Pergamon

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 2/3, pp. 153–160, 1994
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0960-0760/94 $7.00 + 0.00
0960-0760(94)E0035-J

Interaction of Naturally Occurring Nonsteroidal Estrogens with Expressed Recombinant Human Estrogen Receptor

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The interaction between the recombinant human estrogen receptor and a variety of nonsteroidal estrogens was studied using a transient transfection assay in mammalian cells. Eight naturally occurring compounds were confirmed to stimulate the transcriptional activity of the human estrogen receptor and to compete for the binding of radiolabeled 17β-estradiol to this protein. In order of biological potency, these were zearalenone, β-zearalenol, coumestrol, genistein, daidzein, phloretin, formononetin, and biochanin A. As with steroidal estrogens, the hormonal activity of these compounds was specific for the estrogen receptor and sensitive to inhibition by 4-hydroxytamoxifen and ICI-164,384. Evidence is also presented to indicate that the stimulatory activity of genistein is unrelated to the protein tyrosine kinase inhibitory activity of this isoflavone. These results demonstrate that a significant number of structurally diverse plant and fungal secondary metabolites exist in nature that may contribute to the total estrogen exposure of the human population.

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INTRODUCTION

Estrogens play an important hormonal role among all vertebrates. These hormones are crucial for normal gestation and embryonic development and occupy a central position in the regulation of fertility in adult females. Animal estrogens are exclusively steroidal compounds and the principal physiological estrogen in most species is 17β-estradiol, although both estrone and estriol also contribute to the total pool of estrogenic hormones [1]. In some mammalian species (equines), estrone is further metabolized to estrogenic derivatives such as equilenin, equilin, and dihydroequilin prior to their excretion [2]. It has long been appreciated that many plants and some fungi are also able to elaborate compounds which possess estrogenic activity in animals (phytoestrogens and mycoestrogens). Phytoestrogens are generally believed to be without a direct effect on the physiology of their host plant species, but it is frequently speculated that they may play a defensive role as chemo-deterrents for foraging herbivores [3] or in the response of plants to foliar pathogens [4].

Received 20 Sep. 1993; accepted 9 Feb. 1994.

There have been isolated reports that selected plant species are capable of synthesizing estrone [5, 6]; however, these reports have been contested. The majority of estrogens produced by plants appear to be secondary metabolites which are nonsteroidal in nature. The structural diversity of compounds which have been suggested to possess estrogenic activity in animals is enormous [7, 8], but for only a limited number of these compounds has their estrogenicity been thoroughly documented. Members of this latter group fall into one of four chemical categories: (1) coumestans (e.g. coumestrol), (2) resorcylic acid lactones (e.g. zearalenone, β-zearalenol) (3) isoflavones (e.g. genistein, daidzein), and (4) dihydrochalcones (e.g. phloretin) [4, 9]. Recent studies from this laboratory have shown that a number of additional multiply hydroxylated flavonoids (in particular apigenin, naringenin, isoliquiritigenin, and 4,4′-dihydroxychalcone) are also inherently estrogenic [10]. Hydroxystilbenes such as diethylstilbestrol (DES), while they are potent estrogens, are synthetic products which have not been reported to occur in nature. Other putative estrogens that have been less well studied with respect to their hormonal properties in animals include bisphenols (e.g. nordihydroguaiaretic acid, curcumin), plant saponins

(e.g. diosgenin, sarsasapogenin), sterols (e.g. β-sitosterol), and various organic acids (e.g. podocarpic acid, gibberellic acid) [7, 8, and references therein].

The purpose of this report was to perform a systematic analysis of secondary metabolites from plants and fungi which have been reported to possess estrogen agonist activity. This study was undertaken using a cell culture system in which these compounds were tested for their ability to directly stimulate the transcriptional activity of the human estrogen receptor. Many of the earlier reports pertaining to these compounds relied on the use of animal models to assess estrogenicity [11–13], raising the complication of possible species differences in their hormonal activity. This problem is avoided in the present study by comparing the effects of all compounds on the human receptor expressed in cell culture. In addition, the selection of a cell culture system in preference to a whole animal model tends to minimize the possibility of conversion of the test compounds to metabolites with altered activity. Those compounds which displayed estrogenic activity during the initial screening were subjected to more detailed analysis to further characterize their agonist activity *in vivo* and their ability to interact with the expressed estrogen receptor *in vitro*.

EXPERIMENTAL

Chemical reagents

The chemicals used in this study were obtained from the following sources: Sigma Chemical Co., St Louis, MO (17β-estradiol, DES, curcumin, nordihydroguaiaretic acid, β-zearalenol, zearalenone, phloretin, gibberellic acid), Aldrich Chemical Co., Milwaukee, WI (biochanin A, sarsasapogenin, diosgenin, podocarpic acid), Indofine Chem ical Co., Sommerville, NJ (coumestrol, daidzein, formononetin), Steraloids, Inc., Wilton, NH (hexestrol), ICN Biomedicals, Inc., Costa Mesa, CA (genistein), and GibcoBRL, Gaithersburg, MD [lavendustin A, calyculin, 2-hydroxy-5-(2,5-dihydroxybenzyl)-aminobenzoic acid]. Purity of the chemicals tested for their estrogenicity was confirmed by thin layer chromatography on silica G plates (Machery-Nagel, Düren, Germany) developed in chloroform–acetic acid (9:1 v/v) or toluene–ethyl acetate–formic acid (9:7:4 v/v). ICI-164,384 and 4-hydroxytamoxifen were kind gifts from A. Wakeling, ICI Pharmaceuticals.

Cell culture and transfection

The biological activity of putative nonsteroidal estrogens was determined using a transient transfection assay in HeLa cells. A wild-type, recombinant estrogen receptor cDNA was expressed from the plasmid pER-18* and activation of receptor was assessed using the estrogen-responsive reporter plasmid pERE-TK-CAT [14]. Conditions used for cell culture and transfection have been described previously [10]. Hormones and chemicals were added to the final concentrations indicated, as 1000× stock solutions prepared in 80% ethanol. Chloramphenicol acetyl transferase (CAT) activity was measured as described by Gorman *et al.* [15] and was calculated as pmol of chloramphenicol acetylated per min per mg of cytosolic protein. Plasmids used for the expression of additional steroid receptors were generously provided by R. Miesfeld (pRSV-GR [16] and p6R-AR [17]) and H. Gronemeyer (cPR0 [18]).

Competition binding analysis

The relative affinities of hormonally active nonsteroidal estrogens were determined by their ability to compete for binding of 17β-[^{3}H]estradiol to estrogen receptor expressed in COS-7 cells using a high copy expression vector (pCMV-ER*). Transfection, preparation of cellular extracts, and competition binding analysis were performed as described previously [10]. Binding reactions were incubated for 2 h at room temperature with 10 mM 17β-[2,4,6,7,16,17(*N*)-^{3}H]estradiol (170 Ci/mmol; New England Nuclear) in the presence of increasing concentrations of unlabeled competitor, followed by the addition of dextran-coated charcoal (DCC) and further incubation for 15 min at 4°C on ice. Hormone remaining bound to the receptor was defined as radioactivity resistant to adsorption by DCC.

RESULTS

A survey of literature on the topic of estrogenic principles found in plants and fungi [7, 8] suggested a list of 15 naturally occurring, nonsteroidal compounds (Table 1) which represent putative agonists for the estrogen receptor. To determine which among these compounds is actually able to stimulate the transcriptional activity of this receptor, they were tested at various concentrations in a HeLa cell culture system in which the human estrogen receptor was transiently expressed by transfecting the cells with a cloned receptor cDNA. Ligand-dependent activation of transcription was assessed by cotransfecting these cells with an estrogen-responsive reporter plasmid pERE-TK-CAT [14] whose efficient expression depends on the presence of the estrogen receptor and its activation by agonist [10]. Typical results from a preliminary screen of these chemicals is given in Table 1, confirming that compounds belonging to at least four classes (resorcylic acid lactones, dihydrochalcones, coumestans, and isoflavones) do indeed possess a significant degree of estrogenic activity in this cell culture system. Among those compounds tested, the most active nonsteroidal estrogens were zearalenone, β-zearalenol, coumestrol (all of which were active at 10^{-7} M), and genistein

*Wang Y. and Miksicek R. J., unpublished plasmid. Structures are available upon request.

Table 1. Stimulation of the transcriptional activity of the estrogen receptor by putative plant and fungal estrogens

Compound	Concentration (Mr)	Fold induction	Illustrative species[a]
17β-Estradiol	10^{-9}	8.0 ± 1.0	NA
β-Zearalenol	10^{-7}	8.2 ± 1.1	*Fusarium graminearum*
Zearalenone	10^{-7}	11.3 ± 1.1	*Fusarium graminearum*
Sarsasapogenin	10^{-6}	1.1	*Smilax ornata*
Diosgenin	10^{-6}	1.7	*Trillium erectum*
β-Sitosterol	10^{-6}	1.3	*ubiquitous*
Phloretin	10^{-6}	7.9 ± 0.9	*Malus sylvestris*
Coumestrol	10^{-7}	12.3 ± 2.1	*Trifolium repens, Medicago sativa*
Genistein	10^{-6}	9.9 ± 1.8	*Podocarpus spicata*
Biochanin A	10^{-6}	4.0 ± 1.0	*Trifolium praetense*
Daidzein	10^{-6}	7.6 ± 2.5	*T. praetense, Glycine max*
Formononetin	10^{-6}	4.7 ± 1.6	*T. praetense, T. subterraneum, G. max*
Curcumin	10^{-6}	0.4	*Curcuma longa*
Podocarpic acid	10^{-6}	2.7	*Podocarpus cupressina*
Gibberellic acid	10^{-6}	0.6	*Gibberella fujikuroi*
Nordihydroguaiaretic acid	10^{-6}	1.6	*Larrea divaricata*

HeLa cells were cotransfected with pER-18 and pERE-TK-CAT, followed by treatment for 48 h with the following compounds at the indicated concentrations. Estrogenic activity was assessed by induction of expression of the CAT reporter plasmid relative to identical plates treated with the ethanol vehicle alone. Data were compiled from 10 transfection experiments and are presented as mean ± standard error.

[a]The examples listed are plant and fungal species in which these compounds have been reported according to Farnsworth *et al.* [7, 8]. This list is not intended to represent a comprehensive description of the distribution of these compounds. NA, not applicable.

(which was effective at 10^{-6} M). Phloretin and daidzein appeared to be less active, but still significantly above the level observed for the remaining compounds or vehicle alone. None of the bisphenols, saponins, sterols, or organic acids induced expression of the reporter plasmid more than 2- to 3-fold above control levels. Generalizing from these results, the resorcylic acid lactones, isoflavones, coumestrol, and phloretin were judged to possess significant estrogenic activity and were selected for more detailed analysis.

The specificity of these compounds for the estrogen receptor was further examined in a series of cotransfection assays using plasmids to express receptors for three other classes of steroid hormones. As shown in Fig. 1, coumestrol, zearalenone, genistein, daidzein, and phloretin were able to support a significant induction of CAT enzymatic activity when tested with the estrogen receptor using pERE-TK-CAT as the reporter plasmid. None of these agents, however, had any effect on the activities of the androgen, glucocorticoid, or progesterone receptors tested in conjunction with an appropriate reporter plasmid (pMMTV-CAT [19]) under conditions where the cognate steroid hormones (testosterone, dexamethasone, and progesterone, respectively) were effective in inducing accumulation of CAT enzyme. These results indicate that each of these nonsteroidal compounds display strong specificity for the estrogen receptor. This contrasts with 17β-estradiol and estrone themselves which paradoxically possess a low affinity for the androgen receptor [20] as well as an ability to partially activate the androgen receptor in a HeLa cell cotransfection assay*.

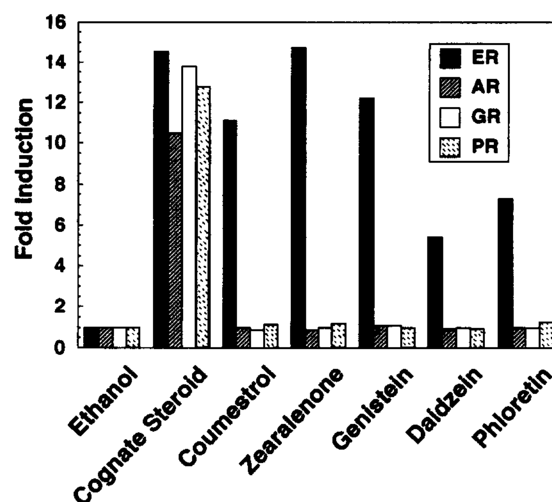

Fig. 1. Receptor specificity of transcriptional activation by nonsteroidal estrogens. HeLa cells were cotransfected with an estrogen receptor expression plasmid and pERE-TK-CAT or vectors expressing the androgen, glucocorticoid, or progesterone receptors along with pMMTV-CAT, as indicated. Prior to analyzing cultures for CAT enzymatic activity, the cells were cultured 48 h in the absence or presence of 10 nM estradiol (ER), 100 nM testosterone (AR), 100 nM dexamethasone (GR), or 100 nM progesterone (PR), indicated as "cognate steroid." In addition, parallel cultures containing one of the four steroid receptors were treated individually with coumestrol (0.1 μM), zearalenone (0.1 μM), genistein (1 μM), daidzein (1 μM), or phloretin (1 μM). Shown are induction ratios obtained by normalizing CAT specific activity in treated cells to that measured in parallel cultures given the ethanol vehicle alone.

*Miksicek R. J., unpublished observations.

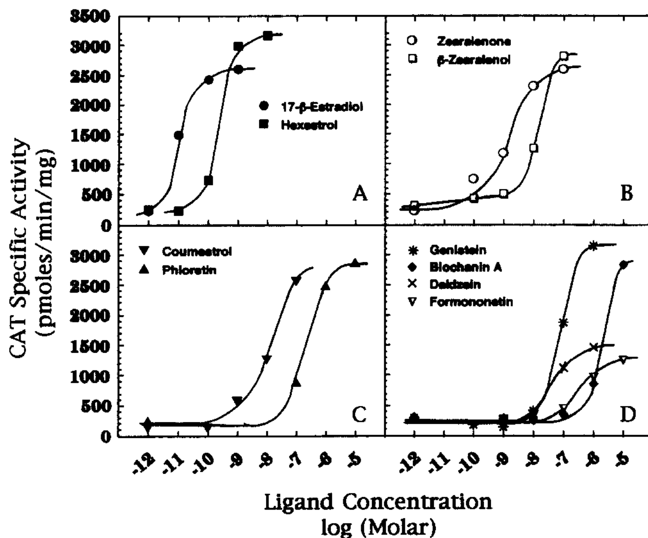

Fig. 2. Concentration dependence of the transcriptional stimulatory effect of nonsteroidal estrogens on the estrogen receptor. HeLa cells, cotransfected with pER-18 and pERE-TK-CAT, were treated with increasing concentrations of 17β-estradiol (●), hexestrol (■), zearalenone (○), β-zearalenol (□), coumestrol (▼), phloretin (▲), genistein (a), biochanin A (◆), daidzein (x), or formononetin (▽), as indicated. CAT specific activity was measured following 48 h of hormone treatment. Shown are individual determinations from representative experiments.

The potencies of these nonsteroidal estrogens were next examined by comparing the concentration dependence of their ability to stimulate the transcriptional activity of the estrogen receptor in the HeLa cell transient transfection assay. Results from these experiments are summarized in Fig. 2. They indicate that, while all of the plant and fungal estrogens are less potent on a molar basis that 17β-estradiol, most of them can stimulate the activity of the estrogen receptor to the same maximal extent as the natural hormone. Daidzein and formononetin would appear to be exceptions to this generalization since they behave as only partial agonists in this assay. A rank ordering of the estrogenic potencies of these compounds based on the inflection points of their dose–response curves (EC_{50}s) is presented in Table 2. These experiments indicate that the potencies of naturally occurring nonsteroidal estrogens cover a 1000-fold range of concentrations and even the most potent (zearalenone) is 200-fold less active on a molar basis than 17β-estradiol in stimulating a transcriptional response through the human estrogen receptor.

A hallmark of compounds that exert their effect through the ligand-binding domain of the estrogen receptor is their sensitivity to the inhibitory effects of estrogen antagonists such as 4-hydroxytamoxifen [21] or ICI-164,384 [22]. An experiment was designed to test the ability of these antagonists to interfere with the stimulatory activity of these nonsteroidal estrogens following their addition to HeLa cells in culture. As depicted in Fig. 3, both the class I estrogen antagonist (4-hydroxytamoxifen) and the class II antagonist (ICI-164,384) blocked the increase in CAT enzymatic activity not only in response to 17β-estradiol, but also in response to all of the nonsteroidal agonists tested. In most cases, these antagonists actually reduced expression from the pERE-TK-CAT reporter plasmid below the basal level observed when cells were treated with the ethanol vehicle alone. This result demonstrates that activation of transcription in response to these nonsteroidal compounds is clearly mediated by the estrogen receptor. A further inference of this data is that these nonsteroidal estrogens and 17β-estradiol

Table 2. Nonsteroidal estrogens ranked by potency as determined in a HeLa cell cotransfection assay

Agonist	EC_{50} ($M \times 10^9$)
17β-Estradiol	0.01
Hexestrol	0.2
Zearalenone	2
β-Zearalenol	15
Coumestrol	15
Genistein	90
Daidzein	90
Phloretin	300
Formononetin	300
Biochanin A	2000

Concentrations of the following compounds that give half maximal induction of transcription have been calculated from the dose–response curves shown in Fig. 2.

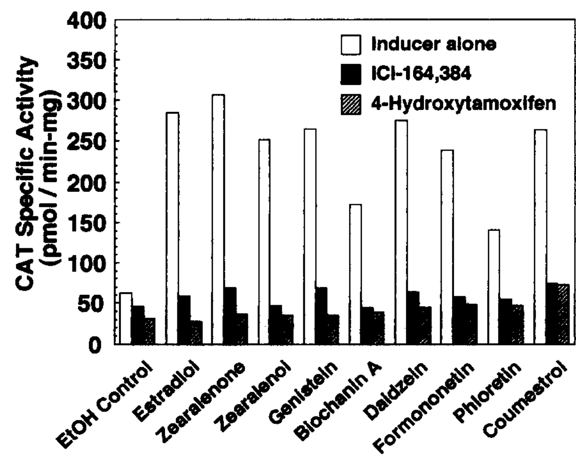

Fig. 3. Estrogen antagonists block the stimulatory effect of nonsteroidal estrogens on the estrogen receptor. HeLa cells were cotransfected with pER-18 and pERE-TK-CAT, followed by treatment for 48 h with 17β-estradiol or the nonsteroidal estrogens shown, either alone or together with 0.1 μM ICI-164,384 or 0.1 μM 4-hydroxytamoxifen. Concentrations of the inducers used in this experiment were the same as those shown in Table 1.

are likely to interact with a common site in the ligand-binding domain of the estrogen receptor, since both 4-hydroxytamoxifen and ICI-164,384 are competitive antagonists for binding of 17β-[^{3}H]estradiol to this protein [21, 22].

This prediction has been directly confirmed by competition binding analysis. Extracts prepared from COS-7 cells induced by transient transfection to overexpress the estrogen receptor were incubated with 10 nM 17β-[^{3}H]estradiol together with increasing concentrations of unlabeled estradiol or the nonsteroidal estrogens indicated. As shown in Fig. 4, 10- to 1000-fold molar excesses of all of the nonsteroidal estrogens tested (except for biochanin A and formononetin, data not shown) effectively competed with 17β-[^{3}H]estradiol for binding to the estrogen receptor. Relative binding affinities (RBAs) obtained from these data are compiled in Table 3.

The ability of the isoflavonoids (genistein, daidzein, biochanin A, and formononetin) to stimulate the activity of the estrogen receptor is complicated by the fact that genistein, in particular, has been reported to be a potent inhibitor of a variety of protein tyrosine kinases [23]. This compound binds competitively with ATP to the nucleotide binding site of these kinases. Autophosphorylation of the EGF receptor can be inhibited by genistein with an IC_{50} of 2.5 μM, while slightly higher concentrations are required to block the phosphorylation of exogenous substrates [23, 24]. The estrogen receptor has also been reported to be phosphorylated on tyrosine residues by a calcium calmodulin-dependent protein kinase present in calf uterine cytosol [25, 26]. The question therefore occurs whether the activating effect of genistein on the estrogen receptor is mediated by direct interaction with the receptor, or indirectly by an alteration in receptor phosphorylation.

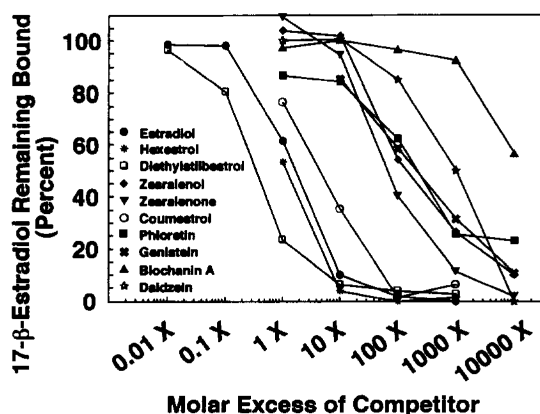

Fig. 4. Competition binding analysis of nonsteroidal estrogens. Extracts from COS-7 cells over expressing the human estrogen receptor were incubated for 2 h at room temperature with 10 nM 17β-[^{3}H]estradiol in the presence of increasing concentrations of the unlabeled ligands shown. 17β-[^{3}H]estradiol remaining bound to the estrogen receptor was determined as radioactivity resistant to adsorption by DCC.

Table 3. Affinities of nonsteroidal estrogens for the human estrogen receptor

Compound	RBA
Diethylstilbestrol	0.4
Hexestrol	0.9
17β-Estradiol	1.0
Coumestrol	7.5
Zearalenone	80
β-Zearalenol	150
Genistein	250
Phloretin	250
Daidzein	1000
Biochanin A	20000

RBAs were determined for the following compounds from the competition binding curves presented in Fig. 3. The RBAs were calculated as the concentration excess, relative to 17β-estradiol, required to give 50% inhibition of specific binding of 17β [^{3}H]estradiol to the estrogen receptor over expressed in COS-7 cells.

To answer this question HeLa cells expressing the estrogen receptor and the estrogen responsive CAT reporter plasmid were treated with two additional inhibitors of protein tyrosine kinase [lavendustin A and 2-hydroxy-5-(2,5-dihydroxybenzyl)-aminobenzoic acid] [27] alone or in combination with estradiol or genistein. In addition, this analysis also included the protein phosphatase inhibitor calyculin A [28]. If genistein promotes estrogen receptor activation solely through a decrease in phosphorylation of the estrogen receptor (or of another component of the transcription machinery required for receptor function), then this effect might be mimicked by other protein tyrosine kinase inhibitors. Conversely, if this mechanism holds, then inhibition of protein dephosphorylation by calyculin A would be predicted to interfere with the stimulatory activity of genistein. As shown in Table 4,

Table 4. Effect of tyrosine kinase and phosphatase inhibitors on activation of the estrogen receptor by 17β-estradiol and genistein

Inhibitor	Agent alone	With 10 nM estradiol	With 1 μM genistein
EtOH Vehicle	32	170	223
Lavendustin A	47	146	217
BABA*	29	155	195
Calyculin A	42	187	194
Genistein	222	213	NA

HeLa cells, cotransfected with pER-18 and pERE-TK-CAT, were treated for 48 h without hormone, with 10 nM 17β-estradiol, or with 1 μM genistein, as indicated. In addition, duplicate plates received 0.5 μM lavendustin A, 1 μM 2-hydroxy-5-(2,5-dihydroxybenzyl)-aminobenzoic acid (BABA), or 5 nM calyculin A. Values refer to CAT specific activity, reported as pmol of [^{14}C]chloramphenicol acetylated per min per mg of cytosolic protein. NA, not applicable.

genistein was unique among the tyrosine kinase inhibitors in being able to stimulate the transcriptional activity of the estrogen receptor. Furthermore, no evidence of synergism was observed when either of these agents was combined with 17β-estradiol or genistein, as might be expected if a block in tyrosine phosphorylation were involved in the activation of this receptor. Calyculin A also failed to interfere with the stimulatory activity of genistein. Together, these data strongly argue that a change in phosphorylation of the estrogen receptor on tyrosine residues or of another intracellular factor required for receptor function is not involved in the hormonal effect of isoflavones such as genistein.

DISCUSSION

There have been many reports that compounds unrelated to the estrogenic steroids, but isolated from natural sources are able to mimic the effects of 17β-estradiol in animals [7, 8, and references therein]. Many of these claims have been amply substantiated by controlled studies of the hormonal effects of these compounds in well established animal [11–13] or cell culture [29] assays for the biological activity of estrogens. Other claims remain anecdotal or poorly documented. Few studies have been performed that compare the activities of a diverse variety of naturally occurring nonsteroidal estrogens. The present study attempts to address some of these deficiencies by systematically examining the biological activities and binding affinities of putative nonsteroidal estrogens in assays that utilize the recombinant human estrogen receptor.

Traditional bioassays used to assess estrogenicity rely on relatively complex biological responses such as the stimulation of water imbibition and growth of the uterus, cornification of the vagina in laboratory rodents [12, 13] or the stimulation of DNA synthesis and cell division in selected breast tumor cell lines [29]. While these phenomena clearly depend on activation of endogenous estrogen receptor in the respective tissues, they all represent long-term responses to hormones which are incompletely characterized and remain poorly understood. Little if any information is available concerning the genes that are targets for activation by the estrogen receptor in these systems or the interplay that exists between this receptor and receptors for other steroids, peptide hormones and growth factors.

The deduced sequence of the estrogen receptor is a available from six species (human, mouse, rat, chicken, frog and trout) as a result of the successful isolation of cDNA clones encoding these receptors [30–36]. It should be noted that a comparison of the sequences of even closely related receptors (human and murine) reveals that seven amino acid substitutions distinguish the primary sequence of the ligand-binding domains of these proteins [30, 31, 36]. While there is no evidence that these sequence differences influence the affinities of these receptors for 17β-estradiol, it is unknown what effect they may have when the behaviors of structurally diverse estrogens are compared. In short, there is insufficient evidence at present to conclude that the behavior of nonsteroidal estrogens in bioassays using laboratory rodents will invariably reflect their biological activity in man. Additionally, there remains a paucity of data on the behavior of these compounds in model systems of estrogenic activity that utilize the human receptor.

The selection of a transient transfection assay to assess agonist activity circumvents many of the limitations described above. This assay is based on the accumulation of a heterologous gene product (CAT) whose expression has been placed under transcriptional control of an estrogen regulated promotor. Induction of CAT enzyme in this system depends both on coexpression of the estrogen receptor and on the presence of a consensus receptor binding site in the promoter used to drive CAT expression [10]. These experiments have confirmed that β-zearalenol, zearalenone, coumestrol, phloretin, genistein, daidzein, biochanin A, and formononetin are able to mimic the ability of 17β-estradiol to stimulate the transcriptional activity of the estrogen receptor in cell culture. In contrast, sarsasapogenin, diosgenin, β-sitosterol, curcumin, podocarpic acid, nordihydroguaiaretic acid, and gibberellic acid appear not to possess a significant degree of inherent estrogenic activity, although the possibility remains that they may be metabolized to hormonally active derivatives *in vivo*. Combined with other studies from this laboratory [10], these results indicate that naturally occurring nonsteroidal estrogens include members belonging to six chemical classes: resorcylic acid lactones, coumestans, chalcones, isoflavones, flavones, and flavanones.

Structural features shared between all of these compounds and the steroidal estrogens include a polycyclic hydrocarbon backbone (C15–C18) with the potential to assume a planar configuration. They also possess at least one aromatic ring containing a free phenolic hydroxyl group, and a second oxygen (usually a hydroxyl group, but occasionally carbonyl or methoxyl groups) positioned approx. 12 Å from the phenolic oxygen. A model proposing the alignment of representative examples of these nonsteroidal estrogens with 17β-estradiol is given in Fig. 5. According to this model, phenolic hydroxyl groups in positions 3 of coumestrol, 7 of isoflavones (e.g. genistein), and 4 of chalcones and dihydrochalcones (e.g. phloretin) can be considered to be equivalent to the 3-hydroxyl group of 17β-estradiol. While the structural disimilarity of resorcylic acid lactones complicate their alignment with 17β-estradiol, it is likely that the 4-hydroxyl substituent serves a comparable role in β-zearalenol and its relatives.

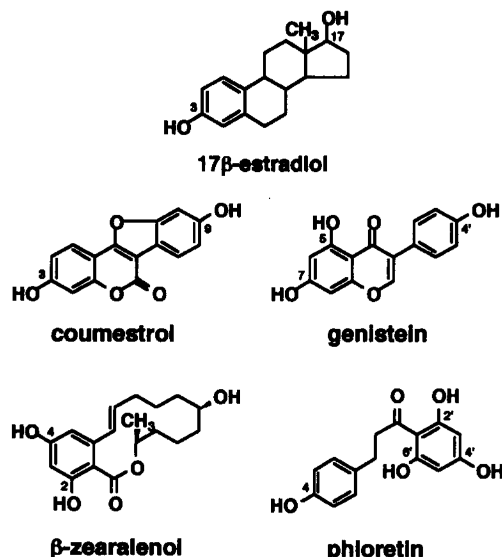

Fig. 5. Comparison between the structures of 17β-estradiol and selected examples of the nonsteroidal estrogens analyzed in this study. Four classes of naturally occurring nonsteroidal estrogens are represented as follows: coumarins (coumestrol), isoflavones (genistein), resorcylic acid lactones (β-zearalenol), and dihydrochalcones (phloretin) While these structures are not intended to depict the actual three dimensional configurations of these molecules, they have been drawn to emphasize the structural similarities between themselves and 17β-estradiol.

The nonsteroidal estrogens described in this study display a significant range of potencies, from compounds that are effective at nanomolar concentrations (e.g. β-zearalenol, zearalenone, and coumestrol) to compounds that must be present at a concentration $>1\,\mu M$ for activity (e.g. biochanin A and formononetin). Slight differences that are evident when comparing the relative potencies of these compounds (Table 2) with their RBAs (Table 3) are likely to result from variations in their uptake, bioavailability, or metabolism in cell culture. The reduced activity of biochanin A and formononetin relative to genistein and daidzein, respectively, can be easily rationalized by the inhibitory effect of methoxylation. Millington *et al.* [37] have in fact concluded that the biological activity displayed by biochanin A and formononetin in whole animals derives largely from their conversion to genistein and daidzein *in vivo*. The data reported here confirm that these two isoflavones bind only weakly to the human estrogen receptor *in vitro*, and have only a modest ability to stimulate this receptor in cell culture.

Among the nonsteroidal estrogens, the isoflavone genistein has been identified to be an effective inhibitor of several protein tyrosine kinases, including the EGF receptor, and the *src* and *fes* oncogenes [24]. Several observations suggest that the ability of genistein to activate the estrogen receptor is unrelated to its tyrosine kinase inhibitory activity. While the estrogen receptor has been reported to be a substrate for tyrosine phosphorylation [26, 27], two independent inhibitors of protein tyrosine phosphorylation [lavendustin A and 2-hydroxy-5(2,5-dihydroxybenzyl)-aminobenzoic acid] have no apparent effect on the basal or estradiol-induced activity of the estrogen receptor. Conversely, calyculin A (a specific inhibitor of protein phosphatases 1 and 2A) fails to interfere with the stimulation of this receptor by genistein. The concentration at which genistein causes half-maximal activation of the estrogen receptor (approx. 10^{-7} M) is substantially less than the IC_{50} of genistein for inhibition of protein phosphorylation ($0.2\text{–}2 \times 10^{-5}$ M) [24]. Finally, genistein can be shown to interact directly with the human estrogen receptor using a competition binding assay. For these reasons, there is no cause to invoke inhibition of protein tyrosine phosphorylation as a mechanism for the ability of genistein to activate the estrogen receptor. It can be concluded that the estrogen receptor stimulatory and tyrosine kinase inhibitory activities of genistein represent independent properties of this isoflavone.

It is hoped that studies such as this will contribute to the growing recognition of the diversity of nonsteroidal estrogens that occur in nature. As the list of these compounds increases, so does our appreciation of the prevalence of nonsteroidal estrogens in the plant kingdom. A thorough understanding of the importance of plant and fungal estrogens as potential human toxicants should include an examination of the consequences of over exposure to environmental estrogens on prenatal development of the reproductive tract, their impact on human fertility, and their potential to promote the growth of hormone-dependent tumors. This will require better awareness of the number of such compounds, their distribution among species used for human consumption, as well as additional information on their abundance in the human diet and their metabolic fate following consumption.

Acknowledgements—This work was supported by grants from the National Cancer Institute (R29 CA 47384) and the American Cancer Society (CD 479).

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