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Oxidative Stress, Mammospheres and Nrf2 – New Implication for Breast Cancer Therapy?

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Abstract

Mammosphere culture of breast cancer cell lines is an important approach used for enrichment of cancer stem cells (CSCs), which exhibit high tumorigenicity and chemoresistance features. Evidence shows that CSCs maintain lower ROS levels due to elevated expression of ROS-scavenging molecules and antioxidative enzymes, which favors the survival of the SLCs and their chemoresistance. The transcription factor NF-E2-related factor 2 (Nrf2) has emerged as the master regulator of cellular redox homeostasis, by up-regulating antioxidant response element (ARE)-bearing genes products. Although Nrf2 has long-term been regarded as a beneficial defense mechanism, accumulating studies have revealed the “dark side” of Nrf2. High constitutive levels of Nrf2 was observed in many types of tumors and cancer cell lines promoting their resistance to chemotherapeutics. In this study, we report a high expression of Nrf2 and its target genes in mammospheres, compared to corresponding adherent cells. In MCF-7 and MDA-MB-231 mammosphere cells, the Nrf2-mediated cellular protective response is significantly elevated, which is associated with increased resistance to taxol and anchorage-independent growth. Brusatol, an inhibitor of the Nrf2 pathway, suppressed the protein level of Nrf2 and its target genes, enhanced intracellular ROS and sensitized mammospheres to taxol and reduced the anchorage-independent growth. These results suggest that mammospheres rely on abnormal up-regulation of Nrf2 to maintain low intracellular ROS levels. Nrf2 inhibitors, such as brusatol, have the potential to be developed into novel adjuvant chemotherapeutic drug combinations, in order to combat refractory tumor initiating CSCs.

Keywords

Nrf2; cancer stem cell; chemoresistance

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Introduction

The balance between ROS production and elimination is crucial for the cell to remain viable and maintain its vital function. Excess ROS produced under pathological conditions will lead to reversible or irreversible tissue injury and consequently induce various diseases, including cancer [1–3]. The biological effects of ROS in cancer are numerous and complicated. On one hand, elevated ROS levels have been detected in almost all cancers, where they promote many aspects of tumor development and progression. On the other hand, tumor cells also express increased levels of antioxidant proteins to detoxify ROS, suggesting that a delicate balance of intracellular ROS levels is necessary for cancer cell function [4]. Therefore, it becomes evident that an in-depth understanding of ROS-mediated signaling in tumor cells is necessary to develop new intervention strategies targeting redox modulation.

The transcription factor Nrf2 has emerged as a master regulator of intracellular antioxidant response through transcriptional activation of a battery of genes including intracellular redox balancing proteins, phase II detoxifying enzymes, and xenobiotic transporters that act to protect cells from environmental toxicants and carcinogens [5–8]. Nrf2 is ubiquitously expressed in all human tissues, but kept at low levels under basal conditions, due to tight regulation by Keap1, a substrate adaptor protein for the Cullin3-based E3 ubiquitin ligase complex [6,9–11]. Under basal conditions, Keap1 constantly targets Nrf2 for Keap1-mediated ubiquitination and subsequent proteasomal degradation [6,12]. Upon oxidative stress, modification of critical cysteine residues in Keap1 imposes a conformational change that disrupts Keap1-Nrf2 binding, leading to diminished Nrf2 degradation and subsequent activation of Nrf2 downstream genes [13–15].

Since Nrf2 is a potent transcription activator of genes that are involved in glutathione synthesis, elimination of ROS, and xenobiotic metabolism, Nrf2 activation has always been viewed as “beneficial” for a host against oxidative stress-associated diseases, including cancer. However, prolonged Nrf2 activation due to genetic mutations in either Nrf2 or Keap1 is frequently seen in cancer cells to promote malignant growth against deleterious microenvironments [16]. For example, many Keap1 mutations or loss of heterozygosity (LOH) at the Keap1 locus have been identified in lung cancer cell lines or cancer tissues. Keap1 mutations or LOH resulted in increased protein level/transactivation of Nrf2 and production of its downstream genes. Tumor tissues have significantly higher levels of both NQO1 and total GST activities than their normal counterparts [17,18]. Also, the level of Nrf2 correlates with chemoresistance in cancer cells [16,19–21]. Activation of the Nrf2–ARE pathway has also been observed in MCF7 breast cancer cell line, which developed resistance to tamoxifen following a prolonged incubation [22]. Collectively, the emerging notion of the “dark side” of Nrf2 has clearly illustrated the urgent need to further understand the role of the Nrf2-ARE pathway in cancer and has provided opportunities for intervention using Nrf2 inhibitors.

Culturing mammospheres has recently become an accepted method to study the molecular biology of stem-like cancer cells because of the properties acquired transitioning from the anchorage-dependent state to the anchorage-independent state [23]. To date, the redox state

of SLCs remains unclear. In this study, we provide evidence showing that Nrf2 is essential for maintaining low ROS levels in mammospheres, and for promoting chemoresistance and colony formation *in vitro*. Inhibition of Nrf2 using brusatol sensitizes mammospheres to taxol-induced cytotoxicity.

Material and Methods

Cell culture and mammosphere preparation

The initial MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC). As adherent cell culture, MCF-7 cells and MDA-MB-231 cells were routinely maintained in RPMI 1640 or in Minimum Essential Medium Eagle's medium (MEM) (Cellgro) media, both supplemented with 10% fetal calf serum (Life Technologies), respectively.

For mammosphere culture, cells were cultured in 6 well ultra-low attachment plates (Corning) at a concentration of 25,000 cells/ml in 2ml/well DMEM/Ham's F-12 (1:1) with 15mM HEPES supplemented with B27 (Invitrogen), 20 ng/ml EGF (Invitrogen), 20 ng/ml bFGF (Invitrogen), antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 ug/ml streptomycin sulfate and 0.25 ug/ml amphotericin B) (Invitrogen), 2 µg/ml Heparin (Stem Cell Technologies), 0.5 µg/ml Hydrocortisone (Stem Cell Technologies) and 5 ug/ml Insulin (Sigma) in a humidified incubator (5% CO₂, 95% air, 37°C). A minority population of cells began to grow as multicellular spheres, as opposed to the majority of cells, which did not survive. Cells were examined microscopically on a daily basis and growth rates recorded. Spheres were collected by gentle centrifugation (1000 rpm) after 7 days and dissociated enzymatically for 10 minutes in 2 ml 0.05% trypsin, 0.53 mM EDTA (Invitrogen) mechanically, using a fire-polished Pasteur pipet or a syringe needle with 90° blunt ends (Fisher Scientific). The cells obtained from dissociation were sieved through a 40-µm cell strainer and analyzed microscopically for single-cellularity. The cells were then suspended at a density of 25,000 cells/ml in new serum free Mammosphere Culture Media to generate the secondary mammospheres. 7 days after the second passage, tertiary mammosphere cells were dissociated and harvested for the subsequent assays.

Detection of intracellular reactive oxygen species (ROS)

To measure intracellular ROS levels, adherent cells or tertiary mammospheres were collected, suspended at a density of 10⁶ cells/ml in PBS containing dichlorofluorescein (DCF) (Sigma) at working concentration of 1 µg/ml. Cells were incubated at 37 °C with 5% CO₂ for 20 min with gentle agitation every 5 min, and were washed three times with PBS and analyzed by flow cytometry.

Antibodies and immunoblot

Anti-Nrf2, anti-HO-1, anti-β-actin, anti-NQO1, anti-GCLM, and anti-Keap1 were purchased commercially from Santa Cruz. To detect protein expression in total cell lysates, cells were lysed in sample buffer (50 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate (SDS), 10% glycerol, 100 mM dithiothreitol (DTT), 0.1% bromophenol blue) and subjected to immunoblotting.

mRNA extraction and qPCR

Total mRNA was extracted from cells using TRI Reagent (Sigma). Equal amounts of RNA were used for reverse transcription using a Transcriptor First-Strand cDNA synthesis kit (Roche). The following TaqMan probes from the universal probe library (Roche) were used: human Nrf2 (no.70), human NQO1 (no. 87), human HO-1 (no. 25), and human GAPDH (no. 25). The following primers were synthesized by Integrated DNA Technologies: hNrf2, forward (5'-ACACGGTCCACAGCTCATC-3') and reverse (5'-TGTCATCAAATCCATGTCCTG-3'); hNQO1, forward (5'-ATGTATGACAAAGGACCCTTCC-3') and reverse (5'-TCCCTTGACAGAGAGTACATGG-3'); hHO-1, forward (5'-AACTTTCAGAAGGGCCAGGT-3') and reverse (5'-CTGGGCTCTCCTTGTG-3'); hGAPDH, forward (5'-CTGACTTCAACAGCGACACC-3') and reverse (5'-TGCTGTAGCCAAATTCGTTGT-3'). Quantitative real-time PCR (qRT-PCR) was performed on the LightCycler 480 system (Roche) as follows: one cycle of initial denaturation (95°C for 4 min), 45 cycles of amplification (95 °C for 10 s and 60 °C for 30 s), and a cooling period. The data presented are relative mRNA levels normalized to the level of GAPDH, and the value from the untreated cells was set as 1. PCR assays were performed three times with duplicate samples, which were used to determine the mean $\pm$ standard deviations. The Student *t*-test was used to evaluate statistically significant differences.

NQO1 activity assay

Cells were washed with PBS twice and harvested in 0.5 ml of homogenization buffer (20 mM Tris-HCl, 2 mM EDTA, pH 7.4), and subjected to three cycles of freezing in a -80 °C freezer and thawing. Cell debris was removed by centrifugation at $12,000 \times g$ for 5 min at 4 °C. The supernatants were transferred to new micro-centrifuge tubes for determination of protein concentration using the BCA Protein Assay Kit (Thermo Scientific), following the manufacturer's instruction. NQO1 activity was determined by the continuous spectrophotometric assay to quantitate the dicumarol-inhibited reduction of its substrate DCPIP (Sigma). The rate of DCPIP reduction was monitored over 1.0 min at 600 nm with an extinction coefficient of $2.1 \text{ mM}^{-1}\text{cm}^{-1}$. The NQO1 activity was calculated as the decrease in absorbance per min per mg total protein of the sample in the presence or absence of the NQO1 enzyme inhibitor, dicumarol (Sigma).

Intracellular glutathione level

The intracellular glutathione concentration was measured using a QuantiChrom glutathione assay kit (BioAssay Systems) according to the manufacturer's instructions. All of the experiments were repeated three times with triplicate samples. The results are presented as mean $\pm$ SD.

MTT Assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, approximately 2×10^4 cells per well were seeded in a 96-well plate and incubated overnight. Cells were treated with indicated compounds or combination

for 48 h followed by the addition of 20 μ l of 2 mg/ml MTT directly into the medium. After incubation (37 °C for 0.5–3 h), the plate was centrifuged and the medium removed. 100 μ l of isopropanol/HCl was added into each well and crystals were dissolved by shaking the plate at room temperature. Absorbance was measured by a plate reader at 570 nm. Triplicate wells were used for each sample and the experiments were repeated at least three times to acquire means and standard deviations.

Colony Formation Assay

To assess colony formation, complete DMEM containing 0.7% low-melting-point (LMP) agarose was added to a 24-well culture plate as a base agar. MCF-7 or MDA-MB-231 cells (adherent or mammosphere, 4000 per well) in RPMI 1640 or MEM containing 0.3% LMP were placed over base agar and allowed to harden at room temperature. Media containing PBS, brusatol, taxol, or a co-treatment was added on top of the agar. Media was changed every week. The cells were allowed to grow for 2 wk before staining with crystal violet. Visible colonies were counted. The results represent the average from three independent experiments with duplicate samples.

Statistical Analysis

Experiments were conducted in triplicate, and data are shown as mean $\pm$ SD. Statistical analysis was performed using two-tailed Student's t tests to compare means. Significance was set at $p < 0.05$.

Results

Mammospheres have higher tumorigenicity and chemoresistance, but lower intracellular ROS levels than their adherent counterparts

Because mammospheres have been implied in previous studies to be resistant to chemotherapy and radiation therapy [24,25], and to have a higher potential of colony formation *in vitro* [26], we collected single cell suspension from tertiary mammspheres and adherent breast cancer cells, and compared their tumorigenicity and chemoresistance using MTT assay and colony formation assays, respectively.

To test the effect of taxol on mammosphere and adherent breast cancer cells, 2×10^4 cells per well were seeded in a 96-well plate and incubated overnight. Taxol was added to the cell cultures at indicated concentrations for 48 hours. As expected, mammosphere originated cells were much more resistant to taxol-mediated toxicity even at the highest dose (Fig. 1A and 1B), suggesting an enriched sub-population of more aggressive breast cancer cells.

Next, a colony formation assay was conducted. Taxol alone reduced the number of colonies formed in both mammosphere and adherent cells; however, mammospheres had to have more colonies in taxol treated groups (Fig. 1C and 1D). These results indicate that mammospheres are more tumorigenic, and more resistant to taxol-mediated cytotoxicity.

It has recently been shown that normal CSCs contain lower levels of ROS than their more mature progeny and that lower ROS appears to be critical for maintaining stem-like cell function and mediating the resistance to chemotherapy and radiotherapy [27,28]. Therefore,

we evaluated intracellular ROS levels in mammospheres and adherent cells from MCF-7 and MDA-MB-231 cell lines. As shown in Fig. 1E and 1F, mammospheres from both cell lines contained lower ROS levels than the corresponding monolayer cells. Taken together, these results suggest that mammospheres with higher tumorigenicity and radioresistance may be associated with lower intracellular ROS levels.

Nrf2 and its downstream gene expressions are elevated in mammospheres

It has been well documented that cellular ROS levels are regulated by the Nrf2 transcription factor that belongs to the CNC family [5,8]. Therefore, we speculated the difference in ROS levels observed in mammospheres and adherent counterparts may due to enhanced Nrf2 protein expression. Indeed, enhanced Nrf2 levels were detected in both MCF-7 and MDA-MB-231 mammospheres, while Keap1 showed no change. Furthermore, the protein levels of Nrf2-target genes *NQO1* and *GCLM* both significantly increased in mammospheres (Fig. 2A and 2B).

As expected, mammospheres and adherent cells have roughly equal *Nrf2* and *Keap1* mRNA levels, suggesting that the different expression level of Nrf2 protein in mammospheres is not due to transcriptional regulation of Nrf2 or Keap1, but controlled at the protein level of Nrf2 (Figure 2C and 2D). Increased mRNA levels of *NQO1*, *GCLM* and *Mrp2* were observed in both MCF-7 and MDA-MB-231 mammospheres. It is worth mentioning that *Mrp2* overexpression has long been associated with enhanced chemoresistance in both breast cancer cell lines and patients [29,30]. Our results indicate that the Nrf2-mediated response is enhanced in the mammospheres, which allows lower intracellular ROS levels and high resistance to taxol treatment.

Brusatol suppresses Nrf2-mediated response in both mammospheres and adherent cells

Our previous study identified brusatol, a quassinoid that inhibits the Nrf2 pathway, is able to sensitize a broad spectrum of cancer cells and A549 xenografts to cisplatin and other chemotherapeutic drugs [31]. To evaluate the effect of brusatol on the Nrf2 pathway in mammospheres, we performed immunoblot assays to detect protein expression of Nrf2 and its downstream genes with or without brusatol treatment. As shown in Figure 3A, a non-cytotoxic concentration of brusatol at 40 nM, suppressed Nrf2 protein expression in both MCF-7 adherent cells and mammospheres. Keap1 protein level did not change, whereas protein expressions of all three Nrf2 downstream gene products, NQO1, GCLM and HO-1, diminished in the presence of brusatol. Similar effects were observed in MDA-MB-231 cells (Fig. 3B). qPCR assay confirmed the inhibitory effect of brusatol on the *NQO1* mRNA level, with no significant change in *Nrf2* or *Keap1* at the transcriptional level (Fig. 3C and 3D).

In accordance with suppressed protein levels of Nrf2 and its downstream genes in the brusatol treated groups, decreased activities of NQO1 (Fig. 3E) and a lower level of intracellular glutathione (Fig. 3F) were observed in brusatol treated cells. It is notable that more prominent differences were observed in the mammospheres, suggesting that mammospheres with higher Nrf2 levels are more sensitive to the suppressive effect of brusatol.

Inhibition of Nrf2 by brusatol increases intracellular ROS level, suppresses mammosphere anchorage-independent growth and enhances cytotoxicity of taxol

To determine whether brusatol treatment resulted in elevated intracellular ROS and hence increased sensitivity to taxol treatment, we measured intracellular ROS levels in mammospheres and adherent cells from MCF-7 and MDA-MB-231 cell lines using flow cytometry. Both brusatol-treated mammospheres or adherent cells displayed a significantly increased ROS level compared to non-treated cells (Fig. 4A and 4B). Taxol itself increases intracellular ROS slightly, which is consistent with previous studies [32], and co-treatment with taxol and brusatol increased ROS dramatically. Again, a more prominent increase in the ROS level was observed in both MCF-7 and MDA-MB-231 mammospheres compared to their adherent counterparts.

Next, MTT and colony formation assays were carried out to evaluate whether co-treatment with brusatol could enhance the cytotoxic effect of taxol. Brusatol at a concentration of 40 nM did not show visible toxicity alone. However, co-treatment significantly reduced the percentage of cell viability and colony formation in mammospheres (Fig. 4C to 4F). In MDA-MB-231 cells, mammospheres were almost eradicated in the co-treatment group (Fig. 4D). Collectively, these results show that brusatol is able to enhance the chemotherapeutic efficacy of taxol through specific inhibition of the Nrf2-dependent cellular defense mechanism *in vitro*.

Discussion

The cancer stem cell theory states that tumors arise from a small sub-population of cells with the capacity for self-renewal. This is widely accepted because it provides an attractive cellular mechanism to account for the origin and could explain resistance against therapeutics found in most tumors. Mammosphere generation of established breast cancer cell lines is considered to be an important method for propagation of tumorigenic breast cancer cells with stem/progenitor cell properties [33].

Studies have shown that redox balance plays an important role in the maintenance of stem cell self-renewal and in differentiation. Very little is known about the redox status in cancer stem cells. One of the key questions that have yet to be validated asks whether cancer stem cells have similar redox properties as their less-tumorigenic descendant cells. Although the redox status of cancer stem cells is not well characterized, it has been suggested that cancer stem cells share some characteristics of normal stem cells. For instance, CD44⁺/CD24^{-/low} breast cancer stem cells are more tumorigenic and are relatively resistant to radiation-induced DNA damage, due to significantly lower levels of basal and radiation-induced ROS in these cells, indicative of higher levels of ROS scavengers [34]. Diehn *et al* reported that ROS levels are lower in human and murine breast cancer stem cells compared to non-stem breast cancer cells [28]. In leukemic stem cells (LSCs), Ito *et al* demonstrated the critical role of the tumor suppressor promyelocytic leukemia protein (PML) in the maintenance of quiescent chronic myelogenous leukemia (CML) stem cells, explaining the effectiveness of eradicating CML stem cells with arsenic trioxide (As₂O₃), a ROS generator that is used to treat CML [35]. Human gastrointestinal cancer stem cells with a high level of CD44 expression showed an enhanced capacity of GSH synthesis along with an increased

expression of a cysteine-glutamate exchange transporter [36]. The mechanism whereby ROS levels are kept low in breast cancer stem cells appears to involve up-regulation of ROS-scavenging molecules, thereby contributing to tumor resistance of chemotherapy and radiation therapy.

In this study, we confirmed the elevated level of Nrf2 and its downstream response, which maintains the low levels of ROS in the CSC enriched mammosphere subpopulation has been confirmed in our study. Previous work from our lab has demonstrated that brusatol inhibited the Nrf2 pathway through enhanced ubiquitination and degradation of Nrf2 [31]. Brusatol treatment led to decreased expression of Nrf2 at the protein level and a suppressed antioxidant response (Fig. 3), which results in marked elevation of intracellular ROS, especially in mammospheres (Fig. 4A and 4B). We demonstrated that mammospheres with higher Nrf2 expression are more susceptible to brusatol-mediated sensitization to taxol treatment (Fig. 4E and 4F). The mechanism underlying the upregulation of the Nrf2 pathway in mammospheres is still unclear. In a lung cancer model, Pan *et al.* reported that 26S proteasome activity is down-regulated in lung cancer stem-like cells propagated *in vitro* [37]. Since Nrf2 is also subjected to 26S proteasomal degradation, it is highly possible that the reduced Nrf2 degradation may give rise to high level of Nrf2, conferring mammospheres a growth advantage and resistance to chemo drugs. Further studies are needed to delineate the detailed molecular regulation of ROS signaling in cancer stem cells.

In summary, our research provides mechanistic insights into the role of Nrf2 in a breast cancer stem cell enriched population, which may offer important clues to understand how Nrf2 promotes breast cancer development and resistance to therapeutic treatment. The incorporation of a newly discovered Nrf2 inhibitor, brusatol, to eradicate cancer stem cells, thus overcoming chemoresistance, represents a novel approach to cancer treatments. Further *in vivo* studies are needed to investigate the effect of Nrf2 inhibitors in sensitizing cancer stem cells to chemotherapeutic drugs. Nrf2 inhibitors may be used in a broad spectrum of tumors and chemotherapeutic drugs to increase the effectiveness of cancer treatments. Development of Nrf2 inhibitors into for clinical use to sensitize many cancer types to current therapies and overcome chemo- or radio-resistance will have an enormous translational impact on cancer treatment.

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Figure 1.

Mammospheres have higher tumorigenicity and chemoresistance, but lower intracellular ROS levels than their adherent counterparts. (A–B) Mammospheres were more resistant to the cytotoxicity of taxol. Single cell suspension from tertiary mammospheres and adherent MCF-7 (A) and MDA-MB-231 (B) cells were seeded in a 96-well plate and incubated overnight. Cells were treated with indicated concentrations of taxol for 48 h followed by MTT assay. (C–D) Mammospheres have an enhanced ability of colony formation. Single cell suspension from tertiary mammospheres and adherent MCF-7 (C) and MDA-MB-231 (D) cells were treated with PBS or indicated concentrations of taxol for 2 wk before visualized with crystal violet. (E–F) Mammospheres have lower intracellular ROS levels than adherent cells. Single cell suspension from tertiary mammospheres and adherent MCF-7 (E) and MDA-MB-231 (F) cells were collected, suspended at a density of 10^6 cells/ml in PBS containing DCF and subject to flow cytometry analysis. Data are shown as mean $\pm$ SD (n=3 independent experiments). The symbol * indicates $P < 0.05$ vs control group.

Figure 2.

Nrf2 and its downstream gene expressions are elevated in mammospheres. (A–B) Immunoblot analysis of the indicated proteins with cell lysates from tertiary mammospheres (Mammo.) and adherent (Ad.) MCF-7 (A) and MDA-MB-231 (B) cells. (C–D) qPCR analysis of the mRNA levels of indicated genes with cell lysates from tertiary mammospheres and adherent MCF-7 (C) and MDA-MB-231 (D) cells. For qRT-PCR, results are expressed as mean $\pm$ SD. (n=3 independent experiments). The symbol * indicates $P < 0.05$ vs adherent cells.

Figure 3.

Brusatol suppresses Nrf2-mediated response in both mammospheres and adherent cells. (A–B) Immunoblot analysis of the indicated proteins from cell lysates from tertiary mammospheres (Mammo.) and adherent (Ad.) MCF-7 (A) and MDA-MB-231 (B) cells, treated with PBS or brusatol. (C–D) qPCR analysis of the mRNA levels of indicated genes with cell lysates from tertiary mammospheres and adherent MCF-7 (C) and MDA-MB-231 (D) cells with indicated treatments. For qRT-PCR, results are expressed as mean $\pm$ SD. (n=3 independent experiments). NQO1 activity (E) and intracellular glutathione levels (F) were measured in single cell suspension from tertiary mammospheres and adherent MCF-7 and MDA-MB-231 cells. Results are expressed as mean $\pm$ SD. (n=3 independent experiments).

Figure 4.

Inhibition of Nrf2 by brusatol increases intracellular ROS levels, suppresses mammosphere anchorage-independent growth and sensitizes cells to taxol treatment. (A–B) Brusatol increases intracellular ROS level. Single cell suspension from tertiary mammospheres and adherent MCF-7 (A) and MDA-MB-231 (B) cells were collected, suspended at a density of 10^6 cells/ml in PBS containing DCF and subjected to flow cytometry analysis. Data are shown as mean $\pm$ SD (n=3 independent experiments). The symbol * and # indicates $P < 0.05$ vs control group. (C–D) Brusatol sensitizes mammospheres to taxol-induced cytotoxic effect. Single cell suspension from tertiary mammospheres and adherent MCF-7 (C) and

MDA-MB-231 (D) cells were seeded in a 96-well plate and incubated overnight. Cells were treated with treated with PBS, brusatol alone, taxol alone or co-treatment for 48 h followed by MTT assays. (E–F) Brusatol suppresses colony formation in mammospheres. Single cell suspension from tertiary mammospheres and adherent MCF-7 (E) and MDA-MB-231 (F) cells were treated with PBS, brusatol alone, taxol alone or co-treatment for 2 wk before visualized with crystal violet.

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