Dual Effects of Phytoestrogens Result in U-Shaped Dose–Response Curves

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Endocrine disruptors can affect the endocrine system without directly interacting with receptors, for example, by interfering with the synthesis or metabolism of steroid hormones. The aromatase that converts testosterone to 17β-estradiol is a possible target. In this paper we describe an assay that simultaneously detects aromatase inhibition and estrogenicity. The principle is similar to that of other MCF-7 estrogenicity assays, but with a fixed amount of testosterone added. The endogenous aromatase activity in MCF-7 cells converts some of the testosterone to 17β-estradiol, which is assayed by quantifying differences in the expression level of the estrogen-induced pS2 mRNA. Potential aromatase inhibitors can be identified by a dose-dependent reduction in the pS2 mRNA expression level after exposure to testosterone and the test compound. Using this assay, we have investigated several compounds, including synthetic chemicals and phytoestrogens, for aromatase inhibition. The phytoestrogens, except genistein, were aromatase inhibitors at low concentrations (< 1 μM) but estrogenic at higher concentrations (≥ 1 μM), resulting in U-shaped dose–response curves. None of the tested synthetic chemicals were aromatase inhibitors. The low-dose aromatase inhibition distinguished phytoestrogens from other estrogenic compounds and may partly explain reports about antiestrogenic properties of phytoestrogens. Aromatase inhibition may play an important role in the protective effects of phytoestrogens against breast cancer.
Key words: **aromatase inhibitors, endocrine disruptors, estrogenicity, phytoestrogens, U-shaped dose–response curves.** *Environ Health Perspect* **110:743–748 (2002). [Online 11 June 2002]**
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In recent years, attention has been drawn to chemicals that exhibit estrogenic effects, and screening programs have been initiated to identify chemicals that act as endocrine disruptors. Most attention has focused on industrial xenobiotics, herbicides, and pesticides (*1,2*), but many naturally occurring compounds are also potential endocrine disruptors, including plant-derived phytoestrogens. However, estimating the effects of phytoestrogens is complex because they can be more or less estrogenic, possess an aromatase inhibitory effect, inhibit other enzymes involved in steroidogenesis, inhibit tyrosine kinases, and have other properties as well (*3,4*). Moreover, the bacterial flora in the gut affects the metabolism of phytoestrogens, and the actual content of phytoestrogens in a plant is affected by a variety of factors, including stress (*5*). Combined, these complications make it difficult to estimate exactly the actual exposure to phytoestrogens, as well as the outcome of the exposure.

Although most attention has been on compounds that directly interact with the estrogen receptors, chemicals can also affect the endocrine system by interfering with the synthesis or metabolism of steroid hormones, for example, by inhibiting inactivation of estradiol by estrogen sulfotransferases (*6*). Another obvious target is the enzyme cytochrome P450arom (aromatase), which catalyzes the conversion of testosterone to 17β-estradiol and Δ4-androstenediol to estrone, which can be further processed to 17β-estradiol by P450c17 (17β-hydroxysteroid dehydrogenase) (*7*). That the aromatase plays a crucial role in the synthesis and availability of the female sex hormone 17β-estradiol has been illustrated by the sex reversal observed in developing turtle embryos after treatment with an aromatase inhibitor (*8*). Because sex hormones are involved in regulatory processes throughout a mammalian organism, the aromatase also plays a critical role here, both in the ovary, where most of the circulating estradiol in premenopausal women is produced, and in peripheral tissues that are the sites for estradiol synthesis in men and postmenopausal women. The synthesis in peripheral tissues is important because the endogenous, so-called intracrine (*7,9*), 17β-estradiol production is the main determinant of proliferation of estrogen-dependent breast cancer cells and breast carcinomas (*10–12*) as well as of many natural effects of estrogens.

In this paper we present a novel assay that estimates estrogenicity and aromatase inhibitory effects of a compound simultaneously. The assay is based on human breast cancer MCF-7 cells that routinely are used in estrogenicity assays in which estrogenicity of a compound can be measured by increased cell proliferation or by the induction of estrogen-induced endogenous genes (*13–15*). To assay aromatase inhibitors, we modified the standard estrogenicity assay by including testosterone in the media, which, because of the endogenous aromatase activity in the MCF-7 cells (*16–18*), is converted to 17β-estradiol that subsequently induces an estrogenic response in the cells. The response can be assayed by increased proliferation or by the induction of the estrogen-regulated pS2 mRNA (*17*). Thus, compounds that inhibit the conversion can be identified by a reduced estrogenic response in the presence of testosterone and the inhibitor. At the same time, reasonably potent estrogens will induce a stronger response than will the added testosterone, and thus increase the estrogenicity also in the presence of testosterone.

Materials and Methods

Cell culturing and exposure. The procedures for cultivation and exposure have been described previously (*14,15*). In brief, we grew human breast cancer MCF-7 cells in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum, 1X nonessential amino acids, 2 mM L-glutamine, 25 IU/mL penicillin–streptomycin, and 1 nM insulin. DMEM and all supplements were from Life Technologies (Rockville, MD, USA). We transferred cells to 25-mL cell culture flasks containing steroid-depleted media, and after 6 days, with change of media after 3 days, we replaced the medium with steroid-depleted media containing different concentrations of the test compounds, with and without 100 nM testosterone added. After 24 hr, we harvested the cells and isolated total RNA.

Test compounds. Test compounds were 17β-estradiol (Sigma-Aldrich, St. Louis, MO, USA), testosterone (Sigma-Aldrich), ICI 182.780 (AstraZeneca Pharmaceuticals,

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Westborough, MA, USA), 4-hydroxy-4-androstene-3,17-dione (4-OHA; Sigma-Aldrich), Anastrozole (kindly provided by AstraZeneca, Basel, Switzerland), biochanin A (Apin Chemicals, Abingdon, UK), genistein (Sigma-Aldrich), formononetin (Apin Chemicals), naringenin (Sigma-Aldrich), chrysin (Sigma-Aldrich), red clover flowers [dried powder, suspended at 10% (v/w) in ethanol; Naturdrogeriet, Aarhus, Denmark], nonylphenol (technical grade; Sigma-Aldrich), bisphenol A (Sigma-Aldrich), dibutylphthalate (Sigma-Aldrich), and dieldrin (Sigma-Aldrich). (Chemical structures of the compounds are shown in Figure 1.) We tested each compound in a range of concentrations with and without 100 nM testosterone.

Quantitation of mRNA expression. We analyzed differences in expression levels of endogenous estrogen-regulated genes by competitive polymerase chain reaction (PCR) and displayed them as for differential display reverse transcriptase PCR. We quantified differences in expression levels by phosphor imaging analysis of the polyacrylamide gels. All procedures related to competitive PCR have been described previously (*14,19*) and detailed manuals can be found on the Internet (*20*). We determined the estrogenic response by quantifying the levels of pS2 mRNA (*21*), which served as a marker for estrogenicity (*14,15*).

Statistical analysis. In a pilot experiment using two independent samples for each data point, some phytoestrogens showed a U-shaped dose–response curve. To allow statistical analysis and verification, we repeated selected experiments with four independent samples at each concentration. Because the U-shaped dose–response curves were quite surprising, we designed the new set of experiments to confirm or reject the hypotheses generated from the results of the pilot experiment. The two-stage procedure, involving a pilot study in the first stage and the main experiments in the second stage, implied that the analysis of the results from the main experiments involved testing a prespecified hypothesis, and therefore no multiple testing problems need to be considered. All curves shown with error bars represent experiments with four independent samples at each data point. All other curves represent data from the pilot experiments with only two independent samples at each concentration, except for the reference compound (testosterone), which in all experiments represents four independent measurements. To adjust for lane-to-lane variation, we normalized each analysis as described previously (*14*). This involved first subtracting the background level (the reading in the lane at a position where no band is present) followed by division with the background level [normalized measurement = ("value of band" – background) ÷ background]. Because the normalized measurements varied with the level of the normalized measurements, we used a logarithmic transformation. This both corrected for the heteroskedacity and provided a better approximation to normality of the model residuals from the subsequent analysis. Normalization by simple division of the measurements by the background (i.e., without first subtracting the background) gave similar results.

We performed the statistical analysis of the dose–response relationship for each compound separately, using a classic analysis of variance (ANOVA) for comparing mean response levels between groups, here the means of the normalized measurements for the different concentrations of the compounds. We made the response levels of the different compounds comparable in the analysis of each compound by using testosterone as the reference level. If the estimated dose–response relationship was not distinctly U-shaped, we used a trend test by entering the logarithm of the dose as a covariate in

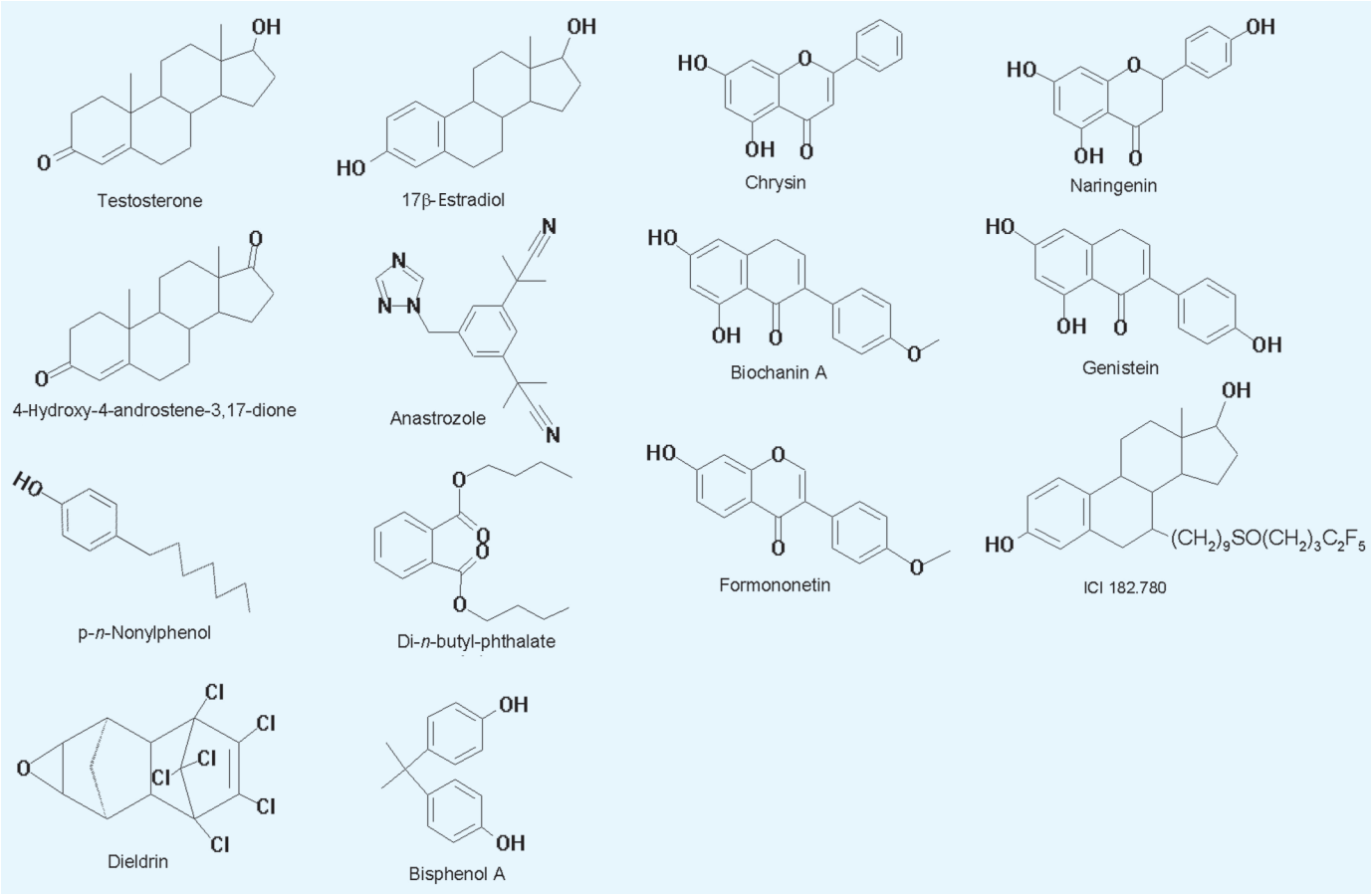

Figure 1. Chemical structures of the compounds used in the study.

the model. *p*-Values are based on *F*-tests in the model. We confirmed all dose–response relationships by nonparametric Kruskal-Wallis tests confirming that the results are robust toward possible deviations from the ANOVA assumption of normality.

Results

Establishment of aromatase inhibitor assay. We tested human breast cancer MCF-7 cells for aromatase activity by assaying the induction of the estrogen-regulated pS2 mRNA after addition of testosterone to the cells. Adding 100 nM testosterone to the culture medium resulted in induction of the pS2 mRNA similar to the induction following addition of 50–100 pM 17β-estradiol to the medium (Figure 2) (*14*). Most likely, this was caused by testosterone being aromatized to 17β-estradiol because the up-regulation could be blocked completely by the potent antiestrogen ICI 182.780 (Figure 2). Next, we tested whether known aromatase inhibitors could abolish the testosterone-mediated pS2 mRNA induction (Figure 3). Both 4-OHA and Anastrozole dose-dependently reduced the testosterone-induced pS2 mRNA expression level to a level similar to that in cells treated with ICI 182.780 (Figures 2–4). Neither 4-OHA nor Anastrozole alone influenced the expression level of the pS2 mRNA, and these compounds did not reduce the induction by 17β-estradiol, showing that they do not possess estrogenic or antiestrogenic activity (not shown). We concluded that MCF-7 cells could be used for identifying potential aromatase inhibitors because such compounds will lead to a down-regulation similar to that resulting from exposure to Anastrozole and 4-OHA.

Testing compounds. We assayed a range of compounds, including synthetic estrogenic chemicals and phytoestrogens (Figure 1, Table 1), in a pilot experiment with two samples per data point. From the observed responses, we selected several compounds for further analysis with four independent samples per data point.

In the presence of testosterone, either the synthetic chemicals had no effect (low-potency compounds), or they slightly increased the expression level of the pS2 mRNA (Figure 3, Table 1). In contrast, and to our surprise, most of the phytoestrogens reduced the level of the pS2 mRNA at concentrations below those where they showed an estrogenic response (Figures 3 and 4). At higher concentrations, the estrogenicity of the phytoestrogens again increased the estrogenicity in the cultures (Figures 3 and 4). Because we measured aromatase inhibition and estrogenicity simultaneously, the resulting curves were the sum of two curves, one similar to that of Anastrozole showing decreasing expression due to aromatase inhibition, and one showing increasing expression due to the estrogenicity of the phytoestrogens. Combined, this resulted in U-shaped dose–response curves (Figures 3 and 4). This U-shaped dose response was most pronounced with biochanin A, with an estimated level of 97.2% (SE, 90.1–105.0%) of the testosterone response at 1 nM, 64.5% (SE, 59.8–69.7%) at 0.1 µM, and 109.5% (SE, 102.0–117.5%) at 10 µM. The statistical significance of the difference between the response level at 1 nM and 0.1 µM concentrations gave a *p*-value of 0.00013. A similar test of the difference between levels at 0.1 µM and 10 µM gave a *p*-value of 0.000003. Genistein did not act as an aromatase inhibitor in this assay (Figure 3), which agrees with previous results, showing that genistein is not an inhibitor of the human aromatase enzyme (*22*). Similar results have previously been reported for formononetin (*3*), which, however, did act as an aromatase inhibitor in this study (Figure 4). We found some compound-specific differences in the results; the most potent aromatase inhibitor was biochanin A, followed by naringenin and chrysin, and we found the smallest effect for formononetin (Figure 4), which agrees with previous studies (*3*). Phytoestrogens are derived from various plants, which often contain many different forms. To analyze whether a plant product (which includes different phytoestrogens) showed the same response, we made a 10% (w/v) ethanol suspension of dried red clover flowers and tested the extract. The resulting dose–response curve was U-shaped, showing that combined aromatase inhibition and estrogenicity also is present in extracts from plants.

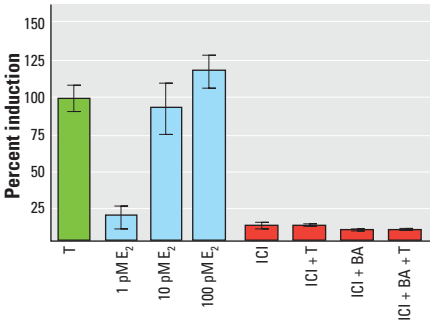

Figure 2. Induction of pS2 mRNA by testosterone (T; 100 nM) and 17β-estradiol (E_2) and reversal of induction by the antiestrogen ICI 182.780 (100 nM). BA, biochanin A (10 µM). All values represent the average of four independent experiments; error bars indicate SD.

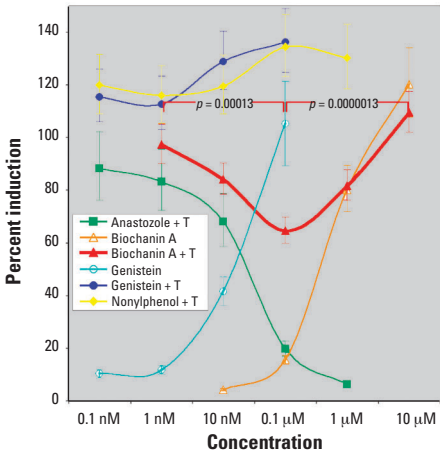

Figure 3. Expression levels of the estrogen-induced pS2 mRNA in MCF-7 cells after incubation with the indicated concentrations of the different compounds in the culture media. All values are shown as percentage relative to the response to 100 nM testosterone (T), which we set to 100%. Each point represents the mean of four independent samples; error bars indicate SEM. The *p*-values indicate the statistical significance of the decline between the 1 nM and 0.1 µM data points of the biochanin A plus testosterone curve and the increase between 0.1 µM and 10 µM data points, thus representing the U shape.

Discussion

We modified the MCF-7 estrogenicity assay to also test for potential aromatase inhibitors. The resulting bioassay is based on features that are inherent to the cells, and as such, it is more "*in vivo*-like" than are standard estrogenicity assays that measure only the estrogenicity of a compound. The modification is important because the intracrine 17β-estradiol production in peripheral tissues, by aromatization of androgens, is the only 17β-estradiol source in men and postmenopausal women. In accordance, the intracrine estradiol synthesis is the main contributor to estrogen-dependent processes in peripheral tissues, including bone growth (*23*) and early artherogenesis (*24*). Moreover, it is the main determinant for the proliferation of breast cancer cells *in vivo* (*10–12*). Because this assay measures aromatase inhibition and estrogenicity simultaneously, it should give a more realistic evaluation of the effect of a given compound in peripheral tissues *in vivo*. Similar *in vitro* MCF-7 cell-based aromatase inhibitor assays have previously been suggested (*25*), and proliferation of MCF-7 cells, transfected into nude mice, is sensitive to aromatase inhibitors (*26*). However, we found no reports on the effects of phytoestrogens, in the presence of androgens, on the proliferation of MCF-7 cells *in vitro* or *in vivo*.

When we tested phytoestrogens in our assay, most of them reduced the estrogenicity at low concentrations, despite their intrinsic estrogenicity. The reduced estrogenicity at low concentrations most probably was caused

by the 10^4–10^5-fold difference in potency between 17β-estradiol and the most potent phytoestrogens (*14,15*). Because of the large difference in potency, a small reduction in the synthesis of 17β-estradiol resulted in a much larger reduction of the expression level of the pS2 mRNA than the potential increase induced by the estrogenicity of the low concentration of phytoestrogens that was needed to inhibit the aromatase. Nevertheless, with regard to the estrogenic response of the cells, most of the phytoestrogens acted as antiestrogens at low concentrations (they reduced the estrogenic response), but they acted as estrogens at high concentrations, resulting in U-shaped dose–response curves. To our knowledge, this is the first time that a simple *in vitro* assay, based solely on functions that are intrinsic to the cells, has shown a U-shaped dose–response curve, and it demonstrates that compounds, in a cellular context, can have opposite effects at different concentrations. Interestingly, we observed U-shaped dose–response curves only for phytoestrogens. None of the tested synthetic chemicals showed aromatase inhibitory effects, suggesting that phytoestrogens can have properties that clearly distinguish them from other estrogenic compounds (Figures 3 and 4, Table 1).

Aromatase inhibition by relatively high concentrations of phytoestrogens has previously been reported (*3,22,27–30*). However, because of the relatively high IC_{50} values (concentration that inhibits 50%) (in the micromolar range), the low-dose inhibition that we report was not evident from the results of other aromatase inhibition assays (*3,22,30*). Nevertheless, both our assays and previous assays detected aromatase inhibitory properties for chrysin, biochanin A, and naringenin, whereas genistein was not an aromatase inhibitor in any of the assays using human aromatases (*3,22*). Formononetin did not act as an aromatase inhibitor in the assay of Le Bail et al. (*3*); however, formononetin was the least potent aromatase inhibitor in our assay, and the divergent results could be caused by a higher sensitivity of our assay (Figure 4). Alternatively, Kao et al. (*22*) have shown that a single amino acid substitution in the active site of the human aromatase can change its sensitivity to compounds and, for example, make it sensitive to genistein. Thus, the aromatase either in our cells or in the cells used by Le Bail et al. (*3*) could have point mutations. Moreover, the sequence in the active site of aromatase enzymes differs among species, and rodent (mouse and rat) aromatases may actually be sensitive to genistein because they have a different amino acid in a position where changes in the human aromatase resulted in sensitivity to genistein (results not shown) (*22*).

Many reports have described estrogenic effects and some also antiestrogenic effects of phytoestrogens *in vivo*, and most of the affected end points are most properly dependent on their estrogenic properties, including increased uterine and mammary glands in animal models (*31–33*). Estrogen-like effects of phytoestrogens have also been observed in humans, including increased bone density, alleviation of postmenopausal symptoms, and effects on the breast (*34,35*). The reports on antiestrogenic effects are less clear, but antiestrogen-like effects have been observed when phytoestrogens were administered together with potent estrogens to immature or ovariectomized female animals, where the phytoestrogens decreased the uterotropic effect of the potent estrogen (*31,32*). This could have been caused by aromatase inhibition because the aromatase is expressed in the uterus (*36*), and inhibition would thus lead to a reduced intracrine estradiol synthesis that subsequently must be "subtracted" from the estrogenic response induced by the administered potent estrogens (*31,32*). Antiestrogenic effects were also observed on the prostate of male mice treated with diethylstilbestrol (DES), where phytoestrogens reduced the dysplastic changes induced by DES (*31*). As described above, this may be the result of aromatase inhibition because the aromatase is expressed in the prostate (*37*), and aromatase inhibitors seem to have similar effects (*38*). The evidence for antiestrogenic properties *in vitro* is scarce. However, a recent report suggested that some phytoestrogens, glyceollins, have antiestrogenic properties because they reduced the estrogenic response when they were added to the culture medium together with 17β-estradiol (*5*). We have not tested any glyceollins in our assay, and their putative aromatase inhibitory properties are not known. However, the design of the assay in Burow et al. (*5*) suggests that glyceollins do posess antiestrogenic properties because the effect most probably cannot be explained by aromatase inhibition.

Another possible example of *in vivo* aromatase inhibition is the protective effects of phytoestrogens against breast cancer that has been demonstrated both experimentally and by epidemiology (*34,39–41*). Breast cancer is often treated with aromatase inhibitors because the intracrine estradiol production in tumor cells is more important for proliferation of estrogen-dependent breast cancer cells

Table 1. Estrogenicity and aromatase inhibitory properties of the tested compounds.

Compound	Estrogenic	Aromatase inhibitor at low concentration
Phytoestrogens		
Biochanin A	Yes	Yes
Genistein[a]	Yes	No
Formononetin[b]	Yes	Yes
Naringenin	Yes	Yes
Chrysin	Yes	Yes
Red clover flowers[c]	Yes	Yes
Synthetic chemicals		
Nonylphenol	Yes	No
Bisphenol A	Yes	No
Dibutylphthalate	Yes	No
Dieldrin	Yes	No
Aromatase inhibitors		
Anastrozole	No	Yes
4-Hydroxy-4-androstene-3,17-dione	No	Yes

[a]Not aromatase inhibitor in humans (*19*). [b]Aromatase inhibition was not detected by Le Bail et al. (*3*). [c]Dried powder suspended at 10% (w/v) in ethanol; red clover flower is a rich source of biochanin A.

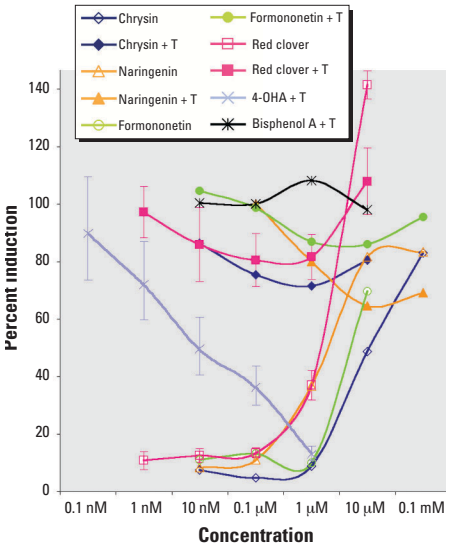

Figure 4. Dose–response curves for selected compounds. Abbreviations: 4OHA, 4-hydroxy-4-androstene-3,17-dione; T, testosterone. The values represent the expression levels of the pS2 mRNA after incubation with the indicated concentrations of the compounds. The values are relative to the response from 100 nM T. Each value without error bars corresponds to the mean of two samples; otherwise, each point represents the mean of four independent samples, and error bars indicate SEM. Dilutions of the 10% w/v red clover suspension were between 10^{-3}- (highest concentration) and 10^{-7}-fold.

than are serum 17β-estradiol levels (*11*). In fact, treatment with aromatase inhibitors was more effective than treatment with antiestrogens, emphasizing the importance of the intracrine estradiol production (*42*). The aromatase inhibition by biochanin A in MCF-7 breast cancer cells was only about one order of magnitude less than that of an aromatase inhibitor used with benefit in breast cancer treatment (Anastrozole) (*42,43*) (Figure 3), and it occurred at concentrations that have been observed for phytoestrogens in humans (*44*). Therefore, we suggest that a major part of the observed beneficial effect of phytoestrogens on breast cancer most likely is due to their aromatase inhibitory properties.

Like pharmaceutical aromatase inhibitors (*45*), phytoestrogens may affect humans and animals differently, depending on the age when the exposure occurs. Several reports describe adverse effects with *in utero* or perinatal exposure, including a higher prevalence of malformed genitalia in boys born from vegetarians, and evidence for cancer-promoting effects in rodents (*46–48*). The apparent differential effects may correlate with the intracrine estradiol production: In children and fetuses with low estradiol production, aromatase inhibition cannot counteract a high exposure by lowering the estradiol production because it is already very low, and they therefore experience adverse effects. In adults, phytoestrogens reduce the intracrine estradiol production, which thereby compensates for the exposure. The same could be the case for tissues with and without intracrine estradiol production. Sensitivity of the aromatase to inhibition by phytoestrogens could even be an evolutionary adaptation to an occasional high exposure to phytoestrogens because such a mechanism would protect against a dangerously high estrogenicity level by reducing the intracrine estradiol synthesis.

Conclusions

Based on our study, we conclude that MCF-7 cells can be used for identifying aromatase inhibitors. Phytoestrogens act as aromatase inhibitors at low concentrations and are estrogenic at higher concentrations, resulting in U-shaped dose–response curves. Aromatase inhibition may partly explain previous reports about antiestrogenicity of phytoestrogens, and low-concentration aromatase inhibition most likely contributes to the cancer-protecting properties of phytoestrogens.

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