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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 progestin 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 has 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

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Abbreviations: CBP, cAMP response element-binding protein; CFP, cyan fluorescent protein; ChIP, chromatin immunoprecipitation; DES, diethylstilbestrol; E_2, estradiol; ER, estrogen receptor; ERE, estrogen response element; FRET, fluorescence resonance energy transfer; GRIP1, glucocorticoid interacting protein 1; HT, hormone therapy; Luc, luciferase; SERM, selective estrogen receptor modulator; tk, thymidine kinase; WHI, Women's Health Initiative; YFP, yellow fluorescent protein.

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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/NCT00119665). 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.

Materials and Methods

MF101 preparation and extraction

MF101 is an ethanol/aqueous extract of the 22 herb species listed in supplemental Table 1 (published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) that was manufactured by Kaiser Pharmaceuticals, Ltd. (Taichong, Taiwan) for Bionovo, Inc. (Emeryville, CA) under U.S. FDA investigational new drug no. 58, 267. Herbs are harvested at the proper season and geographical location to ensure proper botanical identification and reduce variability in yield for pharmaceutical application. The herbs are identified by a botanist before processing. Based on traditional Chinese medical application and pharmacology, each plant part used in the extract is dried and cut for extraction. Half the amount (6 g) of the herb *Anemarrhenae asphoeloides* is extracted in 60 ml of 95% EtOH (1:10) for 72 h at room temperature. The extract and the plant material are than mixed with the rest of the 22 herbs (261 g remaining) with 2670 ml of distilled H_2O (1:10) and heated at 70–72 C for 60 min in a reverse steam vat. The extract is concentrated by vacuum drying at below freezing temperature to avoid crystallization. The concentrated extract is spray dried with corn or rice starch and ground up dry plants to form a powder. The dry soluble solids to nonsoluble solids ratio is 53.3% soluble solids to 46.7% starch and cellulose from the plants. The powder is tested for heavy metal, microbiological, and chemical contamination to comply with FDA, cGMP drug manufacturing standards. The extract is again tested with the chemical standards using HPLC to ensure their presence in the final extract. Two chemical standards, nyasol and bakuchiol, as well as the whole extract were quantified using an API-4000 MS/MS system (Sciex/Applied Biosystems, Foster City, CA) in negative multiple reaction monitoring mode. For experiments, MF101 is extracted in water. Details on the preparation (supplemental Appendix 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) and composition (supplemental Tables 1 and 2) and botanical names (supplemental Table 2) of MF101 are provided in supplemental material.

Cell culture

The MCF-7, U2OS, and HEK293 cell lines were cultured and maintained as previously described (20, 23). The U2OS cells expressing a tetracycline-inducible ERα or ERβ cDNA were prepared and maintained as previously described (20).

Transfection assays

U2OS cells were collected, transferred to a cuvette, and then electroporated with a gene pulser (Bio-Rad Laboratories, Hercules, CA) as previously described using 3 μg ERE-tk-Luc and 1 μg of ERα or ERβ expression vectors (22). Transfection of the HEK293 with CFP-ERα-YFP or CFP-ERβ-YFP was done as previously described (24).

ER binding assays

The relative binding affinity of MF101 to 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 (five readings per well; 0.1 sec integration time; entire plate read eight times; G factor = 0.91) using the Analyst AD plate-reader (LJL Biosystems, Sunnyvale, CA) with fluorescein excitation (485 nM) and emission (530 nM) filters. Each MF101 dose was performed in triplicate, and the relative error was determined by calculating the SE of three values from the mean.

Protease protection assay

^{35}S-labeled ERα and ERβ were synthesized *in vitro* with the TnT T7 quick coupled transcription/translation system (Promega Corp., Madison, WI). One microliter of *in vitro* translation product was diluted to 16 μl with 50 mM Tris-Cl (pH 7.6) containing estradiol (E_2), MF101, or 0.01% ethanol control. The samples were incubated on ice for 30 min. Varying concentrations of elastase (0, 0.065, 0.125, 0.25, 0.5, or 1 μg) were added to each sample and incubated for 5 min at room temperature. The assay was terminated with 20 μl SDS-PAGE sample buffer. The samples were heated for 10 min at 98 C and resolved with a 10% polyacrylamide gel. Gels were fixed and dried, and radiolabeled proteolytic fragments were detected by autoradiography.

Chromatin immunoprecipitation (ChIP)

After a 45-min treatment with MF101, U2OS-ERα and U2OS-ERβ cells were fixed in 1% formaldehyde solution and ChIP was done as previously described (21). Immunoprecipitations were performed overnight at 4 C with anti-ERα (1D5; Dako, Carpinteria, CA), anti-ERβ (6A12, 14C8, and 7B10, GeneTex, San Antonio, TX), anti-cAMP response element-binding protein (CBP) (A-22, Santa Cruz Biotechnology, Santa Cruz, CA), anti-glucocorticoid interacting protein 1 (GRIP1) (ab9261, Abcam, Cambridge, MA), and anti-RNA polymerase II (Santa Cruz Biotechnology) antibodies. PCR was done with keratin 19 primers, 5′-TCCAGC-CTGGGTGACAGAGC and 5′-TCCAAGTTCACCCCAACCTGA, which span the consensus estrogen response element (ERE) and half-ERE in the keratin 19 enhancer region (25).

Cell proliferation assays

MCF-7 breast cancer cells were cultured in phenol-free DMEM/F-12 media containing 4% stripped fetal bovine serum for 1 wk before treatment. Five thousand cells were plated in 24-well plates and treated with vehicle, E_2, or MF101 for 7 d. ^3H-thymidine incorporation was used to quantify DNA synthesis.

Xenograft studies in nude mice

MCF-7 (250,000) cells were aggregated in suspension and then resuspended in 25 μl neutralized collagen (26). The cells were then grafted

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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′-GCCCCTCAACGTTAGCTTCA-3′, reverse 5′-TTCCAGATATCCTCGCTGGG-3′; cyclin D1 forward 5′-AACTACCTGGACCGCTTCCT-3′, reverse 5′-CCACTTGAGCTTGTTCACCA-3′; Gus forward 5′-CTCATTTGGAATTTTGCCGATT-3′, reverse 5′-CCGAGTGAAGATCCCCTTTTTA-3′; keratin 19 forward 5′-CCAGGTCAGTGTGGAGGTGG-3′, reverse 5′-TTGGCTTCGCATGTCACTCA-3′; TNFα forward 5′-GAGTGACAAGCCTGTAGCCCATGTTG-3′, reverse 5′-GCAATGATCCCAAAGTAGACCTGCCC-3′; IL-6 forward 5′-TACCCCCAGGAGAAGATTCC-3′, reverse 5′-TTTTCTGCCAGTGCCTCTTT-3′; and β-actin forward 5′-AGCCTCGCCTTTGCCGA-3′, reverse 5′-CTGGTGCCTGGGGCG-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 E_2 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 (24).

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 E_2 (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 E_2 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). E_2 activated the *keratin 19* gene in both U2OS-ERβ (Fig. 1E) and U2OS-ERα 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 E_2 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 E_2 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 (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 E_2

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 E_2 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 E_2 (Fig. 3B) and MF101 (Fig. 3C). These results provide additional evidence that

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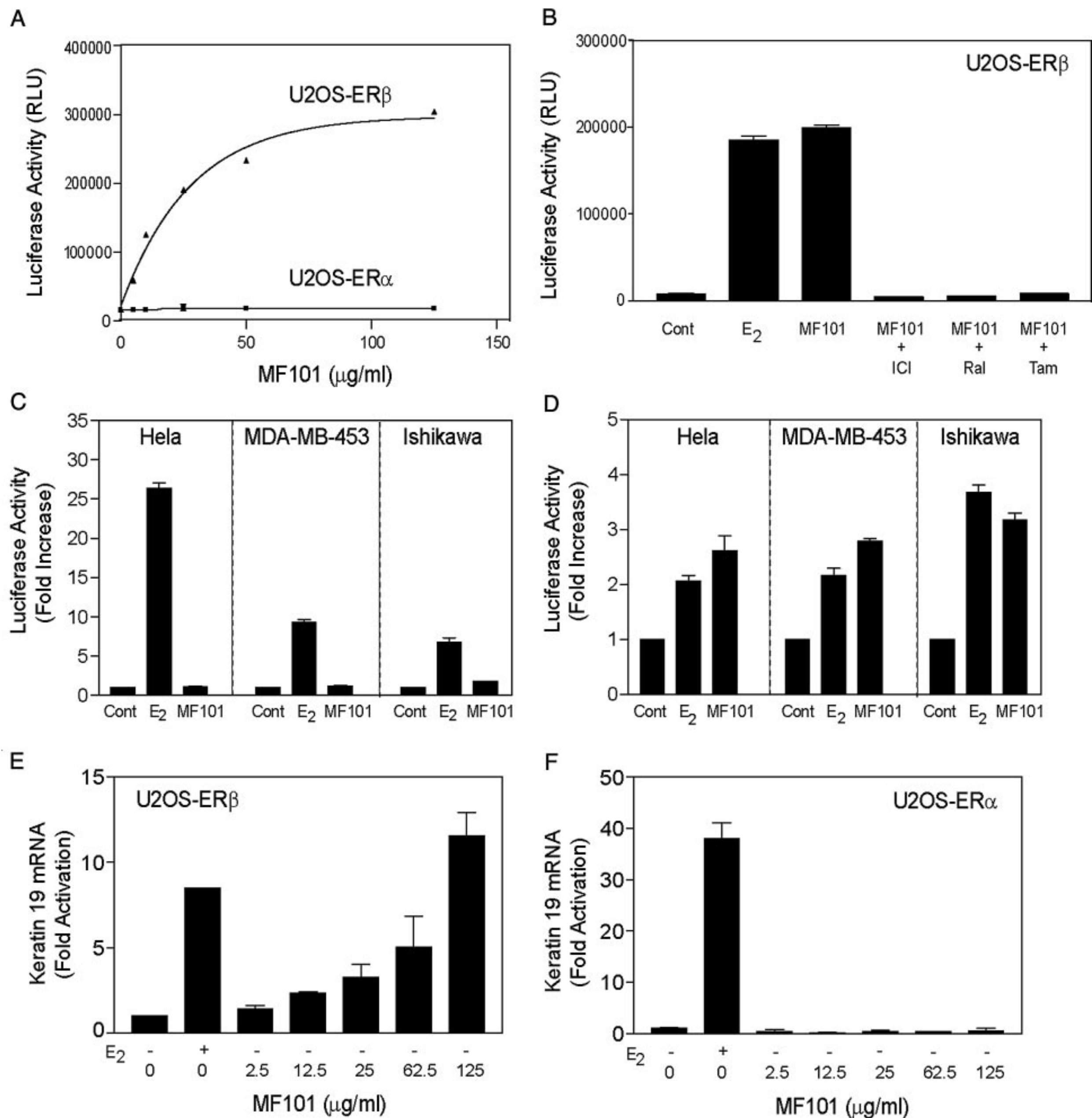

FIG. 1. MF101 selectively activates transcription through ERβ. A, A single copy of the vitellogenin A2 ERE upstream of the minimal tk promoter (ERE tk-Luc) was cotransfected into U2OS cells with expression vectors for human ERα or ERβ. After transfection, the cells were treated for 18 h with increasing amounts of MF101 and luciferase activity was measured. RLU, Relative light units. B, MF101 activation of ERE tk-Luc is blocked by antiestrogens. U2OS cells were cotransfected with ERE tk-Luc and an expression vector for ERβ. The cells were treated with 10 nM E_2 or 125 μg/ml MF101 in the absence or presence of 1 μM ICI, raloxifene (Ral), or tamoxifen (Tam) for 18 h. Cont, Control. C and D, MF101 is an ERβ-selective agonist in human cervical (HeLa), breast (MDA-MB-453), and endometrial (Ishikawa) cell lines. Cells were cotransfected with ERE tk-Luc and an expression vector for human ERα (C) or ERβ (D). The cells were treated with 10 nM E_2 or 125 μg/ml MF101 for 18 h, and luciferase activity was measured. E and F, MF101 selectively activates transcription of the endogenous *keratin 19* gene through ERβ. U2OS cells stably transfected with ERβ (E) or ERα (F) were treated with 10 nM E_2 or increasing amounts of MF101 for 18 h. The level of keratin 19 mRNA was measured by real-time PCR. Each data point is the average of triplicate determinations ± SEM.

MF101 binds equally to ERα and ERβ. Because ERα and ERβ have different primary structures, we compared the conformational responses produced by MF101 *vs.* E_2. Compared with the 10 nM E_2 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 E_2, 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 E_2 in ERβ, whereas MF101 produces a markedly different

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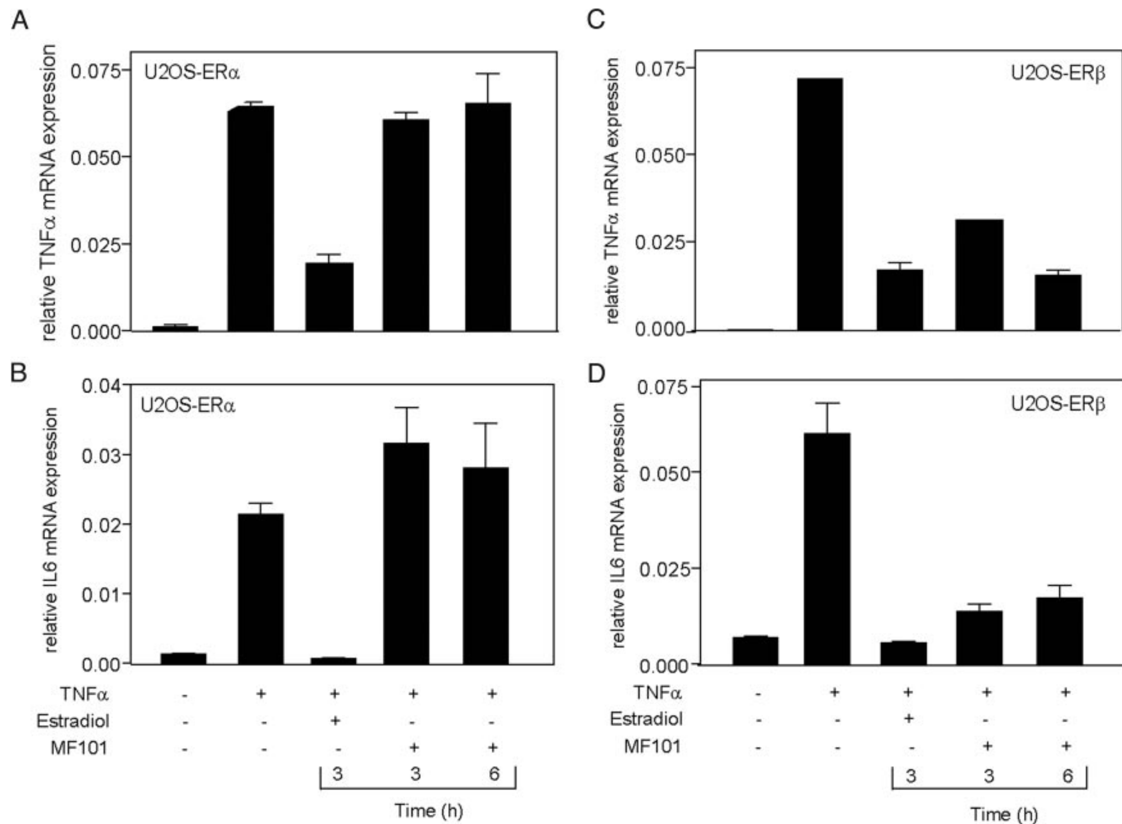

FIG. 2. MF101 selectively represses transcription of the *TNFα* and *IL-6* genes through ERβ. U2OS-ERα (A and B) and U2OS-ERβ (C and D) cells were treated with 1 μg/ml doxycycline for 18 h to induce ER expression. Cells were then treated with 5 ng/ml TNFα for 2 h and 100 nM E_2 or 125 μg/ml MF101 for the indicated times. TNFα (A and C) and IL-6 (B and D) mRNA levels were determined by real-time PCR. Each data point is the average of triplicate determinations ± SEM.

conformation in ERα, compared with the one produced by E_2.

To further investigate whether MF101 changes the conformation of ERα and ERβ without the fluorescent tags, we performed limited proteolysis to probe the conformational features of ERα and ERβ when bound with MF101 or E_2. Radiolabeled ERα and ERβ were synthesized in an *in vitro* transcription and translation system and digested with elastase for increasing times. MF101 and E_2 produced a distinct digestion pattern of ERβ (Fig. 4A) and ERα (Fig. 4B), compared with the control. The digestion pattern of ERβ was different with E_2 and MF101 (Fig. 4A). The strongest protection is observed when ERβ is bound with E_2 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, ERα demonstrates a slight increase in protection to elastase, compared with the control, but less than that observed with E_2 (Fig. 4B). MF101 also produced a distinct pattern, compared with the control or E_2. The *two arrows* show several protected fragments of ERα with E_2, compared with the MF101-treated ERα (Fig. 4B). The FRET and protease digestion studies demonstrate that MF101 binds to ERα and ERβ and induces conformational changes in both ER subtypes.

MF101 causes the selective recruitment of ERβ and coregulatory proteins to target genes

Our studies suggests that upon MF101 binding, ERβ adopts a different overall conformation from ERα, which could prevent ERα 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_2 recruits ER to the keratin 19 ERE in both U2OS-ERα and U2OS-ERβ cells as well as RNA polymerase II and coregulatory proteins (20, 21). ChIP shows that MF101 recruited ERβ but not ERα 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-ERβ cells. These results demonstrate that MF101 produces a conformation in ERβ but not in ERα that allows the MF101-ERβ 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 ERα. Unlike E_2, MF101 did not stimulate cell

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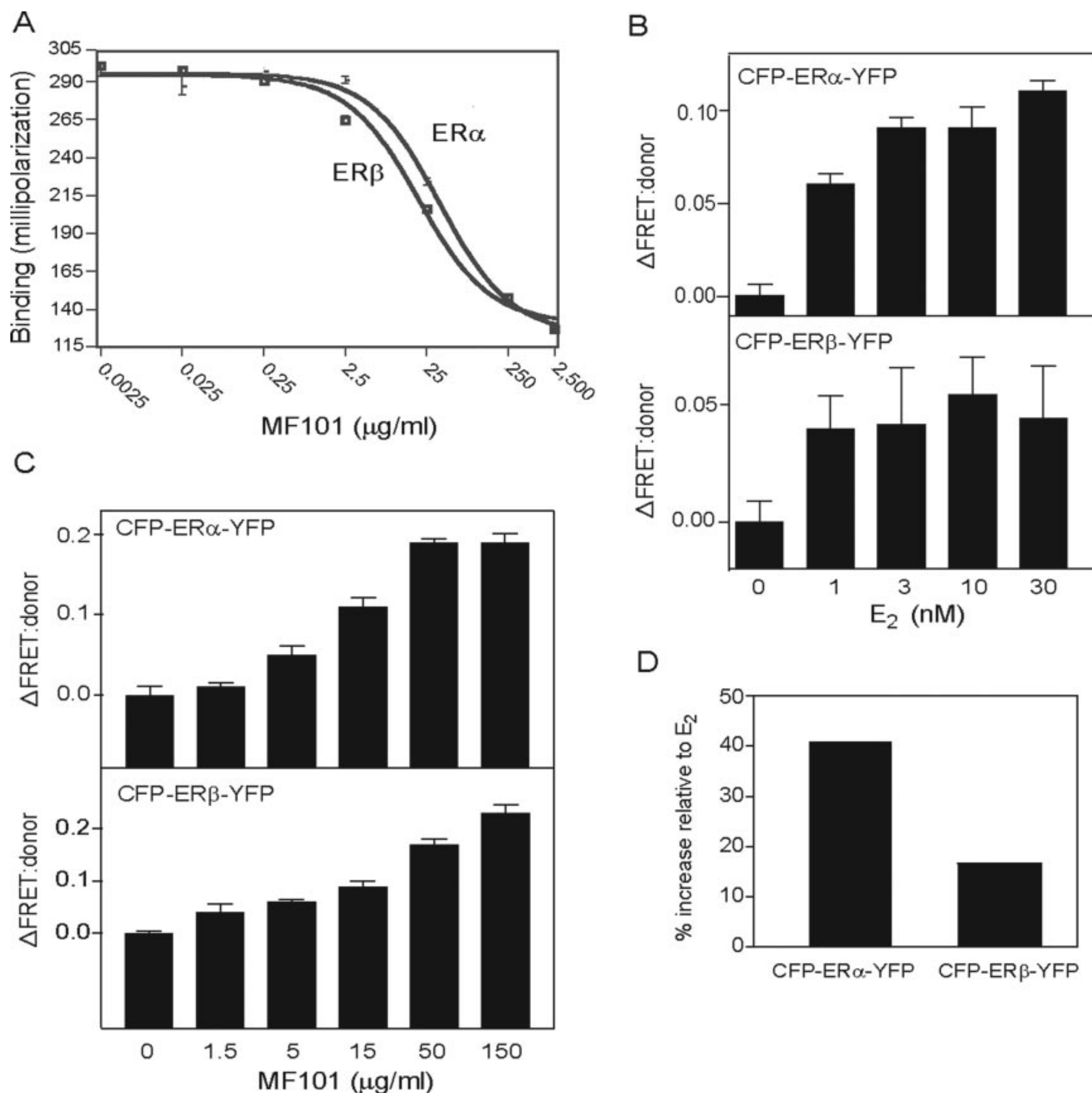

FIG. 3. ERβ-selectivity of MF101 results from the formation of a functional conformation that allows ERβ binding to an ERE. A, Purified ERα or ERβ were incubated with fluorescent E_2 in the absence or presence of increasing amounts of MF101. B and C, HEK293 cells were transfected with CFP-ERβ-YFP or CFP-ERα-YFP and then treated with increasing amounts of E_2 (B) or MF101 (C). After 24 h FRET:donor was measured. D, FRET:donor with MF101 (150 μg/ml) expressed relative to the FRET:donor produced by 10 E_2 nM. The results shown in D are an average of four experiments and are not derived directly from the data in B and C.

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 E_2 (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 E_2 (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 of effects of HT make it worthwhile to begin to explore the potential benefits of herbs for menopausal symptoms. However, it is possible

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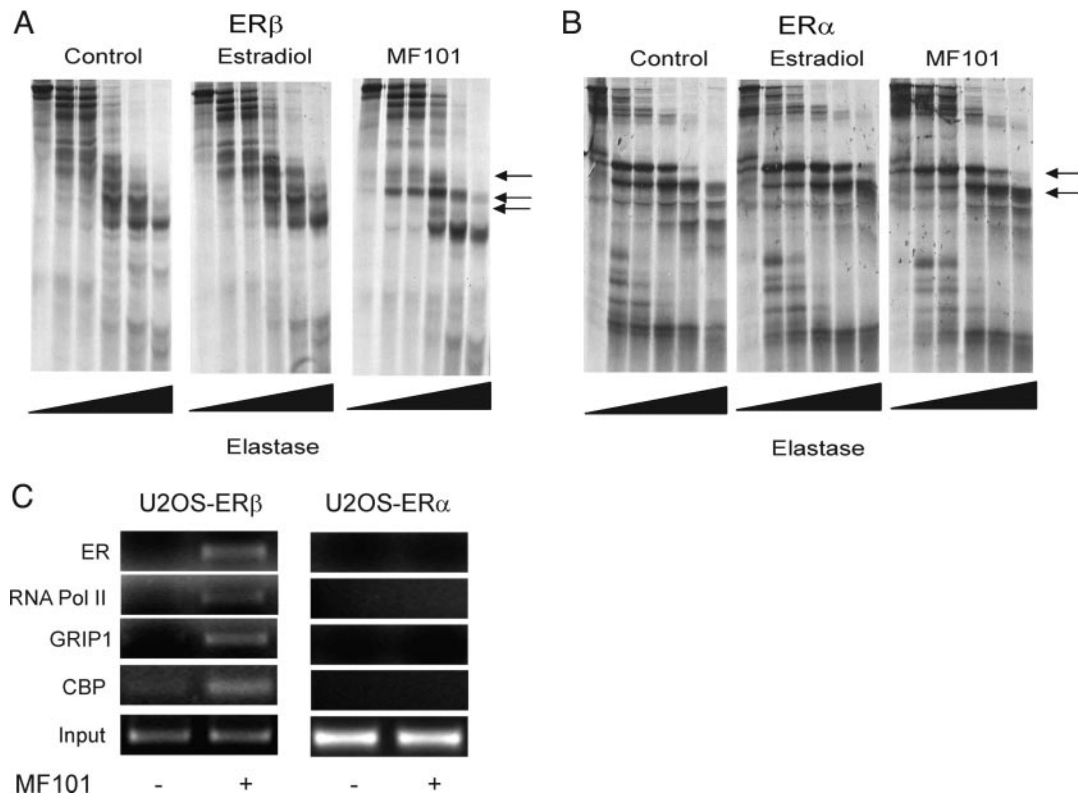

FIG. 4. MF101 changes conformation in ERα and ERβ and selectively recruits ERβ and coregulators to the *keratin 19* gene. ^{35}S-methionine-labeled ERβ (A) or ERα (B) was synthesized in an *in vitro* transcription/translation system and then incubated with increasing amount of elastase in the presence of vehicle (control), E_2, or MF101. The protein fragments were separated by SDS-PAGE and the dried gels were exposed to x-ray film. C, U2OS-ERβ or U2OS-ERα cells were treated with MF101 for 45 min, and then ChIP was performed using antibodies to RNA polymerase (pol) II, GRIP1, CBP, or ERs. The PCR product spans the ERE of the *keratin 19* gene.

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 E_2, the dose of MF101 needed for ERβ activation or repression is about 50,000-fold greater than that of E_2 on a weight basis. However, because MF101 is a crude extract, the dose of the estrogenic components in the mixture in comparison with E_2 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 E_2 (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 E_2, compared with the conformational change of ERα when MF101 *vs.* E_2 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 coregulator 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 coregulators, 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).

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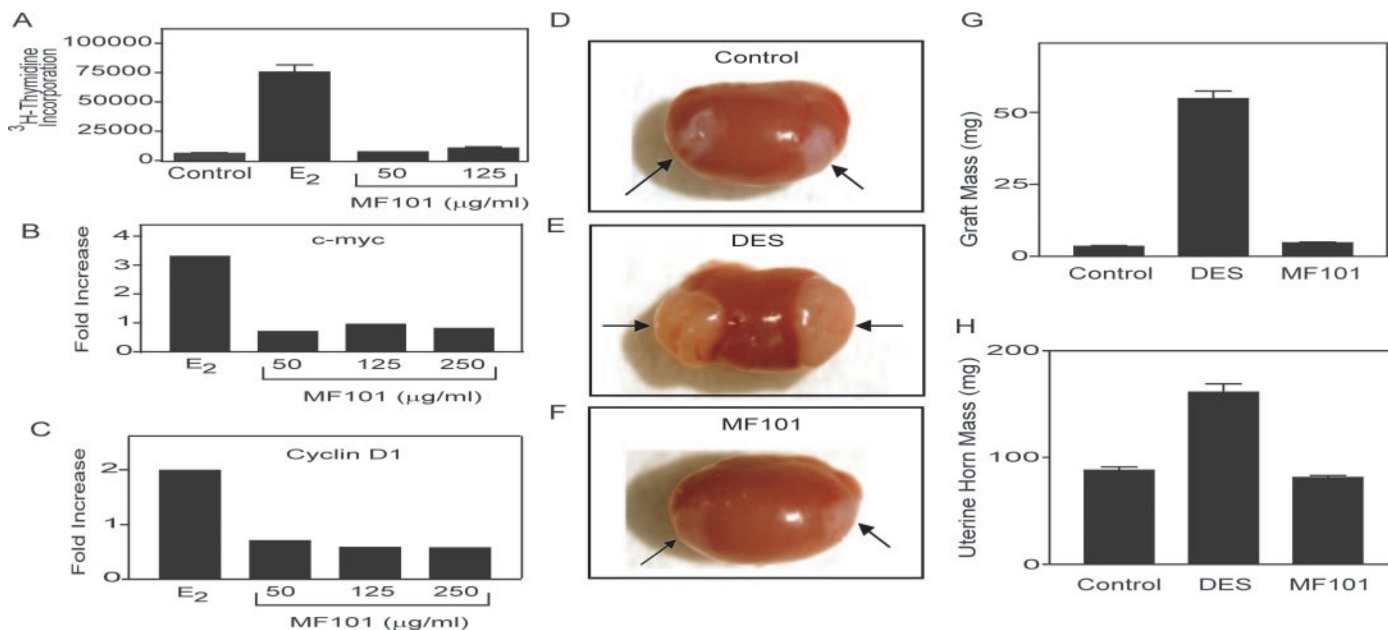

FIG. 5. MF101 does not stimulate proliferation or tumor formation of MCF-7 breast cancer cells. A, MCF-7 cells in stripped serum were treated with E_2 or MF101 for 7 d, and the amount of ^{3}H-thymidine incorporation was measured to determine DNA synthesis. B and C, MF101 does not activate c-*myc* or *cyclin D1* gene transcription. MCF-7 cells were treated with 10 nM E_2 or increasing amounts of MF101. After 1 or 3 h, total RNA was isolated and the level of c-*myc* (B) or cyclin D1 (C) mRNA, respectively, was determined by real-time PCR. D–H, MCF-7 cells were grafted under the kidney capsule of intact female nude mice. Mice were untreated (control) or treated with a sc DES pellet (2 mg) or 0.5 ml MF101 every other day by oral gavage. After 1 month, the tumors and uterus were removed and analyzed for size and weight. Gross morphology of the xenografts in control (D), DES (E), and MF101 (F) treated mice is shown. The *arrow* points to the site of grafting. Average weights ± SEM of tumor grafts (G) and uterus (H) are from five mice in each group.

Our results demonstrate that ERβ-selective drugs, such as MF101, do not activate the ERα-mediated proliferative pathways but trigger the antiinflammatory pathway by activating ERβ. The repression of inflammatory genes by ERβ, such as TNFα and IL-6, is consistent with the observations that a synthetic ERβ-selective agonist is effective at treating several inflammatory conditions in animal models (42) and that ERβ is important for the protective effect of estrogens on the vascular system (43). Our findings suggest that drugs targeted to ERβ will preserve the antiinflammatory action but will be devoid of the proliferative effects of estrogens used in HT.

Whereas the role of ERβ in hot flashes is unknown our findings provide proof of principle that ERβ-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 ERβ-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 ERβ-selective drugs will promote breast or uterine cancer, it is critical to translate our basic research findings by determining whether ERβ-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 ERβ, it is possible MF101 might work through ERα in the brain rather than ERβ because we have not examined the selectivity of MF101 in neurons.

We demonstrated that despite containing many different herbs, the MF101 extract is ERβ 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 ERβ-selective estrogens. Our studies have found that MF101 contains at least six different ERβ-selective compounds by thin-layer chromatography, HPLC, and liquid chromatography/mass spectrometry (data not shown). Our results suggest that MF101 or pure ERβ-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 ERα-mediated breast cancer cell proliferation or uterine growth. Our study also provides a scientific foundation to explore whether MF101 and other ERβ-selective agonists from herbs prevent menopausal symptoms, breast cancer, and inflammatory diseases asso-

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ciated with menopause, such as osteoporosis and cardiovascular disease.

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