Dietary estrogens are believed to exert their estrogenic or antiestrogenic (chemopreventive) action in estrogen responsive cells by interacting with the estrogen receptor (ER). The present study was undertaken to evaluate a direct role of ER in estrogenic or antiestrogenic activities of three dietary estrogens (coumestrol, genistein and zearalenone). HeLa cells were transiently co-transfected with an expression vector for ER and an estrogen-responsive reporter gene construct. Coumestrol, genistein, and zearalenone all increased the activity of the reporter gene, only in the presence of the ER, and the activation was blocked with the ER antagonist ICI 164,384, demonstrating an ER-specific, agonist response. In addition, in MCF-7 cells, coumestrol and zearalenone increased the expression of the estrogen-responsive pS2 gene. Coumestrol and genistein inhibited the purified estrogen-specific 17β-hydroxysteroid oxidoreductase enzyme and the conversion of estrone to 17β-estradiol in T-47D cells, which contain this enzyme. However, they did not inhibit the estrogen-induced proliferation of T-47D cells. In conclusion, coumestrol, genistein, and zearalenone are all potent estrogens in vivo, and they act through ER mediated mechanism. Our findings give no evidence to support the idea that these compounds act as antiestrogens through competition for the binding sites of ER or by inhibition of the conversion of estrone to 17β-estradiol in breast cancer cells, since this effect was nullified by their agonist action on cell proliferation. Therefore, their suggested chemopreventive action in estrogen-related cancers must be mediated through other mechanisms. Key words: antiestrogens, breast cancer, estrogen metabolism, estrogen receptor, gene expression, phytoestrogens. Environ Health Perspect 102:572-578 (1994)

Human diets are known to contain various plant-derived nonsteroidal weakly estrogenic compounds (phytoestrogens). Structurally, they are divided into three classes: isoflavonoids, coumestans, and mammalian lignans. Isoflavonoids (e.g., biochanin A and genistein) and coumestans (coumestrol) are formed in numerous edible plants, especially in soy and other leguminous plants. Mammalian lignans (e.g., enterolactone and enterodiol) are mainly derived from unrefined grain as precursors, which are activated by intestinal bacteria. Trace amounts of estrogenic mycotoxins (e.g., zearalenone) are also found in human diets. Structurally, zearalenone is resorcylic acid lactone. A synthetic derivative of zearalenone, zeronol, has been used as a hormonal substitute growth promoter in cattle. The chemical structures of coumestrol, genistein, and zearalenone are shown in Figure 1.

Phytoestrogens may be excreted in amounts hormonally comparable to the endogenous steroidal estrogens (1). The intake of dietary estrogens is significantly higher in countries where the incidence of breast and prostate cancers is low, suggesting that they may act as chemopreventive agents (1). The chemopreventive effect of dietary soy which is rich in phytoestrogens has recently been demonstrated on the development of estrogen-dependent mammary tumors (dimethylbenzanthracene- and nitrosomethylurea-induced mammary tumors in rats) (2). These compounds may also account for the soy-effect seen as a delayed development of dysplastic changes in the prostate of neonatally estrogenized mice (3).

Dietary estrogens are believed to exert their estrogenic or antiestrogenic (chemopreventive) action in estrogen-responsive cells by interacting with the specific intracellular estrogen receptor (ER). In agreement with this, the compounds (coumestrol, genistein, and zearalenone) which are the most active biologically (e.g., enhance the proliferation of estrogen-responsive tumor cells), also have high binding affinities for ER (4) and have been shown to exert estrogenic activity in vitro (5). To exert antiestrogenic activity, it is postulated that at proper concentrations, the phytoestrogens could compete for the same receptor sites, displace endogenous estrogens from receptor sites and block the action of the hormonally more potent, endogenous estrogens. In addition to acting through the ER, other factors, such as inhibition of tyrosine protein kinase, inhibition of DNA topoisomerase, inhibition of angiogenesis, stimulation of sex hormone binding globulin, and suppression of growth of both ER positive and negative cancer cells, may be operative in mediating the influence of dietary estrogens (6-10).

The present study was undertaken to provide direct support for the role of ER in estrogenic and antiestrogenic action of dietary estrogens (coumestrol, genistein, biochanin A, zearalenone, and zeronol). Their capability to interact with ER and exert a specific, ER-mediated response was studied in HeLa cells, which were transiently co-transfected with an expression vector for ER and an estrogen-responsive chloramphenicol acetyl transferase (CAT) reporter construct. In addition, the judgement of their estrogenicity was based on the proliferative response of two ER-positive breast cancer cell lines, MCF-7 and T-47D cells, and the expression of estrogen-responsive gene, pS2, in MCF-7 cells. Antiestrogenicity was analyzed in MCF-7 and T-47D cells by testing the proliferative response to the phytoestrogens in the presence of 17β-estradiol or estrone and the inhibition of 17β-reduction of estrone by the estrogen-specific 17β-hydroxysteroid oxidoreductase, an enzyme which

Figure 1. Chemical structures of coumestrol, genistein, and zearalenone.

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has been reported to be an indicator and possible regulator of estrogen action in breast tissue (13).

Materials and Methods
All reference steroids were purchased from Steraloids, Inc. (Wilton, New Hampshire). Stock cultures of MCF-7 cells were grown in Dulbecco’s modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, Maryland) supplemented with 5% fetal bovine serum (FBS; Gibco BRL), insulin (from bovine pancreas, 10 μg/ml; Sigma, St. Louis, Missouri) and 17β-estradiol (1 nM; Sigma). For T-47D cells DMEM was supplemented with 10% FBS and insulin (7.5 μg/ml).

Cell Proliferation Experiments
For MCF-7 cell proliferation experiments, phenol red-free DMEM (Sigma) supplemented with 5% dextran charcoal stripped FBS, insulin (10 μg/ml) and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) was used. The stock cultures were harvested by trypsinization, resuspended with medium, and seeded on 96-well culture plates at 2000 cells/well. The test compounds 17β-estradiol, estrone (Sigma), coumestrol (Eastman Kodak, Rochester, New York), genistein (Gibco BRL), biochanin A (Sigma), zearetalone (Sigma) and zeronol (International Minerals and Chemical Corporation, Terre Haute, Indiana) were dissolved in ethanol, diluted with medium and added to culture medium with final concentration of ethanol of 0.1%. The test compounds were added at day 2 and the cell proliferation assay was performed at day 9. For antiestrogen experiments, the test compounds were added 1 hr before estradiol. The media which included the test compounds changed every other day.

Cell proliferation was quantified by colorimetric MTT assay. The use and validity of the MTT assay in MCF-7 cells is described by Martikainen et al. (14). We added 30 μl of MTT (thiazolyl blue; Sigma) solution (5 mg/ml in phosphate buffered saline) to culture wells and incubated them for 4 hr. The media was removed and insoluble formazan (converted by viable cells from MTT) was solubilized by adding 100 μl of dimethyl sulfoxide per well. The absorbance of converted dye was measured at a wavelength of 540 nm by a plate reader (Labsystems Multiskan, Elfab OY, Helsinki, Finland). Each culture plate had its own control (no hormone), and the values (mean of one row of eight wells) are expressed as percentages of the mean of control (16 wells).

For T-47D cell proliferation experiments, we used phenol red-free DMEM with 10% dextran charcoal stripped FBS, insulin (7.5 μg/ml) and antibiotics as above. The stock cultures were harvested by trypsinization, resuspended with medium, and seeded on culture dishes (3 cm diameter). Cells were allowed to attach overnight, the media was changed, and the test compounds were added as above. The medium plus test compounds was changed every other day. At day 7, cells were counted in a Coulter counter.

Measurement of 17β-Hydroxy-steroid Oxidoreductase Activity
The capability of dietary estrogens to inhibit the estrogen-specific 17β-hydroxy-steroid oxidoreductase type I (E.C. 1.1.1.62) was tested by using the enzyme purified from human placenta (12). An aliquot of the purified enzyme in 15 mM phosphate buffer, pH 7.4 with 2 mM DTT, 1 mM EDTA, 1 mM PMSF, and 10% glycerol was preincubated with the NADPH-generating system (50 μl 4 mM NADP, 50 μl glucose-6-phosphate, 50 μl glucose-6-phosphate dehydrogenase, 50 U/ml) in a final volume of 250 μl with dietary estrogen (final concentration 1.2 μM) for 20 min. Thereafter, [3H]-estrone was added to the incubation medium to give the final concentration of 0.72 μM. Incubation was continued for 2 hr and stopped by adding cold carriers (17β-estradiol and estrone).

The steroids were extracted twice with 3 ml of methylene chloride. The combined methylene chloride solutions were dried over sodium sulfate and evaporated to dryness under nitrogen. Extracted steroids were dissolved in absolute ethanol, and ethanol solutions were filtered and evaporated. The residue was dissolved in 50 μl of acetonitrile before HPLC run. The steroids were separated and determined with HPLC. The column system consisted of a guard column followed by a C 18 150 x 3.9 mm ID analytical column (Techopak C18 HPLC Technology, Wellington House, Cheshire, UK). The mobile phase was acetonitrile/water (35/65). Retention times for estrone, 17β-estradiol, 16α-17β-estradiol, 2-OH-estradiol, 2-OH-estrone, 4-OH-17β-estradiol, 4-OH-estrone, androstenedione, and testosterone were 12.2, 9.8, 2.4, 4.9, 6.3, 5.4, 7.3, 13.0, and 10.8 min, respectively. For inline detection of radioactive metabolites, the eluant of the HPLC column was continuously mixed with liquid scintillant and then monitored with inline radioactivity detector (Beta-Flow, Pharmacia/LKB/Wallac, Turku, Finland). Separation and quantification of [3H]-labelled steroids have been described in detail earlier (16). Relative velocities were calculated for each compound.

The type of enzyme inhibition was determined with a graphical method according to Dixon (17). The reaction velocities were determined with a series inhibitor concentrations (1.2 nM to 0.12 μM) and with two substrate concentrations (0.24 and 0.72 μM). Straight lines were obtained on plotting 1/v versus i. The point of intersection of the two lines gave -K<sub>i</sub>.

The 17β-oxidoreductase activity of the MCF-7 cells was assessed by determining the ability of intact monolayers to convert added [3H]-estrone to [3H]-estradiol (13,18). For each experiment, MCF-7 cells were harvested by trypsinization and seeded at a concentration of 2 x 10<sup>5</sup> cells per culture dish (60 x 15 mm). The cells were grown for 5 days in 5 ml of the DMEM with phenol red, supplemented with 5% FBS, 1 nM 17β-estradiol, and insulin (10 μg/ml). The medium was changed every other day. On day 5 of culture, the growth medium was removed and the test compound was added in 3 ml of phenol red-free DMEM without serum or hormones. Control cultures were run in parallel without the test compound. After the preincubation with the test compound for 1 hr, [3H]-estrone (2 nM) was added to each dish, which were then incubated for 4 to 24 hr. The number of cells in each dish was determined by counting released nuclei with a Coulter counter.

In addition to interconversion of estrone and estradiol, no other metabolites (17β-estradiol, 16α-17β-estradiol, 2-hydroxy- or 4-hydroxyl derivatives of estrone or estradiol) were formed under conditions used for the assay of estradiol 17β-oxidoreductase activity.

Transfection of HeLa Cells with Estrogen Receptor and Expression of pS2
The estrogen-responsive reporter vector, ERET81CAT, and the pRSV vector containing the mouse ER cDNA (without the neomycin resistance cassette) was constructed as previously described (19). The HeLa cells were cotransfected with these two vectors, p+ER, or with only the ERET81CAT reporter vector, p-ER, to test the ER-mediated estrogenic activity of the phytoestrogens. The HeLa cells were grown in phenol red-free DMEM/F12 medium (Sigma) supplemented with 5% FBS and penicillin-streptomycin. Cells were harvested by trypsination and mixed with the ERET81CAT DNA, 70 μg/ml, and the pRSV-ER DNA, 10 μg/ml or ERET81CAT only, 80 μg/ml. The cells were electroporated at 960 μF, 0.4 kV in 0.8 ml of cells. Cells were then pooled and plated on fibronectin treated plates in DMEM/F12 plus insulin-transferrin-selenium (Sigma). Four hours after transfection, the serum-free medium was replaced and 1 nM 17β-estradiol (E<sub>2</sub>) or varying concentrations of the dietary estrogen.
gens were added as indicated. For incubation with the estrogen antagonist ICI 164,384 (ICI), the cells were pretreated for 1 hr with 100 nM ICI before co-incubating with the other hormones (20). Triplicate samples for each hormone concentration were harvested at 28 hr after transfection; the cells were harvested in lysis buffer and then assayed for CAT (chloramphenicol acetyltransferase) protein with the CAT-ELISA kit (Boehringer Mannheim, Indianapolis, Indiana) according to the manufacturer's instructions. CAT levels were standardized to total cellular protein levels, measured by the BCA protein assay reagent (Pierce, Rockford, Illinois). Fold stimulation was calculated from the induction over basal CAT levels (vehicle only) within each transfection.

To determine expression of pS2 in MCF-7 cells, the stock cultures were harvested by trypsinization, resuspended with medium and seeded on 6-cm diameter culture dishes at 200,000 cells/dish. After 4 days in culture, the media was changed to serum-free and phenol red-free DMEM with varying concentrations of 17β-estradiol (E2), coumestrol, genistein, or zearalenone. After 24 hr, the cells were harvested with guanidinium thiocyanate, and the RNA was extracted with single-step method (21). The expression of pS2 gene was studied by Northern blot technique as described by Dubik and Shiu (22).

Data were analyzed using ANOVA and Tukey's test on a BMDP 7D computer program.

Results

Effects of Dietary Estrogens on Cell Proliferation

Proliferation of MCF7 cells was analyzed with the various dietary estrogens. The data in Figure 2 show that coumestrol, genistein, biochanin A, zearalenone, and zeranol all significantly enhanced the proliferation of MCF-7 cells. The response was dose dependent in each case, and no inhibition was seen at the concentrations of 1 μM or less. Coumestrol, zearalenone, and zeranol were potent at 10 pM while higher concentrations (more than 1 nM) were needed to show a significant estrogenic activity by genistein or biochanin A. For comparison with the dietary estrogen responses, the dose dependency of MCF-7 cell proliferation on 17β-estradiol and estrone is shown in Figure 3. The stimulation became significant at 10 pM concentration and reached its maximum at 1 nM concentration of 17β-estradiol. Considerably higher concentrations of estrone were needed to get a significant stimulation.

MCF-7 cell proliferation was analyzed for these same compounds in the presence of 10 nM 17β-estradiol, a concentration which results in submaximal stimulation, to test these compounds for antiestrogenic activity. None of the tested dietary estrogens, at concentrations below 10 nM, reduced the rate of cell proliferation. On the contrary, coumestrol, biochanin A, zearalenone, and zeranol all had additive effects with 17β-estradiol (Fig. 4). A similar additive effect could not be demonstrated with genistein at any tested concentration. In contrast, genistein slightly reduced the rate of the cell proliferation at the 1 and 10 pM concentrations. However, this effect was not statistically significant. MCF-7 proliferation was also tested with these compounds in the presence of 1 nM estradiol, which is the lowest concentration...
to give maximal stimulation of proliferation. As with the 10 pM estradiol concentration, no inhibition of cell growth by the phytoestrogens was observed (data not shown).

**Expression of pS2 Gene**

The expression of pS2, which is an estrogen-regulated gene and hormonally responsive in breast cancer cells, was analyzed for stimulation by the compounds. Estradiol was stimulatory at 1 and 100 nM (Fig. 5). Coumestrol and zearalenone also demonstrated an increase in pS2 expression at the concentration of 100 nM, while zearalenone was active at 1 nM (Fig. 5). Genistein had no effect at concentrations of 1 and 100 nM.

**Transactivation of Estrogen-responsive Gene**

At 100 nM, coumestrol, genistein, and zearalenone were able to increase the levels of CAT protein over basal CAT levels in HeLa cells co-transfected with the mouse ER and an estrogen-responsive CAT reporter gene (Fig. 6). In addition, 1 nM zeranol produced the maximal CAT induction of 103.4 ± 10.4-fold stimulation, whereas 2 μM biochanin A was required to achieve the 7.4 ± 0.4-fold increase over untreated CAT levels. In comparison with maximal 17β-estradiol-induction (1 nM), coumestrol (100 nM) produced an equivalent fold stimulation, however, both zeranol (1 nM) and zearalenone (100 nM) exceeded 17β-estradiol-induced activation. To ensure that the estrogenic activity of these compounds was directly mediated by the ER, the transfected cells were co-cultured with the estrogen antagonist ICI 164,384 (100 nM) and the dietary estrogens. No induction of CAT was observed with 100 nM coumestrol, 100 nM genistein, and 100 nM zearalenone (Fig. 6) as well as with 1 nM zeranol (0.94 ± 0.47). The activity of these phytoestrogens was also tested in HeLa cells transfected with the estrogen-responsive CAT vector, but not with the ER. No stimulation was detected with estradiol, coumestrol, genistein, and zearalenone (Fig. 6) or zeranol and biochanin A (data not shown). Therefore, the ER is required for transcriptional activation of CAT by all of these dietary estrogens.
Table 1. Effect of dietary estrogens and triphenylethylene antiestrogens (tamoxifen, toremifene, and 4-OH-toremifene) on the NADPH-dependent conversion of \(^{3}H\)-estrone (0.72 μM) to \(^{3}H\)-estradiol in vitro

| Inhibitor       | Concentration (μM) | N  | Relative velocity |
|-----------------|-------------------|----|------------------|
| Coumestrol      | 1.2               | 6  | 18.8 ± 7.3       |
|                 | 0.12              | 11 | 49.0 ± 15.4      |
|                 | 12                | 8  | 75.6 ± 27.3      |
|                 | 1.2               | 6  | 106.3 ± 39.6     |
| Genistein       | 1.2               | 10 | 62.8 ± 29.9      |
|                 | 0.12              | 5  | 90.6 ± 38.2      |
| Biochanin A     | 1.2               | 8  | 91.3 ± 28.2      |
| Zearalenone     | 1.2               | 12 | 84.6 ± 31.5      |
| Tamoxifen       | 1.2               | 4  | 66.8 ± 8.1       |
| Toremifene      | 1.2               | 7  | 92.1 ± 24.9      |
| 4-OH-Toremifene | 1.2               | 7  | 91.3 ± 31.6      |

*Values are the means ± SD. Relative velocity for control = 100.

Inhibition of 17β-Estradiol Oxidoreductase Activity

Even though only agonist activity was observed in the transfection, expression, and cell proliferation studies, we considered that dietary estrogens might act as antiestrogens through an alternative mechanism, other than through the ER, by inhibiting the 17β-estradiol oxidoreductase enzyme. 17β-estradiol oxidoreductase is the enzyme responsible for the 17β-reduction of estrone to estradiol and has been reported to be an indicator and possible regulator of estrogen action in breast tissue (13). The capability of dietary estrogens to inhibit the estrogen-specific 17β-hydroxysteroid oxidoreductase type I (E.C. 1.1.1.62) was tested by using the enzyme purified from human placenta. At a concentration of 12 nM, coumestrol significantly inhibited the enzyme, while considerably higher concentrations were needed to show inhibition with genistein (Table 1). Zearalenone was inactive at the concentration of 1.2 μM. The kinetic studies of the inhibition showed that the inhibition of the enzyme by coumestrol was competitive with K₀ 9 nM.

The effects of dietary estrogens on the oxidoreductase activity of MCF-7 cells which do not contain the placental form of the 17β-estradiol oxidoreductase (23), was assessed by determining the conversion of \(^{3}H\)-estrone (2 nM) to \(^{3}H\)-estradiol during the 37 °C incubation for 4 to 24 hr. The reduction of \(^{3}H\)-estrone was linear up to 8 hr after the addition of the substrate (Fig. 7). Thereafter, the rate of reduction resulting in the formation of \(^{3}H\)-estradiol decreased and the ratio of estradiol to estrone changed little. Neither coumestrol nor genistein had a significant effect on the conversion of \(^{3}H\)-estrone and \(^{3}H\)-estradiol in MCF-7 cell cultures at the concentration of 1.2 μM (Fig. 7). Zearalenone inhibited the reduction of \(^{3}H\)-estrone at 4 to 8 hr after the start of \(^{3}H\)-estrone addition (Fig. 7). At 24 hr, the \(^{3}H\)-estradiol accumulation had reached the control level, i.e., the level found in cells cultured without phytoestrogens. In contrast to the findings in MCF-7 cells, coumestrol and genistein both inhibited the reduction of estrone to 17β-estradiol in T-47D breast cancer cells which contain a similar form of the enzyme as found in the human placenta (Fig. 8). Zearalenone had no effect on the conversion at any tested concentration.

To determine if inhibition of estrone conversion to 17β-estradiol by coumestrol and genistein in T-47D cells could effect an estrogen-induced physiological response, cell proliferation of the ER-positive breast cancer cells was analyzed. The effects of the phytoestrogens coumestrol and genistein, alone or cotreatments with the estrogen agonist estrone, on the proliferation of T-47D cells are shown in Figure 9. Both coumestrol and genistein stimulated the proliferation of the T-47D cells at 1 μM and 1 nM concentrations, showing agonist activity. Similarly with the MCF-7 proliferation data, antiestrogenicity was not shown, since coumestrol and genistein had an additive effect with estrone. With 1 pM estrone, which is the lowest concentration giving significant stimulation of proliferation, neither compound inhibited the stimulatory effect of estrone.

Discussion

Coumestrol, genistein, zearalenone, zeranol, and biochanin A acted as estrogen agonists in transiently transfected HeLa cells, with zearalenone and zeranol showing potent estrogenic activities. This transcriptional activation of CAT was blocked by the pure estrogen antagonist ICI 164,384 and CAT induction did not occur in HeLa cells not transfected with the ER. Therefore, these dietary estrogens function as transcriptional activators through ER-mediated mechanisms. They have also been shown to exhibit agonist activity in mouse L cells transfected with a similar CAT reporter gene construct (5), although ER dependence was not demonstrated in this study.

In accordance with the transfection results in HeLa cells, coumestrol, genistein, biochanin A, zearalenone, and zeranol all acted as agonist and increased the proliferation rates of MCF-7 breast cancer cells in the absence of endogenous estrogens. Coumestrol, zearalenone, and zeranol were also shown to increase the expression of the estrogen-responsive gene, pS2. Coumestrol, zearalenone and zeranol were active at 10 pM (in terms of cell proliferation rate) while higher concentrations (100 nM) were needed to show the activity of genistein and biochanin A. These concentra-
the most significant method to rank these compounds, since high concentrations of the dietary estrogens, such as concentrations above the maximal 1.2 μM concentration used in this study, may exert biological effects through mechanisms other than directly with the ER. For example, at high concentrations, genistein is a potent growth inhibitor of both ER positive and negative breast cancer cells (6). Isoflavonoids, particularly genistein, are also potent inhibitors of the tyrosine protein kinase activity of several growth factor receptors and oncogenes, which may be associated with tumor cell growth (7,10). Genistein has also been reported to inhibit angiogenesis in vitro (11), to stimulate sex hormone binding globulin formation and suppress cell proliferation of human hepatocarcinoma cells (12). To show these effects, high concentrations are required. The findings of this study clearly indicate that, at lower concentrations (less than 1 μM), these nonsteroidal compounds, coumestrol, genistein, and zearalenone, which represent three structurally different classes, all act in a similar ER-mediated mechanism as endogenous estrogens.

None of the compounds showed any antiestrogenicity in the presence of 17β-estradiol at an agonist concentration giving maximal or submaximal stimulation of cell proliferation. This suggests that they do not act as antiestrogens in breast cancer cells at the concentrations of 10 nM or below. There are at least two possible explanations for these findings. First, because there is a lower binding affinity by the receptor for the environmental compounds, they are not able to compete, at the doses tested, with 17β-estradiol for the receptor ligand binding sites. Second, binding to the receptor may displace 17β-estradiol from the receptor sites, but the binding of nonsteroidal estrogen would also trigger the estrogen action seen as an increased rate of cell proliferation. At present, there is no way to differentiate between these two possibilities; however, considering the lower biological activity of some of these compounds, they might be expected to show some attenuation of the proliferative response.

The interconversion of estrone and 17β-estradiol and formation of their biologically inactive derivatives have a potentially important role in the regulation of estrogen action at defined sites in the organism (13). Coumestrol was a remarkably good competitive inhibitor of 17β-reduction of estrone when the estrogen-specific enzyme purified from human placenta was tested (Table 1). The other compounds (genistein, biochanin A, and zearalenone) were weaker inhibitors or were inactive. This means that the structural requirements for the binding to the ligand site on the ER differ significantly from the substrate active site on the estrogen-specific 17β-oxidoreductase enzyme. In the case of zearalenone, the difference was most striking. Zearalenone was a potent estrogen, but was not able to inhibit the conversion of 3H-estrone to 3H-estradiol by the purified 17β-hydroxysteroid oxidoreductase enzyme. In contrast to the results with the placental 17β-estradiol oxidoreductase enzyme, the conversion of 3H-estrone to 3H-estradiol in MCF-7 cells was not inhibited by coumestrol or genistein, while the conversion was slightly inhibited by zearalenone. The obvious explanation would be the structural properties for the inhibitor required by the different forms of the enzyme.

There is evidence for the presence of multiple forms of 17β-hydroxysteroid oxidoreductase enzyme in breast cancer cells (13). The placental form of the enzyme has not been found in MCF-7 cells, but T-47D cells are known to have this form (23). The proliferative response of the T-47D cells to estrone occurs at much lower concentrations than MCF-7 cells, which may be explained by the more effective conversion of estrone to estradiol due to the higher concentration of the oxidoreductase enzyme in the T-47D cells. However, we were not able to show estrone antagonism (antiestrogenicity) with the phytoestrogens because they were effective at stimulating cell proliferation, presumably by direct, agonist regulation of estrogen-responsive genes; this effect may mask the inhibitory effect on estrone activation of proliferation. Whether the enzyme inhibition would play a role in other in vivo conditions cannot be judged by these results. Generally, the most abundant circulating endogenous estrogen is estrone, and this enzyme inhibition may be important in tissues which have this enzyme (placenta, breast, endometrium, prostatic urethra, and collecting ducts).

The relevance of all these in vitro findings for the in vivo conditions remains unclear. There is little information on the possible action of dietary estrogens in the human population. In a recent study of postmenopausal women, plant foods were found to have significant estrogenlike effect on vaginal cell maturation, which is a sensitive and specific indicator of estrogenicity (24). There is no evidence, to date, for their antiestrogenicity in women. In agreement with this, we found no evidence to support the hypothesis that dietary estrogens act as antiestrogens through competition for the binding sites of ER or through a metabolic step involving the inhibition of the interconversion of estrone to 17β-estradiol, since this effect could be counteracted by their
ability to act as an estrogen agonist and stimulate a biological response. Furthermore, we conclude that all of these compounds can act as agonists to induce endogenous estrogen-specific responses or to transactivate an exogenous estrogen-responsive reporter construct by acting directly through the ER.

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