Published in final edited form as:
Mol Cell Endocrinol. 2008 February 13; 283(1-2): 49–57.

Liquiritigenin is a plant-derived highly selective estrogen receptor β agonist

Jennifer E. Mersereau[a], **Nitzan Levy**[a], **Richard E. Staub**[b], **Scott Baggett**[b], **Tetyana Zogric**[a], **Sylvia Chow**[b], **William A. Ricke**[c], **Mary Tagliaferri**[b], **Isaac Cohen**[b], **Leonard F. Bjeldanes**[d], and **Dale C. Leitman**[a,d,*]

a *Departments of Obstetrics, Gynecology and Reproductive Sciences and Center for Reproductive Sciences, Cellular and Molecular Pharmacology, University of California, San Francisco, CA, United States*

b *Bionovo Inc., Emeryville, CA, United States*

c *Departments of Urology, Pathology and Laboratory Medicine, and James P. Wilmot Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, NY, United States*

d *Department of Nutritional Sciences and Toxicology, University of California, Berkeley, CA, United States*

Abstract

After the Women's Health Initiative found that the risks of hormone therapy outweighed the benefits, a need for alternative drugs to treat menopausal symptoms has emerged. We explored the possibility that botanical agents used in Traditional Chinese Medicine for menopausal symptoms contain ERβ-selective estrogens. We previously reported that an extract containing 22 herbs, MF101 has ERβ-selective properties. In this study we isolated liquiritigenin, the most active estrogenic compound from the root of *Glycyrrhizae uralensis* Fisch, which is one of the plants found in MF101. Liquiritigenin activated multiple ER regulatory elements and native target genes with ERβ but not ERα. The ERβ-selectivity of liquiritigenin was due to the selective recruitment of the coactivator steroid receptor coactivator-2 to target genes. In a mouse xenograph model, liquiritigenin did not stimulate uterine size or tumorigenesis of MCF-7 breast cancer cells. Our results demonstrate that some plants contain highly selective estrogens for ERβ.

1. Introduction

Since the average age of the population in the United States is rising, there are an increasing number of menopausal women. Menopause is often associated with hot flashes, night sweats, mood changes, urogenital atrophy and loss of bone density that have traditionally been treated with hormone therapy (HT) to restore estrogen levels. The Women's Health Initiative (WHI) trial, however, found that estrogen plus progestin increased a woman's risk of heart disease, breast cancer, and dementia [1–5]. In addition, a second arm of the WHI found that using estrogen alone increased the risk of stroke and dementia [6,7]. While some of the unfavorable

Correspondence should be addressed to D.C.L: University of California, San Francisco, MS 1258 P.O. Box 0556 San Francisco, CA 94143-0556 Tel. (415) 502-5262 FAX (415) 753-3271 email: dale@leitmanlab.com.

J. E. M. and N. L. contributed equally to this work

Disclosure Statement: J.E.M., N.L., T.Z. and W.A.R. have nothing to declare. R.E.S, S.B., S.C., M.T., and I.C., are employed by and have equity interests in Bionovo, Inc. L.F.B. is on the Scientific Advisory Board of Bionovo, Inc. D.C.L. is on the Scientific Advisory Board and has received financial support for research from Bionovo, Inc.

results on heart disease from the WHI are likely due to the late time when HT was initiated in relation to the onset of menopause [8] the adverse impact of HT on breast cancer and blood clots indicated that new strategies are needed to treat menopausal symptoms.

Alternative drugs to traditional HT could potentially include selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene. However, while current SERM therapy has some favorable effects, such as improved bone mineral density [9,10] and the prevention of breast cancer, SERMs exacerbate hot flashes [11]. Other pharmacological options for hot flashes include antidepressant therapy using serotonin and norepineprhine reuptake inhibitors, as well as other neuro-modulators, such as gabapentin [12]. However, the overall benefit of these treatments is unclear considering their moderate efficacy [13], potential significant side effects [14,15] and lack of benefits on other menopausal symptoms, such as vaginal atrophy and osteoporosis.

Many patients rely on botanical dietary supplements (BDS) used in Traditional Chinese Medicine (TCM) to relieve their menopausal symptoms. It has been reported that about 25% of women use botanical extracts to treat menopausal symptoms [16]. Recently, there has been an increased interest in using isoflavones, which are one of several classes of phytoestrogens, as an alternate therapy for menopausal symptoms. Daidzein and genistein, the major isoflavones found in soy products, have been studied at length with inconclusive results from clinical trials. Some studies with daidzein have shown modest improvement in menopausal symptoms as compared to placebo [17,18], while a large meta-analysis did not show that daidzein-rich isoflavones improved symptoms over placebo [19]. Similarly, studies with genistein, have shown inconclusive results [19,20]. The selective estrogen receptor (SERM) DT56a is an enzymatic isolate of soybeans that has been shown to improve vasomotor symptoms and increased bone mineral density in post-menopausal women, with no effect on sex hormone levels or endometrial thickness [21–23]. While these data suggest that phyto-SERMs from soy have promise to safely treat menopausal symptoms and osteoporosis their effectiveness still needs to be evaluated in larger placebo-controlled randomized trials.

In addition to soy, it has been found that Chinese herbs contain compounds that have estrogenic activity [24,25]. We have been performing basic and clinical research on one botanical extract, MF101, which is composed of 22 individual plants [25]. A Phase 2 clinical trial with 217 postmenopausal women found that 5 gm and 10 gm MF101 were safe for short term use and more effective than placebo after 12 weeks of treatment (data not shown). We also demonstrated that MF101 acted as an ERβ-selective agonist by regulating gene transcription via ERβ pathways [25]. In addition, we showed that MF101 does not stimulate MCF-7 breast cancer cell proliferation or uterine growth in a mouse xenograft model [25]. These findings suggested that plants used in TCM might be a source for the discovery of estrogen receptor subtype selective drugs to safely treat menopausal symptoms.

The estrogens used in current HT regimens activate both known estrogen receptor subtypes, ERα and ERβ. Although the precise roles of both ERs are not known, the specific activation of each subtype results in different biological outcomes. ERα and ERβ knockout mice have different phenotypes [26]. In addition, estradiol (E_2,) activation of ERα versus ERβ results in different gene regulation patterns [27]. Estrogen acts as an agonist on ERα and ERβ in all tissue types, which likely explains the beneficial aspects of HT, but this non-selective action also likely causes the adverse side effects unveiled by the WHI. On the other hand, drugs that selectively activate ERα or ERβ might mimic some of the beneficial effects while avoiding the untoward effects. Since ERα has been shown to cause the proliferation of breast cancer cells and ERβ has been demonstrated to be a tumor suppressor [28,29], it is conceivable that ERβ-selective agonists may serve as safer long-term alternative treatment to traditional HT.

Due to the large unmet need encountered as a result of the WHI, we have developed a parallel strategy to discover drugs for the treatment of menopausal symptoms. Our first approach was to determine the mechanism of action of standardized crude plant extracts and to test them in controlled clinical trials. To this end, we showed that MF101 had ERβ-selective properties [25] and a Phase 2 clinical trial with MF101 for the treatment of hot flashes was done to further evaluate its safety and efficacy (http://clinicaltrials.gov/show/NCT00119665). Our next step was to isolate and characterize individual chemical entities from MF101 as potential drugs for menopausal symptoms. This paper summarizes the results from a study representing our second approach as we isolated an ERβ-selective compound, liquiritigenin, from the root of *G. uralensis*, and determined its biologic activity on estrogen receptors in cells and animal models.

2. Materials and methods

2.1. Isolation and structural identification of liquiritigenin from Glycyrrhiza uralensis

Dry, powdered *G. uralensis* roots were extracted with 9:1 water—methanol (18 h, constant mixing) at a 10:1 solvent to mass ratio. The filtrate was recovered after suction filtration (Whatman #1 filter), concentrated by rotary evaporation to remove the methanol, and partitioned with an equal volume of ethyl acetate (repeated once). The combined ethyl acetate layers were dried with anhydrous sodium sulfate, concentrated to dryness by rotary evaporation *in vacuo*, and resuspended in a small volume of ethyl acetate. The sample was loaded onto a fritted glass column packed with silica gel (200–400 mesh, 60Å) and eluted with a hexane/ethyl acetate gradient, starting with 100% hexane. Liquiritigenin eluted from the silica column with 60–80% ethyl acetate in hexane. The liquiritigenin fractions recovered off the silica column were further purified by preparative reverse phase HPLC (Delta 600 system, Waters Corporation, Milford, MA) on a C_8 column (SymmetryPrep, 19 × 150 mm, Waters Corporation) with UV detection (λ = 254 nm). A gradient elution from 35–40 % acetonitrile in water over 15 min at a flow rate of 12 mL/min was utilized to isolate liquiritigenin. Mass spectrometry analysis was performed on a HP 1100 LC/MS (Agilent Technologies, Santa Clara, CA), and yielded the expected molecular ion of *m/z* 255 $[M\text{-}H]^-$. The ^1H and ^{13}C spectra were recorded on a Bruker 500 MHz nuclear magnetic resonance spectrometer (Bruker, Fallanden, Switzerland). NMR spectra were acquired in methanol-d_4 and were consistent with published data for liquiritigenin isolated from *Glycyrrhiza* species [30].

2.2. Cell culture, Transfection, and Luciferase Assays

The human osteosarcoma U2OS cell line, human breast MCF-7 cancer cell line, and human cervical HeLa cancer cell line were obtained from the cell culture facility at the University of California, San Francisco. The MCF-7 cells express ERα, whereas the U2OS and WAR5 do not express endogenous ERs. The prostate cancer cell line, WAR5 cells was prepared as previously described [31]. All cell lines were maintained and subcultured as previously described [32]. Transfections were carried out with a Bio-Rad gene pulser. Cells were electroporated with 3 μg of ERE, CECR6, NKD, or NKG2 tk-Luciferase reporter vectors along with 1 μg of ERα or ERβ expression vectors. After electroporation, the cells were plated and treated with estradiol (E_2), liquiritigenin (Liq), 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN, Tocris Bioscience, Ellisville, Missouri) or 4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT, Tocris Bioscience, Ellisville, Missouri) for 18 h. Cells were then solubilized and luciferase activity was determined with an assay kit (Promega, Madison, WI). Experiments were performed at least three times and the mean ± S.E. was calculated and statistical analysis was performed using the Prism curve-fitting program (Graph Pad Software, version 3.03).

2.3. Real-time PCR

U20S cells expressing a tetracycline-inducible ERα or ERβ cDNA were prepared as previously described [27]. Cells were treated with doxycycline (1 μg/ml) for 18 hours and then with E_2

or liquiritigenin for 3 hours. Total RNA was isolated using the Aurum Total RNA kit (Bio-Rad, Hercules, CA) and reverse transcription (RT) reactions were performed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time quantitative PCR was performed using SYBR Green Supermix with an iCycler thermal cycler (Bio-Rad). Experiments were performed at least three times and the mean ± S.E. was calculated and statistical analysis was performed using the Prism curve-fitting program (Graph Pad Software, version 3.03). We used the following PCR primers:

NKD	Forward 5′-CAGCCAGAGCAAGAGGAGCGTC-3′ Reverse 5′-CCGGCGAGATCTAAGTAGTGGT-3′
NKG2E	Forward 5′-GCCAGCATTTTACCTTCCTCAT-3′ Reverse 5′-AACATGATGAAACCCCGTCTAA-3′
CECR6	Forward 5′-ACAGTCGGTGTGGAATGTC-3′ Reverse 5′-AGAAGGGAGAAGGGGAAACA-3′

2.4. ER Binding Assays

The relative binding affinity of liquiritigenin to pure full-length ERα and ERβ was determined using ERα and ERβ competitor assay kits, according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Fluorescence polarization of the fluorophore-tagged estrogen bound to ERα and ERβ in the presence of increasing amounts of competitor ligand or extract was determined (10 readings per well; 0.02 millisecond integration time; G factor = 1.1087) using the GENios Pro microplate reader (Tecan Systems Inc., San Jose, CA) with fluorescein excitation (485nM) and emission (530 nM) filters. Each liquiritigenin dose was performed in triplicate and the relative error was determined by calculating the standard error of three values from the mean.

2.5. Chromatin immunoprecipation (ChIP)

Following treatment with liquiritigenin or E_2, stably transfected U2OS-ERα and U2OS-ERβ cells were fixed with 1% formaldehyde and ChIP was done as previously described [33]. Immunoprecipitations were performed overnight at 4 °C with anti-SRC-2 (ab9261, Abcam, Cambridge, MA) antibodies. DNA fragments were purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) and PCR-amplified. The primers used for ChIP are: CECR6 forward 5-TGATAAATGCTAGTGAGGTGCC-3, reverse 5-AGAACCGCCTGCTCCTAACAAT-3. NKD forward 5-GGGTCAGGACGAGTGTTTTCTT-3, reverse 5-ACCCCGGACCAAATTTCAGTTA-3. NKG2E Forward 5′-AGCCACCCAAAGTCTCCTAT-3′; Reverse 5′-TTCAGTGGAGAGGTCAGGTT-3′. PCR reactions for non-immune assays served as negative controls (data not shown).

2.6. Xenograft studies in nude mice

MCF-7 (250,000) cells were aggregated in suspension and then resuspended in 200 μL neutralized collagen, as previously described [34]. The cells were then grafted under the kidney capsule of nude mice as described and illustrated in detail at: http://mammary.nih.gov/tools/mousework/Cunha001/index.html. Five mice per group were treated with a continuous infusion using osmotic pumps (Alzet, Cupertino, CA) containing vehicle, E_2 (0.4 mg) or liquiritigenin (2 mg) that infused 2.5 μl/h for 1 month. After one month of treatment, the tumors and uteri were removed and analyzed. These animal studies were carried out with approval from the University of California, San Francisco Committee on Animal Research.

3. Results

Our previous results showed that MF101 contains ERβ-selective activity [25]. The 22 herbs constituting MF101 were individually screened for estrogenic activity in transfection assays. Of the 22 herbs, *G. uralensis* contained high estrogenic activity. Activity-guided isolation of the compounds from the *G. uralensis* was initiated using ERE tk-Luc and an expression vector for ERβ. These studies resulted in the identification of the flavanone, liquiritigenin (Fig. 1). To assess the relative activity of liquiritigenin via ERα or ERβ, we used transfection assays with increasing concentrations of liquiritigenin. Liquiritigenin produced a dose-response activation of ERE tk-Luc in the U2OS cells transfected with ERβ, but not ERα (Fig. 2A). The activation first occurred at 1 nM and the maximal activation was observed at 500 nM. The EC_{50} of liquiritigenin (36.5 nM) for the activation of ERE tk-Luc was about 80-fold less than the EC_{50} of E_2 (0.45 nM) (Fig. 2B). The ER subtype selectivity of liquiritigenin was compared to the ERβ-selective agonist, DPN, and the ERα agonist, PPT. Whereas no activation of ERE tk-Luc by ERα was observed with liquiritigenin, DPN produced a 20-fold activation and PPT produced a 74-fold activation, which was similar to the activation by E_2 (Fig. 2C, left panel). In contrast, E_2, liquiritigenin and DPN produced a similar activation of ERE tk-Luc with ERβ, whereas PPT had no effect (Fig. 2C, right panel). The activation of ERE tk-Luc by liquiritigenin in the presence of ERβ was blocked by the ER antagonists, ICI 182780, raloxifene and tamoxifen, demonstrating that the activation is mediated by ERβ (Fig. 2D). The ERβ-selectivity of liquiritigenin was also observed in HeLa cells and the prostate cancer WAR5 cell line (Fig. 2E, 2F, respectively). Liquiritigenin did not activate other nuclear receptors including the androgen receptor, progesterone receptor B, glucocorticoid receptor or thyroid hormone receptor in transfection assays (Fig. 3A, 3B, 3C and 3D, respectively). These results demonstrate that liquiritigenin is more selective than DPN with ERβ in U2OS cells and is an ERβ-selective agonist in multiple cell lines.

3.1. Liquiritigenin selectively activates native target elements and genes through ERβ

In addition to the traditional ERE, it is important to determine if liquiritigenin selectivity activates ERβ in elements derived from native ER target genes. We used ER regulatory elements from the *cat eye syndrome chromosome region candidate 6 (CECR6), killer cell lectin-like receptor (NKG2E)* and the *naked cuticule homolog* (*NKD)* genes that are activated by E_2 [35,36]. The *CECR6* element has an ERE and the NKG2E regulatory region contains a composite element containing a c-jun, heat-shock factor 2, and CCAAT/enhancer-binding protein beta and a unique variant ERE [35,36]. The NKD element does not contain an ERE or other known alternative regulatory elements [36]. These elements were transfected into U2OS cells with expression vectors for ERα or ERβ. Liquiritigenin produced a dose-dependent activation of the CECR6 (Fig. 4A), NKG2E (Fig. 4B) and NKD (Fig. 4C) with ERβ but not with ERα. To determine if liquiritigenin exhibits ERβ-selectivity on native genes, we examined its effect in the previously characterized U2OS cell lines that are stably transfected with ERα or ERβ[27]. Liquiritigenin produced a time-dependent increase in CECR6 (Fig. 4D), NKG2E (Fig. 4E) and NKD (Fig. 4F) mRNA in the U2OS-ERβ cells, but not the U2OS-ERα cells. These studies demonstrate that liquiritigenin is an ERβ-selective agonist on multiple ER regulatory elements and native target genes.

3.2. Liquiritigenin exhibited similar binding affinities for ERα and ERβ, and caused the recruitment of SRC-2 to target genes selectively in ERβ cells

Estrogen receptor ligands have been shown to have different affinities for ERα or ERβ. For example, E_2 binds with equal affinity to both ERα and ERβ, whereas some phytoestrogens, such as the isoflavone genistein bind with a higher affinity to ERβ compared to ERα[37,38]. One of the possible mechanisms for the ERβ-selectivity of liquiritigenin is that it binds with higher affinity to ERβ than to ERα. However, competition binding curves show that ERβ only

has only a 20-fold greater affinity for liquiritigenin compared to ERα (Fig 5A), which is not likely sufficient to explain the differences in transcriptional regulation. A more plausible explanation for the ERβ-selectivity is that liquiritigenin recruits coactivators only to ERβ. To test this hypothesis, U2OS-ERα and U2OS-ERβ cells were incubated with liquiritigenin for increasing times and then ChIP was done with an antibody to the coactivator SRC-2. We chose to focus on SRC-2 because we previously showed that E_2 recruited only SRC-2 to multiple ER regulatory elements, such as *NKG2E, CECR6* and *NKD* genes [35]. Liquiritigenin caused the recruitment of SRC-2 to the *CECR6* (Fig 5B), *NKG2E* (Fig 5C), and *NKD* (Fig 5D) genes in the U2OS-ERβ cells, but not the U2OS-ERα cells. These results demonstrate that liquiritigenin acts as an ERβ-selective agonist because it only recruits coactivators to ERβ.

3.3 In mouse xenograft models, liquiritigenin does not have proliferative effects on breast cancer cells or mouse uterus

The major concern with estrogens for menopausal symptoms is the proliferation of breast and endometrial cells causing an increased risk for breast and uterine cancer. To determine if liquiritigenin has a proliferative effect on breast cancer cells, we grafted MCF-7 breast cancer cells under the kidney capsule of nude mice. Using a subcutaneous osmotic pump designed to deliver a steady dose of drug, we treated the mice for 30 days with vehicle, E_2, or liquiritigenin. Large tumors developed in the mice treated with E_2 (Fig. 6B), while there was essentially no tumor growth in the mice treated with vehicle (Fig. 6A) or liquiritigenin (Fig. 6C). There were also no differences in the weights of the tumors in mice treated with liquiritigenin compared to the control mice (Fig. 6D). In addition, after 30 days of treatment, liquiritigenin did not increase uterine horn mass, whereas E_2 did (Fig. 6E).

4. Discussion

We have been exploring plants as a source for ER subtype selective estrogens based on their historical use for treating menopausal symptoms in TCM and on the findings that soybeans contain isoflavones that selectively bind to ERβ [38] and preferentially activate it in transfection studies [39]. We first tested a crude plant extract, MF101, for ER subtype selectivity. Despite the fact that MF101 is comprised of 22 different botanical agents and a multitude of compounds, our studies found MF101 exhibited ERβ-selectivity and did not exhibit proliferative effects on human breast cancer cells or the mouse uterus [25]. The purpose of this study was to isolate active compounds from the individual plant components of MF101 as potential drugs to treat menopausal symptoms. Individual compounds have the potential to be safer than the crude herbal formulation since some of the non-therapeutic compounds might elicit adverse affects. In clinical trials, however, there were no major adverse effects detected after MF101 treatment (data not shown). Active compounds can also be synthesized and quantified, allowing for the administration of known amounts and higher doses of the active drug. In this study, we isolated the ERβ-active compound, liquiritigenin, from *G. uralensis*, one of the herbs present in MF101. We further detailed the binding and transcriptional activity of this purified compound through the ER.

Liquiritigenin induced only ERβ-specific pathways in transfection assays. Liquiritigenin activated ERE-tk-luciferase, as well as three native ER regulatory elements (NKG2E, CECR6, and NKD) in cells transfected with ERβ but not with ERα. The ERβ-selectivity was also observed with the native *NKG2E, CECR6,* and *NKD* genes since these genes were activate activated in the U2OS-ERβ cells, but not in the U2OS-ERα cells. The mechanism for the ERβ-selectivity is unlikely related to differences in the binding to ERα and ERβ, because ERβ only has a 20-fold higher affinity for liquiritigenin compared to ERα. Our ChIP studies showed that liquiritigenin recruits SRC-2 to the *NKG2E, CECR6,* and *NKD* genes only in

U2OS-ERβ cells. These findings suggest that the selectivity of liquiritigenin is due to the differential recruitment of coactivators to ERβ.

The major problem with HT is not a lack of efficacy, but rather its proliferative effects on breast cancer cells. Therefore, it is essential to rule out a proliferative action for any alternative drug for HT. In a mouse xenograft model, liquiritigenin did not stimulate breast cancer tumor formation after 30 days of treatment, as compared to therapeutic doses of E_2, which caused the formation of large tumors. In addition, unlike E_2, liquiritigenin did not increase the size of the uterus. The lack of stimulation of breast and uterine cells by liquiritigenin is consistent with the findings that the synthetic ERβ-selective drug, ERB-041 also does not elicit any proliferative effects on mammary and uterine tissue in rats [40]. The data with liquiritigenin, MF101 and ERB-041, as well as the findings that ERβ acts as a tumor suppressor in breast cancer cells [28,29], indicate that ERβ-selective agonists might have a safer profile than the estrogens currently used in HT that activate both ERα and ERβ.

While plants are known to contain many estrogenic compounds [24], their selectivity for the ER subtypes remains largely unstudied. The isoflavone genistein binds better to ERβ than ERα [38], and exhibits ERβ-selectivity in transfection studies [39]. Liquiritigenin was more selective than genistein in our studies. Genistein at 1 μM produced a large activation of ERE-tkLuc [39] and activated numerous genes in U2OS-ERα cells (data not shown). In contrast, liquiritigenin did not activate multiple ER regulatory elements or endogenous genes at the same concentration (data not shown). A related compound, isoliquiritigenin has been shown to activate ERα in MCF-7 cells [41]. We found that isoliquiritigenin is a non-selective agonist that activates both ERα and ERβ transcriptional pathways (data not shown). The structural differences between liquiritigenin, genistein, and isoliquiritigenin that result in higher ERβ selectivity for liquiritigenin are currently being investigated.

We previous showed that the crude botanical mixture MF101 acted as a selective ERβ-agonist by inducing a functional conformational change in the ERβ receptor that causes the recruitment of coactivators [25]. Here we identified liquiritigenin as a major ERβ-selective compound from one of the plants in MF101. These studies suggest that liquiritigenin might be a viable drug candidate to selectively activate ERβ in humans. However, the major question that remains to be answered is whether ERβ-selective estrogens, like liquirtigenin, will be effective at treating menopausal symptoms. In a rat hot flash model ERB-041 did not show any efficacy, suggesting that ERβ-selective compounds might not be useful for treating hot flashes [40]. However, it is possible that other ERβ-selective ligands, such as liquirtigenin will exert different biological effects than ERB-041. Whereas our studies with the U2OS osteosarcoma cells indicate that liquirtigenin regulates genes in bone cells it is unclear if they will increase bone mineral density. Future clinical trials with liquiritigenin are required to determine if it is effective at treating hot flashes, other menopausal symptoms or osteoporosis. While the precise effects that are mediated by ERβ are unclear and the indications of ERβ-selective ligands need to be determined, our study indicates that plants are a source of highly selective ERβ compounds.

Acknowledgements

We thank Pierre Chambon and Jan-Åke Gustafsson for providing plasmids and Roanna Medina for technical assistance. This work was supported by a grant from the National Center for Complementary and Alternative Medicine (AT002173).

Abbreviations

HT

hormone therapy

TCM

traditional Chinese medicine

WHI
Women's Health Initiative

BDS
botanical dietary supplements

E_2
estradiol

ER
estrogen receptor

ERE
estrogen response element

SRC-2
glucocorticoid interacting protein 1

ChIP
chromatin immunoprecipitation

NKG2E
killer cell lectin-like receptor

CECR6
cat eye syndrome chromosome region candidate

NKD
naked cuticule homolog

RT-PCR
real-time polymerase chain reaction

SRC-2
steroid receptor coactivator-2

References

1. Wassertheil-Smoller S, Hendrix SL, Limacher M, Heiss G, Kooperberg C, Baird A, Kotchen T, Curb JD, Black H, Rossouw JE, Aragaki A, Safford M, Stein E, Laowattana S, Mysiw WJ. Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. Jama 2003;289:2673–84. [PubMed: 12771114]
2. Chlebowski RT, Hendrix SL, Langer RD, Stefanick ML, Gass M, Lane D, Rodabough RJ, Gilligan MA, Cyr MG, Thomson CA, Khandekar J, Petrovitch H, McTiernan A. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. Jama 2003;289:3243–53. [PubMed: 12824205]
3. Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones BN 3rd, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. Jama 2003;289:2651–62. [PubMed: 12771112]
4. Manson JE, Hsia J, Johnson KC, Rossouw JE, Assaf AR, Lasser NL, Trevisan M, Black HR, Heckbert SR, Detrano R, Strickland OL, Wong ND, Crouse JR, Stein E, Cushman M. Estrogen plus progestin and the risk of coronary heart disease. N Engl J Med 2003;349:523–34. [PubMed: 12904517]

5. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. Jama 2002;288:321–33. [PubMed: 12117397]

6. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hsia J, Hubbell A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R, Wassertheil-Smoller S. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. Jama 2004;291:1701–12. [PubMed: 15082697]

7. Shumaker SA, Legault C, Kuller L, Rapp SR, Thal L, Lane DS, Fillit H, Stefanick ML, Hendrix SL, Lewis CE, Masaki K, Coker LH. Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women: Women's Health Initiative Memory Study. Jama 2004;291:2947–58. [PubMed: 15213206]

8. Rossouw JE, Prentice RL, Manson JE, Wu L, Barad D, Barnabei VM, Ko M, LaCroix AZ, Margolis KL, Stefanick ML. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. Jama 2007;297:1465–77. [PubMed: 17405972]

9. Delmas P, Bjarnason N, Mitlak B, Ravoux AC, Shah A, Huster W, Draper M, Christiansen C. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med 1997;337:1641–1647. [PubMed: 9385122]

10. Love R, Mazess R, Barden H, Epstein S, Newcomb P, Jordan V, Carbone P, DeMets D. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992;326:852–6. [PubMed: 1542321]

11. Cranney A, Adachi JD. Benefit-risk assessment of raloxifene in postmenopausal osteoporosis. Drug Saf 2005;28:721–30. [PubMed: 16048357]

12. Loprinzi L, Barton DL, Sloan JA, Zahasky KM, Smith de AR, Pruthi S, Novotny PJ, Perez EA, Christensen BJ. Pilot evaluation of gabapentin for treating hot flashes. Mayo Clin Proc 2002;77:1159–63. [PubMed: 12440550]

13. Evans ML, Pritts E, Vittinghoff E, McClish K, Morgan KS, Jaffe RB. Management of postmenopausal hot flushes with venlafaxine hydrochloride: a randomized, controlled trial. Obstet Gynecol 2005;105:161–6. [PubMed: 15625158]

14. Sicat BL, Brokaw DK. Nonhormonal alternatives for the treatment of hot flashes. Pharmacotherapy 2004;24:79–93. [PubMed: 14740790]

15. Loprinzi CL, Kugler JW, Sloan JA, Mailliard JA, LaVasseur BI, Barton DL, Novotny PJ, Dakhil SR, Rodger K, Rummans TA, Christensen BJ. Venlafaxine in management of hot flashes in survivors of breast cancer: a randomised controlled trial. Lancet 2000;356:2059–63. [PubMed: 11145492]

16. Upchurch DM, Chyu L, Greendale GA, Utts J, Bair YA, Zhang G, Gold EB. Complementary and alternative medicine use among American women: findings from The National Health Interview Survey, 2002. J Womens Health (Larchmt) 2007;16:102–13. [PubMed: 17324101]

17. Petri Nahas E, Nahas Neto J, De Luca L, Traiman P, Pontes A, Dalben I. Benefits of soy germ isoflavones in postmenopausal women with contraindication for conventional hormone replacement therapy. Maturitas 2004;48:372–80. [PubMed: 15283929]

18. Uesugi S, Watanabe S, Ishiwata N, Uehara M, Ouchi K. Effects of isoflavone supplements on bone metabolic markers and climacteric symptoms in Japanese women. Biofactors 2004;22:221–8. [PubMed: 15630287]

19. Williamson-Hughes PS, Flickinger BD, Messina MJ, Empie MW. Isoflavone supplements containing predominantly genistein reduce hot flash symptoms: a critical review of published studies. Menopause 2006;13:831–9. [PubMed: 16932241]

20. Nelson HD, Vesco KK, Haney E, Fu R, Nedrow A, Miller J, Nicolaidis C, Walker M, Humphrey L. Nonhormonal therapies for menopausal hot flashes: systematic review and meta-analysis. Jama 2006;295:2057–71. [PubMed: 16670414]

21. Somjen D, Yoles I. DT56a (Tofupill/Femarelle) selectively stimulates creatine kinase specific activity in skeletal tissues of rats but not in the uterus. J Steroid Biochem Mol Biol 2003;86:93–8. [PubMed: 12943748]
22. Yoles I, Yogev Y, Frenkel Y, Nahum R, Hirsch M, Kaplan B. Tofupill/Femarelle (DT56a): a new phyto-selective estrogen receptor modulator-like substance for the treatment of postmenopausal bone loss. Menopause 2003;10:522–5. [PubMed: 14627860]
23. Somjen D, Katzburg S, Knoll E, Hendel D, Stern N, Kaye AM, Yoles I. DT56a (Femarelle): a natural selective estrogen receptor modulator (SERM). J Steroid Biochem Mol Biol 2007;104:252–8. [PubMed: 17428655]
24. Oerter Klein K, Janfaza M, Wong JA, Chang RJ. Estrogen bioactivity in fo-ti and other herbs used for their estrogen-like effects as determined by a recombinant cell bioassay. J Clin Endocrinol Metab 2003;88:4077–9. [PubMed: 12970265]
25. Cvoro A, Paruthiyil S, Jones JO, Tzagarakis-Foster C, Clegg NJ, Tatomer D, Medina RT, Tagliaferri M, Schaufele F, Scanlan TS, Diamond MI, Cohen I, Leitman DC. Selective activation of estrogen receptor-beta transcriptional pathways by an herbal extract. Endocrinology 2007;148:538–47. [PubMed: 17095596]
26. Hewitt SC, Harrell JC, Korach KS. Lessons in estrogen biology from knockout and transgenic animals. Annu Rev Physiol 2005;67:285–308. [PubMed: 15709960]
27. Kian Tee M, Rogatsky I, Tzagarakis-Foster C, Cvoro A, An J, Christy RJ, Yamamoto KR, Leitman DC. Estradiol and Selective Estrogen Receptor Modulators Differentially Regulate Target Genes with Estrogen Receptors {alpha} and {beta}. Mol Biol Cell 2004;15:1262–1272. [PubMed: 14699072]
28. Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res 2004;64:423–8. [PubMed: 14729654]
29. Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci U S A 2004;101:1566–71. [PubMed: 14745018]
30. Fu B, Li H, Wang X, Lee FS, Cui S. Isolation and identification of flavonoids in licorice and a study of their inhibitory effects on tyrosinase. J Agric Food Chem 2005;53:7408–14. [PubMed: 16159166]
31. Ricke WA, Ishii K, Ricke EA, Simko J, Wang Y, Hayward SW, Cunha GR. Steroid hormones stimulate human prostate cancer progression and metastasis. Int J Cancer 2006;118:2123–31. [PubMed: 16331600]
32. An J, Ribeiro RC, Webb P, Gustafsson JA, Kushner PJ, Baxter JD, Leitman DC. Estradiol repression of tumor necrosis factor-alpha transcription requires estrogen receptor activation function-2 and is enhanced by coactivators. Proc Natl Acad Sci U S A 1999;96:15161–6. [PubMed: 10611355]
33. Cvoro A, Tzagarakis-Foster C, Tatomer D, Paruthiyil S, Fox MS, Leitman DC. Distinct Roles of Unliganded and Liganded Estrogen Receptors in Transcriptional Repression. Mol Cell 2006;21:555–564. [PubMed: 16483936]
34. Parmar H, Young P, Emerman JT, Neve RM, Dairkee S, Cunha GR. A novel method for growing human breast epithelium in vivo using mouse and human mammary fibroblasts. Endocrinology 2002;143:4886–96. [PubMed: 12446616]
35. Levy N, Zhao X, Tang H, Jaffe RB, Speed TP, Leitman DC. Multiple Transcription Factor Elements Collaborate with ER{alpha} to Activate an Inducible Estrogen Response Element in the NKG2E gene. Endocrinology. 2007
36. Levy N, Tatomer D, Herber CB, Zhao X, Tang H, Sargeant T, Ball LJ, Summers J, Speed TP, Leitman DC. Differential regulation of Native Estrogen Receptor Regulatory Elements by Estradiol, Tamoxifen, and Raloxifene. Mol Endocrinol. 2007In Press
37. Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S. Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol Pharmacol 1998;54:105–12. [PubMed: 9658195]
38. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139:4252–63. [PubMed: 9751507]

39. An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC. Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J Biol Chem 2001;276:17808–14. [PubMed: 11279159]

40. Harris HA, Albert LM, Leathurby Y, Malamas MS, Mewshaw RE, Miller CP, Kharode YP, Marzolf J, Komm BS, Winneker RC, Frail DE, Henderson RA, Zhu Y, Keith JC Jr. Evaluation of an estrogen receptor-beta agonist in animal models of human disease. Endocrinology 2003;144:4241–9. [PubMed: 14500559]

41. Maggiolini M, Statti G, Vivacqua A, Gabriele S, Rago V, Loizzo M, Menichini F, Amdo S. Estrogenic and antiproliferative activities of isoliquiritigenin in MCF7 breast cancer cells. J Steroid Biochem Mol Biol 2002;82:315–22. [PubMed: 12589938]

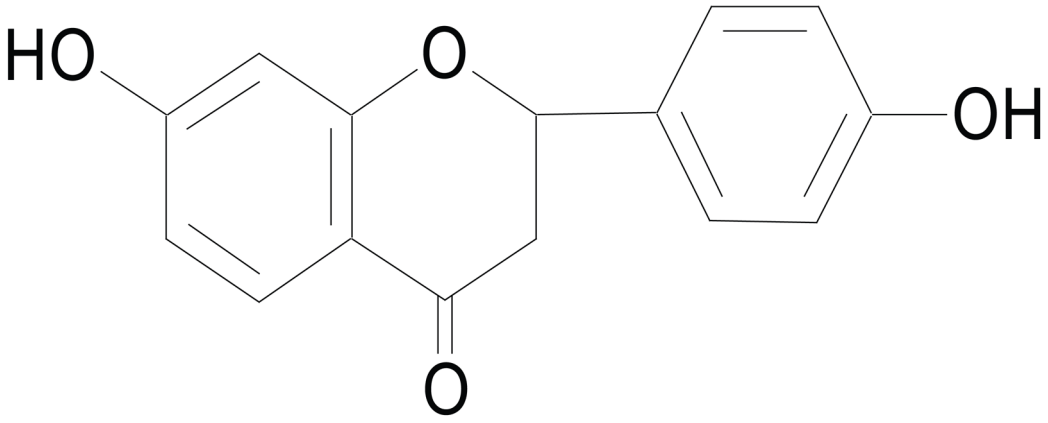

Fig. 1.
Structure of liquiritigenin. The active compound was isolated from dry, powdered *G. uralensis* and identified by NMR.

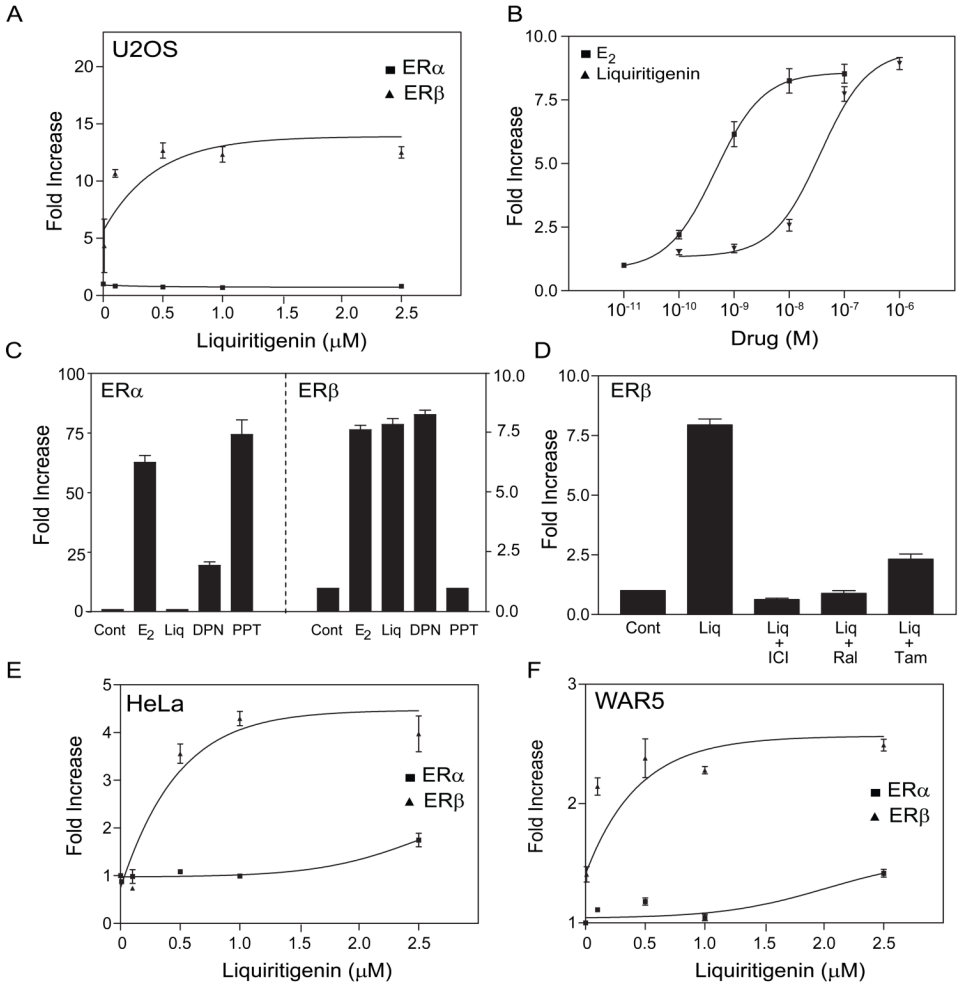

Fig. 2.

Liquiritigenin selectively activates transcription through ERβ. (A) ERE tk-Luc was cotransfected into U2OS cells with expression vectors for ERα or ERβ. After transfection, the cells were treated for 18 h with liquiritigenin and luciferase activity was measured. Dose-response curves for E_2 and liquiritigenin in U2OS cells. Following transfection the cells were treated with increasing amounts of E_2 or liquiritigenin for 18 h (B). ERE tk-Luc was cotransfected into U2OS cells with expression vectors for ERα (left panel) or ERβ (right panel) and then treated with 10 nM E_2, 1 μM liquiritigenin, 1 μM DPN or 1 μM PPT for 18 h and then luciferase was measured (C). The activation by liquiritigenin is blocked by anti-estrogens. (D) ERE tk-Luc was cotransfected into U2OS cells with an expression vector ERβ and the cells were treated with 1 μM liquiritigenin in the absence or presence of 1 μM ICI 182780 (ICI), raloxifene (Ral) or tamoxifen (Tam). Liquiritigenin selectively activated the ERE tk-Luc with ERβ in HeLa cervical (E) and WAR5 prostate cancer (F) cell lines. Each data point is the average of triplicate determinations ± S.E.M. An activation by the drug was significant ($p < 0.05$) when it was 2-fold greater than the control values.

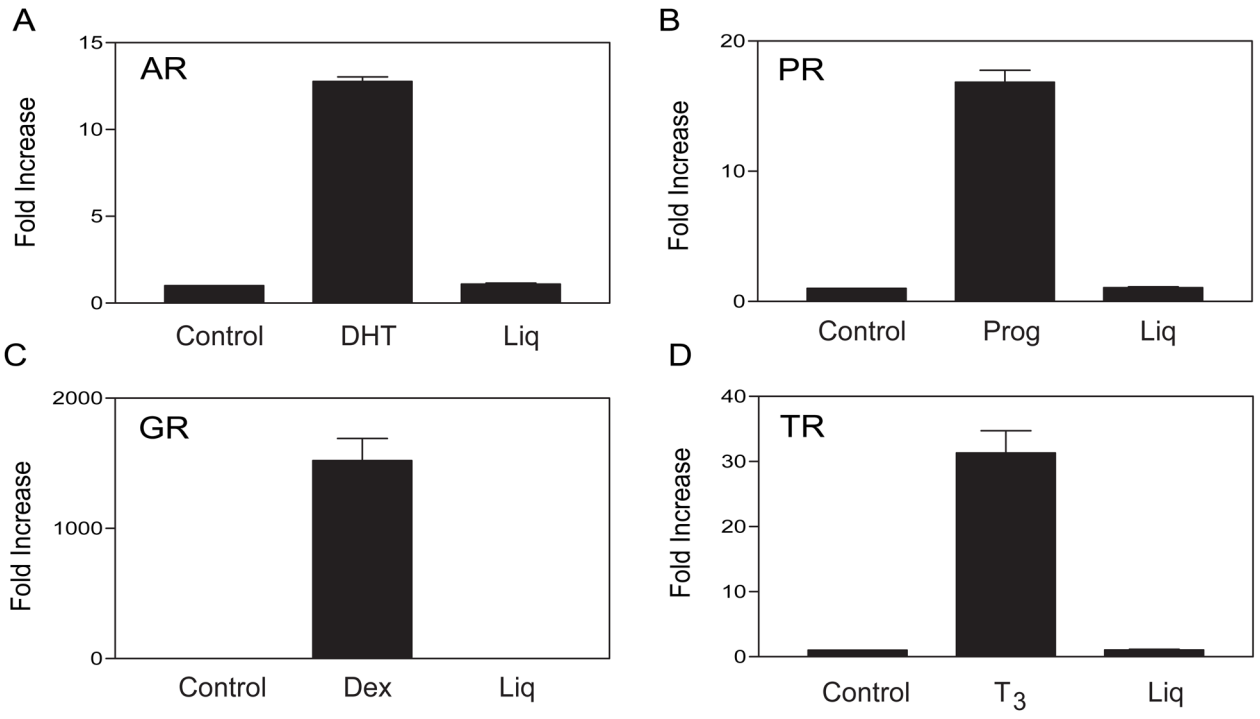

Fig. 3.

The activation by liquiritigenin is selective for the estrogen receptor. U2OS were transfected with TAT3-luciferase and androgen receptor (AR) (A), MMTV-luciferase and glucocorticoid receptor (GR) (B), TAT3-luciferase and progesterone receptor B (PR) (C), or F2 tk-Luc and thyroid hormone receptor β1 (TR) (D). The cells were treated for 18 h with 1 nM dihydrotestosterone (DHT), or 1 nM dexamethasone (Dex), or 1 nM progesterone (Prog), or 10 nM triiodothyronine (T_3) (A, B, C, D, respectively) or 2.5 μM liquiritigenin (Liq). Each data point is the average of triplicate determinations ± S.E.M. An activation by the drug was significant ($p < 0.05$) when it was 2-fold greater than the control values.

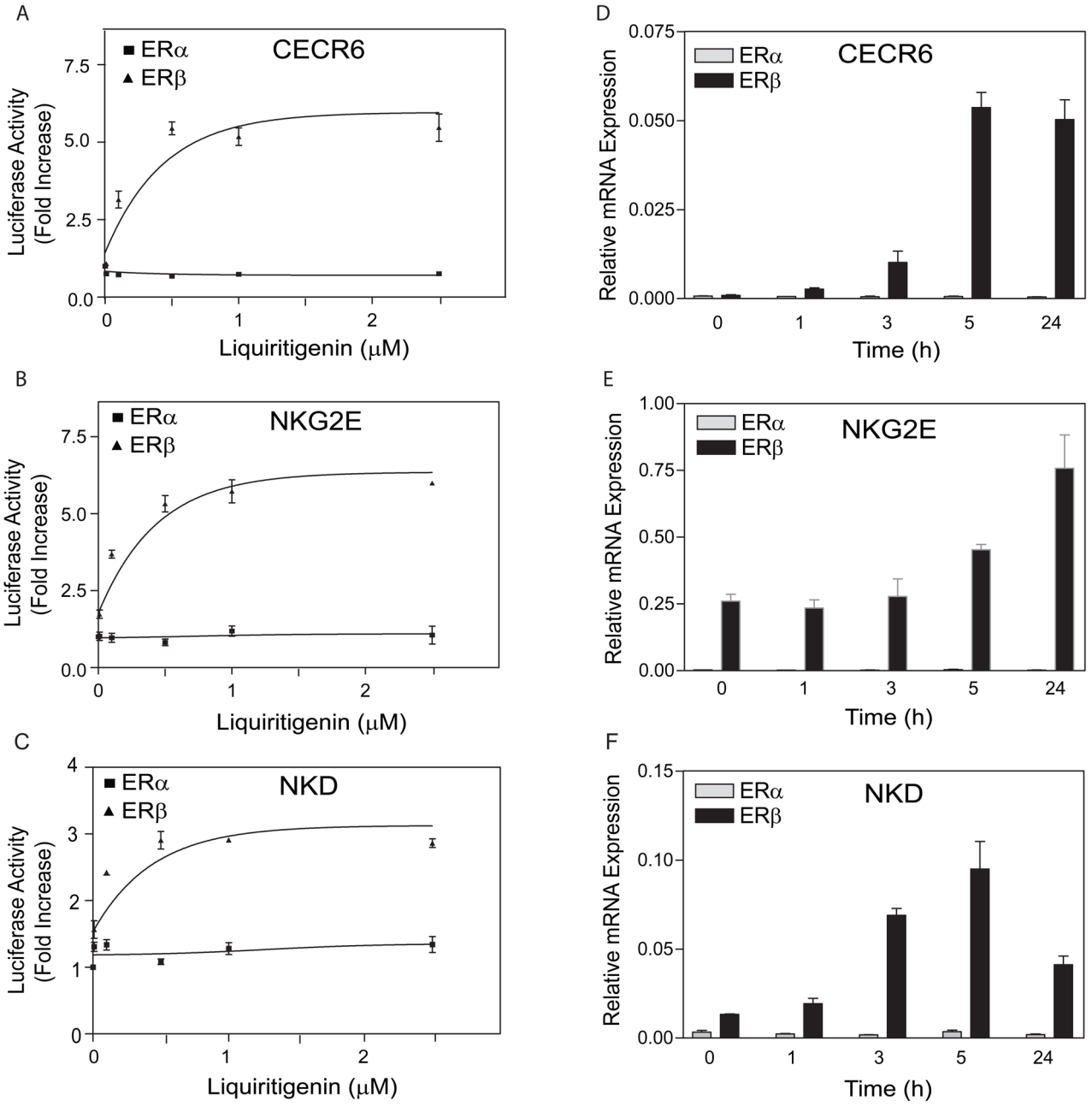

Fig. 4.

Liquiritigenin selectively activates transcription of the native ER regulatory elements and genes through ERβ. CECR6 tk-Luc (A), NKG2E tk-Luc (B), and NKD tk-Luc (C) were cotransfected into U2OS cells with expression vectors for human ERα or ERβ. After transfection, the cells were treated for 18 h with increasing amounts of liquiritigenin and luciferase activity was measured. U2OS cells stably transfected with tetracycline inducible ERβ or ERα were treated with 1 μg/ml doxycycline for 18 h to induce ER expression. The cells were then treated for increasing times with liquiritigenin. The level of CECR6 (D), NKG2E (E), and NKD (F) mRNA was measured by real-time PCR. Each data point is the average of triplicate determinations ± S.E.M. An activation by the drug was significant ($p < 0.05$) when it was 2-fold greater than the control values.

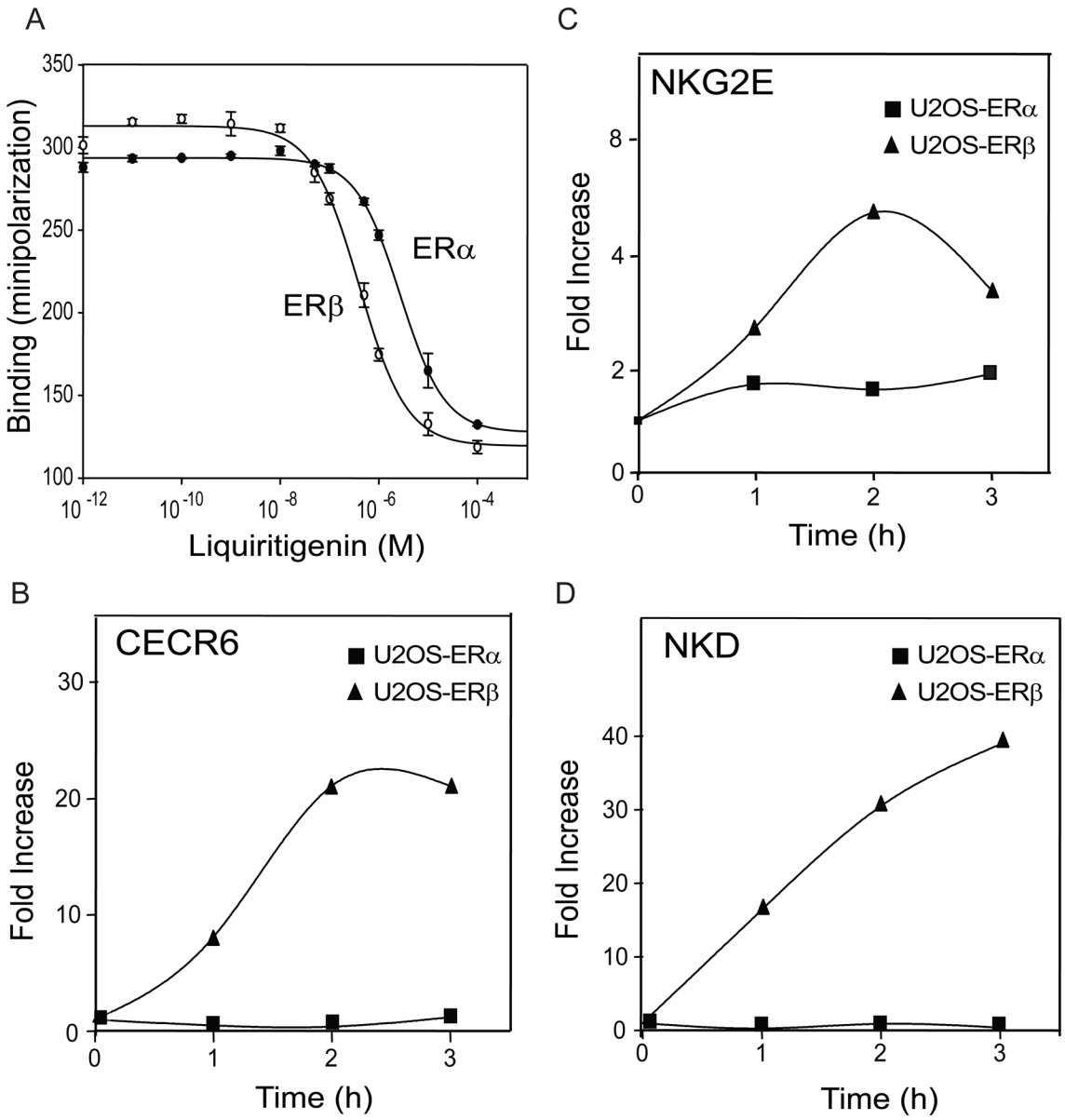

Fig. 5.

Liquiritigenin selectively recruits SRC-2 to the ER target genes. (A) Purified ERα or ERβ were incubated with fluorescent E_2 in the absence or presence of increasing amounts of liquiritigenin. U2OS-ERβ or U2OS-ERα cells were treated with liquiritigenin for increasing times and ChIP was performed using antibodies to SRC-2. Real-time PCR was performed to amplify the ER regulatory element in the *CECR6* (B), *NKG2E* (C), and *NKD* (D) genes.

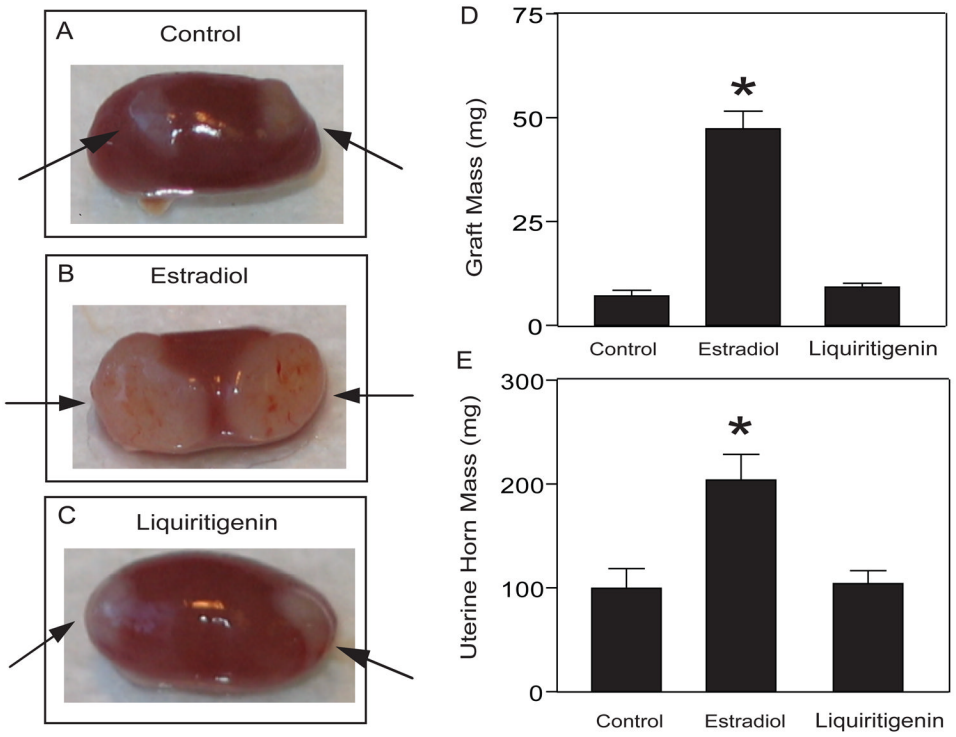

Fig. 6.

Liquiritigenin does not stimulate tumor formation of MCF-7 breast cancer cells or cause uterine growth in a *mouse xenograft model*. MCF-7 cells were grafted under the kidney capsule of intact female nude mice. Mice were continuously infused with vehicle (Control), E_2 (0.4 mg) or liquiritigenin (2 mg) using a subcutaneous osmotic pump. After one month, the tumors and uterus were removed and analyzed for size and weight. Gross morphology of the xenografts in control (A), E_2 (B) and liquiritigenin (C) treated mice. The arrow points to the site of grafting. Average weights ± S.E.M of tumor grafts (D) and uterine horns (E) from 5 mice in each group. * indicates a $p<0.05$ difference between control and drug treatment using the student's t-test.