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Design and synthesis of azaisoflavone analogs as phytoestrogen mimetics

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ABSTRACT

A series of azaisoflavone analogs were designed and synthesized and their transactivation activities and binding affinities for ERα and ERβ were investigated. Among these compounds, **2b** and **3a** were the most potent with 6.5 and 1.1 μM of EC_{50}, respectively. Molecular modeling study showed putative binding modes of the compound **3a** in the active site of ERα and ERβ, which were similar with that of genistein and provided insight of the effect of *N*-alkyl substitution of azaisoflavones on ERβ activity. Also, a biphasic effect of azaisoflavone analogs on MCF-7 cell growth depending on their concentrations was investigated.

1. Introduction

Estrogen receptors (ERs), a member of the steroid nuclear hormone receptor family, are ligand-activated transcription factor which play a crucial role in the development, maintenance, and function of the female reproductive system as well as other tissues such as colon, prostate, bone, cardiovascular system and central nervous systems [1]. There are two isoforms, ERα and ERβ. ERα is expressed in nearly all tissues whereas ERβ is mainly expressed in the ovaries, uterus and oviduct of the female reproductive tract but not in breast tissue [2]. ERα and ERβ have different biological functions including a tissue-specific regulation as well as their different gene expression patterns [3].

The hormone 17β-estradiol (E2) binds to ligand binding domain (LBD) of ERs, enters the nucleus of the cell, and regulates the target gene expression related to proliferation and differentiation of the cell by recruiting a various co-regulators. A deficiency of estrogen is associated with hot flashes, sweating, heart attacks and bone loss and hormone replacement therapy (HRT) is a medical treatment for older postmenopausal women in order to reduce these symptoms [4]. However, Women's Health Initiative (WHI) study showed beneficial effects of estrogen could increase risk of certain cancers such as breast [5]. Selective ER modulators (SERMs) are therapeutic agents for treating osteoporosis and breast cancer by modulating both ERs [6]. Tamoxifen, the first clinically relevant SERM, antagonizes the ER in breast tissue via active metabolite, hydroxytamoxifen and inhibits estrogenic effects such as proliferation of cancer cell [7].

Recently, a number of researches have been demonstrated ERα is more active in driving breast cancer cell proliferation than ERβ [8]. Meanwhile, it has been demonstrated that ERβ has anti-proliferative effect in breast cancer cells [9] and preventive effects for prostate adenocarcinoma [10] and also modulates the immune response [11]. Therefore, discovery of selective ERβ agonists may provide high potential to develop therapeutic agents for the various diseases such as breast cancer, prostate cancer, endometriosis, and inflammation. Recently, non-steroidal selective ERβ agonists SERBA-1, ERB-041 and WAY-202196 have been reported their anti-inflammatory activities and estrogenic activities (Fig. 1).

Phytoesterogens, naturally occurring estrogenic plant compounds, have been considered as an alternative method of menopause treatment. Among them, isoflavones are the well-known ER agonistic phytoestrogens and exhibit a number of biological properties including the prevention of cancers [12], coronary heart diseases [13], and osteoporosis [14]. Genistein is one of the potent

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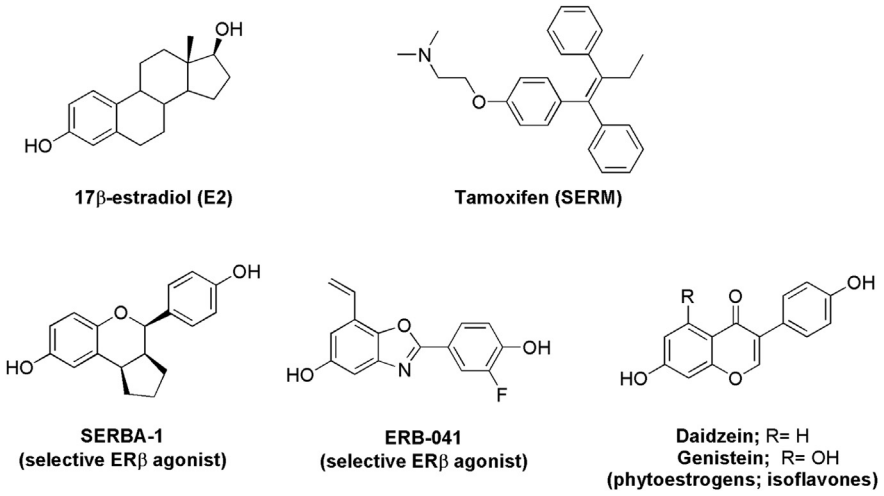


Fig. 1. Structures of ER modulators.

phytoestrogenic isoflavone, reveals 10–40 fold greater affinity for ERβ than for ERα [15], and inhibits cell proliferation in various breast cancer cell lines including MCF-7, T47D, BT20, MD-MBA-231, and SKBR3 [16]. The biphasic effect of genistein on cell growth has been reported in several studies in which genistein increased the MCF-7 cell growth at low concentrations but inhibited at higher concentrations [16a,17].

ERα and ERβ are similar in sequence with 58% identity and both ligand biding domains (LBDs) are nearly conserved differing by only two amino acids; Leu384 and Met421 in ERα are replaced by Met336 and Ile373 in ERβ, respectively (Fig. 2) [18]. The hydroxy groups in both ends of E2 and genistein essentially interact with key residues such as Glu353, Arg394 and His524 of ERα by hydrogen bonding within 2–3 Å (Glu305, Arg346 and His475 of ERβ). Their different residues Leu384/Met336 are positioned above the C-ring of genistein in the distance of 4–5 Å, and Met421/Ile373 are located below the A-ring near C-5 hydroxy group of genistein. These two pairs of amino acids within the LBD of ERα and ERβ make slight differences and mainly contribute to ligand selectivity [9c,19] (Fig. 3).

Regarding the previous findings, we became interested in modifying C-ring of isoflavone to identify a novel selective ER agonist. In this point of view, a previously reported compound azaisoflavone became the object of our attention. In fact, azaisoflavones were originally derived from isoflavone scaffold and designed as an iNOS inhibitor since inhibitory activity of several naturally occurring isoflavone for NO production was reported [20]. We supposed that the azaisoflavone analogs could function as phytoestrogens. Furthermore, *N*-substitution of azaisoflavone ring may give selectivity for ligand binding domains of ERα and ERβ. Herein we report synthesis of azaisoflavone analogs and their ERα/β transactivation activities. Structure activity relationship study was focused on the influence of phenolic OH of A and B-ring and *N*-substitution of C-ring of azaisoflavones. The effect of azaisoflevones on MCF-7 cell proliferation was also evaluated.

2. Chemistry

Synthetic routes for the preparation of azaisoflavones **1a**-**3d** are outlined in Scheme 1. Aniline or methoxyaniline was acylated with acetonitrile in the presence of boron trichloride and aluminum trichloride by Friedel–Craft's reaction. Condensation of the resulting ketones **4a**–**4c** with *p*-anisaldehyde resulted in 2'-aminochalcones **5a**–**5c** which were followed by *N*-acetylation to afford 2'-acetamidochalcones **6a**–**6c**. Rearrangement of 2'-acetamidochalcone **6a**–**6c** in the presence of thallium nitrate and trimethyl orthoformate gave β-ketoacetal **7a**–**7c** which were subsequently cyclized under acidic condition to yield quinolones **8a**–**10a**. The resulting quinolones **8a**–**10a** were alkylated with various alkyl halides in the presence of base to afford *N*-alkylated quinolones **8b**–**10d**. The final hydroxyquinolones **1a**–**3d** were obtained by deprotection of methoxyquinolones **8a**–**10d** under HBr and acetic acid conditions, respectively.

3. Results and discussion

3.1. ER transactivation activity and binding affinities

ER agonist activities of the prepared compounds were evaluated by in vitro transient transactivation assay and described in Table 1. The efficacy of the tested compounds was compared to the reference compounds E2 and genistein.

At first, an importance of phenolic OH on A and B-ring of azaisoflavones was investigated. Mono-hydroxy analogs **1a**–**d**, dihydroxy of daidzein analogs **2a**–**c** and tri-hydroxy of genistein analogs **3a**–**c** were compared with the corresponding methoxy compounds **8a, 9a**–**c** and **10a**–**c**, respectively. All methoxy compounds showed lower activities than the respective hydroxy compounds, and mono-hydroxy analog **1a** was less active than daidzein analog **2a** and genistein analog **3a** as expected.

When the oxygen of genistein was simply replaced with nitrogen as depicted in compound **3a**, both ER activities were dropped, whereas the selectivity for ERβ was increased. Genistein type analog **3a** showed the highest ERβ activity with 2.9 fold selectivity over ERα.

Effect of *N*-alkyl substituent of azaisoflavone on ER activity was evaluated. In the case of mono-hydroxy compounds **1a**–**d**, only *N*-propylated compound **1d** showed increased ERα and β activity than the parent compound **1a** while *N*-methyl or ethyl substitution vanished activity. In the daidzein analogs **2a**–**f**, introduction of alkyl substituent at nitrogen of C-ring enhanced ERβ activity and selectivity. Especially, *N*-methyl substituted compound **2b** showed promising ERβ activity with the highest ERβ selectivity at the ratio of 6.4:1. When the size of substituent was increased, the ERβ activity was decreased. In the genistein analogs **3a**–**c**, *N*-alkyl substitution lowered ERβ activity and selectivity. In contrast, *N*-alkyl

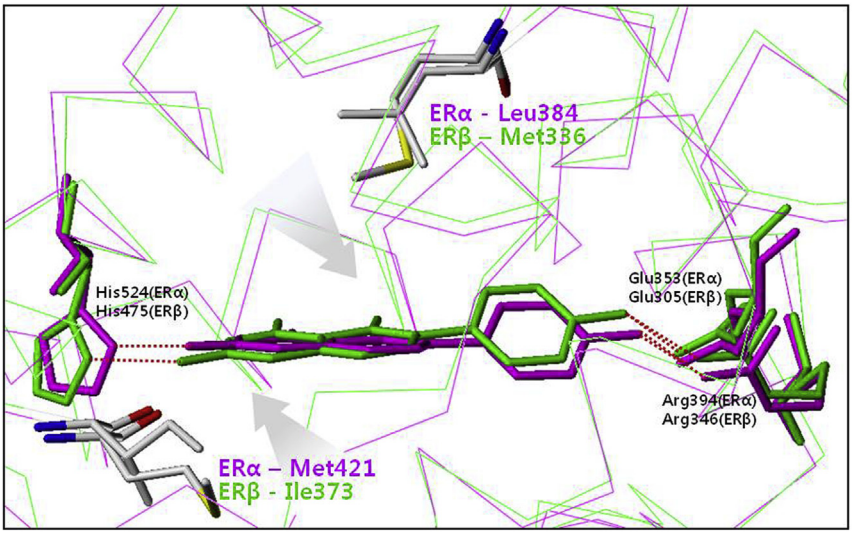
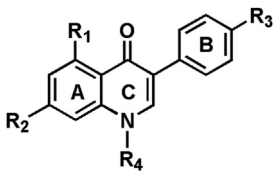


Fig. 2. Differences between the crystal structures of ERα and ERβ complexed with genistein. Two different colors represented complexed structures of genistein with LBDs of ERα (megenta) and ERβ (green) (PDB ID: ERα-1X7R and ERβ-1X7J). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

substitutions have relatively weak influence in the ERα activity of both daidzein and genistein analogs. Among these compounds, *N*-methylated daidzein analog **2b** and non-alkylated genistein analog **3a** were the most potent ERβ agonists showing 6.5 μM and 1.1 μM of EC_{50} value, respectively.

The binding affinities of azaisoflavones for ERα and ERβ were determined by a radiometric competitive binding assay and depicted in Table 1. Most of the azaisoflavone analogs revealed rare ERα binding affinity except tri-hydroxy of genistein analogs. Genistein analogs **3a**–**c** showed remarkable ERβ binding affinity along with relatively lower ERα binding affinity. Introduction and lengthening of *N*-alkyl substituents of genistein analogs increased both ERα and β binding affinities although selectivity was reversely lowered. Thus, non-substituted genistein analog **3a** showed the most selective binding affinity for ERβ. IC_{50} values of genistein analogs were determined; **3a** (74.5 μM for ERα, 4.2 μM for ERβ), **3b** (35.6 μM for ERα, 0.72 μM for ERβ), **3c** (18.4 μM for ERα, 0.10 μM for ERβ), and **3d** (16.6 μM for ERα, 0.15 μM for ERβ). Di-hydroxy of daidzein analogs showed moderate binding affinity for ERβ with no affinity for ERα, and *N*-alkyl substitution slightly enhanced ERβ binding affinity.

Interestingly, these results of ERβ binding affinity were not correlated with transactivation activity mentioned above. There is a quite discrepancy between transactivation activity and binding affinity and it may be ascribed to the causes that the receptor–ligand complex is only present in the binding assays, whereas various interactions with the many coregulators are present in the cellular transcription assays [21]. These interactions with coregulators can modulate the transcriptional activities of ligand.



R_1, R_2, R_3 = H, OH
R_4 = H, Me, Et, Pr

Fig. 3. Representative structure of azaisoflavone analogs.

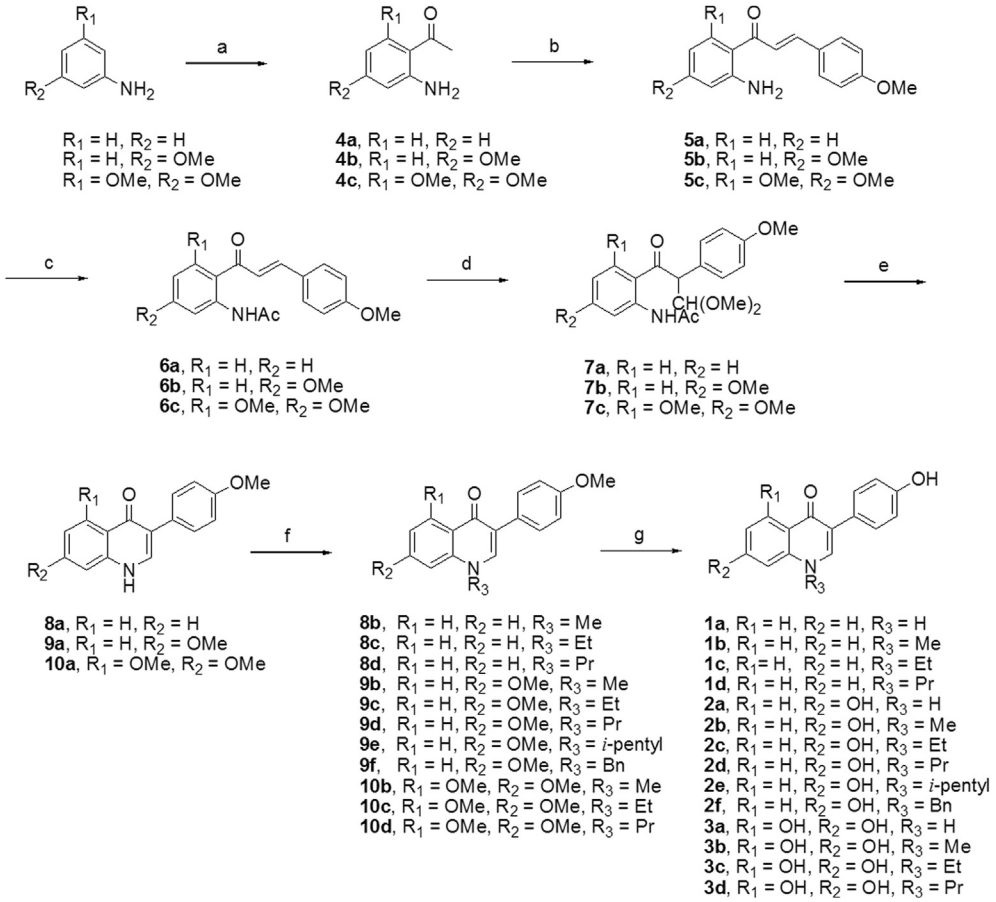
3.2. Docking analysis

To compare binding modes between known isoflavone and azaisoflavone and to explain the effect of *N*-alkyl group on the ERα/β selectivity, A docking analysis of azaisoflavones **2a**–**f** and **3a**–**d** were carried out using Surflex Dock interfaced with SYBYL-X version 2.0. Crystal structures of hERα and hERβ in complex with genistein (PDB code: ERα – 1X7R, ERβ – 1X7J) were employed for the docking study and internal default parameters of Surflex Dock were used for all the variables.

We investigated closely binding modes of azaisoflavones at the active site of ERα/β in comparison with that of genistein. Herein we took note of two points. First, the distance between centroid C-ring of genistein and Met336 in ERβ is closer than that of genistein and Leu384 in ERα. It is known to causes relatively stronger Met-aromatic interaction in ERβ than Leu-aromatic interaction in ERα, so it is consisted with the observed selectivity of genistein for ERβ over ERα. Also, it has been reported genistein C-5 hydroxy group is repulsive to the Met421 sulfur atom in ERα, but not to Ile373 in ERβ [22]. A number of ERβ selective agonists were reported [19]. Among them, benzoxazole analog ERB-041 provides good example for the role of Met421 in ERα and Ile373 in ERβ to distinguish ERα from ERβ. ERB-041 has 200 folds selectivity for ERβ and was proposed that the vinyl group of benzoxazole ring made steric clash with Met421 in ERα, which is relatively longer than Ile373 in ERβ. In contrast, this vinyl substituent appropriately positioned near Ile373 in ERβ, and even forming an additional hydrophobic interaction [23].

Azaisoflavone **3a** showed a similar binding mode with genistein whose hydroxyl groups of A and B-ring interact with the critical residues including His, Glu, Arg, and water molecule within appropriate distance in the active site of both ERs as shown in Fig. 4. Regarding the previous report, we also assume favorable Met336-aromatic interaction of **3a** in ERβ. In fact, C_4 atom of **3a** located at a closer distance of 3.744Å from S_δ of Met336 in ERβ, on the other hand, 3.886 Å from $C_{\delta 2}$ of Leu384 in ERα. Additional H-bonding interaction was observed between C-7 hydroxy group of compound **3a** and Gly472 in ERβ.

Fig. 5 exhibited binding modes of *N*-alkylated daidzein analogs at the active site of ERα and β. *N*-alkylated genistein analogs showed relatively low docking scores compared with *N*-alkyalted



Scheme 1. Synthesis of azaisoflavone analogs. Reaction conditions: a) CH_3CN, BCl_3, $AlCl_3$, dichloroethane, 80 °C, 20 h; b) *p*-anisaldehyde, NaOH, EtOH, rt, 10 h; c) Ac_2O, pyridine, rt, 3 h; d) thallium(III)nitrate, $HC(OCH_3)_3$, rt, 4 h; e) HCl, EtOH, reflux, 2 h; f) R_3-X, NaH, DMF, rt, 3–12 h; g) HBr, AcOH, 120 °C, 3 days.

daidzein analogs in accordance with transactivation activities. In the case of ERα, when alkyl substituents were introduced at the nitrogen of daidzein analogs, they occupied an empty space near Leu384 which might be more acceptable than longer Met336 for these alkyl groups.

On the other hand, binding modes in ERβ LBD were quite different with those in ERα. The daidzein analogs **2b**–**d** were flipped over retaining their key H-bonding interactions with Glu305, Arg346, His475, and water molecule. Methyl, ethyl and propyl groups were positioned near Ile373 instead of Met336 as shown in Fig. 5. The docking scores of **2b**–**d** decreased with lengthened alkyl substituents showing Me (6.8615), Et (6.8290), and Pr (6.6300). Methyl group of azaisoflavone **2b** seemed to be the most suitable substituent for the cavity near Ile373. Taken together, docking result gave an account of the transactivation activity and selectivity of the discussed compounds.

3.3. Effects of MCF-7 cell growth

Some phytoestrogens are known to modulate survival and death of breast cancer cell in an ER-dependent or -independent manner [24]. Interestingly, genistein and daidzein have been found to stimulate breast cancer cell growth in vitro and in vivo studies at low concentrations (0.1–10 μM) while they inhibit the cell proliferation at high concentrations (20–100 μM) [17,25]. However, the mechanism of the biphasic action of genistein on the growth of breast cancer cells is not fully understood yet.

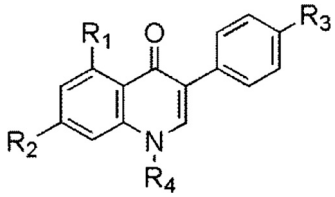
The proliferative and/or anti-proliferative activity of mostly ER active azaisoflavone analogs on MCF-7 cell was also evaluated. MCF-7 (ATCC HTB-22) and CV-1 (ATCC CCL-7) cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS; GE Healthcare). The cells were cultured in phenol red-free DMEM containing 5% charcoal-dextran stripped FBS (CD-FBS) for 3 days to eliminate any estrogenic source before treatment of samples.

Effect of azaisoflavones on cell growth was not correspondingly related to the ER activity. Among tested compounds, azaisoflavones **1d**, **2c**, **2d**, **3a**, **3b** and **3c** showed biphasic effects with stimulation of cell growth at low concentrations and inhibition at high concentrations as genistein did (Fig. 6). Meanwhile, compounds **2e**, **2f** and **3d** were only observed inhibitory activity at the given doses.

4. Conclusion

In summary, we have designed and synthesized a series of azaisoflavones based on phytoestrogenic isoflavone scaffold and evaluated their estrogenic activity and binding affinities in the LBD of ERα and β. We found that several azaisoflavone analogs displayed promising estrogenic activity and subtype selectivity. Also, most of the azaisoflavone analogs were found to modulate the proliferation of MCF-7 breast cancer cells in a dose dependent manner. SAR study indicated that presence of hydroxy group is critical for the ER activity while the potency and selectivity of the compounds were highly influenced within a very narrow structural change. Interestingly, introduction of optimal length of *N*-alkyl substituent in the daidzein anlalogs increased ER activity, meanwhile genistein analogs lowered ERβ activity with *N*-substitution. Molecular modeling

Table 1
In vitro functional transactivation activities and competitive binding affinities of azaisoflavones on human ERα/β.



Compds	R_1	R_2	R_3	R_4	ER transactivation activity			ER binding affinity		
					% max (20 μM)[a]		β/α ratio	% max (20 μM)[b]		β/α ratio
					ERα	ERβ		ERα	ERβ	
Genistein	OH	OH	OH	–	172.8	232.8	1.3	93.4	95.1	1.0
1a	H	H	OH	H	NA[c]	19.6		NA	10.7	
1b	H	H	OH	Me	NA	NA		NA	18.4	
1c	H	H	OH	Et	NA	NA		NA	18.3	
1d	H	H	OH	Pr	79.0	42.4	0.5	NA	23.0	
2a	H	OH	OH	H	24.5	31.9	1.3	NA	24.1	
2b	H	OH	OH	Me	16.1	102.1	6.4	NA	40.6	
2c	H	OH	OH	Et	33.1	95.4	2.9	NA	40.4	
2d	H	OH	OH	Pr	42.3	67.0	1.6	NA	37.2	
2e	H	OH	OH	*i*-pentyl	13.0	44.8	3.4	NA	38.5	
2f	H	OH	OH	Bn	31.7	33.1	1.0	NA	39.2	
3a	OH	OH	OH	H	46.2	132.1	2.9	6.9	82.9	12.0
3b	OH	OH	OH	Me	50.4	63.2	1.3	35.6	93.0	2.6
3c	OH	OH	OH	Et	60.5	47.2	0.8	57.0	92.2	1.6
3d	OH	OH	OH	Pr	56.9	16.7	0.3	78.5	106.6	1.4
8a	H	H	OMe	H	NA	10.1		NA	21.3	
9a	H	OMe	OMe	H	NA	16.2		NA	42.5	
9b	H	OMe	OMe	Me	NA	10.3		NA	NA	
9c	H	OMe	OMe	Et	NA	NA		NA	12.8	
10a	OMe	OMe	OMe	H	10.1	10.9		NA	47.2	
10b	OMe	OMe	OMe	Me	NA	NA		NA	NA	
10c	OMe	OMe	OMe	Et	NA	13.2		NA	13.6	
E2[d]					100	100	1.0	100	100	1.0

[a] Fold activation relative to maximum activation obtained with E2 (20 nM) for ERα and β. The compounds were tested in at least three separate experiments.
[b] Binding affinities were determined by a competitive binding assay using [^{3}H]-E2. Fold affinities relative to maximum activation obtained with E2 (1 nM) for ERα and β. The compounds were tested in at least three separate experiments.
[c] NA means not active, which is for compounds producing transactivation activity or binding to ER lower lower than 10% at 20 μM.
[d] The concentration of E2 for transactivation activity is 20 nM and that of E2 for the competitive binding affinity assay is 1 nM.

also demonstrated that, despite the high homology, ligands can be made to discriminate between the two ER subtypes. These findings suggest that further elaboration of the azaisoflavone scaffold as well as more sophisticate tuning with the appropriate substitution will be the next strategy to explore those applications. We anticipate that these azaisoflavones might work as a useful pharmacological tool for development of therapeutic agents to treat ER mediated disease.

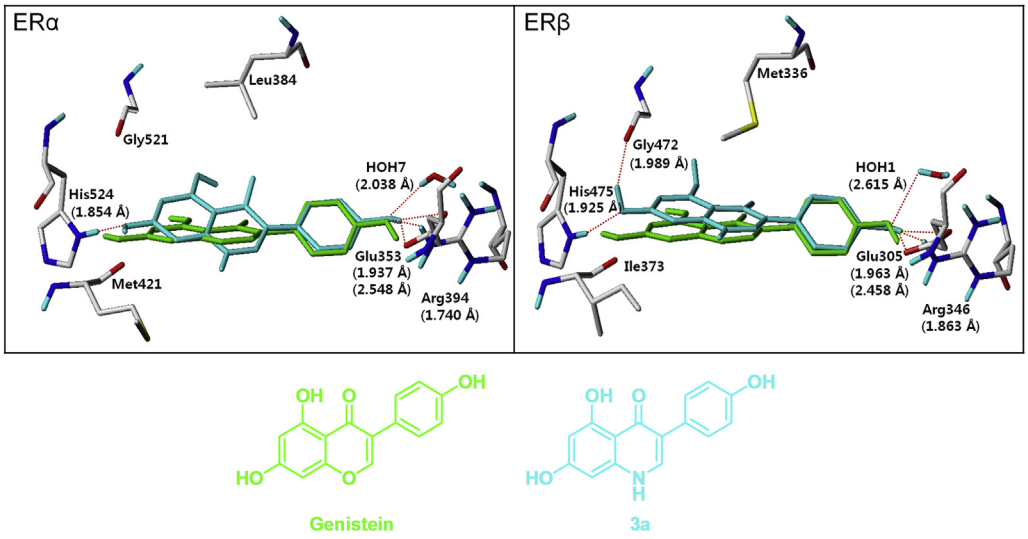


Fig. 4. A comparison of putative binding modes of azaisoflavone **3a** with those of genistein in the active sites of hERα and hERβ.

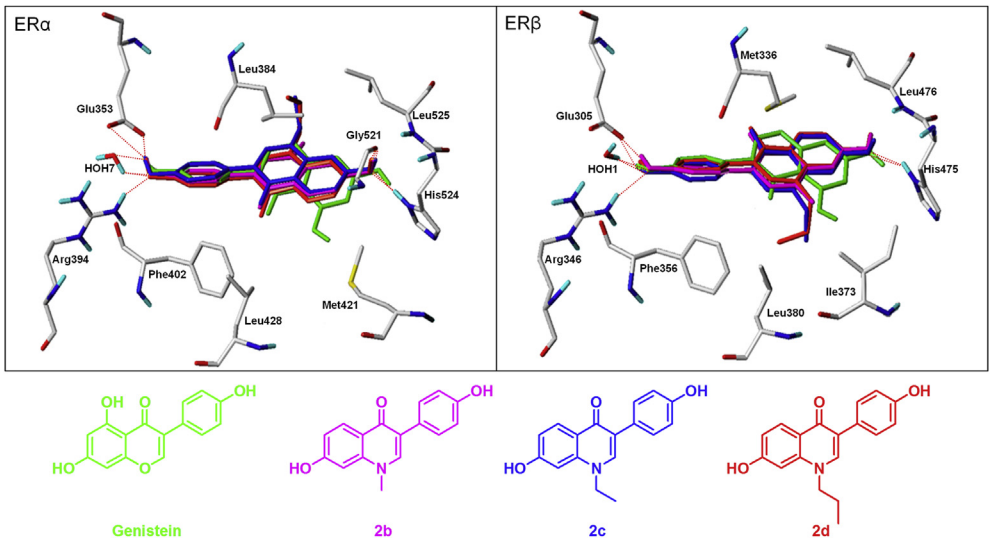


Fig. 5. Putative binding modes of *N*-alkylated daidzein analogs **2b**–**d** when docked into the X-ray crystal structure of ERα and ERβ.

5. Experimental

5.1. General procedures

Most of the reagents and solvents were purchased from Aldrich chemicals and used without purification. Unless indicated, all anhydrous solvents were distilled under nitrogen condition. Acetonitrile, dichloroethane, pyridine and dimethylformamide were distilled from calcium hydride under nitrogen. Column chromatography was performed using silica gel 60 (230–400 mesh, Merck) with the indicated solvents. Thin-layer chromatography (TLC) was performed using Kieselgel 60 F254 plates (Merck). IR spectra were recorded on a JASCO FT/IR 430 spectrophotometer. NMR spectra were recorded on a Varian YH 400 spectrometer (^{1}H 400 MHz and ^{13}C 100 MHz). Chemical shifts are expressed in parts per million (ppm, δ) relative to an internal standard, tetramethylsilane. ^{1}H NMR data are reported in the order of chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and/or multiple resonances), number of protons and coupling constant in hertz (Hz). Mass spectra were recorded on a JEOL GCmate II spectrometer with glycerol as a matrix for the FAB-MS.

5.2. Synthesis of compounds

5.2.1. 3-(4-hydroxyphenyl)-1H-quinolin-4-one (*1a*)

A solution of quinolone **8a** (100 mg, 0.39 mmol), acetic acid and HBr (0.1 ml, 1.984 mmol) was stirred at 120 °C for 3 days. The reaction mixture was cooled to rt to form precipitates. The resulted precipitates were filtered and washed with water and EtOAc thoroughly. The residue was purified by column chromatography (EtOAc/toluene/EtOH = 1:1:1) to give quinolone **1a** (79 mg, 84%): IR (neat, cm^{-1}) 3414, 1614, 1577, 1373, 1262, 1181; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.23 (1H, s), 8.20 (1H, dd, J = 8.4, 0.8 Hz), 8.16 (1H, s), 7.59–7.68 (2H, m), 7.49–7.46 (2H, m), 7.36 (1H, dt, J = 7.2, 1.6 Hz), 6.76 (2H, dt, J = 8.8, 3.0 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.0, 156.8, 139.8, 138.1, 132.1, 130.2, 127.2, 126.2, 126.0, 123.9, 120.6,

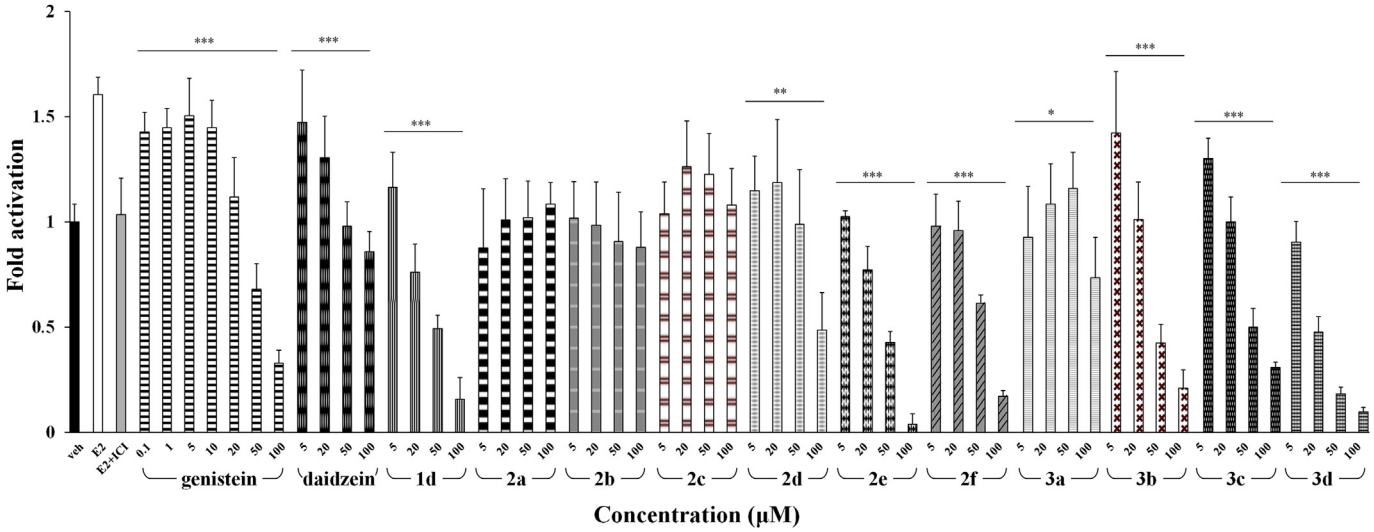


Fig. 6. Cytotoxic effects of genistein, daidzein, and azaisoflavone analogs on MCF-7 breast cancer cells. Each experiment was performed three times independently and results were expressed as means ± SD. One-way ANOVA with Dunnett's posttest was performed for each compound. Treatment means that are significantly different from control are indicated as $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

118.9, 115.4; LRMS (FAB) *m/z* 238.1 (M + H^+); HRMS (FAB) calcd for $C_{15}H_{12}N\,O_2$, 238.0868; found: 238.0865 (M + H)$^+$.

*5.2.2. 1-Methyl-3-(4-hydroxyphenyl)-1H-quinolin-4-one **(1b)***

This compound was prepared from **8b** using the same procedure as described for the preparation of **1a**: Yield 75%; IR (neat, cm^{-1}) 3408, 1644, 1518, 1451, 1350, 1159; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.39 (1H, s), 9.40 (1H, s), 8.10 (1H, s), 7.42 (2H, d, J = 8.4 Hz), 6.75 (2H, d, J = 8.4 Hz), 6.22 (1H, s), 6.09 (1H, s), 3.72 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.0, 164.8, 163.2, 157.0, 144.2, 142.8, 130.3, 129.6, 125.7, 118.6, 115.5, 108.2, 98.4, 91.1, 41.4; LRMS (FAB) *m/z* 252.1 (M + H^+); HRMS (FAB) calcd for $C_{16}H_{14}N\,O_2$, 252.1025; found: 252.1027 (M + H)$^+$.

*5.2.3. 1-Ethyl-3-(4-hydroxyphenyl)-1H-quinolin-4-one **(1c)***

This compound was prepared from **8c** using the same procedure as described for the preparation of **1a**: Yield 81%; IR (neat, cm^{-1}) 3380, 1612, 1515, 1269, 838, 763; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (1H, d, J = 8.0 Hz), 8.22 (1H, s), 7.75–7.69 (2H, m), 7.51 (2H, d, J = 8.4 Hz), 7.36 (1H, t, J = 7.2 Hz), 6.75 (2H, d, J = 8.4 Hz), 4.35 (2H, q, J = 6.8 Hz), 3.72 (1H, s), 1.34 (1H, t, J = 6.8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.5, 156.9, 142.9, 139.1, 132.5, 130.3, 129.0, 127.0, 126.9, 123.9, 120.9, 117.0, 115.3, 47.9, 15.1; LRMS (FAB) *m/z* 266.1 (M + H^+); HRMS (FAB) calcd for $C_{17}H_{16}N\,O_2$, 266.1181; found: 266.1180 (M + H)$^+$.

*5.2.4. 1-propyl-3-(4-hydroxyphenyl)-1H-quinolin-4-one **(1d)***

This compound was prepared from **8d** using the same procedure as described for the preparation of **1a**: Yield 72%; IR (neat, cm^{-1}) 3195, 1610, 1566, 1541, 1512, 1492, 1330, 1231, 840, 750; ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD) δ 8.52 (1H, d, J = 8.4 Hz), 7.67–7.71 (1H, m), 7.51 (1H, d, J = 8.4 Hz), 7.34–7.46 (2H, m), 7.45 (2H, d, J = 8.8 Hz), 6.89 (2H, d, J = 8.8 Hz), 4.19 (2H, dd, J = 7.6, 7.2 Hz), 1.94 (2H, m), 1.03 (3H, dd, J = 7.6, 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$ + CD_3OD) δ 176.3, 156.2, 142.3, 138.9, 130.0, 127.3, 126.9, 126.3, 123.8, 122.0, 115.3, 115.2, 55.0, 22.2, 11.0; LRMS (FAB) *m/z* 280.1 (M + H^+); HRMS (FAB) calcd for $C_{18}H_{18}N\,O_2$, 280.1338; found: 280.1336 (M + H)$^+$.

*5.2.5. 7-Hydroxy-3-(4-hydroxyphenyl)-1H-quinolin-4-one **(2a)***

A solution of quinolone **9a** (9.5 mg, 0.034 mmol), pyridine (0.5 mL) and HBr (1 mL) was stirred at 80 °C overnight. The reaction mixture was cooled to rt, diluted and extracted with EtOAc. The organic layer was washed with water and brine, then dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography ($CHCl_3$/EtOAc/MtOH = 20:1:2) to give **2a** (2.3 mg, 27%): IR (neat, cm^{-1}) 3263, 2924, 1591, 1460, 1235, 840; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.57 (1H, d, J = 5.6 Hz), 10.20 (1H, br s), 9.29 (1H, s), 8.00 (1H, d, J = 8.8 Hz), 7.87 (1H, d, J = 5.6 Hz), 7.48 (2H, d, J = 8.8 Hz), 6.82 (1H, d, J = 2.4 Hz), 6.73–6.78 (3H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.0, 160.6, 156.3, 141.5, 137.0, 129.9, 128.0, 127.4, 119.8, 119.6, 115.0, 114.3, 101.2; LRMS (FAB) *m/z* 254.1 (M + H^+); HRMS (FAB) calcd for $C_{15}H_{12}N\,O_3$, 254.0817; found: 254.0814 (M + H)$^+$.

*5.2.6. 7-hydroxy-3-(4-hydroxyphenyl)-1-methyl-1H-quinolin-4-one **(2b)***

This compound was prepared from **9b** using the same procedure as described for the preparation of **1a**: Yield 58%; IR (neat, cm^{-1}) 3350, 2920, 1621, 1535, 1517, 1331, 1254, 1193; ^{1}H NMR (400 MHz, CD_3OD) δ 8.27 (1H, d, J = 8.8 Hz), 8.00 (1H, s), 7.45 (2H, d, J = 8.8 Hz), 6.97 (1H, dd, J = 2.4, 8.8 Hz), 6.94 (1H, d, J = 2.4 Hz), 6.84 (1H, d, J = 8.8 Hz), 3.89 (3H, s); ^{13}C NMR (100 MHz, CD_3OD) δ 195.7, 180.0, 164.8, 151.7, 144.9, 131.2, 120.9, 116.0, 101.3, 100.7, 65.7, 54.0, 41.1; LRMS (FAB) *m/z* 268.1 (M + H^+); HRMS (FAB) calcd for $C_{16}H_{14}N\,O_3$, 268.0974; found: 268.0975 (M + H)$^+$.

*5.2.7. 7-hydroxy-3-(4-hydroxyphenyl)-1-ethyl-1H-quinolin-4-one **(2c)***

This compound was prepared from **9c** using the same procedure as described for the preparation of **1a**: Yield 83%; IR (neat, cm^{-1}) 3337, 2855, 1734, 1457, 1257, 1031; ^{1}H NMR (400 MHz, CD_3OD) δ 8.26 (1H, d, J = 8.8 Hz), 8.01 (1H, s), 7.44 (2H, d, J = 8.8 Hz), 6.95 (1H, d, J = 2.4 Hz), 6.93 (1H, dd, J = 2.4,8.8 Hz), 6.82 (2H, d, J = 8.8 Hz), 4.31 (2H, q, J = 14.4 Hz), 1.48 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 183.2, 171.4, 161.3, 156.1, 143.8, 135.1, 131.1, 119.4, 116.0, 107.5, 100.5, 97.3, 88.4, 71.5, 59.8, 30.7, 22.8; LRMS (FAB) *m/z* 282.1 (M + H^+); HRMS (FAB) calcd for $C_{17}H_{16}N\,O_3$, 282.1130; found: 282.1129 (M + H)$^+$.

*5.2.8. 7-hydroxy-3-(4-hydroxyphenyl)-1-propyl-1H-quinolin-4-one **(2d)***

This compound was prepared from **9d** sing the same procedure as described for the preparation of **1a**: Yield 82%; IR (neat, cm^{-1}) 3393, 2955, 2922, 2850, 1739, 1462, 1244, 1021; ^{1}H NMR (400 MHz, CD_3OD) δ 8.27 (1H, d, J = 8.8 Hz), 8.01 (1H, s), 7.44 (2H, d, J = 8.8 Hz), 6.96–6.94 (2H, m), 6.84 (2H, d, J = 8.8 Hz), 4.23 (2H, t, J = 7.2 Hz), 1.92 (2H, q, J = 7.2 Hz), 1.02 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 177.2, 162.8, 157.7, 144.3, 142.5, 131.1, 129.8, 128.0, 122.5, 121.4, 116.0, 115.6, 100.7, 55.7, 23.1, 11.2; LRMS (FAB) *m/z* 296.1 (M + H^+); HRMS (FAB) calcd for $C_{18}H_{18}N\,O_3$, 296.1287; found: 296.1280 (M + H)$^+$.

*5.2.9. 7-hydroxy-3-(4-hydroxyphenyl)-1-(3-methylbutyl)-1H-quinolin-4-one **(2e)***

This compound was prepared from **9e** using the same procedure as described for the preparation of **1a**: Yield 52%; IR (neat, cm^{-1}) 3120, 2956, 1617, 1515, 1443, 1267, 1025; ^{1}H NMR (400 MHz, CD_3OD) δ 8.27 (1H, d, J = 8.8 Hz), 8.06 (1H, s), 7.79 (1H, d, J = 2.4 Hz), 7.43 (1H, dd, J = 2.4,8.8 Hz), 7.00–6.93 (4H, m), 4.29 (2H, t, J = 7.2 Hz), 7.00–6.93 (4H, m), 3.97 (3H, s), 1.78 (3H, m), 1.06 (3H, s), 1.04 (3H, s); ^{13}C NMR (100 MHz, CD_3OD) δ 177.1, 163.7, 157.7, 144.0, 142.4, 131.2, 129.7, 128.0, 122.6, 121.1, 116.1, 116.0, 100.7, 52.8, 38.6, 27.2, 22.7; LRMS (FAB) *m/z* 324.2 (M + H^+); HRMS (FAB) calcd for $C_{20}H_{22}N\,O_3$, 324.1600; found: 324.1602 (M + H)$^+$.

*5.2.10. 7-hydroxy-3-(4-hydroxyphenyl)-1-benzyl-1H-quinolin-4-one **(2f)***

This compound was prepared from **9f** using the same procedure as described for the preparation of **1a**: Yield 37%; IR (neat, cm^{-1}) 3423, 3214, 1616, 1516, 1327, 1270, 1222, 837; ^{1}H NMR (400 MHz, CD_3OD) δ 8.24 (1H, d, J = 8.8 Hz), 8.15 (1H, s), 7.46 (2H, d, J = 8.4 Hz), 7.32–7.35 (2H, m), 7.24–7.28 (1H, m), 7.19–7.21 (2H, m), 6.87 (1H, dd, J = 8.8, 2.4 Hz), 6.80–6.83 (3H, m), 5.48 (2H, s)); ^{13}C NMR (100 MHz, $CDCl_3$ + CD_3OD) δ 176.2, 161.0, 156.0, 142.4, 141.3, 134.9, 129.9, 128.9, 128.7, 127.9, 126.3, 125.9, 121.7, 114.9, 114.4, 100.1, 56.4; LRMS (FAB) *m/z* 344.1 (M + H^+); HRMS (FAB) calcd for $C_{22}H_{18}N\,O_3$, 344.1287; found: 344.1285 (M + H)$^+$.

*5.2.11. 5,7-dihydroxy-3-(4-hydroxyphenyl)-1H-quinolin-4-one **(3a)***

This compound was prepared from **10a** using the same procedure as described for the preparation of **1a**: Yield 87%; IR (neat, cm^{-1}) 3294, 1654, 616, 1566, 1514, 1440,1382, 1275, 1199, 884, 829, 695, 655; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.02 (1H, d, J = 6 Hz), 10.11 (1H, s), 7.43-7.41 (1H, m), 7.42 (2H, dd, J = 6.8,2.0 Hz), 6.74 (2H, dd, J = 6.8, 2.0 Hz), 6.26 (1H, d, J = 2.0 Hz), 5.97 (1H, d, J = 2.0 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.7, 163.7, 156.9, 142.2, 138.5, 130.3, 126.1, 118.6, 115.4, 108.1, 97.9, 91.9; LRMS (FAB)

m/*z* 270.1 (M + H^+); HRMS (FAB) calcd for $C_{15}H_{12}N\,O_4$, 270.0766; found: 270.0762 (M + H)$^+$.

5.2.12. 5,7-dihydroxy-3-(4-hydroxyphenyl)-1-methyl-1H-quinolin-4-one (3b)

This compound was prepared from **10b** using the same procedure as described for the preparation of **1a**: Yield 88%; IR (neat, cm^{-1}) 3407, 1644, 1561, 1517, 1451, 1349, 1159; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.39 (1H, s), 9.40 (1H, s), 8.10 (1H, s), 7.43 (2H, d, J = 8.4 Hz), 6.75 (2H, d, J = 8.4 Hz), 6.22 (1H, s), 6.09 (1H, s), 3.72 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.0, 164.8, 163.2, 157.0, 144.2, 142.8, 130.3, 129.6, 125.7, 118.6, 115.5, 108.2, 98.4, 91.1; LRMS (FAB) *m*/*z* 284.1 (M + H^+); HRMS (FAB) calcd for $C_{16}H_{14}N\,O_4$, 284.0923; found: 284.0918 (M + H)$^+$.

5.2.13. 5,7-dihydroxy-3-(4-hydroxyphenyl)-1-ethyl-1H-quinolin-4-one (3c)

This compound was prepared from **10c** using the same procedure as described for the preparation of **1a**: Yield 85%; IR (neat, cm^{-1}) 2971, 1612, 1509, 1459, 1354, 1288, 1245, 1161, 1121, 1073, 1031, 951; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.42 (1H, s), 8.11 (1H, s), 7.44 (2H, d, J = 8.8 Hz), 6.74 (2H, d, J = 8.8 Hz), 6.29 (1H, d, J = 2.0 Hz), 6.07 (1H, d, J = 2.0 Hz), 4.19-4.17 (2H, m),1.31 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.0, 165.0, 163.2, 157.0, 143.2, 141.6, 130.4, 129.6, 128.9, 125.7, 119.0, 115.4, 108.5, 98.2, 90.8, 48.5, 14.5; LRMS (FAB) *m*/*z* 298.1 (M + H^+); HRMS (FAB) calcd for $C_{17}H_{16}N\,O_4$, 298.1079; found: 298.1074 (M + H)$^+$.

5.2.14. 5,7-dihydroxy-3-(4-hydroxyphenyl)-1-propyl-1H-quinolin-4-one (3d)

This compound was prepared from **10d** using the same procedure as described for the preparation of **1a**: Yield 32%; IR (neat, cm^{-1}) 3258, 1642, 1560, 1515, 1446, 1362, 1157, 836; ^{1}H NMR (400 MHz, CD_3OD) δ 7.84 (1H, s), 7.36 (2H, d, J = 8.4 Hz), 6.79 (2H, d, J = 8.4 Hz), 6.27 (1H, s), 6.14 (1H, s), 4.06 (2H, t, J = 7.2 Hz), 1.83 (2H, m), 0.96 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 178.9, 164.0, 162.9, 156.5, 142.9, 141.6, 129.8, 125.5, 119.7, 114.7, 108.3, 97.7, 90.2, 54.8, 21.3, 9.7; LRMS (FAB) *m*/*z* 312.1 (M + H^+); HRMS (FAB) calcd for $C_{18}H_{18}N\,O_4$, 312.1236; found: 312.1243 (M + H)$^+$.

5.2.15. 1-(2-aminophenyl)ethanone (4a)

To a solution of boron trichloride (2.77 g, 23.63 mmol) in dichloromethane was added a solution of aniline (2.00 g, 21.48 mmol) in 1,2-dichloroethane (15 mL) at 0 °C. To the mixture was added aluminum chloride (3.15 g, 23.63 mmol) and acetonitrile (0.88 g, 21.48 mmol) and the mixture was refluxed at 80 °C for 20 h. After the reaction was completed, the reaction mixture was cooled to 0 °C, then 2 N HCl was added. The mixture was stirred at 80 °C for 30 min. The reaction mixture was extracted with dichloromethane. The organic layer was washed with 1 N NaOH and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to give amino phenylketone **4a** (2.67 g, 92%) as a yellow solid: IR (neat); 3460, 3341, 1644, 1615, 1242, 1162; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (1H, dd, J = 8.4, 1.6 Hz), 7.26 (1H, ddd, J = 8.4, 1.6 Hz), 6.65 (2H, m), 6.27 (2H, br s), 2.58 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.8, 150.2, 134.4, 132.0, 118.2, 117.2, 115.7, 27.9.

5.2.16. 1-(2-amino-4-methoxyphenyl)ethanone (4b)

This compound was prepared from *m*-anisidine using the same procedure as described for the preparation of **4a**: Yield 56%; IR (neat, cm^{-1}) 3450, 3332, 1619, 1223; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (1H, d,, J = 8.8 Hz), 6.41 (2H, brs), 6.23 (1H, dd, J = 8.8, 2.4 Hz), 6.06 (1H, d, J = 2.4 Hz), 3.79 (3H, s), 2.51 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.8, 164.2, 152.7, 134.0, 112.7, 104.3, 99.0, 55.0, 27.5.

5.2.17. 1-(2-amino-4,6-dimethoxyphenyl)ethanone (4c)

This compound was prepared from 3,5-dimethoxyaniline using the same procedure as described for the preparation of **4a**: Yield 81%; IR (neat, cm^{-1}) 3435, 3282, 2973, 1615, 1578, 1456, 1426, 1363, 1322, 1247, 1208,1168, 1139, 953, 813; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.34 (2H, s), 5.73 (1H, d, J = 2.4 Hz), 5.68 (1H, d, J = 2.4 Hz), 3.79 (3H, s), 3.75 (3H, s), 2.51 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.4, 164.2, 163.7, 153.8, 105.9, 92.2, 88.5, 55.5, 55.4, 34.1.

5.2.18. 1-(2-aminophenyl)-3-(4-methoxyphenyl)propenone (5a)

A mixture of aminophenyl ketone **4a** (2.50 g, 18.51 mmol), *p*-anisaldehyde (2.52 g, 18.51 mmol), a small amount of sodium hydroxide (1-2 pallets), and ethanol (3 mL) was stirred at rt for 10 h. After the reaction was completed, the solvent was removed *in vacuo*. The residue was diluted and extracted with EtOAc. The organic layer was washed with water and brine, then dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give 2'-aminochalcone **5a** (4.27 g, 91%) as a yellow solid : IR (neat, cm^{-1}) 3463, 2925, 1568, 1259, 1028, 800, 748, 657; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (1H, d, J = 8.4 Hz), 7.65 (1H, d, J = 15.6 Hz), 7.52 (2H, d, J = 7.6 Hz), 7.43 (1H, d, J = 15.6 Hz), 7.20 (1H, m), 6.86 (2H, d, J = 8.8 Hz), 6.63 (2H, d, J = 8.0 Hz), 6.23 (2H, s), 3.79 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0, 151.1, 143.0, 134.3, 131.1, 130.2, 128.2, 120.9, 117.5, 116.0, 114.6, 55.6, 29.9.

5.2.19. 1-(2-amino-4-methoxyphenyl)-3-(4-methoxyphenyl) propenone (5b)

This compound was prepared from **4b** using the same procedure as described for the preparation of **5a**: Yield 81%; IR (neat, cm^{-1}) 3406, 3290, 2964, 2836, 1635, 1568, 1509, 1421, 1380, 1348, 1288, 1250, 1211, 1176, 1144, 990, 957; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (1H, d, J = 8.8 Hz), 7.69 (1H, d, J = 15.2 Hz), 7.57 (2H, d, J = 8.8 Hz), 7.47 (2H, d, J = 15.2 Hz), 6.92 (2H, d, J = 8.8 Hz), 6.56 (2H,brs), 6.27 (1H, dd, J = 8.8, 2.4 Hz), 6.11 (1H, d, J = 3.0 Hz), 3.84 (3H, s), 3.81 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.9, 169.9, 164.8, 161.9, 144.5, 144.4, 132.6, 130.5, 127.8, 120.4, 116.5, 114.7, 109.7, 104.5, 55.8, 55.6, 31.1, 25.9.

5.2.20. 1-(2-amino-4,6-dimethoxyphenyl)-3-(4-methoxyphenyl) propenone (5c)

This compound was prepared from **4c** using the same procedure as described for the preparation of **5a**: Yield 56%; IR (neat, cm^{-1}) 3432, 3333, 2938, 2360, 1571, 1509, 1457, 1419, 1339, 1282, 1245, 1211, 1163, 1026, 970; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (1H, d, J = 15.6 Hz), 7.50 (2H, d, J = 8.8 Hz), 7.36 (1H, d, J = 15.6 Hz), 6.87 (2H, d, J = 8.8 Hz), 5.99 (2H, s), 5.80 (1H, d, J = 2.4 Hz), 5.75 (1H, d, J = 2.4 Hz), 3.82 (3H, s), 3.81 (3H, s), 3.77 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.6, 164.1, 162.8, 161.0, 153.1, 139.5, 129.9, 128.9, 127.6, 114.4, 107.2, 92.5, 89.0.

5.2.21. N-[2-[3-(4-methoxyphenyl)-1-oxo-2-propenyl]phenyl] acetamide (6a)

To a solution of 2'-aminochalcone **5a** (4.20 g, 16.58 mmol) in pyridine (40 mL) was added acetic anhydride (40 mL). The mixture was stirred at rt for 3 h. After the reaction was completed, the reaction mixture was extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to give a yellow solid. The residue was recrystallized with EtOAc and *n*-hexane to give 2'-acetamidochalcone **6a** (3.27 g, 76%) as a pale yellow solid: IR (neat, cm^{-1}) 3158, 1687, 1641, 1576, 1510, 1455, 1308, 1255, 1204, 1012, 838, 812; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.53 (1H, s), 8.66 (1H, d, J = 8.0 Hz), 7.93 (1H, dd, J = 8.0, 1.4 Hz), 7.75 (1H, d, J = 15.6 Hz), 7.59 (2H, d, J = 3.2 Hz), 7.52 (1H, dt, J = 7.8, 1.6 Hz), 7.41 (1H, d, J = 15.6 Hz), 7.13 (1H, dt, J = 8.2, 1.0 Hz), 6.92 (2H, dd, J = 8.8, 4.8 Hz),

3.84 (3H, s), 2.22 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.6, 169.6, 162.2, 145.8, 141.1, 134.6, 130.7, 130.6, 127.5, 123.9, 122.6, 121.4, 120.6, 114.7, 55.7, 25.7.

*5.2.22. N-{5-methoxy-2-[3-(4-methoxyphenyl)acryloyl]phenyl} acetamide **(6b)***

This compound was prepared from **5b** using the same procedure as described for the preparation of **6a**: Yield 61%; IR (neat, cm^{-1}) 1700, 1605, 1512, 1452, 1419, 1291, 1235, 1202, 1176, 1158, 1235, 1202, 978, 835; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.20 (1H, br s), 8.35 (1H, d, J = 2.4 Hz), 7.82 (1H, d, J = 8.8 Hz), 7.64 (1H, d, J = 15.2 Hz), 7.48 (2H, d, J = 8.8 Hz), 7.34 (1H, d, J = 15.2 Hz), 6.83 (2H, d, J = 8.8 Hz), 6.54 (1H, dd, J = 8.8, 2.4 Hz), 3.79 (3H, s), 3.75 (3H, s), 2.17 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.8, 169.9, 164.7, 161.9, 144.5, 144.4, 132.6, 130.5, 127.8, 120.4, 116.5, 114.7, 109.7, 104.5, 55.8, 55.6, 25.9.

*5.2.23. N-{3,5-dimethoxy-2-[3-(4-methoxyphenyl)acryloyl]phenyl} acetamide **(6c)***

This compound was prepared from **5c** using the same procedure as described for the preparation of **6a**: Yield 89%; IR (neat, cm^{-1}) 2941, 1699, 1605, 1578, 1513, 1471, 1365, 1336, 1294, 1262, 1234, 1203, 1176, 1158, 1108, 1031, 975; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.99 (1H, s), 7.88 (1H, d, J = 2.4 Hz), 7.62 (1H, d, J = 15.6 Hz), 7.49–7.52 (2H, m), 7.22–7.27 (1H, m), 6.87–6.91 (2H, m), 6.22 (1H, d, J = 2.0 Hz), 3.86 (3H, s), 3.85 (3H, s), 3.82 (3H, s), 2.17 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.1, 170.3, 164.5, 161.8, 142.3, 141.8, 131.1, 128.2, 126.0, 114.6, 110.2, 97.8, 94.1, 58.1, 57.6, 57.0, 24.1, 38.9.

*5.2.24. N-[2-[3,3-dimethoxy-2-(4-methoxyphenyl)-1-oxopropyl] phenyl]acetamide **(7a)***

A solution of thallium(III) nitrate trihydrate (3.31 g, 7.45 mmol) in trimethyl orthoformate (7 mL) was added to a solution of 2'-acetamidochalcone **6a** (2.00 g, 6.77 mmol) in trimethyl orthoformate (15 mL) over a period of 15 min and the mixture was stirred at rt for 4 h. After the reaction was completed, thallium(I) was removed through the filtration. The filtrate was neutralized by addition of 5% aqueous solution of NaOH and extracted with dichloromethane. The combined extracts were washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to give acetal **7a** (2.37 g, 98%) as a brown semisolid: IR (neat, cm^{-1}) 3445, 1515, 1382, 1243; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.53 (1H, s), 8.66 (1H, dd, J = 8.8, 0.8 Hz), 7.94 (1H, dd, J = 8.4, 1.6 Hz), 7.46 (1H, dt, J = 7.6, 1.6 Hz), 7.28 (2H, dd, J = 6.4, 2.0 Hz), 7.03 (1H, dd, J = 7.8, 1.4 Hz), 6.83 (2H, dd, J = 6.4, 2.0 Hz), 5.02 (1H, d, J = 8.4 Hz), 4.85 (1H, d, J = 8.4 Hz), 4.09 (1H, q, J = 7.2 Hz), 3.74 (3H, s), 3.39 (3H, s), 3.18 (3H, s), 2.21 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 135.2, 131.2, 130.0, 122.6, 121.0, 114.6, 106.7, 56.8, 55.8, 55.4, 54.7, 25.8.

*5.2.25. N-{2-[3,3-dimethoxy-2-(4-methoxyphenyl)propionyl]-5-methoxyphenyl}acetamide **(7b)***

This compound was prepared from **6b** using the same procedure as described for the preparation of **7a**: Yield 32%; IR (neat, cm^{-1}) 2935, 1612, 1512, 1457, 1245, 1110, 818; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.07 (1H, br s), 8.38 (1H, d, J = 2.4 Hz), 7.92 (1H, d, J = 8.8 Hz), 7.31 (2H, d, J = 8.8 Hz), 6.83 (2H, d, J = 8.8 Hz), 6.55 (1H, dd, J = 8.8, 2.4 Hz), 5.05 (1H, d, J = 8.8 Hz), 4.81 (1H, d, J = 8.8 Hz), 3.81 (3H, s), 3.74 (3H, s), 3.41 (3H, s), 3.20 (3H, s), 2.24 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0, 165.0, 159.2, 144.8, 133.4, 129.9, 127.5, 114.8, 114.5, 109.8, 106.7, 104.3, 56.3, 55.8, 55.7, 55.4, 54.8, 51.5, 26.0.

*5.2.26. N-{2-[3,3-dimethoxy-2-(4-methoxyphenyl)propionyl]-3,5-dimethoxyphenyl}acetamide **(7c)***

This compound was prepared from **6c** using the same procedure as described for the preparation of **7a**: Yield 98%; IR (neat, cm^{-1}) 3395, 1700, 1654, 1614, 1578, 1511, 1451, 1430, 1335,1294, 1242, 1161, 1109, 1069, 807; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.64 (1H, s), 7.81 (1H, s), 7.31 (2H, d, J = 8.8 Hz), 6.83 (2H, d, J = 8.8 Hz), 6.11 (1H, d, J = 2.4 Hz), 4.93 (1H, d, J = 8.8 Hz), 4.81 (1H, d, J = 8.4 Hz), 3.80 (3H, s), 3.75 (3H, s), 3.75 (3H, s), 3.13 (3H, s), 2.16 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.6, 169.4, 164.3, 161.1, 158.9, 141.9, 130.2, 128.0, 113.9, 107.8, 97.5, 94.5, 60.5, 55.8, 55.6, 55.4, 55.1, 54.5, 25.7.

*5.2.27. 3-(4-methoxyphenyl)-4(1H)-quinolinone **(8a)***

To a solution of acetal **7a** (2.37 g, 6.63 mmol) in ethanol was added 5% HCl and the mixture was stirred at rt for 10 min, then warmed to 50 °C and stirred for 2 h. The reaction mixture was cooled to rt to form light brown precipitates. The precipitates were filtered and recrystallized with ethanol to give quinolone **8a** (1.52 g, 91%): IR (neat, cm^{-1}) 3062, 2360, 1562, 1515, 1340, 1296, 1244, 1022, 819, 754, 615; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.16 (1H, dd, J = 8.0, 1.2 Hz), 8.06 (1H, d, J = 4.0 Hz), 7.62 (3H, m), 7.53 (1H, dd, J = 7.6, 0.6 Hz), 7.29 (1H, dt, J = 7.4, 1.2 Hz), 6.91 (2H, td, J = 8.0, 3.4 Hz), 3.74 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.4, 158.6, 139.9, 138.1, 132.1, 120.1, 129.1, 126.2, 123.8, 120.2, 118.8, 118.7, 113.9, 55.7; LRMS (FAB) m/z 252.1 (M + H^+); HRMS (FAB) calcd for $C_{16}H_{14}N\,O_2$, 252.1025; found: 252.1017 (M + H)$^+$.

*5.2.28. 3-(4-methoxyphenyl)-1-methyl-1H-quinolin-4-one **(8b)***

To a solution of quinolone **8a** (100 mg, 0.321 mmol) and NaH (60% dispersion in mineral oil, 19.3 mg, 0.482 mmol) in DMF (1.5 mL) was added iodoethane (91.2 mg, 0.642 mmol). The mixture was stirred at rt for 3 h. The reaction was quenched with water and extracted with EtOAc. The organic layer was washed with water, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography ($CHCl_3$/EtOAc/MeOH = 2:1:0.2) to give **8b** (102.4 mg, 98%): IR (neat, cm^{-1}) 3512, 2925, 1613, 1574, 1510, 1460, 1242, 1177, 1070, 1028; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.89 (1H, s), 7.49 (2H, d, J = 8.8 Hz), 6.87 (2H, d, J = 8.8 Hz), 6.45 (1H, s), 6.39 (1H, s), 3.87 (3H, s), 3.75 (3H, s), 3.72 (6H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.6, 162.7, 162.6, 158.5, 144.6, 141.7, 130.3, 129.1, 121.9, 113.7, 112.4, 95.1, 91.2, 56.4, 56.2, 55.7, 41.5; LRMS (FAB) m/z 266.1 (M + H^+); HRMS (FAB) calcd for $C_{17}H_{16}N\,O_2$, 266.1181; found: 266.1173 (M + H)$^+$.

*5.2.29. 3-(4-methoxyphenyl)-1-ethyl-1H-quinolin-4-one **(8c)***

This compound was prepared from **8a** using the same procedure as described for the preparation of **8b**: Yield 81%: IR (neat, cm^{-1}) 2990, 2833, 1571, 1512, 1329, 1241, 1176, 1031, 835, 758; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (1H, d, J = 8 Hz), 8.23 (1H, s), 7.49–7.65 (4H, m), 7.36 (1H, t, J = 7.4 Hz), 6.93 (2H, d, J = 7.2 Hz), 4.35 (2H, q, J = 6.8 Hz), 3.75 (3H, s), 1.35 (3H, t, J = 6.8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.9, 158.7, 142.9, 139.2, 132.8, 130.2, 128.8, 127.5, 127.2, 123.8, 120.4, 117.0, 113.9, 55.9, 47.8, 15.1; LRMS (FAB) m/z 280.1 (M + H^+); HRMS (FAB) calcd for $C_{18}H_{18}N\,O_2$, 280.1338; found: 280.1347 (M + H)$^+$.

*5.2.30. 3-(4-methoxyphenyl)-1-propyl-1H-quinolin-4-one **(8d)***

This compound was prepared from **8a** using the same procedure as described for the preparation of **8b**: Yield 98%: IR (neat, cm^{-1}) 2963, 2933, 1623, 1583, 1512, 1490, 1246, 1178, 1035, 835, 760; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (1H, d, J = 8.0 Hz), 7.60 (1H, d, J = 2.0 Hz), 7.54 (2H, d, J = 8.8 Hz), 7.51–7.56 (1H, m), 7.25–7.33 (2H, m), 6.85 (2H, d, J = 8.8 Hz), 3.99 (2H, t, J = 6.8 Hz), 3.73 (3H, s), 1.79 (2H, m), 0.90 (3H, t, J = 7.6 Hz),; ^{13}C NMR (100 MHz, CDCl) δ 175.7, 158.6, 141.7, 138.9, 131.7, 129.7, 128.0, 127.5, 127.3, 123.3, 121.1, 115.2, 113.6, 55.2, 54.7, 22.2, 11.1; LRMS (FAB) m/z 294.1 (M + H^+); HRMS (FAB) calcd for $C_{19}H_{20}N\,O_2$, 294.1494; found: 294.1495 (M + H)$^+$.

*5.2.31. 7-methoxy-3-(4-methoxyphenyl)-2,3-dihydro-1H-quinolin-4-one **(9a)***

This compound was prepared using from **7b** the same procedure as described for the preparation of **8a**: Yield 30%; IR (neat) 2965, 1632, 1560, 1515, 1350, 1289, 1250, 1180, 1029 cm^{-1}; IR (neat, cm^{-1}) 3398, 3073, 2967, 1634, 1517, 1252, 1181, 1030, 829; ^1H NMR (400 MHz, DMSO-d_6) δ 11.78 (1H, d, J = 3.6 Hz), 8.05 (1H, d, J = 9.2 Hz), 7.99 (1H, t, J = 2.4 Hz), 7.60 (2H, d, J = 8.4 Hz), 6.91 (4H, d, J = 4.8 Hz), 3.83 (3H, s), 3.74 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.9, 162.3, 158.6, 141.6, 141.5, 137.8, 137.6, 130.1, 129.1, 128.1, 120.7, 120.0, 114.1, 113.9, 99.5, 55.1, 55.7; LRMS (FAB) m/z 282.1 (M + H^+); HRMS (FAB) calcd for $C_{17}H_{16}N\ O_3$, 282.1130; found: 282.1134 $(M + H)^+$.

*5.2.32. 7-methoxy-3-(4-methoxyphenyl)-1-methyl-1H-quinolin-4-one **(9b)***

This compound was prepared from **9a** using the same procedure as described for the preparation of **8b**: Yield 95%: IR (neat, cm^{-1}) 3584, 2924, 1618, 1575, 1509, 1482, 1331, 1252, 1213, 1178, 1123, 1065, 1033, 831; ^1H NMR (400 MHz, $CDCl_3$) δ 8.48 (1H, d, J = 8.8 Hz), 7.59 (3H, d, J = 8.8 Hz), 7.00 (1H, dd, J = 2.4,8.8 Hz), 6.94 (2H, d, J = 8.8 Hz), 6.71 (1H, d, J = 2.4 Hz), 3.94 (3H, s), 3.83 (3H, s), 3.78 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.3, 162.4, 162.3, 158.4, 141.7, 129.5, 129.0, 127.8, 121.1, 113.4, 112.2, 97.4, 55.4, 55.1, 40.5; LRMS (FAB) m/z 296.1 (M + H^+); HRMS (FAB) calcd for $C_{18}H_{18}N\ O_3$, 296.1287; found: 296.1284 $(M + H)^+$.

*5.2.33. 7-methoxy-3-(4-methoxy-phenyl)- 1-ethyl-1H-quinolin-4-one **(9c)***

This compound was prepared from **9a** using the same procedure as described for the preparation of **8b**: Yield 85% [1];H NMR (400 MHz, $CDCl_3$) δ 8.49 (1H, d, J = 9.2 Hz), 7.62 (1H, s), 7.60 (2H, d, J = 8.8 Hz), 6.98 (1H, dd, J = 2.4,8.8 Hz), 6.94 (2H, d, J = 8.8 Hz), 6.76 (1H, d, J = 2.4 Hz), 4.15 (2H, q, J = 14.4 Hz), 3.93 (3H, s), 3.83 (3H, s), 1.50 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.3, 162.4, 162.3, 158.5, 140.5, 140.3, 129.7, 129.6, 127.9, 121.7, 121.6, 113.6, 111.8, 104.9, 97.7, 55.5, 55.2, 47.9, 14.2; LRMS (FAB) m/z 310.1 (M + H^+); HRMS (FAB) calcd for $C_{19}H_{20}N\ O_3$, 310.1443; found: 310.1445 $(M + H)^+$.

*5.2.34. 7-methoxy-3-(4-methoxyphenyl)-1-propyl-1H-quinolin-4-one **(9d)***

This compound was prepared from **9a** using the same procedure as described for the preparation of **8b**: Yield 67% [1];H NMR (400 MHz, CD_3OD) δ 8.32 (1H, d, J = 8.8 Hz), 8.02 (1H, s), 7.53 (2H, d, J = 8.8 Hz), 7.05 (1H, dd, J = 2.4,8.8 Hz), 6.99 (1H, d, J = 2.4 Hz), 6.94 (2H, d, J = 8.8 Hz), 4.26 (2H, t, J = 7.2 Hz), 3.94 (3H, s), 3.80 (3H, s), 1.90 (2H, q, J = 14.4 Hz), 1.48 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 175.3, 162.3, 158.5, 141.3, 140.5, 129.6, 129.5, 127.9, 121.6, 121.0, 113.5, 111.6, 98.0, 55.4, 55.2, 54.7, 21.9, 11.1; LRMS (FAB) m/z 324.2 (M + H^+); HRMS (FAB) calcd for $C_{20}H_{22}N\ O_3$, 324.1600; found: 324.1600 $(M + H)^+$.

*5.2.35. 7-methoxy-3-(4-methoxyphenyl)-1-(3-methylbutyl)-1H-quinolin-4-one **(9e)***

To a solution of quinolone **9a** (50 mg, 0.17 mmol) and NaH 60% dispersion in mineral oil (12 mg, 0.53 mmol) in DMF (1 mL) was added dimethylallyl bromide (41 μL, 0.35 mmol). The mixture was stirred at rt for 2 h. The reaction was quenched with water and extracted with EtOAc. The organic layer was washed with water, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography (*n*-hexane/EtOAc = 5:2) to give *N*-isoprenylated compound (53 mg, 85%): IR (neat, cm^{-1}) 3584, 1617, 1576, 1510, 1472, 1258, 1177, 1034, 831; ^1H NMR (400 MHz, $CDCl_3$) δ 8.33 (1H, d, J = 8.8 Hz), 8.13 (1H, s), 7.47 (2H, d, J = 8.8 Hz), 7.18 (1H, dd, J = 2.4,8.8 Hz), 7.08 (1H, d, J = 2.4 Hz), 6.76 (2H, d, J = 8.8 Hz), 5.39, (1H, t, J = 7.2 Hz), 5.14 (2H, d, J = 6.4 Hz), 4.03 (3H, s), 3.64 (3H, s), 1.95 (3H, s), 1.83 (3H, s). And then to a solution of *N*-isoprenylated compound (30 mg, 0.08 mmol) in ethanol was added $Pd(OH)_2$ (24 mg, 80 wt%) and ammoniumformate (64.2 mg, 0.80 mmol) in H_2O (0.5 mL) and stirred at rt for 18 h. The mixture was filtered through Celite pad and washed with ethanol. The liquor was concentrated *in vacuo* and purified by column chromatography (*n*-hexane/EtOAc = 3:2) to give **9e** (28 mg, 92%): ^1H NMR (400 MHz, $CDCl_3$) δ 8.34 (1H, d, J = 8.8 Hz), 8.13 (1H, s), 7.47 (2H, d, J = 8.8 Hz), 7.18 (1H, dd, J = 2.4,8.8 Hz), 7.08 (1H, d, J = 2.4 Hz), 6.76 (2H, d, J = 8.8 Hz), 4.35 (2H, t, J = 7.2 Hz), 4.03 (3H, s), 3.64 (3H, s), 1.80–1.76 (3H, m), 1.95 (3H, s), 1.83 (3H, s); LRMS (FAB) m/z 352.2 (M + H^+); HRMS (FAB) calcd for $C_{22}H_{26}NO_3$, 352.1913; found: 352.1913 $(M + H)^+$.

*5.2.36. 1-methoxy-7-benzyloxy-3-(4-benzyloxyphenyl)-1H-quinolin-4-one **(9f)***

To a solution of quinolone **9a** (65.0 mg, 0.231 mmol) and NaH 60% dispersion in mineral oil (18.5 mg, 0.462 mmol) in DMF (2 mL) was added benzyl bromide (33.6 μL, 0.277 mmol). The mixture was stirred at rt for 5 h. The reaction was quenched with water and extracted with EtOAc. The organic layer was washed with water, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography (*n*-hexane/EtOAc = 2:3) to give **9f** (53.5 mg, 62%): ^1H NMR (400 MHz, $CDCl_3$) δ 8.45 (1H, d, J = 9.2 Hz), 7.71 (1H, s), 7.61 (2H, d, J = 8.8 Hz), 7.29–7.35 (3H, m), 7.17 (2H, d, J = 6.8 Hz), 6.92 (2H, d, J = 8.8 Hz), 6.90–6.93 (1H, m), 6.61 (1H, d, J = 2.0 Hz), 5.26 (2H, s), 3.80 (3H, s), 3.72 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.6, 162.4, 158.7, 141.7, 141.0, 135.3, 129.8, 129.4, 129.2, 128.2, 127.8, 126.1, 121.7, 121.6, 113.7, 112.4, 98.6, 56.7, 55.4, 55.3; LRMS (FAB) m/z 372.2 (M + H^+); HRMS (FAB) calcd for $C_{24}H_{22}N\ O_3$, 372.1600; found: 372.1600 $(M + H)^+$.

*5.2.37. 5,7-dimethoxy-3-(4-methoxyphenyl)-1H-quinolin-4-one **(10a)***

This compound was prepared from **7c** using the same procedure as described for the preparation of **8a**: Yield 93%; IR (neat, cm^{-1}) 3368, 3232, 2592, 1644, 1601, 1514, 1472, 1411, 1310, 1243, 1203, 1183, 1164, 1108, 1073, 1037, 977, 945, 867, 832; ^1H NMR (400 MHz, DMSO-d_6) δ 11.89 (1H, s), 7.89 (1H, s), 7.49 (2H, d, J = 7.2 Hz), 6.89 (2H, d, J = 7.2 Hz), 6.52 (1H, s), 6.35 (1H, s), 3.80 (3H, s), 3.78 (3H, s), 3.73 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.7, 162.8, 161.5, 158.7, 143.9, 137.0, 130.5, 128.7, 121.6, 113.9, 111.2, 95.8, 91.9, 56.6, 56.1, 55.7, 31.4; LRMS (FAB) m/z 312.1 (M + H^+); HRMS (FAB) calcd for $C_{18}H_{18}N\ O_4$, 312.1236; found: 312.1241 $(M + H)^+$.

*5.2.38. 5,7-dimethoxy-3-(4-methoxyphenyl)-1-methyl-1H-quinolin-4-one **(10b)***

This compound was prepared from **10a** using the same procedure as described for the preparation of **8b**: Yield 83%; IR (neat, cm^{-1}) 3512, 2925, 1613, 1574, 1510, 1459, 1344, 1289, 1241, 1177, 1070, 1027; ^1H NMR (400 MHz, DMSO-d_6) δ 7.89 (1H, s), 7.49 (2H, d, J = 8.4 Hz), 6.87 (2H, d, J = 8.4 Hz), 6.45 (1H, d, J = 2.0 Hz), 6.39 (1H, d, J = 2.0 Hz), 3.87 (3H, s), 3.75 (3H, s), 3.73 (3H, s), 3.72 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.6, 162.7, 162.6, 158.5, 144.6, 141.7, 130.3, 129.1, 121.9, 113.8, 112.4, 95.1, 91.2, 56.4, 56.1, 55.7, 41.5; LRMS (FAB) m/z 326.1 (M + H^+); HRMS (FAB) calcd for $C_{19}H_{20}N\ O_4$, 326.1392; found: 326.1391 $(M + H)^+$.

*5.2.39. 5,7-dimethoxy-3-(4-methoxyphenyl)-1-ethyl-1H-quinolin-4-one **(10c)***

This compound was prepared from **10a** using the same procedure as described for the preparation of **8b**: Yield 88%; IR (neat, cm^{-1}) 3284, 1641, 1562, 1515, 1449, 1362, 1159, 836; ^1H NMR

(400 MHz, DMSO-d_6) δ 7.82 (1H, s), 7.49 (2H, d, $J = 8.4$ Hz), 6.89 (2H, d, $J = 8.4$ Hz), 6.50 (1H, d, $J = 2.0$ Hz), 6.45 (1H, d, $J = 2.0$ Hz), 4.21 (2H, q, $J = 7.2$ Hz), 3.91 (3H, s), 3.88 (3H, s), 3.78 (3H, s), 1.42 (3H, t); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.6, 162.8, 162.7, 158.5, 143.3, 140.7, 130.4, 129.1, 122.3, 113.7, 112.8, 94.9, 90.9, 56.4, 56.2, 48.3, 14.6; LRMS (FAB) m/z 340.1 (M + H$^+$); HRMS (FAB) calcd for $C_{20}H_{22}N\,O_4$, 340.1549; found: 340.1550 (M + H)$^+$.

5.2.40. 5,7-dimethoxy-3-(4-methoxyphenyl)-1-propyl-1H-quinolin-4-one (**10d**)

This compound was prepared from **10a** using the same procedure as described for the preparation of **8b**: Yield 84%; IR (neat, cm^{-1}) 2963, 2934, 1632, 1612, 1591, 1463, 1289, 1245, 1163, 1038, 834; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (2H, t, $J = 8.4$ Hz), 7.44 (1H, s), 6.90 (2H, d, $J = 8.4$ Hz), 6.34 (1H, d, $J = 2.0$ Hz), 6.29 (1H, d, $J = 2.0$ Hz), 3.97 (2H, q, $J = 7.2$ Hz), 3.94 (3H, s), 3.90 (3H, s), 3.81 (3H, s), 1.89 (2H, tq, $J = 7.2$ Hz), 1.00 (3H, t); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.7, 163.2, 162.4, 158.5, 143.1, 139.7, 130.1, 128.2, 123.1, 113.4, 112.9, 93.9, 90.0, 56.2, 55.7, 55.3, 21.7, 11.2; LRMS (FAB) m/z 354.2 (M + H$^+$); HRMS (FAB) calcd for $C_{21}H_{24}N\,O_4$, 354.1705; found: 354.1740 (M + H)$^+$.

5.3. Molecular modeling

5.3.1. Minimization of molecules

All the molecules for docking were sketched in the SYBYL-X and energy minimizations were performed using Tripos Force field and Gasteiger-Huckel charge with 100,000 iterations of conjugate gradient method with convergence criterion of 0.05 kcal/mol. After minimization is completed, all molecules are included in a database for docking analysis.

5.3.2. Protein preparation

Crystal structures of genistein bound to ERα and ERβ (PDB code 1X7R and 1X7J) were refined using the following protocol. All hydrogen was added to selected protein as random orientation and the charge was loaded to protein and ligands with MMFF94 and Gasteiger-Huckel, respectively. Sidechain amide was fixed and then staged minimization of biopolymer hydrogens and ligands was performed with 100 iterations of conjugate gradients method to a gradient of 0.5 kcal using MMFF94s Force field and charges. For next docking study, originally bound ligand was extracted from prepared protein.

5.3.3. Docking studies

All molecular modeling calculations were carried out using the Surflex Dock interfaced with SYBYL-X version 2.0. In this automated docking program, the flexibility of the ligands is considered while the protein or biomolecule is considered as a rigid structure. The ligand is built in an incremental fashion, where each new fragment is added in all possible positions and conformations to a pre-placed base fragment inside the active site.

The protomol was generated using the crystallized ligand with a threshold of 0.50 as default settings. All other parameters accepted the default settings. The 3D coordinates of the active site were taken from the X-ray crystal structures of the ERα and ERβ (PDB code 1X7R and 1X7J) reported as complex with genistein.

5.4. ERE-luciferase reporter gene assay

CV-1 cells were seeded in 48-well plate at a density of 1.5×10^5 cells/well. One day after seeding, the cells were co-transfected with 30 ng hERα (or hERβ) and 100 ng pERE-luciferae plasmid in each well for 6 h, and further treated with vehicle and samples for 24 h before harvesting for the luciferase assay. To normalize the transfection efficiency, β-galactosidase plasmid was co-transfected. The luciferase activities were measured using luciferase assay system (Promega Corp., Madison, WI) and the β-galactosidase activities were measured as the absorbance at 410 nm by using an ELISA plate reader. Data are reported as relative luciferase activity divided by the β-galactosidase activity.

5.5. ER competitive binding affinity assays

The competitive ER binding assay was performed as previously described (Arcaro et al., 1999). Briefly, 6 nM of the recombinant human ER (rhER α, β Invitrogen Life Technology, Inc., CA, USA) was incubated with test compounds for 9 h at room temperature in the presence of 2.5 nM of [2,4,6,7-^{3}H]-E2 (81.0 ci/mmol). Subsequently, 100 μL of 50% (v/v) hydroxyapatite slurry was added to the reaction mixture for 20 min at 4 °C. Bound and free radioligand were separated by centrifugation at 12,000 × g for 2 min, and the radioactivity of the supernatant was determined using a liquid scintillation counter (LS-6500, Beckman counter, CA, USA). The amount of the receptor-bound [^{3}H]-E2 in the presence of the test compounds was presented relative to maximum receptor bound ligand (10^{-7} M E2). The data are expressed as the ratio of the receptor-bound [^{3}H]-E2 in the presence of the compounds to the 0.1% DMSO used as a control. The IC_{50} values were determined from dose–response curves obtained from three separate experiments.

5.6. MTT assay

MCF-7 cells were seeded in 96-well plate at a density of 1.5×10^3 cell/well for 24 h and followed by the treatment of 0.1 nM E2, 100 nM ICI and the indicated concentrations of samples. After 4 days, MTT solution (5 mg/mL in phosphate buffered saline) was added to each well and further incubated for 2 h. The absorbance of the formazan crystals dissolved in 100 μL of DMSO was measured at 540 nm using the microplate reader (VERSAmax Molecular Devices, CA, USA).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.07.030.

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