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Deciphering the molecular mechanism underlying anticancer activity of coumestrol in triple-negative breast cancer cells

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ABSTRACT

Triple-negative breast cancer (TNBC) represents the highly aggressive subgroup of breast cancers with poor prognosis due to absence of estrogen receptor (ER). Therefore, alternative targeted therapies are required against ER-negative breast cancers. Coumestrol, a phytoestrogen inhibits cell growth of ER-negative breast cancer MDA-MB-231 cells; the exact mechanism has not yet been reported. Unlike normal cells, cancer cells contain elevated copper which play an integral role in angiogenesis. The current focus of the work was to identify any possible role of copper in coumestrol cytotoxic action against breast cancer MDA-MB-231 cells. Results demonstrated that coumestrol inhibited cell viability, induced ROS generation, DNA damage, G1/S cell cycle arrest, up-regulation of Bax and apoptosis induction via caspase-dependent mitochondrial mediated pathway in MDA-MB-231 cells. Further, addition of copper chelator, neocuproine and ROS scavenger, *N*-acetyl cysteine were ineffective in abrogating coumestrol-mediated apoptosis. This suggests non-involvement of copper and ROS in coumestrol-induced apoptosis. To account for coumestrol-mediated up-regulation of Bax and apoptosis induction, direct binding potential between coumestrol and Bax/Bcl-2 was studied using in silico molecular docking studies. We propose that coumestrol directly enters cells and combines with Bax/Bcl-2 to alter their structures, thereby causing Bax binding to the outer mitochondrial membrane and Bcl-2 release from the mitochondria to initiate apoptosis. Thus, non-copper targeted ROS independent DNA damage is the central mechanism of coumestrol in ER-negative MDA-MB-231 cells. These findings will be useful in better understanding of anticancer mechanisms of coumestrol and establishing it as a lead molecule for TNBC treatment.

1. Introduction

Breast cancer is the leading cause of cancer deaths among women worldwide (Siegel et al., 2013). One of the important types of breast cancer is triple negative breast cancer characterized by lack of estrogen receptor (ER). Treatment of ER-negative breast cancer is difficult due to poor response of drugs in the absence of ER expression (Chen and Russo, 2009; Matossian et al., 2017; Williams et al., 2015). Therefore, alternative therapies are required to identify new chemotherapeutic agents against ER-negative breast cancers.

Coumestrol, a phytoestrogen is involved in a wide spectrum of biological activities including anticancer activity against breast, prostate and ovarian cancer (Dixon-Shanies and Shaikh, 1999; Lim et al., 2016, 2017). In context to anti-cancer activity against breast cancer, it has been demonstrated that coumestrol inhibits cell growth of ER-positive breast cancer MCF-7 cells by increasing ROS generation through CKII inhibition-dependent activation of NADPH oxidase (Lee et al., 2013). In addition, coumestrol showed anti-proliferative and apoptotic effects though ERα signaling in long-term estrogen-deprived MCF7:5C breast cancer cells (Obiorah et al., 2014).

In another study, it has been reported that coumestrol exhibits a potent inhibitory effect on ER-negative MDA-MB-231 human breast cancer cells (Magee et al., 2004). Also, coumestrol inhibits DNA synthesis in MDA-MB-231 cells at high concentrations (20–90 μM) (Wang and Kurzer, 1998). Recently, epidemiological studies suggest that inverse relationship exists between increased soy consumption (coumestrol) and decreased breast cancer risk in Asian countries (Dong and Qin, 2011; Setchell and Cassidy, 1999). Though reports suggest coumestrol inhibits breast cancer MDA-MB-231 cells, the exact mechanism has not yet been reported. The current focus of the work was to delineate the molecular mechanism underlying anticancer action of coumestrol against MDA-MB-231 cells.

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Studies suggest that cancer cells exhibit altered metabolism with elevated copper as compared to normal cells (Ebadi and Swanson, 1988; Nasulewicz et al., 2004; Yoshida et al., 1993). Elevated copper in cancer cells is responsible for angiogenesis and metastasis by acting as a co-factor of several angiogenic molecules such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiogenin (Brewer, 2005; Pan et al., 2002). Previous study by our research group has shown that coumestrol induces oxidative stress, DNA fragmentation and apoptosis in copper supplemented lymphocytes (Zafar et al., 2016). These effects were abolished in the presence of neocuproine, membrane permeant copper chelator and ROS scavengers, thus suggesting that coumestrol targets copper to induce ROS generation which causes oxidative DNA damage and cellular apoptosis (Zafar et al., 2016). Recently, we have shown that in vitro treatment of coumestrol inhibits cell viability and proliferation, induced ROS generation coupled to excessive DNA damage in breast cancer MCF-7 cells (Zafar et al., 2017). Further, we found that excessive DNA damage by coumestrol treatment up-regulates p53 which directs the cell to cell cycle arrest at G1/S phase via p21 inhibitor (CDK inhibitor) and promotes mitochondrial-mediated apoptosis in MCF-7 cells (Zafar et al., 2017). All these effects induced by coumestrol were inhibited by neocuproine and ROS scavengers (Zafar et al., 2017). These results also suggest that coumestrol targets elevated nuclear Cu(II) ions for redox cycling to generate ROS which causes DNA damage and apoptosis of ER-positive breast cancer MCF-7 cells (Zafar et al., 2017). Taken together all these results, it was prudent for us to identify any possible role of copper in coumestrol cytotoxic action in breast cancer MDA-MB-231 cells.

In the present study, we investigated the detailed anticancer mechanism of coumestrol in breast cancer MDA-MB-231 cells. Our results demonstrated that coumestrol does not target Cu(II) to induce apoptosis in ER-negative MDA-MB-231 cells. Thus, non-copper targeted ROS independent DNA damage is the central anticancer mechanism of coumestrol in ER-negative MDA-MB-231 cells. We posit that coumestrol could be used clinically as anticancer agent for the therapy of both ER-positive as well as ER–negative breast cancers.

2. Materials and methods

2.1. Chemicals and antibodies

Coumestrol, neocuproine, bathocuproine, desferoxamine mesylate, histidine, *N*-acetyl-L-cysteine, MTT dye, DAPI, propidium iodide, RNase, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and dihydroethidium (DHE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mycoplasma PCR detection kit and Annexin V-FITC apoptosis kit were procured from Sigma-Aldrich, St. Louis, MO, USA. MitoProbe JC-1 assay kit (mitochondrial depolarization detection) was purchased from Molecular Probes (Life Technologies, USA). Other chemicals were of highest grade purity. Coumestrol was dissolved in dimethyl sulphoxide (DMSO) to make 3 mM stock solution and was kept at − 20 °C. Before starting any experiment, effect of DMSO (vehicle control) was checked on the viability of MDA-MB-231 cells and found to be ineffective in causing viability inhibition. Antibodies to Bax, Bcl-2, phospho-histone H2A.X (Ser139), caspases 3/9, CDK2, cyclin E and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2. Cell line culture

Human breast cancer MDA-MB-231 cell line was obtained from America Type Culture Collection (ATCC, USA) and cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution at 37 °C in a humidified atmosphere with 5% CO_2. Cell line was grown in T25 tissue culture flasks and checked for mycoplasma contamination by mycoplasma PCR detection kit prior to future experiments.

2.3. MTT assay for cell viability

In vitro cytotoxic activity of coumestrol on MDA-MB-231 breast cancer cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Cells (1×10^4 per well) were seeded in 96-well plate and treated with increasing concentrations of coumestrol for 24–72 h. Next, 20 µl of MTT reagent (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. Subsequently, formazan crystals were dissolved in DMSO and the absorbance was read at 560 nm in ELISA microplate reader (Bio-Rad, USA). Data is expressed as percentage cell proliferation in coumestrol-treated cells. Concentration which causes 50% inhibition of cell viability of MDA-MB-231 cells was used to determine the IC50 for coumestrol against such cells. Later, using IC50 value of coumestrol, viability assay was also performed in the presence of metal chelators (bathocuproine or neocuproine (50 µM each): copper chelators, desferoxamine (50 µM): iron chelator and histidine (50 µM): zinc chelator) and ROS scavenger, *N*-acetyl-L-cysteine (NAC) (5 mM).

2.4. Determination of intracellular ROS generation

Intracellular ROS generation in cancer cells was determined using fluorescent probes, DCFH-DA (for H_2O_2) (Wang and Joseph, 1999) and DHE (O_2^-) (Dikalov et al., 2002) via fluorescence microscopy and flow cytometry. Briefly, cells (1×10^4 per well) were seeded and treated with IC50 concentration of coumestrol. Also, MDA-MB-231 cells were treated in different sets of experiments: (1) Coumestrol (IC50) + neocuproine (50 µM) and (2) Coumestrol (IC50) + NAC (5 mM) for both DCFH-DA and DHE assay at 37 °C in 5% CO_2. After complete treatment, cells were washed with PBS and stained with 30 µM DCFH-DA for 1 h or 20 µM DHE for 30 min and visualized under inverted fluorescence microscope (BX43, Olympus, Japan). The production of intracellular ROS was also estimated using flow cytometric analysis and 10,000 events were recorded per sample. Data reported as mean ± SEM of three independent biological replicates.

2.5. Apoptosis detection by Annexin-V FITC/PI double staining

Coumestrol-induced apoptosis was detected using Annexin-V apoptosis detection kit (Sigma, St. Louis, MO, USA). Cells were seeded in a 6-well plate at a density of 1×10^5 cells per well and treated with IC50 concentration of coumestrol, coumestrol (IC50) + neocuproine (50 µM) and coumestrol (IC50) + NAC (5 mM) at 37 °C in 5% CO_2. Later, cell were harvested, washed with PBS and resuspended in 1 × binding buffer. Cells were then stained with Annexin-V FITC/PI and incubated for 15 min in dark. Apoptosis in treated cells was measured using flow cytometer (BD Biosciences FACS, CA, USA) with 10,000 events recorded per sample. FlowJO software (Treestar, Ashland, OR) was used to analyze data and generate quadrant statistics data. Data reported as mean ± SEM of three independent biological replicates.

2.6. Cell cycle arrest using PI staining

Cells were seeded at a density 1×10^5 per well in 6-well culture plate and then treated with IC50 concentration of coumestrol to determine cell cycle arrest phase. Few experiments were performed containing the following treated reactions: (1) Coumestrol (IC50) + neocuproine (50 µM) and (2) Coumestrol (IC50) + NAC (5 mM) at 37 °C in 5% CO_2. Then, cells were harvested and fixed in 70% ethanol at − 20 °C. Cells were washed and stained with 1 ml of the DNA staining reagent containing 0.1% Triton X-100, RNase (100 µg/ml) and 30 µg/ml propidium iodide in dark for 30 min. DNA content in different cell cycle phases was measured with flow cytometer and histograms were plotted using FlowJo software (Treestar, Ashland, OR).