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Enterolactone: A novel radiosensitizer for human breast cancer cell lines through impaired DNA repair and increased apoptosis

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ABSTRACT

Introduction: Radiotherapy is a potent treatment against breast cancer, which is the most commonly diagnosed cancer among women. However, the emergence of radioresistance due to increased DNA repair leads to radiotherapeutic failure. Applying polyphenols combined with radiation is a more promising method leading to better survival. Enterolactone, a phytoestrogenic polyphenol, has been reported to inhibit an important radioresistance signaling pathway, therefore we conjectured that enterolactone could enhance radiosensitivity in breast cancer. To assess this hypothesis, radiation response of enterolactone treated MDA-MB-231 and T47D cell lines and corresponding cellular mechanisms were investigated.

Methods: Cytotoxicity of enterolactone was measured via MTT assay. Cells were treated with enterolactone before X-irradiation, and clonogenic assay was used to evaluate radiosensitivity. Cell cycle distribution and apoptosis were measured by flow cytometric analysis. In addition, DNA damages and corresponding repair, chromosomal damages, and aberrations were assessed by comet, micronucleus, and cytogenetic assays, respectively.

Results: Enterolactone decreased the viability of cells in a concentration- and time dependent manner. Enterolactone significantly enhanced radiosensitivity of cells by abrogating G2/M arrest, impairing DNA repair, and increasing radiation-induced apoptosis. Furthermore, increased chromosomal damages and aberrations were detected in cells treated with enterolactone combined with X-rays than X-ray alone. These effects were more prominent in T47D than MDA-MB-231 cells.

Discussion: To our knowledge, this is the first report that enterolactone is a novel radiosensitizer for breast cancer irrespective of estrogen receptor status. Authors propose enterolactone as a candidate for combined therapy to decrease the radiation dose delivered to patients and subsequent side effects.

1. Introduction

Breast cancer is the most commonly diagnosed type of cancer and the leading cause of cancer related death in women worldwide, with approximately 1.7 million new cases and 521,900 deaths in 2012 (Ferlay et al., 2015, Torre et al., 2015). Conventional radiation therapy is a potent loco-regional control modality against almost every stage of breast cancer (Smith et al., 2009). Nonetheless, several factors, such as development of radioresistance and inevitable side effects, which influence healthy tissues in the target site milieu, could result in failure of radiotherapy (Jameel et al., 2004, Khoram et al., 2016). In addition, the intrinsic radioresistance of breast cancer cells restrains the effectiveness of radiation alone. Therefore, using combined modalities appears as a feasible method to overcome these obstacles (Malik et al., 2016). Some plant polyphenols have been known to preferentially sensitize cancer cells to radiation by abrogating DNA repair mechanisms through

Abbreviations: ENL, enterolactone; ER, estrogen receptor; IGF-I, insulin-like growth factor I; PKB or Akt, protein kinase B; MAPK/ERK, mitogen-activated protein kinase/extracellular-signal regulated kinase; PI3K, phosphatidylinositol 3-kinase; MTT, (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; LMP, low-melting-point agarose; PI, propidium iodide; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; IC50, half maximal inhibitory concentration; PE, plating efficiency; SF, surviving fraction; LQ, linear quadratic; SER, sensitizer enhancement ratio; AO/EB, acridine orange/ethidium bromide; MN, micronuclei; CAs, chromosomal aberrations; SEM, Standard error of mean; IR, ionizing radiation; DSBs, double strand breaks; CAPE, caffeic acid phenethyl ester; GRP, glucose regulator proteins; SSBs, single strand breaks.

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targeting proteins involved in cell signaling pathways. Hence, they have been introduced as radiosensitizers with the potential to be applied in combination with radiotherapy (Huang et al., 2010, Begg et al., 2011, Kesari et al., 2011, Khoram et al., 2016). Although remarkable investigations have been devoted to identify novel naturally-occurring polyphenols, none of them have been proved sufficiently effective in combination with radiotherapy (Garg et al., 2005, Lin et al., 2012, Liu et al., 2013, Taghizadeh et al., 2015, Khoram et al., 2016).

A group of polyphenols known as dietary lignans, which are especially rich in flaxseed, have been suggested to be the effective environmental factor in reduction of breast cancer incidence in Asia as compared to the USA and Northern Europe (Saarinen et al., 2002, Danbara et al., 2005, Chen et al., 2007, Jungeström et al., 2007, Chen et al., 2009). Trans-α,β-Bis(3-hydroxybenzyl) butyrolactone or enterolactone (ENL), a dietary lignin with structural similarity to endogenous estrogens, is classified as a phytoestrogen which exhibits weak estrogenic properties (100 to 1000-fold less effective than natural estrogens) (McCann et al., 2013, 2014). Nonetheless, it has been confirmed that ENL exhibits antiestrogenic effect on estrogen receptor (ER)-positive breast cancer cells (Jungeström et al., 2007). Physiologically relevant concentrations of ENL typically ranges from 0.1 to 10 μM, although considerable variation has been observed based on population and dietary preferences (McCann et al., 2013, 2014).

Biological effects of ENL have been reported recently including inhibition of survival signaling pathways and anti-cancer, anti-proliferative, anti-angiogenic, anti-metastatic, and anti-apoptotic properties (Danbara et al., 2005, McCann et al., 2013, 2014). The proliferation and growth of hormone-related cancers, namely breast and prostate cancers, as well as colon cancer could be inhibited by ENL and flaxseed, as shown in some *in vitro* and *in vivo* studies (Lin et al., 2000, Danbara et al., 2005, Jungeström et al., 2007, Saarinen et al., 2010, McCann et al., 2013, 2014). Besides, inhibition of an important radioresistance pathway (*i.e.*, insulin-like growth factor I (IGF-I)/IGF-I receptor signaling and its downstream targets, including protein kinase B (PKB or Akt) and mitogen-activated protein kinase/extracellular-signal regulated kinase (MAPK/ERK)) by ENL has previously been reported (Chen et al., 2007, 2009). However, MAPK/ERK, phosphatidylinositol 3-kinase (PI3K)/Akt, and IGF-I/ IGF-I receptor have been suggested to play key roles in survival including apoptosis inhibition and repair induction (Chen et al., 2007, Valerie et al., 2007, Chen et al., 2009). Therefore, we hypothesized that inhibition of these signaling pathways by ENL could enhance radiosensitivity of cancer cells through impairing DNA repair and promoting apoptosis. To confirm this assumption, we evaluated whether physiologically relevant concentrations of ENL could enhance the radiation response of breast cancer cells. Moreover, the possible underlying cellular mechanisms were investigated in MDA-MB-231 as ER-negative and T47D as ER-positive breast cancer cells.

2. Materials and methods

2.1. Materials

Enterolactone (ENL), (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), low-melting-point agarose (LMP), penicillin, propidium iodide (PI), cytochalasin B, ethylenediaminetetraacetic acid (EDTA) and colcemid were purchased from Sigma-Aldrich (Germany). Fetal bovine serum (FBS), RPMI 1640, and trypsin were obtained from GIBCO (United Kingdom). Streptomycin and ribonuclease A were gained from Jaberebn-Hayan and Sinaclon (Iran), respectively. In addition, acridine orange was purchased from Hopkins & Williams Ltd. (United Kingdom). Other chemicals were acquired from Merck (Germany). All petri dishes and flasks were obtained from Nunc (Denmark).

2.2. Cell lines and culture conditions

MDA-MB-231 (ATCC® HTB-26™) as ER-negative and T47D (ATCC® HTB-133™) as ER-positive human breast cancer cell lines were purchased from National Cell Bank of Iran (Pasteur Institute, Iran). Both cell lines were cultured at 2×10^4 cells/cm^2 density in the RPMI 1640 culture medium supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin. Cells were cultured as monolayer and incubated in a humidified atmosphere (95%) with 5% CO_2 at 37 °C and routinely sub-cultured upon reaching 80–90% confluence using 0.25% (w/v) trypsin and 0.03% (w/v) EDTA in phosphate buffered saline (PBS) solution.

2.3. MTT assay

Exponentially growing MDA-MB-231 and T47D cells were seeded at a density of 3000 and 6000 cells/well in 96-well plates and incubated for 24 and 48 h, respectively, before adding ENL to the medium. Cells were treated with final concentrations of ENL (1, 10, 50, 100, 200 and 500 μM) dissolved in 0.1% (v/v) dimethyl sulfoxide (DMSO) and incubated for additional 24, 48 and 72 h. Next the medium was aspirated and 100 μl of MTT solution (0.5 mg/ml) was added. Then cells were incubated for another 3 h followed by addition of 100 μl DMSO in order to dissolve the formed formazan crystals. The plates were swirled gently followed by measurement of absorbance at a test wavelength of 570 nm and a reference wavelength of 630 nm to get the sample signal (OD_{570}–OD_{630}) by Elisa reader spectrophotometer (BioTek, USA). Cell viability was calculated by comparing absorbance of the samples with that of the control (Mosmann, 1983, Denizot and Lang, 1986). Besides, half maximal inhibitory concentration (IC50) values were calculated.

2.4. Enterolactone and X-ray treatments

MDA-MB-231 and T47D cells were cultured in T25 culture flasks and incubated for 24 and 48 h, respectively. Thereafter, both cells were treated with particular concentrations of ENL and incubated for additional 72 h. Then, the ENL-containing medium was replaced with fresh medium and X-irradiated at 0–10 Gy with 6 Mev X-ray photon (200 cGy/min), produced by Siemens Primus linear accelerator X-ray machine (Erlangen, Germany) at Pars Hospital (Tehran, Iran). Four groups of cells were considered in the experiments: cells receiving no treatment (control), cells treated by ENL alone (ENL), cells exposed to X-irradiation (X-ray), cells receiving combined treatment (ENL + X-ray). Control and ENL groups were not exposed to radiation. All data were represented as the means of three independent experiments.

2.5. Clonogenic assay

Immediately after X-irradiation, an appropriate number of control and ENL pre-treated cells (1 or 10 μM), depending on the cell line and X-ray dose, were seeded into 35 mm petri dishes in triplicates according to the published procedures (Franken et al., 2006). Afterward, the cells were incubated for 7 (MDA-MB-231) and 12 (T47D) days to allow colony formation. Finally, the colonies were fixed with 2% (v/v) formaldehyde solution and stained with 5% (w/v) crystal violet dye. Cell assemblies containing more than 50 cells were considered and counted as colonies. The plating efficiency (PE) and surviving fraction (SF) were calculated for each dose of X-ray (0–10 Gy) according to the Eqs. (1) and (2):

$$PE = \frac{Number\ of\ colonies\ counted}{Number\ of\ cells\ seeded} \times 100 \quad (1)$$

$$SF = \frac{Number\ of\ colonies\ counted}{Number\ of\ cells\ seeded \times (PE/100)} \quad (2)$$