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Assessment of the Estrogenicity of Isoflavonoids, Using MCF-7-ERE-Luc Cells

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In the current study, our research focused on the estrogenic activity of isoflavonoids, mainly genistein, biochanin A and daidzein. Genistein enhanced the reporter gene expression of MCF-7-ERE-Luc cells, at a concentration as low as 10 nM, with a concentration of 100 nM the achieved gene expression effects were similar to those of 10 pM 17β-estradiol. Based on the estrogenic activities of biochanin A and daidzein, hydroxyl groups at the 4' and 5 positions are needed for the maximal effect of the genistein. The estrogenic effects of these isoflavonoids were inhibited by the concomitant treatment with tamoxifen. The data showed that the estrogenic effects of isoflavonoids were mediated through estrogen receptors. When the isoflavonoids were tested as mixtures, the estrogenic effects were lower than the arithmetic sum of those induced by each individual isoflavonoid. The estrogenic potency of each isoflavonoid was presented at EC50 levels with a 17β-estradiol equivalent concentration (EEQ) based on the dose response of each chemical. The EC50s and EEQs of genistein, biochanin A and daidzein were 4.15, 0.89 and 0.18 μM, and 15.0, 5.12 and 1.83 μM/M, respectively. Our data clearly demonstrated that the pERE-luciferase reporter gene assay was suited for the sensitive and quantitative measurement, and large scale screening, of the estrogenicity of chemicals *in vitro.*

Key words: Genistein, Biochanin A, Daidzein, EEQ, 17β-Estradiol, ERE

INTRODUCTION

Many studies have focused on the soy isoflavones, particularly daidzein and genistein, and their possible anticarcinogenic properties, and have demonstrated estrogenic, as well as antiestrogenic activities, both *in vitro* and *in vivo* (Wang *et al.*, 1996; Hsieh *et al.*, 1998). These compounds have been shown to lengthen the follicular phase of the menstrual cycle *in vivo* (Cassidy *et al.*, 1994), reduce the urinary excretion of 17β-estradiol and the formation of favor 2-hydroxyestrone (Xu *et al.*, 1998), all of which could be associated with a reduction in the risk of breast cancer. Furthermore, there has been increased interest in selective estrogen receptor modulators (SERMs), which could be future candidate medicines for the prevention of osteoporosis in post-menopausal woman, and at the same time reduce the risk of developing breast cancer and atherosclerosis (Ansbacher *et al.*, 1999; Delmas *et al.*, 1999; Weryha *et al.*, 1999). Raloxifen is one SERM, which is now undergoing clinical trials (Aochner-Celnikier *et al.*, 1999). Brzezinski and Debi presented the idea that phytoestrogen may represent a natural SERM. However, whether soy isoflavonoids, as natural SERMs, decrease, increase or have neutral effects, on breast and other estrogen-dependent cancers, is unclear.

Isoflavonoids are members of a large family of compounds; contain a phenyl side-chain, with a variable number of hydroxyl or other groups. These compounds have some structural similarities to the natural estrogen, 17β-estradiol, as well as other steroid hormones, and steroid hormone antagonists. During the last decade, the numbers of studies on flavonoids, and their possible roles in preventing chronic diseases, including heart diseases and cancers, have dramatically increased. These compounds exist in relatively large amounts in our food, especially in fruits, vegetables and teas. *In vitro,* flavonoids have been reported to act as antioxidants (Noroozi *et al.*, 1998), arrest the cell cycle, inhibit topoisomerase I (Kuzumaki *et al.*, 1998; Constantinou *et al.*, 1998) and extend antiproliferative activities (Fotsis

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et al., 1997; Choi *et al.*, 1998; Fioravanti *et al.*, 1998). Many of the observed effects are thought to prevent breast and/ or prostate cancer development, but this relationship remains unclear. There have been reports that isoflavones bind to estrogen receptors (Shutl and Cox 1972; Pierr *et al.*, 1978), and have weak estrogenic activities (Breinholt and Larsen, 1998). Several bioassay methods for the detection, or quantitative evaluation, of estrogenic activity have previously been described (Meyer *et al.*, 1994; Vanderkuur *et al.*, 1998). The firefly luciferase gene is widely used as a reporter gene for the analyses of the mechanisms of transcriptional regulation, due to its high sensitivity and simplicity of determination. We, therefore, have used the plasmid that contains the ERE upstream of the luciferase gene. A stable transfection of this plasmid, into MCF-7 human breast cancer cells, where estrogen receptors are abundant, was obtained. Using these MCF-7-ERE-Luc cells, we have evaluated the relationship of the structure of isoflavonoids and their estrogenic activities.

MATERIALS AND METHODS

Cell Culture

The MCF-7 human breast cancer cells were cultured in Minimum Essential Medium (MEM, Gibco), supplemented with 5% (v/v) fetal bovine serum, insulin and penicillin-streptomycin, at 37°C and in 5% CO_2. The MCF-7-ERE-Luc cells, stably transfected with *pERE-Luc*, were generously provided by Dr. P.G. Seo (POSTECH). MCF-7-ERE-Luc cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 10% (v/v) fetal bovine serum, insulin, penicillin-streptomycin and 0.5 g/L geneticin (G418, Gibco) at 37°C and in 5% CO_2.

Chemicals

The 17β-estradiol, tamoxifen, genistein and daidzein were purchased from Sigma chemical Co. (St. Louis, MO, U.S.A.). The biochanin A was kindly provided from Dr. Peter lee at the FDA. The Micro BCA protein assay kit was purchased from Pierce (Rockland, IL, U.S.A.). The Tfx50, reporter lysis buffer and luciferin were from Promega (Madison, WI, U.S.A.). The Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, insulin, penicillin-streptomycin and geneticin were purchased from GIBCO-BRL (Grand Island. NY, U.S.A.).

pERE-luciferase reporter assay

The MCF-7-ERE-Luc cells were maintained in DMEM, without phenol red, but supplemented with 10% charcoal treated FBS (assay medium), for four days, and then plated in 96 well plates at a density of $5{\times}10^3$ cells per well. The cells were treated with the chemicals for 17 h, lysed, and the luciferase activity determined using luciferin, with a luminometer. The protein content of the lysate was determined using the Micro BCA protein assay kit (Pierce, Rockland, Illinois, U.S.A.). The luciferase activity was normalized to the protein content. The data was represented as the fold induction relative to the control.

Cell proliferation assay (E-screen assay)

The MCF-7 human breast cancer cells were grown in MEM medium, supplemented with 5% charcoal treated FBS, for four days, and then plated in 96 well plates at a density of 10^4 cells per well. Two days after seeding, the cells were treated with various concentrations of the chemicals for six days. The numbers of cells were measured based on the modified SRB assay of Soto *et al.* The cells were fixed with 50% TCA, and incubated with 0.4% SRB. The optical density was measured at 570 nm.

Data analysis and statistics

To determine the EC50 and CALUX EEQ, a complete standard curve of 17β-estradiol was prepared, using the Table curve v1.0 for windows. The EC50s of the 17β-estradiol and the test chemicals were calculated by determining the concentration at which 50% of the maximum luciferase activity was attained. The detection limit was calculated as the luciferase activity elicited by the control, plus three times the standard deviation. To calculate CALUX EEQ, the luciferase response caused by the test chemicals was interpolated over the linear range of the corresponding 17β-estradiol standard curve. The r^2 of the fit of the standard curve was usually 0.99. For each assay, a 10 pM 17β-estradiol calibration standard was measured to correct for assay to assay variation. Differences between group means were tested using one-way ANOVA. The acceptance level was set at $p<0.05$.

RESULTS

Estrogen effect on the MCF-7 human breast cancer cell proliferation

The MCF-7 cells were maintained in minus phenol red medium for 6 days prior to the 17β-estradiol treatment, were then treated with various concentrations of 17β-estradiol for 6 days, and the cell numbers measured based on the SRB assay. As the concentration of the 17β-estradiol increased, the numbers of cells increased, in a dose dependent manner. 1 nM 17β-estradiol showed the maximal stimulation of the MCF-7 proliferation, and the EC50 was calculated to be 94 pM (Fig. 1). 1 μM tamoxifen resulted in a minimal effect on the MCF-7 human breast cancer cell proliferation, however, when 1 μM tamoxifen treatment was combined with 1 nM 17β-estradiol, the cell proliferation that was stimulated by the 17β-estradiol decreased. This data showed that the estrogen action on the MCF-7 cell

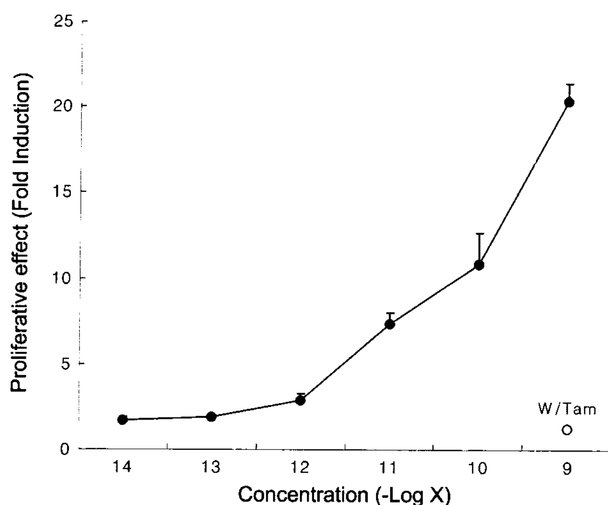

Fig. 1. Cell proliferation effects of 17β-estradiol on the MCF-7 human breast cancer cells. MCF-7 human breast cancer cells were grown in MEM medium, with 5% charcoal treated FBS, for four days, and plated in 96 well plates at 10^4 cells in minus phenol red MEM medium, with 5% charcoal treated FBS. Cells were treated with various concentrations of 17β-estradiol, and 1 nM 17β-estradiol and 1 μM tamoxifen for 6 days. Cell numbers were measured based on the SRB assay, as described in the materials and methods. The data represent the mean ±S.D. (n=4).

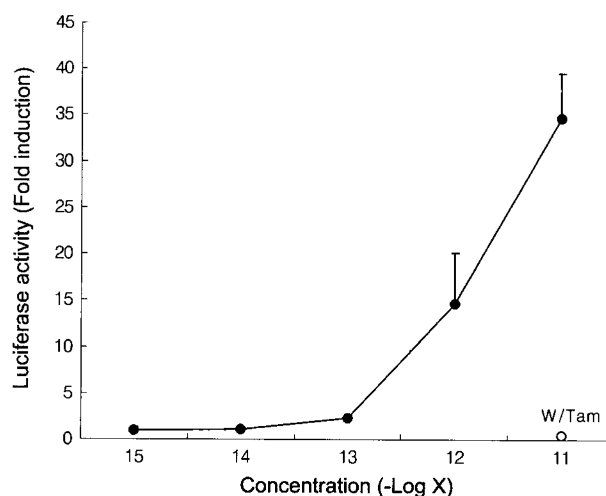

Fig. 2. The effect of 17β-estradiol on pERE-luciferase in MCF-7 cells. MCF-7-ERE-luc cells, stably transfected with *pERE-Luc*, were maintained in minus phenol red DMEM, with 10% charcoal treated FBS, for four days, and plated in 96 well plates at of 5×10^3 cells per well. Cells were treated with various concentrations of 17β-estradiol, and 10 pM 17β-estradiol and 1 μM tamoxifen for 17 h. The luciferase activity was determined as described in the materials and methods, and the data represent the mean±S.D. (n=4).

proliferation was mediated through the estrogen receptor system.

Estrogen effect on pERE-luciferase in MCF-7 cells

MCF-7 cells, stably transfected with *pERE-Luc,* were maintained in minus phenol red medium 4 days prior to treatment with the 17β-estradiol. Various concentrations of 17β-estradiol resulted in an increase in the luciferase activity, in a dose dependent manner (Fig. 2). As shown in Fig. 2, 0.02 pM 17β-estradiol began to increase the luciferase activity, with 10 pM 17β-estradiol giving the maximal luciferase activity. The EC50 value from this experiment was calculated to be 1.42 pM. Concomitant treatment with 1 μM tamoxifen and 10 pM 17β-estradiol inhibited the luciferase activity that was stimulated by the 17β-estradiol treatment alone. This data indicates that the effect of estrogen on the luciferase activity was mediated through an estrogen receptor system. In fact, 1 μM tamoxifen treatment alone further reduced the basal luciferase activity, which suggests that there are trace amounts of estrogenic substances in the charcoal stripped serum.

Effects of isoflavonoids on pERE-Luciferase in MCF-7 cells

We have examined isoflavonoids, such as biochanin A, daidzein and genistein, to see if, in fact, they are estrogenic, using a pERE-luciferase reporter gene system in MCF-7 human breast cancer cells. When various concentrations of genistein were administered to the MCF-7-ERE-Luc cells, the luciferase activities were stimulated in a dose dependent manner (Fig. 3). As shown in Fig. 3, genistein resulted in an 85 fold increase in the luciferase activity over that obtained from untreated control cells. This magnitude of stimulation is much greater than the endogenous estrogen, 17β-estradiol, which resulted in a

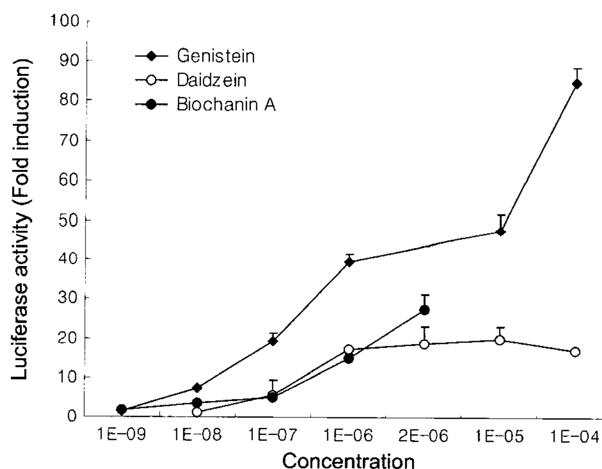

Fig. 3. The effects of isoflavonoids on the pERE-luciferase in MCF-7 cells. MCF-7-ERE-luc cells, stably transfected with *pERE-Luc*, were maintained in minus phenol red DMEM, with 10% charcoal treated FBS for four days, and plated in 96 well plates at of 5×10^3 cells per well. Cells were treated with the indicated concentrations of chemicals for 17 h, and the luciferase activity determined as described in the materials and methods. The data represent the mean±S.D. (n=4).

25-35 fold increase in the maximally activity. Currently, the mechanism for the action of genistein is not understood. The biochanin A also stimulated the luciferase activity, but the magnitude of this stimulation (27 fold) was lower than that of the genistein (85 fold) (Fig. 3). Biochanin A is a derivative of genistein, which is methoxylated at the 4'-OH position. This data suggests the 4'-OH is very important in isoflavonoid for maintaining estrogenic activity. Daidzein, another isoflavonoid, which has no OH group in the 5 position of the A ring, showed a similar luciferase activity stimulation effect to that of the biochanin A (Fig. 3). This data indicated that hydroxyl groups at both the 4' and 5 positions are necessary for the maximal estrogenic activity effect of genistein. As shown in Fig. 3, isoflavonoids, such as genistein, biochanin A and daidzein show estrogenic activity, in a dose dependent manner, in the pERE-luciferase reporter gene system.

Antiestrogen effect on the isoflavonoid action of estrogen

The MCF-7-ERE-Luc cells were treated with 100 μM genistein and 1 μM tamoxifen, and examined for luciferase activity. Tamoxifen inhibited the effect of genistein by 28%, when administered with 100 μM genistein (Fig. 4). When 1 μM tamoxifen was administered, concomitantly, with 10 μM daidzein, the estrogenic effect of the daidzein was abolished. Also, when 1 μM tamoxifen was administered with 2 μM biochanin A the tamoxifen was able to inhibit the biochanin A estrogenic effect (Fig. 4). From these data, it is suggested that isoflavonoids bring about their estrogenic activity through an estrogen receptor system.

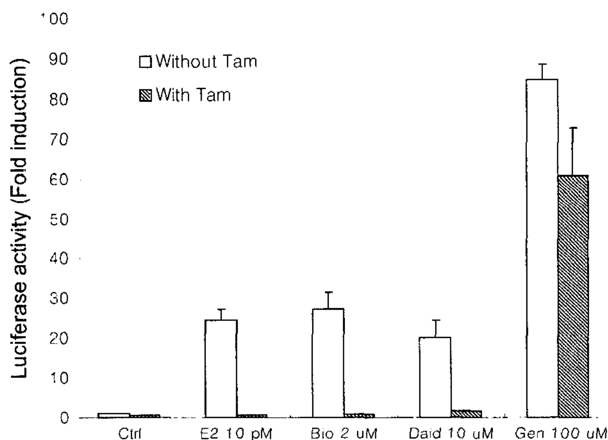

Fig. 4. The effect of tamoxifen on the luciferase activity caused by isoflavonoids in MCF-7-ERE-Luc cells. MCF-7-ERE-luc cells, stably transfected with *pERE-Luc*, were maintained in minus phenol red DMEM, with 10% charcoal treated FBS, for four days, and plated in 96 well plates at of 5×10^3 cells per well. Cells were treated with the indicated concentration of isoflavonoids in the absence, or presence, of 1 μM tamoxifer for 17 h, and luciferase activity determined as described in the materials and methods. The data represent the mean±S.D. (n=4).

Although the superinduction of genistein did not seem to act through an estrogen receptor system, it may work through the tyrosine kinase inhibition, or some other intracellular signaling pathway.

Effects of isoflavonoids mixtures on pERE-Luciferase expression

Isoflavonoids were tested in combination, in order to determine the potential interactivating effects of exposure to these mixtures. As shown in Fig. 5, 5 μM genistein alone resulted in 190% of the activity of 10 pM 17β-estradiol, and 0.2 μM daidzein alone resulted in 50% of the activity of 10 pM 17β-estradiol. A combination of 5 μM genistein and 0.2 μM daidzein, resulted in 195% of the activity of 10 pM 17β-estradiol, instead of the 240% estrogenicity expected from the arithmetic sum of the estrogenicity of 5 μM genistein and 0.2 μM daidzein (Fig. 5A). A combination of 5 μM genistein and 1 μM biochanin A showed 160% of the activity of 10 pM 17β-estradiol, whereas biochanin A alone had 95% of the activity of 10 pM 17β-estradiol, and 5 μM genistein alone had 190% of the activity of 10 pM 17β estradiol. Likewise, the combined estrogenic activity of genistein and biochanin A (160%) appeared to be lower than the arithmetic sum of the individual estrogenic activities (Fig. 5B). 0.2 μM daidzein alone brought about an estrogenic activity 50% that of 10 pM 17β-estradiol, and 1 μM biochanin A alone brought about 95% of the

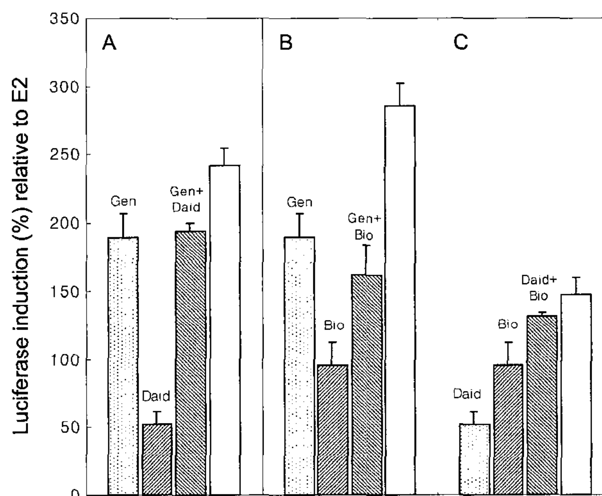

Fig. 5. Interactive effects of isoflavonoids on the pERE-luciferase in MCF-7 cells. MCF-7-ERE-luc cells, stably transfected with *pERE-Luc*, were maintained in minus phenol red DMEM, with 10% charcoal treated FBS, for four days, and plated in 96 well plates at of 5×10^3 cells per well. Cells were treated with isoflavonoids at the EC50 concentration alone, or as a mixture, for 17 h, and the luciferase activity determined as described in the materials and methods. The open bars indicate the arithmetic sum of the individual luciferase activity. All values are shown as percentage relative to the response of 10 pM 17β-estradiol. The data represent the mean±S.D. (n=4).

Table I. Quantification of the estrogenic effects of isoflavonoids, according to the pERE-luciferase reporter and E-SCREEN assays

Compound	EC50 (μM)		ᵃEEQ (μM/M)
	CALUX	E-SCREEN	
Biochanin A	0.890	0.460	5.12
Daidzein	0.179	0.382	1.83
Genistein	4.150	0.069	15.0

[a]EEQ: 17β-Estradiol equivalent concentration (EEQ) is calculated from the concentration of 17β-Estradiol that results in the same luciferase activity of the compound at EC10.

activity of 10 pM 17β-estradiol. When these isoflavonoids were combined, the estrogenic activity became 130% of that of 10 pM 17β-estradiol, which was lower than the arithmetic sum of the two individually (Fig. 5C). These data showed that the luciferase activity stimulated by the isoflavonoids mixture was lower than the arithmetic sum of the luciferase activities of the isoflavonoids when tested individually.

Quantification of estrogenic effect of isoflavonoids

In order to estimate the estrogenic potency of each isoflavonoids, we calculated the EC50 and 17β-estradiol equivalent concentration (EEQ), based on the dose response curve of each chemical. When the EC50s of biochanin A, from the pERE-luciferase reporter and E-screen assays were compared, they turned out to be quite similar (Table I). The EC50s of daidzein from the pERE-luciferase reporter and E-screen assays turned out to be similar. However, the EC50 of genistein from pERE-luciferase reporter assay was 100 fold higher than that of the E-SCREEN assay. At this time, we could not understand why the pERE-luciferase reporter and E-SCREEN assay for genistein should give such different EC50 values. Based on the EEQ, 1 mole of biochanin A turned out to be equivalent to 5.1 μmole of 17β-estradiol, 1 mole of daidzein to 1.8 μmole of 17β-estradiol and 1 mole of genistein to 15 μmole of 17β-estradiol. Thus, the pERE-luciferase reporter assay showed the estrogenicity of genistein to be greater than that of biochanin A. Biochanin A showed a slightly more estrogenicity than daidzein, in terms of the luciferase activity.

DISCUSSION

In this paper, we have demonstrated that the estrogenicity of isoflavonoids can be evaluated by assaying for the ERE driven reporter gene expression using MCF-7-ERE-Luc cells. We also demonstrated that changes in the levels of the reporter gene expression show a quantitative dose-response correlation. There are several short term assays that can be useful to determine estrogenicity, including the cell proliferation assay (E-SCREEN), as described by Soto *et al.* The E-SCREEN assay is based on the dose-response relationship between the proliferation of human estrogen-dependent breast cancer cells, and the concentration of estrogen to which the cells are exposed, over 6 days of incubation (Soto *et al.*, 1995). The sensitivity of the E-SCREEN assay is relatively high, and can discriminate between estrogen agonists and antagonists. However, the proliferation response is an indirect effect, and the assay is complicated by the toxicity of some compounds. Also, it is unclear to what degree pro-estrogen may, or may not, be activated to their estrogenic forms in cultured cells. The direct receptor binding assay can be automated, and thus, scaled to accommodate testing of a large number of compounds. However, the ER binding assay only shows how well the tested compound binds to the ER, but does not define the agonist or antagonist ligand. Furthermore, chemical that chould be metabolize to estrogenic compounds, in mammalian cells, are not detected in the cell free binding assays. In a clinical setting, the radioimmunoassay (RIA) is commonly used to measure the serum estradiol concentration. Because it is antibody-based, the RIA can only measure 17β-estradiol, although a similar assay could probably be developed for other compounds. However, RIA assays have a detection limit of 10-20 pmol/L and can not be used to measure, for example, the concentration of estradiol in the serum of prepubesent children (Andersson and Skakkebæk, 1999). An endogenous gene-based assay can be useful both in cultured cells, and in selected tissues, from exposed animals. However, the endogenous gene expression assay is more time consuming, and therefore, less suited for large-scale screening of chemicals than other more simple *in vitro* assays (Jorgensen *et al.* 2000). Recently, Nishihara *et al.* proposed an assay based on the measurement of yeast two-hybrid protein-protein interactions between ER and coactivators. Because binding of agonist leads to the dissociation of corepressors, and recruitment of coactivators, this assay measures both the ability of a compound to bind to the receptor, and whether it recruits a coactivator; therefore, it yields more information than a simple binding assay. Furthermore, other protein-protein interactions could be included, for example, interactions with corepressors could discriminate between agonist and antagonist, as antagonists generally do not displace corepressors.

The sensitivity of the currently available assays, measure the lowest detectable concentration of estradiol, which is very different. The most sensitive method is the MCF-7 cell proliferation assay, in which concentrations as low as 0.1 pM can be measured (Soto *et al.*, 1995). This assay may actually be able to measure concentrations in the femtomolar range (Masamura *et al.*, 1995). With yeast-based reporter gene assays, which are generally

several orders of magnitude less sensitive (Gaido *et al.*, 1997), measurements of very low estradiol concentrations have been reported (Klein *et al.*, 1994). The fish vitellogenin assay can detect estradiol at approximately 4 pM (Sumpter and Jobling, 1995), whereas the direct binding assay generally requires concentrations in the nanomolar range (Bolger *et al.*, 1998; Andersen *et al.*, 1999). Thus, there is a need for additional, very sensitive, assays that can be used to verify the results obtained by, for example, the MCF-7 cell proliferation assay. In this paper, our data clearly show that the ERE-luciferase reporter assay, using MCF-7-ERE-Luc cells, can be an alternative assay, based on the quantification of the estrogen-induced changes, for detecting the expression level of the reporter gene in MCF-7 cells that have been stably transfected with *pERE-Luc*.

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