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Prenylflavonoids as Nonsteroidal Phytoestrogens and Related Structure–Activity Relationships

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In the search for estrogen receptor (ER) modulators, a series of prenylflavonoids were found to be widely distributed amongst tonic herbal medicines and to possess estrogen-like activity in MCF-7/BOS cells, as evaluated by an estrogen-screening assay. Cell-cycle analysis revealed that the stimulatory effects of these compounds toward cell proliferation were elicited at the G1–S checkpoint and could significantly increase the S-phase population of MCF-7 cells under hormone-free conditions. ER-responsive gene (PS2, PgR) and protein (PgR) expression was also detected; mRNA and protein-expression levels for PS2 and PgR were up-regulated by the compounds in a dose-dependent manner. These effects could be inhibited by the pure ER antagonist ICI 182,780 ((7α-[9-{4,4,5,5,5-pentafluoropentyl}sulfinyl]nonyl)estra-1,3,5(10)-triene-3,17β-diol). It was therefore concluded that the estrogen-like effects of these prenylflavonoids were mediated primarily through ERs. Furthermore, to explore the structure–activity relationship based on the estrogen receptor and detailed molecular mechanisms among the prenylflavonoids, protein–ligand docking simulations were carried out by using the DS-MODELING software package. The binding affinity of each prenylflavonoid toward ERα was scored, and the receptor–ligand interaction was also analyzed to provide the simulation characteristics of virtual molecular recognition mechanisms.

Introduction

Phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogens.[1] Similar to the numerous effects of estrogens on the human body, phytoestrogens are known to exert widely beneficial effects toward human health, especially against cancer, osteoporosis, irregular menopause syndrome, cardiovascular disease, and possibly neurodegenerative diseases, to name a few.[2] Furthermore, phytoestrogens are sometimes considered a 'natural' source of selective estrogen-receptor modulators, eliminating the side-effects of hormone-replacement therapies. Therefore, the discovery of new phytoestrogens may provide strategic ideas for the development of alternatives to estrogen that selectively block the unwanted effects of this hormone, yet which mimic its beneficial effects.[3]

Herein, we report our research on a series of prenylflavonoids as a class of nonsteroid phytoestrogens that was conducted based on the discovery of several estrogenic compounds.[4] The estrogenic activities were tested by a modified MCF-7 cell-proliferation assay (E-SCREEN method).[5,6] Cell-cycle analysis and estrogen-receptor-responsive gene- and protein-expression of PS2 and progesterone receptor (PgR) were examined to explore the initial mechanisms. ICI 182,780 ((7α-[9-{4,4,5,5,5-pentafluoropentyl}sulfinyl]nonyl)estra-1,3,5(10)-triene-3,17β-diol), a pure estrogen receptor (ER) antagonist, was also used as a tool to determine whether the prenylflavonoids exert their effects through an ER-dependent pathway.

It has been revealed that 17β-estradiol (E2) exerts its effects on target cells predominantly through the binding and activation of ERs.[7] Considering the theoretical and experimental evidence, the prenylflavonoids were concluded to exert their activities through an ER-dependent pathway. Therefore, a ligand–protein docking experiment was performed to evaluate the binding characteristics of these prenylflavonoids with estrogen receptor α, an ER subtype that contributes to cell proliferation.[8] This study provided some in silico evidence for stereoselective ligand recognition by human ERα and helped us to explain the structure–activity relationships of these compounds.

Results

Stimulatory effect of the prenylflavonoids toward cell proliferation

The structures of the prenylflavonoids are shown in Figure 1. In the MCF-7 cell-proliferation assay, 17β-estradiol was set as the positive control. The proliferative effects of the compounds relative to that of 17β-estradiol (1 nM, 100%) were expressed as the relative proliferative effect (RPE). Icaritin (**2**), 4′-desme-

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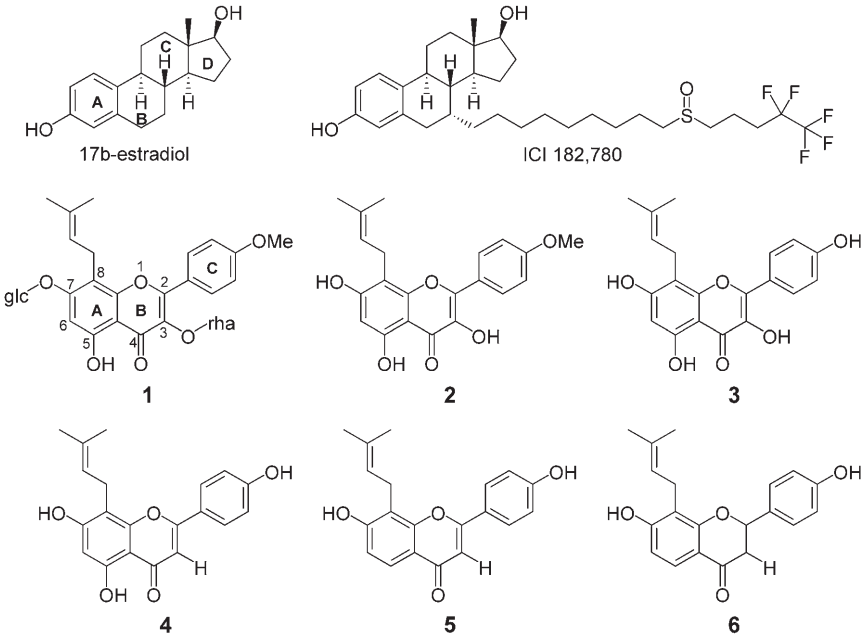

Figure 1. 17β-Estradiol (steroid ring lettering shown) and related compounds: ICI 182,780 (faslodex), icariin (**1**, flavanoid ring lettering and atom numbering shown; glc = glucose, rha = rhamnose), icaritin (**2**), 4′-desmethylicaritin (**3**), 4′,5,7-trihydroxy-8-prenylflavone (**4**), 4′,7-dihydroxy-8-prenylflavone (**5**), and isobavachin (**6**).

thylicaritin (**3**), 4′,5,7-trihydroxy-8-prenylflavone (**4**), 4′,7-dihydroxy-8-prenylflavone (**5**), and isobavachin (**6**) significantly stimulated the proliferation of MCF-7/BOS cells in a dose-dependent manner ($p < 0.01$) (Figure 2). Isobavachin showed the most potent activity, whereas icariin was inactive. The estrogenic potency of these compounds is ranked as follows: 17β-estradiol > **6** > **4** > **5** > **3** > **2** ≫ **1** (inactive) (Table 1). The potency of the compounds differs dramatically; one explanation is that

Table 1. Estrogenic effects of prenylflavonoids on MCF-7/BOS cells.

Compound	EC_{50} [mol L^{-1}]
17β-estradiol	5.0×10^{-11}
1	inactive
2	3.5×10^{-7}
3	1.4×10^{-7}
4	3.1×10^{-9}
5	8.0×10^{-8}
6	2.1×10^{-9}

substitution of the flavan ring system with prenyl and hydroxy groups changes the lipophilic properties of these compounds. Consequently, their stimulatory effects toward cell proliferation is influenced by cell-membrane permeability. Another reason for the variation in potency observed in the cell-proliferation assay may be associated with the binding affinity toward the specific ER subtype and the interaction with key residues in the binding domain, as ligand–receptor binding is the first step to initiate downstream events.

Experiments also showed that the stimulatory effects toward proliferation induced by the prenylflavonoids could be inhibited by the ER antagonist ICI 182,780 (Figure 3). All compounds were also tested on MDA-MB23 cells, another breast cancer cell line that does not express ERα, and they showed no effect (data not shown). Therefore, this indicates that the proliferation stimulatory effects of the prenylflavonoids are ER-dependent.

Cell-cycle analysis

In the current experiment, isobavachin was selected as a representative as the most potent compound. Cell-cycle analysis showed that in hormone-free medium, almost all cells were in G1 phase and that the proportion was even higher after co-

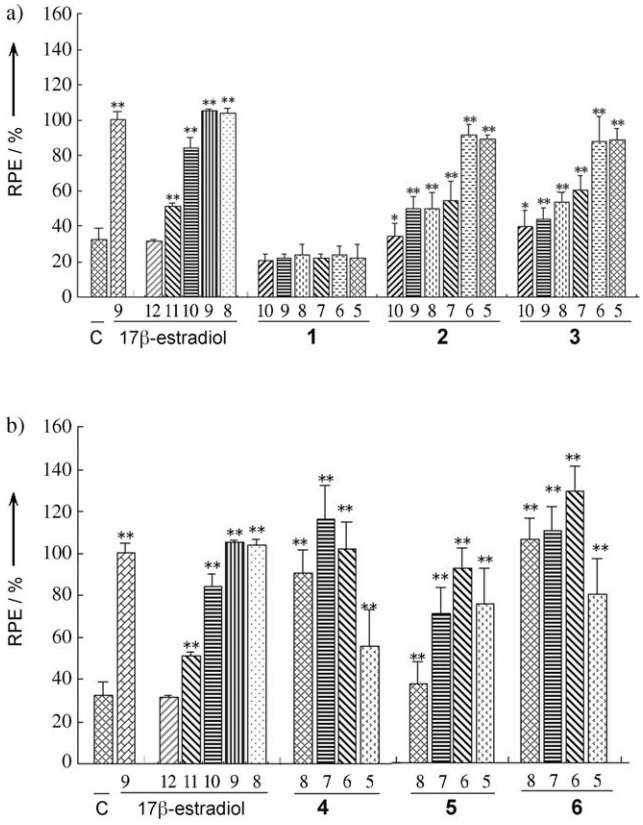

Figure 2. Estrogenic activities of the prenylflavonoids a) **1**, **2**, and **3** and b) **4**, **5**, and **6** in MCF-7/BOS cells. Cells were incubated in phenol-red-free DMEM supplemented with hormone-free serum with 17β-estradiol or other test compounds for 7 days; *x*-axis values report compound concentrations in −log M. Incubation with DMSO vehicle alone was performed as control (C), and the final concentration of DMSO in the medium never exceeded 0.1%. After the 7-day incubation, an MTT assay was performed to measure cell proliferation. The proliferative effect relative to 17β-estradiol (1 nM, 100%) is expressed as the relative proliferative effect (RPE). Results are expressed as the mean ± SD of five separate experiments. Significant difference was set at $*p < 0.05$ and $**p < 0.01$ versus control.

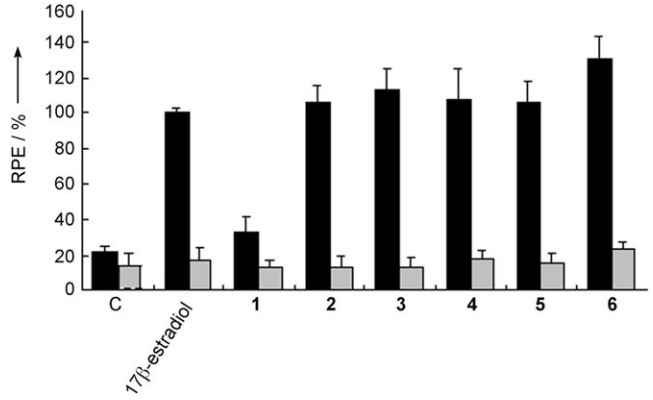

Figure 3. Effect of co-treatment with pure ER antagonist ICI 182,780 on cell proliferation induced by the prenylflavonoids **1**–**6** in MCF-7/BOS cells. Cells were incubated in phenol-red-free DMEM supplemented with hormone-free serum with (■) or without (■) 100 nM ICI 182,780 for 7 days. Incubation with DMSO alone was performed as control (C) and the final concentration of vehicle, DMSO, in the medium never exceeded 0.1%. The compound concentration in each case was 10^{-6} M. After incubation for 7 days, an MTT assay was performed to measure cell proliferation. The proliferative effect relative to 17β-estradiol (1 nM, 100%) is expressed as the relative proliferative effect (RPE). Results are expressed as the mean ± SD of five separate experiments.

exposure to 100 nM ICI 182,780. As shown in Figure 4, a dramatic increase in the number of cells in S phase is observed after treatment with 1 nM 17β-estradiol or 1 μM isobavachin for 24 h relative to the control. The proportion of cells in S phase increased from 3.2% (solvent control) to 17.5% (1 nM 17β-estradiol) and 20.2% (1 μM isobavachin). The effects occur in a dose-dependent manner and can be inhibited by 100 nM ICI 182,780. These results are in accordance with the data obtained from the cell-proliferation analysis and possibly demonstrate that the prenylflavonoids exert their effect on cell proliferation at S-phase entry.

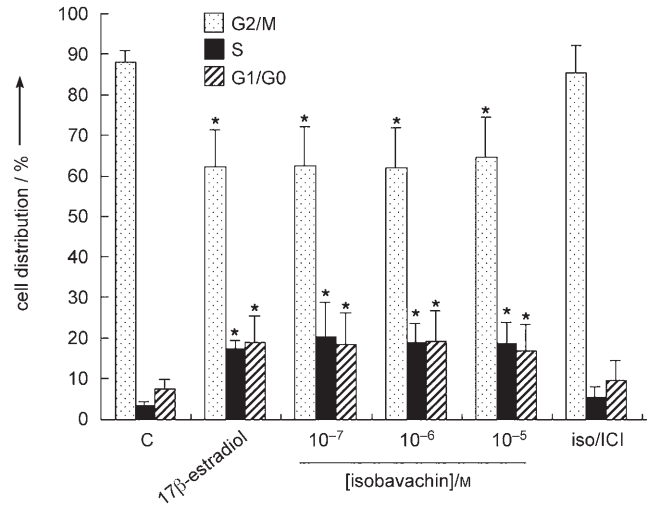

Figure 4. Effects of the prenylflavonoids on cell-cycle distribution of MCF-7/BOS cells. DMSO was used as solvent control. MCF-7 cells were cultured in DMEM with 10% fetal bovine serum for 24 h. The medium was then changed to medium without hormone, and incubation was continued for another 24 h with DMSO (C), 17β-estradiol (1 nM), various concentrations of isobavachin, or a combination of isobavachin (10^{-6} M) and ICI 182,780 (10^{-7} M) (iso/ICI). Cell-cycle analysis was performed by flow cytometry. Results are expressed as percentage of cells in G1 phase, S phase, and G2 phase. These data represent three separate experiments; significant difference was set at $^{*}p < 0.05$ versus control.

Analysis of gene and protein expression

The expression of the progesterone receptor and PS2 genes in MCF-7/BOS cells could be regulated in a characteristic manner by estrogenic compounds. These ER-responsive genes can be stimulated by agonistic estrogens such as the natural ligand estradiol,[9] whereas ICI 182,780 is known to antagonize the stimulatory effect in MCF-7/BOS cells.[10] We studied the effects of the prenylflavonoids on the progesterone receptor and PS2 mRNA expression in MCF-7 breast cancer cells in comparison with 17β-estradiol and ICI 182,780 (Figure 5). As shown, 17β-estradiol (1 nM) and isobavachin (0.1–10 μM) significantly increase the expression of mRNA of PgR and PS2 compared with the negative control. These effects can also be inhibited by 100 nM ICI 182,780. The alteration in protein expression

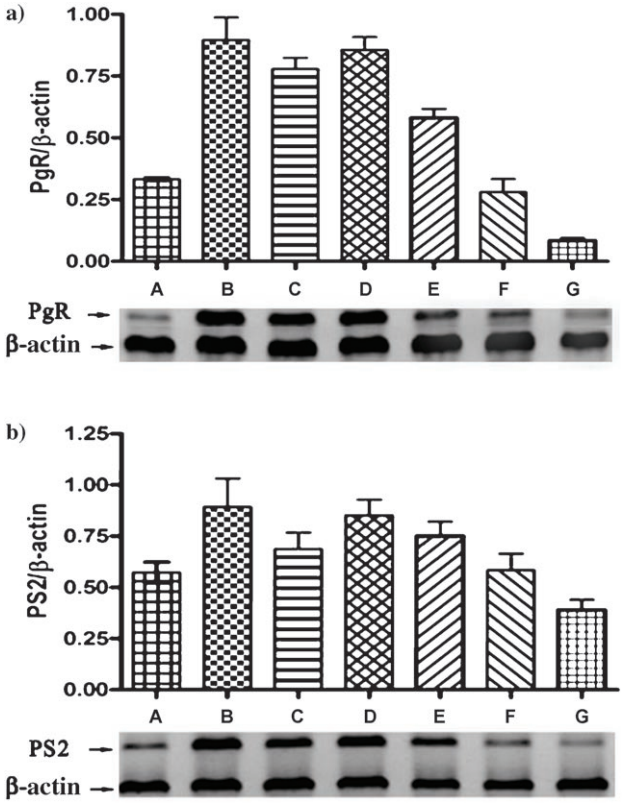

Figure 5. The effect of isobavachin on mRNA expression of estrogen-responsive genes in MCF-7/BOS cells. Lanes: A, solvent control; B, 1 nM 17β-estradiol; C, 0.1 μM isobavachin; D, 1 μM isobavachin; E, 10 μM isobavachin; F, 1 nM 17β-estradiol + 100 nM ICI 182,780; G, 1 μM isobavachin + 100 nM ICI 182,780. After incubating with test compounds for 24 h, total RNA was extracted with Trizol reagent (Gibco BRL). The mRNA levels of a) progesterone receptor (PgR) and b) PS2 were measured by RT-PCR and normalized with β-actin as an internal control. RT-PCR products were separated on an ethidium bromide stained 1.5% agarose gel, which was scanned by using a bio-imaging analyzer (Bio-Rad, USA). The density of the products were quantified with Quantity One version 4.2.2 software (Bio-Rad, USA).

showed a similar tendency. Compared with the negative control, isobavachin stimulated the expression of PgR protein by more than 2-fold (Figure 6). These data confirm that the estrogen-like activity of prenylflavonoids is mediated by the estrogen receptor.

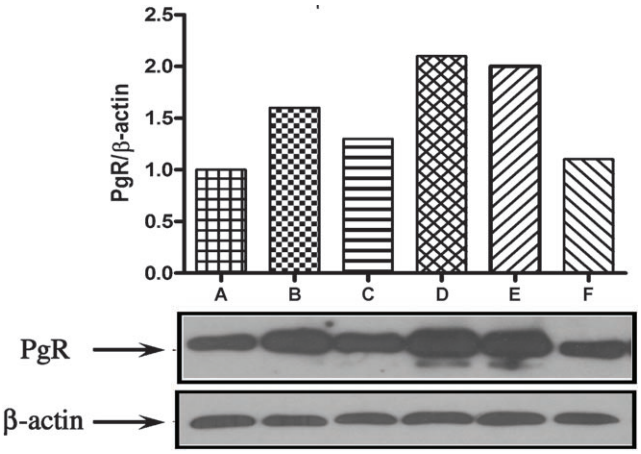

Figure 6. Effect of isobavachin on protein expression of estrogen-responsive PgR in MCF-7/BOS cells. After incubation with test compounds for 24 h, cells were harvested, and Western-blot analysis of the total protein was conducted. Lanes: A, control; B, 1 nM 17β-estradiol; C, 0.1 μM isobavachin; D, 1 μM isobavachin; E, 10 μM isobavachin; F, 1 μM isobavachin + 100 nM ICI 182,780.

Table 2. Docking results between prenylflavonoids and ERα.[a]

Compound	DockScore	Rank	Residue			
			Arg 394	Glu 353	His 524	Gly 521
17β-estradiol	51.695	1	+	+	+	+
6	50.673	2	+	+	+	+
4	49.009	3	+	+	+	+
5	40.045	4	+	+	+	+
3	39.511	5	+	+	+	+
2	30.594	6	+	+	−	−
1	−957.394	7	−	−	−	−

[a] The docking affinity and H-bond interaction between the prenylflavonoids with key amino acid residues in the recognition of ERα activation were carried out by Discovery Studio Modeling software package (Accelrys, USA).

Molecular simulation and docking

According to the docking results, the binding scores ranked as follows: 17β-estradiol > **6** > **4** > **5** > **3** > **2**, and **1** was considered to have no ability to bind with ERα (Table 2). This result was basically in accordance with the results of the biological tests.

The E2 molecule docked in the ER ligand-binding domain was initially recognized by H bonds with active-site residues Arg 394, Glu 353, and His 524, which are known to be essential for the reading of agonistic activity and subsequent nuclear signaling of E2, and are thus referred to as the catalytic triad.[11,12] In our experiment, the interaction between the simulated docked ligands and the key residues was analyzed (Figure 7, Table 2). The results show that the interaction between the ligands and the active-site residues are correlate well with the binding affinity and biological activity.

Discussion

The study presented herein suggests that a variety of prenylflavonoids possess diverse estrogenic activities, the effects of which are related to their structures and are mediated by the ER. Therefore, such compounds could be considered as a series of novel phytoestrogens. These compounds were widely distributed in some tonic herbal medicines,[13] which were used

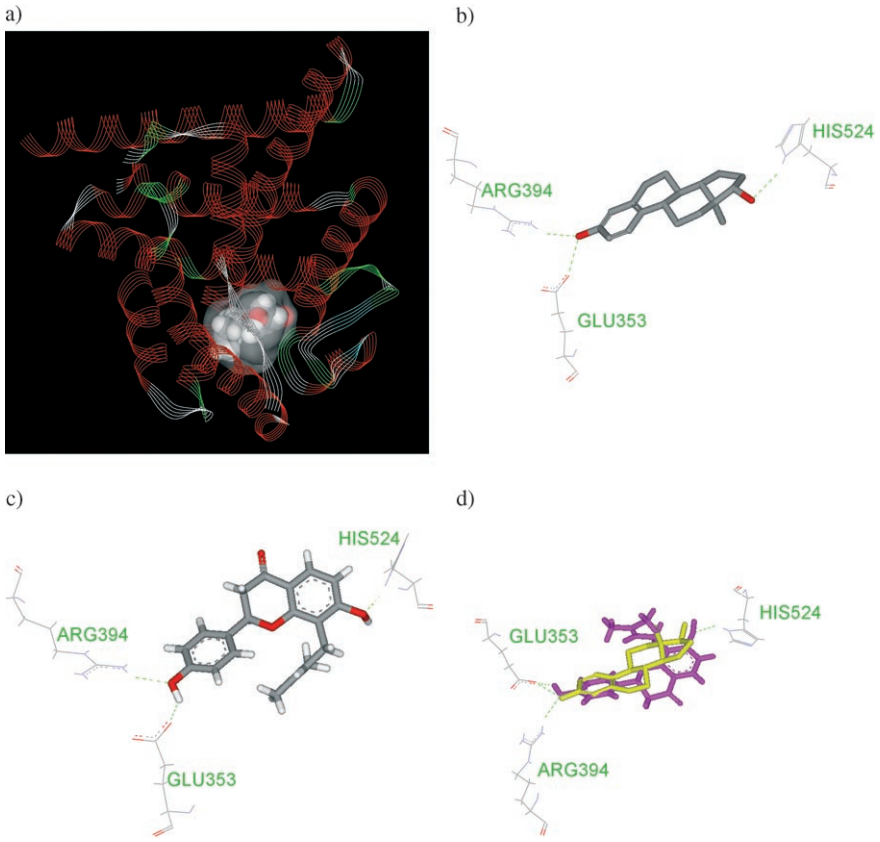

Figure 7. a) ERα-LBD at the ERα binding domain; b)–d) binding mode of energy-minimized 17β-estradiol and isobavachin at the ER binding pocket showing residues involved in their recognition.

to restore harmony and were considered to be vital for invigorating and maintaining balance of the entire endocrine system in close relationship with hormone or hormone-like activity.

According to the results of our experiments, the stimulatory effects toward cell proliferation and the ER-responsive up-regulation of gene and protein expression mediated by the prenylflavonoids could be reversed by co-administration of the pure ER antagonist ICI 182,780. It can be concluded that the prenylflavonoids may exert their estrogenic activity through an ER-dependent pathway. By this, both estrogens and phytoestrogens that bind to the ligand-binding domain (LBD) of the ERs usually interact with chromatin to alter their cytoarchitectural and phenotypic properties and initiate a series of molecular events, including the intervening activities of signal pathways associated with cell-cycle progression and cell proliferation. Cell-cycle analysis strictly proves that the regularity induced by prenylflavonoids is focused on the checkpoint of entry from G1 phase to S phase in MCF-7 cells. Prenylflavonoids may exert control over several key G1-phase cell-cycle regulators, namely Rb, cyclin D1, Myc, Cdk2, Cdk4, among others, which are also required for S-phase entry.[14] However, a detailed molecular mechanism is far from clear and requires further exploration.

According to the present data, the activities of the prenylflavonoids differ dramatically as a result of their structural differences. It is known that hormone recognition is achieved through a combination of specific hydrogen bonds and the complementarity of the binding cavity to the nonpolar character of the molecule.[15] The hydroxy groups play a critical role in the orientation of these compounds in the ligand-binding site of the ER. The prenylflavonoids presented herein display several principal structure variation possibilities, including the position of the prenyl group, configurational changes at stereogenic centers, and functional groups on the aromatic rings. Based on the results, the loss of activity caused by the presence of a glycosyl group on the flavone structure, as exemplified by compound **1**, could be explained by the steric hindrance produced by the bulky group which prevents the required docking to the receptor site. The differences between compounds **3** and **4** show that the absence of the 3-OH group on the B ring (Figure 1) reinforces estrogenic activity. The distinction between compounds **4** and **5** demonstrates that the introduction of a 5-OH group on the A ring results in a dramatic increase in estrogenic activity. The distinction between compound **6** and other compounds also indicates that the maturation of these compounds from **2** increases the activity, showing that the polarity of the B ring may play a crucial role in the interaction with the receptor; the hydrophobicity of the B ring is probably conducive toward the interaction between the active compounds and the binding domain.

The results of simulation experiments on the contribution of key residues also agreed with those of the biological studies, which demonstrated that the docking ability and interactions with key residues are essential for the appropriate agonist interpretations in the binding domain of ERα. The structure–activity relationships demonstrate that the hydroxy groups on the phenolic A ring, which form H bonds with key residues Glu 353 and Arg 394, is critical for ligand recognition and activation. The hydrogen bond between the hydroxy group of the C ring and His 524 also contributes to molecular recognition. The remainder of the molecule also participates in a number of polar and nonpolar interactions the explain the ability of ER to selectively recognize and bind ligands.

Conclusions

The assay for stimulatory effects toward cell proliferation with the hormone-dependent breast cancer cell line MCF-7/BOS shows that the prenylflavonoids have a marked estrogenic effect. They are able to stimulate the expression of ER-responsive genes and proteins. The biological effects of the prenylflavonoids was completely inhibited by treatment with the pure ER antagonist ICI 182,780. These results prove that the prenylflavonoids exert estrogen-like activity through the estrogen receptor.

The results of the docking experiment based on the structure of the LBD of ERα bound to estradiol provide a molecular view of the binding factors. Based on the biological effects and docking experiments, the docking affinity and receptor–ligand interaction of the compounds clearly contribute to the activation of the estrogen receptor.

Experimental Section

Materials and compounds: The prenylflavonoids (Figure 1) were offered in this case by the Department of Pharmaceutical Chemistry, College of Pharmaceutical Sciences, Zhejiang University, China, which were synthesized by regular methods. Structure identification was performed at the Institut für Pharmazeutische Biologie, Heinrich-Heine-Universität, Düsseldorf, Germany. These prenylflavonoids were found as important constituents of some traditional Chinese tonic herbal medicines such as *Epimedium sagittatum maxim* and *Psoralea corylifolia*.[13,16] 17β-estradiol (E2), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenol-red-free Dulbecco's modified Eagle's medium (phenol-red-free DMEM) was purchased from Sigma–Aldrich (St. Louis, USA). Dextron T-70 was provided by Pharmacia (New Jersey, USA). DMEM and fetal bovine serum were obtained from Gibco BRL Life Technologies, Scotland. Human serum was provided by Blood Center of Zhejiang Province (Hangzhou, China). Charcoal–dextran stripped human serum was prepared by an existing modified protocol[6] to remove hormone.

Cell-proliferation assay: The MCF-7/BOS cell line (*ER*+ human breast adenocarcinoma) was a kind gift from professor Ana M. Soto, Tufts University School of Medicine. Confluent MCF-7/BOS cells were washed twice with D-Hanks solution before the addition of trypsin–EDTA (0.25%). The flask was left for ≈2–3 min at room temperature (close to 20 °C), after which the cells were detached, resuspended in full medium, counted, and seeded to 24-well plates at a proper density (1×10^4 cells/well). After 48 h, the cells were completely attached to the well surface. The cells were then washed with D-Hanks solution, and the estrogen-free medium (phenol-red-free DMEM and charcoal–dextran stripped serum (5%)) was added. Following another pretreatment for 48 h, the cells were exposed to increasing concentrations of 17β-estradiol and other test compounds. Cell proliferation was assessed after 7 days, during which the medium was refreshed every 3 days. In the assessment method, cells were incubated with a solution of MTT

(50 μL, 5 mg mL^{-1}) for 4 h. Following this, the medium was discarded, and DMSO was added. The optical density was measured at $\lambda = 570$ nm with a microplate reader (ELx800, Bio-Tek, Winooski, USA). By the MTT method, the results of cell cultures were expressed as fold proliferation relative to that of 17β-estradiol (1 nM).

Cell-cycle analysis: Exponentially growing cells were plated in Petri dishes (150 mm) in phenol-red-free DMEM containing 10% charcoal–dextran stripped human serum for 48 h and treated with test compounds. After 24 h, the cells ($\approx 1 \times 10^6$ cells) were harvested, placed into a polypropylene tube, and centrifuged. The supernatant was removed, and 70% EtOH (4 °C, 1 mL) was added dropwise to the cell pellet while vortexing. The cells were kept at 4 °C until DNA staining. Fixed cells were treated with RNase A (100 μg mL^{-1}) in phosphate-buffered saline (PBS) solution for 1 h, followed by staining with propidium iodide (50 μg mL^{-1} in PBS). Flow-cytometric analysis of cell-cycle distribution and apoptosis was performed with a BD FACSCalibur with a blue ($\lambda = 488$ nm) argon laser (Becton Dickinson, San Jose, USA). Data acquisition was performed with CellQuest v 3.1 software, and data were analyzed with ModFit LT 3.0 software (Variety Software House, Inc., Topsham, USA).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR): MCF-7/BOS cells were grown in Petri dishes (150 mm) in phenol-red-free DMEM containing 10% charcoal–dextran stripped human serum for 48 h and treated with solvent or different concentrations of the prenylflavonoids. After 24 h, the cells were harvested, and total RNA was isolated with Trizol reagent (Gibco BRL) according to the manufacturer's instructions. After extraction, RNA was precipitated by recommended procedures and dissolved in 0.1% diethylpyrocarbonate solution. To synthesize first-strand cDNA, total RNA (2 μg) was incubated with oligo(dT)$_6$ primer (0.5 μg, Sangon, China) in deionized water (5 μL) at 65 °C for 15 min. Reverse transcription reactions were performed with M-MuLV reverse transcriptase (200 units, Gibco BRL) in 5× reaction buffer (250 mM Tris–HCl, pH 8.3 at 25 °C; 375 mM KCl; 15 mM MgCl2; 50 mM dithiothreitol) and a mixture of deoxynucleoside triphosphates (dNTPs, 1 mM each) containing 10× PCR buffer (100 mM Tris–HCl, pH 8.3 at 25 °C; 500 mM KCl; 15 mM $MgCl_2$), 25 units Taq polymerase (Sangon, China), and 30 pmol of each primer. The final volume was adjusted to 50 μL. The specific primer pairs were designed as follows: progesterone receptor sense primer 5′-AGTTGTGAGAGCACTGGATGC-3′, progesterone receptor antisense primer 5′-GATCTGCCACATGGTAAGGC-3′, PS2 sense primer 5′-TGGAGAACAAGGTGATCTGC-3′, PS2 antisense primer 5′-ATCTGTGTTGTGAGCCGAGG-3′, β-actin sense primer 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, and β-actin antisense primer 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′.[17] The PCR reactions were initiated by 3 min denaturation at 94 °C, followed by amplification at 94 °C for 45 s, and 55 °C for 45 s, 72 °C for 45 °C; 30 cycles were carried out for the progesterone receptor, 25 cycles for PS2, and 20 cycles for β-actin by using Mastercycler gradient (Eppendorf, Germany). The PCR products were analyzed by 1.5% agarose gel electrophoresis, visualized with ethidium bromide staining, and quantified with a bio-imaging analyzer (Bio-Rad, USA). The density of the products was quantified using Quantity One version 4.2.2 software (Bio-Rad, USA).

Generation of cell lysates and Western-blot analysis: MCF-7/BOS cells were harvested at 4 °C and lysed on ice in extraction buffer containing Tris–HCl (20 mM, pH 7.5), NaCl (150 mM), EDTA (1 mM), Triton X-100 (1%), sodium deoxycholate (0.5%) plus PMSF (1 mM), leupeptin (10 μg mL^{-1}), and aprotinin (30 μg mL^{-1}). Lysates were cleared by centrifugation (4 °C, 14000 *g*, 30 min). Total protein present in each lysate was quantified by using a modified Lowry assay (DC protein assay; Bio-Rad, Hercules, USA). SDS-PAGE, Western blotting, and densitometric measurement were performed by standard protocols. Total protein content (40 μg lysate per lane) was analyzed, and the proteins were transferred to a nitrocellulose membrane. Transferred membranes were blocked for 1 h in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBS/T). Primary antibodies were added in 5% milk, and the blot was incubated overnight at 4 °C. The blots were washed three times for 5 min each with 10 mL TBS/T and incubated with secondary antibody (anti-mouse HRP or anti-rabbit HRP, 1:3000; Santa Cruz Biotechnology, USA) in 10 mL TBS/T with gentle agitation for 1 h at room temperature. Then the blots were washed three times for 5 min each with TBS/T, exposed to a chemiluminescent detection system using the SuperSignal West Pico Substrate (Pierce, Rockford, USA) and exposed to film. Digital images of the films were captured and quantified using the bio-imaging system (Bio-Rad, USA). The expression levels of PgR in treated cultures were compared with those of untreated control cultures.

Data analysis: All data are expressed as the mean ± SD from three or more independent experiments. Statistical significance was established by ANOVA followed by Student *t* test by using SPSS v.10.1, SPSS Inc., Chicago, USA) or Microsoft Excel software. Cases for which $p < 0.01$ were considered to be statistically significant.

Computational methodology for docking: According to the results of biological experiments, E2 and the prenylflavonoids exert their estrogen-like activities through an ER-dependent pathway. Based on the analysis of the structure–activity relationships of phytoestrogens and other ligands, it was concluded that the differences of the ligand behavior were primarily due to the ligand structure. X-ray crystallography and mutation analysis also suggest that ligand recognition is achieved through a combination of specific hydrogen bonds and complementarities between the binding cavity and the ligands.[11] To further explore the structure–activity relationships of the prenylflavonoids, docking experiments between the estrogen receptor and the compounds were conducted.

Receptor structure: The starting coordinates of human ERα ligand-binding domain in complex with 17β-estradiol (E2–ERα–LBD, PDB code: 1ERE) were obtained from the RCSB Protein Data Bank (www.pdb.org).[12]

Molecular structure of the compounds: The starting structures of compounds were constructed and optimized by the DS CHARMM force field.[18] All conformers were minimized to an rmsd value of 0.01 kcal mol^{-1}. The lowest-energy conformer was accordingly selected for docking and molecular dynamic (MD) simulations. The receptor docking site was defined based on the binding position of E2 in the receptor and specified as all atoms within a radius of 12.0 Å around E2 by using an "eraser" algorithm. MD simulations were carried out for each mode employing this binding site. MD simulation was performed with time steps of 0.001 ps, a distance-dependent dielectric of 4.00, and a nonbonded cutoff distance of 8.0 Å at 300 K. The Monte Carlo method was employed in a conformational search of the ligands. The LigandFit method was used to examine the ligand–site match and the orientation/permutation during docking; the docked ligands were evaluated by DockScore.[19] All modeling work was carried out with Discovery Studio Modeling software package (Accelrys, USA) run on a Dell PowerEdge 2600 Server with default setting values except those explicitly stated.

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