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Commonly Occurring Plant Flavonoids Have Estrogenic Activity

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SUMMARY

A remarkable diversity of naturally occurring and synthetic compounds have been shown to mimic the biological effects of 17β-estradiol by virtue of their ability to bind to and activate the nuclear estrogen receptor. This report extends the family of nonsteroidal estrogens to include several multiply hydroxylated chalcones, flavanones, and flavones. The hormone-like activity of these natural plant products is indicated by their ability to stimulate an estrogen receptor-dependent transcriptional response and to promote growth of estrogen-dependent MCF7 cells in culture. The transcriptional response can be inhibited by the steroidal estrogen antagonist ICI-164,384 and is specific for the estrogen receptor. Evidence is presented to show that selected hydroxylated flavonoids interact directly with the estrogen receptor, based on their ability to compete for the binding of 17β-[^{3}H]estradiol to the receptor in cell-free extracts. These compounds are less active, on a molar basis, than 17β-estradiol or the synthetic dihydroxystilbene estrogens, but they have potencies comparable to those of other known phytoestrogens. Together, these findings broaden our understanding of the structure-activity relationships for nonsteroidal estrogens and present a series of new chemical prototypes for the future development of potentially useful agonists and antagonists for this nuclear receptor. The wide distribution of weakly estrogenic flavonoid pigments in food crops and medicinal plants raises additional questions about the possible health risks and benefits of these compounds, meriting closer examination of their presence in the human diet.

Flavonoids are secondary metabolites that occur naturally in all plant families (1, 2). They can be isolated from many tissues in both flowering and nonflowering plants and they are usually found conjugated to sugars, although the nonconjugated forms (aglycones) have also been identified in nature. One of their principal roles in angiosperms is to provide the characteristic pigmentation of the petals of flowers. In addition, they are thought to serve as insect deterrents, natural fungicides (phytoalexins), and potential regulators of the plant hormone indoleacetic acid (2). The flavonoids occur in several structurally and biosynthetically related classes. Among these the flavones, flavonols (3-hydroxyflavones), and anthocyanins are widely distributed among plant species, whereas the flavanones, isoflavones, and chalcones are characteristic of a more limited number of plant families (1).

The isoflavones genistein, daidzein, and formononetin have previously been reported to be weak estrogens (3-6) and together with coumestrol (a structurally related plant coumarin) are believed to account for the ability of forage plants such as subterranean clover (*Trifolium subterraneum*) and alfalfa (*Medicago sativa*) to interfere with the reproductive capacity of livestock (7). There has also been an isolated report that the dihydrochalcone phloretin possesses modest estrogenic activity (8). A second line of evidence suggesting that structural similarity may exist between the steroidal nucleus of 17β-estradiol and the rigid ring structure that is characteristic of the flavonoids is the observation that several flavones (most notably, 7,8-benzoflavone, chrysin, and apigenin) act as inhibitors of aromatase cytochrome P-450 (9). This enzyme is responsible for synthesizing estrogens from their androgenic precursors, and the flavones exert their inhibition by competing with substrate for binding to the catalytic site of this enzyme. Although no sequence homology is known to exist between aromatase and the steroid receptors, the ability of aromatase to competitively bind both steroidal estrogens and the flavones may reflect a previously unrecognized conformational similarity between these two classes of compounds. For these reasons, experiments were undertaken to examine the ability of representative flavonoids to interact with the human estrogen receptor.

The system chosen for an analysis of the estrogenic activity of selected flavonoids is a transient gene expression assay utilizing HeLa cells in which an acute estrogen response is created by co-transfecting cultures with a recombinant estrogen receptor cDNA and an estrogen-dependent reporter plasmid. The estrogen receptor gene is introduced into these cells under the control of the plasmid pER18. Agonist activity of selected flavonoids is assessed by their ability to induce the expression

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ABBREVIATIONS: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's minimal essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ERE, estrogen response element.

of the plasmid pERE-TK-CAT (10), in which expression of the bacterial CAT gene is placed under the transcriptional control of the *Herpes simplex* thymidine kinase promoter and a palindromic estrogen receptor binding site from the vitellogenin A2 gene of *Xenopus laevis* (11). This assay is therefore based on measurement of a primary transcriptional response in which the accumulation of CAT enzyme reflects ligand-dependent activation of the estrogen receptor. More traditional assays for estrogenic compounds (based on their uterotrophic activity *in vivo* or their mitogenic activity towards several breast tumor cell lines in culture) suffer from the disadvantage that the mechanisms for these biological responses are complex and poorly understood. They require days to weeks of hormone treatment, and their results can be perturbed by metabolism or bioconversion of the estrogenic compounds being tested. Moreover, the fact that eight amino acid differences distinguish the primary sequences of the hormone-binding domains of the human and rodent estrogen receptors (12, 13) raises concern that estrogenicity assays performed in laboratory rodents may not invariably reflect the activity of the same compounds with the human receptor. A final advantage of the co-transfection assay is that it allows the estrogen receptor to be manipulated as a variable, a feature that is useful when controlling for the specificity of the hormone response. Using this assay, it can be shown that a variety of naturally occurring flavonoid pigments possess a heretofore unrecognized estrogen agonist activity.

Materials and Methods

Chemicals. Sources for the chemicals used in this study include Sigma Chemical Co. (17β-estradiol, apigenin, flavone, flavanone, naringenin, phloretin, zearalenone, and β-zearalenol), Fluka (kaempferol), and ICN Biomedical (genistein). Chalcone, isoliquiritigenin, 4,4′-dihydroxychalcone, 2′,4′,4,6′-tetrahydroxychalcone, and 4′,7-dihydroxyflavanone were kindly provided by Dr. Rodney Bednar (State University of New York at Stony Brook). The estrogen antagonist ICI-164,384 was a generous gift from A. E. Wakeling (Imperial Chemical Industries). The purity of each of the chemicals used in this study was confirmed by thin layer chromatography on silica G plates (Machery-Nagel) developed in 90% chloroform/10% acetic acid and visualized under UV illumination.

Plasmids. Expression studies in HeLa cells were performed with the plasmid pER-18,[1] which contains a human estrogen receptor cDNA (*Tth*III 1 to *Sst*I) cloned into the *Hinc*II site of the eukaryotic expression plasmid pHCMV-TK-Blue A$^+$.[2] The cDNA present in pER-18 encodes a nonmutated (i.e., glycine at position 400) estrogen receptor and was isolated in this laboratory by reverse transcription of human myometrial mRNA, followed by amplification of estrogen receptor sequences using the polymerase chain reaction. Higher levels of receptor expression necessary for ligand-binding studies were achieved using the plasmid pCMV-ER in a COS-7 transient expression system. pCMV-ER was constructed by inserting the same region of the human estrogen receptor cDNA into the *Bgl*II site of pCMV-4 (14), with the use of *Bam*HI linkers.

Cell culture. HeLa and COS-7 cells were routinely maintained in phenol red-free DMEM containing 10% (v/v) calf serum and supplemented with 5 mM HEPES, pH 7.4, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine. Cells were plated at a density of 2×10^6/100-mm dish 1 day before transfection.

Transfection studies. Cells were transfected using a standard calcium phosphate coprecipitation method (15). Transactivation studies were performed using HeLa cells transfected with 1 μg of the estrogen receptor expression plasmid pER-18 and 16 μg of the estrogen-responsive reporter plasmid pERE-TK-CAT (10). After 5–6 hr of exposure to the calcium phosphate/DNA coprecipitates, cells were treated for 3 min with 15% (v/v) glycerol in DMEM, to increase the level of CAT enzyme expression. Cells were then maintained for 48 hr in DMEM containing 5% (v/v) charcoal-treated calf serum supplemented at the concentrations indicated with estrogens, flavonoids, or an equivalent volume of the ethanol vehicle. Cell-free lysates of the transfected cultures were prepared by five cycles of freezing and thawing in 0.25 M Tris·HCl, pH 8, and CAT enzymatic assays were performed on aliquots representing 100 μg of protein (16). Enzymatic activity is expressed as pmol of chloramphenicol acetylated/mg of lysate protein/min at 37°.

Competition binding studies. The relative affinities of flavonoids for the estrogen receptor were determined by their ability to compete with 17β-[^{3}H]estradiol for binding to the estrogen receptor expressed in COS-7 cells in culture. COS-7 cells were transfected with 10 μg of pCMV-ER, as described above. Two days after transfection, cells were harvested by scraping and the resulting cell pellets were sonicated in extraction buffer (0.4 M KCl, 20 mM Tris·HCl, pH 7.4, 1 mM EDTA, 2 mM dithiothreitol, 10 mM sodium molybdate, 10%, v/v, glycerol) supplemented with 1 μg/ml concentrations of each of the protease inhibitors aprotinin, leupeptin, pepstatin, and chymostatin. Postmitochondrial supernates were prepared from the sonic extracts by centrifugation at 45,000 rpm for 60 min at 4° in a Ti75 rotor. Estradiol-binding activity was determined using 17β-[2,4,6,7,16,17(N)-^{3}H]estradiol (170 Ci/mmol; New England Nuclear), according to the method of Scatchard (17), and was consistently in the range of 1–5 pmol/mg of protein, with an equilibrium binding constant (K_d) of 0.3–1.2 nM.

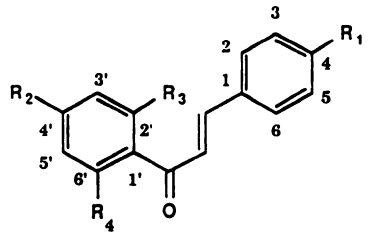
Binding affinities relative to 17β-estradiol were determined by incubating 100 μg of protein from sonic extracts of pCMV-ER-transfected COS-7 cells with 10 nM 17β-[^{3}H]estradiol in the presence of increasing concentrations of the competing flavonoid for 2 hr at room temperature. Specific binding, defined as 17β-[^{3}H]estradiol bound by receptor in the presence of unlabeled competitor, was determined as radioactivity resistant to adsorption by dextran-coated charcoal (18).

MCF7 cell growth curves. To determine the effects of selected flavonoids on the growth of MCF7 cells in estrogen-depleted medium, cells were plated at a density of 5×10^3 cell/cm^2 in 60-mm dishes and grown for 12 days in phenol red-free DMEM containing 5% (v/v) charcoal-treated calf serum supplemented with 5 mM HEPES, pH 7.4, 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM glutamine, and 0.6 μg/ml bovine insulin. Medium was changed every second day throughout the course of the experiment and the cells were maintained in the continuous presence of hormones or flavonoids at the concentrations indicated. Determination of the number of viable cells was performed using a hemocytometer, after dispersion of the cells by Versene-trypsin.

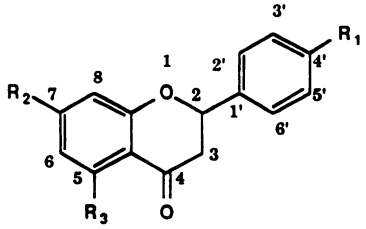
Results

Flavonoids function as weak estrogens. When tested in a transient co-transfection assay, several of the flavonoids and the related open-chain chalcones depicted in Fig. 1 appeared to support an estrogen response nearly as strong as that produced by 17β-estradiol itself (Fig. 2). Transcription of the estrogen-responsive reporter plasmid (pERE-TK-CAT) was induced 15-fold by 17β-estradiol (Fig. 2, *lane 2*), relative to the ethanol vehicle (Fig. 2, *lane 1*). For comparison, transfected cells were also treated with the known mycoestrogens zearalenone and zearalenol (Fig. 2, *lanes 3* and *4*) and the phytoestrogen phloretin (Fig. 2, *lane 6*), which supported inductions of approximately 15-, 9-, and 3-fold, respectively, above the control level. Among the flavonoids tested in this experiment, apigenin (4′,5,7-trihydroxyflavone), naringenin (4′,5,7-trihydroxyflavanone), isoliquiritigenin (2′,4,4′-trihydroxychalcone), and 4′,7-

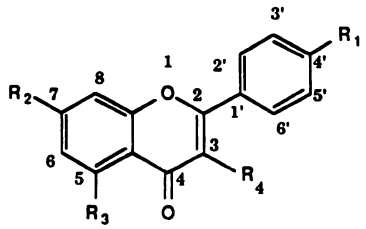
[1] Y. Wang and R. J. Miksicek, unpublished construct.
[2] R. J. Miksicek, unpublished construct.



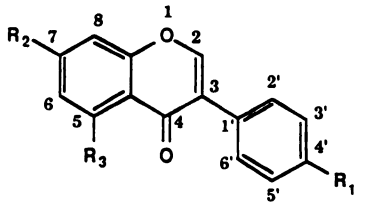
Chalcones	R1	R2	R3	R4
4,4'-dihydroxy	-OH	-OH	-H	-H
2',4,4'-trihydroxy (Isoliquiritigenin)	-OH	-OH	-OH	-H
2',4,4',6'-tetrahydroxy	-OH	-OH	-OH	-OH
2',4,4',6'-tetrahydroxy dihydrochalcone (Phloretin)	-OH	-OH	-OH	-OH



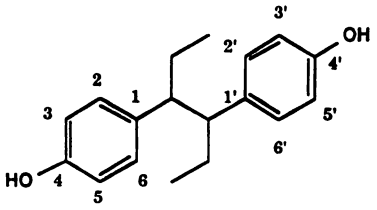
Flavanones	R1	R2	R3
4',7-dihydroxy	-OH	-OH	-H
4',5,7-trihydroxy (Naringenin)	-OH	-OH	-OH



Flavones	R1	R2	R3	R4
4',5,7-trihydroxy (Apigenin)	-OH	-OH	-OH	
3,4',5,7-tetrahydroxy (Kaempferol)	-OH	-OH	-OH	-OH



Isoflavones	R1	R2	R3
4',5,7-trihydroxy (Genistein)	-OH	-OH	-OH



Dihydroxy Stilbenes	Bridge Conjugation
Hexestrol	CH3-CH2-CH-CH-CH2-CH3
Diethylstilbestrol	CH3-CH2-C=C-CH2-CH3

Fig. 1. Structures of estrogenic flavonoids used in this study (hydroxychalcones, flavanones, flavones, and isoflavones) and their similarity to diphenylethylene estrogens.

dihydroxyflavanone increased CAT enzymatic activity noticeably above background levels (Fig. 2, *lanes 7-10*), with inductions ranging from 4- to 10-fold. Naringenin chalcone (2′,4,4′,6′-tetrahydroxychalcone) is typical of a large number of additional flavonoids including chalcone, flavone, flavanone, and several of their monohydroxylated derivatives, which were without effect in this assay (Fig. 2, *lane 5*, and data not shown). The results of this experiment and >10 similar experiments screening these and a number of related compounds consistently indicated that the aromatic 15-carbon frames of flavone, flavanone, and chalcone are able to exert an estrogen response when they carry two or three hydroxyl substituents in an appropriate configuration (see Fig. 1). The estrogenicity of these flavonoids can be understood in view of the superficial similarity between these compounds and the dihydroxystilbene estrogens (diethylstilbestrol and hexestrol).

The biological activity of flavonoids is mediated by the estrogen receptor. In light of recent reports that dopamine and okadaic acid (19) are able to stimulate the transcriptional activity of the estrogen receptor independently of ligand, experiments were designed to further characterize the response of the estrogen receptor to the active flavonoids. Fig. 3A indicates that the induction of CAT activity by the flavonoids (isoliquiritigenin and 4′,7-dihydroxyflavanone) shares with that produced by 17β-estradiol a dual requirement for the estrogen receptor and the presence of an ERE in the co-transfected reporter plasmid. Parallel experiments in which flavonoids were used to treat HeLa cells co-transfected with either the glucocorticoid receptor or the androgen receptor and an appropriate hormone-responsive reporter plasmid (pMMTV-CAT) (20) failed to elicit any increase in CAT expression in response to phloretin, 4,4′-dihydroxychalcone,

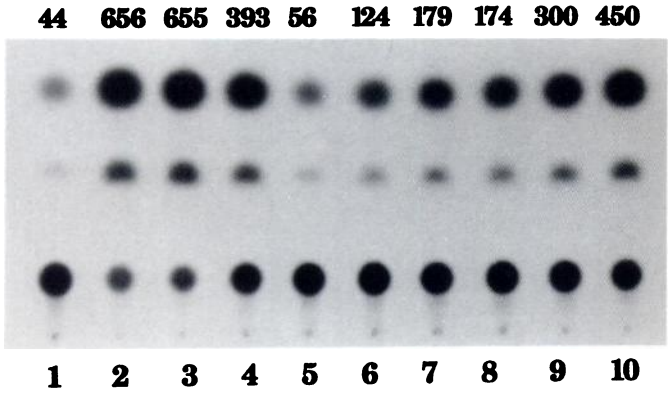
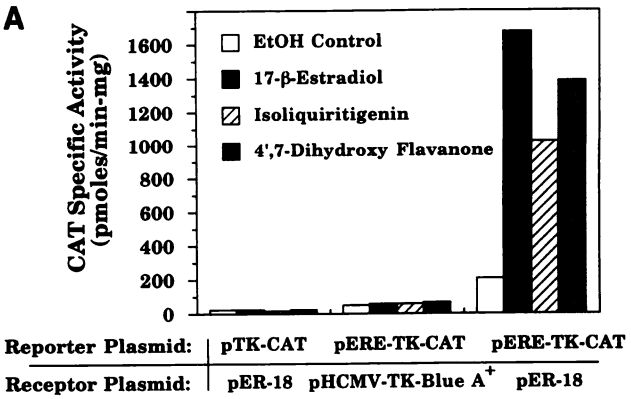


Fig. 2. Induction of an estrogen response by selected flavonoids. HeLa cells were transiently transfected with a $CaPO_4$ coprecipitate containing the estrogen receptor expression plasmid pER-18 (1 μg) and the hormone-responsive reporter plasmid pERE-TK-CAT (16 μg). Individual cultures were treated with ethanol vehicle alone, 5 nM 17β-estradiol, or 1 μM concentrations of the test compounds. *Numbers above the chromatogram*, CAT specific activity, in pmol of product generated/min/mg of cytosolic protein. The species represent the 3-acetylated or 1-acetylated [^{14}C]chloramphenol products and the unacetylated substrate, respectively, from *top* to *bottom*. The compounds tested included ethanol control (*lane 1*), 17β-estradiol (*lane 2*), zearalenone (*lane 3*), β-zearalenol (*lane 4*), 2′,4,4′,6′-tetrahydroxychalcone (*lane 5*), phloretin (*lane 6*), apigenin (*lane 7*), naringenin (*lane 8*), isoliquiritigenin (*lane 9*), and 4′,7-dihydroxyflavanone (*lane 10*).



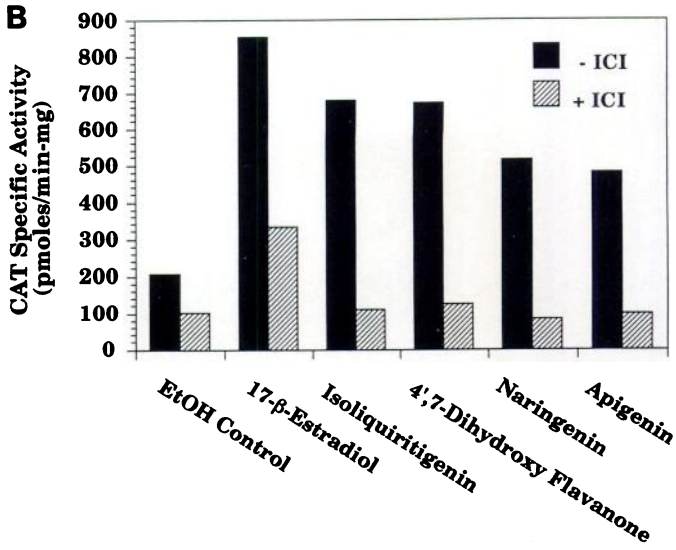


Fig. 3. Dependence of the stimulatory activity of flavonoids on the estrogen receptor and its cognate hormone response element. A, HeLa cells were co-transfected with reporter plasmids lacking (pTK-CAT) or containing (pERE-TK-CAT) a single copy of the vitellogenin A2 ERE and an expression plasmid without (pHCMV-TK-Blue) or with (pER-18) the estrogen receptor cDNA. CAT enzymatic activity was determined after treatment with 5 nM 17β-estradiol or 1 μM concentrations of the indicated flavonoids. B, Parallel co-transfections with pERE-TK-CAT and pER-18 were treated with 17β-estradiol (5 nM) or the indicated inducer (1 μM) in the absence or presence of 100 nM levels of the estrogen antagonist ICI-164,384 (*ICI*). *EtOH*, ethanol.

4′,7-dihydroxyflavanone, or genistein, indicating that these flavonoids are specific agonists for the estrogen receptor (data not shown). These results also rule out the possibility that these compounds have a general stimulatory effect upon the activity of the thymidine kinase promoter or nonspecific effects on the transcriptional machinery of HeLa cells.

If the active flavonoids represent true ligands for the estrogen receptor and occupy the same binding site in the receptor as does 17β-estradiol, one would predict that their stimulatory activity would be sensitive to inhibition by simultaneous treatment of the cells with a steroidal estrogen antagonist. This result is shown in Fig. 3B, which demonstrates that the antiestrogen ICI-164,384 (21) was able to reduce the expression of pERE-TK-CAT induced not only by 17β-estradiol but also by isoliquiritigenin, 4′,7-dihydroxyflavanone, naringenin, and apigenin. Thus, when analyzed for activity using this transient transfection assay, these flavonoids display all of the characteristics of true agonists for the estrogen receptor.

The estrogenic flavonoids show activity at micromolar concentrations. The data presented above (Figs. 2 and 3) suggest that the flavonoids are less potent agonists than 17β-estradiol and that higher concentrations are required before their estrogenic activity becomes manifest. Results from an examination of the concentration dependence of this effect are given in Fig. 4. The synthetic estrogen hexestrol is a highly effective agonist for the human estrogen receptor, producing an induction of CAT enzymatic activity that is half-maximal at 0.2 nM, similar to the potency of 17β-estradiol (data not shown). In contrast, the potencies of the hydroxylated chalcones, flavones, and flavanones are significantly lower, requiring concentrations in the range of 0.1–1 μM for half-maximal responses. These flavonoids therefore display EC_{50} values similar to those previously reported for the known phytoestrogens phloretin and genistein (Ref. 3 and Fig. 4). Based upon these data and additional dose-response curves not shown, a ranking of the estrogenic potency of these compounds is as follows: 17β-estradiol = hexestrol ≫ 4,4′-dihydroxychalcone = isoliquiritigenin = genistein > phloretin = 4′,7-dihydroxyflavanone = apigenin = naringenin. Although these flavonoids are 10^3- to 10^4-fold less potent at inducing a biological response than are the steroidal estrogens or the hydroxystilbenes, they are able to generate an estrogen response of the same magnitude as that produced by the physiological hormone at concentrations in the micromolar range.

Estrogenic flavonoids compete with 17β-estradiol for binding to the estrogen receptor. It is conceivable that the flavonoids characterized above activate the estrogen receptor by a mechanism that does not involve direct binding to the receptor polypeptide. This possibility is given added weight by the observation that the isoflavone genistein, which has been previously shown to mimic the effects of 17β-estradiol by binding to the estrogen receptor (4), is also able to inhibit the activity of several protein tyrosine kinases (22) with an IC_{50} of

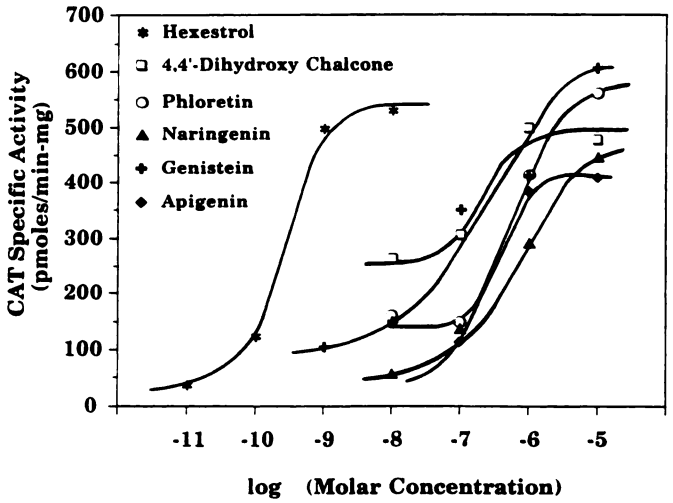


Fig. 4. Concentration dependence of transcriptional activation by selected flavonoids. CAT-specific activity was measured in HeLa cells cotransfected with pER-18 and pERE-TK-CAT. Independent cultures were analyzed after 48 hr of treatment with increasing concentrations of hexestrol or selected flavonoids, as indicated.

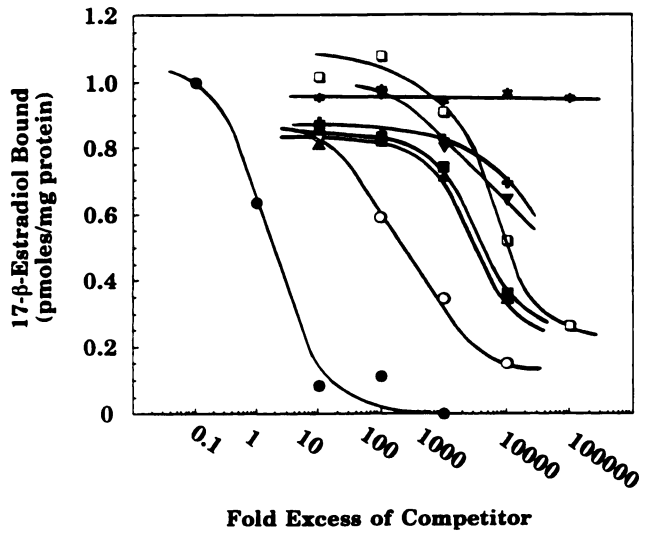


Fig. 5. Competition binding of flavonoids to the human estrogen receptor. A dextran-coated charcoal assay was used to determine the ability of increasing concentrations of flavonoids to compete with 17β-[^{3}H]estradiol for binding to the estrogen receptor expressed in COS-7 cells from the cDNA expression plasmid pCMV-ER. Two days after transfection, cytosolic extracts were incubated for 2 hr at 25° with 10 nM 17β-[^{3}H]estradiol plus the indicated competitor. Protein-bound counts were corrected for nonspecific binding in the presence of a 1000-fold excess of unlabeled 17β-estradiol. ●, 17β-Estradiol; ○, genistein; ■, 4,4′-dihydroxychalcone; ▲, isoliquiritigenin; ▼, 4′,7-dihydroxyflavanone; □, apigenin; +, naringenin; *, flavone.

2–20 μM. It has been reported that the estrogen receptor is a target for tyrosine phosphorylation (23) and that this phosphorylation is able to play a regulatory role in receptor function (24). It was therefore important to establish the mechanism by which the chalcones, flavones, and flavanones activate the estrogen receptor. The ability of the estrogen antagonist ICI-164,384 to inhibit stimulation of pERE-TK-CAT by the flavonoids (Fig. 3B) strongly implies that the flavonoids act by binding directly to the estrogen receptor.

Competition binding experiments were performed to test this hypothesis, and the results are given in Fig. 5. Increasing concentrations of 4,4′-dihydroxychalcone, 4′,7-dihydroxyflavanone, apigenin, and to a lesser extent isoliquiritigenin and naringenin all gave progressive competition for the binding of 10 nM 17β-[^{3}H]estradiol to the human estrogen receptor expressed in COS-7 cells. This competition was somewhat less than that shown by the isoflavonoid genistein, whereas the nonhydroxylated compound flavone was completely unable to compete with estrogen for binding to the receptor. The relative binding affinities shown by the flavonoids (3000–10,000-fold lower than that of 17β-estradiol) predict equilibrium dissociation constants (K_d values) that cluster in the range of 0.9–3 μM. This is in good agreement with the *in vivo* potencies of these compounds in a transient transfection assay (Fig. 4). Taken together, these data indicate that di- and trihydroxylated derivatives of chalcone, flavone, and flavanone are able to bind to the human estrogen receptor at micromolar concentrations and to serve as effective estrogen agonists. The ability of these flavonoids to bind competitively with 17β-estradiol to the ligand binding site of the estrogen receptor makes it less likely that their activating effect involves post-translational modification of the estrogen receptor, such as a change in its phosphorylation state. It does not, however, rule out the possibility that some or all of these compounds may also share the tyrosine kinase-inhibitory activity of genistein and exert secondary effects through this mechanism.

Selected flavonoids satisfy the estrogen requirement for growth of MCF7 cells. To independently confirm the observation that hydroxylated flavonoids such as 4,4′-dihydroxychalcone and apigenin are able to mimic the activity of 17β-estradiol, an experiment was undertaken to assess their effect on the proliferation of MCF7 cells. MCF7 represents an established human breast tumor cell line whose growth in cell culture depends upon the presence of estrogens. When these cells were maintained under steroid-depleted conditions, normal rates of growth could be restored by supplementation with physiological concentrations of 17β-estradiol, but not by estrogen antagonists such as ICI-164,384 (Fig. 6). Both 4,4′-dihydroxychalcone and apigenin had equivalent stimulatory effects on the proliferation of MCF7 cells, whereas flavone itself was without activity. Table 1 shows that continuous growth of these cells for 7 days in the presence of 17β-estradiol, 4,4′-dihydroxychalcone, or apigenin resulted in a significant increase in total cell number, relative to cells treated with the ethanol vehicle alone, and conversely that ICI-164,384 caused a significant inhibition of cell growth. These effects reflect an ability of both 17β-estradiol and the active flavonoids to reduce the doubling time of MCF7 cells nearly in half.

Discussion

The observations cited above demonstrate that a much wider spectrum of hydroxylated flavonoids possess weak estrogenic activity than has previously been appreciated. Although these estrogenic flavonoids are less potent than either 17β-estradiol or the estrogenic stilbenes, they appear to possess a pharmacological efficacy at optimal concentrations equivalent to that of the biological hormone (Figs. 4 and 6). It is well established that weak estrogens of plant origin, although they possess a potency only 10^{-3} to 10^{-4} times that of steroidal estrogens, can nonetheless have dramatic effects on the reproductive physiology of animals when they are present in sufficient quantities

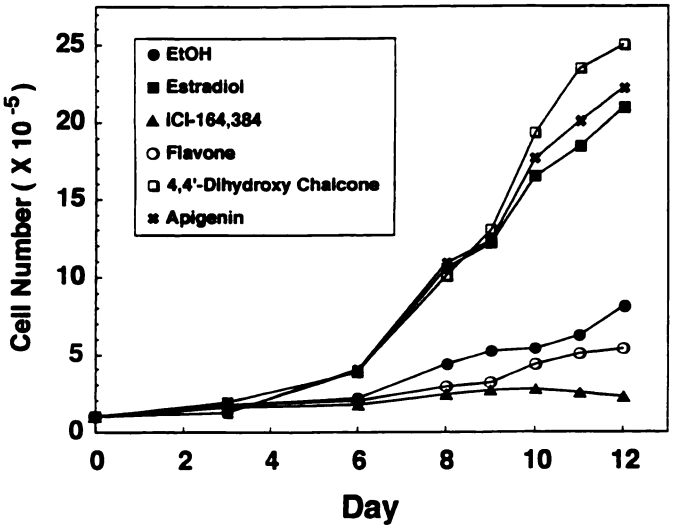


Fig. 6. Effects of steroids and flavonoids on the growth of MCF7 cells. MCF7 cells (1×10^5) were plated in 60-mm dishes, in phenol red-free DMEM containing 5% (v/v) charcoal-treated calf serum, on day 0 and were re-fed every second day. Cultures were harvested at the indicated times by trypsinization and counted. Each *point* represents the total number of viable cells per dish, averaged from duplicate determinations. Cells were maintained, as shown, in the continuous presence of 10 nM 17β-estradiol or 1 μM ICI-164,384, flavone, 4,4′-dihydroxychalcone, or apigenin. Control cultures contained 0.1% ethanol alone.

TABLE 1

Stimulation of MCF7 cell growth by flavonoids

MCF7 cells were plated in 60-mm dishes at a density of 5×10^3/cm² and cultured in estrogen-depleted medium as described for Fig. 6. One week after the addition of the indicated steroid or flavonoid, cells were suspended by trypsinization and counted. Values represent the mean ± standard deviation of total cell numbers from four independent dishes.

Supplement	Concentration	Cell number ($\times 10^{-5}$)	Cell doublings	Generation time
	M			*hr*
Ethanol		4.84 ± 0.59	2.3	73
17β-Estradiol	10^{-8}	14.0 ± 2.45[a]	3.8	44
ICI-164,384	10^{-6}	2.63 ± 0.22[b]	1.4	120
Flavone	10^{-6}	3.92 ± 0.93	2.0	84
4,4′-Dihydroxy-chalcone	10^{-6}	12.6 ± 1.23[b]	3.7	45
Apigenin	10^{-6}	12.7 ± 0.71[b]	3.7	45

[a] $p < 0.01$ versus ethanol control, by Student's *t* test.
[b] $p < 0.005$ versus ethanol control, by Student's *t* test.

in the diet (7). They are generally dismissed as important human toxicants because the diversity of the human diet normally ensures that no single plant estrogen is present in sufficient amounts to have physiological consequences. It may be appropriate, however, to qualify this conclusion for individuals who habitually consume vegetarian or semivegetarian diets (25). Neither is it understood to what extent individual phytoestrogens can cooperate to activate the estrogen receptor when they are present simultaneously at concentrations that only partially activate the estrogen receptor. Prolonged supplementation of the diets of 25 otherwise asymptomatic postmenopausal women with a combination of soy flour, red clover sprouts, and linseed (species that are rich in flavonoids) was found to produce a significant degree of vaginal maturation and a modest depression in the serum concentration of follicle-stimulating hormone (26).

Concern has previously been voiced regarding the possible health consequences caused by the presence of estrogenic substances in the human diet (4). It had been thought that the naturally occurring estrogens of plant origin (which include coumestrol, phloretin, estrone, and the isoflavones) tend to be sporadic in their natural distribution and are largely limited to members of the family *Leguminosae* (Lotoideae and Trifolieae), which are not typically used for human consumption (2). Among the phytoestrogens described in this report, naringenin and its glycosides, although not widely distributed, represent the major bitter components of grapefruit, where they are concentrated mainly in the rind (2). Typical of most other flavanones, naringenin is restricted primarily to members of the citrus family (27).

The flavones and flavonols, however, include members that are much more widely distributed among plants, including many fruits, vegetables, and crop species. Flavones (e.g., apigenin) are an abundant group of pale yellow pigments characteristically found in flower petals, leaves, seeds, and fruits of flowering plants, with a high concentration (up to 7% of dry weight) in leafy vegetables (27). Flavonols are among the most ubiquitous of all flavonoids, being prominent constituents of the flowers and leaves of at least 80% of higher plant species. It has been estimated that the intake of 4-oxoflavonoids (i.e., flavones, flavanones, and flavonols) typically averages 160–175 mg/day in the United States and that total flavonoid consumption is likely to be as high as 1.0–1.1 g/day when all classes of flavonoids are considered (27). In particular, apigenin and kaempferol (3,4′,5,7-tetrahydroxyflavone), both of which are estrogenic (Figs. 2–4 and 6, and data not shown), are regarded as "major flavonoids" (1) because of their common occurrence among plants and their significant concentrations when they are present.

These findings renew questions regarding the possible effects that phytoestrogens, including the estrogenic flavonoids described here, may have when they are present in the human diet. Considerable controversy exists regarding the consequences of long term exposure to dietary estrogens when they are present at concentrations that fail to fully saturate binding to the estrogen receptor. Pharmacological doses of estrogens can in some cases induce mammary carcinogenesis in animal models (28) but they may also have an inhibitory effect on the growth of some transplantable breast tumors. The growth of breast tumors and tumor-derived cell lines that show a typical pattern of estrogen dependence (e.g., MCF7 cells) is stimulated by physiological concentrations of estrogens (Fig. 6). A divergent opinion is that weak estrogens, such as those found in plants, may function paradoxically as antiestrogens *in vivo* (4, 29) by protecting estrogen receptors from activation by the more potent steroidal agonists. This is inconsistent with the ability of two estrogenic flavonoids (4,4′-dihydroxychalcone and apigenin) to satisfy the estrogen requirement of MCF7 cells cultured in estrogen-depleted medium (Fig. 6). Indeed, these data suggest that dietary flavonoids have the potential to contribute to the growth of estrogen-dependent tumors in postmenopausal women, under conditions where 17β-estradiol is limiting.

This report also provides motivation for a more detailed look at the content of individual flavonoids in the human diet and an analysis of the pharmacology of flavonoid uptake and metabolism in humans, to better assess their potential benefits and risks. Among the health risks posed by inappropriate estrogen stimulation are unwanted antifertility effects and

possible promotion or progression of malignancies of the breast and female reproductive tract.

Compounds in this study that display estrogenic activity (including the dihydrochalcone phloretin) share at least two phenolic hydroxyl substituents located at positions 4′ and 7 of the flavone nucleus (corresponding to positions 4 and 4′, respectively, of the chalcone ring system). All of these flavonoids can be regarded as either open-chain or cyclized versions of 1,3-diphenyl-1-propene-3-one (Fig. 1), and in this respect they are reminiscent of the dihydroxystilbene estrogens (e.g., diethylstilbestrol) and the triphenylethylene estrogen antagonists (e.g., tamoxifen). Differences among the flavonoids that appear to be tolerated without compromising their estrogenic activity include closure of the central pyran ring and saturation of the bridge that links the two phenolic groups.

Structural parallels have been drawn between the estrogenic isoflavones (genistein and daidzein) and the principal physiological estrogen (17β-estradiol). These similarities include a planar ring system that contains a *p*-hydroxy-substituted A-ring and a second in-plane hydroxyl group located at a distance of approximately 12 Å from the first (30). All three features are also conserved among the active chalcones, flavones, and flavanones characterized in this study, as well as a number of flavanols (3-hydroxyflavones) that possess a similar degree of estrogenic activity (data not shown). The lower affinity of the flavonoids for the estrogen receptor, relative to the steroidal estrogens, is likely to reflect deviations in the ring system or the disposition of hydroxyl substituents from the optimal pattern present in 17β-estradiol. Further analysis of derivatives with alterations of the ring system, including changes in the heterocyclic substitution of the central ring and the addition of axial alkyl groups, will be required to determine whether congeners with inhibitory activity or increased affinity for the estrogen receptor can be identified. The various classes of weakly estrogenic flavonoids described above represent a fertile starting point for such studies (31).

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