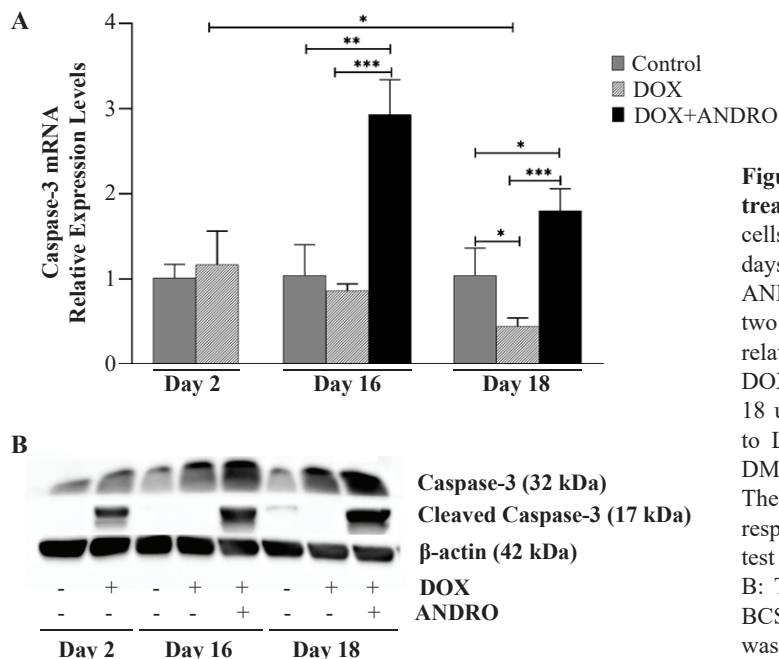
Until now, drug resistance remains a main unresolved problem in cancer chemotherapy. Despite its great potential for breast cancer chemotherapy, DOX treatment can induce drug resistance via several signaling pathways that induce cell proliferation and inhibit cell death. Since the cytotoxic mechanisms of DOX are mostly through its metabolite, semiquinone radical, that causes oxidative stress,



**Figure 5. ANDRO increased caspase-9 expression in BCSCs treated with repeated DOX.** DOX and DOX+ANDRO cells were repeatedly treated with 0.1  $\mu\text{M}$  DOX every two days until day 20. In addition to DOX treatment, 0.3 mM ANDRO was supplemented to DOX+ANDRO cells every two days starting from day 14 until day 20. Control cells were treated with 0.01% DMSO as vehicle. The mRNA relative expression levels of caspase-9 in BCSCs treated with DOX or DOX+ANDRO were determined on day 2, 16, and 18 using qRT-PCR assay and presented as  $2^{-\Delta\Delta Ct}$  using Livak formula. Data were presented as the mean  $\pm$  SD. The significance differences between treated cells and their respective control were analyzed using ANOVA and Tukey's test and shown as \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

suppression of oxidative stress in cancer cells may stimulate the signaling pathways of DOX resistance. In this study, the DOX-induced oxidative stress in BCSCs was compared with that induced by ROT that is known as an oxidative stress inducer. DOX, likewise ROT, induced oxidative stress, in which superoxide anion, but not peroxide, was enhanced. ROT inhibits electron transfer of mitochondrial respiratory chain from the iron-sulfur centers in complex I to ubiquinone leading to an increase of superoxide production. (25,26) Meanwhile, DOX can produce superoxide anion through several mechanisms, particularly the enhancement of NADPH oxidase activity.(10,11)

The present study is consistent with previous study which indicates that during oxidative stress induction, BCSCs attempt to eliminate superoxide radicals using the endogenous mitochondrial enzyme, MnSOD, which in turn reduced its activity.(27) There can be multiple causes of reduced MnSOD activity in the overaccumulation of superoxide. One possible cause is the direct inhibition of MnSOD activity by the excess superoxide, which can contribute to the production of hydroxyl radicals, the most toxic ROS, or react with mitochondrial nitric oxide to produce peroxynitrite, a reactive nitrogen species (RNS); both radicals further attack mitochondrial enzymes including MnSOD.(28) Another possible cause is the downregulation of MnSOD gene expression, which can be



**Figure 6. ANDRO increased caspase-3 expression in BCSCs treated with repeated DOX.** DOX and DOX+ANDRO cells were repeatedly treated with 0.1 μM DOX every two days until day 20. In addition to DOX treatment, 0.3 mM ANDRO was supplemented to DOX+ANDRO cells every two days starting from day 14 until day 20. A: The mRNA relative expression levels of caspase-3 in BCSCs treated with DOX or DOX+ANDRO were determined on day 2, 16, and 18 using qRT-PCR assay and presented as 2<sup>-ΔΔCt</sup> according to Livak formula. Control cells were treated with 0.01% DMSO as vehicle. Data were presented as the mean ± SD. The significance differences between treated cells and their respective control were analyzed using ANOVA and Tukey’s test and shown as \**p*<0.05, \*\**p*<0.01, and \*\*\**p*<0.001. B: The pro- and cleaved caspase-3 protein expressions in BCSCs were analyzed using Western blotting assay. β-actin was used as the internal control.

mediated by various signaling pathways activated by the excess superoxide.(29)

The role of MnSOD in cancer is still subject to debate. While it has been proposed that MnSOD acts as a tumor suppressor during tumorigenesis or in the early stages of cancer growth, many studies have shown evidence of MnSOD functioning as an oncogene, with its expression level positively correlated with aggressive and invasive cancer phenotypes.(30) Moreover, overexpression of MnSOD has been associated with DOX resistance, promoting cancer cell survival in various types of cancer, including circulating breast cancer cells.(31,32) Thus, the reduced sensitivity of BCSCs to DOX observed in this study may be due to the increased activity of MnSOD, which in turn leads to decreased lipid peroxidation.(27) The mechanism underlying MnSOD overexpression in DOX resistance involves the activation of Nrf2 signaling.(32) Nrf2 is a DNA-binding transcription factor that promotes the expression of multiple antioxidant genes, including MnSOD. It has been reported that the disruption of Nrf2 signaling leads to impaired antioxidant defense.(33) However, upstream mediators, such as natural compounds, may regulate Nrf2 signaling, potentially influencing DOX resistance or offering chemoprotection against DOX-induced toxicity in organs.(34)

In addition to modulating MnSOD activity, this study highlights the impact of oxidative stress induced by ROT and DOX on the gene expression of survivin, a member of the inhibitor-apoptosis-protein (IAP) family, in BCSCs. Notably, during the early phase of DOX treatment, survivin

mRNA expression was significantly upregulated, despite a reduction in BCSC viability (Figure 1). This trend was comparable to the results observed with ROT treatment. It is postulated that BCSCs respond to the increase in cell death caused by oxidative stress by elevating survivin at both mRNA and protein levels. BCSCs constitute a minor yet highly aggressive cell population in breast cancer, characterized by tumorigenic capacity attributed to the overexpression of MnSOD and survivin, among other factors.(35,36) As evidenced in recent publication, human BCSCs (CD24<sup>-</sup>/CD44<sup>+</sup>) exhibit enhanced resistance to oxidative stress compared to their non-BCSCs counterpart (CD24<sup>+</sup>/CD44<sup>-</sup>), in line with their higher expressions of MnSOD and survivin.(13)

However, following repeated DOX treatment, the overwhelming upregulation of survivin, coupled with the downregulation of pro-apoptotic proteins caspase-9 and caspase-3, surpassed the cytotoxic effect of DOX, allowing BCSCs to sustain their viability. These gene expression modulations may correspond to the aforementioned overexpression of MnSOD. MnSOD-induced survivin expression is mediated by AMP-activated kinase (AMPK) activation which shifts cancer cell metabolism towards glycolysis.(37) Therefore, we speculate that the MnSOD/AMPK pathway could be a potential mechanism responsible for DOX resistance in BCSCs, the most aggressive cell population in breast cancer, contributing to disease progression. Furthermore, a link between ROS production and the downregulation of survivin expression through the ROS/AKT/FOXO/survivin signaling axis has been

suggested.(38) Thus, it is also plausible that the observed overexpression of survivin in the present study was triggered by decrease in superoxide anion levels as a consequence of increased of MnSOD activity.

Consistently, numerous studies have demonstrated that the association between DOX sensitivity and MnSOD activity is supported by ANDRO supplementation, which has the ability to reverse DOX resistance by reducing MnSOD activity and increasing oxidative stress.(27) However, the present study highlights that the action mechanism of ANDRO in re-sensitizing BCSCs to DOX also involves the modulation of anti- and pro-apoptotic gene expressions, specifically survivin, caspase-9 and caspase-3, in BCSCs with reduced viability. To elaborate, ANDRO supplementation after 14-day repeated DOX treatment suppressed survivin and enhanced caspase-9 and caspase-3 mRNA synthesis, leading to the reversal of DOX resistance. This was supported by a significant increase in apoptotic cell percentages, a decrease in live cell percentages in BCSCs, and an increase in both pro-caspase-3 and cleaved caspase-3 protein expression. Despite upregulating the gene expression of pro-caspase-3, the present study also suggests that ANDRO also activated the cleavage of pro-caspase-3 into cleaved caspase-3. In a previous *in silico* study, it has been demonstrated that ANDRO can directly bind to survivin, caspase-9, and caspase-3, thereby preventing the activation of anti-apoptotic proteins and promoting pro-apoptotic proteins.(21) This molecular interaction further strengthens the contribution of ANDRO in re-sensitizing BCSCs to DOX.

Overall, this study emphasize that the cytotoxic mechanisms of DOX, likewise ROT, generate ROS, mainly the superoxide anion, rather than peroxide. This leads to the suppression of MnSOD levels and subsequently induces oxidative stress. BCSCs are capable of compensating for the cytotoxic effects of DOX by increasing survivin expression, which allows them to survive oxidative stress. The dramatic increase in survivin expression observed during repeated DOX treatment in BCSCs highlights the crucial role of BCSCs in developing DOX resistance due to their aggressive nature. Additionally, ANDRO supplementation sensitizes human BCSCs to DOX treatment by downregulating survivin and upregulating caspase-9 and caspase-3 mRNA expressions, thereby activating intrinsic apoptosis. Further studies are needed to investigate the upstream signaling pathways modulated by ANDRO that regulate the redox status, aiming to overcome DOX resistance in BCSCs. Moreover, it is essential to evaluate the efficacy of ANDRO supplementation in combination with DOX therapy *in vivo*.

## Conclusion

This study sheds light on the role of ANDRO in regulating apoptotic gene expressions, such as survivin, caspase-9, and caspase-3, which is likely achieved by reversing oxidative stress status through MnSOD downregulation. Therefore, this study propose that combining ANDRO supplementation with DOX chemotherapy can not only combat DOX resistance in breast cancer, particularly in BCSCs, but also prevent its development.

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## Authors Contribution

SIW, RAS, and ML were involved in conceptualization. SIW and RAS were involved in methodology. RAS, AS, and EY were involved in data curation. SIW, RAS, and ML were involved in formal analysis. SIW, RAS, AS, EY, and ML were involved in investigation. SIW were involved in funding acquisition. SIW and RAS were involved in writing original draft, reviewing, and editing. All authors discussed the results and commented on the manuscript.

## References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2021; 71(3): 209-49.
2. Azamjah N, Soltan-Zadeh Y, Zayeri F. Global trend of breast cancer mortality rate: A 25-year study. *Asian Pac J Cancer Prev.* 2019; 20(7): 2015-20.
3. Parsons J, Francavilla C. 'Omics approaches to explore the breast cancer landscape. *Front Cell Dev Biol.* 2019; 7: 395. doi: 10.3389/fcell.2019.00395.
4. Moo TA, Sanford R, Dang C, Morrow M. Overview of breast cancer therapy. *PET Clin.* 2018; 13(3): 339-54.



5. Yang L, Shi P, Zhao G, Xu J, Peng W, Zhang J, *et al.* Targeting cancer stem cell pathways for cancer therapy. *Signal Transduct Target Ther.* 2020; 5(1): 8. doi: 10.1038/s41392-020-0110-5.
6. Shibata M, Hoque MO. Targeting cancer stem cells: A strategy for effective eradication of cancer. *Cancers.* 2019; 11(5): 732. doi: 10.3390/cancers11050732.
7. Nadia YF, Syahrani RA, Arumsari S, Sadikin M, Wanandi SI. Effect of cell culture medium on the proliferation and stemness of CD24-/CD44+ human breast cancer stem cells. *Indones Biomed J.* 2021; 13(4): 355-63.
8. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA.* 2003; 100(7): 3983-8.
9. Lindeman GJ, Visvader JE. Insights into the cell of origin in breast cancer and breast cancer stem cells. *Asia Pac J Clin Oncol.* 2010; 6(2): 89-97.
10. Bandyopadhyay A, Wang L, Agyin J, Tang Y, Lin S, Yeh IT, *et al.* Doxorubicin in combination with a small TGFbeta inhibitor: A potential novel therapy for metastatic breast cancer in mouse models. *PLoS One.* 2010; 5(4): e10365. doi: 10.1371/journal.pone.0010365.
11. Micallef I, Baron B. Doxorubicin: An overview of the anti-cancer and chemoresistance mechanisms. *Annals of Clinical Toxicology.* 2020; 3(2): 1031.
12. Syahrani RA, Louisa M, Wanandi SI. Sensitivity of breast cancer stem cells (Cd24-/Cd44+) to Doxorubicin is associated with oxidative stress status. *Int J Appl Pharm.* 2019; 11(6): 91-6.
13. Wanandi SI, Syahrani RA, Jusman SWA. 8P - The impact of rotenone-modulated oxidative stress on the survival of human breast cancer stem cells (CD24-/CD44+). *Ann Oncol.* 2017; 28 (Suppl 5): v3.
14. Chen X, Duan N, Zhang C, Zhang W. Survivin and tumorigenesis: Molecular mechanisms and therapeutic strategies. *J Cancer.* 2016; 7(3): 314-23.
15. Mobahat M, Narendran A, Riabowol K. Survivin as a preferential target for cancer therapy. *Int J Mol Sci.* 2014; 15(2): 2494-516.
16. Rahman MN, Wijaya CR, Novalentina M. Survivin clinical features in cervical cancer. *Mol Cell Biomed Sci.* 2017; 1(1): 6-16.
17. Sandra F. Survivin Ser81 plays an important role in PI3K/Akt/mTOR signaling pathway. *Mol Cell Biomed Sci.* 2018; 2(2): 55-9.
18. Jarukamjorn K, Nemoto N. Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent andrographolide. *J Health Sci.* 2008; 54(4): 370-81.
19. Chao WW, Kuo YH, Lin BF. Isolation and identification of *Andrographis paniculata* (Chuanxinlian) and its biologically active constituents inhibited enterovirus 71-induced cell apoptosis. *Front Pharmacol.* 2021; 12: 762285. doi: 10.3389/fphar.2021.762285.
20. Adiguna SP, Panggabean JA, Swasono RT, Rahmawati SI, Izzati F, Bayu A, *et al.* Evaluations of andrographolide-rich fractions of *Andrographis paniculata* with enhanced potential antioxidant, anticancer, antihypertensive, and anti-inflammatory activities. *Plants.* 2023; 12(6): 1220. doi: 10.3390/plants12061220.
21. Wanandi SI, Limanto A, Yunita E, Syahrani RA, Louisa M, Wibowo AE, *et al.* In silico and in vitro studies on the anti-cancer activity of andrographolide targeting survivin in human breast cancer stem cells. *PLoS One.* 2020; 15(11): e0240020. doi: 10.1371/journal.pone.0240020.
22. Shirraishi A, Tachi K, Essid N, Tsuboi I, Nagano M, Kato T, *et al.* Hypoxia promotes the phenotypic change of aldehyde dehydrogenase activity of breast cancer stem cells. *Cancer Sci.* 2017; 108(3): 362-72.
23. Wanandi SI, Syahrani RA, Arumsari S, Wideani G, Hardiany NS. Profiling of gene expression associated with stemness and aggressiveness of ALDH1A1-expressing human breast cancer cells. *Malays J Med Sci.* 2019; 26(5): 38-52.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(delta delta C(T)) method. *Methods.* 2001; 25(4): 402-8.
25. Heinz S, Freyberger A, Lawrenz B, Schladt L, Schmuck G, Ellinger-Ziegelbauer H. Mechanistic investigations of the mitochondrial complex I inhibitor rotenone in the context of pharmacological and safety evaluation. *Sci Rep.* 2017; 7: 45465. doi: 10.1038/srep45465.
26. Ochi R, Dhagia V, Lakhkar A, Patel D, Wolin MS, Gupte SA. Rotenone-stimulated superoxide release from mitochondrial complex I acutely augments L-type Ca<sup>2+</sup> current in A7r5 aortic smooth muscle cells. *Am J Physiol Heart Circ Physiol.* 2016; 310(9): H1118-28.
27. Rachman AT, Suraduhita A, Syahrani RA, Louisa M, Wanandi SI. Suppression of MnSOD by andrographolide and its relation to oxidative stress and viability of breast cancer stem cells treated with repeated doxorubicin administration. 1st Jenderal Soedirman International Medical Conference (JIMC) 2020. Proceedings of the 1st Jenderal Soedirman International Medical Conference in conjunction with the 5th Annual Scientific Meeting (Temilnas) Consortium of Biomedical Science Indonesia 2020, November 28 – 29, 2020. Setúbal, Portugal: SCITEPRESS; 2021. p. 28-34.
28. Holley AK, Bakthavatchalu V, Velez-Roman JM, St Clair DK. Manganese superoxide dismutase: Guardian of the powerhouse. *Int J Mol Sci.* 2011; 12(10): 7114-62.
29. Li M, Chiu JF, Mossman BT, Fukagawa NK. Down-regulation of manganese-superoxide dismutase through phosphorylation of FOXO3a by Akt in explanted vascular smooth muscle cells from old rats. *J Biol Chem.* 2006; 281(52): 40429-39.
30. Miriyala S, Spasojevic I, Tovmasyan A, Salvemini D, Vujaskovic Z, St Clair D, *et al.* Manganese superoxide dismutase, MnSOD and its mimics. *Biochim Biophys Acta.* 2012; 1822(5): 794-814.
31. Cho SJ, Park JW, Kang JS, Kim WH, Juhn YS, Lee JS, *et al.* Nuclear factor-kappaB dependency of doxorubicin sensitivity in gastric cancer cells is determined by manganese superoxide dismutase expression. *Cancer Sci.* 2008; 99(6): 1117-24.
32. Fu A, Ma S, Na W, Tan BXX, Tan EY, Luo KQ. High expression of MnSOD promotes survival of circulating breast cancer cells and increases their resistance to doxorubicin. *Oncotarget.* 2016; 7(31): 50239-57.
33. Miller CJ, Gounder SS, Kannan S, Goutam K, Muthusamy VR, Firpo MA, *et al.* Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle. *Biochim Biophys Acta.* 2012; 1822(6): 1038-50.
34. Mirzaei S, Zarrabi A, Hashemi F, Zabolian A, Saleki H, Azami N, *et al.* Nrf2 Signaling pathway in chemoprotection and doxorubicin resistance: Potential application in drug discovery. *Antioxidants.* 2021; 10(3): 349. doi: 10.3390/antiox10030349.
35. Yu C, Ou J, Wang M, Jialielihan N, Liu Y. Elevated survivin mediated multidrug resistance and reduced apoptosis in breast cancer stem cells. *JBUON.* 2015; 20(5): 1287-94.
36. Yousefnia S, Seyed Foroootan F, Seyed Foroootan S, Nasr Esfahani MH, Gure AO, Ghaedi K. Mechanistic pathways of malignancy in breast cancer stem cells. *Front Oncol.* 2020; 10: 452. doi: 10.3389/fonc.2020.00452.
37. Hart PC, Mao M, de Abreu AL, Ansenberger-Fricano K, Ekoue DN, Ganini D, *et al.* MnSOD upregulation sustains the Warburg effect via mitochondrial ROS and AMPK-dependent signalling in cancer. *Nat Commun.* 2015; 6: 6053. doi: 10.1038/ncomms7053.
38. Wani TH, Chowdhury G, Chakrabarty A. Generation of reactive oxygen species is the primary mode of action and cause of survivin suppression by sepantonium bromide (YM155). *RSC Med Chem.* 2021; 12(4): 566-78.