Selective Activation of Estrogen Receptor-β Transcriptional Pathways by an Herbal Extract

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Novel estrogenic therapies are needed that ameliorate menopausal symptoms and have the bone-sparing effects of endogenous estrogens but do not promote breast or uterine cancer. Recent evidence suggests that selective activation of the estrogen receptor (ER)-β subtype inhibits breast cancer cell proliferation. To establish whether ERβ-selective ligands represent a viable approach to improve hormone therapy, we investigated whether the estrogenic activities present in an herbal extract, MF101, used to treat hot flashes, are ERβ selective. MF101 promoted ERβ, but not ERα, activation of an estrogen response element upstream of the luciferase reporter gene. MF101 also selectively regulates transcription of endogenous genes through ERβ. The ERβ selectivity was not due to differential binding because MF101 binds equally to ERα and ERβ. Fluorescence resonance energy transfer and protease digestion studies showed that MF101 produces a different conformation in ERα from ERβ when compared with the conformations produced by estradiol. The specific conformational change induced by MF101 allows ERβ to bind to an estrogen response element and recruit coregulatory proteins that are required for gene activation. MF101 did not activate the ERα-regulated proliferative genes, c-myc and cyclin D1, or stimulate MCF-7 breast cancer cell proliferation or tumor formation in a mouse xenograft model. Our results demonstrate that herbal ERβ-selective estrogens may be a safer alternative for hormone therapy than estrogens that nonselectively activate both ER subtypes. (Endocrinology 148: 538–547, 2007)

Menopause is associated with the onset of hot flashes, night sweats, mood changes, and urogenital atrophy, which many women find distressing enough to seek medical management for relief. Estrogens in the form of hormone therapy (HT) have been the standard treatment for menopausal symptoms for decades. Although HT is the most effective treatment for hot flashes, the Women’s Health Initiative (WHI) trial found that the combination of estrogen and progesterin increases a woman’s risk for heart disease, stroke, breast cancer, venous thromboembolic events, and dementia (1–5) and does not improve quality of life indices such as emotional and sexual functioning and vitality (6, 7). In a second arm of the WHI study, estrogen only failed to demonstrate any cardiovascular benefit in older women and was found to increase the risk of stroke, venous thromboembolism, and dementia (8, 9).

The adverse effects of HT have caused considerable concern among postmenopausal women, and many of them reluctantly stopped taking estrogens despite the lack of effective alternatives to treat hot flashes (10). The WHI findings created a large unmet need for effective alternatives to HT for menopausal symptoms. Selective estrogen receptor modulators (SERMs) have been introduced as an alternative to estrogens (7). The SERMs, raloxifene and tamoxifen, enhance bone mineral density (11, 12), and raloxifene is an approved drug for osteoporosis prevention (13). Unlike estrogens, raloxifene and tamoxifen decrease the incidence of breast cancer (14, 15). Despite these important effects, raloxifene and tamoxifen increase the incidence of hot flashes (16).

Thus, the only effective estrogens for hot flashes are those that cause cancer.

The discovery of safer estrogens for HT requires a greater understanding of the role of estrogen receptor (ER) subtypes in causing clinical effects. Estrogen signaling pathways are mediated by two ERs, ERα and ERβ (17, 18). Whereas the exact physiological roles of the two ER subtypes remain unknown, it is clear that ERα and ERβ have different biological roles. The ERα and ERβ knockout mice exhibit different phenotypes (19) and the genes regulated by estradiol and SERMs with ERα are distinct from those regulated by ERβ (20). These studies suggest that drugs targeted selectively to only ERα or ERβ will produce more selective clinical effects, rather than the global effects elicited by estrogens used in current HT regimens that regulate both ER subtypes. Based on the observation that ERα promotes proliferation of...
breast cancer cells, whereas ERβ acts as tumor suppressor and that both forms are effective in transcriptional repression of inflammatory genes responsible for osteoporosis (21), we hypothesize that ERβ-selective agonists might be a safer alternative for long-term HT.

Our finding that soy contains phytoestrogens that selectively trigger ERβ transcriptional pathways (22) suggests that other botanical products might be a source of ERβ agonists. We have been investigating the effects of a botanical extract, MF101, on hot flashes in postmenopausal women. Preliminary findings in a phase 1 trial with 22 postmenopausal women found that MF101 reduced hot flashes and did not produce any adverse effects (data not shown). MF101 is approved by the Food and Drug Administration (FDA) for an ongoing phase 2, randomized, placebo-controlled trial for the treatment of hot flashes (http://clinicaltrials.gov/show/NCT00196655). MF101 is composed of 22 individual plant species. The entire formula and its individual herbs were selected based on the known pharmacology of the herbs and traditional therapeutic uses in Chinese medicine for the treatment of vasomotor symptoms. An attempt was also made to select herbs that do not promote cancer while simultaneously treating symptoms. We used a molecular approach to determine whether MF101 in its native mode of administration has selective ER activity as a scientific basis for the mechanism of action that can be potentially translated clinically to prevent hot flashes. In this study, we demonstrate that MF101 is a selective ERβ agonist on gene regulation and does not stimulate breast cancer cell proliferation or uterine growth. These results suggest that ERβ-selective estrogens might be safer than current estrogens in HT that activate both ERα and ERβ.
under the kidney capsule of intact nude mouse as described and illustrated in detail on the Web site (http://mammary.nih.gov/tools/mouse-work/Cunha001/index.html). Animals were untreated (control), treated with a sc diethylstilbestrol (DES) pellet (2 mg) or 0.5 ml (25 mg/dose) MF101 every other day by oral gavage. Tumors were analyzed 1 month after grafting. The animal studies were carried out with approval from the University of California, San Francisco, Committee on Animal Research.

Real-time RT-PCR

Total RNA was isolated using Trizol (Invitrogen Life Technologies) and reverse transcription reactions were performed using iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative PCR was performed using SYBR Green Supermix with an iCycler thermal cycler (Bio-Rad). We used the following primers: c-myc forward 5'-GCCCTCAACGT- TACCTICA-3'; reverse 5'-TTCCAGATATCCTCCTGGA-3'; cyclin D1 forward 5'-AAGTTAAGGAAGTGAGGGC-3'; reverse 5'-GGGCACTGG- TAGCCTGTTCAACA-3'; Gus forward 5'-CTCATTTGGAATATTTCG- CGATT-3'; reverse 5'-CCGAGTGAAGATCCCCTTTTTA-3'; keratin 19 forward 5'-CCAGCTGACGTGGGAGGTGG-3'; reverse 5'-TGGCCT- TCCGATGTCACCTACA-3'; TNFa forward 5'-GGATGACAGCTG- TAGCCGGATTG-3'; reverse 5'-GAATACTGGACCGCTTCCT-3'; keratin 19 forward 5'-CTTCTGCGATGCTCCCTTTT-3'; and β-actin forward 5'-AGCCTCAGGTCAGTGTGGAGGTGG-3'; reverse 5'-AACTACCTGGACCGCTTCCT-3'.

The data were collected and analyzed using the comparative cycle threshold method using Gus or β-actin expression as the reference gene.

Fluorescence resonance energy transfer (FRET) analysis

A day before transfection, HEK293 cells (n = 200,000) were plated into each well of a six-well dish and grown in DMEM-H21 supplemented with 5% charcoal-stripped fetal calf serum. CFP-Erα-YFP (24) or CFP-ERβ-YFP (500 ng/well) was transfected into cells using Lipofectamine Plus (Invitrogen). The day after transfection, 100,000 cells/well were replated in black, clear-bottomed, 96-well plates (Costar, Cambridge, MA) in the presence or absence of 10 nM E2 or MF101. Cells were fixed in 4% paraformaldehyde in PBS before reading on the fluorescence plate reader. For FRET detection on the fluorescence plate reader (Safire; Tecan, Durham, NC), measurements were taken from the bottom of the plate with the following settings: yellow fluorescent protein (YFP), excitation at 485 nm/emission at 527 nm; cyan fluorescent protein (CFP), excitation at 435 nm/emission at 485 nm; and FRET, excitation at 435 nm/emission at 527 nm. Each plate contained an untransfected cell control (background), and each data point was collected in quadruplicate. FRET to donor ratios were calculated after background subtraction and correction for acceptor (YFP) contribution into the FRET spectrum.

Results

The herbal extract, MF101, selectively activates transcription with ERβ

The individual herbs and the formulation of MF101 are shown in supplemental Table 1. We initially examined the relative contributions of ERα and ERβ to MF101 activity in standard luciferase (Luc) reporter assays. U2OS osteosarcoma cells were cotransfected with a classical ERE upstream of a minimal thymidine kinase (tk) promoter (ERE-tk-Luc) and expression vectors for human ERα or ERβ. MF101 produced a dose-dependent activation of ERE-tk-Luc with ERβ but not with ERα (Fig. 1A). One hundred twenty-five micrograms of MF101 caused the activation equivalent to 10 nM E2 (Fig. 1B). The ER antagonists, ICI 182,780, raloxifene, and tamoxifen, blocked the activation by MF101 (Fig. 1B), indicating that the effect of MF101 is mediated through ERβ. MF101 also did not activate ERE-tk-Luc with ERα in other cell types, including HeLa, MDA-MB-453, and Ishikawa cells (Fig. 1C). Similar to U2OS cells, MF101 activated ERE-tk-Luc with ERβ to the same magnitude as E2 in these three cell lines (Fig. 1D). The ER-subtype selectivity was examined in U2OS cells stably transfected with a tetracycline-inducible ERα or ERβ (20) using the keratin 19 gene, which contains an ERE (25). E2 activated the keratin 19 gene in both U2OS-ERα (Fig. 1E) and U2OS-ERβ cells (Fig. 1F), whereas MF101 produced a dose-dependent increase in keratin 19 mRNA only in the U2OS-ERβ cells.

Estrogens possess antiinflammatory properties by repressing the expression of inflammatory genes (21). The repression of the TNFα or IL-6 genes might be an important mechanism by which estrogens prevent inflammatory conditions, such as osteoporosis (27). To investigate whether MF101 represses the expression of the TNFα and IL-6 genes, the tetracycline-inducible ERα or ERβ cells were treated with E2 or MF101. Because the basal expression of these genes is very low, it is necessary to activate these genes with TNFα to observe repression. TNFα produced a large increase in TNFα and IL-6 mRNA (Fig. 2, A–D), which was inhibited by E2 in both the U2OS-ERα (Fig. 2, A and B) and U2OS-ERβ (Fig. 2, C and D) cells. MF101 repressed the TNFα activation of the TNFα and IL-6 genes in the U2OS-ERβ cells (Fig. 2, C and D) but not in the U2OS-ERα cells (Fig. 2, A and B) cells. These studies demonstrate that MF101 selectively triggers ERβ-mediated transcriptional pathways.

MF101 binds equally to ERα and ERβ and induces conformational changes distinct from E2

Phytoestrogens found in soybeans, such as genistein, bind to ERβ with a 7- to 30-fold higher affinity, compared with ERα (28, 29). These data suggest that MF101 may act as an ERβ-selective agonist by virtue of a higher binding affinity to ERβ. However, competition binding curves show that MF101 binds equally to ERα and ERβ (Fig. 3A). The findings that MF101 binds to ERα but does not stimulate it to activate genes suggest that MF101 induces a functional conformation only with ERβ. Schaufele et al. (24) demonstrated that FRET could be used to discriminate conformational changes in the androgen receptor and ERα that correlate with ligand binding. The FRET signal is derived from intramolecular conformational changes that alter the orientation of the N and C termini of the fluorescent tags, bringing the donor (CFP) and the acceptor (YFP) in close proximity.

Conformational changes induced by MF101 in ERα and ERβ were examined by FRET in HEK293 cells transfected with vectors containing the cDNA for ERα or ERβ fused between CFP and YFP to create the chimeric proteins, CFP-ERα-YFP and CFP-ERβ-YFP. HEK293 cells were used because high-transfection efficiency is required for FRET analysis. After cells were transfected, they were treated with E2 or MF101 and FRET/donor was measured (see Materials and Methods). A slightly higher basal FRET/donor was observed with CFP-ERβ-YFP, compared with CFP-ERα-YFP (Fig. 3, B and C), which is probably due to differences in basal conformation. An increase in FRET/donor was observed with both ERα and ERβ at similar doses of E2 (Fig. 3B) and MF101 (Fig. 3C). These results provide additional evidence that
MF101 binds equally to ERα and ERβ. Because ERα and ERβ have different primary structures, we compared the conformational responses produced by MF101 vs. E2. Compared with the 10 nM E2 response, MF101 increased FRET:donor by 42% with ERα, whereas the same dose of MF101 increased FRET:donor by 15% with ERβ (Fig. 3D). The FRET studies indicate that MF101 brings the N and C termini of ERα into a much closer proximity than that induced by E2, which leads to the higher FRET:donor observed with ERα. These results demonstrate that MF101 produces a conformational change in ERβ that closely resembles the active conformation elicited by E2 in ERβ, whereas MF101 produces a markedly different
conformation in ER\(\alpha\), compared with the one produced by E\(\alpha\).

To further investigate whether MF101 changes the conformation of ER\(\alpha\) and ER\(\beta\) without the fluorescent tags, we performed limited proteolysis to probe the conformational features of ER\(\alpha\) and ER\(\beta\) when bound with MF101 or E\(\alpha\). Radiolabeled ER\(\alpha\) and ER\(\beta\) were synthesized in an in vitro transcription and translation system and digested with elastase for increasing times. MF101 and E\(\alpha\) produced a distinct digestion pattern of ER\(\alpha\) (Fig. 4A) and ER\(\alpha\) (Fig. 4B), compared with the control. The digestion pattern of ER\(\alpha\) was different with E\(\alpha\) and MF101 (Fig. 4A). The strongest protection is observed when ER\(\alpha\) is bound with E\(\alpha\) as demonstrated by the presence of several protected fragments (three arrows) at the highest elastase concentrations, which are less prominent in the control and MF101 samples sample. When bound with MF101, ER\(\beta\) demonstrates a slight increase in protection to elastase, compared with the control, but less than that observed with E\(\alpha\) (Fig. 4B). MF101 also produced a distinct pattern, compared with the control or E\(\alpha\). The two arrows show several protected fragments of ER\(\alpha\) with E\(\alpha\), compared with the MF101-treated ER\(\alpha\) (Fig. 4B). The FRET and protease digestion studies demonstrate that MF101 binds to ER\(\alpha\) and ER\(\beta\) and induces conformational changes in both ER subtypes.

MF101 causes the selective recruitment of ER\(\beta\) and coregulatory proteins to target genes

Our studies suggest that upon MF101 binding, ER\(\beta\) adopts a different overall conformation from ER\(\alpha\), which could prevent ER\(\alpha\) from binding to the regulatory elements or recruiting coregulatory proteins that are required for gene activation (30–32). To investigate these possibilities, we performed ChIP on the keratin 19 gene because E\(\alpha\) recruits ER to the keratin 19 ERE in both U2OS-ER\(\alpha\) and U2OS-ER\(\beta\) cells as well as RNA polymerase II and coregulatory proteins (20, 21). ChIP shows that MF101 recruited ER\(\beta\) but not ER\(\alpha\) to the keratin 19 gene (Fig. 4C). MF101 also induced recruitment of RNA polymerase II, GRIP1 and CBP to the keratin 19 gene selectively in U2OS-ER\(\beta\) cells. These results demonstrate that MF101 produces a conformation in ER\(\beta\) but not in ER\(\alpha\) that allows the MF101-ER\(\beta\) complex to bind an ERE and recruit coregulatory proteins that activate the keratin 19 gene.

MF101 does not stimulate MCF-7 cell tumor formation or uterine growth in mouse xenograft models

A critical feature of an alternative estrogen for menopausal symptoms is that it does not increase the risk for breast and uterine cancer. We investigated whether MF101 has growth-promoting properties in MCF-7 breast cancer cells, which express only ER\(\alpha\). Unlike E\(\alpha\), MF101 did not stimulate cell
proliferation of MCF-7 cells (Fig. 5A). MF101 also did not activate c-myc (Fig. 5B) or cyclin D1 (Fig. 5C) genes, which are key genes involved in breast cancer induced by E2 (33, 34).

To determine whether MF101 causes tumor formation, MCF-7 cells were grafted under the kidney capsule of nude mice. In control mice, only small tumors were formed after 1 month (Fig. 5D). In contrast, large tumors developed in mice treated with DES (Fig. 5E). At a dose comparable with the amount used to treat hot flashes in women, MF101 did not increase the size of the tumor graft (Fig. 5, F and G) or uterine weight (Fig. 5H), compared with control mice. These data demonstrate that MF101 does not promote proliferation of MCF-7 cells or uterine growth and are consistent with the hypothesis that ERα mediates the proliferative effects of E2 (22, 23, 35).

**Discussion**

Hot flashes are experienced by most menopausal women. Estrogens are the most effective and widely used therapy for hot flashes in the United States. However, the findings of the WHI (3, 8) have caused many women to stop taking HT (10) and to explore alternative therapies in search of a safer treatment for vasomotor symptoms (36). Herbal formulas, which have been used in China for centuries, might provide a platform of potential drugs to treat hot flashes. Whereas it is known that some herbs have estrogenic activity (37, 38), it is unclear whether they are effective for treating hot flashes and other clinical symptoms. Clearly, the adverse effects of HT make it worthwhile to begin to explore the potential benefits of herbs for menopausal symptoms. However, it is possible
that such estrogenic herbs may elicit the same adverse effects associated with estrogens currently used in HT, unless those herbs possess some form of selective ER action. Here we examined whether the herbal formula, MF101, primarily designed to treat hot flashes, has selective estrogen receptor activity.

We demonstrated that MF101 triggers only ERβ-mediated transcriptional pathways because it activated ERE-tk-Luc and the endogenous keratin 19 gene in U2OS-ERβ cells but not in U2OS-ERα cells. MF101 also repressed TNFα and IL-6 genes only in U2OS-ERβ cells. Whereas the magnitude of regulation was equivalent to E2, the dose of MF101 needed for ERβ activation or repression is about 50,000-fold greater than that of E2 on a weight basis. However, because MF101 is a crude extract, the dose of the estrogenic components in the mixture in comparison with E2 is not known. Surprisingly, MF101 binds equally to purified ERα and ERβ. The binding of MF101 to ERα was also demonstrated with FRET and protease digestion studies by showing that MF101 changed the conformation of ERα. However, we found that MF101 did not antagonize the activation of ERE-tk-Luc or the keratin 19 gene, even though it binds equally to ERα and ERβ.

The lack of antagonist activity is due to the much weaker binding of MF101 to ERα compared with E2 (data not shown). The pure compounds isolated from MF101 will allow us to evaluate the ERα antagonist activity in MF101. The FRET studies also demonstrated when MF101 is bound to ERβ, the overall conformation more closely resembles the active conformation of ERβ produced by E2 compared with the conformational change of ERα when MF101 vs. E2 is bound. The ChIP studies showed that, even though MF101 produces a conformational change in both ERα and ERβ, only the conformation induced in ERβ is capable of binding to an ERE and recruiting coactivator proteins. These results demonstrate that the ERβ-selectivity of MF101 results from its capacity to create a distinct conformation that allows ERβ to bind to an ERE and recruit coactivators, such as GRIP1 and CBP. The selective recruitment of coactivators to ERβ by MF101 is clinically important because ERα mediates proliferation and tumor formation of MCF-7 breast cancer cells, whereas ERβ acts as a tumor suppressor in ER-positive breast cancer cells (13, 29). The inability of MF101 to promote the interaction of ERα with regulatory elements and recruit coactivators can account for the observation that MF101 did not activate c-myc and cyclin D1 genes in MCF-7 cells or stimulate tumor formation and uterine size in a mouse xenograft model.

Whereas the activation function of ERα is associated with the proliferative effects of estrogens, the repression of inflammatory genes (21) is likely an important mechanism by which estrogens prevent osteoporosis (39) and possibly coronary heart disease in younger women (40). Estrogens repress inflammatory genes through both ERα and ERβ, but the repression is about 20 times more effective with ERβ (41).

![Figure 4](https://academic.oup.com/endo/article/148/2/538/2501313)
Our results demonstrate that ER\(\beta\)/H9252-selective drugs, such as MF101, do not activate the ER\(\beta\)/H9251-mediated proliferative pathways but trigger the antiinflammatory pathway by activating ER\(\beta\)/H9252. The repression of inflammatory genes by ER\(\beta\)/H9252, such as TNF\(\alpha\)/H9251 and IL-6, is consistent with the observations that a synthetic ER\(\beta\)/H9252-selective agonist is effective at treating several inflammatory conditions in animal models (42) and that ER\(\beta\)/H9252 is important for the protective effect of estrogens on the vascular system (43). Our findings suggest that drugs targeted to ER\(\beta\) will preserve the antiinflammatory action but will be devoid of the proliferative effects of estrogens used in HT.

Whereas the role of ER\(\beta\) in hot flashes is unknown, our findings provide proof of principle that ER\(\beta\)/H9252-selective drugs should not have the same toxicity profile as the currently available estrogens, which have been shown to increase the risk of both breast and uterine cancer. Moreover, our results are consistent with previously reported data showing that a synthetic ER\(\beta\)-selective ligand (ERB-041) did not elicit a proliferative response in the mammary gland or uterus of rats (42). Whereas these preclinical studies indicate that it is unlikely ER\(\beta\)-selective drugs will promote breast or uterine cancer, it is critical to translate our basic research findings by determining whether ER\(\beta\)/H9252-selective agonists are safe and effective at relieving hot flashes in women. Phase 1 studies indicated that MF101 was well tolerated and provided a preliminary indication that hot flashes were reduced (data not shown). However, these uncontrolled studies await confirmation in a larger clinical trial. To begin this process, we launched a multicenter, randomized, double-blind, placebo-controlled phase 2 trial in a group of 180 postmenopausal women to evaluate the efficacy of two different doses of MF101 to reduce hot flashes (http://clinicaltrials.gov/show/NCT00119665). Whereas our findings suggest that any potential benefits of MF101 on hot flashes would be mediated through ER\(\beta\), it is possible MF101 might work through ER\(\alpha\) in the brain rather than ER\(\beta\) because we have not examined the selectivity of MF101 in neurons.

We demonstrated that despite containing many different herbs, the MF101 extract is ER\(\beta\)/H9252-selective. Examining the effects of the crude MF101 on estrogenic activity has several advantages. First, it is important to study MF101 because it is currently being studied in clinical trials as a drug under an FDA investigational new drug. Second, MF101 might be more effective at preventing hot flashes and be a better drug than the individual compounds because of synergy among many compounds. Third, MF101 provides a starting point to isolate pure ER\(\beta\)/H9252-selective estrogens. Our studies have found that MF101 contains at least six different ER\(\beta\)/H9252-selective compounds by thin-layer chromatography, HPLC, and liquid chromatography/mass spectrometry (data not shown). Our results suggest that MF101 or pure ER\(\beta\)-selective agonists from MF101 might be a safer approach to manage menopausal symptoms because, unlike estrogens used currently in HT, they do not cause the ER\(\alpha\)-mediated breast cancer cell proliferation or uterine growth. Our study also provides a scientific foundation to explore whether MF101 and other ER\(\beta\)-selective agonists from herbs prevent menopausal symptoms, breast cancer, and inflammatory diseases associated with ER\(\beta\)/H9252.
ciliated with menopause, such as osteoporosis and cardiovascular disease.

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