Advances in the Development of Prodrugs as Selective Modulators of Estrogen Receptors

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Abstract
Due to the complexity of estrogen signaling mediated by estrogen receptors (ERs) in a variety of biological environments, there is great interest in the identification and optimization of selective estrogen receptor ligands. Prodrugs that can be activated in specific environments allow for tissue selectivity. Therefore, there have been recent advances in the development of prodrugs for ERs that can be released through enzymatic reactions, chemical reactions (eg, oxidation by reactive oxygen species or reduction by ascorbic acid), or light-mediated processes. In addition, researchers have linked ER ligands to additional drugs for selective cellular targeting. In this review, we highlight the compounds that have been generated and their potential uses in disease states such as breast cancer, inflammation, and menopause.

Key Words: estrogen receptor ligand, prodrug, masked ligand, estrogen receptor, SERM

Abbreviations: 4-OHT, 4-hydroxytamoxifen; DHED, 10(17)-dihydroxyestra-1,4-dien-3-one; DOX, doxorubicin; ER, estrogen receptor; LSD1, lysine specific demethylase 1; SERM, selective estrogen receptor modulator.

Literature for this review was retrieved from searches of the PubMed, Google Scholar, and Web of Science electronic databases between March and June 2022, using search terms prodrugs of estrogen receptor, enzyme-activated estrogen receptor ligands, masked estrogen receptor ligands, activation of estrogen receptor ligands. Only articles written in English were included and there were no restrictions in year of publication. The references were selected based on the judgment of the authors and citations and bibliographies of the original references were also examined.

Estrogen Receptor
Estrogen signaling is a complex and essential biological process that mediates reproduction, cardiovascular health, bone development, and neurological functions. Estrogens influence cellular processes through molecular interactions with estrogen receptors (ERs) found in the nucleus, cytoplasm, or plasma membrane. Two isoforms of ER exist: ERα and ERβ, which have distinctive patterns of tissue distribution and transcriptional regulation [1]. There are high ERα levels in reproductive cells, such as uterus, ovary, and mammary gland cells, and lower levels in bone, brain, liver, and vascular cells; ERβ, however, is typically found in reproductive tissues at lower levels than ERα but is the predominant receptor in the brain, lung, and colon [2]. Loss of estrogen or its receptors as well as increased activity of ERs are linked to the development and progression of a variety of diseases, including cancers (breast cancer, ovarian cancer, colon cancer, endometrial cancer, and prostate cancer), osteoporosis, neurodegenerative diseases (stroke, Parkinson disease, Alzheimer disease, multiple sclerosis, etc), cardiovascular disease, and obesity [3]. Estrogens have been used clinically as fertility treatments, contraceptives, and hormone therapies in menopause. However, they can cause issues in some patients due to unintended regulation of ER function in other target tissues, including the development of breast and uterine cancer [4]. Because of conflicting positive and negative effects of estrogens on different tissues, there has been much interest in the development of selective estrogen receptor modulators (SERMs) and subtype-selective ligands that are context-selective as safer and more effective pharmaceuticals. Unlike estrogen itself, which functions as an agonist in multiple contexts, these molecules have the ability to selectively act as agonists or antagonists in tissue-specific manners. The specificity of these ligands is derived from the expression levels of ERα vs ERβ as well as the differential expression of co-regulatory proteins and varying conformational changes of ER induced by ligand binding [5]. Development of these ligands has been very advantageous for the treatment of breast cancer and osteoporosis as well as ameliorating menopausal symptoms. However, adverse effects, including blood clotting and carcinogenesis, have been observed [4]. Therefore, due to the complexity of ER signaling and unavoidable side effects, context-specific estrogens are continually being developed as potential chemical probes or pharmacophores.

Prodrugs
Prodrugs are inactive derivatives that undergo an enzymatic or chemical transformation within a biological environment to release the active parent drug. It is estimated that...
approximately 10% of drugs worldwide can be classified as prodrugs [6]. Typically, prodrugs are designed to increase solubility, stability, and selectivity of an active drug or improve absorption and time profiles or decrease off-target effects. As seen in Fig. 1, there are many strategies for prodrug design. The simplest scheme (Fig. 1A) utilizes a masking group that interferes with the activity of the drug that upon release generates an inert byproduct and the active drug. In addition, the masking group itself can be a secondary active ingredient that upon release provides synergistic activity (Fig. 1B). Alternatively, a new functional group can be installed (Fig. 1C) as a result of a biological transformation which provides the intended activity. In all cases, the prodrug must be modified in a biological context to generate the active drug(s). Strategies for activation include general metabolism such as oxidation and reduction, hydrolysis, specific enzymatic reactions, chemical reactions, and light (Fig. 1D) [6].

The idea of using prodrugs to target ER is not a new approach. Indeed, some of the commonly used regulators of ER are delivered as prodrugs. In 1957, the U.S. Food and Drug Administration (FDA) approved the first hormonal contraceptive pill which contained mestranol, a methyl ether version of ethinyl estradiol that is demethylated to ethinyl estradiol in the liver [7]; see Fig. 2. In addition, estrogen sulfamates have been developed as orally active and long-acting estrogens with high systemic activity requiring hydrolysis for ER binding [8]. The SERM tamoxifen, first approved in 1977 by the FDA for the treatment of metastatic breast cancer and currently used to treat all stages of hormone-responsive breast cancers, is readily metabolized by cytochrome P450 enzymes to 4-hydroxytamoxifen (4-OHT) as illustrated in Fig. 2; 4-OHT has a 30- to 100-fold higher affinity for the ERs than tamoxifen itself and is significantly more potent in inhibiting estrogen-dependent cell proliferation [9-11]. However, these historical prodrugs of ER ligands were serendipitous and not necessary intentionally designed. In this review, we will highlight recent advances in the development of purposely designed prodrugs of ER ligands for context-specific regulation of receptor activity. ER ligands are primed for prodrug design because of the necessity of a phenol to mimic the A-ring of 17β-estradiol; in examination of various estrogenic compounds bound to the ERs, this phenol forms
important hydrogen bonding interactions [12]. By blocking this site with a masking group that can be released in a specific context (Fig. 1), a prodrug can be designed.

**Boronic Ester–Masked Estrogen Receptor Ligands**

Boronic esters have been developed as masking groups for a variety of small molecules because they readily react with reactive oxygen species such as hydrogen peroxide, hypochlorite, and peroxynitrite to release the corresponding alcohols or phenols [13]. This prodrug strategy has promise for inflammatory diseases such as multiple sclerosis, rheumatoid arthritis, amyotrophic lateral sclerosis, ischemia-reperfusion injury, and Alzheimer disease, which typically have an overproduction of certain reactive oxygen species, including hydrogen peroxide [14]. In addition, there is increased oxidative stress in tumor microenvironments that results in increased levels of reactive oxygen species [15]. Due to the role of ER in both cancer and inflammatory diseases, boron-based prodrugs of ER ligands have recently been developed.

Jiang et al designed boron-based prodrugs of 4-OHT that were able to inhibit growth of estrogen-dependent breast cancer cell lines (MCF-7 and T47D) but not the triple-negative breast cancer cell line (MDA-MB-231) similar to 4-OHT itself [16]. They observed sufficient conversion of the prodrugs into 4-OHT after incubation with the elevated concentrations of H₂O₂ in the breast cancer cell lines. Further evaluation in a mouse xenograft model of MCF-7 cells showed that the compound 1 (Fig. 3) had increased oral bioavailability in comparison to tamoxifen, and because it does not require metabolic hydroxylation by cytochrome P450s, the plasma concentrations of 4-OHT were 30- to 40-fold greater upon intraperitoneal or oral administration, allowing for a lower treatment concentration for inhibition of tumor growth [17]. The same group similarly masked the SERM endoxifen as a boronic ester (compound 2, Fig. 3) and observed potent inhibition of cell growth after activation in MCF-7 and T47D cells, increased plasma levels of endoxifen after oral administration to mice, and effective inhibition of MCF-7 breast tumors in mice [18]. The authors have not reported direct binding affinities of compound 1 or 2 with ERα, but the size and characteristics of the boronic ester in place of the A-ring phenol suggest that the interaction would be diminished. The group then applied a similar strategy to the selective estrogen receptor downregulator (SERD) fulvestrant, designing a boronic acid prodrug (compound 3, Fig. 3) that showed superior bioavailability and efficacy in mouse and rat studies [19, 20]. Interestingly, compound 3 was able to bind to ERα with a similar affinity as fulvestrant without conversion to the phenol; presumably, the boronic acid is able form similar interactions in the binding pocket as the phenol. The preclinical trials with these compounds show promise for boronic ester masked estrogens in breast cancer contexts, especially regarding prolonged bioavailability.

Our group expanded this strategy to the ERβ-selective agonist diarylproprionitrile (DPN) by masking both phenols as boronic esters as seen in compound 4 in Fig. 3 [21]. Masking decreased in vitro binding affinity for ERβ by approximately 20-fold and transcriptional activation of the receptor by greater than 500-fold. However, in the presence of exogenous hydrogen peroxide at pathological concentrations, the agonistic activity was restored. Selective activation of ERβ could have promising implications for neuroinflammation and the development of additional boron-based prodrugs could allow for regulation of ER activity in contexts where there is increased reactive oxygen species. In any context, a better understanding of the mechanism and kinetics of activation in vivo as well as the selectivity for specific tissues must be examined; previous reviews on prodrugs activated by reactive oxygen species have outlined limitations and issues to address moving forward in the development of this technique [14, 15].

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Figure 2. Historically used prodrugs of ER with masking groups highlighted in blue. A, Mestranol is demethylated in vivo to generate ethinyl estradiol. B, Tamoxifen is hydroxylated by the cytochrome P450 enzymes, CYP2D6, to generate the stronger ER ligand 4-hydroxytamoxifen.
Enzyme-Activated Release of Estrogen Receptor Ligands

Some recent work has focused on identifying and optimizing molecules that can be catabolized by specific enzymes present in the target location where ER activity is to be modulated. The identification of enzymes to utilize in a specific context can be challenging; however, initial reports for both menopause symptoms and breast cancer have illustrated that enzyme-activated release of ER ligands could be a promising strategy.

In both surgically induced and natural menopause, women experience a sharp decrease in circulating estrogen and are often faced with significant neurological and psychiatric side effects due to decreased levels of 17β-estradiol in the brain. Therapies that reintroduce estrogen can have significant side effects, including risk for thromboembolism, coronary heart disease, stroke, breast cancer, and the reactivation of estrogen-modulated organs [22]. Therefore, an estrogen prodrug, 10β,17β-dihydroxyestra-1,4-dien-3-one (DHED) (compound 5, Fig. 4) that is converted into 17β-estradiol selectively in the brain has recently been investigated [23]. The para-quinol of the A-ring is rapidly reduced back to an estrogen via a short chain NADPH-dependent dehydrogenase/reductase (SDR) only found in the central nervous system [24]; this SDR has previously been linked to neuroprotection [25]. Prokai et al found that DHED treatment decreased symptoms of brain estrogen deficiency in rats with surgically induced menopause but it remained inert in the rest of the body [26]. The DHED was converted into 17β-estradiol after incubation with various homogenates of rat brain structures but not rat uterus homogenate. In addition, the authors concluded that DHED has increased water solubility, decreased lipophilicity, and decreased binding to plasma protein in comparison to 17β-estradiol that allowed for easy transport across the blood-brain-barrier. They did not observe increased uterus weight nor proliferation of breast cancer tissues upon treatment of mice with DHED. In a follow-up study, Merchenthaler et al reported that DHED significantly reduced basal tail temperature in a rat model of menopause heat flushes, implying that DHED acts similarly to estrogen treatment; however, DHED treatment was not accompanied by typical estrogenic effects in the periphery, such as uterus stimulation, expression of galanin in the anterior pituitary, and tumor growth [27].

The use of enzymes that are overexpressed in cancer to help release drugs is gaining interest although a general strategy has not been established [28]. Recently, Ota et al designed a novel prodrug (compound 6, Fig. 4) that linked 4-OHT to a FAD-dependent lysine specific demethylase (LSD1) inhibitor trans-2-phenylcyclopropylamine [29]. LSD1 is a transcription factor involved in cancer cell growth, and high expression levels have been found in tumor tissues of breast cancer patients; previous work illustrates that inhibition of LSD1 itself can slow estrogen-mediated breast cancer cell growth [30-35]. After linking the 2 drugs together, they detected release of 4-OHT in the presence of LSD1, observed decreased levels of the ERα-target gene pS2 after treatment, and noted synergistic inhibition of MCF-7 cell growth without general cytotoxicity in HMEC cells. It is important to note that compound 6 is both an enzyme-activated prodrug as well as a dual-action prodrug (discussed below).

Photocaged Estrogen Ligands

Another strategy recently employed to selectively release ER ligands is through photocaging, which allows for rapid activation and localized control in specific tissue regions by exposure to a certain wavelength of light. When exposed to a specific wavelength of light, the ligand is released from the chemical cage, allowing for modulation of gene expression. Issues with photocaged estrogen analogues include the diffusion of the activated ligands out of the target area, solubility
problems, potential toxicity issues, and optimization of the wavelength and duration of light application.

First, Cruz et al reported success with a photocaged analogue of 17β-estradiol appended with a nitroveratryl group (compound 7, Fig. 5) [36] that masked agonist activity. In the absence of UV light, the ligand remained caged within the cells, resulting in no transcriptional activity in a luciferase reporter gene assay. However, when exposed to UV light, the compound uncaged and reached 86% of the maximum activity inducible by 17β-estradiol. This indicated successful photocaging that could potentially allow for a rapid and controlled method of regulating ER-mediated gene expression. Shi and Koh expanded the same caging method to tamoxifen, including compound 8 (Fig. 5); the compound was a successful antagonist of ERα and Erβ, mediating gene transcription and repression when 4-OHT was released from the photocage using UV light irradiation [37]. Similarly, Zhang et al reported successful control of a modulatory protein fused to an estrogen receptor via the uncaging of the ER antagonist cyclofen, compound 9 (Fig. 5) [38]. They observed ER-mediated gene expression in experiments with zebrafish embryos on both the global (entire embryo gene activation) and local (activation of a single cell via two-photon illumination) scale. However, researchers reported issues related to the extended length of time the compound remained in the cell and the lack of subcellular control due to

Figure 4. Enzyme-activated ER ligands with the masking groups highlighted in blue. DHED (compound 5) is converted to 17β-estradiol by short chain NADPH-dependent dehydrogenase/reductase enzymes found exclusively in the central nervous system. Compound 6 is cleaved by the histone demethylase LSD1 to release tamoxifen.

Figure 5. Photocaged ER ligands with the masking group highlighted in blue. Compounds 7-9 release 17β-estradiol, 4-hydroxytamoxifen, and cyclofen, respectively.
Overall, the photocaged ligands are interesting tools to examine gene regulation; however, there has not been any effort that we are aware of to examine these types of compounds in disease states. Nevertheless, there has been much effort to develop other photoactivatable chemotherapies and the challenges to address moving forward. Therefore, considerations such as the strength of the light source and penetrability are as important as the design of the prodrug [39, 40].

**Dual-Action Prodrugs**

An alternative strategy for selective targeting of estrogen-dependent tissues, specifically in cancer, has been to append an ER ligand to a known pharmaceutical agent. Although not traditional prodrugs, these dual-action molecules have similar effects. The ER ligand part is designed to help target a cytotoxic molecule to the specific tumor cells and ameliorate side effects; then upon unmasking within the cell, the released...
molecules would exhibit synergistic activity. Specifically, Sadeghi-Aliabadi and Brown linked estrone, at a variety of positions, to doxorubicin (DOX), an anthracycline antibiotic that is in clinical use for metastatic breast cancer [41]. They determined that the most favored linkage to achieve selective toxicity against estrogen-dependent cancer cell lines was at position 17 with a long alkyl chain of 12 carbons (compound 10, Fig. 6). More work is necessary to characterize the prodrug aspects of this molecule, including ER affinity, metabolic release, and degradation pathways. Dao et al also set out to append DOX to an ER ligand; they selected the steroidal antiestrogen RU39411 as their ER targeting component and connected DOX using a pH-dependent hydrolysable hydrazone linkage as seen in compound 11 in Fig. 6 [42]. They observed that their compound was 70-fold more potent than DOX alone in MCF-7 cells and microscopy studies suggested that drug uptake was mediated by membrane ER. Elevated levels of free DOX were found in the nucleus suggesting successful pH-sensitive hydrazone release; however, the exact mechanism and quantification of metabolites was not examined.

With the discovery of the platinum (Pt)-based anticancer drug, cisplatin, there has been a push to design and optimize additional metal-based drugs [43]. Cisplatin is an effective chemotherapeutic because it crosslinks with purine bases on DNA, interferes with DNA repair mechanisms, and causes DNA damage that results in apoptosis; however, there are many undesirable side effects, including kidney problems, decreased immune functions, hemorrhages, and hearing loss [44]. Therefore, metal-based prodrugs have advantages because these cytotoxic compounds can be released from the metal through light exposure, changes in pH, or the redox environment within a cancer cell and potentially avoid side effects. Most work has focused on redox activation because low levels of oxygen in hypoxic tumors result in a more reducing environment compared to normal tissues; large concentrations of cellular reductants such as glutathione and ascorbic acid are present within the cytoplasm of tumor cells [45]. The Lippard group first examined estrogen-tethered Pt(IV) compounds 12 (Fig. 6) that were hypothesized to deliver 2 equivalents of a linked-modified estrogen and 1 equivalent of cisplatin. They observed upregulation of HMGB1, a protein important for inhibiting repair of DNA damage. Unfortunately, they did not observe increased cytotoxicity in comparison to cotreatment of cisplatin and 17β-estradiol. However, their work set the stage for development of additional metal-based masked estrogens.

Hu et al designed a series of tamoxifen-Pt(IV) complexes with the more potent compound 13 (Fig. 6) that allowed for increased cellular uptake in comparison to cisplatin [46]. They observed a slight decrease in binding affinity for ERs in comparison to tamoxifen but when subjected to ascorbic acid to mimic the intracellular reducing environment of cancer cells, they saw complete release of the tamoxifen group over the course of 3 hours. The complexes showed cytotoxicity against ER-positive cell lines (MCF-7 and A2780), significantly less potency against other human cancer cell lines and normal cell lines, and impressive cytotoxicity against tamoxifen-resistant TamR-MCF-7 cells. More recently, Song et al developed a series of multifunctional Pt(IV) prodrugs (compound 14, Fig. 6) that simultaneously target DNA, melatonin membrane receptor (MT1), and subsequently ER functionality [47]. Although compound 14 did not interact directly with ER, melatonin is known to downregulate estrogen responsive genes in breast cancer cells through MT1 binding [48]. By appending 2 equivalents of melatonin to the metal, they were able to retain MT1 binding which subsequently inhibited ERα transcriptional activation and downregulated expression levels of ERα protein. A reducing environment of 1mM ascorbic acid resulted in release of the melatonin. Finally, in an MCF-7 xenograft mouse model, compound 14 showed effective tumor inhibition with increased survival rate in comparison to melatonin treatment. Zhao et al reported a tamoxifen-modified ruthenium complex (compound 15, Fig. 6) that displayed enhanced cellular uptake and upon photoactivation within ER+ breast cancer cells served as a singlet oxygen-generating photosensitizer; the use of the ER ligand to target the photodynamic therapeutic agent to specific cells is very promising [49].

In addition to dual-action prodrugs for cancer therapy, there has been recent work on the development of prodrugs that would simultaneously release a compound to help alleviate adverse side effects. Bechmann et al hypothesized that they could link the vasoactive nitric oxide to a SERM (compound 16, Fig. 7) in order to diminish side effects such as hot flushes and endothelial dysfunction [50]. Interestingly, the compounds showed antagonistic activity for ERβ but no interaction with ERα. They observed that release of nitric oxide weakened the antiproliferative and proinflammatory effects of the SERM in normal vascular tissue and proposed that these molecules could be used as radioisensitizers. Similarly, Wang et al appended 2 nitric oxide donating groups to the phytoestrogen genistein as seen in compound 17 (Fig. 7) [51]. They observed slow and continually release of nitric oxide in the osteoblastic cell line MC3T3-E1, with increased mineralization, suggesting potential use for the treatment of postmenopausal osteoporosis.

**Conclusions**

There is continuing interest in the development of selective ligands for ER activation or inhibition in different contexts. These compounds can be probes for ER function in different contexts, to doxorubicin (DOX), an anthracycline antibiotic that is in clinical use for metastatic breast cancer [41]. They determined that the most favored linkage to achieve selective toxicity against estrogen-dependent cancer cell lines was at position 17 with a long alkyl chain of 12 carbons (compound 10, Fig. 6). More work is necessary to characterize the prodrug aspects of this molecule, including ER affinity, metabolic release, and degradation pathways. Dao et al also set out to append DOX to an ER ligand; they selected the steroidal antiestrogen RU39411 as their ER targeting component and connected DOX using a pH-dependent hydrolysable hydrazone linkage as seen in compound 11 in Fig. 6 [42]. They observed that their compound was 70-fold more potent than DOX alone in MCF-7 cells and microscopy studies suggested that drug uptake was mediated by membrane ER. Elevated levels of free DOX were found in the nucleus suggesting successful pH-sensitive hydrazone release; however, the exact mechanism and quantification of metabolites was not examined.

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**Conclusions**

There is continuing interest in the development of selective ligands for ER activation or inhibition in different contexts. These compounds can be probes for ER function in different contexts.
tissues and disease states as well as potential pharmaceutical agents. There are advantages to developing prodrugs for ER, such as selective activation in only the desirable location. There has been recent progress in this field using chemical activation (eg, hydrogen peroxide), enzymes, reducing environments, and light to release ligands from their masked counterparts, as discussed here. However, much of the work so far has focused on breast cancer and specifically the modification of tamoxifen. Therefore, there is potential for this field to grow, through the masking of other known ER ligands and the identification of masking groups that can be released in desirable contexts. These studies have highlighted the amenability of ER ligands for modification and attachment of masking groups through chemical transformations of the A-ring phenol and other sites. Moving forward, it would be advantageous to apply knowledge from other studies, including pH-sensitive prodrugs, glutathione-responsive prodrugs, antibody-drug conjugates, and enzyme-activatable prodrugs [52, 53]. Optimized masked ER ligands that are only activated when and where regulation is required have the potential to circumvent side effects of traditional estrogen or SERM treatments.

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Disclosures

The authors have nothing to disclose.

Data Availability

Data sharing is not applicable to this article as no datasets were generated or analyzed when compiling the review.

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