Estrogen-Specific 17β-Hydroxysteroid Oxidoreductase Type 1 (E.C. 1.1.1.62) as a Possible Target for the Action of Phytoestrogens (43831)

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Abstract. **Several plant estrogens, especially coumestrol and genistein, were found to reduce the conversion of [^{3}H]estrone to [^{3}H] 17β-estradiol catalyzed by estrogen-specific 17β-hydroxysteroid oxidoreductase Type 1 (E.C. 1.1.1.62) *in vitro*. Coumestrol, the most potent inhibitor in our experiments, is the best inhibitor of the enzyme known to date. All compounds with inhibitory effects were also estrogenic. However, structural demands for 17β-HSOR Type 1 inhibition and estrogenicity of tested compounds in breast cancer cells (judged by increased cell proliferation) were not identical. Zearalenone and diethylstilbestrol, both potent estrogens, did not inhibit 17β-HSOR Type 1. Thus, changes in the estrogen molecule may discriminate between active sites of 17β-HSOR Type 1 and estrogen binding sites of the ER. The effects of these compounds *in vivo* cannot be predicted on the basis of these results. Inhibition of 17β-HSOR Type 1 enzyme could lead to a decrease in the availability of the highly active endogenous estrogen. However, these compounds are estrogenic per se, and they may thus replace endogenous estrogens. Additional studies are needed to further understand the role of these plant estrogens in the etiology of hormone-dependent cancers. It is not easily conceivable how the chemopreventive action of Asian diets, possibly mediated by phytoestrogens in soya products, can be based on the inhibition of estrone reduction at the target cells by phytoestrogens or related compounds, unless they are "incomplete estrogens" (i.e., unable to induce all effects typical of endogenous estrogens).** [P.S.E.B.M. 1995, Vol 208]

Marked geographic differences have been found in the incidence rates of estrogen-related cancers such as those of breast and prostate (1–5). Generally, the incidence rates are high in Northern Europe and North America and low in Asia. Epidemiological studies indicate that environmental factors, especially diets, are important determinants of cancer risk (2, 6–9). Dietary factors, at least to a certain extent, are thought to act via changes in hormonal, especially estrogen, concentrations. As an indication of this, obesity (10) and diets high in calories and fats (11–14), which are risk factors of postmenopausal breast cancer and prostate cancer, also increase estrogen concentrations in postmenopausal women and men.

Epidemiological studies also suggest that plant-based diets may delay the development of prostate and breast cancers (2, 15, 16). Soya products widely used in Asian countries, with a low incidence of breast and prostate cancers, are considered to be chemopreventive in experimentally induced cancers (17, 18). The serum concentrations and urinary excretion of phytoestrogens, found in substantial amounts in soya, are much higher in men and women living in Japan, suggesting that the effect of soy could very well be based on the antiestrogenic action of phytoestrogens (19, 20).

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The mechanism of the possible antiestrogen action of phytoestrogens is not known. They may exert their activity in estrogen-responsive cells by interacting with a specific intracellular estrogen receptor (ER). Compounds with high binding affinities for ER are generally the most active biologically and enhance proliferation of estrogen-responsive tumor cells (21). They have also been shown to be capable of transactivating an estrogen responsive reporter gene in cells co-transfected with an expression vector for the estrogen receptor (22, 23). None of the phytoestrogens we studied earlier (coumestrol, genistein, biochanin A, and zearalenone) reduced the proliferation rate of 17β-estradiol-stimulated breast cancer cells (i.e., they had no antiestrogenic effects) (23).

In addition to their interaction with ER, dietary estrogens or structurally related compounds might compete with endogenous estrogens for the active site of the estrogen biosynthesizing and metabolizing enzymes and thus reduce the concentration of biologically active endogenous estrogens. Changes in 17β-oxidoreduction status of endogenous estrogens (estradiol and estrone) would considerably modify the biological activities of these hormones. The reversible reaction between weak estrone (oxidized form) and highly active estrogen, estradiol, is catalyzed by several 17β-hydroxysteroid oxidoreductase enzymes (17β-HSORs).

The present study was carried out to determine whether phytoestrogens and structurally related plant-derived and synthetic compounds (Table I) inhibit the reduction of estrone to 17β-estradiol by estrogen-specific 17β-hydroxysteroid oxidoreductase Type 1 (E.C. 1.1.1.62, also known as 17β-hydroxysteroid dehydrogenase Type 1 or 17β-HSD Type 1). This enzyme is expressed in both steroidogenic cells, such as ovarian granulosa cells (24, 25) and placental trophoblasts (26), as well as in some target tissues of estrogen action, such as normal and malignant breast and endometrium (27–29) and in the epithelium of the prostatic urethra and prostatic collecting ducts adjacent to estrogen receptor positive stroma (30). If such inhibition were present, it would lead to a decreased estrogen effect by interfering with estradiol biosynthesis at the ovarian and target cell level.

Table I. Plant-Derived Polycyclic Compounds Grouped by Chemical Structure and Sources

Compound[a]	Sources
Coumestans	Leguminous plants
Coumestrol	(e.g., soy, alfalfa)
Stilbenes	Wood
4,4'-Dihydroxystilbene	
3,5-Dihydroxystilbene	
Coumarins	Beans
Coumarin	
Isoflavonoids	Leguminous plants (soy),
Genistein	clover
Biochanin A	
Flavonoids	Fruit and vegetables
Apigenin	
Chrysin	
Quercetin	
Kaempferol	
Plant sterols	Plant oils, leguminous
α-Sitosterol	plants, wood
β-Sitosterol	
β-Sitostanol	
Saponins	Licorice
α-Glycyrrhetinic acid	
β-Glycyrrhetinic acid	
Resorcylic acid lactones	Grain and feeds infected by
Zearalenone	Fusarium (mold)

[a] See Materials and Methods for the producers and purity of the test compounds.

Materials and Methods

Phytoestrogens and Related Compounds. The plant-derived compounds were: apigenin, biochanin A, chrysin, coumarin (1,2-benzopyrone), kaempferol, quercetin, α-glycyrrhetinic acid and β-glycyrrhetinic acid (95% pure), zearalenone (all from Sigma Chemical Co., St. Louis, MO); coumestrol (Eastman Kodak, Rochester, NY) 3,5-dihydroxystilbene (pinosylvin) and 4,4'-dihydroxystilbene (both kindly provided by Prof. Bjarne Holmbom, Laboratory of Forest Products Chemistry, Åbo Akademi, Turku, Finland); genistein (Boehringer); α-sitosterol (66% pure), β-sitosterol (91% pure) and β-sitostanol (all kindly provided by Kaukas OY, Research Center, Lappeenranta, Finland). The synthetic compounds were diethylstilbestrol (DES) and tamoxifen (both from Sigma); 3,3'-diethylstilbestrol and 4,4'-dihydroxybiphenyl (97% pure) (both kindly provided by Dr. Manfred Metzler, University of Kaiserslautern, Department of Food Chemistry and Environmental Toxicology, Kaiserslautern, Germany); and toremifene and 4-OH-toremifene (kindly provided by Orion Pharmaceuticals, Farmos Research, Turku, Finland).

Breast Cancer Cell Lines. Three breast cancer cell lines with different concentrations of 17β-hydroxysteroid oxidoreductase Type 1 were used. In MCF-7 cells (kindly provided by Dr. Pirkko Härkönen, University of Turku, Turku, Finland) 17β-HSOR Type 1 concentrations were under the detection limit of our immunoassay (31). In addition, two types of T-47D cell were used. The concentrations of 17β-HSOR Type 1 in wild type T-47D (kindly provided by Dr. Pirkko Härkönen, University of Turku, Turku, Finland) and T-47D_{21} cells were 2.5 ng/mg and 235 ng/mg of cytosolic proteins, respectively. T-47D_{21} cells were stably transfected with 17β-HSOR Type 1 cDNA and over 95% of the total 17β-HSOR activity present in these cells results from the transfected recombinant enzyme. These cells will be described in

detail elsewhere. Stock cultures were grown in phenol red free Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD) with antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml, Gibco BRL). The medium of MCF-7 cells was supplemented with 5% fetal calf serum (FCS) (Gibco BRL), insulin (from bovine pancreas; Sigma) 10 μg/ml and 17β-estradiol (1 n*M*; Sigma). To the medium of T-47D and T-47D_{21} cells, 10% FCS and 7.5 μg/ml of insulin was added.

MCF-7 Cell Proliferation Assays. The stock cultures were harvested by trypsinization, suspended in DMEM medium containing antibiotics, 5% dextran charcoal stripped FCS (dcFCS) and 10 μg/ml insulin, and seeded on 96-well culture plates at 2000 cells/well (200 μl medium per well). On the second day of culture, the medium was changed and test compounds were added. The test compounds were diluted in ethanol. Equal amounts of ethanol (final concentration 0.1%) were added to the control wells. The cells were cultured for 7 days in the presence of test compounds, and the medium was changed every other day. Cell proliferation was quantified by a colorimetric MTT assay as described by Martikainen *et al.* (32). The values of each row (eight wells with same treatment) were combined and calculated as percentages of the control values; each culture plate had its own control (16 wells). Twelve to 16 replicate rows were done for each test compound concentration.

T-47D Cell Proliferation Assays. The stock cultures were harvested by trypsinization, suspended with medium (DMEM with 5% dcFCS and 7.5 μg/ml insulin) and seeded on 30 × 10 mm culture dishes at 10,000 cells/dish. On the second day of culture, the medium was changed and test compounds were added. Estrone and test compounds (coumestrol and genistein) were diluted in ethanol, and equal amounts of vehicle (final concentration 0.1%) were added to the control dishes. The concentrations used were 0.1 p*M* for estrone and 0.1 μ*M* and 1 n*M* for both coumestrol and genistein. The media were changed every other day. The cells were cultured for 10 days in the presence of test compounds. The number of cells was determined by counting the released nuclei with Coulter Counter.

Measurement of 17β-Reductase Activity Using Purified Human 17β-HSOR Type 1. The final volume of the assay medium for the study of 17β-HSOR Type 1 activity was 250 μl, consisting of 100 μl of purified enzyme (26) in 50 m*M* phosphate buffer, pH 7.4, with 2 m*M* dithiotreitol (DTT), 1 m*M* EDTA and 20% glycerol, 150 μl of NADPH generating system (50 μl 4 m*M* NADP, 50 μl 40 m*M* glucose-6-phosphate, 50 μl 50 U/ml glucose-6-phosphate dehydrogenase), and 3 μ*M* of test compound in 50% ethanol. After preincubation at 37°C for 20 min, 3 μCi of [^{3}H]labeled substrate (estrone) was added, and incubation was continued for 60 minutes. The [^{3}H]estrone (specific activity 98 Ci/mmol) was purchased from Amersham International (Buckinghamshire, England). The final substrate concentration was 0.72 μ*M*.

After incubation, unlabeled carriers (estradiol and estrone) were added. All reference steroids were purchased from Steraloids, Inc. (Wilton, NH). 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, the ethanol solutions were filtered and evaporated, and the residues dissolved in the mobile phase. HPLC was used for separation and quantification of the products. The column was a C18 150 × 3.9 mm ID analytical column (Technopak 10 C18 HPLC Technology; Wellington House, Cheshire, UK). The mobile phase was acetonitrile/water (35/65) and the flow rate was 1.2 ml/min. For in-line detection of radioactive metabolites, the eluent of the HPLC column was continuously mixed with liquid scintillant and then monitored with an in-line radioactivity detector. Details of separation and quantification of [^{3}H]labeled steroids have been described previously (30). Reductase activities were calculated as percentages of [^{3}H]estrone converted to [^{3}H]estradiol. The amount of enzyme in each assay was adjusted so that the conversion of the substrate to product was 5%–30% during incubation. Product formation was proportional to incubation time up to 2 hr. In addition to conversion of estrone to estradiol no other metabolites (17α-estradiol, 17β-estriol, 2-hydroxy-, or 4-hydroxyl derivatives of estrone or estradiol) were detected.

Measurement of Interconversion of Estrone and Estradiol in MCF-7 and T-47D Cells. The enzyme activity of wild type T-47D breast cancer cells was assessed by determining the ability of intact monolayers to convert added [^{3}H]estrone to [^{3}H]estradiol (33, 34). For each experiment, stock cultures were harvested by trypsinization and seeded on culture dishes (60 × 15 mm, 2 × 10^5 cells per dish) and cultured for 5 days in phenol red-free DMEM with 5% dcFCS and insulin (as in stock cultures). The medium was changed every other day. On the fifth day, growth medium was removed and the test compound diluted in ethanol was added to 3 ml of serum-free DMEM. Control dishes, containing the vehicle alone, were run in parallel in each experiment. After a 1-hr preincubation with the test compound, [^{3}H]estrone (final concentration 2 n*M*) and nonlabeled estrone (final concentration 2 n*M*) were added and the dishes were incubated for 4 hr. In these cells conversion was linear up to 8 hr. After incubation, 2 ml of medium was used for extraction of the steroids which were analyzed as described above. The number of cells per dish was de-

termined by counting released nuclei with a Coulter Counter.

A double-isotope labeling method was used to study the effects of coumestrol and genistein (estrogenic compounds with the strongest inhibitory activity on estrone reduction) and zearalenone (a potent estrogen with no inhibitory activity on estrone reduction) on interconversion between estrone and estradiol in breast cancer cells (MCF-7 cells and T-47D_{21} cells). With this method both estrone reduction and estradiol oxidation can be analyzed simultaneously. The cells were cultured and preincubated with test compounds as described above. After preincubation, [^{3}H]estrone (final concentration 2 n*M*), [^{14}C]estradiol (final concentration 800 n*M*) and nonlabeled estrone (final concentration 800 n*M*) were added, and the dishes were incubated for 1 hr (T-47D_{21} cells) or 8 hr (MCF-7 cells). In T-47D_{21} cells conversion was linear up to 90 min and in MCF-7 cells up to 8 hr. The [^{14}C]estradiol (specific activity 52 mCi/mmol) was purchased from DuPont NEN (Les Ulis Cedex A, France). The steroids were extracted as described above and separated by HPLC, and 0.6 ml fractions were collected with an LKB collector over 30-sec periods. Two milliliters of scintillation liquid (HiSafe 3, Wallac, Turku, Finland) were added to each fraction, and the activity of fractions was counted with a Wallac 1409 liquid scintillation counter. DPM values from fractions within estrone and estradiol peaks were used to calculate percentages of [^{3}H]estrone converted to [^{3}H]estradiol and [^{14}C]estradiol converted to [^{14}C]estrone.

Results

Estrogenicity of Phytoestrogens and Structurally Related Compounds. Assessment of the estrogenicity of individual phytoestrogens or structurally related compounds was based on their capability to enhance proliferation of estrogen-dependent MCF-7 or T-47D breast cancer cells in culture (Table II).

As we have shown earlier (23), the compounds had following order of estrogenic efficacy: 17β-estradiol > zearalenone > coumestrol > genistein > biochanin A. Additionally, 3,3′-diethylstilbestrol, 3,5-dihydroxystilbene (pinosylvine), 4,4′-dihydroxystilbene, kaempferol, coumarin, β-sitosterol, β-sitostanol, and α-glycyrrhetinic acid significantly enhanced cell proliferation, but they all showed weaker estrogenic activity than zearalenone and coumestrol. β-Sitosterol was active only in T-47D cells, but not in MCF-7 cells. β-Glycyrrhetinic acid and several flavones (apigenin, chrysin, and quercetin) were not active in the assay. Coumestrol, genistein and zearalenone induced dose-

Table II. Estrogenicity of Phytoestrogens and Structurally Related Compounds

Compound	LEC[a]	e_{max}[b]	HC[c]
Coumestans			
Coumestrol[d]	10 p*M*	78	1 μ*M*
Coumestrol[e]	10 p*M*	98	1 μ*M*
Stilbenes			
4,4′-dihydroxystilbene[d]	0.5 μ*M*	48	0.5 μ*M*
3,5-dihydroxystilbene[e]	1 μ*M*	27	1 μ*M*
3,3′-diethylstilbestrol[e]	0.1 n*M*	70	1 μ*M*
Coumarins			
Coumarin[d]	0.7 μ*M*	37	0.7 μ*M*
Isoflavonoids			
Genistein[d]	0.1 μ*M*	72	1 μ*M*
Biochanin A[d]	0.1 μ*M*	64	1 μ*M*
Flavonoids			
Apigenin[d]	Inactive	—	0.12 μ*M*
Chrysin[d]	Inactive	—	0.17 μ*M*
Quercetin[d]	Inactive	—	0.1 μ*M*
Kaempferol[d]	0.3 μ*M*	12	0.35 μ*M*
Plant sterols			
α-Sitosterol[d]	Inactive	—	0.25 μg/ml
β-Sitosterol[d]	Inactive	—	0.1 μg/ml
β-Sitosterol[e]	0.4 μg/ml	71	0.4 μg/ml
β-Sitostanol[e]	0.4 μg/ml	67	0.4 μg/ml
Saponins			
α-Glycyrrhetinic acid[e]	1 μ*M*	79	1 μ*M*
β-Glycyrrhetinic acid[e]	Inactive	—	1 μ*M*
Resorcylic acid lactones			
Zearalenone[d]	10 p*M*	79	1 μ*M*

[a] LEC = the lowest effective concentration.
[b] e_{max} = the maximal estrogenic effect of the substance relative to 17β-estradiol given as percentage of the maximal E_2 response.
[c] HC = the highest concentration studied.
[d,e] Estrogenicity was defined as capability to enhance proliferation of MCF-7[d] or T-47D[e] breast cancer cells in culture.

dependent responses (23). Coumestrol and zearalenone were potent at 10 p*M* concentrations, while higher concentrations (100 n*M*) were needed to show the estrogenicity of genistein (23).

Inhibition of Conversion of [^{3}H]-Estrone to [^{3}H]-Estradiol by Phytoestrogens and Structurally Related Compounds (Experiments with Purified 17β-HSOR Type 1). The capability of phytoestrogens and structurally related compounds to inhibit conversion of [^{3}H]estrone to [^{3}H]estradiol catalyzed by estrogen-specific 17β-HSOR Type 1 was studied by using the enzyme purified from human placenta (Table III). Coumestrol was the most potent inhibitor of estrogen-specific 17β-hydroxysteroid oxidoreductase. As we have shown earlier (23), coumestrol had significant inhibitory effect at a concentration of 0.12 μ*M*. Genistein was weakly inhibitory. However, zearalenone was completely inactive at a concentration of 1.2 μ*M*. β-Sitosterol, like genistein, was slightly inhibitory, while α-sitosterol was inactive. The remaining compounds (coumarin, flavones, and α- and β-glycyrrhetinic acids) were inactive at the concentration 1.2 μ*M*.

Several diphenyl ethenes and ethanes were studied to show the chemical requirements of the inhibition. Diethylstilbestrol, 3,3′-diethylstilbestrol, 3,5-dihydroxystilbene, and dihydroxybiphenyls were inactive, whereas 4,4′-dihydroxystilbene showed moderate inhibition. Synthetic triphenylethylene antiestrogens (tamoxifen, toremifene, and 4-OH-toremifene) were also inactive.

Inhibition of [^{3}H]-Estrone Reduction and [^{14}C]-17β-Estradiol Oxidation in MCF-7 and T-47D Cells. The capability of phytoestrogens to inhibit estrone reduction in cells was tested by using wild type T-47D breast cancer cells. Only compounds showing the strongest inhibitory activity on the purified enzyme (coumestrol and genistein) were able to reduce the conversion, as shown in Table IV.

The interconversion of estrone and estradiol in T-47D$_{21}$ cells was studied with the double-isotope labeling method. In these cells, estrone is rapidly converted to estradiol (60% within 1 hr), while only 20% of estradiol is converted to estrone (Fig. 1). The conversion was much slower in MCF-7 cells and showed no difference between reduction and oxidation (Fig. 2). Both coumestrol and genistein inhibited estrone reduction in T-47D$_{21}$ cells, but they had no effect on estradiol oxidation. Zearalenone (used as a negative control) was completely inactive in these cells (Fig. 1). None of the three compounds tested affected the interconversion in MCF-7 cells (Fig. 2).

Effects of Coumestrol and Genistein on Estrone-Induced Proliferation of Transfected T-47D Cells. The effects of coumestrol and genistein on estrone-induced cell proliferation of T-47D$_{21}$ cells were studied in an attempt to find out whether these compounds, which are potent inhibitors of estrone reduction, counteract the effect of estrone. However, these compounds were not seen to have any estrone antagonism (Fig. 3). At concentrations effective in enzyme inhibition, both coumestrol and genistein significantly increased the cell proliferation when added alone, and, when combined with estrone, an additive effect was seen (Fig. 3).

Table III. Inhibition of Conversion of [^{3}H]Estrone to [^{3}H]Estradiol by Phytoestrogens and Structurally Related Compounds (Tested Using the Purified 17β-HSOR Type 1 Enzyme)

Compound	IC[a] (μ*M*)	Conversion of [^{3}H]Estrone to [^{3}H]Estradiol (control = 100) (Mean ± SD)
Coumestans		
Coumestrol	1.2	18.3 ± 7.5[c]
	0.12	49.0 ± 15.4[c]
	0.012	75.6 ± 27.3
	0.0012	106.0 ± 39.6
Stilbenes		
Diethylstilbestrol	1.2	96.8 ± 11.7
4,4′-Dihydroxystilbene	1.2	65.1 ± 14.2[c]
3,5-Dihydroxystilbene	1.2	98.3 ± 12.0
3,3′-Diethylstilbestrol	1.2	76.1 ± 27.6
Coumarins		
Coumarin	1.2	86.4 ± 10.0
Isoflavonoids		
Genistein	1.2	62.8 ± 29.9[c]
	0.12	90.6 ± 38.2
Biochanin A	1.2	91.3 ± 28.2
Flavonoids		
Chrysin	1	96.0 ± 23.9
Quercetin	1	123.0 ± 11.8[c]
Plant sterols		
α-Sitosterol	1.2	74.4 ± 32.3
β-Sitosterol	1.2	69.2 ± 19.8[b]
β-Sitostanol	1.2	68.0 ± 14.3[b]
Saponins		
α-Glycyrrhetinic acid	1	86.7 ± 10.0
β-Glycyrrhetinic acid	1	102.4 ± 17.6
Resorcylic acid lactones		
Zearalenone	1.2	84.6 ± 31.5
Biphenyls		
4,4′-Dihydroxybiphenyl	1.2	109.3 ± 22.9
Triphenyl ethylenes		
Tamoxifen	1.2	86.8 ± 8.1
Toremifene	1.2	92.1 ± 24.9
4-OH-toremifene	1.2	91.3 ± 31.6
Steroids		
Androstenedione	1.2	94.2 ± 29.0
Progesterone	1.2	107.2 ± 13.4

[a] IC = inhibitor concentration.
[b,c] Differs significantly from the controls at 5% and 1% levels, respectively (Tukey's studentized range method).

Discussion

Several plant-derived compounds (coumestrol, genistein, 4,4′-dihydroxystilbene, and β-sitosterol) were

Table IV. Inhibition of Conversion of [^{3}H]Estrone to [^{3}H]Estradiol by Phytoestrogens and Structurally Related Compounds in Wild-Type T-47D Breast Cancer Cells

Compound	IC[a] (μM)	Conversion of [^{3}H]Estrone to [^{3}H]Estradiol (control = 100) (Mean ± SD)
Coumestans		
Coumestrol	1.2	42.1 ± 21.3[b]
	0.12	67.4 ± 10.6[b]
	0.012	91.5 ± 4.5
	0.0012	89.4 ± 1.4
Stilbenes		
4,4'-Dihydroxystilbene	1.2	106.0 ± 2.6
Diethylstilbestrol	1.2	89.2 ± 6.8
	0.12	92.4 ± 10.0
	0.012	100.7 ± 9.3
Coumarins		
Coumarin	1.2	108.6 ± 8.0
Isoflavonoids		
Genistein	1.2	39.4 ± 8.4[b]
	0.12	88.8 ± 5.2
	0.012	92.6 ± 9.9
Biochanin A	1.2	85.4 ± 2.1
	0.12	104.2 ± 2.1
	0.012	104.2 ± 3.6
Flavonoids		
Quercetin	1.2	106.0 ± 2.6
Plant sterols		
β-Sitosterol	1.2	93.2 ± 10.3
Resorcylic acid lactones		
Zearalenone	1.2	79.3 ± 21.2
	0.12	122.5 ± 9.1
	0.012	111.3 ± 12.3

[a] IC = inhibitor concentration.
[b] Differs significantly from the controls at 1% level (Tukey's studentized range method).

found to reduce the conversion of [^{3}H]estrone to 17β-[^{3}H]estradiol catalyzed by purified estrogen-specific 17β-hydroxysteroid oxidoreductase Type 1 (17β-hydroxysteroid dehydrogenase Type 1) *in vitro*. Inhibition by coumestrol and genistein was dose dependent and evident in the presence of NADPH. To our knowledge, coumestrol is the most potent inhibitor of 17β-HSOR Type 1 known to date. It can be asked whether this inhibition of 17β-HSOR Type 1 by phytoestrogens is a mere "accident" or whether it reflects a co-evolutionary phenomenon, as recently suggested by Dr. Michael E. Baker (35).

Coumestrol and genistein also inhibited reduction of estrone in T-47D cells known to contain 17β-HSOR Type 1 enzyme (31). Inhibition by genistein was statistically significant at the concentration which is about 5-fold the serum concentration found in Japanese men (20). Experiments with [^{3}H]estrone and [^{14}C]estradiol showed that the reaction proceeded in T-47D$_{21}$ cells from estrone to 17β-estradiol. Conversion of 17β-estradiol to estrone was slower and unaffected by coumestrol or genistein. These compounds were equally effective in wild-type T-47D cells, even though coumestrol had considerably higher effects in

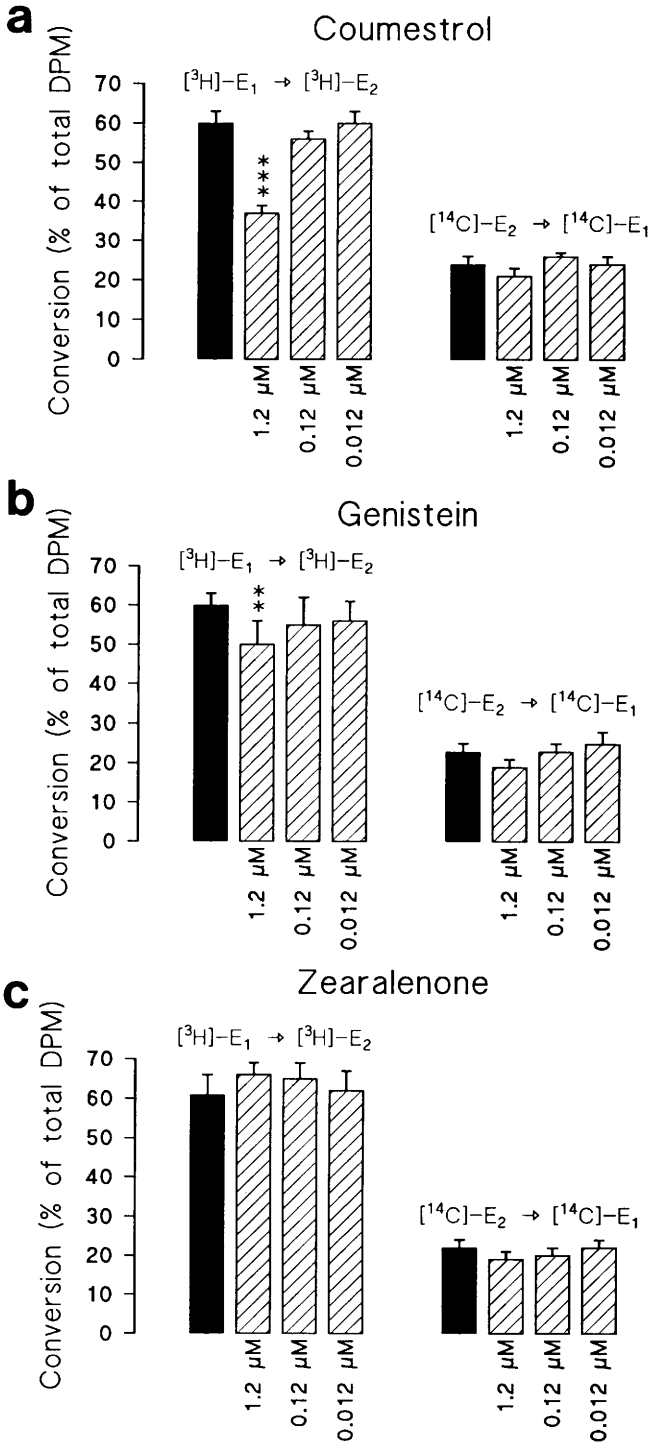

Figure 1. The effect of coumestrol (a), genistein (b), and zearalenone (c) on interconversion of estrone and estradiol in T-47D$_{21}$ cells. Values indicate means of six to eight parallel dishes ± standard deviation. Solid bars indicate control levels. The concentrations of test compounds are shown below the bars. Bars on the left show conversion of [^{3}H]estrone to [^{3}H]estradiol and bars on the right conversion of [^{14}C]estradiol to [^{14}C]estrone. **, ***Differs significantly from controls, $P < 0.01$ or $P < 0.001$, respectively (Student's *t*-test with Bonferroni correction).

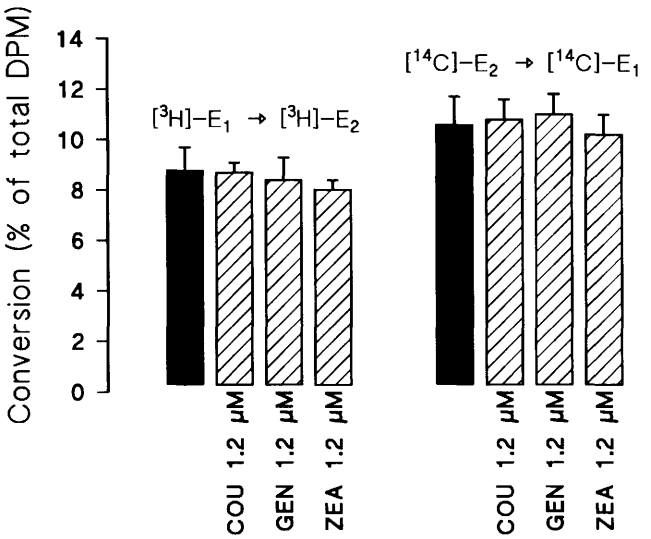

Figure 2. Effect of coumestrol (COU), genistein (GEN), and zearalenone (ZEA) on interconversion of estrone and estradiol in MCF-7 cells. Values indicate means of six parallel dishes ± standard deviation. Solid bars indicate control levels. Bars on the left show conversion of [^{3}H]estrone to [^{3}H]estradiol and bars on the right conversion of [^{14}C]estradiol to [^{14}C]estrone.

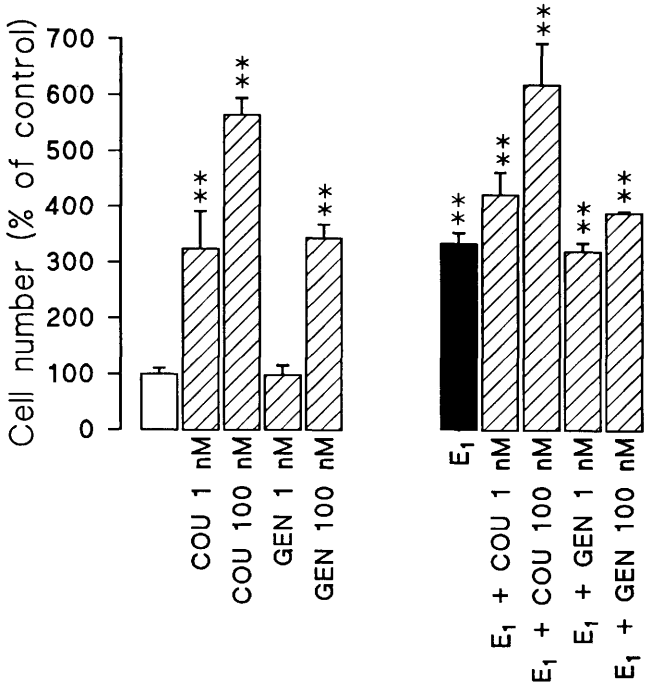

Figure 3. Effect of coumestrol (COU) and genistein (GEN) on proliferation of T-47D$_{21}$ cells alone (bars on the left) and in the presence of estrone (E$_1$; 0.1 p*M*; bars on the right). Values indicate means of six to eight parallel dishes ± standard deviation. The open bar indicates the control level and the solid bar the effect of estrone alone. Concentrations of test compounds are shown below the bars. **Differs significantly from controls at 1% level (Tukey's studentized range method).

assays with purified enzyme. In T-47D$_{21}$ cells coumestrol was more potent than genistein, yet less effective than in tests with purified enzyme. At present, no explanation is available for this diminution of coumestrol action in cell cultures.

In addition to these phytoestrogens, some structurally related compounds (4,4'-dihydroxystilbene and β-sitosterol) found in edible plants or wood were slightly inhibitory. On the basis of our findings, structural demands for the 17β-HSOR Type 1 inhibition were the following: (i) (at least) one phenolic hydroxyl group on each of two rings is needed (4,4'-dihydroxystilbene versus, 3,5-dihydroxystilbene); (ii) a proper distance between the two hydroxyl groups is a prerequisite for the inhibition (4,4'-dihydroxystilbene versus 4,4'-dihydroxybiphenyl); (iii) isoflavonoid structure makes the compound a potential inhibitor, while flavonoids studied so far have been inactive; (iv) groups substituted at C-atoms 1 and 2 of the ethenes make the compound more effective (coumestrol versus 4,4'-dihydroxystilbene); (v) bulky side chains at C-atoms 1 and 2 of ethenes (such as in tamoxifen and toremifene) may inhibit the binding of the molecule to the active site of the enzyme. These structural properties are also relevant for estrogenicity (36). However, when estrogenic activity and enzyme inhibition are correlated (Fig. 4), the requirements appear not similar. Zearalenone and diethylstilbestrol, both potent estrogens, did not inhibit 17β-HSOR Type 1. Thus, changes in the estrogen molecule may discriminate between active sites of different estrogen biosynthesizing and metabolizing enzymes and estro-

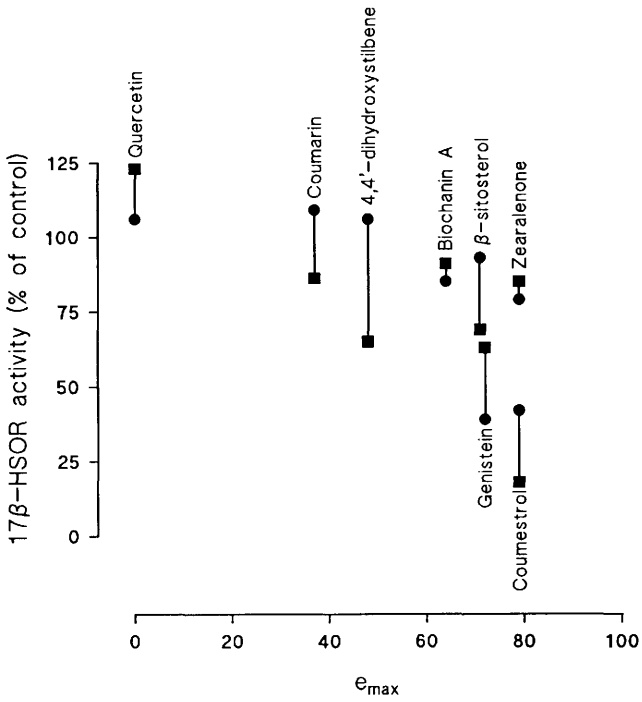

Figure 4. Correlation between estrogenic activity and inhibition of 17β-HSOR activity of eight plant-derived compounds (coumestrol, 4,4'-dihydroxystilbene, coumarin, genistein, biochanin A, quercetin, β-sitosterol, and zearalenone). 17β-HSOR activity was determined by measuring the conversion of [^{3}H]estrone to [^{3}H]estradiol by the purified enzyme (indicated by solid squares) and in T-47D cells (solid circles) and expressed as percentages of control level.

gen binding sites of the ER. None of the compounds studied and exerting an inhibitory action on the enzyme was completely without estrogenicity. However, by analyzing these different requirements in detail, it might be possible to design a nonestrogenic 17β-HSOR inhibitor.

The enzyme specificity of the inhibition is not known. A number of 17β-hydroxysteroid oxidoreductases occur in mammalian cells. The soluble enzyme of the human placenta, which was the target of the present study, does not occur in MCF-7 cells (31), and coumestrol and genistein did not interfere with the oxidoreduction of endogenous estrogens in these cells. This may indicate that inhibition by plant estrogens is not necessarily a universal property of 17β-hydroxysteroid oxidoreductases or other estrogen-metabolizing enzymes.

It is likely that 17β-HSOR Type 1 is an essential enzyme for fluctuating secretion of estradiol from the developing Graafian follicles of premenopausal women and for local production of estradiol from weak circulating precursors in the peripheral tissues of postmenopausal women (24, 25, 29, 37). Thus, inhibition of 17β-HSOR Type 1 could modify both the serum concentration of estradiol and the concentration of the hormone in the target tissue. As intracellular estradiol concentration is one of the key factors regulating hormone action, a specific 17β-HSOR inhibitor could be useful in the prevention and treatment of estrogen-dependent diseases, such as breast cancer.

However, compounds capable of inhibiting estrone reduction in T-47D_{21} cells, such as coumestrol and genistein, were not able to inhibit estrone-induced proliferation of the same cells. Such a static experiment may not adequately reflect *in vivo* conditions. In addition, inherent estrogenic properties of these compounds could mask a decrease in availability of endogenous estradiol.

It is not easily conceivable how the chemopreventive action of soya could be based on inhibition of estrone reduction at target cells by phytoestrogens or related compounds, unless they are "incomplete estrogens" (i.e., incapable of inducing all effects typical of endogenous estrogens). Theoretically, the binding of highly different estrogenic ligands could influence the ER and, concomitantly, the interaction of ER with estrogen responsive elements (EREs) or transcription factors. These structural variations could modify the effect of the receptor complex on transcription on one responsive gene more than another. Whether these mechanisms explain the estrogenicity and the possible antiestrogenic properties of phytoestrogens remains to be established.

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