Introduction

Estrogens administered to postmenopausal women exert beneficial effects on bone, vasomotor symptoms, and vulvovaginal atrophy, but potentially induce adverse actions on breast, the venous system, and the uterus via endometrial hyper-stimulation in the absence of a progestogen. Other menopausal signs and symptoms such as arthralgia, sleep disorders, mood changes, depression, and cognition may also be improved but the evidence is less compelling. To enhance the beneficial effects and reduce the potential side-effects and toxicity of estrogens, the class of agents called selective estrogen receptor modulators (SERMs) was developed. As with other pharmacological agents developed for treatment of patients, the SERMs may mimic the effects of similarly acting endogenous estrogens. The best examples of drugs which mimic endogenous factors are opioids such as morphine, which bind to the same receptor and act similarly to endogenous opioids such as the endorphins.

Recent studies identified estetrol or E₄, an endogenous fetal estrogen with tissue-specific properties analogous to the selective tissue effects of SERMs. This estrogen, made exclusively in the liver of the human fetus during pregnancy, circulates at very high levels in the mother and in the fetus. Discovered by Diczfalusy in 1965, the precise effects of E₄ on various tissues during pregnancy remain unknown. A series of recent studies elucidated various tissue-specific properties of E₄ including differential effects on brain, the vascular nitrous oxide system, and membrane actions as opposed to nuclear. Estetrol was identified as a potential drug for human use by Coelingh Bennink in 2001. Clinical studies in premenopausal and postmenopausal women have demonstrated possible use as an estrogen in combined oral contraception and for reduction of hot flashes and bone resorption. As a potential beneficial property, this estrogen exerts limited effects on the liver compared with estradiol (E₂). Specifically, E₄ causes minimal changes in liver proteins and coagulation factors and does not stimulate triglyceride levels. These biomarker data suggest that E₄ might be associated with a lesser enhancement of deep venous thromboses (DVT) or pulmonary emboli (PE), which represent stimulation of clotting factors in the liver. However, ongoing clinical studies are not sufficiently mature to confirm the possibility of limited DVT or PE effects.
In this study, we postulate that E₄ might be beneficial as treatment of breast cancer. Postmenopausal women with ER + breast cancer, who have initially responded to tamoxifen or an aromatase inhibitor, but later became resistant to anti-estrogen treatment, can respond to estrogen with tumor regression. However, the estrogenic effects on liver proteins with concomitant DVT and PE can be problematic with this therapy. Estetrol might then provide similar benefits on tumor regression but with lesser toxicity.13–16 The effects of estrogen in postmenopausal women would appear to be paradoxical, as this sex steroid can also stimulate breast cancer growth. However, modeling studies have demonstrated that breast cancer cells, deprived of estrogen long term, develop the ability to respond to estrogen with programmed cell death (apoptosis). Our studies of long-term deprived MCF-7 cells (long-term estrogen-deprived [LTED] cells) and the in vivo studies of Jordan et al demonstrate that E₂ induces apoptosis both by death receptor and mitochondrial mechanisms.17,18 This study examined whether E₄ might exert similar effects and was conceived to provide preclinical data supporting a subsequent clinical trial. The other pregnancy estrogen, estriol (E₃), was also investigated in this study to be systematic in our assessments.

We designed these studies to systematically compare the effects of E₂, E₃, and E₄ on cell proliferation and apoptosis in wild-type MCF-7 and LTED cells. The data demonstrate strong pro-apoptotic effects of each estrogen in LTED cells and dose-dependent agonistic versus antagonistic actions of E₃ and E₄ in wild-type MCF-7 cells.

Materials and Methods

Materials

Estetrol (E₄) was provided by Pantarhei Bioscience (Zeist, The Netherlands); 17β-estradiol (E₂) and estriol (E₃) were purchased from Steraloids (Newport, RI); caspase inhibitor Z-VAD-FMK from ApexBio (Houston, TX); and fluorescein diacetate (FDA) and propidium iodide (PI) were from Sigma-Aldrich (St Louis, MO).

Cell culture

The human breast cancer cell line, MCF-7, was routinely maintained in Improved Minimum Essential Medium (IMEM) with 5% fetal bovine serum (FBS). T47D cells were cultured in RPMI 1640 with 10% FBS.

Long-term estrogen-deprived MCF-7 cells were developed from MCF-7 cells as described by Masamura et al.19 The cells were maintained in IMEM with 5% dextran charcoal-stripped serum (DCC-FBS) in the absence of phenol red. Fully adapted LTED cells grow in estrogen-deprived medium at the same rate at which MCF-7 cells grow in estrogen-containing medium (Supplementary Figure S1).

Growth assay

For assay of cell number, MCF-7 or T47D cells were plated in 6-well plates at the density of 30000 cells per well in their culture media containing FBS. Two days later, the culture medium was replaced with phenol red-free media supplemented with 5% DCC-FBS containing treatment agents. Treatments from day 1 were renewed on day 3 by aspirating medium from wells and replacing with fresh medium and treatments. Long-term estrogen-deprived cells were treated in their culture medium. On day 6, cell numbers were counted using a Coulter counter.20

Determination of cell proliferation

Proliferation assays were carried out using 5-Bromo-2′-deoxyuridine (BrdU) Labeling and Detection Kit I (Roche Diagnostics, Indianapolis, IN) following the manufacturer’s instructions. Briefly, cells were plated into 6-well plates on sterile cover slips at the density of 2×10⁵ cells per well. One day after seeding, the cells were washed with phosphate-buffered saline (PBS) once and treated with estrogens in phenol red-free media with 5% DCC-FBS for 24 hours. BrdU was added to the culture medium at the concentration of 10 µM and incubated for 1 hour followed by incubation with anti-BrdU antibody and secondary fluorescent antibody. The cover slips were mounted to glass slides using VECTASHIELD Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Images of the cells were acquired using Olympus IX81 microscope and Metamorph software. BrdU positive cells were quantified by manual counting using ImageJ software. Three to five fields (20× objective) of each treatment were counted.

Determination of apoptotic cell death

Apoptosis was measured using the Cell Death Detection ELISA Kit (Roche Diagnostics) following the manufacturer’s instructions. Briefly, cells were plated into 12-well plates at the density of 8×10⁴ cells per well. Two days later, the cells were treated with testing compounds for desired periods of time. The cell lysates were prepared by incubation of the cell monolayer with 0.5 mL lysis buffer at room temperature for 30 minutes followed by centrifugation at 1400 r/min for 10 minutes at 4°C. A parallel set of plates with identical treatment was prepared for cell counting. The result was expressed as absorbance at 405 nm normalized by cell number.

Determination of gene expression by quantitative real-time polymerase chain reaction

Cells grown in 60 mm dishes were cultured in phenol red-free media with 5% DCC-FBS for 24 hours and treated with E₄ or E₂.
for 24 hours before RNA extraction. Total RNA was extracted and purified using the Qiagen RNeasy Mini Kit (Valencia, CA). Transcription of estrogen-regulated gene pS2 was determined by quantitative real-time polymerase chain reaction (q-PCR) using the SYBR Green method. GAPDH was used as a house-keeping gene for quantification. Relative mRNA copies were calculated by comparing with vehicle control using ΔΔCt method.21

Sequences of primers used were as follows: pS2 = forward 5′-ACGACACCGTGGGGTC-3′; reverse 5′-ACGGCACCGTGAGATG-3′; GAPDH = forward 5′-ACCCCTCCACCTTTG-3′; reverse 5′-CTCTTGTGCTCTTCGGG-3′.

Live/dead cell analysis of LTED cells

Long-term estrogen-deprived cells were seeded in 6-well plates at a density of 2 × 10^5 per well in IMEM with 5% DCC-FBS. Three days later, the cells were treated with estrogens for 3 days. On the day of analysis, the culture media were collected, the cells were trypsinized (5 minutes, at 37°C), and combined with the medium. The cells in suspension were passed through a strainer (45 µm) and then spun down at 300 g for 5 minutes at room temperature. Cells were resuspended in 200 µL PBS. An aliquot of 100 µL FDA (0.02 mg/mL) and 30 µL PI (0.02 mg/mL) were added to cell suspension and incubated at room temperature for 3 minutes in dark and then placed on ice. Live/dead cells were detected using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA).

Statistics

Differences in average cell number and apoptosis were analyzed by Student t-test. The differences are considered significant if the value of P is less than .05.

Results

Differential effects of E2, E3, and E4 on growth of MCF-7 and LTED cells

It is well documented that proliferation of hormone-depend-ent breast cancer cells is stimulated by E2 via activation of ERα. To determine the effect of E3 and E4, we initially carried out growth assays in ERα-positive MCF-7 cells and a derivative of MCF-7 cells, LTED.

Estetrol dose-dependently stimulated the growth of MCF-7 cells at the dose range of 10^{-12} to 10^{-8} M (Figure 1A) with peak stimulation at the dose of 10^{-8} M. There was no significant reduction in cell number when MCF-7 cells were exposed to higher concentrations of E4 up to 10^{-4} M (data not shown) suggesting that E4 might be tolerable in patients. Estriol exhibited similar stimulatory effects on MCF-7 cells (Figure 1B). Growth promotion effects of E4 and E3 were then compared with that of E2. As shown in Figure 1C and Table 1, E2 was 100-fold and 2000-fold more potent than E3 and E4, respectively. T47D, another ER+ breast cancer cell line, showed similar growth responses to E3 as MCF-7 (Supplementary Figure S2).

In striking contrast, both E3 and E4 inhibited growth of LTED cells in a dose-dependent fashion (Figure 2A and B). In LTED cells, the potency of E3 and E4 was similar but about 1000-fold lower than that of E2 (Figure 2C and Table 1).

Table 1. Concentrations of estrogens causing 50% growth stimulation in MCF-7 or inhibition in LTED cells.

|        | EC50 (MCF-7) | IC50 (LTED) |
|--------|--------------|-------------|
| E2     | 1.5 × 10^{-13} M | 7.3 × 10^{-13} M |
| E3     | 1.4 × 10^{-11} M | 3.4 × 10^{-10} M |
| E4     | 3.1 × 10^{-10} M | 2.8 × 10^{-10} M |

Abbreviation: LTED, long-term estrogen-deprived.

Figure 1. Effects of E2, E3, and E4 on growth of MCF-7 cells. (A) Dose-response stimulation of E4 on cell growth. (B) Dose-response stimulation of E3 on cell growth. (C) Comparison of growth stimulatory effects of 3 estrogens. ∗P < .05, ∗∗P < .005, ∗∗∗P < .0005 compared with the vehicle control. The cells were exposed to various concentrations of E2, E3, or E4 for 5 days before cell counting. Each treatment was in duplicate. The experiment was repeated for at least 3 times with similar results. The figure shown here is a representative result.

Table 1. Concentrations of estrogens causing 50% growth stimulation in MCF-7 or inhibition in LTED cells.
Differences between MCF-7 and LTED cells were also reflected in their responses to these estrogens on proliferation. Two concentrations of E4 and E3 were used and $10^{-10}$ M of E2 was as a positive control. In MCF-7 cells, low concentrations of E4 ($10^{-12}$ M) and E3 ($10^{-14}$ M) did not stimulate cell proliferation. Higher concentrations significantly increased the number of proliferative cells shown by higher percentage of cells which were labeled with BrdU (Supplementary Figure S3). These results are consistent with those of growth assay. Long-term estrogen-deprived cells had a higher proliferation rate (36.8%) than that of MCF-7 cells (16.5%) and were not further stimulated by any of 3 estrogens (Supplementary Figure S3).

To confirm the role of estrogen receptor \( \alpha \) (ER\( \alpha \)) in mediating growth stimulation of E4, expression of estrogen-inducible gene, pS2, was determined by q-PCR in both MCF-7 and LTED cells. Differing from the results of growth and proliferation, the response pattern of MCF-7 and LTED cells to E4 and E2 was similar. Estetrol dose-dependently stimulated pS2 expression in both cell lines except at the concentration of $10^{-12}$ M. Fold of stimulation is higher in LTED cells at any given concentration (Supplementary Figure S4). These results were confirmed in T47D, another ER\(+\) breast cancer cell line (Supplementary Figure S4).

**Effects of E4 and E3 on apoptosis in LTED cells**

Our prior studies have demonstrated that long-term estrogen deprivation causes adaptation of MCF-7 cells such that E2 induces apoptosis.\(^{17}\) We hypothesized that reduction in cell number in LTED cells exposed to E4 or E3 was a result of apoptosis. Using an ELISA assay we found that both E4 and E3 dose-dependently induced apoptosis in LTED cells (Figure 3A and B). The apoptotic effect of these estrogens can be partially blocked by pan-caspase inhibitor Z-VAD (Figure 3C) suggesting that both caspase-dependent and caspase-independent pathways are involved.

We next used FDA/PI staining to further demonstrate that E4 and E3 caused death of LTED cells. FDA is cleaved by esterase in

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**Figure 2.** Effects of E2, E3, and E4 on growth of LTED cells. (A) Dose-response inhibition of E4 on cell growth. (B) Dose-response inhibition of E3 on cell growth. (C) Comparison of growth inhibitory effects of 3 estrogens. *\( P < .05 \), **\( P < .0005 \) compared with the vehicle control. The cells were exposed to various concentrations of E2, E3, or E4 for 5 days before cell counting. Each treatment was in duplicate. The experiment was repeated for at least 3 times with similar results. The figure shown here is a representative result.

**Figure 3.** Induction of apoptosis in LTED cells by E3 and E4. (A) Dose-response effect of E4 on cell death. (B) Dose-response effect of E3 on cell death. (C) Inhibition of E4-induced apoptosis by caspase inhibitor Z-VAD. **\( P < .005 \), ***\( P < .0005 \), ****\( P < .00005 \) compared with the vehicle control; \( \dagger\dagger\dagger\dagger P < .00005 \) compared with E4 10\(^{-10}\) M. The experiment was repeated for at least 3 times with similar results. The figure shown here is a representative result.
live cells and becomes fluorescent and accordingly, live cells exhibit green fluorescence. Dead cells, in contrast, cannot cleave FDA but have increased membrane permeability to PI. Binding of PI to DNA will label the dead cells red. Both E4 and E3 dose-dependently increased the number of dead cells. This result is consistent with the cell counts (Figure 2) and further confirms the pro-apoptotic effects of these estrogens. Surprisingly, at a similar low concentration (10^{-10} M), E4 and E3 were more potent than E2 (Supplementary Figure S5). The mechanism behind this observation requires further investigation.

**Biphasic effect of E4 and E3 in MCF-7 cells**

An unexpected finding of our studies was the biphasic effect of E4 and E3 on growth of MCF-7 cells. In addition to growth stimulation at physiological concentrations, both E4 and E3 inhibited cell growth at sub-physiological concentrations (Figure 4A and B). Growth of MCF-7 cells was reduced by 50% with E4 10^{-12} M and E3 10^{-14} M. The inhibitory effects of low doses of E4 and E3 resulted from induction of apoptosis (Figure 4C and D). Z-VAD completely abolished the apoptotic effect of E3 (Figure 4D) and E4 (data not shown) indicating the effects are caspase-dependent.

To determine whether the biphasic effect of E4 and E3 is cell line-specific, another ER+ positive cell line, T47D, was employed. Estetrol at lower concentrations reduced the number of T47D cells (Supplementary Figure S6A). The inhibitory effect was observed at the concentration starting from 10^{-14} M. It was further demonstrated that the inhibitory effect of low-dose E4 in T47D cells was due to increased apoptosis (Supplementary Figure S6B). These results indicate that the inhibitory effect of low-dose E4 is not restricted to MCF-7
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In contrast, the effect of E3 in T47D is predominantly stimulatory on cell growth (Supplementary Figure S2) and additionally prevents the cells from apoptotic death (data not shown) even at low concentrations (10^{-14} and 10^{-12} M).

Our prior studies have shown that E2-induced apoptosis in LTED cells is partially mediated by Fas/FasL death receptor pathway.\textsuperscript{17} We found that E4 also increased FasL protein in LTED, MCF-7, and T47D cells (Supplementary Figure S7). These results suggest that similar mechanism might be involved in the pro-apoptotic effect of E4 in these ER\textsuperscript{+} breast cancer cells.

Combination of E4 or E3 with E2

To determine whether E4 or E3 exert antagonistic effects on E2-stimulated growth, assays using E4 or E3 in combination with E2 were carried out in both MCF-7 and LTED cells. In MCF-7 cells, E2 at a 10^{-10} M concentration stimulated cell growth by 4.4-fold compared with the vehicle control. Addition of E4 at concentrations from 10^{-12} to 10^{-6} M did not alter cell proliferation in response to E2 (Figure 5A). Similarly, E3 did not alter E2-stimulated cell growth in LTED cells (data not shown). In contrast to MCF-7 cells, 10^{-10} M E2 caused 86% reduction in cell number in LTED cells. Combinations with various concentrations of E4 did not change this inhibitory effect of E2 (Figure 5B). We then examined the effect of E2 and E4 combination on apoptosis of LTED cells. In this assay, a lower concentration of E2 (10^{-12} M) was used which caused 2-fold increase in apoptosis. Addition of E4 to E2 further increased apoptosis (Figure 5C). However, the apoptotic effects in LTED cells treated with E2 plus higher concentrations of E4 (10^{-10} M and 10^{-8} M) were similar to those with E4 alone.

Discussion

Approximately 80\% of breast cancers express ER\textsubscript{\alpha} and 70\% progesterone receptor.\textsuperscript{22} These hormone-dependent tumors often regress in response to aromatase inhibitors or antiestrogens such as tamoxifen. However, resistance develops after 12 to 18 months of treatment and tumors regrow. Intensive searches for new approaches to treat relapsing breast cancer remain a current area of research. One of the strategies is to use estrogenic compounds to induce apoptosis.

Clinical data have shown that treatment with the high-dose synthetic estrogen, diethylstilbestrol (DES), induced tumor regression in women with advanced breast cancer.\textsuperscript{12,23} However, the use of DES fell into disfavor after a randomized controlled trial demonstrated reduced side-effects and toxicity with tamoxifen compared with DES.\textsuperscript{12,23} Later studies demonstrated that E2 caused tumor regression in animal models of breast cancer when the tumors became resistant to tamoxifen. Clinical trial data in women confirmed that high-dose estrogens are effective for treatment of advanced breast cancer after multiple prior hormone therapies failed. However, all clinically used estrogens including DES, E2, and ethinyl estradiol (EE) have side-effects that lead to discontinuation of the treatment.\textsuperscript{12}

To search for a safer alternative estrogen for breast cancer treatment, we evaluated E4 and E3 in ER\textsuperscript{+} breast cancer cell lines, MCF-7, T47D, and LTED cells. In general, E4 and E3 act similar to E2 but with lower potency. These two estrogens are stimulatory for MCF-7 cells but inhibitory for LTED cells. However, as demonstration of cell-specific effects of E3 and E4, these steroids, as opposed to E2, inhibited growth of MCF-7 cells at low concentrations (10^{-14} to 10^{-12} M), a phenomenon which is at least partially due to induction of apoptosis. These biphasic effects of E3 and E4 never been reported before. A similar biphasic effect of E4 was confirmed in T47D cells. There was much less of an inhibitory effect of E3 in T47D cells. Whether the difference is due to the unique property of E4 or differential responsiveness of the cell lines is
unclear. Many hormones and endocrine disrupting chemicals exhibit non-monotonic dose-response curves. Estrogens are among these hormones. In ER-positive breast cancer cells, E3 stimulates proliferation at physiological concentrations (10⁻¹² to 10⁻⁸ M) but inhibits cell growth at higher concentrations. It is not clear whether this effect represents apoptosis or non-specific toxicity of high-dose estrogens. We have not seen any inhibitory effects of E4 and E2 in MCF-7 cells at higher concentrations up to 10⁻⁵ and 10⁻⁴ M. Notwithstanding potency differences, the dose-response curves of E3 and E4 are mirror images of that of E2. These differential phenomena between E3 versus E2 and E4 could be due to differences of these estrogens in receptor selectivity and affinities. For example, activation of membrane ER, GPR30 (now called GPRE1), stimulates proliferation of Eκ-α-negative breast cancer cells but inhibits ERα-positive cells. Although GPRE1 is reported to block estrogen-related membrane signaling, the role of this receptor in breast cancer growth is not well known. While the precise mechanism of the pro-apoptotic effect remains to be defined, our findings suggest that E3 and E4 could be used as therapeutic agents not only for postmenopausal patients who have relapsing cancer after primary endocrine therapies but also as a choice of treatments for hormone-sensitive breast cancer.

Some but not all studies have shown that E4 may act as an antagonist on mammary glands and breast cancer. When used alone, E4 exerts weak estrogenic effects on proliferation of mammary epithelial cells and ductal elongation and end bud development of immature mouse mammary glands but antagonized stimulatory effects of E2 on these parameters when these two steroids are combined. The antagonistic effect of E4 was also reported by studies with breast cancer models. Visser et al. found that E4 prevented and inhibited the growth of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumor in Sprague-Dawley rats. The antitumor effect of E4 was similar to that of tamoxifen. However, there was no antagonistic effect of E4 on the uterus in same animals.

In vitro results with LTED cells have shown promising apoptotic effects of E4. A pertinent in vivo model is aromatase inhibitor-resistant human breast cancer. We have inoculated aromatase expressing MCF-7 cells in nude mice and treated the animal with aromatase substrate androstenedione plus or minus letrozole. Unfortunately, this model did not work as expected. It took too long to develop letrozole resistance (41 weeks on average) and not all tumors developed resistance. Tumors that regrew while on letrozole treatment were still stimulated rather than suppressed by E4, suggesting that the “letrozole-resistant tumors” in this mouse model behave differently from LTED cells. Therefore, more research should be done to develop a proper preclinical model to evaluate antitumor effects of estrogens in vivo. Currently, for E4, a dose escalation proof of concept study in postmenopausal women with advanced breast cancer refractory to hormone treatment (ABCE4 study) is ongoing in Germany.

In summary, compared with E2, E3 and E4 are weak estrogens that stimulate MCF-7 but inhibit LTED cells. The pro-apoptotic effects of E3 and E4 on LTED cells and at low doses on MCF-7 cells indicate potential usage of these steroids for hormone-sensitive breast cancer.

Author Contributions
Experimental design: WY, RS, CV and HCB; Experiment performance: WY and JW; Data analysis: WY; Manuscript preparation: WY, CV and RS.

Supplemental Material
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