Breast Cancer Research and Treatment 10: 169–175, 1987

Report

Stimulation of breast cancer cells *in vitro* by the environmental estrogen enterolactone and the phytoestrogen equol

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Key words: dietary estrogens, enterodiol, enterolactone, equol, estrogen-stimulated growth, hormone-dependent breast cancer, phytoestrogens

Summary

The phenolic lignans enterolactone and enterodiol appear periodically in women's urine, dependent upon synthesis from plant-derived lignans by the intestinal microflora. The phytoestrogen equol is also present in women's urine, and is also derived from a vegetarian diet. Antiestrogenic or antiproliferative actions of these compounds have been postulated and related to the observation that there is a reduced incidence of breast cancer associated with diet. We evaluated the estrogenic and antiestrogenic activity of these compounds using four sensitive assays in tissue culture, including the use of human breast cancer cell lines T47D and MCF-7. Unexpectedly, we found that enterolactone and enterodiol, as well as equol, are weak estrogens, and that enterolactone and equol could stimulate the growth of estrogen-dependent breast cancer cell lines. We suggest that these environmental agents can promote the growth of breast cancer, particularly hormone-dependent metastases that may be located near the gut or in the mesenteries or liver, where the concentration of these intestinally produced compounds would be highest. Treatment with an antiestrogen such as tamoxifen blocks the estrogenic activity of these compounds. In the absence of treatment with an antiestrogen such as tamoxifen, hormonal therapy to block steroidal estrogen synthesis in a patient with breast cancer could conceivably be circumvented by a vegeterian diet rich in the precursors to estrogenic compounds such as enterolactone and equol.

Introduction

During the past decade there has been an extensive investigation of the chemistry, biosynthesis and periodic appearance of phenolic lignans in women's urine [1–10]. The description of enterolactone (2,3-bis-(3-hydroxybenzyl) butan-4-olide, or HPMF) and enterodiol (2,3-bis-(3-hydroxybenzyl)butane-1,4-diol) was particularly interesting because lignans, which occur extensively in plants, had not previously been obtained from an animal source [1, 2]. Subsequent studies demonstrate that the urinary levels of phenolic lignans are dependent upon modification of plant-derived lignans by the intestinal microflora, so that, for example, the addition of linseed to the normal diet can substantially increase the production of both enterolactone and enterodiol, a precursor in the biosynthesis of enterolactone [6, 10].

The possibility of antiestrogenic or antiproliferative actions of the phenolic lignans has been postulated [2, 5], supported somewhat indirectly by a number of reports [7, 11] including the description of anticancer activity of other lignans [12, 13]. Un-

fortunately, progress in elucidating the biology and pharmacology of these compounds has been hampered by the lack of adequate quantities for large-scale studies.

To address these questions, we have evaluated the estrogenic and antiestrogenic activity of enterolactone and enterodiol using four sensitive assays in tissue culture. We used the phytoestrogen equol for comparison because equol has also been shown to be present in the urine of normal women [14] and patients with breast cancer [7]. The estrogenic actions of phytoestrogens have previously been described *in vitro* [15].

Methods

The structures of the compounds used in the study are shown in Figure 1. 17β-Estradiol was obtained from Sigma Chemical Co., St. Louis, Missouri. Equol was obtained from the Medical Research Council steroid reference collection, Department of Chemistry, Queen Mary College of the University of London, England. Enterolactone was obtained from two sources; Dr. G.F. Woods, Scientific Development Group, Organon International, Newhouse, Lanarkshire, Scotland, provided one sample, and Dr. K.D.R. Setchell, Childrens Hospital, Cincinnati, Ohio, provided the second sample, Enterodiol was obtained from Dr. Setchell. Tamoxifen and N-desmethyltamoxifen were obtained from ICI Pharmaceuticals Division, Macclesfield, England.

MCF-7 cells were maintained in Minimum Essential Medium (MEM; Gibco) with phenol red, containing nonessential amino acids, 10 mM Hepes, insulin 6 ng/ml, penicillin 100 units/ml, streptomycin 100 μg/ml, and 5% calf serum (Gibco) that was charcoal stripped. Cells were transferred to phenol red-free medium (using Sigma phenol red-free MEM) for a total of 3–7 days before beginning exposure to compounds. To measure stimulation of progesterone receptor or growth, cells were plated at 25,000 to 50,000 per well in 16-mm multiwell plates, and after attachment for 1–3 days, the cells were treated for 3 or 4 days with the compounds in the phenol red-free medium containing 0.1% solvent ethanol with daily medium changes, 1 ml per well.

T47D cells were maintained in RPMI 1640 (with phenol red; Gibco) with insulin 6 ng/ml, penicillin 100 units/ml, streptomycin 100 μg/ml, and 10% whole fetal calf serum (Gibco). Cells were transferred to medium with charcoal-stripped serum and without phenol red (using indicator-free RPMI 1640 from Gibco) for 9 days before use. After plating at 250,000 cells per 16-mm well and attachment for 2 days, the cells were grown for 6 days further in the phenol red-free medium containing compounds and 0.1% solvent ethanol, with medium changes every 3 days.

Progesterone receptor was measured in the attached cells by whole cell uptake of [^{3}H] R5020 (New England Nuclear), 5 nM in Hanks' balanced salt solution with 25 mM Hepes (HBSS), 0.5% ethanol, and 25 nM dexamethasone to reduce binding of R5020 to glucocorticoid receptor. One hundred-fold excess of nonradioactive R5020 was added to measure nonspecific binding in separate wells. After incubation with 0.5 ml of the tritiated ligand for 45 min at 37° C, the wells were washed 4 times with 1 ml of HBSS containing bovine serum albumin 2 mg/ml. If only DNA was to be determined, the wells were sonicated in 1 ml of the wash buffer diluted 1:9 with water, and samples were taken for radioactivity and/or DNA. DNA was measured fluorometrically using Hoechst 33258 (Calbiochem-Behring) according to Labarca and Paigen [16].

The stimulation of prolactin in primary pituitary cells from the immature rat, and the stimulation of progesterone receptor in primary uterine cells also from the immature rat, have been described in detail elsewhere [17–19].

Results

Enterolactone, enterodiol, and equol have close structural similarities with estrogens and antiestrogens (Fig. 1). As shown, the orientation of the phenolic groups of the compounds is similar to the orientation of the hydroxyl groups of 17β-estradiol, an important arrangement in the structure-activity

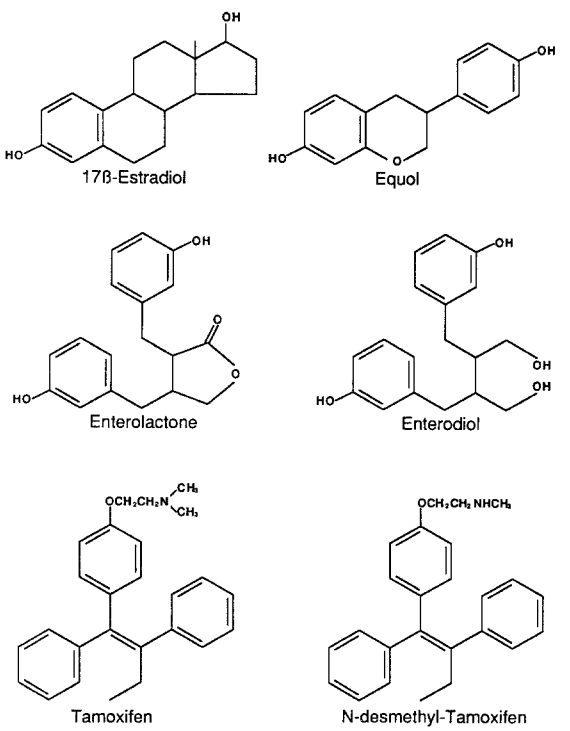

Fig. 1. Structures of the compounds used in this study.

relationships of estrogenic molecules [20]. In contrast, tamoxifen (Fig. 1) is the principal antiestrogen used in the treatment of hormone-dependent breast cancer, and N-desmethyltamoxifen is the major circulating metabolite in man, present at a ratio of 2:1 to tamoxifen in the circulation of women treated with the drug [21].

To study the estrogenic activities of the lignans, we used four tissue culture systems: two primary cultures of normal cells and two breast cancer cell lines. Pituitary cell prolactin synthesis in primary culture has been validated to study the structure-activity relationships of estrogens and antiestrogens [17, 18]. Progesterone receptor stimulation by estrogens in primary cultures of rat uterine cells was used because of the prior report of possible antiestrogenic activity of enterolactone on rat uterine estrogen-stimulated RNA synthesis [11]. The assay has also been validated for the study of estrogens and antiestrogens [19].

We were also able to use estrogen-stimulated responses in two human breast cancer-derived cell lines: T47D cell growth [22], and induction of progesterone receptor [23] and growth [24] in MCF-7 cells, both cultured in phenol red-free medium [25]. As in the primary cell assays, estradiol stimulated the markers while antiestrogens inhibited the action of estradiol.

Enterolactone, enterodiol, and equol showed weak estrogenic properties in each assay when compared with estradiol (Fig. 2). The half-maximal stimulation for all three responses was approximately 10^{-11} M by estradiol, 10^{-7} M by equol, and 10^{-5} M by enterolactone. Enterodiol was approximately 1/10 as active as enterolactone (Fig. 2A, B). The stimulation of MCF-7 cell progesterone receptor by enterolactone or by enterodiol was inhibited by antiestrogens, for example, by tamoxifen + N-desmethyltamoxifen (Table 1A, B).

The estrogenic properties were confirmed by our study of the growth of the estrogen receptor positive cell lines T47D and MCF-7. Estradiol, entero-

Table 1. Estrogenic and antiestrogenic actions on MCF-7 cells.

	Compounds	*Response*
		PgR (pmol/mg DNA)
A:	Control	0.94 ± 0.09
	TAM	0.54 ± 0.03
	Enterolactone 10^{-5} M	3.60 ± 0.16
	Enterolactone + TAM	0.68 ± 0.10
B:	Control	0.32 ± 0.04
	TAM	0.35 ± 0.05
	Enterodiol 10^{-4} M	2.43 ± 0.19
	Enterodiol + TAM	0.37 ± 0.03
		µg DNA/well
C:	Control	6.6 ± 0.33
	Enterolactone 10^{-4} M	4.1 ± 0.07
	+ E2 10^{-10} M	4.0 ± 0.06
	+ E2 10^{-7} M	4.1 ± 0.20
	E2 10^{-10} M	9.8 ± 0.14
	E2 10^{-7} M	10.7 ± 0.05

MCF-7 cells were cultured and treated for 3 days with compounds as described in Methods. Progesterone receptor (PgR) is shown in A and B; DNA content is shown in C. TAM: tamoxifen 1 µM + N-desmethyltamoxifen 2 µM. E2: estradiol. Range is the standard error, n = 4.

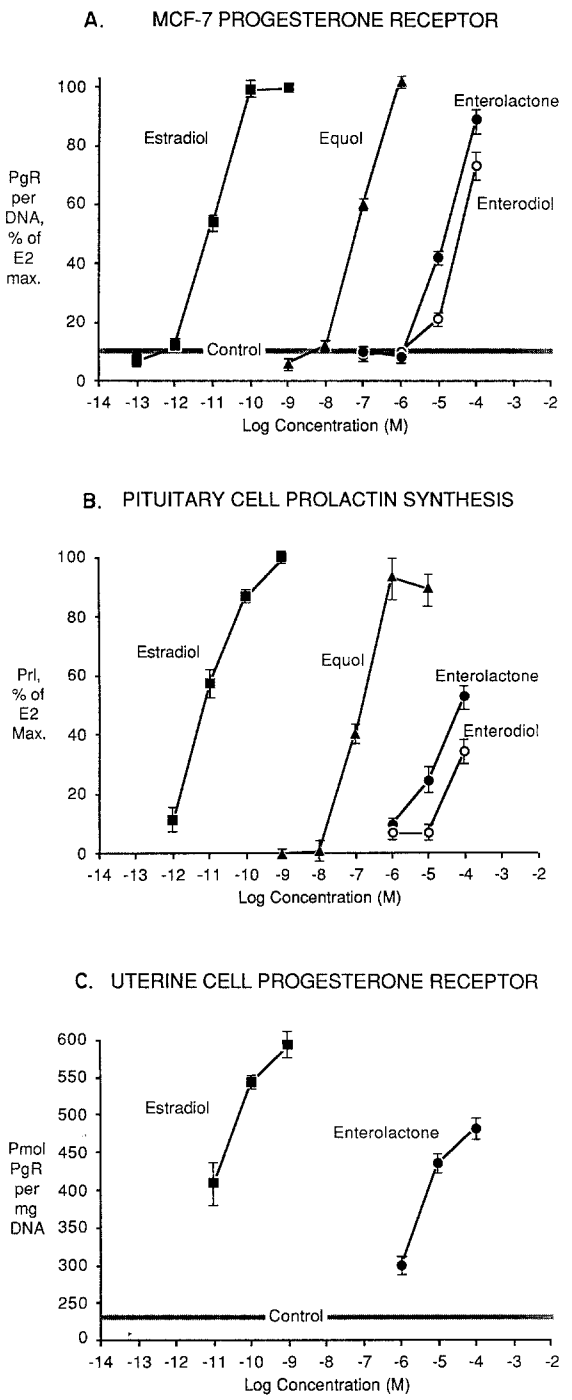

Fig. 2. Dose-response curves for estradiol, equol, enterolactone, and enterodiol.

A. Progesterone receptor stimulation (PgR) in the human breast cancer cell line MCF-7. Progesterone receptor is shown as percent of maximal stimulation by estradiol (E2), in order to show responses for enterolactone and enterodiol on the same figure. Maximal stimulation was 4.8 pmol/mg DNA for estradiol, equol, and enterolactone curves, and 3.3 pmol/mg DNA for the enterodiol experiment. Bars show the propagated standard error from n = 2 for PgR and n = 4 for DNA.

B. Stimulation of prolactin synthesis in primary cultures of rat pituitary cells. Primary cultures were treated with compounds for 6 days, and the rate of prolactin synthesis was measured by 1-hr incorporation of [^{3}H] leucine into immunoprecipitable prolactin, and expressed as a percentage of total incorporation of [^{3}H] leucine into trichloracetic acid-precipitable material. This assay is described elsewhere [17]. Measurements are shown as percentage of maximal stimulation by estradiol (E2). For the estradiol, enterolactone, and enterodiol curves, control and maximal estrogen-stimulated prolactin synthesis were, respectively, 5.0% and 13.5% of total protein synthesis. For the equol curve, the control and maximal stimulation values were respectively 3.9% and 13.8% of total protein synthesis. Bars show the standard error, n = 3.

C. Stimulation of progesterone receptor (PgR) in primary cultures of immature rat uterine cells. Primary cultures were tested for 24 h with the indicated compounds and PgR received by [^{3}H]R5020 binding as described [19], N = 3.

lactone, and equol stimulated the growth of both cell lines in a concentration-related manner (Figs 3, 4). Tamoxifen (1 μM) + N-desmethyltamoxifen (2 μM) inhibited both estradiol and enterolactone-stimulated growth (shown for MCF-7 cells in Fig. 4), but inhibition by tamoxifen was reversed ("estrogen rescue", ref. 22) by an excess of estradiol (10^{-7} M, Fig. 4), indicating that the tamoxifen operated through a specific and competitive mechanism, and not through generalized toxicity.

Interestingly, enterolactone concentrations of 10^{-4} M inhibited cell growth in the MCF-7 cells (Table 1C) and in the T47D cells (not shown). However, this did not seem to be through an anti-estrogenic mechanism, because an excess of estradiol could not reverse the inhibition (Table 1C). The inhibition of growth by high concentrations of enterolactone would therefore appear to be due to nonspecific toxicity. Similar concentrations of enterolactone have been reported to bind to and inhibit the Na^+, K^+ pump [26, 27] which may bear on

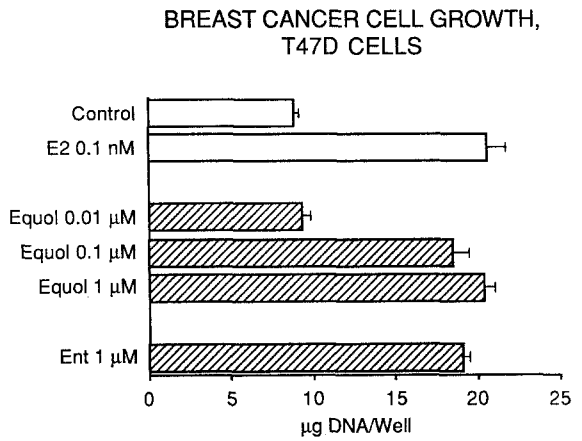

Fig. 3. Stimulation of T47D human breast cancer cells. T47D cells were treated 6 days with the indicated concentration of enterolactone (Ent), estradiol (E2), or equals with medium changes every 3 days. At the end of the growth period, the cells were washed and sonicated in the wells, and samples were taken for DNA as described in Methods. Cross-hatching indicates growth in the presence of equol or enterolactone. Error bars represent the standard error, n = 4 wells per determination.

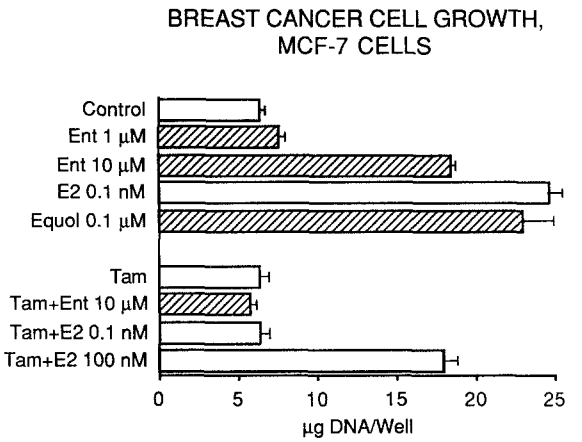

Fig. 4. Stimulation of MCF-7 human breast cancer cells. MCF-7 cells were treated with compounds for 4 days with daily changes. DNA was measured as described in Methods. Ent: enterolactone, E2: estradiol, Tam: tamoxifen 1 μM + N-desmethyltamoxifen 2 μM (which are the approximate circulating levels during tamoxifen therapy in the treatment of breast cancer [21]). After treatment, wells were washed once and sonicated, and DNA was determined as described. Cross-hatching indicates the presence of enterolactone or equol. Error bars represent the standard error, n = 4 wells per determination.

the toxicity we observed. The toxicity observed in the MCF-7 and T47D cells was not observed in the two rat primary cells (not shown). This may indicate that the breast cancer cell lines are more sensitive to the toxic effects of enterolactone than normal cells, an observation which may merit further study.

Discussion

The lignans enterolactone and enterodiol were estrogenic in several response assays, contrary to prior suggestions that the compounds might have antiestrogenic properties. The possibility that the hormonal activity of these compounds may affect estrogen-dependent breast cancer is of interest, especially for enterolactone which is the predominant lignan that has been identified. The growth of T47D and MCF-7 cells was stimulated by 1–10 μM enterolactone in the medium. The concentrations of enterolactone in body fluids have been reported at approximately 10 nM in peripheral serum, 100 nM in semen, and 1 μM in urine [7, 8]. The peripheral circulating levels are thus probably too low to affect cell growth. However, levels of the compound in the intestine, mesenteries (portal circulation), and liver are probably higher. A combination of local environment (e.g., intestine, liver, bladder), individual diet (e.g., high in linseed), or individual metabolism (efficient formation of lignans in the gut) could provide conditions for enterolactone-stimulated growth of breast cancer metastases. In this regard, it should be noted that the production of equol (which was also estrogenic in the stimulation of breast cancer cell growth) has been reported to increase 1000-fold in some but not all individuals whose diet was supplemented with soya flour [28], and significant individual variation in the production of other dietary estrogens may also occur.

These findings may have important implications for the clinical management of breast cancer in postmenopausal women who seek to improve their diet and general health by becoming vegetarians. High intake of estrogens of dietary origin may reac-

tivate the microscopic metastatic disease in patients not taking long-term tamoxifen therapy as an adjuvant [28]. The estrogens in the diet may circumvent therapy with aromatase inhibitors (e.g., aminoglutethimide). The mechanism of action of these drugs is to prevent the production of estrone from androstenedione, thereby denying the tumor a source of endogenous estrogens. This approach will not affect the action of dietary estrogens; however, nonsteroidal antiestrogens (which block the actions of all estrogens in the tumor) will be effective.

In contrast, dietary estrogens may not act solely as described above. The concentration of enterolactone and other phytoestrogens in the peripheral circulation may ultimately be insufficient to drive tumor growth *in vivo*, but high concentrations in the hepatic portal circulation may be adequate to stimulate an increase in the production of sex hormone binding globulin (SHBG) by the liver [30]. This has been described in the comparison of replacement estrogen administered by oral vs. subcutaneous route [31, 32]. It has been suggested that a rise in sex hormone binding globulin may decrease the concentration of free steroidal estrogens in the peripheral circulation in postmenopausal patients and act indirectly to prevent estrogen-dependent breast cancer recurrence [30]. Future studies should be aimed at resolving these direct vs. indirect alternatives. Clearly this second alternative may link diet and cancer [33] such that vegetarians have a lower incidence of breast cancer.

Our own observations, however, bear on the direct effects of enterolactone and equol, which are estrogenic in all *in vitro* systems we tested. While indirect effects of the phenolic lignans and other phytoestrogens may be important, it is clear that the compounds have the potential to stimulate breast cancer proliferation *in vitro*, and this should be considered as a factor in the treatment of vegetarian women with breast cancer.

Acknowledgements

We would like to thank Drs. G.F. Woods and K.D.R. Setchell for providing samples of enterolactone to use in these studies and Dr. Setchell for the sample of enterodiol. Professor D.N. Kirk provided the sample of equol from the MRC steroid reference collection at Queen Mary College of the University of London. We thank Kate Strunk and Patrick O. Williams, Jr., for technical assistance. These studies were supported by ACS grant PDT 290, USPHS grants CA-32713, and CA-20432, and NIH training grant CA-09471 in Human Cancer Biology. Dr. Gloria Calaf was supported by a grant from the Pan American Health Organization.

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