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Regulation of specific target genes and biological responses by estrogen receptor subtype agonists

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Estrogenic effects are mediated through two estrogen receptor (ER) subtypes, ER α and ER β . Estrogens are the most commonly prescribed drugs to treat menopausal conditions, but by non-selectively triggering both ER α and ER β pathways in different tissues they can cause serious adverse effects. The different sizes of the binding pockets and sequences of their activation function domains indicate that ER α and ER β should have different specificities for ligands and biological responses that can be exploited for designing safer and more selective estrogens. ER α and ER β regulate different genes by binding to different regulatory elements and recruiting different transcription and chromatin remodeling factors that are expressed in a cell-specific manner. ER α -selective and ER β -selective agonists have been identified that demonstrate that the two ERs produce distinct biological effects. ER α and ER β agonists are a promising new approach for treating specific conditions associated with menopause.

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Introduction

Estrogens have important actions in non-reproductive tissues, including the brain, urogenital tract, and bone. Because of their actions in these tissues, estrogens have been used for over 50 years to prevent and treat a variety of conditions affecting postmenopausal women, including hot flashes, urogenital atrophy, and osteoporosis. Estrogens would be the clear drug of choice for treating menopausal symptoms if they did not cause some serious

adverse effects. The most troublesome side-effect of estrogens is the increased risk of breast and endometrial cancer [1*,2]. Estrogens also increase blood clotting that can lead to venous thromboembolisms, and possibly strokes and heart disease, particularly in older women [1*].

Estrogens in hormone therapy (HT) were formulated long before there was a significant understanding of the mechanism of action of estrogens. The identification of ER α and ER β (Figure 1) and the crystal structures of their ligand binding domain (LBD), the discovery of a variety of coregulatory proteins involved in the genomic pathway and the demonstration of the nongenomic actions of estrogens [3,4] provide an extraordinary opportunity to design a new generation of estrogens that are safer and more selective. Estrogen receptor subtype agonists (ERSAs) [5,6*,7,8*,9,10*] have been identified (Figure 2) that might represent new classes of drugs to treat menopausal conditions. Here we will review ER α and ER β regulation of genes and the actions of several ERSAs and their potential clinical applications.

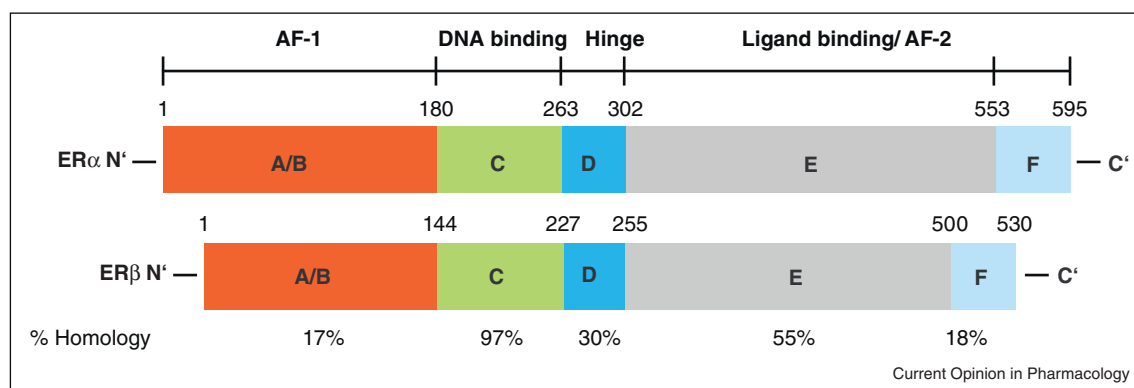
Differences in ER α and ER β are important for designing ERSAs

ERs are composed of three major modular domains; an A/B domain, a DNA binding domain (DBD), and a LBD. Several features differ between ER α and ER β that might be important for designing ERSAs. First, the sizes of the ER α and ER β binding pocket for ligands are different, providing a structural basis for designing ligands that selectively bind to each ER. Second, the two activation function (AF-1 and AF-2) domains that are responsible for regulating gene transcription are located in the least homologous regions (Figure 1). The A/B domain containing AF-1 has only 17% homology, whereas the LBD that containing the AF-2 is 55% homologous. Differences in AF-1 and AF-2 could allow drugs to be designed that recruit different cofactors to ER α and ER β , thereby causing a different pattern of genes regulated.

ER α and ER β have distinct cellular actions, which provide a rationale for developing ERSAs. This has been demonstrated with microarrays that showed ER α and ER β regulate different genes [11,12,13*,14]. Only 40% of genes regulated by estradiol (E₂) in U2OS cells that express ER α were also regulated by ER β [12]. Furthermore,

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Figure 1



Comparison of the structures and homology between ER α and ER β . Human ER α contains 595 amino acids whereas ER β contains 530 amino acids. The DNA binding domains are nearly identical whereas the A/B domain and LBD, which contains AF-1 and AF-2, respectively, have the least homology.

ER α and ER β regulate different classes of genes suggesting that the two ERs have distinct physiological roles. Another feature that distinguishes ER β from ER α is that ER β regulates three classes of genes, whereas ER α regulates a single class of genes [15^{*}]. U2OS cell lines stably transfected with a doxycycline-inducible ER α or ER β [15^{*}] were used to measure the effects of unliganded ER in cells treated only with doxycycline or liganded-ER when cells were treated with both doxycycline and E₂. Unliganded ER α produced a small upregulation of only 1 gene and downregulation of 3 genes, whereas the addition of E₂ to doxycycline treated U2OS-ER α cells resulted in the activation of 518 genes and repression of 157 genes. These data indicate that ER α requires the ligand to regulate gene transcription in U2OS cells. By contrast, three classes of genes were regulated in U2OS-ER β cells. 453 genes were regulated by unliganded ER β (Class I genes). 258 genes were not regulated by unliganded ER β , but regulated by E₂-bound ER β (Class II genes). 83 genes were regulated by unliganded ER β and potentiated by the addition of E₂ (Class III genes). The unliganded effect of ER β is mediated by AF-2, because it is lost when the ER β AF-2 is replaced by the ER α AF-2 [16]. These results demonstrate that intrinsic differences in AF-2 of ER α and ER β can lead to a different set of regulated genes regulated.

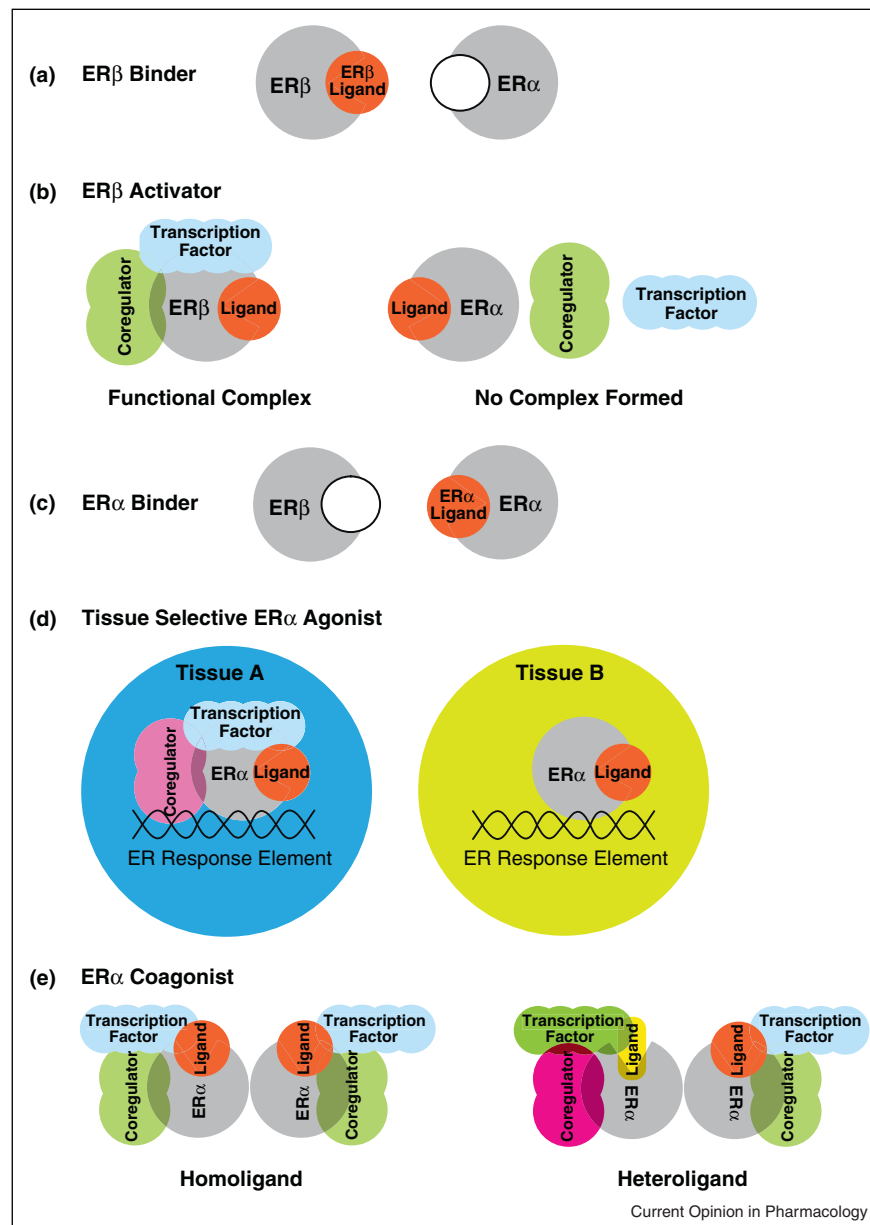
ER α and ER β regulate different genes by binding to distinct regulatory elements

A major question is how do ER α and ER β regulate different genes. The first step required for estrogens to regulate gene transcription involves the binding of ligand to the LBD. This causes a conformational

change that allows the ligand-ER complex to bind to regulatory elements in target genes. ER α and ER β might regulate different genes by binding to different regulatory elements on target genes. To explore this possibility, ChIP-seq was performed in U2OS cells that express a stably transfected ER α or ER β to identify ER binding sites. 11,975 binding sites were found for ER β in response to E₂ [15^{*}] and 15,947 binding sites for ER α (unpublished data). There was approximately a 30% overlap between ER α and ER β binding sites. Different ER α and ER β binding sites were also observed in MCF-7 cells [17^{*},18]. There were 4405 ER α and 1897 ER β binding sites, of which 1386 binding sites were common. These results demonstrate that many ER α and ER β binding sites are unique in U2OS and MCF-7 cells.

Tiling arrays [19,20^{*},21] and ChIP-seq [15^{*},22] studies demonstrated that many ER binding sites are more diverse and complex than the classical estrogen responsive element (ERE), requiring multiple different transcription factors for activity, such as AP1, FoxA1, and Sp1 [15^{*},19,20^{*},21–23]. The complexity is exemplified by the regulatory element in the NKG2E gene that requires a collaboration between c-jun, heat-shock factor 2, and CCAAT/enhancer-binding protein beta and a unique variant ERE for full activation by E₂ [24]. In MCF-7 cells T-cell factor and p53 motifs were present only in ER α binding sites [17^{*}], whereas forkhead transcription factors and Sp1 sites were enriched in ER α and ER β sites, respectively [18]. These observations suggest that transcription factor binding elements are a major determinant of whether ER α or ER β will bind to a particular gene.

Figure 2



Potential classes of estrogen receptor subtype agonists (ERSAs) for drug therapy. Potential ERβ-selective estrogens. **(a)** ERβ binders (ERB-041) are estrogens that are selective because they bind to ERβ with a much higher affinity than ERα. **(b)** ERβ activators (MF101, liquiritigenin) bind to ERα and ERβ with a similar affinity, and form a functional complex when bound to ERβ (left panel), but not ERα (right panel). An ERβ binder/activator (DPN) selectively binds to **(a)** and activates ERβ **(b)**. Potential ERα-selective agonists. **(c)** ERα binders (PPT) bind to ERα with a much higher affinity than ERβ. **(d)** Tissue selective ERα agonists (Radix Glycyrrhiza and Radix Pueraria) form a functional transcription complex at response elements with ERα in some tissues (left panel), but not in other tissues (right panel). **(e)** A ligand such as E₂ binds to both ERα subunits that leads to the recruitment of coregulators and transcription factors (left panel). In the presence of an ERα coagonist (chalcone) E₂ binds to one subunit and the coagonist binds to the other subunit (right panel). The heteroliganded ERα could create a different conformation than the homoliganded ERα that leads to the recruitment of different coregulators and/or transcription factors.

ERα and ERβ regulate different genes by recruiting distinct coregulators and chromatin remodeling factors

Once the ER complex attaches to a regulatory element it functions as a docking site for the recruitment of

coregulatory proteins, and transcription and chromatin remodeling factors to form a large protein complex that regulates transcription [25,26]. Even if ERα and ERβ bind to the same site they could regulate different genes because differences in their conformation might lead to

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the recruitment of different coregulatory proteins at the same genes. For example, liquiritigenin (LIQ) caused the recruitment of the coactivator, NCOA2 to the *CECR6*, *NKG2E*, and *NKD* genes in U2OS-ER β cells, but not in U2OS-ER α cells [9]. Furthermore, GIOT-4 has been identified as an ER β specific coactivator [27], whereas a member of the SWI/SNF chromatin remodeling complex, BAF57 selectively regulates ER α -mediated transcription [28].

Identification of three classes of ER β -selective agonists

Multiple ER β -selective agonists have been synthesized [10^{*}]. 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) has 70-fold higher relative binding affinity and 170-fold higher relative potency in transfection assays with ER β compared to ER α [7]. Wyeth synthesized a number of ER β -selective compounds [29]. ERB-041 has been the most studied. It has over a 200-fold greater selectivity for binding to ER β compared to ER α [6^{*}]. In addition to synthetic compounds, a plant extract, MF101 contains ER β -selective agonists [8^{*}], several of which have been identified, including liquiritigenin and nysol [9,13^{*}]. Based on binding and functional studies, we proposed that these compounds can be grouped into three classes [13^{*}] (Figure 2). One class is represented by ERB-041 that is selective because it binds to ER β at a much higher affinity than ER α (Figure 2a). We termed it an ER β binder. MF101, LIQ, and nysol bind to both ER α and ER β similarly, but they only activate ER β [13^{*}]. When these compounds bind to ER α they produce an inactive conformation that prevents ER α from forming a functional complex and recruiting coactivators [8^{*},9] (Figure 2b). These are termed ER β activators. DPN is selective because it not only binds ER β with higher affinity, but also more potently activates ER β than ER α . We termed it an ER β binder/activator. While most genes regulated by DPN, ERB-041, MF101, LIQ, and nysol are the same, these three classes of ER β agonists regulate some different genes [13^{*}]. Importantly, many genes regulated by these ER β agonists in U2OS-ER β cells are distinct from those regulated by E $_2$. This observation is consistent with the finding that ER β binding sites are different when it is bound to ERB-041 compared to E $_2$ in MCF-7 cells [17^{*}]. From these results, it can be expected that different classes of ER β agonists will produce different biological and clinical effects from one another and non-selective estrogens used in HT.

ER β -selective agonists for hot flashes

Estrogens are the most effective treatment for hot flashes. However, it is unclear if this effect is mediated through ER α , ER β , or both ERs. This has been difficult to address experimentally because of inadequate animal models to test drugs on spontaneous hot flashes. Most studies used rat models that measure tail skin temperature as a surrogate marker for hot flashes. In a morphine-

addicted rat model two ERB-041 analogs were ineffective [30], whereas DPN was effective in another rat model [31]. A Phase II clinical trial with 217 postmenopausal women having moderate to severe hot flashes was conducted with the ER β -selective plant extract, MF101. After 12 weeks, there was a statistically significant median 11.9% reduction in hot flashes and a 67% reduction in night sweats in women treated with MF101 compared to those treated with placebo [32]. Taken together, these results suggest that ER β agonists might have beneficial effects on hot flash prevention.

ER β -selective agonists for breast cancer prevention

Multiple studies showed that ER α mediates the proliferative effects of estrogens in breast cells. Anti-proliferative effects of ER β have been demonstrated in breast cancer cells [33,34]. In MCF-7 breast cancer cells, ER β causes a G2 cell cycle arrest [34] by inhibiting the activity of cyclin dependent kinase 1 (CDK1) that is essential for cells to progress from G2 phase to mitosis. The major activator of CDK1 is cyclin B1. ER β inhibits the transcription of the cyclin B1 gene that leads to a reduction in cyclin B1 protein levels (submitted). CDK1 is inhibited by the tumor suppressor proteins, GADD45A and BTG2. ER β binds to the promoter of these genes leading to increased transcription (submitted). Ultimately, the reduction in cyclin B1 and increased production of GADD45A and BTG2 leads to the inactivation of CDK1 and a G2 cell cycle arrest.

ERB-041 did not produce proliferative effects in the rat mammary gland [6^{*}]. MF101 did not stimulate growth promoting genes, such as c-myc and cyclin D1 in MCF-7 cells [8^{*}]. Furthermore, MF101 or LIQ did not increase MCF-7 cell tumor formation in mouse xenograft models [8^{*},9]. These results demonstrate that ER β agonists do not promote proliferation of normal mouse mammary epithelial and human breast cancer cells. ER β inhibits ER α -mediated activation of reporter genes in transfection assays [35], suggesting that one mechanism whereby ER β exerts an anti-proliferative action is by interfering with the action of ER α . This was examined in MCF-7 cells that express ER α , ER β , or both ERs [17^{*}]. These studies showed that ER α and ER β competed for the same genomic binding sites and that the presence of both ERs produced new binding sites for ER α and ER β homodimers, which probably leads to a different gene expression profile that is observed when the two ERs are coexpressed in cells [36]. These findings suggest that ER β agonists might be useful for preventing breast cancer by antagonizing the proliferative action of ER α .

ER β -selective agonists for inflammatory diseases

One important action of estrogens that is relatively unappreciated is their anti-inflammatory effect. A number of

diseases during menopause have an inflammatory component to their pathogenesis. These conditions include osteoporosis, cardiovascular disease, Alzheimer's disease, obesity, and atrophic vaginitis. Estrogens in HT are very effective at preventing osteoporosis and atrophic vaginitis, but controversy exists regarding their effects on cardiovascular disease, obesity, and Alzheimer's disease. The anti-inflammatory action of ERB-041 has been examined in multiple inflammatory rodent models, including endometriosis, rheumatoid arthritis, inflammatory bowel disease, and sepsis [6*,37,38]. These studies demonstrated that ERB-041 was very potent at blocking inflammation in these models and suggested that ER β -selective agonists might be important drugs to treat a variety of disorders associated with inflammation. MF101 and synthetic ER β agonists, including ERB-041 are potent repressors of pro-inflammatory genes [8*,39], indicating that estrogens can produce anti-inflammatory actions through ER β . The effects of ER β on inflammatory conditions associated with menopause, such as osteoporosis, obesity, cardiovascular disease, and atrophic vaginitis are unclear. ERB-041 did not prevent ovariectomy-induced bone loss or weight gain in rats [6*], suggesting that ER α mediates these effects. DPN decreases the size of infarcts in mouse hearts subjected to ischemia and reperfusion similar to E₂ [40]. This cardioprotective effect of DPN was abolished in ER β knockout mice [40]. These findings indicate that ER β agonists might be useful for preventing cardiovascular disease. Another possible clinical indication for ER β agonists, where an anti-inflammatory effect could be therapeutic is atrophic vaginitis. Our pre-clinical studies with mice indicate that ER β agonists may play a role in the treatment of postmenopausal vaginal atrophy and dryness.

ER α is important for preventing osteoporosis, weight gain and insulin resistance

ER α is essential for preventing osteoporosis because a rare genetic mutation that inactivates ER α leads to severe osteoporosis in humans [41]. The observation that PPT, but not ERB-041 prevents bone loss in rats after ovariectomy provides additional evidence that ER α mediates the beneficial effects of estrogens in bone [6*,42]. ER α also probably mediates the beneficial effects of estrogens in adipose tissue and on insulin resistance, because ERKO mice have increased weight gain, greater adipose tissue, insulin resistance, and impaired glucose tolerance [43]. PPT prevents weight gain in rats and exerts anti-diabetic effects by improving insulin sensitivity and glucose intolerance [44].

ER α -selective agonists

The major concern for developing ER α agonists is that they will cause cell proliferation and increase the risk of cancer. In fact, PPT stimulates the proliferation of HC11 mouse mammary epithelial cells [45] and increases uter-

ine weight in rats [42]. These findings indicate that ER α -selective binders (Figure 2c), like PPT might not be useful drugs for hormone therapy. Another strategy would be to design tissue selective ER α agonists that activate ER α in some tissues, such as the bone and adipose tissue, but not in the mammary gland and uterus (Figure 2d). An alternative strategy is to combine estrogens with other compounds that block the proliferative effects of estrogens in the mammary gland and uterus (Figure 2e). Progestins are effective at blocking the proliferative effects of estrogens in the uterus, but unfortunately they exacerbate the proliferative effects in the mammary gland.

Tissue selective ER α agonists prevent weight gain without promoting cell proliferation

It is well established that estrogens exert tissue-specific effects, but the mechanism is unclear. Tiling arrays identified 1090 ER α binding sites on chromosomes 1 and 6 in MCF-7 cells whereas 1137 ER α binding sites were found in U2OS cells [46*]. Only 172 ER α binding sites were common to both cell types. The cell-specific recruitment of ER α is mediated by the binding of the pioneer factor, FoxA1 that recognizes monomethylated and dimethylated histone H3. Once FoxA1 recognizes these methylated histones near an ER binding site it interacts with ER to open up chromatin structure and facilitate the recruitment of transcription factors leading to increased transcription [46*]. Because FoxA1 is expressed in MCF-7 cells, but not U2OS cells, the genes regulated by ER α are different [46*]. These findings suggest that it might be possible to design tissue selective ER α modulators that mimic the agonist activity of E₂ in some tissues, but not in other tissues.

We identified two plant extracts (PEs), Radix Glycyrrhiza and Radix Pueraria that behave as tissue selective ER α agonists (Figure 2d). These PEs activate ER α in transfection assays using an ERE upstream of the luciferase reporter and bind to purified ER α (In preparation). To test the effects of the PEs on weight loss, ovariectomized mice were fed a high fat diet (HFD). After the mice gained weight, they were treated orally for 6 weeks with the PEs separately while being maintained on the HFD. The vehicle treated control mice continued to gain weight, whereas the E₂-treated mice, which served as positive controls, lost 20.5% of their weight. The body weight and abdominal fat of both PE treated mice was significantly reduced to levels similar to mice treated with E₂. By contrast to E₂ no significant proliferative effects were found in the mammary gland and uterus. While further characterization and studies are needed with the PEs these studies suggest that it might be possible to develop tissue selective ER α agonists that retain the beneficial effects mediated by ER α without promoting breast and endometrial cancer.

ER α coagonists change the gene expression profile and proliferative response of E₂

Another potential way to make estrogens safer for drug therapy is to add a second drug to alter the biological properties of estrogens after they interact with ER α . We screened plant extracts and found that a chalcone derivative dramatically changed the gene expression profile by E₂ in U2OS cells expressing ER α . We termed the chalcone an ER α coagonist (Figure 2e), because it was inactive by itself, but it caused E₂ to regulate genes that it did not activate in its absence and it potentiates the regulation of E₂ on some genes (In preparation). The coagonist blocked E₂-mediated proliferation of MCF-7 cells, suggesting that the coagonist changes the proliferative response of E₂ by causing ER α to regulate a different set of genes. While the mechanism of the coagonist is unclear, our studies suggest the possibility that it binds to ER α as hetero-ligand with one subunit binding to E₂ and the other subunit binding to the chalcone (Figure 2e, right panel). The combination of two different ligands bound to ER α simultaneously probably produces a different conformation than when ER α is bound to only E₂ (Figure 2e, left panel) or the chalcone. While the effects of the coagonist on E₂-mediated bone loss, weight loss, and mammary gland and endometrial cell proliferation in animals need to be investigated, it may be possible that coagonist compounds can alter the clinical responses to estrogens and make them safer.

Concluding remarks

Gene expression data, tiling arrays, and ChIP-seq data show that ER α and ER β regulate different genes by binding to distinct regulatory elements and interacting with different coactivators and transcription factors. Animal studies demonstrated that ER α -selective and ER β -selective agonists produce different biological effects. Three classes of ER β -selective agonists have been identified; ER β binder, ER β activator, and ER β binder/activator. ER β -selective agonists might be clinically useful for preventing breast cancer and treating hot flashes and inflammatory conditions associated with menopause. Because the proliferative effects of estrogens are mediated through ER α , the impetus to design ER α -selective agonists for clinical use has not been strong as for ER β -selective agonists. However, ER α is clearly important for preventing osteoporosis, weight gain, and insulin resistance. Tissue selective ER α agonists or ER α coagonists may provide a safer approach if proven to activate ER α in tissues that are beneficial, such as the bone and adipose tissue, but not the mammary gland and uterus. While many additional studies are needed to evaluate the safety and efficacy of ER α -selective and ER β -selective agonists they offer a new therapeutic approach for preventing and treating specific menopausal conditions.

Conflict of interest

O.I.V. and C.B.H. have nothing to declare. S.P., E.F.S., I.C., and M.T., are employees of Bionovo, Inc. D.C.L. and T.P.S. are on the Scientific Advisory Board of Bionovo, Inc. D.C.L. has received financial support for research from Bionovo, Inc.

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