Phytoestrogens and Mycoestrogens Bind to the Rat Uterine Estrogen Receptor[1]

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ABSTRACT Consumption of phytoestrogens and mycoestrogens in food products or as dietary supplements is of interest because of both the potential beneficial and adverse effects of these compounds in estrogen-responsive target tissues. Although the hazards of exposure to potent estrogens such as diethylstilbestrol in developing male and female reproductive tracts are well characterized, less is known about the effects of weaker estrogens including phytoestrogens. With some exceptions, ligand binding to the estrogen receptor (ER) predicts uterotrophic activity. Using a well-established and rigorously validated ER-ligand binding assay, we assessed the relative binding affinity (RBA) for 46 chemicals from several chemical structure classes of potential phytoestrogens and mycoestrogens. Although none of the test compounds bound to ER with the affinity of the standard, 17β-estradiol (E_2), ER binding was found among all classes of chemical structures (flavones, isoflavones, flavanones, coumarins, chalcones and mycoestrogens). Estrogen receptor relative binding affinities were distributed across a wide range (from ~43 to 0.00008; E_2 = 100). These data can be utilized before animal testing to rank order estimates of the potential for in vivo estrogenic activity of a wide range of untested plant chemicals (as well as other chemicals) based on ER binding. J. Nutr. 132: 658–664, 2002.

KEY WORDS: • *uterus* • *estrogen receptor* • *phytoestrogen* • *mycoestrogen* • *structure-activity relationship* • *rats*

Phytoestrogens contained in the diet and in food supplements have the potential for both risks and benefits with respect to human health. Estrogenic chemicals consumed as constituents of plants (phytoestrogens) or fungi (mycoestrogens) exert a variety of adverse effects in animals including reduction in fertility in sheep grazing on phytoestrogen-containing clover (1–3), alteration of uterine (4–6) and behavioral (7,8) development in rodents, and increases in the volume of the sexually dimorphic nucleus in female rats (9). More recently, neonatal exposure to genistein has been shown to induce both uterine and vaginal abnormalities similar to those caused by the potent estrogen, ethinyl estradiol (10). Indeed, some researchers suggest that studies involving laboratory animals ideally should use phytoestrogen-free diets to ensure that no inadvertent estrogen exposure occurs (11,12). Despite these adverse effects observed in animals, dietary phytoestrogens might be beneficial in humans (13–16).

Although the hazards of human exposure to potent synthetic estrogens (17,18) or antiestrogens with estrogen agonist activity (19–21) are well documented, the potentially hazardous or beneficial effects of estrogens of plant origin are less well studied. Sonnenschein and Soto (22) indicate that exogenous estrogens of environmental origin can alter an organism or population by disrupting normally functioning endocrine or reproductive systems by inappropriately mimicking endogenous hormone actions, inhibiting hormone action, modulating hormone production or altering hormone receptor populations.

Under normal conditions, the estrogen receptor (ER)[3] is either unliganded or bound by its major endogenous ligand, 17β-estradiol (E_2). This ligand-ER binding results in conformational changes and activation of the ER that allows binding of the ER-ligand complex to nuclear estrogen response elements. This sequence ultimately leads to gene transcription and translation (23–25). The ER-ligand competitive binding assay quantitatively assesses a chemical's ability to bind to the ER. Work in our laboratory (26) and others (27) seeks to construct in vitro test systems to assess large numbers of environmental estrogens. The data from these in vitro assays can be used to rank order chemicals that may act via the ER system in vivo in a manner similar to that of the endogenous estrogen (28).

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[3] Abbreviations used: CAS, Chemical Abstracts Service; E_2, 17β-estradiol; ER, estrogen receptor; HAP, hydroxylapatite; IC_{50}, the molar concentration of test compound that inhibits E_2 binding by 50%; RBA, relative binding activity; SAR, Structure-Activity Relationship; QSAR, Quantitative Structure-Activity Relationship.

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The purpose of this study was to assess the ER binding of several groups of compounds of plant (flavones, isoflavones, flavanones, coumarins, chalcones) or fungal origin (zearalanone derivatives). These data and others (26) are being utilized to develop a battery of computational models to predict ER binding of additional plant- and fungus-derived chemicals as well as synthetic environmental chemicals. Quantitative Structure Activity Relationship (QSAR) and other Structure Activity Relationship (SAR) models have shown the utility of this assay approach to provide quantitative information for priority setting for conducting further evaluations of estrogenicity (29,30).

MATERIALS AND METHODS

Animals. Adult Sprague-Dawley–derived rats, bred and maintained as an out-bred colony at the National Center for Toxicological Research, were ovariectomized under light ether anesthesia. After an interval of at least 10 d, uteri were removed from groups of 10–12 rats immediately after they were killed by CO_2 asphyxiation. To produce a pool of uterine cytosol, the uteri were weighed and homogenized using a Polytron homogenizer at 4°C in TRIS-EDTA-dithiothreitol-glycerol buffer (0.01 L/g tissue) consisting of 10 mmol/L TRIS; 1.5 mmol/L EDTA; glycerol (0.1 L/L buffer); and dithiothreitol (0.154 g/L), pH 7.4. The homogenates were transferred to chilled ultracentrifuge tubes and centrifuged at 105,000 × *g* for 1 h at 4°C. The supernatants were decanted and frozen at −70°C until used. All procedures involving animals were conducted with strict adherence to guidelines and procedures reviewed and approved by the Institutional Animal Care and Use Committee both within the National Center for Toxicological Research and by an independent review panel.

Assay validation. All assay procedures outlined here were previously detailed (26). Uterine cytosol concentration (ER), [^{3}H]-E_2, and incubation time and temperature are key parameters for validation of the ER competitive binding assay (27,31,32). Scatchard analyses were used to obtain K_d and B_{max} for cytosol concentrations ranging from 10 to 100 g uterine tissue/L of buffer. B_{max} was linear with cytosol concentrations in the range of 2–50 g/L (data not shown). At a cytosol concentration of 17 g/L (B_{max} = 0.22 nmol/L), a stable K_d of ~0.1 nmol/L was observed. This K_d is consistent with current literature values (33). In addition, at a fixed concentration of 1 nmol/L [^{3}H]-E_2, binding increased linearly with increasing receptor concentration. Therefore, the final assay incubation conditions were chosen to be 20 h, 4°C using 17 g/L uterine tissue with 1 nmol/L [^{3}H]-E_2.

Assay procedures. The commercial sources, CAS (Chemical Abstracts Service) designations and chemical purities of the chemicals used are shown in **Table 1**. All test compounds were dissolved in 100% ethanol at the highest concentrations achievable, typically 10^{-2} to 10^{-3} mol/L. Serial dilutions of the stock solutions in 100% ethanol were made to provide a range of assay concentrations. Each competition assay consisted of 27 tubes; tubes 1 and 26 ("zero competitor" tubes) had no competing E_2. Tubes 2 and 27 had a final concentration of 1×10^{-7} mol/L E_2, a two orders of magnitude excess of E_2 to saturate the ER with nonradiolabeled competitor. The placement of an assay control tube at both the beginning and end of the assay controls for any "tube rack position" effects. The zero competitor tubes containing [^{3}H]-E_2 only were assayed to determine total binding, whereas the tubes containing 1×10^{-7} mol/L E_2 were assayed to correct for nonspecific binding. Also, in each assay, a complete competition curve for E_2 (tubes 2–7 containing a final E_2 concentration of 1×10^{-7} mol/L to 3.3×10^{-11} mol/L) was included as an internal assay standard. The test compounds were also dissolved in 100% ethanol, serially diluted in ethanol and assayed at six concentrations for each chemical in assay tubes 8–25. Standards or test compounds (1×10^{-5} L) were combined with 1×10^{-5} L of [^{3}H]-E_2 (final concentration of 1 x 10^{-9} mol/L), 2.3×10^{-4} L of TRIS buffer (50 mmol/L, pH 7.4) and 5×10^{-5} L of stock cytosolic ER in prechilled 12 × 75 borosilicate glass assay tubes. Each assay tube was run in duplicate and each assay was repeated at least once.

After incubation at 4°C for 20 h, 7.5×10^{-4} L of a hydroxyapatite (HAP) slurry (60% HAP in 50 mmol/L TRIS, pH 7.4) was added to the reaction mixture tubes. The tubes were vortexed briefly at 5-min intervals for 20 min and centrifuged for 4 min at 600 × *g*. The supernatant was discarded and the pellet was resuspended in 2 mL of cold 50 mmol/L TRIS buffer, and vortexed as above. This wash was repeated two times. After the third wash, the pellet was resuspended in 0.002 L of cold 100% ethanol and vortexed every 5 min for 15 min. After centrifugation at 600 × *g*, the ethanolic supernatant was decanted directly into scintillation counting vials for determination of [^{3}H]-E_2 remaining in the assay tube.

The percentage of binding of the test compounds to the ER was calculated by first subtracting the nonspecific binding (mean dpm in tubes 2 and 27) from the dpm of all of the other tubes. This provides the specifically bound dpm. This number is then expressed as the percentage of [^{3}H]-E_2 bound, i.e., specifically bound dpm of the sample/specifically bound dpm of the [^{3}H]-E_2 only tubes × 100. The data for each compound are expressed as an IC_{50}, i.e., the molar concentration of the test compound that produces a 50% inhibition of [^{3}H]-E_2 binding to the ER. The relative binding activity (RBA) is defined as the ratio of the E_2 IC_{50} to the test compound IC_{50} multiplied by 100.

RESULTS

Binding curves for the 29 compounds that competed with E_2 for ER binding are shown in **Figures 1-6**; data for the 12 compounds that did not bind to ER or which bound only slightly to ER (5 compounds) are not shown. In many cases, competing ligands were dissolved to the limits of solubility in an attempt to achieve complete binding curves. Nonbinders are defined as chemicals for which an IC_{50} was not obtained at the highest assay concentration achievable. Several of the phytoestrogens exhibited slight binding at the highest concentrations, but an IC_{50} concentration was not reached. Most of the curves appeared to be parallel to the E_2 standard curve throughout the log-linear portion of the curves. Slight deviations from parallelism were observed with the 3′- and 4′-hydroxyflavanones (Fig. 2) and the 4- and 4′-hydroxychalcones (Fig. 5) particularly at concentrations giving >50% competition. These deviations from parallelism did not substantially affect their IC_{50} values. In all assay replicates, the IC_{50} values for individual chemicals did not vary by >0.5 log units of concentration. Also, the flavones 6,4′-dihydroxyflavone, 3,6,4′-trihydroxyflavone and apigenin (Fig. 1), and the chalcones 4- and 4′-hydroxychalcone (Fig. 5) exhibited an increase in the percentage of [^{3}H]-E_2 bound at concentrations that were ≤15% of maximum E_2 binding. This effect did not alter the IC_{50} values.

The basic structures and ring designations for the flavanoids, chalcones, coumarins and mycoestrogens are shown in **Figure 7**. Chemical information (CAS number, source, purity) and experimental data derived from the competition curves (IC_{50}, RBA and log RBA) are shown in Table 1. Of the 46 chemicals tested, 34 exhibited competition for the ER; 3 were strong binders (log RBA >0), 14 were moderate binders (log RBA between 0 and −2), and 17 were weak binders (log RBA ≤−2). Some level of competition for ER binding of [^{3}H]-E_2 was seen in some members of all chemical classes; however, the highest affinity phytoestrogen (3,6,4′-trihydroxyflavone) competed with [^{3}H]-E_2 binding to the ER with ~0.05% lower affinity than E_2. Some of the mycoestrogens competed with affinities approaching that of E_2. Specific results are described below.

All of the flavones that bound ER are hydroxylated at the 4′ carbon with the exception of quercetin, a nonbinder. All other nonbinders lacked 4′ hydroxyls. Flavones with hydroxyls at the 6,4′ or 3,6,4′ carbons exhibited the strongest ER binding. Although neither the parent flavone structure nor 7-hy-

TABLE 1

Chemical information and comparisons of rat uterine cytosolic estrogen receptor (ER) binding data

Compound Name	CAS #	Source[1–8]	Purity[9]	Mean[10] IC_{50}	Mean[10] RBA	Log RBA
17β-Estradiol (standard)	50-28-2	U.S. Biochem	NA[11]	9.0×10^{-10}	1.0×10^{2}	2.00
			Flavones			
3,6,4′-Trihydroxyflavone		Indofine	Pure	2.0×10^{-7}	4.5×10^{-1}	−0.35
6,4′-Dihydroxyflavone		Indofine	Pure	5.9×10^{-7}	1.5×10^{-1}	−0.82
7,4′,5-Trihydroxyflavone (Apigenin)	520-36-5	Sigma	95%	3.2×10^{-6}	2.8×10^{-2}	−1.55
3,5,7,4′-Tetrahydroxyflavone (Kaempferol)	520-18-3	Fluka	>96%	3.7×10^{-6}	2.5×10^{-2}	−1.61
3,3′,4′,7-Tetrahydroxyflavone (Fisetin)	528-48-3	Aldrich	NA[11]	2.0×10^{-5}	4.5×10^{-3}	−2.35
3,5,7,3′,4′,5′-Hexahydroxyflavone (Myricetin)	529-44-2	Indofine	Pure	5.1×10^{-5}	1.8×10^{-6}	−2.75
5,6,7-Trihydroxyflavone (Baicalein)	491-67-8	Aldrich	NA	1.0×10^{-4}	9.0×10^{-4}	−3.05
3,5,7,4′,6′-Pentahydroxyflavone Dihydrate (Morin)	480-16-0	Sigma	NA	2.0×10^{-4}	4.5×10^{-4}	−3.35
Rutin	153-18-4	Sigma	95%	1.1×10^{-3}	8.2×10^{-5}	−4.09
6-Hydroxyflavone	6665-83-4	Indofine	Pure	NB[11]	—	—
6-Hydroxy-2′-methoxyflavone		Indofine	NA	NB	—	—
5,7-Dihydroxyflavone (Chrysin)	480-40-0	Sigma	NA	NB	—	—
3,5,7,3′,4′-Pentahydroxyflavone dihydrate (Quercetin)	6151-25-3	Sigma	NA	NB	—	—
Flavone	525-82-6	Sigma	NA	NB	—	—
7-Hydroxyflavone	6665-86-7	Indofine	Pure	NB	—	—
			Falvanones			
5,7,4′-Trihydroxyflavanone (± Naringenin)	93602-28-9	Sigma	95%	1.2×10^{-5}	7.5×10^{-3}	−2.13
4′-Hydroxyflavanone	6515-37-3	Indofine	Pure	4.0×10^{-5}	2.3×10^{-3}	−2.65
3′-Hydroxyflavanone		Indofine	Pure	5.4×10^{-5}	1.7×10^{-3}	−2.78
6 Hydroxyflavanone	4250-77-5	Indofine	Pure	SB[11]	—	—
7 Hydroxyflavanone	6515-36-2	Indofine	Pure	SB	—	—
Flavanone	487-26-3	Aldrich	95%	NB	—	—
± Catechin	7295-85-4	Sigma	NA	NB	—	—
5,7,3′-Trihydroxy-4′-methoxyflavanone (Hesperetin)	520-33-2	Sigma	NA	NB	—	—
Naringenin-7-neohesperidoside (Naringin)	10236-47-2	Sigma	≥95%	NB	—	—
3,5,7,3′,4′-Pentahydroxyflavanone (Taxifolin)	480-18-2	Sigma	≥98%	NB	—	—
			Isoflavones			
5,7,4′-Trihydroxyisoflavone (Genistein)	446-72-0	Toronto	>99%	2.0×10^{-7}	4.5×10^{-1}	−0.35
7,4′-Isoflavandiol (Equol)	531-95-3	Spectrum	NA	6.0×10^{-7}	1.5×10^{-1}	−0.82
4′,7-Dihydroxyisoflavone (Daidzein)	486-66-8	Sigma	≥98%	4.0×10^{-6}	2.3×10^{-2}	−1.65
7,3′,4′-Trihydroxyisoflavone	485-63-2	Indofine	Pure	2.0×10^{-5}	4.5×10^{-3}	−2.35
5,7-Dihydroxy-4-methoxyisoflavone (Biochanin A)	491-80-5	Sigma	NA	2.1×10^{-5}	4.3×10^{-3}	−2.37
5,4′-dihydroxy-7-methoxyisoflavone (Prunetin)	552-59-0	Indofine	Pure	SB	—	—
7-hydroxy-4′-methoxyisoflavone (Formononetin)	485-72-3	Indofine	Pure	SB	—	—
6,7,4′-Trihydroxyisoflavone	17817-31-1	Indofine	Pure	SB	—	—
4′,5,7-Trihydroxyisoflavone 7-glucoside (Genistin)	529-59-9	Sigma	≥95%	NB	—	—
			Chalcones			
4,2′,4′,6′-Tetrahydroxychalcone (Phloretin)	60-82-2	Sigma	NA	1.3×10^{-6}	6.9×10^{-2}	−1.16
4,2′,4′-Trihydroxychalcone	961-29-5	Indofine	Pure	1.6×10^{-6}	5.4×10^{-2}	−1.26
4′-Hydroxychalcone	2657-25-2	Indofine	97%	2.4×10^{-5}	3.7×10^{-3}	−2.43
4-Hydroxychalcone	20426-12-4	Indofine	97%	3.2×10^{-5}	2.8×10^{-3}	−2.55
Chalcone	94-41-7	Indofine	97%	6.0×10^{-5}	1.5×10^{-3}	−2.82
			Coumarins			
Coumestrol	479-13-0	Spectrum	NA	1.1×10^{-7}	8.2×10^{-1}	−0.05
4-Ethyl-7-hydry-3-(p-methoxyphenyl)-dihydro-1-benzopyran-2-one	5219-17-0	NCI	NA	1.1×10^{-7}	8.2×10^{-1}	−0.05
			Mycoestrogens			
α-Zearalenol	36455-72-8	Sigma	NA	2.9×10^{-9}	4.3×10^{1}	1.63
α-Zearalanol	26538-44-3	Sigma	NA	3.0×10^{-9}	3.0×10^{1}	1.48
Zearalanone	5975-78-0	Sigma	NA	4.3×10^{-8}	2.1×10^{0}	0.32
β-Zearalanol	42422-68-4	Sigma	98%	1.4×10^{-7}	6.4×10^{-1}	−0.19
β-Zearalenol	71030-11-0	Sigma	NA	4.4×10^{-7}	2.0×10^{-1}	−0.69

[1] Aldrich, Milwaukee, WI.
[2] Fluka Chemical, Milwaukee, WI.
[3] Indofine Chemical, Somerville, NJ.
[4] Sigma, St. Louis, MO.
[5] Spectrum Chemical, Gardena, CA.
[6] Toronto Research Chemicals, North York, Ontario, Canada.
[7] United States Biochemical, Cleveland, OH.
[8] Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.
[9] Purity information as provided by the vendor/manufacturer.
[10] The data represent the means of at least two independent binding assays.
[11] CAS, Chemical Abstracts Service; RBA, relative binding affinity; NA, not available; NB, nonbinder; SB, slight binder.

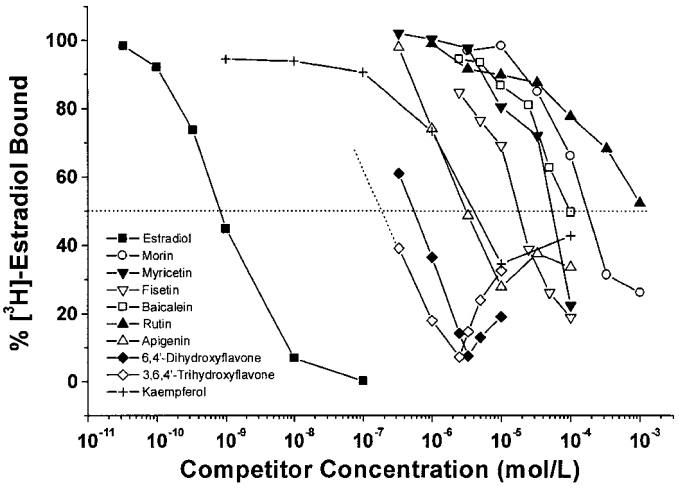

FIGURE 1 Competition curves for flavone binding to rat uterine cytosolic estrogen receptor (ER). In this assay, [^{3}H]-17β-estradiol (E_2) is competing for binding to the ER with increasing concentrations of either nonradiolabeled E_2 (assay standard) or the flavones. Each datum represents the mean of at least 2 independent binding assays. The competitor concentration at 50% reduction in [^{3}H]-E_2 binding (IC_{50}) is found at the intersection of the binding curves with the 50% binding line (· · · · · ·).

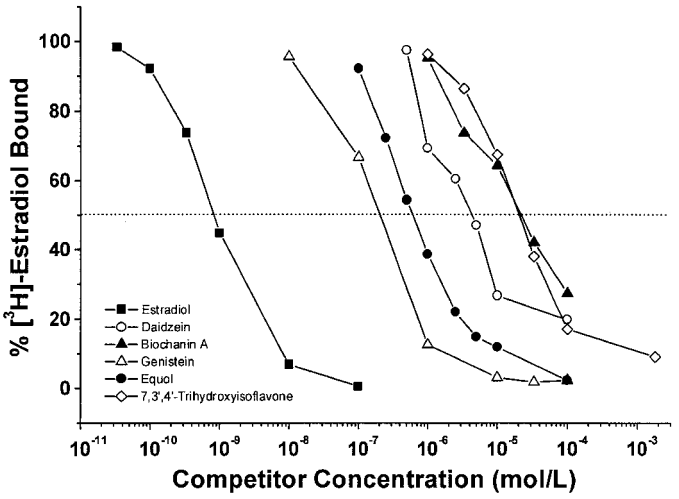

FIGURE 3 Competition curves for isoflavone binding to rat uterine cytosolic estrogen receptor (ER). In this assay, [^{3}H]-17β-estradiol (E_2) is competing for binding to the ER with increasing concentrations of either nonradiolabeled E_2 (assay standard) or the isoflavones. Each datum represents the mean of at least 2 independent binding assays. The competitor concentration at 50% reduction in [^{3}H]-E_2 binding (IC_{50}) is found at the intersection of the binding curves with the 50% binding line (· · · · · ·).

droxyflavone bound to ER, hydroxylation of carbon-6 conferred weak binding. Placement of hydroxyls at the 5 and 7 positions (apigenin) or an additional hydroxyl at the 3 position (kaempferol) reduced ER binding by ~90% compared with 3,6,4′-trihydroxyflavone. Removal of hydroxyls from the C-ring reduced ER binding (baicalein) or eliminated ER binding (chrysin) as did increasing the number of hydroxyls (fisetin, myricetin, morin, quercetin). Only one methoxyflavone (6-hydroxy-2′-methoxyflavone), which failed to bind ER, was assessed.

Five of the ten flavanones bound to the ER. Single hydroxylation on either the A- or C-rings (6- and 7-hydroxyflavanone and 3′- and 4′-hydroxyflavanone) conferred weak ER binding. This suggests that the phenolic A-ring in steroids is crucial for binding because it can form three hydrogen bonds with ER amino acids and water. Naringenin (the strongest ER-binding flavanone) differs from the flavone apigenin only by the absence of the double bonds at the 2 and 3 carbons of the B-ring. This marginally reduced naringenin ER binding by ~75%.

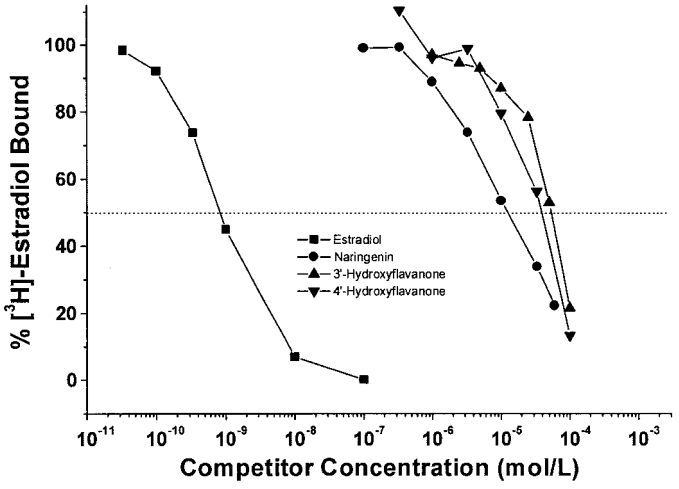

FIGURE 2 Competition curves for flavanone binding to rat uterine cytosolic estrogen receptor (ER). In this assay, [^{3}H]-17β-estradiol (E_2) is competing for binding to the ER with increasing concentrations of either nonradiolabeled E_2 (assay standard) or the flavanones. Each datum represents the mean of at least 2 independent binding assays. The competitor concentration at 50% reduction in [^{3}H]-E_2 binding (IC_{50}) is found at the intersection of the binding curves with the 50% binding line (· · · · · ·).

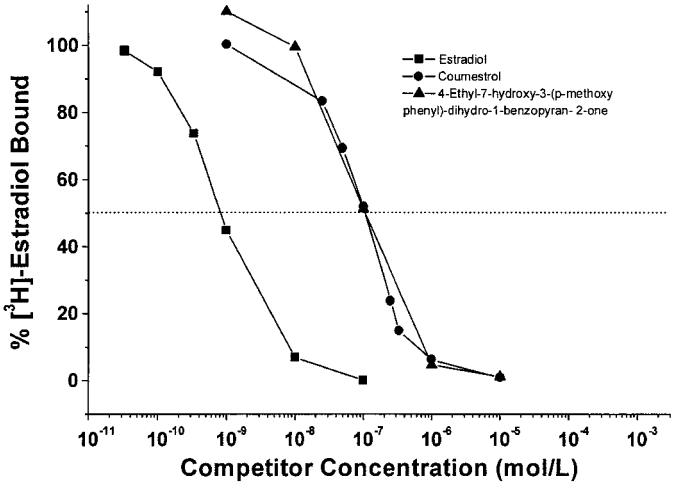

FIGURE 4 Competition curves for coumarin binding to rat uterine cytosolic estrogen receptor (ER). In this assay, [^{3}H]-17β-estradiol (E_2) is competing for binding to the ER with increasing concentrations of either nonradiolabeled E_2 (assay standard) or the coumarins. Each datum represents the mean of at least 2 independent binding assays. The competitor concentration at 50% reduction in [^{3}H]-E_2 binding (IC_{50}) is found at the intersection of the binding curves with the 50% binding line (· · · · · ·).

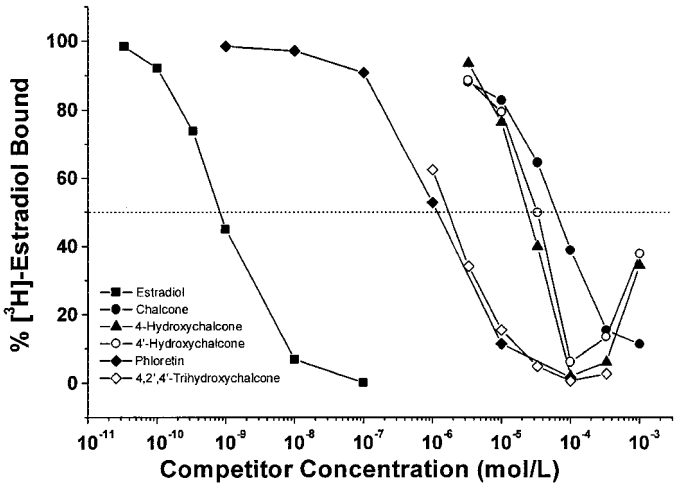

FIGURE 5 Competition curves for chalcone binding to rat uterine cytosolic estrogen receptor (ER). In this assay, [^{3}H]-17β-estradiol (E_2) is competing for binding to the ER with increasing concentrations of either nonradiolabeled E_2 (assay standard) or the chalcones. Each datum represents the mean of at least 2 independent binding assays. The competitor concentration at 50% reduction in [^{3}H]-E_2 binding (IC_{50}) is found at the intersection of the binding curves with the 50% binding line (· · · · · ·).

Hydroxylation of the basic isoflavone structure at the 5,7,4′ position (genistein) or at the 7,3′,4′ positions (7,3′,4′-trihydroxyisoflavone) resulted in a binding affinity of about 0.45% or 0.00045% of E_2, respectively. Conversion of genistein to daidzein by removal of the hydroxyl at the 5 position reduced the affinity for the ER by ~95%. Removal of the ketone oxygen at the 4 position and the double bond between C-2 and C-3 in the B-ring (equol) increased ER binding compared with daidzein. Replacement of the 7-hydroxyl of genistein by a methoxy group, thus converting genistein to prunetin, reduced binding to 0.4% of E_2.

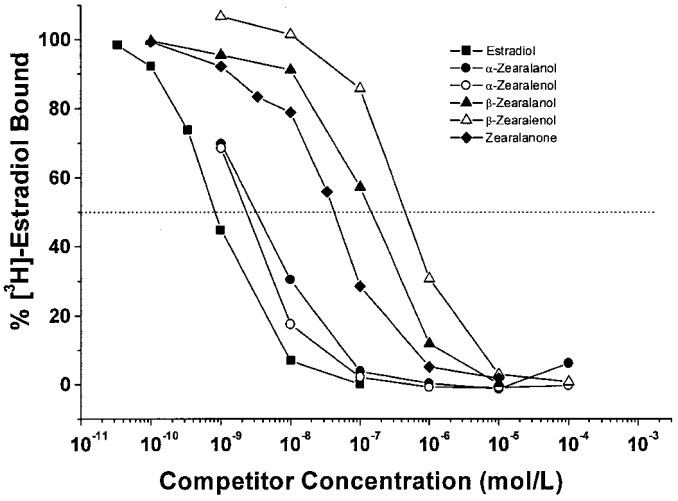

FIGURE 6 Competition curves for mycoestrogen binding to rat uterine cytosolic estrogen receptor (ER). In this assay, [^{3}H]-17β-estradiol (E_2) is competing for binding to the ER with increasing concentrations of either nonradiolabeled E_2 (assay standard) or the mycoestrogens. Each datum represents the mean of at least 2 independent binding assays. The competitor concentration at 50% reduction in [^{3}H]-E_2 binding (IC_{50}) is found at the intersection of the binding curves with the 50% binding line (· · · · · ·).

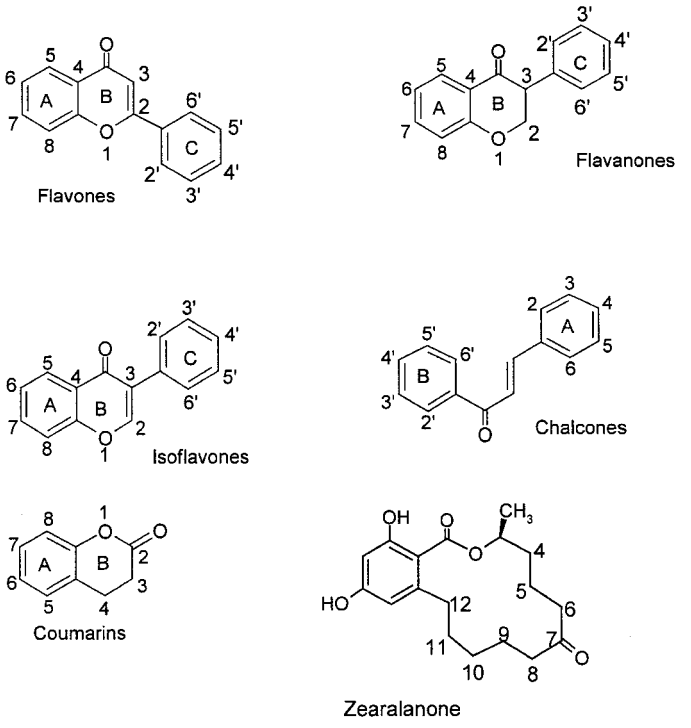

FIGURE 7 Generalized molecular structures for the six categories of chemicals examined. Ring labeling of carbon atoms allows identification and location of substituent groups.

Chalcone, a nonhydroxylated chemical, exhibited weak binding to the ER, i.e., 0.0015% that of E_2. Monohydroxylation of the chalcone structure at the 4- or 4′-carbons bound to ER with ~0.003% of the affinity of E_2, a slight increase in ER binding. Chalcones with multiple hydroxyls such as 4,2′ 4′-trihydroxychalcone and 4,2′,4′,6′-tetrahydroxychalcone (prunetin) competed with [^{3}H]-E_2 for ER with ~0.069% and 0.054% lower affinity than E_2, respectively.

Only two coumarins [coumestrol and 4-ethyl-7-hydroxy-3-(p-methoxyphenyl)-dihydro-1-benzopyran-2-one] were assessed for ER binding. These each bound to ER with an affinity 0.9% that of E_2.

In the group of mycoestrogens selected for assay, the "-anones" differ from the "-enones" by the absence of a double bond between carbons 11 and 12. Zearalanone bound ER with ~2% lower affinity than E_2. Conversion of the C-7 ketone oxygen to a hydroxyl in the α stereochemical arrangement increased the ER binding to ~43% (α-zearalenol) and 30% (α-zearalanol) that of E_2. Shifting these hydroxyls to the β arrangement reduced ER binding to about 0.6% that of E_2 (β-zearalanol) and 0.2% that of E_2 (β-zearalenol).

DISCUSSION

We used a competitive binding assay for the rat uterine ER, in which receptor and ligand concentrations were optimized for chemical throughput (26). Each assay included a complete competition curve for E_2 (positive control) and a negative control (ethanol). The in vitro ER binding assay measures a single bimolecular event, i.e., the reversible binding of a compound to the ER under equilibrium binding conditions established under tightly controlled chemical and physical parameters. This is demonstrated by the simple sigmoid curve on a semilog plot of the reference standard, E_2. Two features of the

binding curves for several of the phytoestrogens warrant comment. First, four compounds (3′-hydroxyflavanone, 4′-hydroxyflavanone, 4-hydroxychalcone and 4′-hydroxychalcone) yielded binding curves that exhibited an increase in slope in the linear portion of the curve below 50% binding compared with both the E_2 standard and their chemical class counterparts. Whether or not this represents evidence of a multistep ER binding mechanism is not known. However, there was little or no effect on the IC_{50}.

Second, several of the flavones (6,4′-dihydroxyflavone, 3,6,4′-trihydroxyflavone and apigenin) and chalcones (4-hydroxychalcone and 4′-hydroxychalcone) exhibited biphasic curves in which there was an increase in the percentage of [^{3}H]-E_2 bound at concentrations above 85% of maximal competition. This biphasic curve suggests that very high concentrations of some competitors caused more [^{3}H]-E_2 to bind to the ER. Borgna and Ladrech (34) showed that the dissociation rate of the ER-[^{3}H]-E_2 complexes increases at high concentrations of competitor. Although this would establish a different equilibrium for [^{3}H]-E_2, this should result in a lowering of apparent [^{3}H]-E_2 binding instead of an increase. It has been suggested (personal communication, Dr. V. J. Kramer, Rohm and Haas, Spring House, PA) that the higher apparent ER binding could be an artifact caused by precipitation of these chemicals at high concentrations, carrying [^{3}H]-E_2 into the HAP pellet. However, the HAP pellet was washed 4 times, which should dilute the high competitor concentrations. Also, because the ER is present in the assay as a high speed supernatant of a cytosolic extract, the high competitor concentrations may cause [^{3}H]-E_2 to bind to other cytosolic proteins, resulting in greater nonspecific binding. Clearly, this phenomenon is not understood.

Recent crystal structure analysis of four ligand-receptor complexes has indicated that the phenolic ring, normally the A-ring in steroids or phytoestrogens, is crucial for binding because it forms three hydrogen bonds with ER amino acids and water (35–37). However, a recent crystal structure for ER-β and genistein showed that the phenolic C-ring served to form the hydrogen bonds (37). This suggests that a chemical may bind in two orientations that differ by 180°. Our data showing that 3′-hydroxyflavanone and 4′-hydroxyflavanone have a phenolic C-ring important in binding suggests the possibility that "flipping" of these chemicals may occur in a manner similar to the situation for genistein binding to ER-β.

Although there are no comprehensive analyses of phytoestrogen or mycoestrogen binding to rat ER, our data are consistent with values for several of the more commonly studied compounds. Verdeal et al. (38), using rat uterine cytosol incubated for 2 h at 20°C and dextran-coated charcoal to remove unbound components, found the following relative binding affinities: E_2, 100; coumestrol, 4.9; genistein, 1.3; daidzein, 0.09; biochanin A, 0.07; and zearalenone, 4.0. Similarly, Wang et al. (39) reported an RBA of 0.9 for genistein and no ER binding for quercetin. Our data are generally within 20% of these values. Gutendorf and Westendorf (40) reported relative binding affinities for genistein (0.01) and coumestrol (0.12) that are lower than the results reported here and those cited above. Comparison of their ER binding data for "non-phytoestrogen" compounds with our previously published data (26) show their RBA to be consistently lower. Although these differences are small in some cases, we cannot explain them.

Mycoestrogens have been shown to be relatively potent inducers of uterine weight in rodents (41). Zearalanol, marketed as Ralgro (zeranol), has long been used as a growth promoter in cattle (42). The mycoestrogens examined here exhibited the greatest binding to ER of all chemical classes examined. The affinity of α-zearalanol to ER is ~30% lower than that for E_2, which is in agreement with previous assays (43,44). Using the yeast cell estrogen screening assay, Coldham et al. (45) showed that α-zearalanol induces an estrogen-responsive reporter gene at a level that is only 1.3% of that induced by E_2.

Measurement of estrogen binding to ER correlates with estrogenicity (46). For chemicals that do not show good correspondence for these two measures, differences in absorption, distribution, metabolism and elimination as well as ligand-specific differences in gene expression may provide an explanation. The estrogenicity of many of the more common phytoestrogens has been established by several measures. Using an in vitro rat endometrium–derived adenocarcinoma cell line, Hopert et al. (47) showed that coumestrol, genistein and daidzein induce production of the estrogen-regulated uterine protein, complement C3 (48,49). Similarly, genistein, biochanin A, daidzein, apigenin, kaempferol and coumestrol have been shown to increase DNA synthesis in MCF-7 cells (50,51). Our analyses indicate that these phytoestrogens are generally the more active ER binders. Although the estrogenic activity of phytoestrogens in humans is still under investigation, daily consumption of soy by postmenopausal women significantly increases the serum levels of several phytoestrogens (52). The serum levels of genistin, daidzin and equol increased by 756, 593 and 1008%, respectively.

Although other publications report ER RBA for structurally diverse data sets (27,53–56), these data, combined with another data set from our laboratory (26), represent the most comprehensive published data set for naturally occurring chemicals competing with [^{3}H]-E_2 for ER assessed under conditions of a single, rigorously validated assay. In addition, these data are sufficiently complete, comprising both ER binders and ER nonbinders, to allow determination of the effects of substituent groups on binding. As such, these data are ideal for input into various computer-based models for QSAR and SAR modeling (29,30). We are currently assessing binding of these dietary estrogens to both rat alphafetoprotein (in amniotic fluid) and human sex hormone binding globulin (in plasma from pregnant women). Together, these data sets, in the appropriate computer-based models, will be useful to predict the risk of estrogen exposure.

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