

Effects of 7-O Substitutions on Estrogenic and Anti-Estrogenic Activities of Daidzein Analogues in MCF-7 Breast Cancer Cells

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Daidzein (**1**) is a natural estrogenic isoflavone. We report here that **1** can be transformed into anti-estrogenic ligands by simple alkyl substitutions of the 7-hydroxyl hydrogen. To test the effect of such structural modifications on the hormonal activities of the resulting compounds, a series of daidzein analogues have been designed and synthesized. When MCF-7 cells were treated with the analogues, those resulting from hydrogen substitution by isopropyl (**3d**), isobutyl (**3f**), cyclopentyl (**3g**), and pyrano- (**2**) inhibited cell proliferation, estrogen-induced transcriptional activity, and estrogen receptor (ER) regulated progesterone receptor (PgR) gene expression. However, methyl (**3a**) and ethyl (**3b**) substitutions of the hydroxyl proton only led to moderate reduction of the estrogenic activities. These results demonstrated the structural requirements for the transformation of daidzein from an ER agonist to an antagonist. The most effective analogue, **2**, was found to reduce in vivo estrogen stimulated MCF-7 cell tumorigenesis using a xenograft mouse model.

1. Introduction

Daidzein (**1**) is one of many known isoflavones that naturally occur in plants such as soybeans and kudzu vines (*Peruraria lobata*).¹ Like other constitutive isoflavones found in soy tissues (e.g., genistein),² daidzein has been shown to act as a variety of biologically active agents ranging from anti-oxidant, estrogen at doses lower than 20 μ M, and breast cancer cell growth and proliferation promoter.^{3–5} The estrogenic effect, however, can be reversed when daidzein concentration exceeds 20 μ M where it has been found to inhibit the growth of different cancer cells.^{4,6,7} For example, daidzein was found to antagonize the inhibitory effects of tamoxifen on pS2 expression⁸ and proliferation⁹ of breast tumor cells. The diverse functionality of daidzein is also reflected in its glucosyl derivative, 7-O-glucosyl-4'-hydroxyisoflavone (daidzin), which acts as an antialcohol-addiction agent,¹⁰ presumably through binding to the enzyme aldehyde dehydrogenase.

The exact mechanism underlying the cellular inhibitory effects of high dose daidzein and its closely related analogue genistein is not completely understood. Previous studies suggest their role as weak agonists competing with estradiol

for estrogen receptor (ER)^a binding.⁵ Additional studies have proposed several possible signaling pathways that may be responsible for these effects, such as binding to the epidermal growth factor receptor kinase¹¹ and interfering with transforming growth factor signaling.¹²

Interestingly, in soy, daidzein is also a key intermediate in the biosynthesis of the anti-estrogenic glyceollins in response to bacterial infection.^{13,14} This naturally occurring transformation of estrogenic to anti-estrogenic function prompted us to examine the structural features that distinguish the estrogenic activity of daidzein from the anti-estrogenic activity of the glyceollins. As previously published, the biosynthesis of glyceollin I from the daidzein precursor leads to the incorporation of (1) a 7,8-phenol ring of the chromanone structure where a six-membered ring is formed via an ether linkage^{13,14} and (2) a central moiety where a freely rotating biphenyl bond in daidzein becomes a rigid pterocarpan structure in glyceollin I. Both or one of the structural modifications could be responsible for the functional reversal of ER activity. Molecular modeling using the crystal structure of ER α indicates that daidzein can fit in the binding pocket of ER α when the receptor is optimized with both 17 β -estradiol (E₂) and 4-hydroxytamoxifen (4-OH-TAM), whereas glyceollins can only fit the pocket when the crystal structure of ER α is optimized with 4-OH-TAM. The modeling results¹⁵ suggest that the structural features in the 7-phenolic position may be the critical one responsible for the anti-estrogenic effects of glyceollin I.

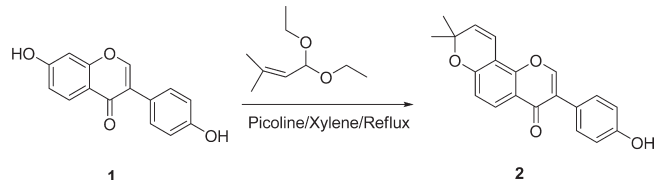
In this study, we explored the potential anti-estrogenic properties of daidzein analogues by modifying the 7-hydroxyl

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^a Abbreviations: E₂, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen responsive element; ICI (ICI182,780), fulvestrant; 4-OH-TAM, 4-hydroxytamoxifen; PgR, progesterone receptor; 3ERT, human estrogen receptor alpha ligand-binding domain in complex with 4-hydroxytamoxifen; 3ERD, ER α bound to the agonist diethylstilbestrol; MOPAC, molecular orbital package.

functional group while keeping the daidzein skeleton intact. We started with an analogue that is structurally identical with glyceollin I on the 7 and 8 ring substitutions but without the pterocarpan moiety. Using molecular modeling as a guiding tool, we designed and synthesized an additional eight daidzein analogues that vary in the 7-O substitution. We also evaluated the effect of daidzein and daidzein analogues on estrogen responsive element (ERE) transcriptional activity, ER α binding affinity, MCF-7 breast cancer cells colonial survival, PgR gene expression, and ER α docking. Finally, we conducted in vivo studies of the anti-estrogenic effects of the most potent analogue on MCF-7 cell tumorigenesis using a xenograft

Scheme 1. Synthesis of **2** from Daidzein



Scheme 2. Synthesis of Daidzein Analogues **3a–3h**



mouse model. The structure activity relationship studies on these daidzein analogues reveal the progressive loss of an ER agonist activity concurrent to the acquisition of ER antagonistic activity with the substitution of the 7-OH hydrogen with varying hydrophobic groups.

2. Results and Discussion

2.1. Chemistry. The synthetic route of 3-(4-hydroxyphenyl)-8,8-dimethyl-8*H*-pyrano[2,3-*f*]chromen-4-one (**2**) is diagrammed in Scheme 1. Briefly, following a literature method,¹⁶ a condensation reaction between **1** and an unsaturated aldehyde masked as its acetal in refluxing *p*-xylene in the presence of picoline as base brought about the desired ring closure in position 8 of **1** to give the target compound **2** as the major product. The minor byproduct formed by substitution and ring closure on daidzein's 4' and 5' positions was negligible and did not present a problem in the isolation and purification of **2**.

This strategy, however, did not work as hoped for all other daidzein analogues from **3a** to **3h** (Scheme 2). The main problems appeared to be associated with the much higher proportion of disubstitution on both hydroxyl groups. The challenge was overcome by adopting a synthetic strategy¹⁷ that used the appropriate alkyl halide in a DMSO suspension of potassium carbonate. The facile reactions proceeded at room temperature giving the desired compounds as the main products with varying amounts of byproducts from substitution on the other hydroxyl group (4'-OH). Notably, in some cases, it was necessary to carefully control reaction time and substrate ratio in order to achieve the desired outcome of majority 7-O substitution. In fact, for several compounds

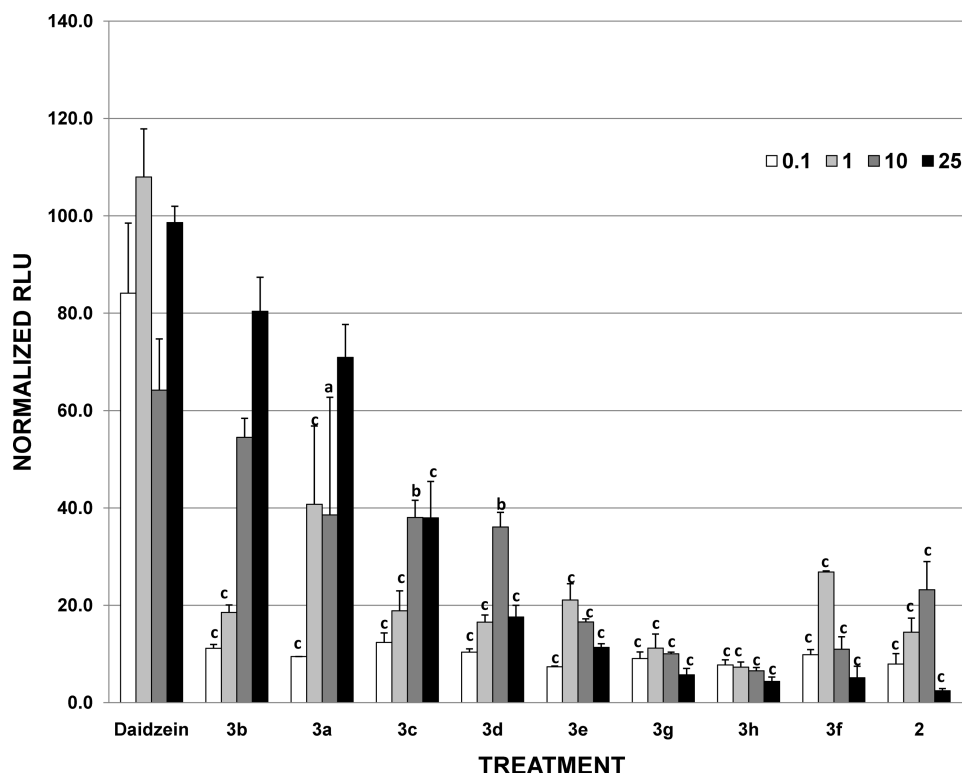
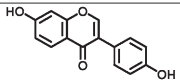
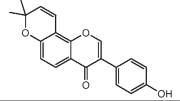
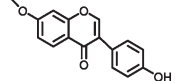
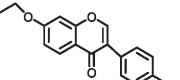
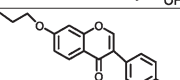
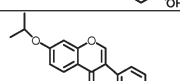
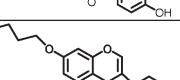
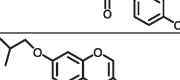
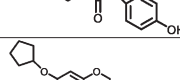
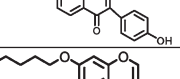


Figure 1. Effects of daidzein and daidzein analogues on ERE transcriptional activity in MCF-7 cells. MCF-7 cells were transiently transfected with pGL2-ERE2x-TK-luciferase plasmid. After a 6 h transfection, cells were treated with compounds (DMSO, daidzein, and daidzein analogues) and incubated overnight. Data are represented as relative light units (RLUs) normalized to E₂ (100 ± SEM). The values are the means and the SEM of triplicates from a single experiment and representative for at least three independent experiments. a, significant difference from daidzein, $p < 0.05$, b, significant difference from daidzein, $p < 0.01$, c, significant difference from daidzein, $p < 0.001$.

Table 1. Summary of Biological Activities of 7-*O*-Substituted Daidzein Derivatives^a

Analogue Structure	Name	Max % Agonist	Max % Inhibition	ER α BINDING MAX %
	Daidzein	108.5% @ 1 μ M	17.2% @ 10 μ M	112
	2	17.7% @ 10 μ M	100% @ 25 μ M 54.6 @ 10 μ M	44.1
	3a	68.5% @ 25 μ M	33.2% @ 25 μ M	82.7
	3b	79% @ 25 μ M	18.8% @ 25 μ M	66.6
	3c	33.4% @ 10 μ M	41.1% @ 25 μ M	44.9
	3d	30.6% @ 10 μ M	68.1% @ 25 μ M 33.4% @ 10 μ M	42.9
	3e	16.5% @ 10 μ M	49.8% @ 25 μ M	81.9
	3f	22.0% @ 1 μ M	91.3% @ 25 μ M 60.5 @ 10 μ M	38.3
	3g	5.4% @ 1 μ M	89.8% @ 25 μ M 54.1 @ 10 μ M	19.2
	3h	0.8% @ 0.1 μ M	46.4% @ 25 μ M	88.5

^aData are represented as relative agonistic and antagonists activities for individual compounds with double normalization in which E2 (0.1 nM) was set at 100% with vehicle (DMSO) set at 0%.

(e.g., **3f** and **3g**), isolation of the target 7-*O*-substituted product from their 4'-*O*-substituted byproducts was rather difficult, and separation by semipreparative HPLC was necessary to afford greater than 98% purity of these analogues.

2.2. Biological Activities of Daidzein Analogues. Effects of Daidzein Analogues on ER Transcriptional Activation in MCF-7 Cells. We have previously described the estrogenic and anti-estrogenic activities of individual flavonoids and phytochemicals using a luciferase-based ERE reporter assay.^{2,5,14} These studies have demonstrated the potent estrogenic activities of certain isoflavones such as genistein or daidzein, as well as the ability of glyceollins to antagonize estrogen-stimulated ERE activity.¹⁵ To test how structural modifications on the 7-hydroxyl group of daidzein affect estrogenic properties, the ER transcriptional activity induced by the daidzein analogues was measured and compared to daidzein using the ERE-based luciferase reporter gene assay. As shown in Figure 1, the estrogenic activities of synthetic daidzein analogues were plotted as dose–response curves when MCF-7 cells were treated with increasing concentrations of the test compounds. At 1 μ M concentration where daidzein induced maximal estrogenic effect in MCF-7 cells, the relative estrogenic potencies of the analogues are seen to decrease in roughly the following order: daidzein > **3a** > **3f** > **3e** > **3b** \cong **3c** > **3d** > **2** > **3g** > **3h**. While this

order varied slightly as analogue concentrations were increased further to 25 μ M (Table 1), several trends have emerged in the dose-dependent data. First, the parent compound daidzein showed more potent estrogenic activity than any of its derivatives when hydrogen was replaced with alkyl groups with varying chain lengths and configurations. Second, shorter alkyl groups appeared to have less effect on the reduction of ER agonist activity than their longer, bulkier counterparts. For example, at 25 μ M concentration, **3b** (ethyl-), **3a** (methyl-), and **3c** (*n*-propyl-) daidzein analogues induced ERE transcriptional activity at 80.4, 70.9, and 38.4%, respectively, retaining greater estrogenic activities than other analogues with longer chain or bulkier substituting groups. Finally, the analogue showing little or no estrogen activity, **2**, resembled the naturally occurring glyceollin I¹⁵ in the chromanone moiety, suggesting that the unique ring substitution on the 7*O*- and 8-chromanone ring was responsible for the diminished estrogenic activity of the modified daidzein.

Antagonist Activities of Daidzein Analogues on ER Transcriptional Activity. To evaluate the anti-estrogenic activities of daidzein analogues, MCF-7 cells were treated with the synthesized compounds at increasing concentrations in the presence of 0.1 nM E₂. The luciferase activities of the treated cells were then measured by the ERE reporter gene assay.

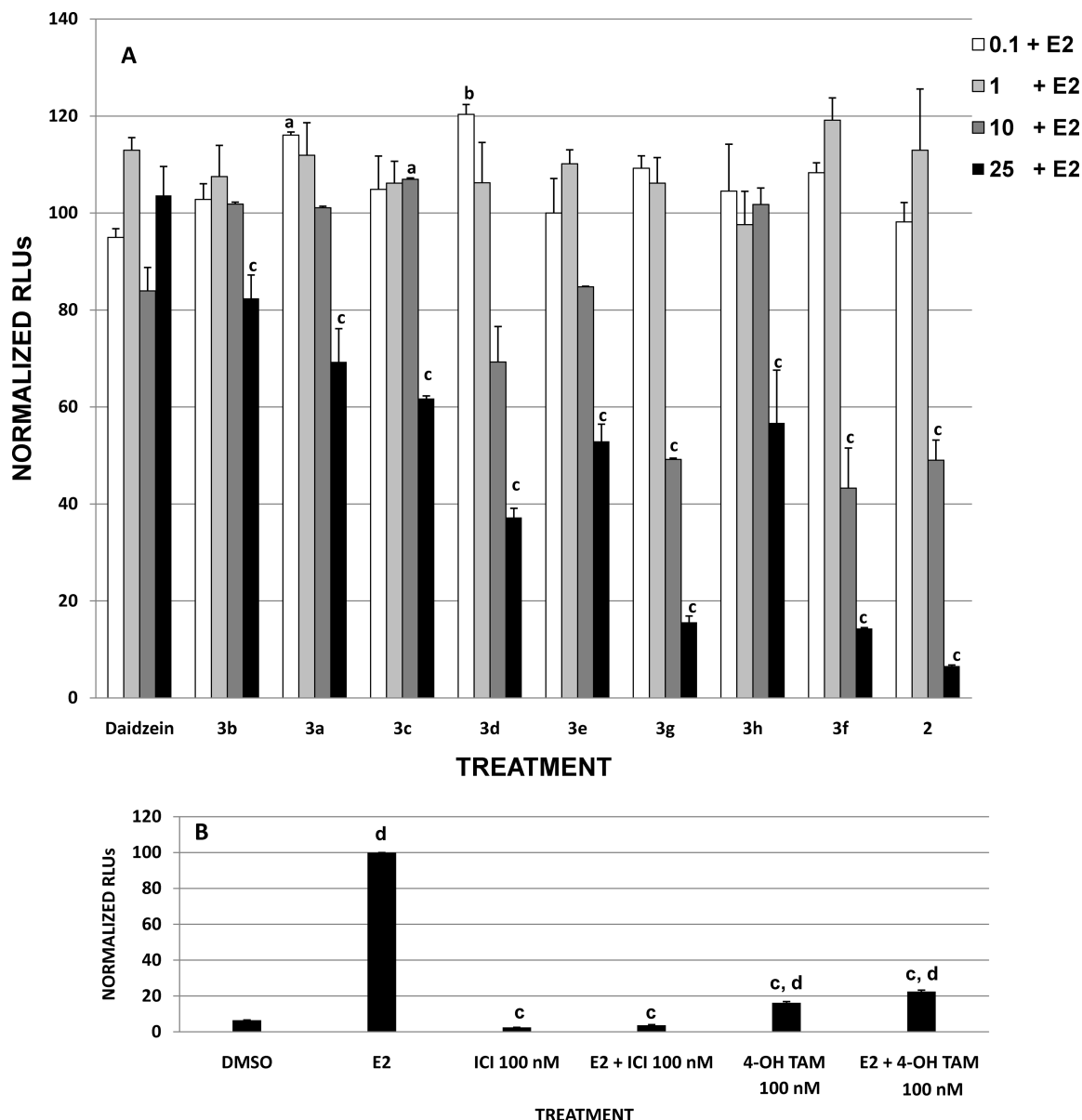


Figure 2. Effects of daidzein and daidzein analogues on E₂ stimulation on ERE transcriptional activity in MCF-7 cells. MCF-7 cells were transiently transfected with pGL2-ERE2x-TK-luciferase plasmid. After a 6 h transfection, cells were treated with (A) compounds (DMSO, E₂, daidzein and daidzein analogues plus E₂) or (B) with compounds (DMSO, E₂, ICI, ICI + E₂, 4-OH-TAM, 4-OH-TAM + E₂) and incubated overnight. Data are represented as relative light units (RLUs) normalized to E₂ (100 ± SEM). The values are the means and the SEM of triplicates from a single experiment and representative for at least three independent experiments. a, significant difference from E₂, $p < 0.05$, b, significant difference from E₂, $p < 0.01$, c, significant difference from E₂, $p < 0.001$, and d, significant difference from DMSO $p < 0.001$.

Figure 2 shows the dose–response curves of MCF-7 cells treated with E₂ plus daidzein and its synthetic analogues where the luciferase activity is normalized to 100 for cells treated with 0.1 nM E₂ alone. The inhibitory effect of some analogues on E₂ became more pronounced as their concentration was increased to 10 μM. At 25 μM (Table 1), the relative anti-estrogenic potencies of the analogues were observed to increase in the following order: daidzein < 3b < 3a < 3c < 3h < 3e < 3d < 3g < 3f < 2. The analogues that reduced estradiol-dependent luciferase activity by greater than 60% at 25 μM included 2, 3f, 3g, and 3d, of which 2 was the most effective anti-estrogen, completely blocking ERE activity at 25 μM. In contrast, analogues 3a, 3b, 3c, 3h, and 3e decreased E₂ induced transcriptional activity by less than 47% at the highest ligand concentration. Compared with estrogenic activity assay results, the analogues showing

strong anti-estrogenic activity exhibited little or no estrogen activity. For example, 2, 3f, and 3g all induced negligible ERE activity, but they behaved as potent estrogen inhibitors (Figures 1 and 2A). Treatment with fulvestrant (ICI) or 4-OH-TAM alone and in combination with estrogen caused a decrease in ERE transcriptional activity over 90% and 80%, respectively (Figure 2B). On the other hand, the strongest estrogenic analogues, 3a, 3b, and 3c, failed to inhibit 50% ERE activity in the presence of E₂ at all concentrations tested (Figure 2A). Taken together, the alkyl substituted daidzein analogues can be viewed in two groups in terms of their ER-mediated transcriptional activities. The first group (3a, 3b, and 3c) where the 7-OH of daidzein is alkoxyated by the methyl, ethyl, and *n*-propyl group, respectively, has retained much of the estrogenic function of daidzein. The second group, consisting of 2, 3g, 3f, and to a

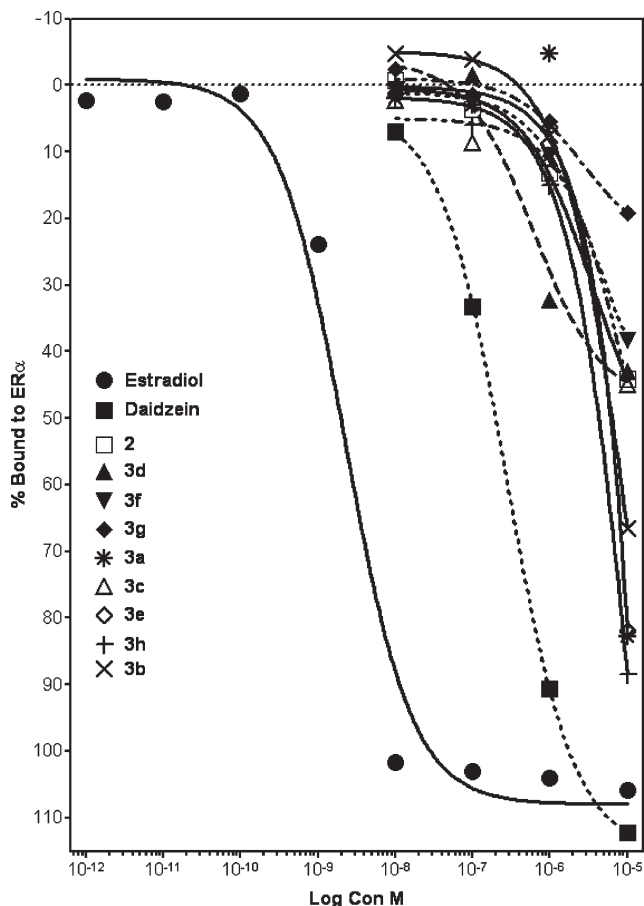


Figure 3. Relative binding affinity of daidzein and daidzein analogues for ER α . Increasing concentrations of daidzein analogues were added to the ER complex and compared to E₂. Data points and error bars represent the mean \pm SEM of at least three experiments ($n = 3$) for each concentration tested ($p < 0.05$).

lesser extent **3d**, has been transformed into potent anti-estrogens. This complete reversal from estrogenic to anti-estrogenic activity in certain daidzein analogues highlights the importance of the structure of 7-O substitution in determining the ligand's interaction with the estrogen receptor. It is also interesting to note the significant difference in ERE activity between the *i*-propyl (**3d**) and *n*-propyl (**3c**) substituted analogue, suggesting that a branched alkyl group may provide a better binding mechanism toward the ER.

Relative Affinity of Daidzein Analogues for ER α . In an attempt to understand the biological activity of the synthesized daidzein analogues, we examined the ability of each daidzein analogues to bind to ER α using a competitive binding assay with fluorescent detection (Figure 3). Analysis of the competitive binding curve yielded IC₅₀ values, representing the concentration of unlabeled ligand required to displace 50% of the tracer from the ER α . Unlabeled E₂ was used as a reference and its displacement of 50% tracer E₂ bound to ER α occurred at 1.5 nM. Compared to daidzein, which has an IC₅₀ value of 0.45 μ M, none of the analogues displayed higher binding affinities toward the estrogen receptor. The maximal % bound to ER α for the analogues is summarized in Table 1. At 10 μ M ligand concentration, four analogues showed over 50% bound ER α , that is, **3a**, **3b**, **3e**, and **3h**, while the rest of analogues appeared to have lower binding affinities toward the ER. The binding data are partially consistent with the observation that structural

modifications on the 7-O position of daidzein reduced more estrogenic potency of the analogues to various degrees at lower concentrations (10 μ M) than at higher doses (25 μ M). However, the reason for higher binding affinities exhibited by **3h** and **3e** which are neither estrogenic nor anti-estrogenic is unclear. The binding curves suggest that at higher concentrations (e.g., 25 μ M as used in the luciferase reporter assay), the degree of binding to ER by several analogues may rapidly approach that of daidzein. This is also consistent with the fact that both estrogenic (**3a**, **3b**, and **3c**) and anti-estrogenic (**2**, **3f**, **3g**) activities of the analogues reached maximal levels at 25 μ M (Figures 1 and 2). For example, **3a** reached 38% ERE activity at 10 μ M; at 25 μ M, its ERE activity increased dramatically to 70.9% (Figure 1). The binding curve for **3a** confirmed this rapid increase in estrogenic activity as its % bound value appears to approach that of daidzein. Notably, for anti-estrogenic compounds, binding affinities to ER α did not appear to correlate well with their ability to inhibit E₂-induced ERE activity.

Effect of Daidzein Analogues on Estrogen-Stimulated Clonogenic Proliferation. To further evaluate the estrogenic or anti-estrogenic activities of the daidzein analogues determined from the ERE transcriptional assay, we selected two analogues, **3a** and **2**, for further test by a clonogenic assay for their ability to inhibit the estradiol-induced proliferation of MCF-7 cells. As discussed earlier, **3a** behaved as an estrogen with a slightly lower potency than daidzein, whereas **2** acted as an anti-estrogen that was able to inhibit 100% ER-mediated transcriptional activity. In the colony assay, MCF-7 cells were treated with increasing concentrations of daidzein, **2**, **3a**, and 4-OH-TAM in the presence or absence of 0.1 nM E₂. Results are illustrated in Figure 4 where estrogen-dependent cell growth was normalized to 100 for the control (DMSO) (Figure 4A) or for E₂ alone (Figure 4B). The addition of 0.1 nM E₂ to the culture media resulted in an approximately 3-fold increase in the clonogenicity of the MCF-7 cells compared to DMSO-treated controls. At 1 μ M, daidzein also promoted the clonogenicity of MCF-7 cells by over 3-fold, while **3a** increased cell proliferation by 43%, confirming that **3a** is a weaker estrogen than daidzein, a direct consequence of the replacement of 7-hydroxy group by 7-methoxy group. In contrast, **2** inhibited the clonogenicity by 15% at the same concentration, demonstrating its anti-estrogenic property at 1 μ M concentration. When the concentration was raised to 10 μ M, daidzein and **3a** continued to increase the clonogenicity of MCF-7 cells by 2.5 times and 24%, respectively. Analogue **2**, however, completely blocked cell clonogenicity at 10 μ M, similar to treatment with 4-OH-TAM at 100 nM (Figure 4B). Thus, the colony assay results clearly demonstrated the divergence of hormonal activities in daidzein analogues by simple modifications of the 7-OH group of daidzein.

Regulation of ER-Mediated PgR Gene Expression by Daidzein Analogues. To further examine the differing hormonal activities of daidzein analogues, we monitored ER-mediated gene expression of progesterone receptor (PgR) by quantitative real time RT-PCR in MCF-7 cells treated with daidzein, **2** (an anti-estrogenic analogue), and **3a** (an estrogenic analogue) (Figure 5). Progesterone receptor (PgR) is an estrogen-responsive gene, whose expression has been shown to indicate a responsive estrogen receptor pathway. The expression of PgR mRNA in MCF-7 cells that were treated with DMSO (control), daidzein, **2** and **3a** either in the presence or absence of E₂ were determined. When treated with ligands only,

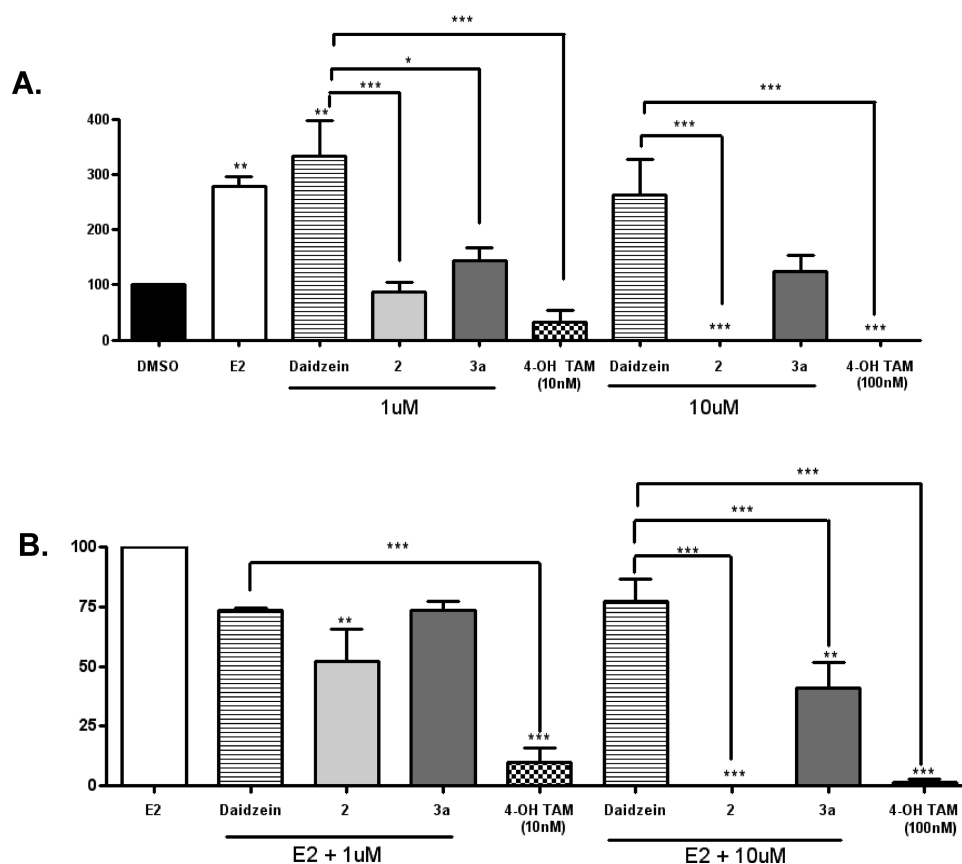


Figure 4. Effects of daidzein, **2** and **3a** on E₂ stimulation on colony formation on MCF-7 cells. MCF-7 cells were placed in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS for 48 h before plating. MCF-7 cells (1000) were plated in six well plates. Forty-eight hours later, cells were treated with DMSO (vehicle), E₂, daidzein, daidzein analogues (at 1 μ M and 10 μ M) and 4-OH-TAM (at 10 nM and 100 nM) (A) and E₂, E₂ + daidzein, E₂ + daidzein analogues (at 1 μ M and 10 μ M) and E₂ + 4-OH-TAM (at 10 nM and 100 nM) (B). Colonies of > 50 cells were counted as positive. Results are normalized to percentage of clonogenic survival from DMSO control cells. The values are the means and the SEM of triplicates from a single experiment and representative for at least three independent experiments. Significant difference from vehicle control (A) and E₂ (B), **p* < 0.05, ***p* < 0.01 and ****p* < 0.001; Tukey test.

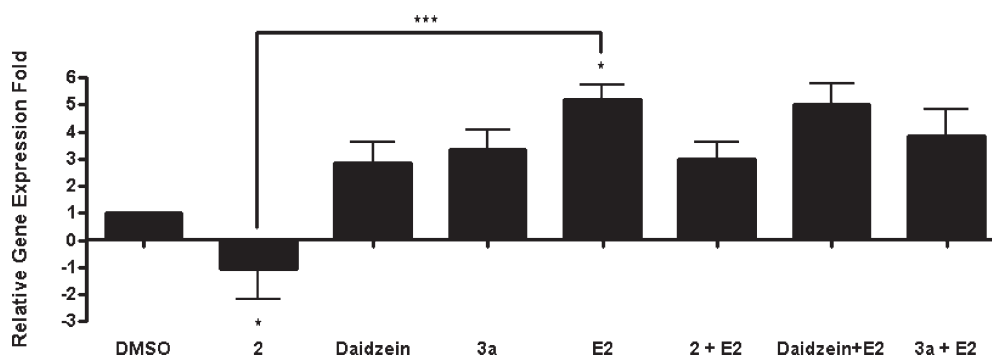


Figure 5. Effects of daidzein, **2** and **3a** on PgR expression. Total RNA was isolated from MCF-7 cells, reverse-transcribed into cDNA and subjected to real-time RT-PCR analysis for quantitation. Treatment on MCF-7 cells was as follows: DMSO (vehicle), daidzein, **2**, **3a**, E₂, daidzein + E₂, **2** + E₂, **3a** + E₂ (10 μ M and 10 μ M + E₂). Results are expressed as the mean unit \pm SEM (****p* < 0.05; ***p* < 0.01; **p* < 0.001) with significant differences from vehicle control.

daidzein and **3a** induced a 3-fold increase in PgR expression compared with control, while a 5-fold increase was induced by E₂. However, **2** caused a decrease in PgR expression compared with DMSO, demonstrating its non-estrogenic nature. When MCF-7 cells were treated with the ligands in the presence of 0.1 nM E₂, both daidzein and **3a** were able to promote the level of PgR expression by 5- and 4-fold, respectively. In contrast, **2** inhibited the E₂ induced PgR expression by reducing its up-regulation from 5- to 3-fold. Recently, we have demonstrated the

ability of the known anti-estrogens fulvestrant (100 nM) and 4-OH-TAM (100 nM) to suppress E₂-stimulated PgR expression by ~75% and ~64% respectively in our MCF-7 cell system.¹⁵ In comparison, analogue **2** resulted in a ~40% suppression of E₂-stimulated PgR expression (Figure 5).

In Vivo Anti-Estrogenic Effects of Analogue 2 on MCF-7 Cell Tumorigenesis. Because the anti-estrogenic effects of analogue **2** on MCF-7 cells observed in our in vitro biological assays, this compound was selected for further testing of

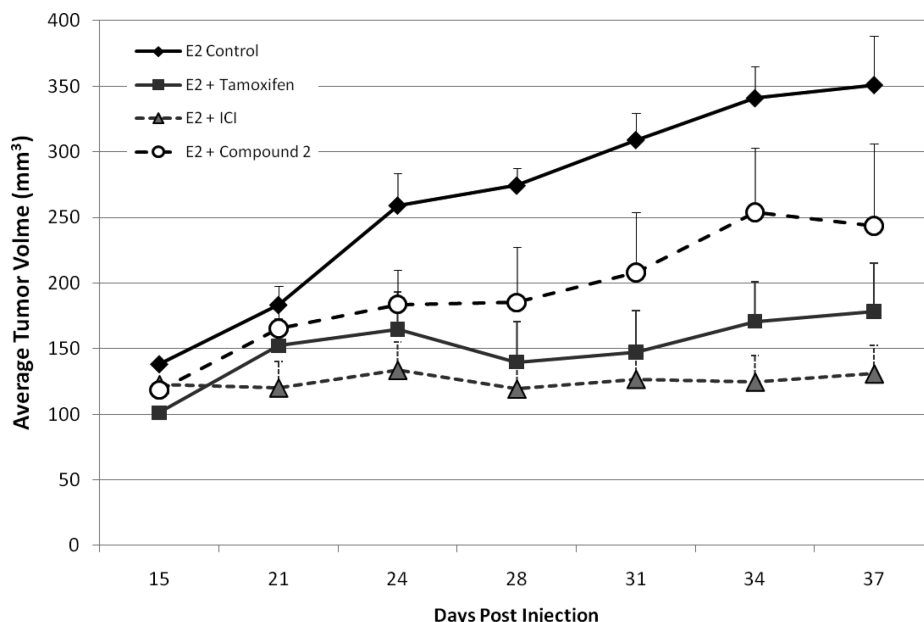


Figure 6. Anti-estrogenic effects of analogue **2** on MCF-7 cell tumorigenesis in vivo. 4–6 week old ovariectomized female Nu/Nu mice were injected bilaterally in the MFP with 5×10^6 MCF-7 cells in matrigel (reduced factor). All animals were implanted with a 17β -estradiol pellet (0.72 mg, 60-day release) subcutaneously in the lateral area of the neck at the time of cell injection. Tumors were allowed to form and at day 15 post cell injection mice were randomized into treatment groups ($n = 5$). Animals were treated daily with i.p. injections of either vehicle (1:5 DMSO/PBS) or analogue **2** (50 mg/kg/animal). Tamoxifen animals were implanted with a slow release tamoxifen pellet (5mg, 60-day release) in the lateral area of the neck (opposite side from E₂ pellet). ICI animals were given a one-time i.p. injection of ICI 182,780 (5 mg in castor oil). Tumor size was measured 3 times weekly using digital calipers. Data are represented as mean tumor volume + SEM.

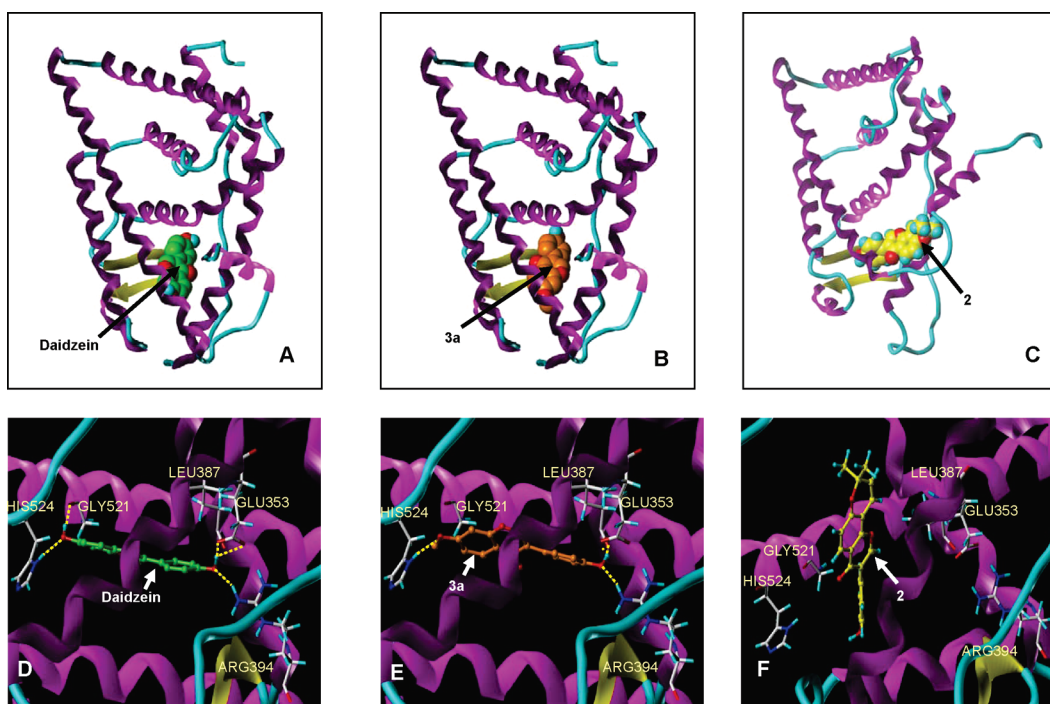


Figure 7. Molecular modeling of daidzein and its analogues **2** and **3a**. Binding modes of daidzein (A), **3a** (B) to estrogenic conformer of ER α and **2** (C) to anti-estrogenic conformer of ER α , wherein, the protein is depicted as ribbon/tube model colored by secondary structure and the compounds are shown as spacefill models. D, E, and F depict the hydrogen bond interactions made by the compounds daidzein, **3a** and **2** with the protein as a ribbon/tube model colored by secondary structure and the compounds as ball and stick models.

estrogen-stimulated MCF-7 cell tumorigenesis using a xenograft model. Ovariectomized female immunocompromised mice were injected with MCF-7 cells and supplemented with exogenous estrogen pellets. Once tumors were palpable animals were treated with analogue **2**, tamoxifen, or ICI and tumor volume compared to vehicle control animals. As shown in

Figure 6, at day 37 post cell injection, a decrease in tumor volume was observed in all treatment groups compared to control (351.17 ± 43.88). Compound **2** treatment resulted in reduced tumor volumes similar to tamoxifen treated animals (241.53 ± 68.29 and 178.12 ± 41.18 mm³, respectively), while treatment with the pure anti-estrogen ICI 182,780 was most effective in

attenuating estrogen-induced tumorigenesis (130.95 ± 23.49). These results confirm that the anti-estrogenic properties of compound **2** observed in vitro extend to in vivo conditions as well.

Molecular Modeling. The estrogenic compound **3a** and anti-estrogenic compound **2**, along with the parent compound daidzein were subjected to docking studies. To understand the nature of their interactions with ER α , two protein conformations were selected as template coordinates for the docking studies. It has been well established that the binding mode of ligand to the estrogen receptor causes structural perturbations in and around the ligand binding pocket resulting in two protein conformations that contribute to the estrogenic or anti-estrogenic effects.^{18–20} Two such representative structures, 3ERD (ER α bound to the agonist diethylstilbestrol) and 3ERT (ER α bound to the antagonist 4-OH-TAM) were taken as protein templates. The compounds were docked to the template protein structures using the Surflex docking program. The Surflex scoring²¹ and the binding postures for the compounds daidzein and **3a** were comparable for the estrogenic and anti-estrogenic protein conformations with a slight preference to the estrogenic conformation. The binding modes of daidzein and **3a** for the estrogenic protein conformation are represented in Figure 7A,B. Daidzein forms hydrogen bonds with GLU353, ARG394, GLY521, and HIS524 (Figure 7D). **3a** forms hydrogen bonds with LEU387, ARG394, and HIS524 (Figure 7E), resulting in fewer hydrogen bonding interactions than daidzein which could account for its weaker estrogenic activity. Compound **2** docked to the anti-estrogenic conformation of the protein with a significantly better Surflex score than when docked to the estrogenic conformation of the protein which implies that it can have anti-estrogenic property. The binding posture of **2** (Figure 7C,F) for the anti-estrogenic protein conformation was similar to that of other ER α antagonists tamoxifen and raloxifene. These docking studies further confirm the nature of estrogenic and anti-estrogenic activity exhibited by the compounds **3a** and **2**.

3. Conclusion

Previous studies in our laboratory demonstrated the diverse estrogenic and anti-estrogenic activity of naturally occurring flavonoid phytochemicals. Through these studies, we identified glyceollin I, a naturally occurring phytoalexin in activated soy, behaved as a potent anti-estrogen.¹⁵ However, daidzein, the precursor in the biological synthesis of glyceollin I was strongly estrogenic. The contrasting hormonal activity of daidzein and glyceollin I provided the rationale to design and synthesize a series of daidzein analogues that have modified structures on the 7-hydroxyl position of daidzein. In particular, one such analogue, **2** was designed to answer the central question: what is the structural requirement that is responsible for the regulation of the agonistic and antagonistic activities of isoflavone phytochemicals. **2** has retained the daidzein skeleton with the addition of a chromanone moiety. Results from ERE luciferase assay, colony assay, and PgR expression revealed that **2**, in contrast to daidzein, acted predominantly as an anti-estrogen with limited agonist activity. The in vitro anti-estrogenic potency of **2** was found comparable to glyceollin I,¹⁵ strongly suggesting that substitution on the chromanone structure, not the presence of pterocarpan skeleton, is responsible for the loss of estrogenic and acquisition of anti-estrogenic activity from daidzein to glyceollin I. To further test the in vivo

efficacy of **2** as an ER antagonist, nude mice xenografted with E₂-dependent MCF-7 cells was treated with the analogue **2**, resulting in significant tumor regression.

By synthesizing and testing additional daidzein analogues we have further demonstrated the effect of varying 7-O-substitution groups on estrogenic or anti-estrogenic activities of the compounds. Short chain alkyl substitutions (**3a**, **3b**, **3c**) weakened the estrogenic potency of daidzein but did not render the compounds anti-estrogenic; longer chain alkyl substitutions (**3e**, **3h**) appeared to further diminish the estrogenic activity. On the other hand, **3d**, **3f**, and **3g** where branched alkyl or cycloalkyl substitutions were introduced, exhibited significant anti-estrogenic property. Molecular modeling study of representative daidzein analogues using the crystal structure of ER α revealed that daidzein and **3a** fitted well in the ligand binding pocket of ER in its estrogen bound configuration. The anti-estrogenic **2** was found to dock better in ER in its anti-estrogen-bound configuration. These results demonstrated the structural requirements for the transformation of daidzein from an estrogen to an anti-estrogen. The most effective compound, **2** may serve as a useful novel anti-estrogen for breast cancer treatment and prevention.

4. Experimental Section

4.1. Chemistry. All reagents and solvents were purchased from either Aldrich Chemical Co. (WI, USA) or Acros organics (NY, USA) and were used as received. All organic solvents used were of reagent grade quality and were used without further purification. Daidzein (**1**) was synthesized by Tyger Scientific Inc. (NJ, USA). ¹H spectra were recorded at 400 MHz on a Varian Unity-400 spectrometer (Varian Inc., Palo Alto, CA), using DMSO-*d*₆ as solvent and as internal standard (δ 2.49). GC-MS analyses were performed on an Agilent Technologies 5975C inert MSD mass spectrometer. Melting points were determined with a Mel-temp II point apparatus and are uncorrected. Crude synthetic products were purified by the following methods: chromatography on Silica Gel (60–100 mesh, Fisher Scientific) column and recrystallization from acetone. Analytical thin layer chromatography (TLC) was performed on 250 μ fluorescent plates Whatman, Germany) and visualized by using UV light. For all products, the purity was ascertained to be greater than 95% by the HPLC method using a Shimadzu (Columbia, MD) 2010 HPLC-UV/MS system with a C-18 reverse phase column.

4.1.1. 3-(4-Hydroxyphenyl)-8,8-dimethyl-8H-pyrano[2,3-f]-chromen-4-one (2**).** According to the literature method,¹⁶ to a solution of **1** (1.9 g, 7.2 mmol) in 360 mL of xylene was added 1,1-diethoxy-3-methyl-2-butene (2.4 g, 14.7 mmol) in 15 mL of xylene and 3-picoline (3.7 mmol in 43 mL xylene). The reaction mixture was refluxed at 130 °C for 18 h. The solvent was evaporated under a vacuum and the residue was chromatographed over silica gel using hexanes as the eluant. Final product was recrystallized from acetone to give 0.34 g of **2** as colorless needle crystalline, with an overall yield of 15%. mp: 262–264 °C (Dec.). GC-MS (*m/z* 320 M⁺, *m/z* 305, *m/z* 254, *m/z* 187, *m/z* 152, *m/z* 118). ¹H NMR (DMSO-*d*₆, 400 MHz, δ): 1.45 (6H, s), 5.94 (1H, d, *J* = 10.0 Hz), 6.79 (1H, d, *J* = 10.0 Hz), 6.80 (2H, d, *J* = 8.8 Hz), 6.92 (1H, d, *J* = 8.4 Hz), 7.38 (2H, d, *J* = 8.8 Hz), 7.88 (1H, d, *J* = 8.4 Hz), 8.36 (1H, s), 9.57 (1H, s, D₂O exchangeable).

4.1.2. 3-(4-Hydroxyphenyl)-7-methoxychromen-4-one (3a**).** Following a literature method,¹⁷ to a solution of **1** (1.27 g, 5 mmol) in 10 mL of DMSO was added anhydrous K₂CO₃ (0.9 g, 6.5 mmol) and iodomethane (0.71 g, 5.0 mmol). The reaction mixture was stirred at room temperature for 2 h and then poured into ice water. The resulting mixture was extracted with ethyl acetate. The organic phases were combined, washed with brine, dried

over sodium sulfate, filtered, and concentrated under a vacuum. The residue was chromatographed over silica gel using hexanes/ethyl acetate (4:1) as eluant. The product was recrystallized from acetone to provide 0.36 g of **3a** as a white powder in 27% isolated yield. mp: 210–211. (lit.¹⁷ 210–211). GC-MS (m/z 268 M^+ , m/z 225, m/z 151, m/z 118). ¹HNMR (DMSO- d_6 , 400 MHz, δ): 3.91 (3H, s), 6.81 (2H, d, J = 8.8 Hz), 7.08 (1H, dd, J = 8.8 Hz, J = 2.4 Hz), 7.16 (1H, d, J = 2.4 Hz), 7.40 (2H, d, J = 8.8 Hz), 8.03 (1H, d, J = 8.8 Hz), 8.38 (1H, s), 9.58 (1H, s, D₂O exchangeable).

4.1.3. 3-(4-Hydroxyphenyl)-7-ethoxychromen-4-one (3b). Following the procedure used to prepare **3a**, reaction of **1** with bromoethane provided 0.44 g of **3b** as a pale yellow solid in 31% yield. mp: 131–132 °C. (lit.²² 131–133 °C). GC-MS (m/z 282, M^+ , m/z 253, m/z 225, m/z 165, m/z 137, m/z 118). ¹HNMR (DMSO- d_6 , 400 MHz, δ): 1.38 (3H, t, J = 6.8 Hz), 4.19 (2H, q, J = 6.8 Hz), 6.82 (2H, d, J = 8.8 Hz), 7.06 (1H, dd, J = 8.8 Hz, J = 2.4 Hz), 7.14 (1H, d, J = 2.4 Hz), 7.40 (2H, d, J = 8.8 Hz), 8.02 (1H, d, J = 8.8 Hz), 8.37 (1H, s), 9.57 (1H, s, D₂O exchangeable).

4.1.4. 3-(4-Hydroxyphenyl)-7-propoxychromen-4-one (3c). According to the procedure used to prepare **3a**, reaction of **1** with 1-bromopropane provided 0.57 g of **3c** in 38% yield as a white powder. mp: 173–175 °C (Dec.). GC-MS (m/z 296 M^+ , m/z 253, m/z 225, m/z 137, m/z 118). ¹HNMR (DMSO- d_6 , 400 MHz, δ): 0.99 (3H, t, J = 7.0 Hz), 1.77 (2H, sextet, J = 7.2 Hz), 4.08 (2H, t, J = 7.0 Hz), 6.81 (2H, d, J = 8.4 Hz), 7.06 (1H, dd, J = 8.8 Hz, J = 2.4 Hz), 7.13 (1H, d, J = 2.4 Hz), 7.39 (2H, d, J = 8.4 Hz), 8.01 (1H, d, J = 8.8 Hz), 8.35 (1H, s), 9.56 (1H, s, D₂O exchangeable).

4.1.5. 3-(4-Hydroxyphenyl)-7-isopropoxychromen-4-one (3d). According to the procedure used to prepare **3a**, reaction of **1** with 2-bromopropane provided 0.26 g of **3d** in 18% yield as a white powder. mp: 179–180 °C (Dec.). GC-MS (m/z 296, M^+ , m/z 253, m/z 225, m/z 137, m/z 118). ¹HNMR (DMSO- d_6 , 400 MHz, δ): 1.32 (6H, d, J = 6.4 Hz), 4.82 (sept, 1H, J = 6.0 Hz), 6.796 (2H, d, J = 8.4 Hz), 7.03 (1H, dd, J = 8.8 Hz, J = 2.4 Hz), 7.13 (1H, d, J = 2.4 Hz), 7.38 (d, 2H, J = 8.4 Hz), 8.00 (1H, d, J = 8.8 Hz), 8.35 (1H, s), 9.56 (1H, s, D₂O exchangeable).

4.1.6. 7-Butoxy-3-(4-hydroxyphenyl)chromen-4-one (3e). Using the procedure used to prepare **3a**, reaction of **1** with 1-bromobutane provided 0.66 g of **3e** as a white powder in 43% yield. mp: 162–164 °C. GC-MS (m/z 310, M^+ , m/z 254, m/z 253, m/z 226, m/z 225, m/z 197, m/z 137, m/z 118). ¹HNMR (DMSO- d_6 , 400 MHz, δ): 0.94 (3H, t, J = 7.2 Hz), 1.45 (2H, sextet, J = 7.2 Hz), 1.737 (2H, quint, J = 7.2 Hz), 4.12 (2H, t, J = 7.0 Hz), 6.80 (d, 2H, J = 8.4 Hz), 7.06 (1H, dd, J = 8.8 Hz, J = 2.4 Hz), 7.13 (1H, d, J = 2.4 Hz), 7.39 (2H, d, J = 8.4 Hz), 8.00 (1H, d, J = 8.8 Hz), 8.35 (1H, s), 9.57 (1H, s, D₂O exchangeable).

4.1.7. 3-(4-Hydroxyphenyl)-7-isobutoxychromen-4-one (3f). By the same procedure used to prepare **3a**, reaction of **1** with 2-bromobutane provided 0.39 g of **3f** in 25% yield as a white powder. mp: 176–178 °C. GC-MS (m/z 310 M^+ , m/z 253, m/z 137, m/z 118). ¹HNMR (DMSO- d_6 , 400 MHz, δ): 0.990 (6H, d, J = 6.4 Hz), 2.05 (1H, nonet, J = 6.40 Hz), 3.89 (2H, d, J = 6.4 Hz), 6.792 (2H, d, J = 8.8 Hz), 7.061 (1H, dd, J = 8.8 Hz, J = 2.4 Hz), 7.119 (1H, d, J = 2.4 Hz), 7.381 (2H, d, J = 8.8 Hz), 8.001 (1H, d, J = 8.8 Hz), 8.350 (1H, s), 9.57 (1H, s, D₂O exchangeable).

4.1.8. 7-Cyclopentyl-3-(4-hydroxyphenyl)chromen-4-one (3g). According to the procedure used to prepare **3a**, reaction of **1** with bromocyclopentane provided 0.35 g of **3g** as a colorless needle crystalline in 22% yield. mp: 204–206 °C. GC-MS (m/z 322 M^+ , m/z 254, m/z 225, m/z 137, m/z 118). ¹HNMR (DMSO- d_6 , 400 MHz, δ): 1.61 (2H, m), 1.74 (4H, m), 1.99 (2H, m), 5.01 (1H, m), 6.81 (2H, d, J = 8.4 Hz), 7.02 (1H, d, J = 8.8 Hz, J = 2.0 Hz), 7.09 (1H, d, J = 2.0 Hz), 7.39 (2H, d, J = 8.4 Hz), 8.01 (1H, d, J = 8.8 Hz), 8.35 (1H, s), 9.57 (1H, s, D₂O exchangeable).

4.1.9. 7-Hexyloxy-3-(4-hydroxyphenyl)chromen-4-one (3h). According to the procedure used to prepare **3a**, reaction of **1**

with 1-bromohexane provided 0.68 g of **3h** as a colorless crystalline in 40% yield. mp: 147–149 °C (lit.²³ 145–148 °C). GC-MS (m/z 338 M^+ , m/z 254, m/z 226, m/z 137, m/z 118). ¹HNMR (DMSO- d_6 , 400 MHz, δ): 0.87 (3H, t, J = 7.2 Hz), 1.31 (4H, m), 1.42 (2H, quintet, J = 7.2 Hz), 1.75 (2H, quintet, J = 7.2 Hz), 4.11 (2H, t, J = 7.0 Hz), 6.80 (2H, d, J = 8.4 Hz), 7.05 (1H, dd, J = 8.8 Hz, J = 2.4 Hz), 7.13 (1H, d, J = 2.4 Hz), 7.39 (2H, d, J = 8.4 Hz), 8.00 (1H, d, J = 8.8 Hz), 8.352 (1H, s), 9.57 (1H, s, D₂O exchangeable).

4.2. Biological Assays. Cell Culture. Human cancer cell lines derived from breast, MCF-7 (ER-positive cells) were cultured in 75 cm² culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Co.) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc., Gaithersburg, MD), basic minimum MEM essential (50 \times , Invitrogen Co.) and MEM nonessential (100 \times , Invitrogen, Co.) amino acids, sodium pyruvate (100 \times , Invitrogen Co.), antimycotic-antibiotic (10 000 U/mL penicillin G sodium; 10 000 μ g/mL streptomycin sulfate; 25 μ g/mL amphotericin B as Fungizone), and human recombinant insulin (4 mg/mL, Invitrogen Co). The culture flasks were maintained in a tissue culture incubator in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. For estrogen studies, cells were washed with PBS 3 times and grown in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% CS-FBS) for 72 h before plating for each particular experiment.

ERE-Luciferase Assay. As previously described,^{15,24} MCF-7 cells, grown for 2 days in 5%-CS-FBS containing phenol-red free DMEM, were plated in 24-well plates at a density of 5×10^5 cells/well in the same media and allowed to attach overnight. After 18 h, cells were transfected with 300 μ g of pGL2-ERE2X-TK-luciferase plasmid (Panomics) for 6 h according to the manufacturer's protocol using Effectene (Qiagen) and treated with vehicle DMSO, E₂, various concentrations of daidzein analogues (all with and without estrogen), ICI and 4-OH-TAM overnight. Media was removed and cells were lysed with reporter lysis buffer. Relative light units (RLUs) were measured in an Opticom II luminometer (MGM Laboratories) using luciferase reagent (Promega).

ER α Binding Assays. Receptor binding determinations of daidzein analogues were achieved using the method of Bolger²⁵ et al. as was applied by Burrow¹⁴ et al. In this method, recombinant ER is in equilibrium with a fluorescent ligand (ES2) and a concentration of the competitor (daidzein analogues). The relative displacement of the ES2 is measured as a change in polarization anisotropy. Serial dilutions of competitors (daidzein analogues and estradiol) were prepared from DMSO stock solutions in screening buffer at the desired concentrations. The ER and ES2 were combined with each competitor aliquot to a final concentration of 2 nM ER and 3 nM ES2, respectively. In addition, both a no binding control (ER + ES2, equivalent to 0% competitor inhibition) and a 100% binding control (only free ES2, no ER, equivalent to 100% competitor inhibition) were prepared. All competitor and controls were prepared in duplicate within a binding experiment. After 2-h incubation at room temperature, the anisotropy values for each sample and control were measured using the Beacon 2000. Anisotropy values were converted to percent inhibition using the following formula: $I_{\%} = (A_0 - A) / (A_0 - A_{100}) \times 100$, where $I_{\%}$ is the percent inhibition, A_0 is 0% inhibition, A_{100} is 100% inhibition, and A represents the observed value. This conversion to percent inhibition makes the data more intuitive and normalizes the experiment-to-experiment differences in the range of anisotropy values. The percent inhibition versus competitor concentration curves were analyzed by nonlinear least-squares curve fitting (Prism 5.0a, GraphPad Software, San Diego, California USA, www.graphpad.com) to yield IC₅₀ values (the concentration of competitor needed to displace half of the bound ligand). To compare binding affinities of the test compounds to those reported in the literature, IC₅₀ values were converted to

relative binding affinities (RBA) using E₂ as a standard. The E₂ RBA was set equal to 100 RBA = (IC₅₀/IC₅₀ of E₂) × 100.

Colony Formation Assay. Similar to our previous studies,^{15,24} cells were cultured in 5% FBS-DMEM and media was changed to phenol red-free 5% CS-DMEM for 48 h prior to assay. MCF-7 cells were seeded at a density of 1 × 10³ cells/well in a six-well plate containing phenol red-free 5% CS-DMEM. The cells were allowed to attach overnight and treated on the following day with DMSO vehicle, 1 nM E₂, the individual daidzein analogues (with and without estrogen) and 4-OH-TAM. Cells were incubated at 37 °C, 5% CO₂ in a humidified incubator. Media was replaced every 7 days and treated with appropriate drug for 3 weeks. After 3 weeks, the media were removed and the cells were fixed with formaldehyde and dried overnight. The cells were then washed and stained with crystal violet and dried. The colonies were counted.

Real Time RT-PCR. Total cellular RNA was extracted using the RNeasy mini column (Qiagen), following the manufacturer's instructions. Briefly, a maximum of 1 × 10⁷ cells are disrupted in buffer containing guanidine isothiocyanate and homogenized. Ethanol is added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. The sample is then applied to the RNeasy mini-column. Total RNA binds to the membrane, and high-quality RNA is then eluted in RNase-free water. All binding, wash, and elution steps were performed by centrifugation in a microcentrifuge. The concentration of RNA was determined using an ultraviolet spectrophotometer. Reverse transcription (RT) was carried out using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). One microgram of total RNA was reverse transcribed to cDNA following the manufacturer's instructions. For each RT, a blank was prepared using all the reagents except the RNA sample (for which an equivalent volume of DEPC treated water was substituted). This blank was used as a nontemplate control in the real-time PCR experiments. The level of PgR transcripts was determined using a real-time quantitative PCR. Primers for PCR were designed to span intron/exon junctions to minimize amplification of residual genomic DNA. The primer sequence for PgR is (5'-TACC-CGCCCTATCTCAACTACC-3'; 5'-TGCTTCATCCCCACAG-ATTAAACA-3'). PCR mix contained optimal concentrations of primers, cDNA and SYBR Green PCR Master Mix (Bio-Rad). Quantification and relative gene expression were calculated with internal controls. The ratio between these values obtained provided the relative gene expression levels.

In Vivo Assay of Anti-Estrogenic Activities of Analogue 2. Xenograft experiments were conducted using previously published protocols (Salvo et al 2006, Rhodes et al 2010). Briefly, 4–6 week old female ovariectomized NU/Nu mice were obtained from Charles River Laboratories (Wilmington, MA). The animals were allowed a period of adaptation in a sterile and pathogen-free environment with phytoestrogen-free food and water ad libitum. MCF-7 cells were harvested in the exponential growth phase using a PBS/EDTA solution and washed. Mice were injected bilaterally in the mammary fat pad (MFP) with 5 × 10⁶ viable cells suspended in 50 μL sterile PBS mixed with 100 μL Matrigel (reduced factor; BD Biosciences, Bedford, MA). 17β-Estradiol pellets (0.72 mg, 60-day release; Innovative Research of America, Sarasota, FL) were implanted subcutaneously in the lateral area of the neck using a precision trochar (10 gauge) at the time of cell injection. All procedures in animals were carried out under anesthesia using a mix of isoflurane and oxygen delivered by mask. Tumors were allowed to form and at day 15 post cell injection mice were randomized into groups of 5 mice each. Mice were treated daily with intraperitoneal injections of either vehicle (1:5 DMSO/PBS) or analog 2 (50 mg/kg/animal) for 21 days. Tamoxifen animals were implanted with a slow release tamoxifen pellet (5mg, 60-day release) in the lateral area of the neck on the opposite side from the estrogen pellet. ICI animals were given a onetime intraperitoneal injection of ICI 182,780 (5 mg in castor oil) on day 15 post cell injection.

Tumor size was measured 3 times weekly using digital calipers. The volume of the tumor was calculated using the following formula: $4/3 \pi LS^2$ (L = larger radius; S = shorter radius). All procedures involving these animals were conducted in compliance with State and Federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by Tulane University Animal Care and Use Committee. The facilities and laboratory animals program of Tulane University are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Molecular Modeling. The coordinates for the estrogen receptor alpha (ERα) bound to the agonist diethylstilbestrol (3ERD.pdb)¹⁸ and bound to the antagonist 4-OH-TAM (3ERT.pdb)²¹ were taken from the Protein Data Bank (<http://www.rcsb.org>). Oxygen atoms representing water were removed and hydrogen atoms were added to the template proteins using Amber99 force field. The representative compounds daidzein, **3a** and **2** were utilized for the docking studies. The 3D structures of the molecules were built using the Tripos SYBYL 8.1 Program (Tripos, St. Louis, MO). Initial geometric optimizations of the ligands were carried out using the standard MMFF94 force field, with a 0.001 kcal/mol energy gradient convergence criterion and a distance-dependent dielectric constant employing Gasteiger and Marsili charges. Additional geometric optimizations were performed using the semiempirical method molecular orbital package (MOPAC). The compounds were docked into the binding pockets of 3ERD and 3ERT using the Surflex docking program. Surflex²¹ is a fully automatic flexible molecular docking algorithm that combines the scoring function from the Hammerhead docking system²⁶ with a search engine that relies on a surface-based molecular similarity method as a means to rapidly generate suitable putative poses for molecular fragments. A protomol²⁷ representing the binding site was first generated using the position of the native ligand of the crystal structures. This protomol consists of the preferred locations of various molecular probes (CH₄, C=O, N–H), which are then used by the docking engine to search for the best three-dimensional morphological similarity between the protomol and the ligand to dock. A proto-thresh value of 0.5 and a proto-bloat value of 0 were used to generate a compact protomol to which the compounds were docked. The Surflex scores and visual inspection of docking results were considered for obtaining the optimal binding posture. The resulting protein–ligand complexes were then subjected to staged minimization using 200 steps of Steepest descent and 1000 steps of Conjugate gradient methods (with Amber99 force fields).

Statistical Analysis. Data were summarized as the mean ± standard error of the mean (SEM) using the Graph Pad Prism V.4 software program. Analysis of variance models were employed to compare relative ERE transcriptional reporter assays between controls versus treatment. A Tukey post-test was performed to compare differences between groups where a *p* value < 0.05 was considered significant.

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