Research Communication

Evidence of Pomegranate Methanolic Extract In Antagonizing the Endogenous SERM, 27-Hydroxycholesterol

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Abstract

The direct relationship between obesity and breast cancer has been elucidated recently with the identification of a cholesterol derivative 27-hydroxycholesterol (27HC), an endogenous SERM that can act through estrogen receptor (ER)-mediated mechanisms. Our recent research shed light on the possible SERM-like property of methanol extract of pericarp of pomegranate (PME) by using human breast (MCF-7, MDA-MB-231), endometrial (HEC-1A), cervical (SiHa, HeLa), ovarian (SKOV3) cancer cell lines, normal breast fibroblasts (MCF-10A) and also by *in vivo* models (ovariectomized Swiss albino mice). Our findings demonstrated that PME binds to ER and downregulates the Estrogen response elements (ERE)-mediated transcription in breast cancer cells without being agonistic in the uterine endometrium and has cardioprotective effects comparable to that of 17-β-estradiol. This preliminary work indicates the ability of PME to antagonize the activity of 27HC. We hypothesize that PME can compete with 27HC for ERα and reduce 27HC-induced proliferation of MCF-7 cells. Relevant estrogen-regulated genes such as pS2, PR and ERα were checked to evaluate the ability of PME to abrogate 27HC-induced genes. This study is significant, being the first report describing that bioactive components of the methanolic extract of pericarp of PME, a proven SERM could plausibly compete for 27HC. © 2016 IUBMB Life, 68(2):116–121, 2016

Keywords: *27-hydroxycholesterol; SERM; pomegranate; obesity; breast cancer*

Introduction

Selective estrogen receptor modulators (SERMs) which are a class of estrogen receptor (ER) ligands exhibiting tissue-specific agonistic or antagonistic activities, used in the hormonal therapy for estrogen-dependent breast cancers (1), often seems ineffective and elicit drug resistance in postmenopausal women. Of late, hypercholesterolemia, a recognized co morbidity of obesity, has been branded as an independent risk factor for breast cancer in postmenopausal women. Along these lines, the discovery of oxysterol 27-Hydroxycholesterol(27HC) as endogenous selective estrogen receptor modulator provides new insight into obesity associated post menopausal breast cancers and their response to various therapies. Recent studies describe how this primary metabolite of cholesterol, 27HC, impacts the growth and metastasis of tumors in established animal models of breast cancer (2). Recognition of 27HC as an endogenous SERM reveals the unidentified ER-mediated mechanism in breast cancer patients where synthetic SERM or aromatase inhibitor or hormone replacement failed to demonstrate its effect (3). Limer and Speirs (4) reported that high consumption of phytoestrogens, which are very weak mimics of natural oestrogens, is associated with a lower incidence of breast cancer. Pomegranate (PME) fruit is such an entity, and several studies have been conducted to evaluate the efficacy of this fruit endowed with a

Abbreviations: *27HC, 27-hydroxycholesterol; E2, 17-β-estradiol; PME,, methanolic extract of pericarp of pomegranate; SERM, selective estrogen modulator*

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Conflict of interest: The views and opinions expressed in the paper are those of the authors. The authors declare that there are no conflict of interests in the publication of this paper.

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very high antioxidant activity antiproliferative, anti-invasive, antiaromatase and proapoptotic agents in various breast cancer cell lines and animal models (5,6). We have earlier (7) demonstrated that the methanolic extract of PME can act as a SERM in breast cancer cell lines and ovariectomized mice and had a strong lipid-lowering action, that is, it decreased the plasma levels of total cholesterol (TC) and triglyceride (TG) in ovariectomized animals and was comparable to tamoxifen and estradiol. 27HC induces proliferation and expression of ERα and ERβ in responsive genes breast cancer cell lines (3). Also, the SERM is also reported to increase cyclin D1 expression and consequently accumulation of cells in S phase of cell cycle (8). As SERM property of PME is already established (7), we hypothesized that the extract could plausibly compete with 27HC to bind to ER. This study is important since it is the first report describing that bioactive components of the methanolic extract of pericarp of PME, a proven SERM could plausibly compete for 27HC. In this study, we attempted to evaluate the ability of PME to compete with 27HC and reduce its proliferative effect in breast cancer cell line, which could possibly open a new horizon for the development of a better SERM for the treatment of breast cancer patients with obesity.

Materials and Methods

Materials

Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), charcoal-treated serum (CTS), 3-(4,5-dimethylthiazolyl-2)−2,5-diphenyltetrazolium bromide (MTT) (Calbiochem, San Diego, CA, USA), BrdU assay Biovision (Milpitas, USA) kit, CDNA superscript assay kit Invitrogen, primers specific to ER-α, PS2, progesterone receptor (PR), steroid receptor coactivator-1 (SRC-1), nuclear receptor corepressor-1 (NCOR-1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1) were used.

TABLE 1 *Primers used for PCR*

SL. No.	*Gene*	*Primer sequence (5′ to 3′)*	T_m *(°C)*
1	ERα	F:CCACCAACCAGTGCACCATT R:GGTCTTTTCGTATCCCACCTTTTC	57.5
2	pS2	F:TTTGGAGCAGAGAGGAGGCAATGG R:TGGTATTAGGATAGAAGCACCAGGG′	57.5
3	PR	F:CGCGCTCTACCCTGCACTC R:TGAATCCGGCCTCAGGTA	61.2
4	NCOR-1	F:CCCAGCAACGAGAGGAATCA R:GTCCATGGGAGGAGTGCTTGT	56.1
5	SRC-1	F:TGGCACTGGAATCAATCCTCAG R:TGGCACTGGAATCAATCCTCAG	61.2
6	GAPDH	F:TGCACCACCAACTGCTTAGC R:GGCATGGACTGTGGTCATGAG	53.4

Cell Culture

ERα-positive MCF-7 cells were maintained in DMEM supplemented with 10% FBS. Cells were cultured in 5% CTS containing DMEM before experiment.

MTT Assay

The MTT assay Calbiochem (San Diego, CA, USA) was used to measure cell viability. Briefly, cells were plated at a density of 4000 cells per well in 96-well flat-bottomed plates. Once the cells attached and attained the morphology, they were treated with 5% CTS containing phenol red free DMEM. After that, the cells were treated with PME (100 μg), 27-Hydroxy cholesterol (10^{-7}M and 10^{-8}M) and combinations of PME and 27-Hydroxy cholesterol. After 48-h incubation at 37°C, 85% humidity and 5% CO_2 atmosphere, the medium was replaced with MTT dissolved at a final concentration of 2 mg/mL in serum-free, phenol red-free medium and incubated for 2 h at 37°C. Then, MTT–formazan was solubilized in lysis buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide), and the optical density was measured spectrophotometrically at 570 nm. Cell survival (CS) was expressed as percentage over the untreated control. CS was calculated as (OD drug-exposed cells/mean OD control cells) $\times$100.

BrdU Cell Proliferation Assay

The rate of proliferation of cells was determined as the rate of incorporation of bromodeoxyuridine (BrdU) into cellular nucleic acids using BrdU proliferation assay kit (Biovision). For BrdU assay cells were seeded in 96-well plate at a density of 4×10^3 cells/well. After treating the cells in 5% CTS for 48 hours treated cells. The cells were further treated with different concentrations of PME (100 μg), 27-Hydroxy cholesterol (10^{-7}M and 10^{-8}M) and combinations of PME and 27-Hydroxy cholesterol for 48 hours.

The cells were then pulsed with BrdU (100 μM) 3 hour before termination of the treatment. The cells were fixed, denatured and incubated with anti-BrdU primary antibody, secondary antibody and substrate according to the manufacturer's protocol. The absorbance was read at 450 nm with 590 nm background subtraction with appropriate blanks on with the help of microplate reader (Bio-Rad).

RNA Isolation and qRT-PCR

For RNA analysis, MCF7 cells were seeded in 60-mm dish plates in phenol red-free media containing 10% charcoal-stripped serum. After 48 hours, cells were treated with the appropriate ligand, that is, PME (100 μg/mL), E2 (10nM), 27HC (10 μM) and combination of PME (100 μg/mL) and 27HC (10 μM). After 24 h, cells were harvested and total RNA was isolated using Trizol method. One microgram of RNA was reverse transcribed using the Superscript cDNA First Strand Synthesis System (Invitrogen).The AB-Biosystems Cycler Real time PCR System was used to amplify and quantitate levels of target

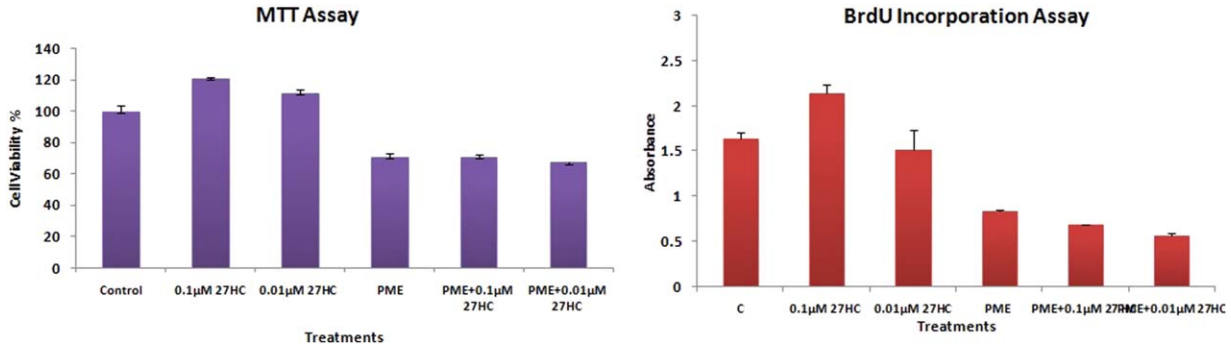

FIG 1 *Cell viability was determined in ER positive MCF-7 cells after treating the cells with 27HC(0.1 μM,0.01 μM),PME (100 μg/ml), PME(100 μg/ml) +27HC(0.1 μM), PME(100 μg/ml) +27HC(0.01 μM) by using a)MTT assay. b) BrdU assay. Both the assays indicate that PME reduced 27HC induced proliferation.*

gene cDNA. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed with 1 μL cDNA, 0.5 μM specific primers (Table 1) and Dynamo SYBR Green qPCR Kit (F-410S/L). Data are normalized to the GAPDH housekeeping gene and presented as fold induction over control. Data are the mean ± SEM for triplicate amplification reactions from one representative experiment. The expression of selected estrogen-responsive genes pS2, PR and ER-α were analyzed.

Reverse-Transcriptase PCR

Total RNA was isolated from untreated and treated MCF-7 cells. Treatments included PME (100 μg/mL), E2 (10 nM), 27HC (10 μM) and combination of PME (100 μg/mL) and 27HC (10 μM). Total RNA was isolated with Trizol reagent (Invitrogen) and converted to first-strand cDNA using Superscript III First Strand Synthesis System (Invitrogen) according to the prescribed procedure. The expression of specific genes was assessed by amplification using the specific primers (Sigma) (Table 1) and the transcripts were normalized by endogenous control GAPDH. The coregulators of ERs, SRC-1 and NCOR-1 were checked for mRNA level expression.

Results

PME Inhibits the Proliferation Induced by 27HC in ER-Positive Cell Line

Our primary aim was to determine the ability of 27HC to induce cell proliferation and that of PME to thwart it. For this purpose, cells were seeded in 96-well plate and treated with PME (100 μg), 27HC (0.1 μM, 0.01 μM), 17-β-estradiol (10 nM) and combination of PME and 27HC, and the proliferative, inhibitory and antagonistic effects were studied by MTT and BrdU assay. As shown in Fig. 1a, PME inhibits the growth of MCF-7 cells and also thwarts the cell proliferation induced by 27HC. PME also decreased the incorporation of BrdU induced by 27HC (Fig. 1b).

PME Downregulate the Expression of Genes Induced by 27HC

The effect of PME on expression of genes involved in estrogen signaling induced by 27HC was studied by RT-PCR. Cells were treated with PME, 27HC and combination of PME and 27HC and estradiol, and the expression of ERα and other 27HC-regulated genes, pS2 and PR, were assessed by qRT-PCR by normalizing with endogeneous control GAPDH. pS2 and PR were upregulated upon 27HC and estradiol treatment, which was consistent with reports of Dusell et al. (8), but we obtained a higher upregulation of PS2 by 27HC than E2, which can possibly be attributed to the higher concentration of 27HC, that is, 10 μM when compared to 1 μM by Dusell et al. (8). Importantly, PME could downregulate the expression of 27HC induced genes, of PR and pS2. 27HC, being a SERM, was checked for ERα degradation as the transcript and protein levels of ERα are reported to be time dependent and ligand

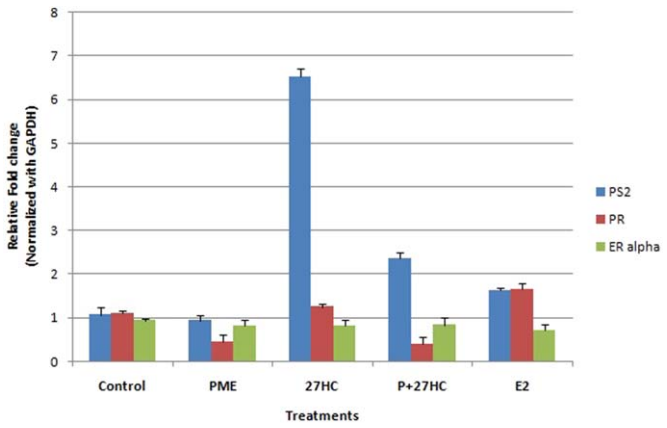

FIG 2 *qRT-PCR. mRNA levels of ERα, pS2 and PR were estimated by real-time PCR after treatments for 24 h. The treatments were PME (100 μg/mL), 27HC (10 μM), PME (100 μg/mL)+ 27HC (10μM) and E2 (10 nM). After treatment, cells were harvested, total RNA was isolated and cDNA was prepared for use as a template for gene expression analysis. All values were normalized to the housekeeping gene GAPDH. Data are presented as the fold induction over untreated cells. Data are the mean ± SEM of triplicate amplification reactions from one representative experiment that was repeated with similar results three independent times.*

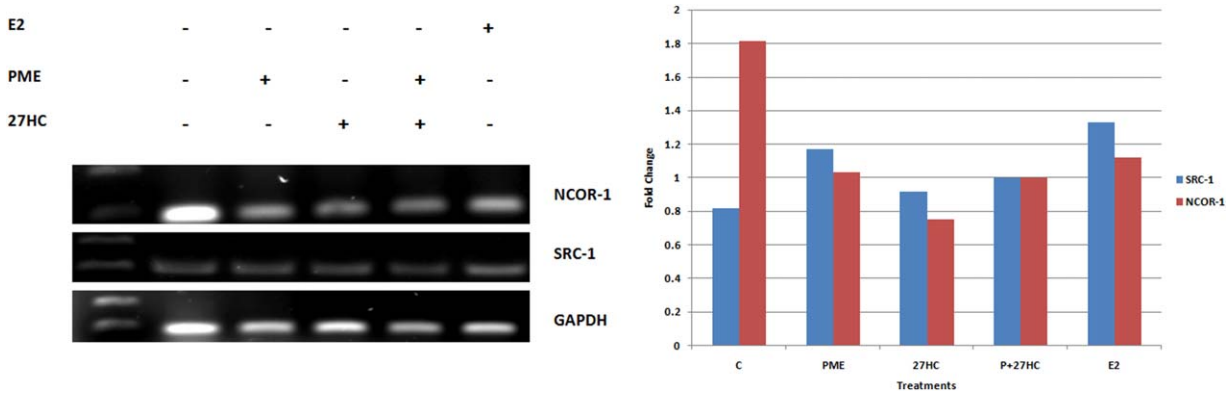

FIG 3 *(a) Reverse-transcriptase PCR. mRNA levels of NCOR-1 and SRC-1 were estimated by reverse-transcriptase PCR after 24-h treatments with PME (100 μg/mL), 27HC (10 μM), PME (100 μg/mL) + 27HC (10 μM) and E2 (10 nM). (b) Representation of relative levels of the transcripts NCOR-1 and SRC-1 when subjected to these treatments.*

regulated (8,9). mRNA levels of ERα were found to slightly reduce upon all the treatments, the greatest reduction being in the cells treated with estradiol (Fig. 2). This points out to the similarity between SERMS and their ligand-mediated action. The changes in the expression pattern of coregulators such as SRC-1 and NCOR1 were also evaluated by RT-PCR followed by amplification with specific primers (Fig. 3). All the treatments increased the levels of SRC-1 mRNA levels, the highest being in estradiol-treated cells (Fig. 3). Contradictorily, treatment with PME decreased the levels of the NCOR1, the highest reduction being in 27HC treated cells. Although 17-β-estradiol is reported to retain the mRNA levels of NCOR1 and degrade the protein NCOR1 with the help of Siah2 (10), we found a small reduction in NCOR1 mRNA levels. It is possible that the balance between the coregulators, that is, both coactivators and corepressors, decides the response of cells to a hormone or hormone-like compound. The exact role of these coregulators in 27HC induction can be elucidated only with further experiments after considering the expression of all relevant coregulators.

Discussion

Cholesterol and breast cancer seem to be closely connected, most often as a probable risk factor (2). There have been reports suggesting that cholesterol can act as inhibitors and activators (11) of ERs and their alternative forms (12). But recent discovery of an endogenous SERM, 27HC, has brought new insight into postmenopause obesity-associated breast cancers. 27HC, the most abundant oxysterol, was found to produce a unique conformational change in both ERα and ERβ, distinguishing it from E2 and other SERMs and has been proven to have proliferative action in breast cancer cellular models (8,13). Also, 27HC increases ER-dependent growth and LXR-dependent metastasis in mouse models of breast cancer (2). This endogenous SERM has been reported to have deleterious effects that include inhibiting protective effects of estradiol in cardiovascular diseases (14,15), promoting atherosclerosis (16,17), negatively regulating bone homeostasis (18) and also in inducing and promoting breast cancer. Thus, in this context, an exogenous SERM, with potential to compete with this endogenous SERM and reducing its deleterious effects, is of

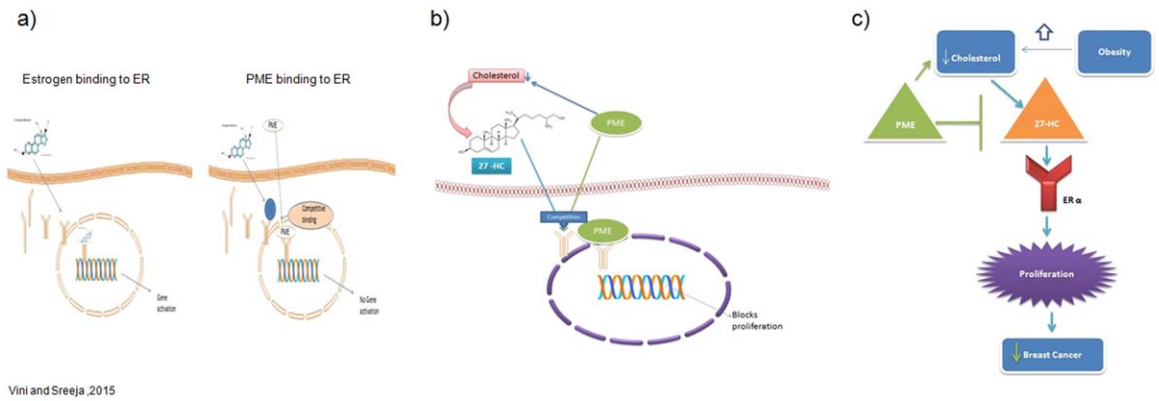

FIG 4 *(a) PME was proposed to compete with 17-β-estradiol to bind to ERα. (b) PME probably follows a similar mechanism of competitive binding with 27HC to reduce its proliferative effect. (c) A proposed summary of action of PME in antagonizing 27HC.*

profound importance. It would be ideal if such an SERM may be natural so that there would be minimal side effects. A few significant findings in this study are worthy for further comments. First, this finding provides the first evidence for SERM activity of PME in antagonizing the endogenous SERM, 27HC. Importantly, our earlier investigation demonstrated that PME (7) could act as SERM, binds to ER and translocates the ligand receptor complex to ERE and downregulates the ER-mediated transcription in breast cancer without being any agonistic effect in the mouse endometrium and endothelial cells compared with E2 and tamoxifen. This study through MTT assay and BrdU assay (Fig. 1) indicates that PME could potently inhibit the 27HC-stimulated proliferation in breast cancer cells, MCF-7, which was comparable to its antiproliferative activity against estradiol (7). Second observation of the study by RT-PCR confirms that PME could diminish the expression of 27HC-upregulated genes that include pS2 and PR (Fig. 2). Also all the treatments could significantly downregulate ERα mRNA levels including the cells in which PME and 27HC was treated in combination. Similar action was observed when PME was treated in combination with 17β - estradiol (7). From the reports of Umetani et al. (14) and our earlier studies (7), it is clear that both PME and 27HC can compete with estradiol in binding to ERα though this was proven in two different cell lines, MCF-7 and EA.hy926 cells. This led us to hypothesize that PME can compete with 27HC and reduce its proliferative effects in breast cell line, MCF-7. It has been already proven that PME could inhibit MCF-7 proliferation and reduce the proliferative effects of estradiol (7). In this study, proliferation assays clearly indicate that PME inhibits the proliferative effects of 27HC. Also the PCR results indicate that 27HC-induced genes are clearly downregulated upon combination of PME and 27HC when compared to MCF-7 treated with 27HC alone. This is indicatory of PME competing with 27HC with ERα. Thus, these experiments point to the ERα-dependent action of these PME in antagonizing the proliferative actions of 27HC.

Although 27HC shows similar actions as that of 17-β-estradiol, it has distinct tissue-specific activity probably owing to difference in ER-E2 and ER-27HC structure and hence its recruitment of cofactors (8). On that account, the mode of action of PME for competing with 27HC might be different from E2. The mRNA transcript levels communicate the fact that SRC-1 levels are upregulated in all the treatments but slightly less when compared to 17-β-estradiol treatment (Fig. 3). The NCOR-1 levels were also downregulated in all the treatments. It has been reported that levels of coregulators relative to each other often dictate the response of hormones (19). It is plausible that this applies to SERMs also. Hence, to understand the role of coregulators, it requires analysis of expression profiles of other coregulators also. These varying expression levels of this coactivator and corepressors and recruitment of other cofactors might be responsible for the differential actions and tissue specificity of PME, 27HC and combination of both when compared to 17-β-estradiol.

Additionally, our earlier studies have shown that PME, as such in ovariectomized mice, can bring down levels of LDL, TC and TG similar to the observed action of 17-β-estradiol while maintaining the HDL levels (5–7).

The competitive activity of the extract discussed here against 27HC-induced events and cell proliferation in MCF-7 model highlights the potential specificity of the extract to attenuate postmenopausal obesity-linked breast cancer risk wherein the estrogen levels drop and 27HC seems to be relatively high. Hence, both the SERM property and its potential to reduce the cholesterol levels might together reduce the breast cancer risk.

Pharmaceutical industries have been testing natural product extracts for developing new drugs for breast cancer. The concept of herbal synergy in case of PME may open new treatment strategies to overcome the consequences of currently using exogenous SERM and also relieve the complications associated with obese breast cancer patients and probably be developed into an ideal oral SERM. The mechanism of action of the extract has to be further explored to understand the exact targets and mode of action.

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