Phytoestrogens Induce Differential Estrogen Receptor Alpha- or Beta-Mediated Responses in Transfected Breast Cancer Cells

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Increased intake of phytoestrogens may be associated with a lower risk of cancer in the breast and several other sites, although there is controversy surrounding this activity. One of the mechanisms proposed to explain the activity of phytoestrogens is their ability to bind and activate human estrogen receptor α (ERα) and human estrogen receptor β (ERβ). Nine phytoestrogens were tested for their ability to transactivate ERα or ERβ at a range of doses. Mammary adenocarcinoma (MCF-7) cells were co-transfected with either ERα or ERβ, and an estrogen-response element was linked to a luciferase reporter gene. Dose-dependent responses were compared with the endogenous ligand 17β-estradiol. Purified genistein, daidzein, apigenin, and coumestrol showed differential and robust transactivation of ERα- and ERβ-induced transcription, with an up to 100-fold stronger activation of ERβ. Equol, naringenin, and kaempferol were weaker agonists. When activity was evaluated against a background of 0.5 n*M* 17β-estradiol, the addition of genistein, daidzein, and resveratrol superstimulated the system, while kaempferol and quercetin were antagonists at the highest doses. This transfection assay provides an excellent model to evaluate the activation of ERα and ERβ by different phytoestrogens in a breast cancer context and can be used as a screening bioassay tool to evaluate the estrogenic activity of extracts of herbs and foods. Exp Biol Med 230:558–568, 2005

Key words: estrogen receptor β; phytoestrogens; isoflavonoids; MCF-7; bioassay

Introduction

Phytoestrogens are secondary metabolites produced in a wide variety of plants that induce biologic responses in vertebrates and can mimic or modulate the actions of endogenous estrogens, usually by binding to estrogen receptors (ERs; Ref. 1). The broad classes of phytoestrogenic compounds include isoflavonoids, coumestans, lignans, and stilbenes (2, 3). Specific classes of isoflavonoids include the flavones (e.g., apigenin), flavonols (e.g., quercetin, kaempferol), flavanones (e.g., naringenin), isoflavones (e.g., genistein, daidzein, equol), and anthocyanidins. Coumestrol is a coumestan and resveratrol, a stilbene (3–5). All are biphenolic compounds with structures resembling natural and synthetic estrogens. Soybeans and clover, alfalfa sprouts, and oilseeds (e.g., flaxseed) are the most significant dietary sources of isoflavones, coumestans, and lignans, respectively, but phytoestrogens are found in a wide number of foodstuffs such as soy beans, black beans, onions, and citrus fruits, as illustrated in Table 1 (3).

Epidemiologic evidence supports a protective effect of high phytoestrogen diets to reduce the incidence of certain hormone-responsive cancers, such as breast and prostate cancer (6). Relative to Asian countries, the incidence and mortality from breast cancer is much higher in the Western world. The contributing factors to this differential effect are various; however, several studies have focused on the environmental, and specifically dietary, differences among these two parts of the world. Immigration studies have shown that rates of breast cancer in first-generation immigrants are low, but they increase in the second and subsequent generations, presumably due to intake of an increasingly Western diet (7, 8). Much of the epidemiologic studies have focused on isoflavones and soy, but interest is increasing in the protective effects of lignans in breast cancer (9). Average daily dietary intake of soy and isoflavones in Asian countries is estimated to be 50 g daily and 30 mg daily, respectively, while in the West the intake is closer to 1 g daily and 1 mg daily of each (10). The lower breast cancer incidence in Asian countries has

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Table 1. Phytoestrogens Tested and Food Sources

Phytoestrogen common name	Chemical name	Chemical class	Common food sources	Maximal reported range of plasma levels in humans *(M)*
Apigenin	5,7,4′-trihydroxyflavone	Flavone	Celery (69), chamomile, licorice (70), parsley (71)	None reported
Coumestrol	7,12-dihydroxycoumestan	Coumarin	Alfalfa, broccoli (72), red clover, sprouts (73)	None reported
Daidzein	7,4′-dihydroxyisoflavone	Isoflavone	Soy products, black beans (73)	5×10^{-7} (74, 75)
Equol	7,4′-isoflavandiol	Isoflavone	Metabolite of daidzein (76)	8×10^{-7} (75)
Genistein	5,7,4′-trihydroxyisoflavone	Isoflavone	Tofu, black beans (73)	9×10^{-7} (74, 75)
Kaempferol	3,5,7,4′-tetrahydroxyflavone	Flavonol	Kale, endive, leek, turnip tops (69)	7×10^{-8} (77)
Naringenin	5,7,4′-trihydroxyflavanone	Flavanone	Grapefruit and other citrus (78)	1.5×10^{-5} (79)
Resveratrol	trans-3,4′-trihydroxystilbene	Stilbene	Peanuts, grapes, Itadori tea, red wine (80)	2×10^{-6} (81, 82)
Quercetin	3,5,7,3′4′-pentahydroxyflavone	Flavonol	Widespread including apple, onions, kale, broccoli, French beans (69)	7×10^{-7} (77, 83)

also been correlated to early exposure to phytoestrogens through breast milk (11).

The mechanisms for the protective effect of phytoestrogens are unclear. One theory is that the presence of these low-affinity, low-potency ligands for ERs can reduce the effect of potent endogenous estrogens such as estradiol when they are present in sufficient quantities, with the net effect of antagonizing the estrogen-responsive system (12). The abundance of isoflavones relative to endogenous estrogens could explain their bioactivity despite their weaker binding. Setchell and Cassidy (13) showed that consumption of 50 mg/day of isoflavones in an adult can give rise to plasma concentrations ranging from 200-3200 n*M*, whereas endogenous plasma estradiol levels range from 0.14–0.28 pmol/ml (14). In addition, phytoestrogens have a weaker affinity for serum proteins such as albumin and sex hormone–binding globulin, which renders a relatively greater proportion of unbound phytoestrogens (14). A number of the pleiotropic molecular effects of phytoestrogens, including modulation of cell-signaling pathways, regulation of the cell cycle, stimulation of apoptosis, and antioxidant activities, may occur independently of ER binding (15, 16).

However, the health consequences of phytoestrogen exposure is perhaps not universally beneficial and, in certain situations, could increase disease risk, particularly with use of dietary supplements that could provide exceedingly high levels of phytoestrogens relative to those provided in whole foods (12). The potential adverse effects could include procarcinogenic effects in peri- and postmenopausal women taking supplements to ameliorate menopausal symptoms, potential effects of natural antiestrogens on reproduction, and developmental effects on children prenatally (in the diet of the mother) and postnatally (e.g., in soy-based infant formulas; Ref. 17). The known divergent chemical and biologic properties already identified of the phytoestrogens make it important to evaluate each individually and in the context of delivery as food or dietary supplement.

Phytoestrogens have the ability to bind the ER due to their biphenolic structure, which is required for the ligand-receptor association (18). They can act like partial ER agonists or antagonists. Several phytoestrogens show a pattern of differential binding to the two ER subtypes, ERα and ERβ, with stronger binding to ERβ as evaluated by radioligand-binding assay (19). The ER is part of the steroid/retinoid receptor gene superfamily, a class of soluble DNA-binding proteins that acts as ligand-activated enhancer factors (20). On ligand binding, the ER initiates transcriptional activation by binding to specific palindromic sequences called estrogen-response elements (EREs) in the promoters of target genes (21). However, non–ERE-driven transcriptional activation (e.g., *via* AP-1) has also been described (22). The differential effects of agonists and antagonists on receptor activity in a given cell context have been ascribed to different conformations of the receptor-ligand complex, as well as by differences in interaction with transcriptional coactivator and corepressor proteins and other transcription factors (23). Compounds showing varying degrees of agonist-antagonist activity, depending on the cell type and target gene, are referred to as *selective ER modulators* (22, 24). Many phytoestrogenic compounds have additional cellular activities not ascribed to activation of the ERs, such as regulation of cell-signaling pathways, and can have activity in inhibiting proliferation and inducing apoptosis in ER-negative breast cancer cell lines (e.g., MDA-MB-231), as well as in ER-positive lines (e.g., MCF-7; Ref. 25).

The two ER subtypes have significant sequence homology in amino acids in their DNA-binding domains (96%), but lesser in the ligand-binding domain (53%). Based on these homologies it is predicted that ERβ recognizes the same EREs as ERα, but that the receptors bind distinct sets of ligands and, indeed, ERα- and ERβ-

specific ligands have been identified (genistein and coumestrol are considered ERβ-selective agonist ligands; Refs. 20, 26). The "classical" ER, ERα, has been detected in the uterus, mammary glands, liver, central nervous system, cardiovascular system, bones, and urogenital tract in humans (27, 28). Originally cloned from rat prostate, ERβ has been found to have a wide tissue distribution with the richest expression in the central nervous system, cardiovascular system, lungs, kidneys, urogenital tract, mammary glands, colon, immune system, and reproductive organs (27, 29). Estrogen receptor β may negatively regulate cellular proliferation and have a protective role in normal breast (30). Currently, there is great interest in identifying ERβ-specific agonists for therapeutic uses in several diseases, including cancer (31). In addition, as use of pharmacologic hormone-replacement therapy for the treatment of menopausal symptoms is waning, interest in the identification of natural sources of estrogens has increased (24).

We have adapted a transfected breast cancer bioassay system that has sensitivity up to 1 p*M* for 17β-estradiol. In this assay system, either ERα or ERβ is overexpressed by transient transfection. When the receptor is activated by the test ligand, the receptor forms a dimer that binds the estrogen response element, activating transcription of the luciferase reporter gene. In the studies presented we compared the abilities of genistein, daidzein, equol, apigenin, naringenin, kaempferol, quercetin, coumestrol, and resveratrol to activate a luciferase gene reporter system in two sets of transfected MCF-7 breast cancer cells. By altering the expression of ERα and ERβ in a breast cancer environment, this assay may be useful to evaluate the activity of not only individual isolated compounds, but also of undefined extracts of whole foods and dietary supplements in a physiologically relevant model. Because of the endogenous expression of both ERs (weaker expression ERβ), it is expected that necessary ER coactivators and corepressors are present in this cell line, something that may not be found in ER-negative cell lines. Conducting the assay with ligand alone versus against a background level of 17β-estradiol allowed assessment of estrogenic versus antiestrogenic activity of this panel of phytoestrogens. This assay represents a flexible, sensitive, reproducible, and selective screening assay for transactivation capacity of various naturally derived estrogenic ligands in which activity on ERα and ERβ can be compared in a parallel system.

Materials and Methods

Chemicals. Genistein, daidzein, equol, apigenin, naringenin, kaempferol, quercetin, resveratrol, and 17β-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO); coumestrol was purchased from Indofine (Somerville, NJ). Phytoestrogens were dissolved in 100% ethanol (EtOH; Aldrich Chemical Co., Milwaukee, WI) and stored at –80°C. Purity of the stock solutions was verified for most of the phytoestrogens by high-performance liquid chromatography in the UCLA/NIH Clinical Nutrition Research Unit Nutritional Biomarker Core Laboratory. Concentrations were confirmed by absorbance measurements at the appropriate wavelength where the extinction coefficient was known.

Plasmids. The laboratory of Thomas Scanlan at the University of California, San Francisco, graciously provided plasmids containing either ERα (psG5-HE0), ERβ (psG5-hERβ, 485 aa), or an estrogen-response element (ERE; EREII-Luciferase GL450) linked to the luciferase reporter gene. The HE0 plasmid contains a point mutation that elicits a lower background response. Use of these plasmids in cell transient transfection systems, including in HeLa and MCF-7 cells, has been previously described (22). We transformed *Escherichia coli* strain DH5α with these plasmids and used bacteria to amplify the plasmids to obtain microgram quantities. An additional β-galactosidase–expression plasmid (CMV-β; Promega, Madison, WI) was obtained from a commercial source and co-transfected in the assay system as an internal standard. Plasmid DNA was prepared using a commercially available kit (Qiagen Mega-kit; Qiagen, Valencia, CA).

Cell Culture, Transfection, and Luciferase Assays. The MCF-7 cells (ATCC HTB-133; American Type Culture Collection, Rockville, MD) were maintained and subcultured in media containing Dulbecco's modified Eagle's medium/F12 (DMEM/F12), with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Grand Island, NY), and bovine insulin (10 μg/ml; Sigma Chemical). They were kept in nongelatinized 75 cm^2 flasks (Corning Incorporated, Corning, NY) under humidified conditions at 5% CO_2. One day before transfection, the cells were plated in 24-well plates (Corning Incorporated) at a concentration of 8×10^4 cells per well. For transfection, maintenance media was aspirated and 100 μl of transfection mix was added. Transfection mix consisted of 0.5 μg DNA divided over the three plasmids (i.e., ERα or ERβ, ERE-Luc, and CMV-β), cationic liposome-based TransFast transfection reagent (1.5 μl/well; Promega), and phenol red-free DMEM/F12 medium. After an incubation of 1 hr for transfection, experimental medium was added without removal of the transfection media. The experimental medium was prepared with phenol red-free DMEM/F12, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT), 10 μg/ml insulin (Sigma Chemical), and the purified phytoestrogens. Phytoestrogen stocks were prepared and stored at concentrations of 1–6 $\times 10^{-3}$ *M*, depending on the compound. Phytoestrogen stocks were diluted at 10-fold serial dilutions using cell-culture media to produce treatment media containing compounds at 10^{-5} *M* to 10^{-10} *M*. A 17β-estradiol standard was also added in every plate at 10^{-6} *M*, and every experiment included an estradiol standard curve with 10-fold dilutions from 10^{-6} *M* to 10^{-12} *M*. A vehicle control containing 0.3% ethanol, which was the maximal level of ethanol used in the treatment

media, was included for each dose-response set. Each level of compound was assayed in triplicate. Cells were incubated for 48 hrs at 37°C, then media was aspirated and cells were lysed with 100 µl of Reporter Gene Lysis Buffer (Promega). After a freeze-thaw cycle to enhance lysis, lysates were transferred to microcentrifuge tubes and stored at –80°C. For the luciferase assay, 20 µl of lysate was added to 100 µl of Luciferase Assay System (Promega). Luminescence was measured with a single tube luminometer (Type TD 20/20; Turner Designs, Sunnyvale, CA). For the internal standard, a β-galactosidase assay kit (Promega) was used to assay enzyme activity, using a 50:50 ratio of lysate to reagent.

To evaluate the antiestrogenic activity of the various ligands, the previously described protocol was used in competition experiments whereby a background dose of 5×10^{-10} *M* 17β-estradiol was included in the full range of phytoestrogen doses. The chosen level of estradiol represents approximately 80% of the maximal activation of the bioassay system (a subsaturating level).

Data Analysis. The software program Prism 4.0 (GraphPad Software Inc., San Diego, CA) was used for data analyses. Levels of luciferase transcription for each phytoestrogen tested were compared with 17β-estradiol (E_2) at 10^{-6} *M* (positive) and vehicle (0.3% ethanol; negative) controls. The levels of relative luciferase activity were divided by the β-galactosidase values to correct for well-to-well variation. The maximal achievable response of the native ligand (17β-estradiol) was set at 100%, and responses for individual phytoestrogens were calculated as a percentage of the estradiol response. Mean values were calculated from triplicate wells averaged over three experiments. The effective concentration that elicits a half-maximal response (EC_{50}) was estimated for each compound using nonlinear regression analysis with a sigmoidal dose-response model provided by the software package. Inter- and intraassay coefficients of variation (CVs) were calculated based on the error on multiple measurements of the 17β-estradiol positive control included in three wells on every plate. For the competition assays, the response of 1 µ*M* 17β-estradiol was set at 100%, and responses for different levels of added phytoestrogens were calculated as a percentage of the estradiol response. A one-way ANOVA was performed for each compound at each ER, with Dunnett's posttest used to compare each treatment level to untreated control.

Results

The aim of the study was to evaluate differential transcriptional activation of the two ERs, ERα and ERβ, by phytoestrogens commonly found in foods. Cells transiently transfected with either ERα or ERβ and the ERE-reporter plasmid were incubated with either 17β-estradiol or one of a panel of phytoestrogens/metabolites. An overview of the chemical names, chemical class, common natural source of the phytoestrogens tested, and reported plasma values is given in Table 1.

The natural ligand 17β-estradiol induced a reproducible response in our system in a range from 10^{-6} to 10^{-12}*M;* the response with either ERα or ERβ activation was equivalent (Fig. 1). The sensitivity of the assay under the given conditions reaches 1 p*M* for 17β-estradiol, and the 17β-estradiol response reaches a plateau beginning at 1 n*M*. The interassay CV was calculated to be 8%, and the intraassay CV was calculated to be 7% for the ERα system and 6% for the ERβ system. A validation experiment conducted with reporter gene only, devoid of supplemented ER, showed that the maximal response at the highest dose of 17β-estradiol was only 38% that of the response with ERα (data not shown).

Activation of luciferase transcription after treatment with the phytoestrogens genistein, daidzein, equol, apigenin, naringenin, kaempferol, quercetin, resveratrol, or coumestrol at log doses from 10^{-10} *M* to 10^{-5} *M* was compared with the maximal response of luciferase transcription at 1 µ*M* 17β-estradiol. Transcriptional activity at each dose is shown in Figure 2 as a percentage of the maximal response with 17β-estradiol treatment. The baseline response, representing background responsiveness in the absence of ligand, was <20% of the estradiol response in almost all cases. We found that genistein, daidzein, coumestrol, and apigenin each elicited a robust full dose–response in ERE-mediated gene transcription in the range of 10^{-10} *M* to 10^{-5} *M*, especially at ERβ (Fig. 2). However, coumestrol did not fully stimulate the system relative to estradiol (V_{max} was

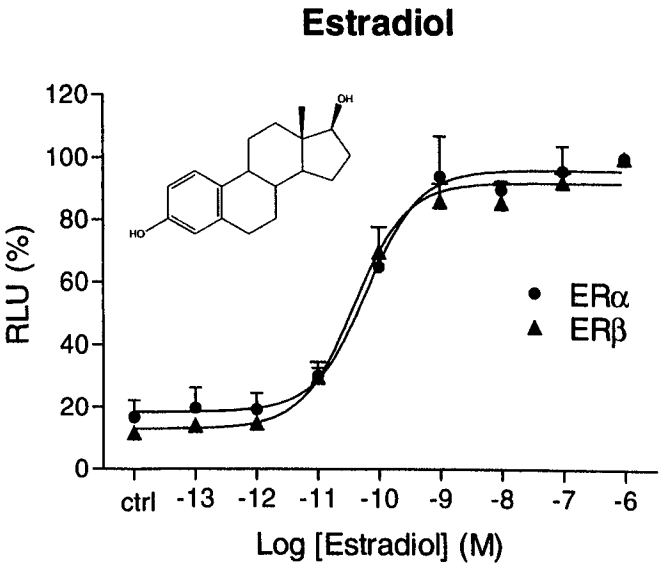

Figure 1. Dose-response curve for 17β-estradiol. The MCF-7 cells were transiently transfected with one of two expression plasmids containing ERα (circle) and ERβ (triangle) plus a luciferase reporter plasmid containing the ERE. Stock 17β-estradiol was diluted 10-fold from 10^{-6} *M* to 10^{-12} *M* and included in the experimental media. Transcriptional activity is represented as relative light units (RLUs) calculated as percentage of the maximal induction by estradiol (10^{-6} *M*) and presented normalized to β-galactosidase activity. Nonlinear regression analysis was performed using a sigmoidal dose-response model. Data presented are the means + SEM of three experiments, with each dose assayed in triplicate wells.

90%), and the major metabolite of daidzein, equol, was only a partial agonist (V_{max} was 57%). Naringenin and quercetin were also weak agonists, as was resveratrol (except for the 10^{-5} *M* dose at ERβ).

The EC_{50} was estimated for each of the phytoestrogens with a full dose-response curve and these values are summarized in Table 2. The EC_{50}s ranged from 10^{-6} to 10^{-7}*M* and from 10^{-7} to 10^{-11}*M* for ERα and ERβ, respectively. For ERα, we were only able to calculate an EC_{50} for the response to coumestrol and genistein; each had an approximately 100-fold greater sensitivity for activating transcription *via* ERβ compared with ERα.

When a background of estradiol was present, individual phytoestrogens displayed differing activities (Figure 3). In the case of kaempferol and quercetin, which had minimal agonist activity, the highest doses were able to compete with estradiol for receptor binding and transcriptional activation (about 42% inhibition relative to 0 *M* phytoestrogen for both kaempferol and quercetin at ERβ). However, the compounds genistein and daidzein, which had robust estrogenic-activat-

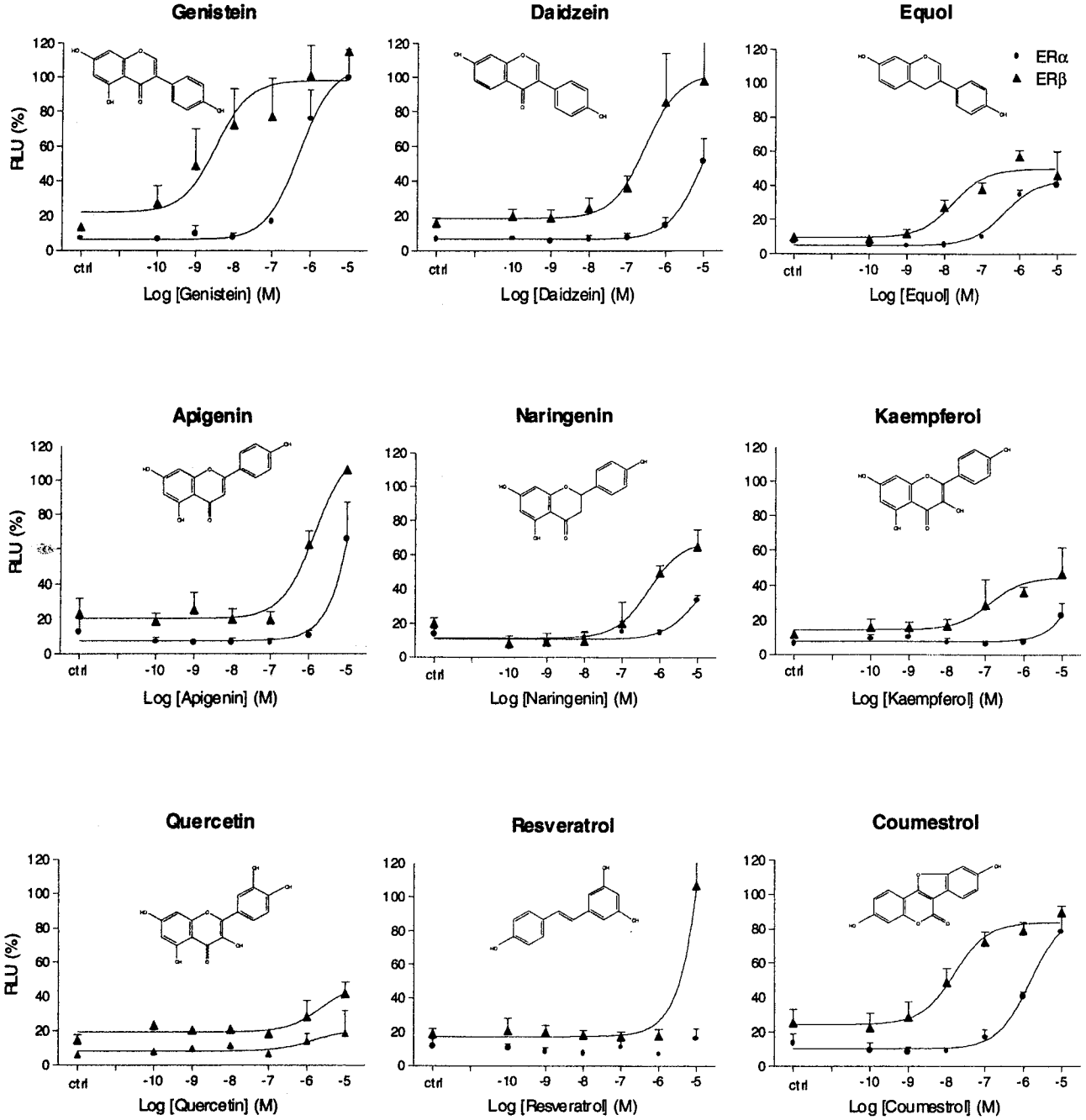

Figure 2. Dose-response curves for isolated phytoestrogens. Cells containing either ERα (circle) or ERβ (triangle) and the ERE reporter plasmid were incubated with either genistein, daidzein, equol, apigenin, naringenin, kaempferol, quercetin, resveratrol, or coumestrol at levels between 10^{-5} *M* and 10^{-10} *M*. Transcriptional activity is represented as RLUs calculated as percentage of the maximal induction by estradiol (10^{-6} *M*) and presented normalized to β-galactosidase activity. Nonlinear regression analysis was performed using a sigmoidal dose-response model. Data points represent the means + SEM of three experiments, with each dose assayed in triplicate wells.

Table 2. The EC_{50} Values for 17β-Estradiol of Various Compounds for ERα and ERβ[a]

Compound	EC_{50} for ERα (*M*)	EC_{50} for ERβ (*M*)
17β-estradiol	6.4×10^{-11}	3.9×10^{-11}
Apigenin	ND	1.4×10^{-6}
Coumestrol	1.6×10^{-6}	1.6×10^{-8}
Daidzein	ND	2.8×10^{-6}
Equol	ND	1.7×10^{-8}
Genistein	4.6×10^{-7}	3.4×10^{-9}
Kaempferol	ND	1.6×10^{-7}
Naringenin	ND	5.1×10^{-7}
Resveratrol	ND	ND
Quercetin	ND	ND

[a] A sigmoidal dose-response curve model was fit to the data shown in Figure 2 to determine EC_{50}. ND indicates EC_{50} not determined due to lack of full dose response to the given ligand.

ing capacity especially at ERβ, at the highest doses showed activity well above the maximal activation capacity of 10^{-6} *M* estradiol alone (up to 218% for genistein and 151% for daidzein at ERβ), indicating that estradiol alone does not maximally stimulate the system. Resveratrol also exhibited additive agonist activity when presented with estradiol. The other phytoestrogens (i.e., apigenin, naringenin, coumestrol) did not compete with estrogen activity, although each had agonist activity when tested in the system alone.

Discussion

In this study, we present a breast cancer bioassay system in which MCF-7 cells are transiently transfected with ERα or ERβ and the estrogenic response was measured through an ERE-response element linked to a luciferase reporter gene. The MCF-7 cell line was chosen because endogenous expression of ERs in MCF-7 cells is predominantly ERα with weak expression of ERβ, suggesting that the full complement of coactivators and corepressors necessary for full ER activity are present in this line (32). The transactivational activity of nine commonly found phytoestrogens was determined in this assay. As others have shown in various transactivation assays in several mammalian cell types, we found that genistein, daidzein, apigenin, and coumestrol selectively promote ERβ-mediated transcription (19, 33–36). We also assessed the agonist activity of equol, a metabolite of daidzein that is produced by the action of intestinal bacterial metabolism in a subset (approximately 33%) of humans (37, 38). In our assay system, equol also exhibited partial agonist activity; however, its maximal activation was only about half that of daidzein at ERβ. The ability to produce equol in the gut has been associated with lower risk of breast cancer in premenopausal women; "equol producers" show plasma steroid hormone profiles associated with a lowered risk of breast cancer (39). Plasma levels up to 8×10^{-7} *M* of equol have been reported in equol producers (14), a value higher than the EC_{50} we found for activity at ERβ (Table 2).

The greater affinity of the phytoestrogens for ERβ is thought to be significant in cancer prevention, as ERβ has been proposed to exert a protective role against cancer. Gustafsson (27) hypothesized that the ratio of ERβ relative to ERα in a given tissue may be important to determine the susceptibility of a tissue to estrogen-induced carcinogenesis. It is also postulated that ERα and ERβ have different or even opposite biologic actions, demonstrating a yin-yang relationship (40). The ERβ may negatively regulate cellular proliferation and have a protective role in normal breast (30); therefore, there is substantial interest in ERβ-selective ligands (27, 40). One molecular mechanism for ERβ selectivity by isoflavones appears to involve their capacity to create an activation function-2 surface of ERβ that has a greater affinity for coregulators than ERα (35). In addition, ERα and ERβ can heterodimerize and, when coexpressed in the same cell, ERβ exerts an inhibitory action on ERα-mediated gene expression (41). We did not complete an ERα and ERβ cotransfection in our system, but it is possible that endogenous ERα or ERβ expression could alter the activity of the exogenously introduced receptor and could account for some differences in results relative to other systems using ER-negative cell types. However, because we are overexpressing the ERs artificially, one might assume that the contribution of the exogenous receptors would supersede that of the endogenous.

The activity of the phytoestrogens is mediated not only through activation of the ER, but also *via* extragenomic actions. For example, other cellular actions of genistein have been described, such as inhibition of tyrosine kinase activity, modulation of transforming growth factor β1 signaling pathways, regulation of cell-cycle checkpoints including G_2M arrest, inhibition of DNA topoisomerase II activity, and antioxidant activity (42–44). The mechanisms of these actions are not well understood, but there is increasing evidence that distinct pools of ERs localize to the plasma membrane and play roles in some of the responses due to estrogenic ligands (41).

An interesting finding of our system is that when genistein and daidzein were coincubated with a background dose of 17β-estradiol, the activity of these phytoestrogens with 17β-estradiol was additive. As other competitive binding assays of these isoflavones have shown that they can compete with 17β-estradiol for binding the receptor, albeit with lower affinity, this result might have been unexpected (45, 46). However this "superagonist" effect has been previously seen in stably transfected T47T breast cancer cells treated with genistein (47) and in MVLN cells (an MCF-7 derivative, which has been stably transfected with a vitellogenin-A2 promoter/luciferase reporter construct) treated with genistein, daidzein, or resveratrol (48). The additive agonist activity shows that the capacity of the assay is not maximized by the highest dose of 17β-estradiol. The mechanisms for this effect is unknown, but because the binding affinities of these compounds to the ERs are thought to be only a fraction of that of 17β-estradiol (especially at ERα; Ref. 19), this activity may potentially be due to extragenomic actions of these ligands, as previously described.

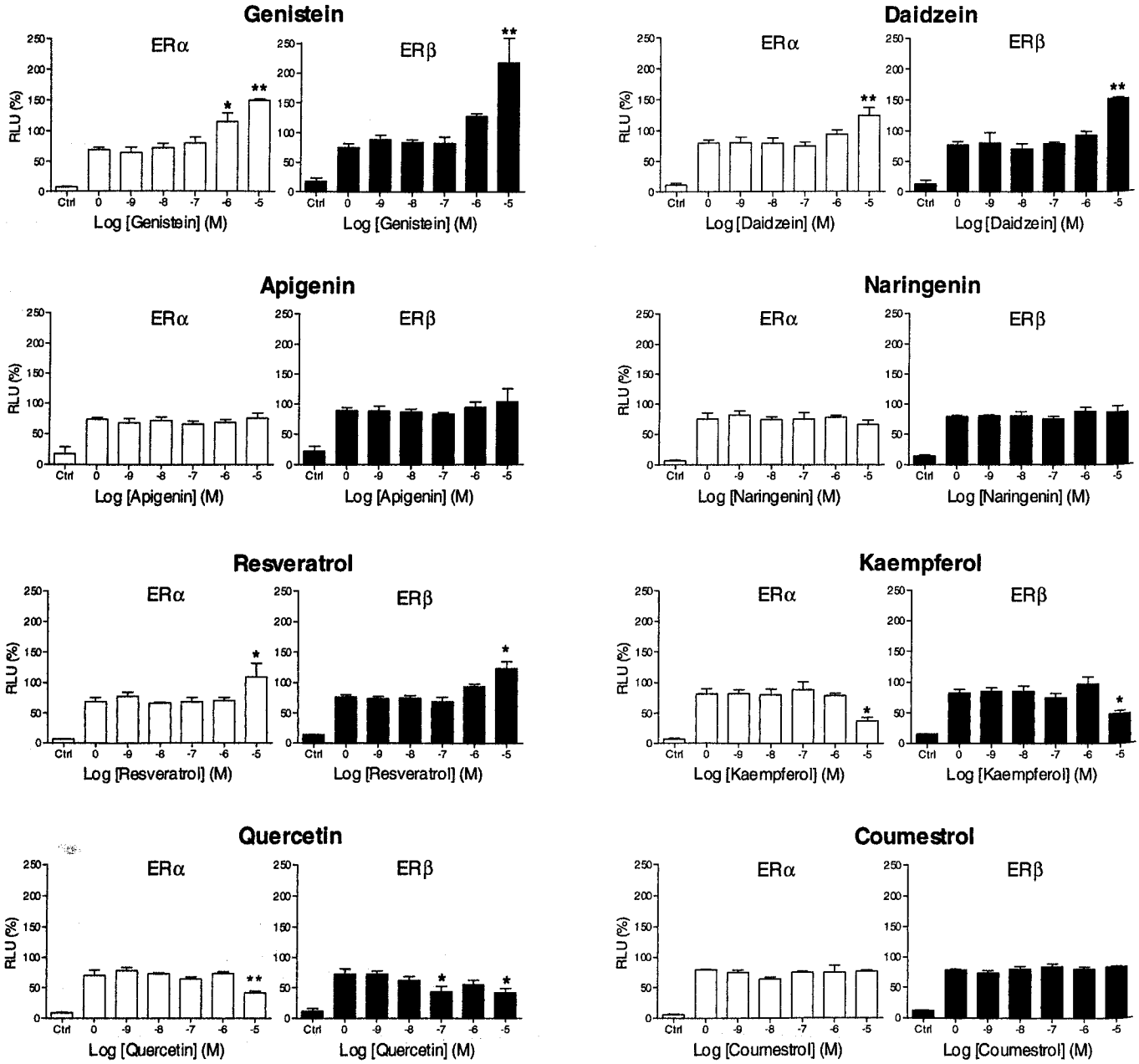

Figure 3. Phytoestrogen response in the presence of estradiol. Cells containing either ERα (light shading) or ERβ (dark shading) and the ERE reporter plasmid were incubated with either genistein, daidzein, apigenin, naringenin, resveratrol, kaempferol, quercetin, or coumestrol, at doses between 10^{-5} *M* and 10^{-9} *M* in media containing 0.5 n*M* estradiol. Transcriptional activity is represented as RLUs calculated as a percentage of the maximal induction by estradiol (10^{-6} *M*) and presented normalized to β-galactosidase activity. The 0 *M* treatment contains estradiol media but no ligand, and the control (ctrl) is a vehicle control (0.3% EtOH). Bars represent the means + SEM of three experiments, with each dose assayed in triplicate wells. One-way ANOVA with Dunnett's posttest was performed for each compound at each ER. Treatment means that are significantly different from control are indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

Resveratrol had no agonist activity except at the very highest dose at ERβ; our data are in agreement with receptor-binding data showing weak affinity of resveratrol for both ERα and ERβ (49). However, resveratrol also exhibited an additive agonist activity in conjunction with 17β-estradiol. Gehm *et al.* (50, 51) studied this effect in MCF cells transiently and stably transfected with a luciferase reporter gene and treated with resveratrol. The additive action was seen at micromolar doses of resveratrol in MCF cells, but not in BG-1 ovarian carcinoma cells. Suboptimal doses of 17β-estradiol and resveratrol were additive, but maximal activation by resveratrol was not further increased by 17β-estradiol, suggesting that both compounds activate the same receptor (50). Conversely, coumestrol, which had strong agonist activity especially at ERβ, had no inhibitory activity when presented with 17β-estradiol.

The other flavonoid compounds naringenin, kaempferol, and quercetin exhibited weak agonist activity at either receptor. The relative magnitude of the maximal agonist response for these three compounds roughly parallels the

relative transactivation activity found in HEK293 cells transiently transfected with ERα and ERβ (19). In addition, naringenin has been previously shown to be only weakly estrogenic in the MVLN assay (52). However, in the competition experiments, kaempferol and quercetin, but not naringenin, inhibited 17β-estradiol activity by some 42% at the highest doses at both receptors, indicating that these two compounds may have estrogen antagonistic activity. Anti-estrogen activity of quercetin has been reported previously; for example, 1 μ*M* quercetin inhibits estrogen-stimulated growth of MCF-7 cells (53).

Our results also support the structure-activity relationships that have been shown to be essential for estrogenic activity. Structural similarity of the phytoestrogens with 17β-estradiol is based on the phenolic ring required for binding to the ER, as well as the presence of two hydroxy groups. The ER-binding ability and transcriptional activation appear to be dependent on the position of the hydroxyl groups, where maximal potency is achieved at positions 4′ hydroxy on the phenolic B ring, in combination with the 7-position hydroxy on the A ring (54). For antagonistic properties, however, the 5′ hydroxy/4′-keto grouping on the A/C ring has been suggested to be of importance (54). The structural difference between genistein and kaempferol (shifting of the phenolic B ring from the 2′ to the 3′ position) results in a 10-fold lower potency in ER binding and in our assay, transcriptional activity. Likewise, oxidation of kaempferol to quercetin results in another 10-fold lower potency in ER binding (54). Our results support a similar relationship.

This system proves to be a sensitive assay to differentiate response to ERα and ERβ by estrogen-like compounds. The sensitivity of the assay under the given conditions reaches the picomolar level for 17β-estradiol. This sensitivity is comparable or greater than reported elsewhere for estradiol response at both ERα and ERβ in several transfected mammalian cell lines (19, 34–36, 55). The reported sensitivity of the MVLN assay is approximately the same for the prototype agonist 17β-estradiol (subnanomolar levels of estradiol), as we found in our assay (56).

Our system is unique in that transcriptional activation by the ERβ can be directly compared with that of the classical receptor ERα. This bioassay system was validated to be specific for the ER in preliminary agonist and antagonist experiments comparing 17β-estradiol (0.1 μ*M*) with diethylstilbestrol (DES; 1 μ*M*), tamoxifen (5 μ*M*), and ICI 182,780 (1 μ*M*). As expected from previous reports, we found robust stimulation of both ERα and ERβ with single doses of 17β-estradiol and DES, with activity at or below control levels with tamoxifen and ICI 182,780 (data not shown). These results were confirmatory of the original report of Paech *et al.* (22) showing activation of both ERα and ERβ in HeLa cells by 17β-estradiol and DES and antagonism by raloxifene, tamoxifen, and ICI 164,384 at the same doses. As previously seen, 17β-estradiol was able to activate both ER isomers with similar potency, as shown in Figure 1. Table 2 shows that the EC_{50}s for activation by 17β-estradiol at both ERs are comparable. The EC_{50}s for 17β-estradiol in similar transfection systems using ERα and ERβ have not generally been reported; therefore, making comparisons to previously published values is difficult. Using a radioligand receptor-binding assay, Kuiper *et al.* (57) reported dissociation constant (K_d) values for 17β-estradiol binding in the nanomolar range, with similar values for both ERα and ERβ. Similarly, Tremblay *et al.* (58) found that Cos-1 cells transfected with murine ERα and ERβ showed similar luciferase activation curves; ligand-binding analysis calculated similar K_ds of 0.2 n*M* for ERα vs. 0.5 n*M* for ERβ. In a system using HepG2 cells transfected with both rat receptors, calculated EC_{50}s were 5-fold different between ERα and ERβ (0.32 n*M* and 1.5 n*M*, respectively; Ref. 36). Our system exhibited greater sensitivity by order of magnitude, as our calculated EC_{50}s were 0.06 n*M* and 0.04 n*M* (Table 2).

The phytoestrogens coumestrol, genistein, daidzein, naringenin, apigenin, and kaempferol were tested in the radioligand-binding system and showed an overall stronger affinity for ERβ (19). The bioassay presented here extends these observations, is unique in offering a direct comparison of the responses of isolated phytoestrogens for ERα and ERβ in a breast cancer context, and incorporates the entire biologic phenomena of activation of transcription. The estrogenic response of compounds has been shown to depend heavily on the cell or tissue type due to the cell-specific presence of corepressors and coactivators (22, 27, 31, 40, 59).

The levels of phytochemicals included in our assay are thought to be achievable *in vivo*, as plasma concentrations of daidzein, genistein, and equol have been determined to be in the μ*M* range after acute soy ingestion (Table 1; Ref. 1). In another study, the plasma concentrations of total phytoestrogens were estimated to be 2–4 μ*M* following the moderate consumption of soy products (60). These levels are dependent on rates of metabolism and clearance, which show considerable individual variation as seen with equol production from daidzein (61). A relevant question is to what levels of phytoestrogens, and in which form, are cells exposed *in vivo*? Most of the phytoestrogens are converted to glucuronide or sulfate conjugates in the small intestinal cell and further metabolized in the liver. Therefore, the main circulating form is the conjugated form (62, 63). However, information in the literature is limited in regards to tissue uptake and metabolism. Most likely the conjugated form is reversed before absorption into the tissue. Based on previous findings outlined in Table 1, which summarizes maximal blood levels of the various phytoestrogens reported in the literature (where available), the concentrations used in our experiments could be physiologically relevant. Note that in many cases these plasma values exceed the EC_{50} for activation of ERβ. Granted, serum

levels do not predict local metabolism at the level of the breast, but we believe most of the conversion occurs at the level of the gut and liver, which gives us some indication that target tissue levels may be somewhat reflected by plasma levels. One study that evaluated breast tissue levels of phytoestrogens in soy-supplemented individuals before aesthetic breast surgery is present in the literature (64). It was found that genistein was undetectable in breast tissue homogenate, but daidzein was present in approximately 100-fold lower levels than serum (64). Equol, however, was present in higher concentrations in breast tissue (and undetectable in serum). This study was run in only nine women, and interindividual variability was great. Also, the time between last supplement ingestion and breast tissue sampling was 12 hrs or more, probably exceeding the half-life of the compounds in blood and making interpretation of the study still complex. In mice, gavage of tritiated naringenin showed that 8 hrs after treatment the compound accumulated in a multitude of tissues, including the uterus and ovary reproductive organs and, to a lesser extent, in the mammary glands (65). These studies suggest that considering their abundance in the diet, phytoestrogens may reach sufficiently high levels in tissues, including breast, to exert biologic effects and that the levels evaluated in our system are physiologically achievable. However, the question of tissue-level bioavailability of phytoestrogens is an issue that merits further study.

This assay is useful not only to evaluate isolated compounds, but also to evaluate extracts of whole foods and herbal supplements. Future studies will identify actions and interactions of sets of compounds as found in natural products. A system that evaluates only ERα activity in transfected yeast identified estrogenic activity in a number of herbs including hops, licorice, soy, red clover, and fo-ti at levels up to one eightieth the activity of estradiol (for soy aglycone; Ref. 66). The system presented here could be useful to detect the estrogenicity of complex mixtures of bioactive compounds as found in dietary supplements, food, serum, and from environmental sources (67, 68). Additional applications could include use in standardizing herbal products before medicinal compounding, which will aid in identifying variability among acquisitions.

In summary, our bioassay system distinguishes differential effects of phytoestrogens on the α and β isoforms of the ER in a breast cancer cell environment. The differential effects of individual phytoestrogens show that the bioactivity of each must be independently evaluated. The direct comparison between responses to the two ER isomers and in the presence or absence of an estradiol background makes this assay an important screening tool for natural estrogen surrogate compounds (to prevent symptoms associated with estrogen deficiency or identify environmental endocrine disruptors), or for compounds that work antagonistically (which may be important for natural selective ER modulator activity in a given tissue). This provides insight into one facet of phytoestrogen action, with the full picture requiring multiple levels of study *in vitro* and *in vivo*.

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1. Setchell KD. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr 68:1333S–1346S, 1998.
2. Cornwell T, Cohick W, Raskin I. Dietary phytoestrogens and health. Phytochemistry 65:995-1016, 2004.
3. Kurzer MS, Xu X. Dietary phytoestrogens. Annu Rev Nutr 17:353–381, 1997.
4. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr 22:19–34, 2002.
5. Yang CS, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu Rev Nutr 21:381–406, 2001.
6. Adlercreutz H. Phyto-oestrogens and cancer. Lancet Oncol 3:364–373, 2002.
7. Ziegler RG, Hoover RN, Pike MC, Hildesheim A, Nomura AM, West DW, Wu-Williams AH, Kolonel LN, Horn-Ross PL, Rosenthal JF, Hyer MB. Migration patterns and breast cancer risk in Asian-American women. J Natl Cancer Inst 85:1819–1827, 1993.
8. Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM. Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. Br J Cancer 63:963–966, 1991.
9. Adlercreutz H. Phytoestrogens and breast cancer. J Steroid Biochem Mol Biol 83:113–118, 2002.
10. Cos P, De Bruyne T, Apers S, Vanden Berghe D, Pieters L, Vlietinck AJ. Phytoestrogens: recent developments. Planta Med 69:589–599, 2003.
11. Lamartiniere CA. Timing of exposure and mammary cancer risk. J Mammary Gland Biol Neoplasia 7:67–76, 2002.
12. Katzenellenbogen JA, Muthyala R. Interactions of exogenous endocrine active substances with nuclear receptors. Pure Appl Chem 75:1797–1817, 2003.
13. Setchell KD, Cassidy A. Dietary isoflavones: biological effects and relevance to human health. J Nutr 129:758S–767S, 1999.
14. Rowland I, Faughnan M, Hoey L, Wahala K, Williamson G, Cassidy A. Bioavailability of phyto-oestrogens. Br J Nutr 89(Suppl 1):S45–S58, 2003.
15. Sarkar FH, Li Y. Mechanisms of cancer chemoprevention by soy isoflavone genistein. Cancer Metastasis Rev 21:265–280, 2002.
16. Magee PJ, Rowland IR. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. Br J Nutr 91:513–531, 2004.
17. Whitten PL, Lewis C, Russell E, Naftolin F. Potential adverse effects of phytoestrogens. J Nutr 125:771S–776S, 1995.
18. Leclercq G, Heuson JC. Physiological and pharmacological effects of estrogens in breast cancer. Biochim Biophys Acta 560:427–455, 1979.
19. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139:4252–4263, 1998.
20. Weihua Z, Andersson S, Cheng G, Simpson ER, Warner M, Gustafsson JA. Update on estrogen signaling. FEBS Lett 546:17–24, 2003.
21. Klein-Hitpass L, Schorpp M, Wagner U, Ryffel GU. An estrogen-responsive element derived from the 5′ flanking region of the *Xenopus* vitellogenin A2 gene functions in transfected human cells. Cell 46:1053–1061, 1986.

22. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 277:1508–1510, 1997.
23. Benassayag C, Perrot-Applanat M, Ferre F. Phytoestrogens as modulators of steroid action in target cells. J Chromatogr B Analyt Technol Biomed Life Sci 777:233–248, 2002.
24. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. receptor interactions. J Med Chem 46:883–908, 2003.
25. Limer JL, Speirs V. Phyto-oestrogens and breast cancer chemoprevention. Breast Cancer Res 6:119–127, 2004.
26. Katzenellenbogen JA, Muthyala R, Katzenellenbogen BS. Nature of the ligand-binding pocket of estrogen receptor α and β: the search for subtype-selective ligands and implications for the prediction of estrogenic activity. Pure Appl Chem 75:2397–2403, 2003.
27. Gustafsson JA. Estrogen receptor β—a new dimension in estrogen mechanism of action. J Endocrinol 163:379–383, 1999.
28. Brandenberger AW, Tee MK, Lee JY, Chao V, Jaffe RB. Tissue distribution of estrogen receptors alpha (ER-α) and beta (ER-β) mRNA in the midgestational human fetus. J Clin Endocrinol Metab 82:3509–3512, 1997.
29. Nilsson S, Gustafsson JA. Estrogen receptor transcription and transactivation: basic aspects of estrogen action. Breast Cancer Res 2:360–366, 2000.
30. Hilakivi-Clarke L, Cabanes A, Olivo S, Kerr L, Bouker KB, Clarke R. Do estrogens always increase breast cancer risk? J Steroid Biochem Mol Biol 80:163–174, 2002.
31. Henke BR, Consler TG, Go N, Hale RL, Hohman DR, Jones SA, Lu AT, Moore LB, Moore JT, Orband-Miller LA, Robinett RG, Shearin J, Spearing PK, Stewart EL, Turnbull PS, Weaver SL, Williams SP, Wisely GB, Lambert MH. A new series of estrogen receptor modulators that display selectivity for estrogen receptor β. J Med Chem 45:5492–5505, 2002.
32. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson JA. Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82:4258–4265, 1997.
33. Collins-Burow BM, Burow ME, Duong BN, McLachlan JA. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. Nutr Cancer 38:229–244, 2000.
34. Breithofer A, Graumann K, Scicchitano MS, Karathanasis SK, Butt TR, Jungbauer A. Regulation of human estrogen receptor by phytoestrogens in yeast and human cells. J Steroid Biochem Mol Biol 67:421–429, 1998.
35. An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC. Estrogen receptor β-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J Biol Chem 276:17808–17814, 2001.
36. Casanova M, You L, Gaido KW, Archibeque-Engle S, Janszen DB, Heck HA. Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors α and β *in vitro*. Toxicol Sci 51:236–244, 1999.
37. Lampe JW, Karr SC, Hutchins AM, Slavin JL. Urinary equol excretion with a soy challenge: influence of habitual diet. Proc Soc Exp Biol Med 217:335–339, 1998.
38. Rowland IR, Wiseman H, Sanders TA, Adlercreutz H, Bowey EA. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr Cancer 36:27–32, 2000.
39. Duncan AM, Merz-Demlow BE, Xu X, Phipps WR, Kurzer MS. Premenopausal equol excretors show plasma hormone profiles associated with lowered risk of breast cancer. Cancer Epidemiol Biomarkers Prev 9:581–586, 2000.
40. Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C. Estrogen receptor (ER)-β reduces ERα-regulated gene transcription, supporting a "Ying Yang" relationship between ERα and ERβ in mice. Mol Endocrinol 17:203–208, 2003.
41. Matthews JB, Gustafsson J. Estrogen signaling: a subtle balance between ERα and ERβ. Mol Interv 3:281–292, 2003.
42. Kim H, Peterson TG, Barnes S. Mechanisms of action of the soy isoflavone genistein: emerging role for its effects via transforming growth factor β signaling pathways. Am J Clin Nutr 68:1418S–1425S, 1998.
43. Piersen CE. Phytoestrogens in botanical dietary supplements: implications for cancer. Integr Cancer Ther 2:120–138, 2003.
44. Cappelletti V, Fioravanti L, Miodini P, Di Fronzo G. Genistein blocks breast cancer cells in the G_2M phase of the cell cycle. J Cell Biochem 79:594–600, 2000.
45. Han DH, Denison MS, Tachibana H, Yamada K. Relationship between estrogen receptor-binding and estrogenic activities of environmental estrogens and suppression by flavonoids. Biosci Biotechnol Biochem 66:1479–1487, 2002.
46. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138:863–870, 1997.
47. Legler J, van den Brink CE, Brouwer A, Murk AJ, van der Saag PT, Vethaak AD, van der Berg B. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicol Sci 48:55–66, 1999.
48. Freyberger A, Schmuck G. Screening for estrogenicity and antiestrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. Toxicol Lett 155:1–13, 2005.
49. Bowers JL, Tyulmenkov VV, Jernigan SC, Klinge CM. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors α and β. Endocrinology 141:3657–3667, 2000.
50. Gehm BD, McAndrews JM, Chien PY, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci U S A 94:14138–14143, 1997.
51. Gehm BD, Levenson AS, Liu H, Lee EJ, Amundsen BM, Cushman M, Jordan VC, Jameson JL. Estrogenic effects of resveratrol in breast cancer cells expressing mutant and wild-type estrogen receptors: role of AF-1 and AF-2. J Steroid Biochem Mol Biol 88:223–234, 2004.
52. Zierau O, Gester S, Schwab P, Metz P, Kolba S, Wulf M, Vollmer G. Estrogenic activity of the phytoestrogens naringenin, 6-(1,1-dimethylallyl)naringenin and 8-prenylnaringenin. Planta Med 68:449–451, 2002.
53. Miodini P, Fioravanti L, Di Fronzo G, Cappelletti V. The two phytoestrogens genistein and quercetin exert different effects on oestrogen receptor function. Br J Cancer 80:1150–1155, 1999.
54. Zava DT, Duwe G. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. Nutr Cancer 27:31–40, 1997.
55. Morito K, Hirose T, Kinjo J, Hirakawa T, Okawa M, Nohara T, Ogawa S, Inoue S, Muramatsu M, Masamune Y. Interaction of phytoestrogens with estrogen receptors α and β. Biol Pharm Bull 24:351–356, 2001.
56. Demirpence E, Duchesne MJ, Badia E, Gagne D, Pons M. MVLN cells: a bioluminescent MCF-7-derived cell line to study the modulation of estrogenic activity. J Steroid Biochem Mol Biol 46:355–364, 1993.
57. Kuiper GG, Shughrue PJ, Merchenthaler I, Gustafsson JA. The estrogen receptor β subtype: a novel mediator of estrogen action in neuroendocrine systems. Front Neuroendocrinol 19:253–286, 1998.
58. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353–365, 1997.
59. Koehler KF, Helguero LA, Haldosen LA, Warner M, Gustafsson JA. Reflections on the discovery and significance of estrogen receptor β. Endocr Rev 26:465–478, 2005.

60. Franke AA, Custer LJ, Tanaka Y. Isoflavones in human breast milk and other biological fluids. Am J Clin Nutr 68:1466S–1473S, 1998.
61. Frankenfeld CL, Atkinson C, Thomas WK, Goode EL, Gonzalez A, Jokela T, Wahala K, Schwartz SM, Li SS, Lampe JW. Familial correlations, segregation analysis, and nongenetic correlates of soy isoflavone-metabolizing phenotypes. Exp Biol Med (Maywood) 229:902–913, 2004.
62. Shelnutt SR, Cimino CO, Wiggins PA, Ronis MJ, Badger TM. Pharmacokinetics of the glucuronide and sulfate conjugates of genistein and daidzein in men and women after consumption of a soy beverage. Am J Clin Nutr 76:588–594, 2002.
63. Zhang Y, Hendrich S, Murphy PA. Glucuronides are the main isoflavone metabolites in women. J Nutr 133:399–404, 2003.
64. Maubach J, Depypere HT, Goeman J, Van der Eycken J, Heyerick A, Bracke ME, Blondeel P, De Keukeleire D. Distribution of soy-derived phytoestrogens in human breast tissue and biological fluids. Obstet Gynecol 103:892–898, 2004.
65. Breinholt VM, Svendsen GW, Dragsted LO, Hossaini A. The citrus-derived flavonoid naringenin exerts uterotrophic effects in female mice at human relevant doses. Basic Clin Pharmacol Toxicol 94:30–36, 2004.
66. Oerter KK, Janfaza M, Wong JA, Chang RJ. Estrogen bioactivity in fo-ti and other herbs used for their estrogen-like effects as determined by a recombinant cell bioassay. J Clin Endocrinol Metab 88:4077–4079, 2003.
67. Harris DM, Besselink E, Potter E, Hirsch AM, Heber D. Estrogenicity of alfalfa and red clover extracts vs. dietary supplements in an estrogen receptor-activated luciferase gene reporter assay (abstract). FASEB J 17:179.2, 2003.
68. Seeram NP, Besselink E, Harris DM, Hardy M, Heber D. Evaluation of cranberry extracts for phytoestrogens by HPLC and LC/MS and their estrogenic properties in an estrogen response element (ERE) assay (abstract). FASEB J 18:114.3, 2004.
69. Hertog MGL, Hollman PCH, Katan MB. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. J Agric Food Chem 40:2379–2383, 1992.
70. Avallone R, Zanoli P, Puia G, Kleinschnitz M, Schreier P, Baraldi M. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla*. Biochem Pharmacol 59:1387–1394, 2000.
71. Justesen U, Knuthsen P, Leth T. Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection. J Chromatogr A 799:101–110, 1998.
72. De Kleijn MJ, van der Schouw YT, Wilson PW, Adlercreutz H, Mazur W, Grobbee DE, Jacques PF. Intake of dietary phytoestrogens is low in postmenopausal women in the United States: the Framingham study. J Nutr 131:1826–1832, 2001.
73. Franke AA, Custer LJ, Cerna CM, Narala KK. Quantitation of phytoestrogens in legumes by HPLC. J Agric Food Chem 42:1905–1913, 1994.
74. Gooderham MJ, Adlercreutz H, Ojala ST, Wahala K, Holub BJ. A soy protein isolate rich in genistein and daidzein and its effects on plasma isoflavone concentrations, platelet aggregation, blood lipids and fatty acid composition of plasma phospholipid in normal men. J Nutr 126:2000–2006, 1996.
75. Wiseman H, Casey K, Bowey EA, Duffy R, Davies M, Rowland IR, Lloyd AS, Murray A, Thompson R, Clarke DB. Influence of 10 wk of soy consumption on plasma concentrations and excretion of isoflavonoids and on gut microflora metabolism in healthy adults. Am J Clin Nutr 80:692–699, 2004.
76. Setchell KD, Zimmer-Nechemias L, Cai J, Heubi JE. Exposure of infants to phyto-oestrogens from soy-based infant formula. Lancet 350:23–27, 1997.
77. de Vries JH, Hollman PC, Meyboom S, Buysman MN, Zock PL, van Staveren WA, Katan MB. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. Am J Clin Nutr 68:60–65, 1998.
78. Ho PC, Saville DJ, Coville PF, Wanwimolruk S. Content of CYP3A4 inhibitors, naringin, naringenin and bergapten in grapefruit and grapefruit juice products. Pharm Acta Helv 74:379–385, 2000.
79. Erlund I, Meririnne E, Alfthan G, Aro A. Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. J Nutr 131:235–241, 2001.
80. Burns J, Yokota T, Ashihara H, Lean ME, Crozier A. Plant foods and herbal sources of resveratrol. J Agric Food Chem 50:3337–3340, 2002.
81. Meng XF, Maliakal P, Lu H, Lee MJ, Yang CS. Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. J Agric Food Chem 52:935–942, 2004.
82. Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 32:1377–1382, 2004.
83. Hollman PC, van Trijp JM, Buysman MN, van der Gaag MS, Mengelers MJ, de Vries JH, Katan MB. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. FEBS Lett 418:152–156, 1997.