Enhanced Antitumor and Anti-Metastasis Efficiency via Combined Treatment with Carvacrol and Doxorubicin

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ABSTRACT

Recent research on treatment of triple-negative breast cancer (TNBC) has focused on drug combinations that can improve their anticancer properties while minimizing adverse effects. This study investigated the efficacy of combined treatment with doxorubicin (Dox) and carvacrol on proliferation, apoptosis, migration and invasion of MDA-MB-231 cells. After co-treatment with varying concentrations of Dox and carvacrol, cell proliferation, migration, and invasion were assessed. The expression levels of proteins associated with the observed effects were measured through Western blotting. The results showed that, co-administration of Dox and carvacrol exhibited a notable dose-dependent reduction in cell proliferation, migration, and invasion. Furthermore, this combination upregulated the expression of Bax and caspase-3 while downregulated Bcl-2 expression. These findings indicated the induction of cell apoptosis through the intrinsic apoptosis pathway. In conclusion, carvacrol may function as a potential chemosensitizer, enhancing Dox-induced activation of intrinsic apoptosis pathway. The combined treatment of Dox and carvacrol holds promise as a potential strategy to enhance the efficacy of future Dox-based breast cancer treatment regimens, offering potential benefits for patients with TNBC.

INTRODUCTION

Breast cancer is a malignancy with an increasing incidence, and a leading cause of cancer-related death among women worldwide (Satsangi et al., 2015). In 2020, 19.3 million new cancers were diagnosed worldwide, including more than 2.26 million women with breast cancer, accounting for around 11.7% of all new cancers. This represents approximately 6.9% of total cancer deaths and 15.5% of total female cancer deaths (Sung et al., 2021). Approximately 20% of patients with breast cancer have negative expressions of ER (estrogen receptor), PR (progesterone receptor) and HER-2/neu (human epidermal growth factor receptor 2), also known as triple-negative breast cancer (TNBC). It is the most resistant to current cancer treatments and is highly aggressive and metastatic, with high rates of recurrence and poor prognosis (Oh et al., 2018; Rakha and Chan, 2011). In 2011, scholars grouped TNBC into six molecular subtypes, which were later refined into four subtypes (Li et al., 2021). Targeting specific characteristics of each subtype is important for precision treatment of TNBC.

Metastasis remains the leading cause of death and disability associated with cancer. Nevertheless, despite various approaches such as cytotoxic and targeted therapies, the effectiveness of current treatment options against corresponding metastatic lesions is frequently insufficient (Steeg, 2006). A possible explanation is that slow-growing micro-metastases may evade the action of cytotoxic agents, which primarily target actively dividing and growing cells (Aguirre-Ghiso, 2007). MMPs (matrix metalloproteinases) are pivotal in the migration and invasion of tumor cells, given that MMPs are extracellular matrix (ECM) zinc-dependent proteases widely involved...
in cell migration and invasion (Brown and Murray, 2015; Folgueras et al., 2004). MMP-2 and -9, as members of the MMP family, are strongly associated with invasion and/or migration of various cancer cells (Yang et al., 2013; Zhang et al., 2012). Drug combination for treating cancer metastasis is the focus of this study, aiming to increase the anti-cancer activity of the available drugs and reducing their undesirable side effects.

Carvacrol has garnered significant interest in recent times, which is presence in various essential oils derived from Lepidium flavum, oregano, wild bergamot, and thyme, among others (Alagawany et al., 2021; Kachur and Suntres, 2020; Güneş Bayir et al., 2019; Salehi et al., 2018). Carvacrol is considered a safe food additive utilized as a flavouring agent in meat products, bakery products, beverages and condiments (Salehi et al., 2018; Solorzano-Santos and Miranda-Novales, 2019). Studies also show carvacrol possesses diverse biological actions, including antioxidant, antibacterial, anti-tumour, liver protection, blood vessel dilation and so on (Burt, 2004; Sharifi-Rad et al., 2018). Multiple investigations have also demonstrated its anti-cancer activity against various cancer types, such as breast cancer (Rathod et al., 2021; Suntres et al., 2015).

Doxorubicin (Dox) is a potent therapeutic agent commonly used for treating breast cancer with high efficacy against both solid and liquid tumors (Aroui et al., 2009; Jawad et al., 2020). However, its usage was limited due to the potential adverse effects, including the risk of cardiac toxicity (Thorn et al., 2011; Almuttaba et al., 2022). Many studies have been done to improve its effectiveness (Liu et al., 2021; Wang and Jiang, 2022), including the combining Dox with certain traditional Chinese medicines can enhance its sensitivity and reduce side effects. For instance, astragaloside IV has been found to markedly reduce Dox-induced cardiotoxicity (Lin et al., 2019), whereas oridonin may be an adjunct to Dox against aggressive breast cancer through promoting apoptosis and suppressing angiogenesis (Li et al., 2019). Tanshinone IIA, a chemical constituent of Danshen plant, exhibits the ability to reverse the resistance of gastric cancer cells to Dox (Xu et al., 2018).

Therefore, this research aimed to examine the synergistic impact and mechanism of carvacrol and Dox on the proliferative, apoptotic, invasive, and migratory traits of MDA-MB-231 cells, with the purpose of establishing whether this combination can lead to an enhancement of the therapeutic effect of Dox.

**MATERIALS AND METHODS**

**Cell lines, main reagents and antibodies**

Human TNBC cell line (MDA-MB-231) was acquired from Xiamen Yimo Biological Technology Co, Ltd. (Xiamen, China), and cultivated in Dulbecco’s modified Eagle’s medium (DMEM) containing 1% penicillin/streptomycin, 10% FBS (fetal bovine serum), and 4500 mg/L glucose at 37°C in 5% CO₂. Dox was dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C. The details of main reagents, antibodies and equipment utilized in this study are provided in Table I.

**Cell cytotoxicity**

Cells were introduced into a 96-well plate in triplicate at 5×10⁴ cells per well (90 µL/well) for 24 h. Subsequently, cells were treated with various concentrations of Dox (0, 1, 1.5, 2.0, 2.5, 5.0, 7.5, 10.0, and 12.5 µM), or carvacrol (0, 100, 150, 200, 250, 300, 400, 500, and 600 µM), concentration 0 µM was used as a control. After being treated for 24 and 48 h, CCK-8 (10 µL) was pipetted into every well for 3 h. The absorbance at 450 nm was determined with a microplate reader, and the IC₅₀ values were determined accordingly.

**Table I. Main reagents, antibodies and equipment used in this study.**

<table>
<thead>
<tr>
<th>Main reagents, antibodies and equipment</th>
<th>Manufactory</th>
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<tbody>
<tr>
<td>Doxorubicin (Dox)</td>
<td>Aladdin Biochemical Technology Co., Ltd., Shanghai, China</td>
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<tr>
<td>Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum</td>
<td>Gibco Life Technologies, Carlsbad, CA, USA</td>
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<tr>
<td>Trypsin and propidium iodide (PI)</td>
<td>Sigma-Aldrich, St. Louis, MO, USA</td>
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<tr>
<td>Primary anti-Bax, anti-Bcl-2, anti-MMP-2, anti-MMP-9 and anti-GAPHH antibodies, and secondary antibodies</td>
<td>Elabscience Biotechnology Co., Ltd., Wuhan, China</td>
</tr>
<tr>
<td>Annexin V-FITC Apoptosis Detection Kit, Caspase-3 Colorimetric Assay Kit, and Cell Counting Kit-8 (CCK-8)</td>
<td>eBioscience, San Diego, CA, USA</td>
</tr>
<tr>
<td>RIPA buffer and all of the other chemicals</td>
<td>Thermo Fisher Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>Nikon D3100 digital camera</td>
<td>Nikon, Tokyo, Japan</td>
</tr>
<tr>
<td>Transwell chambers (8 µm pore size), and flow cytometer</td>
<td>BD Bioscience, Bedford, MA, USA</td>
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Drug sensitivity and colony formation assay

To explore cell sensitivity to Dox, varying concentrations of carvacrol (100, 150, and 200 µM) and Dox (1 µM), alone or in combination, were administered to cells for 24 h, cell without treatment was used as a blank control, while Dox 1 µM was used as positive control. The aforementioned CCK-8 assay was utilized to evaluate cell viability.

To determine the long-term impact of carvacrol combined Dox on MDA-MB-231 cells, a colony formation assay was conducted with some modifications as described earlier (Lu et al., 2018). In brief, 10⁵ cells were seeded in each well of 6-well plates for 24 h, and subsequently cultured with carvacrol (100, 150, 200 µM), in combination with Dox (1 µM), for an additional 24 h, Dox 1 µM was used as positive control. Afterward, the cells were treated with fresh medium to facilitate their growth for 14 days. The cells were then fixed using 100% methanol, stained with Giemsa solution, and the colonies were counted and photographed by a digital camera.

Wound healing assay

The impact of carvacrol in combination with Dox on cell motility was measured using a wound-healing assay (Shen et al., 2013). 2×10⁶ cells were plated onto each well of 6-well plates and cultured overnight until reaching 90% confluence. The scratch wound was created in the monolayers using a pipette tip, following by washing three times with PBS buffer. Then, cells were exposed to various combinations of carvacrol (100, 150, 200 µM) and Dox (1 µM) for 24 h, cell without treatment was used as a blank control, while Dox 1 µM was used as positive control. Finally, images were captured at 0 and 24 h using the microscope at exactly the same point.

Transwell assay

Transwell chambers were utilized to perform cell invasion assays, with membrane top being precoated with 100 µL of Matrigel diluted 1:20 in cold DMEM medium. Following treatment with various combinations of carvacrol (100, 150, 200 µM) and Dox (1 µM) for 24 h, cell without treatment was used as a blank control, while Dox 1 µM was used as positive control. Approximately 5×10⁴ cells in non-serum DMEM medium were added to the upper chamber, while complete medium was utilized in the bottom chamber. Following 24 h incubation, cells were fixed using methyl alcohol and stained with Giemsa. After removing non-invading cells, three arbitrary cell fields in the bottom chamber were selected for photography. The migrated cells were enumerated as previously depicted (Rahman et al., 2013).

Cells cycle assay

Cells (1×10⁵ cells/well) were exposed to various combination of carvacrol (100, 150, 200 µM) and Dox (1 µM) for 24 h, Dox 1 µM was used as positive control. All cells were then fixed for 24 h using 70% cold ethanol, followed by two washes with PBS. Subsequently, they were resuspended in PBS (1 mL) that contained 0.02 mg/mL PI (propidium iodide), followed by incubation (37°C, 15 min). A flow cytometer was applied to quantify DNA content according to previous protocol (Li et al., 2013).

Apoptosis assay

Following a 24-h incubation with varying combinations of carvacrol (100, 150, 200 µM) and Dox (1 µM), Dox 1 µM was used as positive control. The assessment of cellular apoptosis was conducted via Annexin V-FITC/PI double staining assay according to manufacturers specification. The stained cells were analyzed via flow cytometry, and the proportion of apoptotic cells was measured.

Caspase-3 activity assay

After exposing the cells for 24 h with various combinations of carvacrol (100, 150, 200 µM) and Dox (1 µM), as sample groups, Dox 1 µM was used as positive control group. Approximately 3×10⁶ cells were used to assess the activity of caspase-3 using Caspase-3/CPP32 colourimetric assay kit. Caspase-3 activity (%) was calculated by comparison with Dox alone, and estimated as sample OD₄₅₀ divided by control OD₄₅₀.

Western blotting

MDA-MB-231 cells (1×10⁶) were seeded overnight in a 25 cm² flask. After treating for 48 h with varying combinations of carvacrol (0, 100, 150, 200 µM) and 1 µM Dox, cells were lysed using RIPA buffer with protease inhibitor cocktail. Bradford assay was used to quantify protein levels (Kielkopf et al., 2020; Kruger, 2009). Next, protein (20 µg) was denatured in a boiling water bath, electrophoretically separated, and transferred to a polyvinylpolymethacrylate (PVMA) membrane. After blocking with nonfat dry milk (5%, 2 h), the membrane was incubated with primary antibodies (1:1000) overnight using a rotary shaker at 4°C. After incubation, they were washed using 0.1% PBS-Tween (5 times), following by a 3 h incubation with secondary antibody (1:1000) on orbital shaking. GAPDH protein was included as an internal control for equal loading. Image Quant Las 4000 software (GE Healthcare, Buckinghamshire, UK) was used to quantify Western blot images.
Statistical analysis

Mean values with their SD (standard deviations) are presented based on three independent experiments. Statistical analysis was conducted utilizing one-way ANOVA and Student’s t-test via GraphPad Prism 8 Software (* \(p < 0.05\), ** \(p < 0.01\), and *** \(p < 0.001\)).

RESULTS

Inhibitory effects of carvacrol and Dox on MDA-MB-231 cell proliferation

The CCK-8 assay indicated a single treatment of carvacrol or Dox displayed inhibitory effects on cell viability that were both time- and dose-dependent (Fig. 1). Specifically, the values of IC\(_{50}\) of carvacrol were 312.7 ± 13.2 µM at 24 h and 225.2 ± 8.5 µM at 48 h (Fig. 1A). Whereas, the IC\(_{50}\) of Dox were 2.36 ± 0.35 µM at 24 h and 1.90 ± 0.08 µM at 48 h (Fig. 1B). Dox (1 µM) and carvacrol less than or equal to 200 µM had little effect on cell viability, therefore, we prioritised 1 µM Dox and 100, 150, 200 µM carvacrol in the following experiments to better evaluate their effect on cell growth.

Carvacrol sensitizes MDA-MB-231 cells to Dox

To access the effect of carvacrol on cell sensitivity to Dox, cells were exposed to various combinations of carvacrol and Dox to evaluate cell viability and colony formation. The CCK-8 assay demonstrated that combining Dox (1 µM) with carvacrol (100, 150, and 200 µM) for 24 h resulted in the decreased cell viability of 88.8, 81.1, and 76.4%, respectively, as compared to 93.1% observed with Dox alone (Fig. 2A). Furthermore, colony formation assay revealed that treatment with Dox and carvacrol combination obviously reduced colony-forming ability, in comparison to cells untreated or treated with Dox alone (Fig. 2B). These data indicate that carvacrol enhances MDA-MB-231 cell sensitivity to Dox.

Fig. 1. Dose and time-dependent inhibition of MDA-MB-231 cell growth exposed to various concentrations of carvacrol (A) and Dox (B) for 24 and 48 h by CCK-8 assay. Data present mean±SD (n = 3).
Notes: * \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\), vs. Control (concentration 0 µM), Dox, doxorubicin concentration groups.
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Fig. 2. Effect of carvacrol combined with Dox on cell viability (A) and colony formation (B) of MDA-MB-231 cells. A, treatment for 24 h s, data present mean ± SD (n = 3); B, left: Representative images of colony-forming assay (200×); right: Data present mean ± SD (n = 3). Groups 1-8: 1. Control; 2. Dox 1 μM; 3. Dox 1 μM + CA 100 μM; 4. Dox 1 μM + CA 150 μM; 5. Dox 1 μM + CA 200 μM; 6. CA 100 μM; 7. CA 150 μM; 8. CA 200 μM. Dox, doxorubicin; CA, carvacrol; \( p < 0.05 \) vs. Control (without treatment); \( p < 0.05 \) vs. Dox alone; \( p < 0.05 \) vs. Dox 1 μM + CA 100 μM.

Fig. 3. Inhibition of MDA-MB-231 cell migration by Dox and carvacrol co-treatment for 24 h assessed by wound healing assay (A) and Transwell invasion assay (B). Notes: (A) Representative images of wound healing at 0 h and 24 h of Dox and carvacrol co-treatment (100×), Mobility percentage relative to the Control (cells without treatment); (B) Representative image of invaded cells (200×), quantitative analysis of invaded cells in three randomly selected fields; \( p < 0.05 \) vs control; \( p < 0.05 \) vs Dox alone, \( p < 0.05 \) vs Dox 1 μM + CA 100 μM. Dox, Doxorubicin; CA, carvacrol. Groups 1-5: 1. Control; 2. Dox 1 μM; 3. Dox 1 μM + CA 100 μM; 4. Dox 1 μM + CA 150 μM; 5. Dox 1 μM + CA 200 μM.
Carvacrol and Dox combination inhibits migration and invasion of MDA-MB-231 cells

The wound healing assay revealed that cells in the Control group (without treatment) showed a strong ability to migrate, whereas Dox 1 µM alone inhibited cell migration to some extent (Fig. 3A). Carvacrol and Dox combination notably increased wound edge distance, and carvacrol demonstrated a dose-dependent inhibition of cell migration, which may be due to its induced cytotoxicity and decreased motility. Similarly, carvacrol and Dox combination exhibited a notable dose-related inhibition of cell invasion, compared to Dox alone (Fig. 3B).

Carvacrol and dox combination induces cell cycle in MDA-MB-231 cells

The utilization of flow cytometry helped in the analysis of MDA-MB-231 cell cycles, to determine whether cell cycle arrest resulted in the inhibition of cell proliferation and colony formation. As illustrated in Figure 4, cell percentage at G0/G1 phase was higher in carvacrol (100, 150 and 200 µM) combined with Dox groups, compared to Dox alone group, while cell proportion at S and G2/M phases was lower.

![Fig. 4. Carvacrol combined with Dox induce cell cycle arrest in MDA-MB-231 cells. After treatment for 24 h, cell cycle analysis was conducted by flow cytometry via propidium iodide staining. (A) Representative flow-cytometric profiles of cell cycle distribution. (B) Percentages of cells in each cell cycle phase. Notes: Data are expressed as mean ± SD (n = 3); * p < 0.05 vs. Dox alone.](image-url)
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Carvacrol and Doxorubicin combination induces apoptosis in MDA-MB-231 cells

Cell apoptosis induced by various concentrations of carvacrol (0, 100, 150, 200 µM) and Dox (1 µM) was detected by flow cytometer. The result indicated MDA-MB-231 cell apoptosis was enhanced with increasing carvacrol concentrations, indicating that carvacrol induced apoptosis dosage-dependently. Compared with Dox alone, different concentrations of carvacrol (100, 150, 200 µM) in combination with 1 µM Dox exhibited a slight increase in apoptosis of 0.14%, 0.28% and 1.15% (Fig. 5).

Carvacrol and Dox combination induces caspase-3 activation in MDA-MB-231 cells

Caspase-3 is a critical downstream caspase in apoptotic pathway and is considered essential for the execution of programmed cell death. Treating MDA-MB-231 cells for 24 h with carvacrol (100, 150, 200 µM) and Dox, resulted in significant induction of caspase-3 activity. Compared to Dox alone, caspase-3 activity was markedly increased by 23.90%, 61.60% and 86.90% at concentrations of 100, 150 and 200 µM carvacrol, respectively (Fig. 6).

Fig. 5. Apoptosis analysis of MDA-MB 231 cells after carvacrol and Dox co-treatment for 24 h. Cell apoptosis was stained with Annexin V and PI, and analyzed by flow cytometer. Data presented as mean ± SD (n = 3).
Notes: * p < 0.05, ** p < 0.01 vs. Dox alone.

Fig. 6. Dose-dependent activation of caspase-3 in carvacrol and Dox co-treated MDA-MB-231 cells. Caspase-3 activity in cells treated with different concentrations of carvacrol combined with Dox for 24 h determined by a Kit.
Note: ** p < 0.01, *** p < 0.001 *** vs. Dox alone; Groups 2-5: 2. Dox 1 µM; 3. Dox 1 µM + CA 100 µM; 4. Dox 1 µM + CA 150 µM; 5. Dox 1 µM + CA 200 µM.
Effect of carvacrol and Dox on the expression of apoptotic, migration and invasion related proteins in MDA-MB-231 cells

To further investigate the effect of carvacrol combined with Dox on MDA-MB-231 cell, expression levels of apoptotic, migration and invasion-related proteins were analyzed using Western blotting. Specifically, Bcl-2, Bax, MMP-2 and MMP-9 levels were measured upon exposure to carvacrol (0, 100, 150, 200 µM) combined with Dox (1 µM) for 48 h. The groups treated with 100, 150 and 200 µM carvacrol exhibited reduced Bcl-2, MMP-2, MMP-9 protein levels and increased Bax level in comparison to Dox alone (Fig. 7). This suggests that carvacrol in combination with Dox had a downregulating effect on Bcl-2, MMP-2 and MMP-9 proteins, while upregulating the expression of Bax.

DISCUSSION

Triple-negative breast cancer (TNBC) is known to be an aggressive breast cancer subtype associated with worse survival (Masso-Welch et al., 2018). Compared to other subtypes, TNBC has a higher mortality rate with estimates suggesting up to 40% of patients succumbing to the disease within the initial five years of diagnosis (Dent et al., 2007). MDA-MB-231 cells are highly tumorigenic and metastatic, exhibiting a representative EMT (epithelial-mesenchymal transition) associated with cancer progression (Brünen et al., 1993; Yang et al., 1997). While, bevacizumab, in combination with chemotherapy, has been used in some countries to treat TNBC, its survival benefits have been limited (Collignon et al., 2016). Therefore, there is an immediate requirement for the development of new treatments and approaches. Our research shows that carvacrol improves Dox’s efficacy through significantly inhibiting cell growth and cloning while inducing dose-dependent apoptosis. The combined treatment regimen has potential as a novel strategy for treating TNBC.

Further investigation on this combined inhibitory effect showed MDA-MB-231 cells upon exposed for 24 h to carvacrol (100, 150 and 200 µM) combined with Dox tended to accumulate within the G0/G1 phase, compared to that of Dox alone, whereas cell distribution in S and G2/M phase decreased. These results indicated that carvacrol induced G0/G1 phase arrest, inhibited DNA synthesis in S phase, and induced MDA-MB-231 cell apoptosis. These observed anti-tumour effects align with a previous study (Arunasree, 2010), where carvacrol was demonstrated to have the ability to suppress proliferation and provoke apoptosis in breast cancer cells.

Our study further unveiled that carvacrol and Dox co-treatment notably reduced Bcl-2 expression while obviously increasing Bax expression, activating caspase-3-mediated MDA-MB-231 cell apoptosis. This demonstrates carvacrol’s therapeutic potential to induce MDA-MB-231 cell apoptosis via the mitochondrial intrinsic pathway by modulating Bcl-2 family proteins. Similarly, previous studies on carvacrol have also demonstrated its propensity to initiate intrinsic pathway-mediated MCF-7 cell apoptosis (Moradipour et al., 2022).

Metastasis is the final outcome of advanced cancer stages, and once cancer has metastasized, it becomes
incurable. Local intravasation, which involves crossing the ECM, is required for metastatic cancer cells to spread. As members of the MMP family, MMP-2 and MMP-9 have significant functions in the process of migration and invasion of diverse cancer cell types (Yang et al., 2013; Zhang et al., 2012). To investigate whether carvacrol in combination with Dox affects MDA-MB-231 cell motility, protein levels of MMP-2 and MMP-9 were measured. Our findings demonstrated carvacrol co-treatment effectively inhibits cell migration and invasion. Specifically, compared with Dox alone, the 100, 150, and 200 μM carvacrol groups exhibited notable lower MMP-2 and MMP-9 levels. These findings suggest carvacrol combined with Dox inhibit proliferation, invasion and migration of MDA-MB-231 cells.

CONCLUSION

The research employed colourimetric, flow cytometric and Western blot techniques to demonstrate the antiproliferative and apoptotic activities of carvacrol against MDA-MB-231 cells. Carvacrol and Dox co-treatment induced cell death via the intrinsic apoptosis pathway by altering the expression of caspase-3, Bax, and Bcl-2 proteins. Likewise, MMP-2 and MMP-9 protein levels significantly reduced, indicating that the combination treatment inhibited cell invasion and migration. Therefore, carvacrol and Dox co-treatment can effectively inhibit the proliferation, migration and invasion of MDA-MB-231 cells. Carvacrol has exhibited promise as an adjuvant agent that can enhance the sensitivity of MDA-MB-231 cells to Dox treatment. Thus, this approach could potentially be more effective in improving the efficacy of future Dox-based breast cancer treatment regimens.

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IRB approval

Not applicable.

Ethics approval

Not applicable.

Statement of conflict of interest

The authors have declared no conflict of interest.

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