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Estrogen Receptors Alpha (ER α) and Beta (ER β): Subtype-Selective Ligands and Clinical Potential

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Abstract

Estrogen receptors alpha (ER α) and beta (ER β) are nuclear transcription factors that are involved in the regulation of many complex physiological processes in humans. Modulation of these receptors by prospective therapeutic agents is currently being considered for prevention and treatment of a wide variety of pathological conditions, such as, cancer, metabolic and cardiovascular diseases, neurodegeneration, inflammation, and osteoporosis. This review provides an overview and update of compounds that have been recently reported as modulators of ERs, with a particular focus on their potential clinical applications.

Keywords

Estrogen receptors; Estrogen receptor subtype α ; Estrogen receptor subtype β ; modulators; agonists; antagonists; therapeutic applications

1. Estrogen receptors subtypes alpha (ER α) and beta (ER β)

For half a century, menopausal symptoms, such as osteoporosis, hot flashes, and urogenital atrophy, have been treated with hormone therapy (HT), comprising estrogens alone or estrogens and progestins. In fact, because of their beneficial actions in non-reproductive tissues, such as bone, brain, and urogenital tract, estrogens would be ideal drugs if they did not have serious adverse effects, consisting mainly of increasing the risk of breast and endometrial cancer, as well as of thromboembolisms and strokes [1].

The physiological functions of estrogenic compounds are modulated largely by the estrogen receptors subtypes alpha (ER α) and beta (ER β). These proteins have actions in the cell nucleus, regulating transcription of specific target genes by binding to associated DNA regulatory sequences. In humans, both receptor subtypes are expressed in many cells and

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tissues, and they control key physiological functions in various organ systems, such as reproductive, skeletal, cardiovascular and central nervous systems, as well as in specific tissues (such as breast and sub-compartments of prostate and ovary). ER α is present mainly in mammary gland, uterus, ovary (thecal cells), bone, male reproductive organs (testes and epididymis), prostate (stroma), liver, and adipose tissue. By contrast, ER β is found mainly in the prostate (epithelium), bladder, ovary (granulosa cells), colon, adipose tissue, and immune system. Both subtypes are markedly expressed in the cardiovascular and central nervous systems. There are some common physiological roles for the two ERs, such as in the development and function of the ovaries, and in the protection of the cardiovascular system. The alpha subtype has a more prominent role on the mammary gland and uterus, as well as on the preservation of skeletal homeostasis and the regulation of metabolism. The beta subtype seems to have a more profound effect on the central nervous and immune systems, and it generally counteracts the ER α -promoted cell hyperproliferation in tissues such as breast and uterus [2–3].

Estradiol and hormone replacement therapies target both the ERs, but this often leads to an increased risk of breast and endometrial cancers, and thromboembolism. Also, selective estrogen receptor modulators (SERMs) target both receptor subtypes, although they display tissue-selective agonist/antagonist activities through mechanisms distinct from those involving ER subtype-selective binding or activation. An ideal SERMs would possess antagonist activity in the mammary gland and uterus, and agonist activity in other target tissues that benefit from an estrogen-like action, such as the cardiovascular, skeletal, and central nervous systems [4]. Alternatively, subtype-selective ligands could be used to elicit beneficial estrogen-like activities and reduced side effects, based on the different distributions and relative levels of the two ER subtypes in different estrogen target tissues noted above. In this regard, there appears to be particular promise for the use of ER β -selective agonists.

The development of compounds possessing ER subtype specificity still constitutes a major challenge. The amino acid sequence of ER α and ER β displays 59% sequence identity in their respective ligand binding domains (LBD), which represents a significant difference. However, the differences within the ligand binding cavities are at only two amino acid positions: Leu384 and Met421 in ER α are replaced by Met336 and Ile373, respectively, in ER β ; not only are these conservative changes between hydrophobic residues, they are also reciprocal, with each ER subtype having one methionine residue and then either a leucine or isoleucine residue. Perhaps more significant is the fact that in most crystal structures, the ER β binding pocket is smaller and narrower than that of ER α , which is more likely due to the greater sequence diversity beyond the ligand binding pocket than to the small differences at the two pocket residue positions. The overall effect of these very subtle differences makes it quite difficult to design subtype-selective ligands. Nevertheless, such efforts have been greatly aided by major advances in the structural biology of estrogen receptors over the past two decades that have shed light on the molecular basis for the binding modes and the regulation of agonist vs. antagonist activities of ER-ligands [5–6]. Consequently, nowadays investigators have a good context in which to consider ligand interactions with the ERs that are likely to be crucial for good binding affinity, and these, together with accumulating

information on structure-binding and structure-activity relationships, have led to the formulation of reliable ER-pharmacophore models [7].

Generally, a good ER-ligand should possess two OH groups that are linked by a lipophilic central scaffold, which places them at a distance of about 11 Å. At least one of these hydroxyls should be a phenol or a phenol-bioisostere. One of these OH groups forms a strong attractive interaction with an H-bond network comprising residues (ER α / β numbering) Glu353/305, Arg394/346 and a water molecule, where the phenolic OH group of the A-ring of estradiol binds. The antipodal hydroxyl group, instead, mimics the 17 β -OH group of estradiol and forms an additional H-bond with residue His524/475 in the estradiol D-ring pocket. Beyond these two key, energetically important interactions, which appear to be commensurate between the two ERs, subtype-selectivity needs to arise from the different shape and hydrophobicity of the central scaffold, which can only count on weakly differential attraction/repulsion balances with the non-conserved residues Leu384(ER α)/Met 336(ER β), and Met421(ER α)/Ile373(ER β), as well as differences in the propensity of the two ERs to form pockets of different sizes. In any case, the selective activation of ER α or ER β may depend not only on a selective receptor binding affinity, but also on selective activation of each receptor subtype [8].

2. Potential clinical applications of ER α and ER β modulators

2.1. Cancer

In many *breast cancers*, ER α activation by estrogens is generally considered responsible for enhanced proliferation, whereas this is counteracted by the presence of ER β , which exerts an antiproliferative effect [9]. Therefore, theoretically, breast cancer patients with estrogen-responsive disease should respond positively to treatment with ER α -antagonists and/or ER β -agonists. However, the situation is a bit more complicated because responses will depend on the expression level of these two receptors in the cancer tissue and also on the stage of the neoplasia. For example, it was recently demonstrated that ductal breast cancer that is highly proliferative shows a high expression of ER α and a low expression of ER β when it is early stage. High-grade invasive ductal cancer then loses the expression of both receptors. On the other hand, early-stage lobular breast cancer shows abundant levels of both ER α and ER β , whereas advanced stages of this disease present only ER α expression, ER β having been lost [10]. These examples would support the use of ER β -selective agonists only in the case of early-stage ductal cancer, since it may prevent or delay development of more invasive disease. Correspondingly, an ER α -antagonist may be helpful for the treatment of advanced lobular cancer (but not early stage disease), or early-stage ER α -positive ductal cancer.

Activation of ER β in early *prostate cancer*, as well as in *benign prostatic hyperplasia*, leads to an antiproliferative and pro-apoptotic effect [11]. Furthermore, ER β seems to inhibit the epithelial-mesenchymal transition (EMT) in prostate cancer cells by destabilizing hypoxia-inducible factor 1 α (HIF-1 α), thus reducing their invasive potential [12]. Therefore, there is hope that an ER β -selective agonist might be used to treat these pathologies.

In human malignant *pleural mesothelioma*, ER β was found to modulate cell proliferation by effectively inactivating the epidermal growth factor receptor (EGFR), thus exerting a tumor

repressive effect [13], and a similar function was also observed in *renal cell carcinoma* cell lines [14].

Loss of ER β expression is generally found in *colorectal cancer*, and the degree to which it is reduced correlates with increasingly poor prognosis [15]. ER β was demonstrated to have a protective effect against the development and progression of colon cancer by inhibiting cell proliferation *in vitro* and tumor formation *in vivo* [16–17]. Therefore, selective stimulation of ER β might be useful as a preventive and/or therapeutic strategy against this type of neoplasia.

The role of ERs in *lung cancer* has been extensively studied, but it still remains quite controversial. In fact, in some studies ER β was found to be the main receptor mediating the proliferative effects of estrogens in lung cancer cells [18]. Furthermore, ER β appears to be involved with insulin-like growth factor-1 receptor (IGF-1R) in a synergistic promotion of lung adenocarcinoma development in murine models [19]. On the other hand, other studies indicate that ER β may have prognostic value, since its overexpression appears to be a positive prognostic marker in stage II and III non-small cell lung cancer (NSCLC) patients [20], whereas its absence, together with the presence of ER α , was associated with a poorer prognosis in patients affected by the same type of cancer [21]. This effect seems to be gender-dependent, since women with ER β -positive tumors did not show any significant difference in mortality from those with ER β -negative tumors, whereas men with ER β -positive tumors displayed a significant reduction in mortality [22]. In any case, ER β -activation in human non-small cell lung cancer cells *in vitro* produced a stimulatory effect on cell proliferation [23]. So, the real potential for the use of ER β -agonists in lung cancer is still rather puzzling. Other types of tumors that might potentially be treated with ER ligands will be mentioned in the specific sections below.

2.2. Neuropathies

Much evidence underlies the important role of estradiol in the central nervous system (CNS) for different kind of diseases, including pathologies associated with *depression* and *anxiety*. A recent study reports that aged female mice respond favorably to E₂ administration and exhibit greater anti-anxiety and antidepressant behavior [24]. In fact, estrogens were found to modulate the synthesis and degradation of serotonin, as well as the expression of serotonin receptors [25]. Activation of both ER α and ER β caused a neuroprotective effect, in particular against toxicity associated with A β accumulation, therefore suggesting a potential role in the prevention and treatment of *Alzheimer's disease* [26]. In animal models, ER α proved to play a key role in the prevention and treatment of *multiple sclerosis*, which was superior to that of ER β [27]. ER β ligands have some capacity to reverse symptoms of established disease, and the use of selective ER α agonists is controversial, because of the increased risk of breast and endometrial cancers and thromboembolic events. Finally, administration of soy isoflavones, which are known ER β -selective agonists, to healthy male individuals caused a general enhancement of their cognitive abilities [28], thus further supporting the beneficial role of ER β in supporting memory and learning functions.

2.3. Cardiovascular disease

The regular production of estrogens in pre-menopausal women places them at a lower risk for cardiovascular disease, but this cardioprotective effects vanishes after menopause [29]. The mechanisms involved in this cardioprotective role of estrogens are currently under investigation, and both ERs seem to be involved at least to some extent [30]. In particular, ER α is highly expressed in endothelial cells and plays a role in mediating the effects of estrogens in the vascular endothelium, whereas ER β stimulates the production of nitric oxide. Therefore, the activation of both receptors has a beneficial hypotensive effect caused by vascular wall dilation [31–32]. A recent study [33] demonstrated that the non-nuclear fraction of ER α , which is linked to plasma membrane, is selectively responsible for the vascular actions elicited by estrogens, such as rapid dilatation, acceleration of endothelial repair, and endothelial NO production. This observation is particularly useful since a selective cytoplasmic stimulation of ER α would promote a beneficial cardioprotective effect, without eliciting all those side effects associated to nuclear activation of this receptor.

2.4. Osteoporosis

Osteoporosis is a major health problem associated with estrogen deficiency in postmenopausal women due to the marked increase of bone resorption that is not compensated by bone formation. Many studies have demonstrated that estrogens play a crucial role in bone homeostasis, not only in women but also in men [34]. Estrogen replacement therapy (ERT) is clinically used to prevent osteoporosis [35]; however, long-term ERT may stimulate proliferation of breast and uterus through ER α , increasing the risk of cancer in these organs. Bone cells express both estrogen receptor, ER α and ER β , although ER α seems to play the major role [36]. The use of a SERM, such as raloxifene, seems to reduce the risk caused by ERT and, at the same time, to counteract osteoporosis [37].

2.5. Obesity and metabolic disorders

ER β may play an important role in regulating metabolic pathway and adipose tissue function [38]. Obesity and related metabolic diseases represent major problems in modern society, and the few drugs approved for treatment have side effects. Recent studies highlight the beneficial effect of E₂ on hyperglycemia, oxidative stress and on the liver dysfunction in diabetic rats [39]. In fact, estrogen deficiency causes metabolic dysfunction, obesity, metabolic syndrome, and type 2 diabetes. Hence, there is a great potential for the treatment of these pathologies with appropriate ER-activating agents, in particular, those behaving as SERMs or acting through ER β [40].

2.6. Menopause

There are several ongoing investigations aimed at establishing the utility of estrogen-based treatments in reversing menopausal changes in women, such as, loss of bone density, hot flushes and cardiovascular problems. Nowadays, a great deal of attention is being devoted to the administration of selective ER β -activators, since they are considered likely to be safer than non-specific estrogens, due to the lack of stimulation of ER α . One of these studies concerns the administration of an herbal extract, MF101 [41], which proved to reduce the frequency of hot flushes in a phase II clinical trial [42].

3. Update on chemical classes comprising ER α and ER β modulators

Over the past few years, many comprehensive reviews have exhaustively covered progress in the development of ER ligands [43–47]. We herein describe an update of the most recently reported molecules that are able to interact with the ERs, with particular focus on those displaying likely potential as perspective therapeutic agents.

3.1. Steroid derivatives

Several synthetic estradiol analogues were tested in animal models to understand the role of both ER subtypes in pathophysiology. Among the steroid derivatives tested, two molecules have been used in several *in vivo* studies as ERs selective ligands: 8 β -VE₂ (**1**, Fig. 1), a 46-fold ER β -selective compound obtained by the addition of a vinyl group at 8 β position of E₂ scaffold [48], and 16 α -LE₂ (**2**, Fig. 1), an ER α -selective compound obtained with the fusion of a lactone ring across the 16 α and 17 α positions of E₂ [49].

Both ERs are thought to play important roles in cardiovascular diseases, having beneficial short and long-term cardiovascular effects [50], although the respective roles of ER α and ER β have not yet been clarified [51]. In a recent study, ovariectomized atherosclerosis-prone apoE^{-/-} mice were fed a high-cholesterol diet and received daily subcutaneous injections of 8 β -VE₂ for 5 weeks. Compared with controls, treatment with 8 β -VE₂ reduced aortic arch atherosclerotic lesion areas by 34% of total and 75% of dense lesions, without altering the serum lipid profile, while the treatment with fulvestrant, a nonselective ERs antagonist devoid of any agonistic activity, completely abrogated the beneficial vascular effects of 8 β -VE. Furthermore, blood samples collected at regular intervals reveal that 8 β -VE₂ reduces serum levels of proinflammatory cytokines. These results demonstrate that ER β may play a crucial role in maintaining vascular homeostasis [52]. On the other hand, ER α seems to be important in maintaining the physiological cardiac glucose uptake in normotensive and nondiabetic female mice. In fact, administration of 16 α -LE₂ to ovariectomized mice completely restored cardiac glucose uptake, whereas this effect was strongly reduced in ER α knockout mice, although the role of ER β in this process still needs to be investigated [53].

ERs, and ER β in particular, also play important roles in skeletal muscle regeneration processes after injuries. Ovariectomized female Wistar rats with noxetin-induced damage have been used to investigate the role of both ERs in skeletal muscle regeneration using E₂, 8 β -VE₂ and 16 α -LE₂. First, serum levels of creatine kinase (CK) were analyzed to investigate the extent of injury, and although all the compounds decrease CK levels, 8 β -VE₂ proved to be the most potent agent in protecting the cell membrane. Furthermore, β ERKO mice experienced increased muscle damage [54], thus highlighting a predominant role for ER β in this repair process.

There is considerable evidence that endogenous estrogens play a significant role as modulators of immune responses and autoimmune diseases. Therapeutic effects of 8 β -VE₂ and 16 α -LE₂ have been evaluated in ArKO (aromatase-knockout) male mice, in which estrogen deficiency is associated with a significant increase in lipopolysaccharide (LPS)-induced serum interleukin-6 (IL-6), tumor necrosis factor, monocyte chemotactic protein 1 and interferon- γ levels, all of which are significantly abrogated by administration of 16 α -

LE₂, but not 8β-VE₂. Furthermore, only 16α-LE₂ is effective in reducing arthritis severity in ArKO mice with collagen-induced arthritis (CIA), suggesting a dominant role of ERα in this process, although both ligands are effective in LPS-induced cytokine expression and antigen-induced arthritis (AIA) [55].

5-Androstenediol (**3**, Fig. 1) and 3β-androstanediol (**4**, Fig. 1) are two steroid hormone derivatives with an interesting ERβ-selective profile. 5-Androstenediol has a higher ERβ affinity (ERβ-RBA = 17%) with a 3-fold selectivity for ERβ, while 3β-androstanediol has a lower affinity (ERβ-RBA = 7%) and a comparable selectivity [56]; both compounds were tested in animal models.

5-Androstenediol was found to act as an ERβ-selective modulator by suppressing the inflammatory responses of microglia and astrocytes, which are responsible for the maintenance of homeostasis in CNS. Microglia seem to play an important role in experimental autoimmune encephalomyelitis (EAE). Furthermore, anti-inflammatory effects of ER-selective ligands within the CNS have been extensively evaluated in animal models of EAE [57]. After the observation that some ERβ selective with an indazole scaffold are effective in inhibiting inflammatory responses in microglia while E₂ and other ERβ ligands are not, a panel of ERβ natural steroids was screened for their ability to suppress induction of IL-6 in microglia upon LPS stimulation. Although most of the compounds have no activity, 5-androstenediol represses the expression of IL-6 in the substantia nigra, indicating that it can suppress microglia-mediated inflammation *in vivo*. Furthermore, this study demonstrates that the binding of 5-androstenediol to ERβ promotes the interaction of the receptor with the CtBP repressor, resulting in transcriptional repression that regulates microglia-mediated inflammation [58].

3β-Androstanediol modulates the LPS-induced inflammatory response in the arterial vascular wall *in vivo* in castrated mice. Interestingly, as observed above for 5-androstenediol, E₂ is ineffective on the same parameters modified by 3β-androstanediol, suggesting the existence of different ligand-selective pathways for the activity of ERβ [59]. Another study reveals this different behavior between 3β-androstanediol and E₂ in prostate cancer models *in vivo*, by using a bioluminescence imaging (BLI) assay. In this study, PC3-Luc cells were subcutaneously injected in the shoulder of BALB/c nu/nu nude mice, and after three weeks, a silastic pellet continuously releasing 3β-androstanediol was implanted into one group. Interestingly, after two weeks of treatment, tumor proliferation and metastatic spreading was significantly inhibited. It was hypothesized that the different effect of E₂ vs. 3β-androstanediol was due to a different conformation of Helix 12 imposed by the two ligands could lead to a differential co-regulator complex recruitment to the regulatory regions of the target genes [60]. Other evidence supports the important role of ERβ in prostate cancer: its apoptotic function seems to be mediated by the upregulation of FOXO3a, a member of the FOXO family transcription factors, which upregulates PUMA (p53-upregulated modulator of apoptosis), a potent pro-apoptotic factor. Athymic mice were injected with PC3-control or PC3-ERβ cells, and after 40 days visible tumors developed. Apoptotic bodies were found in ERβ-expressing tumors but not in control tumors. Treatment of PC3-cells with 3β-androstanediol led to an increase in the level of pro-apoptotic factor p53-upregulated modulator of apoptosis (PUMA). It is important to note that PC3 cells are

also non-responsive to E₂, as described above for the other two studies. Furthermore, this study shows that when paired with the ER α -selective agonist propyl pyrazole triol (PPT), 3 β -androstenediol no longer increases PUMA expression. The fact that PC3 cells fail to respond to E₂ may be explained by considering that simultaneous activation of the two ER subtypes could lead to opposing effects that neatly cancel out one another [61].

The positive results of these first *in vivo* experiments raise the interesting question concerning the stability of the 3-OH group present in these steroid derivatives, because it could be converted to the corresponding ketone by 3-hydroxysteroid dehydrogenases, thus producing metabolites with a lower affinity for the ERs but increased affinity for androgen receptor (AR) [62]. Further studies will be needed to verify the extent to which these compounds undergo metabolism *in vivo*.

In recent years, one of the main strategies used to selectively targeted estradiol derivatives has been to conjugate them to other specific molecules to try to avoid proliferative side effects due to ER α stimulation in sensitive reproductive tissues, such as breast and uterus. Among these E₂ conjugates are several considered as antiosteoporosis agents. A recent study reports the generation of a new female osteoblast-specific ER α KO mouse (pOC-ER α KO), which was used to clarify the estrogen signaling pathway in bone cells *in vivo*. pOC-ER α KO mice show decreased cancellous bone mass in the proximal tibia, vertebra, and distal femur, and decreased cortical bone mass in the tibial midshaft at 12 and 18 weeks of age, indicating that ER α in osteoblasts is required for appropriate bone mass and strength in female mice [63]. To overcome the ER α -related side effects and to selectively target estrogen effects in bones, different estradiol-conjugates were prepared. BTE₂-A1 (**5**) is a novel bone-targeted estrogen, which was obtained by conjugation of estradiol to a mimic of the tricarbonylmethane system of the tetracycline ring A, a portion that binds very well to the bone mineral hydroxyapatite. BTE₂-A1 shows a slight preference for ER α , with a RBA = 16% and, interestingly, shows a higher binding affinity to hydroxyapatite than that of tetracycline *in vitro*. After subcutaneous administration of this compound *in vivo*, a partial dissociation of the effects of estradiol in bone vs. uterus was noted, indicating that this bone-targeting strategy has some merit, although further studies will be required to clarify the mechanism of action [64].

Novel estrogen conjugates with integrin-binding RGD peptides, notably 3,17-estradiol-RGD tetrapeptides (**6–8**, Fig. 1) and 3-estrone-RGD octapeptides (**9–11**, Fig. 1), show high oral anti-osteoporosis activity in an ovariectomized mouse model. Serum calcium content and alkaline phosphatase (ALP) levels in mice given 110.3 nmol/kg of these compounds orally were significantly lower than those of mice receiving control estrogen, and both femur weight and femur ash and mineral levels of mice receiving control estrogen were lower than those receiving conjugates. Finally, these compounds showed a lower risk of endometrial hyperplasia and thromboembolic events [65]. In addition to these conjugates, 17-estradiol-RGD octapeptides (such as **12**, Fig. 1) and 3-estradiol-RGD octapeptides (such as **13**, Fig. 1) were evaluated and assayed as anti-osteoporosis agents in ovariectomized mice. These conjugates caused a reduction of serum calcium and ALP levels when compared to estrogen control, showed femur weights and femur ash, and higher mineral values. The anti-osteoporosis activity of these octapeptide derivatives was significantly higher than that of

estradiol and tetrapeptide analogues, while the risk of thrombogenesis and endometrial hyperplasia was lower, suggesting that the derivatization of estradiol with RGD peptides may be a good strategy to further increase the potency of these conjugates [66]. Unfortunately, the affinities and selectivities of compounds **6–13** for ERs have not been reported.

Another anti-osteoporosis compound was obtained by conjugation of E₂ with iminodiacetic acid (**14**, SE₂, Fig. 1), a well-known, high-affinity calcium chelator, in order to obtain a preferential distribution to bone. SE₂ shows a greater affinity for bone and a lower affinity for uterus, when compared to E₂, and in osteoblasts, it maintains high affinity for ER α , similar to that of E₂. Furthermore, in this study, SE₂ was effective in preventing OVX-induced bone loss in rats, decreasing bone turnover and improving bone mass and trabecular architecture [67].

Some interesting E₂-conjugates consist in poly(amide)polyamine (PAMAM) dendrimers (EDCs), in particular those linked by shorter tethers to PAMAM through a 17 α -phenylethynyl unit, show good affinity for the ERs, which are often comparable with those of the parent ligands alone, with a slight preference for ER α (for example, compound **15**: ER α -RBA = 3.8 %, ER β -RBA = 1.9 %, Fig. 1) [68]. Recently, EDC **15** was shown to promote cardiovascular protection in mice. In particular, this EDC interacts only with the membrane ER α receptor, thus showing exclusive non-nuclear action. Interestingly, in mice the EDC **15** does not stimulate proliferative effects due to ER α activation in sensitive reproductive tissues and, therefore, does not induce uterine or breast cancer growth. This selective effect is due to the highly preferential activation of the ERs present in extra-nuclear sites [69].

3.2. Benzopyrans

Many efforts to find new SERMs have focused on the design of benzopyran derivatives. In particular, McKie *et al.* [70] screened commercially available phenolic compounds using a cellular assay based on inhibition of the release of the cytokine interleukin-6 (IL-6) from an estrogen receptor-negative osteosarcoma cell line (U2OS), which had been transfected with ER α . The results of this first screening inspired the design and synthesis of a series of new potent benzopyran ER α ligands, with good receptor binding affinities, but devoid of undesirable proliferative effects on breast cancer MCF-7 cells. This study led to **16** (Fig. 2) as a potential lead compound for the prevention and treatment of osteoporosis and breast cancer. Compound **16** preferentially binds to ER α (RBA = 85%), and it showed an IC₅₀ value of 0.38 nM in the IL-6-based cellular assay, thus demonstrating an improvement over reference compounds such as tamoxifen and raloxifene, whereas the anti-proliferative activity on MCF-7 cell line was quite comparable to that of raloxifene (IC₅₀ = 7.7 nM). Moreover, when compound **16** was tested in immature or ovariectomized rat uterine bioassay, it did not increase significantly uterine weight, confirming the lack of unwanted uterine stimulatory activity [71]. In 2004, benzopyranone analogue CC-8490 of undisclosed structure (with a reported molecular weight of 586.05) belonging to this class of compounds, proved able to induce apoptosis in glioma cells in a time- and dose-dependent manner, and also to suppress tumor growth *in vivo* and prolong survival in malignant glioma xenograft

models [72]. Interestingly, the pro-apoptotic effect of CC-8490 seemed to be non ER-related, because the tested cell lines did not express estrogen receptors α or β , suggesting a mechanism which excludes the ER signaling pathway. Otherwise, a positive correlation between nuclear-factor (NF)- κ B activation and resistance to SERM-induced apoptosis was found, suggesting that the NF- κ B pathway acts as a pro-survival mechanism in glioma cells and its inhibition should accentuate the antiglioma activity of CC-8490.

These findings led Celgene Corporation, in partnership with the US National Cancer Institute, to start in 2003 a phase I clinical trial of CC-8490 in patients affected by recurrent or refractory high-grade gliomas [73] in the search for agents to treat brain tumors; this compound was found to be safe and well-tolerated. This trial is currently listed on clinicaltrials.gov as being completed, and CC-8490 is passed to a phase 2 trial, but no additional information was found on the Celgene Corporate website.

Compound **17** (LY3201, Fig. 2) [74] belongs to the cyclopentane benzopyran series, and it showed a high selectivity for ER β ($K_i = 0.44$ nM, $\beta/\alpha = 19$). Recent studies at Eli Lilly investigated the effects of LY3201 in brain and identified this derivative as a possible therapeutic agent for CNS diseases. Benzopyran **17** was administered to mice as subcutaneous pellets (0.04 mg/d), and after 3 days it was found to provoke a shift in the balance between excitatory and inhibitory signaling in favor of inhibition. This effect was mainly attributed to an increased production of γ -aminobutyric acid (GABA), which is one of the main inhibitory transmitters in brain. It also increased the rate of removal of glutamate, the primary excitatory neurotransmitter in central nervous system, which occurs by glutamine synthase in astrocytes that take up glutamate and convert it into glutamine [75]. Considering this potential anxiolytic action of compound **17** and the well-known involvement of estrogens on the serotonin system, further *in vivo* tests were performed to investigate possible effects on serotonergic neurons of the dorsal raphe, which are involved in anxiety and depression. In ovariectomized rats, where the enzyme responsible of serotonin synthesis, tryptophan hydroxylase (TPH), is down-regulated due to the absence of estrogens, treatment with LY3201 in a continuous-release fashion for 3 days caused an increase in the number of TPH-positive neurons; however, if the benzopyran **17** was administered 10 weeks after estrogen deprivation, it was no longer able to restore TPH-positive neurons. These findings suggest a potential use of this ER β -selective agonist in combination with selective serotonin re-uptake inhibitors to shorten the onset of their anti-depressant effect in postmenopause [76].

A further beneficial effect of ER-ligands against neurodegenerative diseases was demonstrated in an animal model of multiple sclerosis, consisting of an experimental autoimmune encephalomyelitis affected mice, where compound **17** caused a down-regulation of inflammatory pathways and led to a decreased mortality and a reduction of severity of symptoms in survived mice. Activated microglia, which express only ER β , are the cells responsible of immunological defense in the CNS. Upon treatment with **17**, microglial activation was markedly reduced, and the production of inflammatory molecules produced by microglia was also reduced. Moreover, T-cell reactivity was compromised by this ER β -selective activator, due to the induction of the enzyme indole-amine-2,3-dioxygenase, which causes T-cell death through depletion of tryptophan [77].

Piperidinoethoxyphenyl-substituted benzopyran **18** (Fig. 2) named CDRI-85/287, an ER ligand which was previously discovered and evaluated for its potent antiestrogenic activity in the uterus and anti-implantation properties in animal models [78–81], recently has attracted great interest for its growth inhibitory activity on breast cancers [82]. CDRI-85/287 in its racemic form displayed a slight preference for ER α over ER β , with low relative binding affinities of 0.25% for ER α and 0.15% for ER β , although previous studies assigned a higher ER binding affinity to the L-enantiomer [83]. Despite the low binding affinity for ERs *in vitro*, compound **18** inhibited the growth of the ER-positive breast cancer cell lines MCF-7 and T47D with IC₅₀ values of about 5 μ M, without exerting toxic effects on healthy cells, and the beneficial activity on breast cancer is reflected *in vivo* where at dose of 20 mg/kg the benzopyran prevented growth of MCF-7 xenografts in nude mice after 8 days of administration. The central role of ER α and ER β in the anti-cancer activity of compound **18** was confirmed by the loss of cytotoxicity when **18** was added to cells in which the expression of the two receptors was knocked down by using *si*RNA. Moreover, it has been demonstrated that CDRI-85/287 reduced the expression of ER α while it increased that of ER β , suggesting that the influence on ER expression may mediate, at least in part, its anti-proliferative activity on ER-positive breast cancer. In any case, the considerable difference between the potent activity *in vivo* and the modest binding to ERs may be attributed to benzopyran metabolites, which, unlike the parent compound, could bind with high affinity to the ERs and have potent biological activity. This compound exerted some anti-proliferative action by interfering with the EGFR/Akt pathway in the ER-negative human breast cancer cell line MDA-MB-231 and inducing apoptosis in cultured cells, while it arrested tumor growth in mice. This additional activity contributes to the ER-mediated mechanism, confirming the anti-tumorigenic potential of CDRI-85/287 in breast cancer [84].

Benzopyranobenzoxapanes **19–22** (Fig. 2) [85] were designed and synthesized as therapeutic agents to treat postmenopausal symptoms, and they are derived from a previous series of benzopyran derivatives discovered in 2006 [86], which were further developed by Johnson & Johnson to improve their stability, pharmacokinetic and pharmacological profiles. Compound **19**, the representative derivative of this class of chromenes, is an ER α -selective ligand with an IC₅₀ of 1.1 nM (ER β -IC₅₀ = 21 nM). This compound displayed excellent activity as an ER antagonist when tested in two cell-based functional assays, Ishikawa endometrial cell alkaline phosphatase secretion and MCF-7 breast cancer proliferation assay, with IC₅₀ values in the low nanomolar range. In the immature rat uterotrophic model, it was administered once daily (1 mg/kg) for 3 days, and it showed antagonistic behavior, exerting a 135% inhibition of uterus weight compared to control animals. Its di-methoxy analogue **22** is about 60-fold less active on ER α and does not bind ER β , with the result that it is inactive in *in vitro* assays, even though it retained strong uterotrophic inhibition. The two mono-methoxy analogues, **20** (R₂ = Me, Fig. 2) and **21** (R₁ = Me, Fig. 2), have modest binding affinities to ERs and weak potencies in cellular assays, which are definitely less than that of **19**. Interestingly, compound **21** has a better binding affinity for ER β (ER β -IC₅₀ = 7 nM) than the parent compound **19**, but it loses beta-selectivity, because it also binds ER α with a similar affinity (IC₅₀ = 9 nM). However, both the mono-methoxy compounds **20** and **21** showed strong inhibitory activities on uterus. Any other replacement of the piperidine ring on the amino side chain inverted activity in the

uterotrophic assay, so this basic moiety appears essential for its *in vivo* antagonism. Furthermore, at least one hydroxyl group (R_1 or R_2) is required for the binding to ERs, as demonstrated by the reduction in binding to ER when hydroxyl substituents R_1 and/or R_2 are methylated. Rather different effects were obtained when these four lead compounds were evaluated in pharmacokinetic studies, in which they were administered intravenously or orally to rats. The bisphenolic compound **19** had poor bioavailability, probably because of rapid metabolism *in vivo*. The di-methoxy analogue **22** by contrast, showed good bioavailability with improved absorption in tissues, and it is metabolized *in vivo* to mono-methoxy derivatives **20** and **21**, which in turn were tested and displayed excellent bioavailabilities, even superior to that of compound **22**.

After these encouraging results, compound **22** and its metabolites **20** and **21** were further evaluated in different animal models to test their tissue selectivity as potential SERMs. In the adult ovariectomized rat model, they were dosed orally once daily for 2 or 6 weeks, and the responses were typical of an ideal SERM: prevention of bone loss, decrease of plasma total cholesterol level and increase in vaginal fluidity without significant stimulation of the uterus. Moreover, in the ovariectomized rat hot flush model, compound **22** was able to reduce the rise in tail temperature, revealing it to be a suitable candidate to treat hot flushes and, generally, an optimal SERM candidate.

3.3. Cyclohexylphenols, cycloalkylene and diphenylmethane derivatives

Fluorinated cyclohexylphenol AC-186 (**23**, Fig. 3) was discovered by Acadia Pharmaceutical as a selective ER β agonist [87–88] and proved to be active *in vivo* against neurodegenerative diseases in mouse models of Alzheimer's disease and Parkinson's disease. In combination with a selective androgen receptor agonist ACP-105, in male gonadectomized triple transgenic Alzheimer's disease mice (3xTg-AD mice), AC-186 decreased amyloid- β levels in the brain by increasing the amyloid- β degrading enzymes neprilysin and insulin-degrading enzyme and improved cognition after 7 months of treatment [89]. Moreover, chronic treatment with the same combination led to increased levels of the androgen receptor in the brain, which is known to be beneficial for Alzheimer's disease, an disorder that is often characterized by a pathological reduction of testosterone and estrogen. The combination of the two androgen and estrogen receptor modulating agents modified spatial memory and decreased anxiety-like behavior in 3xTg-AD mice. In a rat model of Parkinson's disease, AC-186 **23** prevented motor, cognitive and sensorimotor gating deficits when it was administered 1 day prior to induction of substantia nigra lesions. In the same animal model, it exerted a gender-specific effect, protecting the viability and functionality of dopamine neurons in the substantia nigra only in male rats: this selective neuroprotection is under hormonal influence but the exact mechanism needs further investigation [90].

Fluorinated diaryl-substituted cyclic alkylidene derivatives such as compound **24** (Fig. 3) were developed as candidate ligands for positron emission tomography (PET) imaging of ERs [91]. Cyclopentylene **24** showed a good affinity for ER β , similar to that of estradiol, and also a good ER subtype selectivity (RBA = 88.6 %, β/α = 6.4) thanks to the lower affinity to ER α (RBA = 13.8%). Biodistribution studies of compound **24** labeled with ^{18}F

demonstrated a selective uptake in target ER-positive tissues, such as uterus, ovaries, kidneys and spleen, when compared to non-target tissues such as blood, muscle, but it had a certain degree of accumulation in the brain, probably because it is a small lipophilic molecule that can easily pass the blood-brain barrier; in addition, key controls to demonstrate that uptake was due to ER binding were lacking. Micro-PET imaging of [^{18}F]**24** in immature female rats confirmed uptake in liver, kidneys and urinary bladder, suggestive of selective elimination by a renal route.

On the other hand, among the diphenylmethane derivatives, a new ER α -selective antagonist was recently discovered, compound **25** (Fig. 3), showing an IC₅₀ value of 4.9 nM on ER α and of 140 nM on ER β [92].

3.4. Benzoxazole, indole and indazole derivatives

Researchers at Wyeth identified benzoxazole ERB-041(**26**, Fig. 4) as one of the most potent and selective ER β agonists, with a 200-fold selectivity for ER β and a low IC₅₀ value (5.4 nM) [93]. Due to its excellent binding affinity and selectivity, ERB-041 represented one of the most promising ER β ligands for therapeutic uses, and it was tested in two inflammation animal models with excellent results that encouraged its clinical development as a drug for the treatment of inflammatory pathologies [51]. In 2010, the first results of a phase II clinical trial were published [94]. The aim of this study was to verify the efficacy and the safety of ERB-041 in patients with rheumatoid arthritis. Although this compound was found to be safe and well-tolerated, the trial failed to show efficacy after oral administration of ERB-041 for 12 weeks, despite the very promising results previously obtained in animal models [95]. It is interesting to note that the ER α selective ligand ORG37663 (undisclosed structure) also gave disappointing results in a phase IIa clinical trial in postmenopausal female rheumatoid arthritis patients [96].

Another potential application of ERB-041 was for the treatment of endometriosis, which is considered an inflammatory disease characterized by ectopic growth of endometrial-like tissue throughout the peritoneum. ERB-041 showed interesting results in *in vivo* animal models of endometriosis, reducing lesion size in nude mice implanted with human endometrial tissue [97], and it was used in a phase II, double-blind, randomized, placebo-controlled trial in reproductive aged women to evaluate its safety and efficacy in reducing symptoms associated with endometriosis, at 75 mg and 150 mg doses [98]; no results have yet been published, however [99].

Recently, a new study indicates the efficacy of ERB-041 as potent skin cancer chemopreventive. In fact, topical application of ERB-041 substantially decreased UVB-induced skin tumor development in SKH-1 hairless mice. Remarkably, ERB-041 not only reduces cutaneous hyperplasia but also cytokine production, including IL6 and IL10. Probably, the response is not mediated by only estrogen response elements, but other transcription factors could play a significant role in this process. The observation that ERB-041 treatment decreases both WNT3a and WNT7b expression in immortalized human squamous cell carcinoma leads to the presumption that it may act by modulating the WNT signaling pathway [100]. While these results are intriguing, it is unclear whether some of the

protective activities seen in this study were not due to a “sunscreen” effect of the topically applied drug.

The indole scaffold has been exhaustively explored to produce new ERs ligands. Among them worth mentioning is KB9520, a compound patented by Karo Bio [101]. It has an undisclosed structure that belongs to the indole class (whose general structure **27** is depicted in Fig. 4), and it displays a 700-fold ER β selectivity. KB9520 has so far proved to be a promising antineoplastic agent in several *in vitro* and *in vivo* assays. The first published results of KB9520 in *in vivo* studies revealed that this compound showed a strong antiproliferative effect, suppressing lymphoma growth in male C57BL/6J mice subcutaneously engrafted with EG/cells, whereas the ER α -agonist PPT had no effect. Furthermore, KB9520 did not affect growth of the uterus, thus confirming its excellent ER β -selective profile also *in vivo* [102]. Later, KB9520 was found to be active *in vivo* for the treatment of cholangiocarcinoma (CCA), a relatively rare neoplasm expressing both ERs that has poor prognosis in most cases. In fact, daily treatment with KB9520 in a rodent model of CCA resulted in reduction of tumor liver infiltration and tumor-associated collagen deposition, without any significant toxic side effects [103]. Interestingly, in both studies the effect of KB9520 is abolished when it is co-administered with fulvestrant, suggesting an ER β -mediated antiproliferative effect of KB9520. Finally, KB9520 has recently found application in the treatment of malignant pleural mesothelioma (MMe), a rare aggressive and treatment-resistant cancer with very poor prognosis. Mice injected with cancer cells expressing endogenous ER β and treated with KB9520 experienced a decrease in tumor size and again KB9520 treatment was not toxic. Interestingly, ER β seems to interfere with the mitochondrial respiratory chain complex and mitochondrial ATP production in tumor cells [104].

Finally, chloroindazole **28** (Fig. 4), an ER β agonist with remarkably high selectivity [105], was recently found to reduce inflammation in microglia and, therefore, may be potentially useful for the therapy of neurodegenerative diseases driven by inflammatory pathways, such as Parkinson's, Alzheimer's, Amyotrophic Lateral Sclerosis (ALS), and Multiple Sclerosis (MS) [58].

3.5. Diarylethane derivatives

One of the most widely utilized ER β -selective agonist is represented by a diarylethane derivative, diarylpropionitrile (DPN, **29**, Fig. 1). This compound possesses a chiral center, but it has generally been studied as a racemic mixture, which demonstrates a 170-fold greater relative potency for ER β [106–107]. Recently, the single enantiomers of DPN (*S*-, and *R*-DPN, Fig. 5) were obtained by enantioselective synthesis, so that they could be separately assayed for binding affinity and transcriptional activity. The *R*-enantiomer (**30**, Fig. 5) displayed higher affinity (ER β -RBA = 32.6%) and selectivity (β/α ratio = 332) for ER β than its counterpart of *S*-configuration (**31**, Fig. 5, ER β -RBA = 9.7%; β/α ratio = 147) [108]. Therefore, *R*-DPN should now be considered as the preferred enantiomer for biological studies of ER β function.

3.6. Tetrahydrofluorenones

Among the tetrahydrofluorenone derivatives developed by researchers at Merck as ER β -selective ligands [109], two were tested in *in vivo* models for their antidepressant activity: SERM-beta1 (**32**, Fig. 6, chirality not specified, however it is likely to be the *S*-enantiomer) [109], a 129-fold ER β -selective ligand, and SERM-beta2 (**33**, Fig. 6), displaying a 176-fold preference for ER β . Due to their considerable lipophilicity, both compounds proved to be potent, selective and brain penetrant ER β -agonists, exhibiting antidepressant-like effects in the Porsolt forced swim test, by modulating tryptophan hydroxylase 1 (TPH1) expression in raphe nuclei, and stimulating neurogenesis in the dentate gyrus. These data suggest that ER β plays an important role in the regulation of mood disorders, since no increase in TPH1 mRNA expression is found in β -ERKO mice. Interestingly, in contrast to common antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs), which need several days of treatment to begin to exert their effects, SERM-beta1 and SERM-beta2 induce hippocampal neurogenesis after only 4 hours of exposure. Furthermore, these compounds do not have proliferative activity in MCF cells and on the immature rat uterus [110]. A better metabolic profile of SERM-beta2 over SERM-beta1 may be hypothesized, since the phenolic OH group, which generally undergoes an extensive phase II metabolic transformation (glucuronation, sulfation), is made less nucleophilic and less accessible by the Cl and F atoms occupying both ortho positions.

3.7. Isoquinolinones

Two ER β -selective isoquinolinone derivatives, β -LGND1 (**34**, Fig. 7) and β -LNGD2 (**35**, Fig. 7), developed by GTx Inc., when tested in animal models of induced obesity, were found to be potentially useful for the treatment and prevention of obesity. Both compounds show an ER β selectivity greater than 100-fold, without significant stimulation of any of the other receptors belonging to the nuclear receptor superfamily. In fact, β -LGND1 and β -LNGD2 induce proliferation in Ishikawa endometrial cells only at the highest concentration tested (1 μ M), whereas they fail to stimulate uterine growth *in vivo*. The cyano-substituted derivative (β -LGND1) repressed body weight gain induced by high-fat diet in mice, prevented loss in bone mineral content, and reduced the increase in white adipose tissue weight. Furthermore, it decreased serum cholesterol and leptin levels and, at the same time, prevented accumulation of fat in the liver. Moreover, it altered the expression of genes involved in energy homeostasis and adipogenesis. The bromo-substituted analogue (β -LNGD2), when submitted to the same assays, gave similarly good results. In fact, this derivative also showed promising results for the treatment of obesity in mice, being effective not only in the prevention of obesity in high fat diet-fed animals but also in its treatment in obese animals. *In vitro* and *in vivo* mechanistic studies indicated that the anti-obesity effects were due to inhibition of PARR- γ transactivation operated by ER β activation [111].

In addition, β -LNGD2 also showed interesting cardioprotective properties, suggesting that estrogens inhibit cardiac hypertrophy and progression in animal models and humans [58]. In particular, β -LNGD2 substantially prevented AngII-induced hypertension, cardiac hypertrophy and cardiac fibrosis in ovariectomized female mice. Interestingly, this compound was active in wild type (WT) mice but inactive in β -ERKO mice, thus confirming the key role of ER β in mediating this effect. β -LGND2 action prevents phosphorylation of

serine 632 of histone deacetylase 4, which is implicated in the modulation of cardiac disease [112]. More recently, β -LNGD2 was found to promote ER β -mediated *in vitro* and *in vivo* anti-angiogenic effects, highlighting application of this compound for the treatment of diabetic retinopathy and other similar ocular diseases arising from dysregulated angiogenesis [113].

3.8. Schiff bases: oximes and imines

The possibility of modulating ER β /ER α selectivity in a class of oxime derivatives was conducted by analyzing the effects of variously substituted aryl substituents bound to the salicylaldoxime central scaffold [114]. The evolution of these compounds led to the production of two ER β -selective ligands (oxime **36**: ER β -RBA = 4.21 %, β/α = 65; oxime **37**: ER β -RBA = 7.01 %, β/α = 64, Fig. 8) [115], displaying agonist properties on ER β . A further increase in ER β -selectivity was later obtained by a spatial inversion of the relative positions of the phenol and oxime groups of the central salicylaldoxime core of compounds **36** and **37**, generating new oxime derivatives (compounds **38** and **39**, Fig. 8). These two oxime derivatives display a very interesting profile of both ER β affinity and selectivity (**38**: ER β -RBA = 130 %, β/α = 29, **39**: ER β -RBA = 87.1 %, β/α = 46), and profiled as full agonist on ER β , with a remarkable beta-selectivity in the functional assays [116]. In particular, ER β -EC₅₀ values were found to be 0.23 nM for **38** and 1.3 nM for **39**. Furthermore, **39** shows a remarkable functional subtype selectivity, with a β/α transcription potency ratio 50-fold higher than that of estradiol.

A series of triaryl-imines produced compounds with similar binding affinities for ER α and ER β ; however, they generally show a very interesting ER α -agonist and ER β -partial agonist/antagonist profile. In particular, representative imines containing a 2-chlorophenyl substituent on the nitrogen atom (**40** and **41**, Fig. 8) displayed ER α -EC₅₀ values of 1 nM and 0.1 nM, and ER β -IC₅₀ values (in the presence of 10 nM E₂) of 19 nM and 6 nM, respectively. X-ray analysis of these triaryl-imines in the ER α ligand-binding domain revealed that they cause a distortion of this pocket, enlarging the separation of helices 3 and 11, which seems to affect the stability of the C-terminal AF2 portion of ER that is involved in the recruitment of coactivators [117].

3.9. Carboranes

Among carboranes containing phenol substituents were several derivatives having good binding affinities for the ERs, and a recent study showed that *o*-carboranes, but not *m*-carboranes, are ER β -selective ligands. In particular, *n*-propyl-substituted *o*-carborane **42** (Fig. 9) shows an interesting 7.4-fold beta selectivity in ER-binding assays, with RBA values of 11.8% on ER α and of 87% on ER β [118]. Most importantly, this compound displayed only moderate estrogenic activity in MCF-7 cells and, therefore, may constitute a promising starting point for the development of ER β -selective carborane-containing ligands. Furthermore, *C,C'*-difluoro-*p*-carboranyl phenol **43** (Fig. 9) was reported to have a higher beta selectivity (10-fold), though a lower binding affinity (ER β -RBA = 50%) than **42** [119].

3.10. Phytoestrogens

Genistein (**44**, Fig. 10) is an isoflavone usually found as the glucoside genistin in soybeans that is cleaved to aglycone by bacterial β -glucosidases in the gut. This benzopyran-based natural compound is widely reported as an ER β selective ligand (ER β -RBA = 36%, ER α -RBA = 5%) [120], and it has been investigated as a therapeutic agent in different animal models. Some studies were designed to assess the possible beneficial effects of genistein on skeletal muscle structural composition in a model of the decline in skeletal muscle mass and muscle strength that affects postmenopausal women due to decreased estrogen levels. In ovariectomized mice, genistein normalized the expression of isoform I of myosin heavy chain molecule (MHC-I), characterized by slow contracting velocity and low force production, in absence of high intensity exercise, whereas the fast type isoform 2 (MHC-II) expression was increased after treatment with genistein in a group of mice exposed to high intensity exercise. These findings suggested that genistein increased the muscle composition in fast fibers in the presence of a high intensity exercise and that this effect is mainly mediated through ER β [121].

ER β influence on skeletal muscle was further evaluated in an animal model of muscle injury induced by injection of notexin, a myotoxin that provokes skeletal muscle damage and impairment of neuromuscular transmission in ovariectomized rats. A high serum level of creatine kinase activity was used as an indicator of muscle injury, and daily treatment with genistein significantly contributed to its reduction. On the other hand, the same injury provoked in ER β -knockout mice led to an evident increase of creatine kinase activity. When embryonic myosin heavy chain (MHC) was analyzed as a marker for the formation of new muscle fibers and for muscle repair, genistein-treated ovariectomized animals showed a marked increase of MHC expression, whereas it was reduced in ER β -knockout mice. These data collectively underlined the beneficial involvement of ER β activation for skeletal muscle growth and regeneration [54].

There are many reports in the literature about neuroprotection exerted by genistein *in vitro* and *in vivo*, and in a rat model of Alzheimer's disease (AD), where A β -lesioned mice were pre-treated with genistein, the deficits in learning, memory and recognition were evidently reduced. Treatment with the ER antagonist fulvestrant abolished the beneficial effects of genistein, confirming that the tested phytoestrogen acts through an estrogenic pathway [122]. A recent study confirmed the preventive role of genistein in the establishment of AD pathology: genistein reduced the brain deposition of A β plaques by increasing A β clearance in two different transgenic mouse models of AD, APP23 mice with genetic deficiency of aromatase in which the brain contains non-detectable levels of estrogens, and in ovariectomized APP23 mice, which still contain a certain level of estrogens in their brains. The low estrogen concentration in APP23 mice with genetic deficiency of aromatase is characterized by a more severe formation of A β plaques and an extensive AD neuropathology than that found in ovariectomized mice, suggesting that the deficiency of endogenous estrogens in the brain could increase the risk of Alzheimer's disease. This estrogen-deficient mouse model was even more sensitive to the reduction of plaque formation by genistein than by endogenous estrogens, thus supporting the beneficial effect of this exogenous phytoestrogen [123]. An intriguing study of genistein on brains of

ovariectomized rats showed that genistein mimicked the estradiol-induced effects by enhancing place learning, but it also impaired response learning compared to untreated animals. Hence, the estrogenic action of genistein is probably site-specific, according to the brain regions that are critical for each cognitive task [124]. Genistein proved to be effective in protection from neuronal death after ischemia, if given few minutes after injury in an animal model of global cerebral ischemia, and this neuroprotective action involves ER β , as demonstrated by the total loss of the genistein-mediated effect when a selective ER β antagonist was administered before ischemia induction [125].

Certain anti-cancer effects of genistein have been reported in many studies. For example, it showed antiproliferative and antiapoptotic activities via ER β in colorectal cancer *in vivo* [126]. Moreover, genistein administration in mice prevented the formation of well-differentiated prostate carcinoma [127]. Some rather different effects of genistein in cancer have also been found, such as the recent report that genistein increased metastatic spread in two different xenograft models of prostate cancer [128]. These contrasting effects of genistein in prostate cancer models may be understood by considering that *in vitro* genistein at physiological doses has some agonist activity on certain androgen receptor mutants, which are responsible for growth and progression of advanced prostate cancer, whereas at higher doses it inhibits proliferation of the same cells [129]. The inhibitory effects of high genistein concentrations may not be ER dependent, as this compound is known to be a receptor tyrosine kinase inhibitor, though a relatively weak one [130].

A variety of many other biological effects of genistein in animals are well documented in literature, exemplified by its ER β -mediated detrimental effects on mandibular condylar cartilage, which is made thinner after oral administration of genistein to female rats [131]. A protective role of genistein on dental pulp has been observed *in vivo*; so, it might prove useful as a capping agent to decrease the inflammation at the site of pulp exposure [132]. In an animal model, genistein had effects on sexual behavior, which was impaired when male rats were exposed to genistein in the neonatal period [133].

Genistein proved to be effective in preventing and treating diabetes, by maintaining the function and stimulating the proliferation of pancreatic islet β -cells in animal models. However, considering the multiple mechanisms of action of this versatile isoflavone, it is plausible that different non ER-mediated mechanisms may contribute to the anti-diabetic effect [134]. A study of the regulation of energy homeostasis in obese female ovariectomized Wistar rats fed a high-fat diet showed that genistein improved the utilization of glucose and lipids. In fact, triglyceride accumulation was reduced in liver and muscle, and hepatic glucose uptake was increased, without any significant effect in adipose tissue, probably due to the low concentration of genistein in this tissue. Thus, lipid and glucose metabolism is generally affected positively when ER is activated by genistein [135]. Many clinical studies involving genistein are presently ongoing or completed, some involving, for example, the treatment of neurodegenerative diseases, breast, prostate, bladder and colorectal cancer, metabolic syndrome, etc., to further explore the multifaceted pharmacological profile of this phytoestrogen [73].

Many examples of combinations of different phytoestrogens have been explored for the treatment of several pathologies [136]. For example, genistein when administered together with two other ER β -selective ligands, daidzein (**45**, Fig. 10) and equol (**46**, Fig. 10), resulted in a synergistic neuroprotective effect *in vivo*, greater than the effects of the single agents, yet without affecting the reproductive system. Daidzein **45** is a second isoflavone abundant in soybeans, whereas equol **46** is produced from daidzein by bacteria in the gut of certain individuals (in particular the *S*-enantiomer is formed in humans). Many studies were conducted to determine whether the positive health effects of soybeans could be attributed to the metabolic product equol, but the results were often variable, and they did not take into account the variability in equol production by different individuals.

The combination of genistein with daidzein and equol increased mitochondrial bioenergetic function and led to the overexpression of mitochondrial antiapoptotic proteins and of A β -degrading enzymes in the brain of adult female ovariectomized rats. Fortunately, the combination of phytoestrogens had no relevant effect on the uterus. *In vitro*, the ER β binding of isolated genistein is higher than that of the other two phytoestrogens, whereas the ER β selectivity of the equal molar mixture of genistein/daidzein/equol is increased (β/α 82.6) when compared to genistein alone (β/α 60.0), suggesting that the mechanism by which they exert their positive combination effects against neurodegeneration is more complex than a simple sum of individual effects, perhaps revealing some complex competitive and synergistic interactions among them [137].

This phytoestrogenic combination, referred as phyto- β -SERM formulation, was extensively studied and tested *in vivo*. For example, in 2011 the research group of Zhao *et al.* reported that the phyto- β -SERM formulation alleviated the typical menopause symptoms in an ovariectomized mouse model of human menopause. The formulation prevented the rise in skin temperature, hair loss and deficit in spatial memory function, and reversed the decline in neurological functionality caused by hormone loss. Notably, by comparison, a simple diet enriched with soy extract had no effect on these ovariectomy-induced changes [138]. The therapeutic potential of this formulation was confirmed in a transgenic mouse model of AD: when phyto- β -SERM administration was started before the appearance of pathology, the formulation alleviated the typical neurological symptoms associated with the disease and attenuated the formation of A β plaques [139]. All these studies on phyto- β -SERM formulation included racemic equol, but recent findings have shown that no significant differences if only the enantiomer *S*-equol is used in its place [140].

Coumestrol **47** (Fig. 10) is a phytoestrogen derived from sprouting plants like alfalfa, clover and beans, and it belongs to the coumestane family. It is a potent ER β and ER α ligand, with binding affinities comparable to those of 17 β -estradiol [120]. Coumestrol displayed a neuroprotective effect when administered before and after global cerebral ischemia in a mouse model; however, this effect was only partially reversed by an ER-antagonist, suggesting that other non ER-mediated mechanisms may be influencing the neuroprotection [141–142].

ER β -selective flavonone liquiritigenin **48** (Fig. 10), isolated from the root of *Glycyrrhiza uralensis*, is an active component of MF101 (MenerbaTM), a plant extract of 22 herbs

traditionally used in Chinese medicine to treat vasomotor symptoms (hot flushes) associated with menopause. Despite the mixed composition, Cvorovic *et al.* demonstrated that MF101 is ER β -selective, because it specifically activated an ER β estrogen response element leading to specific gene activation, although no binding preference for this receptor subtype was observed with this extract [143]. MF101 at the dose of 10 g/day for 12 weeks induced a significant reduction of frequency of hot flushes in postmenopausal women and appeared to be safe and well tolerated, and without any effect on the uterus level in a phase 2 clinical trial [42]. Presently, MF101 is under evaluation in a phase 3 clinical trial in collaboration with Bionovo Inc. [144], which previously patented this herbal extract [145]. Moreover, MF101 has been clinically evaluated for improving sleep quality in postmenopausal women [146]. Liquiritigenin also has a tumor-suppressor function, demonstrated by the reduction of the tumor size in a xenograft model of glioma, with a concomitant increase of nuclear ER β expression [147].

Caffeic acid phenylethyl ester **49** (Fig. 10), a component of beehive propolis, is a natural compound known for its several pharmacological and biochemical properties [148]. Estrogen receptor binding affinity assays of this ester derivative of caffeic acid revealed an IC₅₀ of 0.12 μ M on ER α and of 0.049 μ M on ER β , thus showing good selectivity for ER β . Moreover, it decreased ER α expression in breast cancer cell lines without promoting cell proliferation in MCF-7 cells, and *in vivo* it caused no increase in uterine weight relative to control [149].

An estrogen-like effect was observed with medicarpin (**50**, Fig. 10), a member of the pterocarpan subgroup of isoflavones, which are infection-induced phytoalexins usually present in many dietary legumes. Medicarpin demonstrated positive effects in skeletal growth *in vivo*, by stimulating osteoblast differentiation and mineralization, an action selectively mediated by ER β , as demonstrated by siRNA knockdown of ER β in osteoblasts. This estrogenic action was devoid of any stimulatory effects on the uterus or stimulation breast cancer cell proliferation. Interestingly, like 17 β -estradiol, medicarpin exerted an osteoprotective effect against bone loss after ovariectomy, preventing premature T cell senescence and enhancing CD28 expression, a membrane glycoprotein considered the phenotypic marker of cellular senescence [150–151].

Zearalenone **51** (Fig. 10) is a mycotoxin produced by numerous *Fusarium* species, and it is found as a contaminant in mold-infected maize, wheat and oats. This structurally intriguing compound is a resorcylic acid lactone, consisting of a phenolic ring fused to a 14-membered macrocyclic lactone, and it has binding affinities for ER α and ER β with IC₅₀ values in the nanomolar range (ER α -RBA = 4.3%, ER β -RBA = 6.0%) [152]. When given to ovariectomized mice, zearalenone increased uterine weight. Orally administered zearalenone is rapidly adsorbed and biotransformed, producing reduced metabolites that likely contribute its estrogenic action, and an interaction between zearalenone and a mixture of soybean isoflavones was reported *in vivo* in pre-pubertal female pigs [153].

Silymarin is an extract of the milk thistle (*Silybum marianum*) composed of a mixture of flavonoids and polyphenols, like silibinin, isosilibinin, silichristin and silidianin. Among them [154], silibinin **52** (Fig. 10), an ER β -selective ligand, is the major bioactive component

of this natural extract, and it is endowed of anticancer, antioxidant and antiinflammatory properties. Male mice fed with a silymarin-enriched diet experienced a reduction in the progression of colorectal cancer and showed increase ER β expression in the intestinal mucosa, suggesting the protective role that this receptor plays in this aggressive tumor. Moreover, the combination of silymarin with lignin showed a more potent, synergistic effect in suppressing intestinal tumorigenesis [155]. Interestingly, other ER-related effects of silymarin *in vivo* are improved endothelial dysfunction, which could prevent cardiovascular diseases in menopause [156], and antiosteoporotic activity in ovariectomized rats, but these are associated with a mild increase in uterine weight [157–158]. In a rat model of Parkinson's disease, silymarin exerted a neuroprotective effect, which appears to be ER mediated because it was partially reversed by fulvestrant administration [159]. However, the complex composition of silymarin makes it difficult to determine which biological effects can be ascribed with certainty to silibinin or to other components of this extract.

Diarylheptanoid **53** (Fig. 10) was isolated from *Curcuma comosa* Roxb., a plant of the Zingiberaceae family [160], and it has been traditionally used by menopausal women in Thailand for its estrogenic activity. The activity of **53** on ER β gene expression in HeLa cells is of 75% relative to estradiol (which is set at 100%), and is similar to that of genistein. This compound was later found to stimulate estrogen-regulated genes in MCF-7 breast cancer cells, predominantly by agonist action on ER α , as determined by several cellular studies and supported by molecular modeling [161–163]. Unfortunately, compound **53** caused marked uterine stimulation in mice, although less so than that of 17 β -estradiol. This action was ER α -mediated, because it was blocked by an antiestrogen and not observed in ER α -knockout mice. ER α is highly expressed in endothelial cells, where it mediates the effects of estrogens in the vascular endothelium. Long-term treatment of ovariectomized rats with diarylheptanoid **53** increased ER α levels and endothelial nitric oxide synthase (eNOS), leading to relaxation of the rat aorta, effects of potential benefit to menopausal women [164].

3.11. ER ligands used in positron emission tomography (PET)

For use in PET studies, particularly imaging ER in breast cancers, some ERs ligands have been purposely modified by introducing radionuclides, such as fluorine-18 or bromine-76 [165–169].

Recent studies, focused on the development of ER β -selective ligands for PET, involved the synthesis of 8 β FEE₂ (**54**, Fig. 11), derived from the ER β selective ligands 8 β VE₂ (**1**, Fig. 1), and its evaluation in *in vivo* model of immature female rodents, and of Br-041 (**55**, Fig. 11), belonging to the class of benzoxazole derivatives with the same scaffold of ERB-041 (**26**, Fig. 4). 8 β FEE₂ maintains good selectivity for ER β ($\beta/\alpha=170$), but its affinity is 10-fold lower than that of 8 β VE₂, whereas Br-041 shows about the same beta-selectivity of ERB-041 ($\beta/\alpha \sim 500$), with a 16-fold higher ER β affinity (ER β -RBA of Br-041 is 50.3% vs. 3.22% of ERB-041) [170]. Efforts PET agents for the *in vivo* imaging of ER α and ER β are motivated by the prognostic value of the expression level of this receptor in cancer patients [165]. It is known, for example, that levels of the two ER subtypes change during the progression of breast cancer, so the independent measurement of both ERs might be useful

in predicting disease outcome and response to endocrine therapies [171–172]. Unfortunately, despite efforts so far to obtain ^{18}F -labeled compound **54** and ^{76}Br -labeled compound **55**, little evidence of ER β -mediated uptake was observed in model systems, underlining the need for improved *in vivo* models to develop ER β -selective radiopharmaceuticals for use as PET imaging agents to measure ER β levels in breast tumors.

4. Conclusions

From a clinical perspective, ER β -selective agonists might be therapeutically useful for some types of cancer, hot flashes and several other inflammatory diseases. Since most of the undesirable proliferative effects of estrogens involve activation of ER α , fewer efforts have been devoted to the development of pharmaceuticals that ER α -selective agonists than for ER β -selective agonists. Nevertheless, ER α undoubtedly has a significant role in preventing osteoporosis, obesity, cardiovascular diseases and diabetes. Therefore, tissue-selective or non-nuclear-selective ER α agonists may prove to be safer, provided they stimulate ER α in tissues such as bone, fat, and vascular endothelium, but not in breast or endometrium. On the other hand, ER α antagonists have already proved to be effective agents for inhibiting cell proliferation in hormone-sensitive breast cancer. Overall, a deeper knowledge of the diverse biological roles of ER α or ER β in different tissues, developed through future studies, will help in the decision of which receptor subtype should best be targeted for the management or cure of various individual pathological disorders.

As efforts to discover and develop new and more specific subtype-selective ligands continue to be made, whether they are agonists or antagonists, several longstanding challenges should be recognized. One relates to the fundamental role played by the phenolic hydroxyl groups that are nearly ubiquitous in ER-binding compounds, since their presence constitutes a major metabolic liability. As a class, phenolic compounds are often characterized by poor pharmacokinetic properties (low bioavailability, short half-life, etc.), because their OH functional groups generally undergo a rapid phase II sulfation and glucuronidation. Adequate consideration should thus be given to bioisosteric replacements for these groups or other strategies, such as the insertion of electron-withdrawing or sterically-hindered groups close to the phenol OH portion, to moderate the phase II metabolic conjugation process, provided these modifications do not compromise the receptor binding. Alternatively, many effective ER-targeted drugs, such as tamoxifen used in breast cancer therapy or mestranol used in oral contraceptives, are prodrugs in which the phenolic function is either missing or masked by derivatization, respectively, but then is revealed by specific hydroxylation or metabolic cleavage. These prodrugs typically have better pharmacokinetic properties than their active metabolites. General guidance from the literature suggests that the development of effective ER β -selective agonists will be most successful by designing non-flat non-symmetric molecules, which are able to fully exploit the opportunities for weak hydrophobic attractive interactions offered by the ER β binding pocket while, at the same time, avoiding promiscuous interactions with other steroid receptors or steroid-modifying enzymes [173].

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ABBREVIATIONS

ER	estrogen receptor
ERα	estrogen receptor subtype alpha
ERβ	estrogen receptor subtype beta
LBD	ligand binding domain
RBA	relative binding affinity

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HIGHLIGHTS

- Estrogens exert beneficial actions in bone, brain, and urogenital tract, but they can have serious adverse effects in breast and uterus.
- The actions of estrogens are mediated by two receptor subtypes (ER α and ER β).
- The development of subtype-selective ligands is challenging due to the similarity of the ligand binding pockets in ER α and ER β .
- The most promising ER-ligands for clinical use are those eliciting an ER β -selective activation.
- So far, in contrast to SERMs, no ER β -selective agonists have been successfully introduced into the clinic.

Fig. 1.
Steroids derivatives.

Fig. 2.
Benzopyran derivatives **16–22**.

Fig. 3.
Cyclohexylphenol **23**, cyclopentylene **24**, and diphenylmethane derivative **25**.

Fig. 4.
Structure of ERB-041 **26**, KB9520 **27** and chloroindazole **28**.

Fig. 5.
Separate enantiomers of DPN.

Fig. 6.
Tetrahydrofluorenone derivatives.

Fig. 7.
 β -LGND1 and β -LNGD2 compounds.

Fig. 8.
Schiff base derivatives: oximes (**36–39**) and imines (**40–41**).

Fig. 9.
Carborane derivatives.

Fig. 10.
Phytoestrogen compounds **44–53**.

Fig. 11.
ERβ selective ligands useful for PET studies.