IDENTIFICATION OF A NOVEL CLASS OF ER-SELECTIVE LIGANDS LACKING CROSS-REACTIVITY TO GPER

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IDENTIFICATION OF A NOVEL CLASS OF ER-SELECTIVE LIGANDS LACKING CROSS-REACTIVITY TO GPER

by

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ABSTRACT

Estrogen plays multiple roles in health and disease, exerting its effects through the classical estrogen receptors (ERα and ERβ) and the G protein-coupled estrogen receptor (GPER). Current ER-targeting ligands, including the therapeutic ERα antagonist tamoxifen, have been shown to cross-activate GPER. This cross-activation is hypothesized to contribute to clinically observed endocrine resistance in breast cancer, highlighting the potential benefit of truly ER-selective antagonists. We report the identification of a novel class of ER-selective ligands that lack cross-reactivity towards GPER, identifying a truly ER-selective agonist (AB-1) and antagonist (AB-82P). Importantly, AB-82P degrades a clinically relevant ERα mutant and exhibits inhibitory effects in cellular models of endocrine resistance. This novel class of ER-selective ligands can aid in improving our understanding of the individual estrogen receptors in estrogen biology and more importantly, provide a structural basis for the development of new, truly ER-selective antagonists for the treatment of ERα-positive breast cancers.
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CHAPTER 1
Introduction

1.1 Estrogen synthesis

Estrogen, often called the female sex hormone, is a small membrane-permeable molecule that plays a role in various systems in the female body. It is present in three forms within the body: estrone (E1), estradiol (E2) and estriol (E3) \[1\] (Fig. 1.1a). Estradiol, specifically 17β-estradiol, is the most prevalent and potent form of estrogen in the body. Therefore, for the remainder of this dissertation, when referring to estrogen, estradiol (E2, 17β-estradiol) will be implied.

Estrogen, originally identified by Allen and Doisy, is mainly synthesized in the ovaries of pre-menopausal women \[2\]. Stimulation of the ovaries by follicle-stimulating hormone, produced by the pituitary gland (as a result of its stimulation by gonadotropin-releasing hormone), results in the secretion of testosterone by the ovaries. Testosterone is subsequently converted (in the ovaries) to estrogen by the enzyme known as cytochrome P450 aromatase (encoded by the \textit{CYP19A1} gene) \[3\]. This conversion step by aromatase is a therapeutic target in breast cancer therapy and will be discussed later in this chapter. In post-menopausal women, the ovaries stop producing estrogen, resulting in a significant decrease in circulating estrogen levels. Although estrogen production strongly decreases in the post-menopausal setting, women do continue to produce lower levels of estrogen, mainly in skin and adipose tissue. This occurs
through local conversion of testosterone and androstenedione to estrogen by the aromatase enzyme (androstenedione is converted to E1 and subsequently to E2). Contrary to the term “female sex hormone”, men also produce estrogen, but at a much lower level than women (circulating estrogen levels in men are comparable to that of post-menopausal women). In men, estrogen is produced in tissues such as the testes (e.g. by Leydig cells) and brain [4].

**Figure 1.1.** Structure of estrogens and the human classical estrogen receptors. (a) Structures of estrone (E1), estradiol (E2, specifically 17β-estradiol) and estriol (E3). (b) Domain structures of human ERα and human ERβ and the locations of important regions of the receptor. Sequence homology (in percentages) of the various ERβ domains versus the corresponding domains in ERα are shown. AF-1: activating function-1, AF-2: activating function-2, DBD: DNA-binding domain, LBD: Ligand-binding domain.
1.2 Physiological roles of estrogen

Estrogen (mainly E2) plays an important role in many physiological processes [5], but its most well characterized role is that in female reproductive organs. In the breast, estrogen is essential for the elongation of epithelial ducts during puberty. This is exemplified by the observation that female ERα knockout mice develop abnormal mammary glands in which no ductal elongation is observed [6]. These mice have a mammary gland phenotype similar to that of a neonatal mouse.

Estrogen is also important in the female reproductive tract. In ERα knockout mice, the reproductive tract develops normally, however these mice are infertile. This is in part due to the impaired role of estrogen in the ovaries of ERα knockout mice. Estrogen plays a role in folliculogenesis and ovulation and ERα has been shown to be the main ER involved in this role [6, 7]. ERβ knockout mice only have partially disrupted folliculogenesis and are subfertile.

Contributing to the infertile phenotype of ERα knockout mice is the role of estrogen in the maturation of the uterus. In ERα knockout mice, the uterus displays a hypoplastic morphology (being small and shriveled) compared to the uterus in wildtype female mice and is unresponsive to estrogen stimulation [6]. The uterus is normally highly sensitive to estrogen and undergoes several changes when stimulated with estrogen, a classical one being its increased absorption and retention of water in a process termed imbibition [8]. Furthermore, uterine epithelial cells also undergo increased cellular proliferation in response to estrogen. The high sensitivity of the uterus to estrogen and its resulting water imbibition is
exploited by researchers to test the estrogenicity of compounds (drugs) in vivo [9].

Aside from its role in female reproductive organs, estrogen also plays a role in many other systems, including the cardiovascular, nervous and immune systems and in the bone and metabolism [10]. For example, in the cardiovascular system estrogen plays several roles including a protective role against arterial hypertension, as is observed with the increase in arterial hypertension (and cardiac disease) in post-menopausal women when the ovaries cease to produce estrogen [11]. In the bone, estrogen plays a role in overall bone health and trabecular bone growth. This is exemplified by the increased risk of developing osteoporosis in post-menopausal women [10].

Overall, estrogen plays a role in many physiological processes in both sexes and exerts its effect through three known estrogen receptors ERα, ERβ and the G protein-coupled estrogen receptor (GPER). The receptors are discussed in the following sections and readers are referred to the following comprehensive references on the wide range of other physiological roles of estrogen in both men and women [4, 5, 10].

1.3 Classical estrogen receptors alpha and beta (ERα and ERβ)

In the late 1950s, Jensen and Jacobson observed that the rat uterus and vagina retained [³H]estradiol, whereas other tissues such as the liver and muscle did not [12]. These observations (and others) led to the idea of the existence of a receptor to estrogen. Later work led to the identification and characterization of an estrogen receptor, which was eventually cloned in 1986 [13-15]. This receptor (the
first hormone receptor to ever be identified), originally called the estrogen receptor, was later termed estrogen receptor alpha (ERα), following the identification of a second estrogen receptor in 1996, termed estrogen receptor beta (ERβ) [16].

1.3.1 Structure

ERα and ERβ, termed the classical estrogen receptors, are ligand (hormone)-activated transcription factors. They belong to the nuclear hormone (steroid) receptor superfamily (which includes e.g. the progesterone receptor and the glucocorticoid receptor) and consist of two main domains: a DNA-binding domain (DBD) and a ligand-binding domain (LBD) (Fig. 1.1b) [17-19]. The LBD is a α-helix bundle, consisting of 12 α-helices; the ligand-binding pocket sitting between helix 3 and 11 [20, 21]. A critical component of the ER-LBD is the C-terminal helix, helix 12, which plays an important role in the activation of the receptor (discussed below). ERα and ERβ share high homology between their DBDs, but not between their LBDs, showing only ~60% homology in the latter [22, 23]. However, the binding cavity of their LBD are highly conserved and differ by two residues [22]. The receptors also contain two functional sites that are involved in transcriptional activity: the constitutively active activation function-1 (AF-1) site and the ligand-dependent activation function-2 (AF-2) site. The latter is localized in the LBD of the receptor and is responsible for ligand-induced transcriptional activity of the receptors. The DBD and the LBD are linked together via a flexible hinge domain which is thought to be important for their function [24]. Wildtype ERα, a 66 kDa protein, has several splice variants: a 46 kDa variant (ER-46) [25] and
the even shorter 36 kDa variant, ER-36 [26]. These splice variants lack the AF-1 region of the wildtype receptor and have been shown to act as inhibitors of ERα signaling and capable of activating rapid signaling pathways [27]. Several isoforms of ERβ have also been identified, but their roles are not well known [28].

1.3.2 Cellular localization

Classically, transcription factors are located in the nucleus where they exert their genomic activity, however there are receptors, like inactive androgen receptors, that are predominantly cytoplasmic, but translocate to the nucleus upon activation [29, 30]. In the case of estrogen receptors, the majority (~95%) of the inactive receptor is localized to the nucleus [31, 32]. However, a fraction of the receptors are found in the cytosol. Interestingly, a fraction of the cytosolic splice variants of ER have been found to localize to the plasma membrane, in particular to caveolae. There, these splice variants have been implicated, at least in part, in E2-induced rapid non-genomic signaling pathways like PI3K and eNOS synthesis [33, 34].

1.3.3 Function

In their inactive, non-ligand-bound state, ERs are bound to chaperone proteins like Hsp90 and p23 in the cell [32, 35]. Following binding to estrogen (E2 binding affinity for ERα and ERβ is 0.05-0.4 nM and 0.09-0.4 nM, respectively [23]), the receptors undergo a conformational change that promotes the release of the bound chaperone proteins and induces receptor dimerization (homodimers and
heterodimers). Activated ER dimers subsequently bind to the promoters of target genes in the nucleus at specific sites on the DNA, termed estrogen response elements (EREs) [28]. EREs have the general consensus sequence 5'-GGTCAnnnTGACC-3' however, most ER binding sites do not have this exact ERE sequence, varying by one or two nucleotides [36]. Known ERα target genes that have an (imperfect) ERE sequence in their promoter include trefoil factor 1 (TFF1, also known as pS2) [37], progesterone receptor [38] and prolactin [39].

DNA-bound ERs subsequently induce or inhibit target gene transcription through the recruitment of coactivators or corepressors, respectively, and initiate proliferative and pro-survival signaling in the cell [18, 40, 41]. These coregulatory proteins bind to AF-1 and/or AF-2 sites on ERs. In an inactive (unliganded) receptor state, the AF-1 and AF-2 sites on ERs are hidden. Only following activation of the ERs, do these sites become exposed and accessible for coregulatory proteins to bind to them via a general LxxLL motif in the coregulatory proteins [42]. The AF-1 region, which functions independently of ligand binding, becomes exposed following activation of ERs by phosphorylation of residues near the AF-1 region. Estrogen has been shown to induce phosphorylation of serines 104 and 106 in the AF-1 domain by cyclin A2-CDK2 [43]. Furthermore, mitogen-activated protein kinase (MAPK) can phosphorylate ER at serine 118 in response to growth factor signaling [44, 45]. Unlike the AF-1 region, the activity of the AF-2 region is ligand-dependent. The AF-2 region is located in the ER-LBD and its accessibility is governed by the location of a structurally important helix, termed helix 12, in the LBD [46]. In an unliganded (or antagonist-bound), inactive
state, helix 12 occupies the AF-2 site, through interaction with its own LxxML sequence, thereby preventing the binding of coregulatory proteins [20]. In the active, estrogen-bound (or agonist-bound) state, helix 12 moves down to lock estrogen in place in the ligand-binding pocket. This conformational change exposes the AF-2 site on the receptor, allowing coregulatory proteins to bind [47]. Thus, whereas AF-2 exposure is dependent on ligand binding, AF-1 can become exposed in a ligand-independent manner; both resulting in transcriptional activity of the receptor. While each activation function site can recruit its own distinct set of coregulatory proteins, several coregulatory proteins can bind to both AF-1 and AF-2 sites (e.g. SRC-1) [18]. Importantly, AF-1 and AF-2 work synergistically with each other and full transcriptional activation of the receptor generally requires that coactivators be recruited to both sites [48]. The recruitment of coregulatory proteins to DNA-bound ERs is a complex phenomenon and leads to the recruitment of basal transcription factors that alter chromatin structures and eventually lead to the recruitment of RNA Polymerase II, ultimately leading to proliferative, survival and migratory cellular responses [49, 50].

Activated ERs bind to DNA at EREs and regulate target gene transcription, however ERs can also regulate transcriptional activity in other ways [51]. Ligand-bound ERs can modulate gene transcription of genes that do not contain an ERE in their promoter region by indirectly binding to these promoters via other DNA-bound transcription factors like activator protein-1 (AP-1) and specificity protein 1 (Sp-1); serving as a docking site for additional regulatory proteins [17, 52].
ERα and ERβ are often said to have opposing effects. In cells expressing both receptor subtypes, ERβ is thought to inhibit ERα-mediated signaling. For example, Matthews et al. showed that ERβ expression reduced ERα-mediated transcription of the progesterone receptor (PR) [53]. Moreover, ERβ expression could reduce ERα protein levels. Similarly, Liu et al. showed that ERβ inhibits ERα-induced transcription of cyclin D1 [54]. The exact mechanism behind the inhibitory effects of ERβ on ERα-mediated signaling is currently not understood, but is thought to involve an ERβ-induced change in the recruitment of coregulatory proteins by ERα [28, 53].

The complexity of ER signaling is further exemplified by the differential responses that binding of different ligands (agonists or antagonists) elicits [55]. Different ligands induce different conformations of the ER LBD, which in turn recruit different coregulatory proteins. Furthermore, differences in expression levels of co-activators and co-regulators also influence the transcriptional activity of ERs [40, 56]. One example of this being 4-hydroxytamoxifen (the active metabolite of tamoxifen). 4-hydroxytamoxifen, an ER antagonist in the breast, acts as an ER agonist in the endometrium. This is believed to be due to lower co-activator expression levels in the breast versus that in the endometrium [40].

Aside from transcriptional signaling, ERs can also induce rapid, non-genomic cellular responses, such as cyclic AMP (cAMP) production, calcium mobilization and protein kinase activation (e.g. ERK), which result in the activation of other pathways that can modulate gene transcription in an ER-independent manner [23, 57]. These rapid signals are believed to, at least in part, originate from
membrane-bound ERs (mentioned earlier, in the case of ER isoforms) [58]. ER-mediated activation of rapid signaling has been reported to occur through binding of ERs to e.g. Src and PI3K, leading to downstream signaling [34].

1.3.4 ER degradation: means to limit ER signaling

Transcriptional activity of hormone receptors is dependent on the intracellular protein levels of the receptor. These levels change with varying cellular states [59]. In the case of the estrogen receptor, the major regulator of ER protein levels is the presence of estrogen itself (a phenomenon shared with other hormone receptors). Estrogen is able to induce ER degradation in an auto-regulatory feedback loop, leading to a decrease in both ER protein and mRNA levels. E2-induced ER degradation is part of how the cell regulates ER function, following activation of the receptor. This degradation occurs through a ubiquitin/proteasome-dependent pathway [60]. Ligand-bound ERs are polyubiquitinated on residues in their C-terminus tail, in a process involving the E1, E2 and E3 ubiquitin/ligase family and others [60, 61]. Lysine residues in the hinge region of ERα (K302 and K303) have also been shown to be ubiquitinated [62]. The polyubiquitinated ER is then trafficked to the 26S proteasome complex for degradation, completing the process of ER-signaling termination. Although ER activity and degradation are linked, and coactivator binding is important, activation of ER is not required for its degradation [61, 63]. One example being ligand-induced ER degradation following binding of the ER antagonist ICI182,780 (discussed later) [64].
1.3.5 Ligand specificity between classical estrogen receptors

Due to the highly conserved binding pocket between ERα and ERβ, subtype-selective ligands have been difficult to develop [22]. Both receptors bind estrogen (E2, 17β-estradiol) with similar affinities (ERα: 0.05-0.4 nM, ERβ: 0.09-0.4 nM) [23]. Furthermore, both receptors can bind other natural and synthetic environmental estrogens that mimic physiological estrogens (estrone, estradiol and estriol). These environmental estrogens consist of e.g. phytoestrogens (such as genistein) and xenoestrogens (such as bisphenol A) [23].

However, to date, no truly ERα-subtype nor truly ERβ-subtype selective ligands exists. There do exist ligands that are biased towards one receptor over the other. For example, 4,4’4’-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) is an ERα agonist that shows ~400-fold binding preference for ERα over ERβ [65]. Inversely, DPN (2,3-bis(4-hydroxyphenyl)propionitrile) acts as an agonist of the classical ERs, but has a ~70-fold higher affinity for ERβ over ERα (Fig. 1.2) [66]. A list of other partially subtype-selective ligands can be found here [22].

1.3.6 Tissue distribution

ERα and ERβ are expressed in multiple tissues. In some, both receptor subtypes are expressed at similar levels, whereas in others one subtype is mainly expressed over the other. In tissues where both subtypes are expressed, the individual subtypes are generally expressed in different cell types within that tissue. ERα is expressed e.g. in breast (luminal epithelial cells), bone, ovary (theca cells), uterus, prostate (Leydig cells) and liver tissue. ERβ is expressed e.g. in breast (stromal
and myoepithelial cells), ovary (granulosa cells), colon and lung tissue. Both receptor subtypes are also expressed in various parts of the brain [1, 17, 67].

![Figure 1.2. Structures of ER subtype-biased compounds.](image)

### 1.4 G protein-coupled estrogen receptor (GPER)

In the late 1990’s/early 2000’s it was already known that estrogen could induce rapid, non-genomic signals (on the scale of seconds to minutes). However, it was debated whether this E2-induced rapid signaling was mediated solely by ERα and ERβ (previously discussed) or whether an unknown receptor was the key mediator [68]. In the late 1990’s several laboratories identified an orphan G protein-coupled receptor (GPCR), later be termed GPR30, that had no known ligand [23, 69, 70]. Estrogen was later identified as the natural ligand of GPR30 (E2 $K_d \approx 3$ nM [71], IC$_{50} \approx 7$ nM [72]), leading to the renaming of the receptor to G protein-coupled estrogen receptor (GPER) [71-75].
1.4.1 Structure

GPCRs are the largest class of signaling receptors in eukaryotes. They consist of 7-transmembrane spanning α-helices and are mainly localized to the plasma membrane, but GPCRs are also present in other membranes inside the cell [76]. GPCRs are organized in the membrane in such that their N-terminus is extracellular while their C-terminus protrudes into the cytosol. The C-terminus of GPCRs plays an important part in translating extracellular signals into the cell. Moreover, it is an important region involved in terminating GPCR signaling.

1.4.2 Function and localization

Upon binding to a ligand, GPCRs undergo a conformational change that allows them to bind intracellular heterotrimeric G proteins. This binding leads to activation of the G proteins (through their binding of GTP) and subsequent downstream activation of second messenger proteins (e.g. synthesis of cAMP and activation of protein kinase A (PKA) and ion channels) [77, 78]. GPCR signaling is terminated in a process involving intracellular phosphorylation of the GPCR by G protein-coupled receptor kinases. These phosphorylated sites act as docking sites for arrestins, which subsequently lead to the internalization, recycling and/or degradation of the GPCR and eventual desensitization of the receptor [78, 79]. Although GPCRs are mainly located on the plasma membrane, they can also be found in intracellular membranes. In the case of GPER, it has been shown to mainly localize to the endoplasmic reticulum [72]. However, GPER has also been detected, at lower levels, in other cellular locations, including the plasma
membrane and the nucleus [80, 81]. Since its ligand (estrogen) is readily membrane-permeable, GPER is not required to be at the plasma membrane to engage with it.

Activation of GPER leads to the activation of an array of downstream rapid signals, including the activation of ERK1/2, PI3K, cAMP synthesis and Ca\(^{2+}\) mobilization (Fig. 1.3) [72, 75, 82]. Filardo et al. showed that E2-induced activation of ERK1/2 occurred through the GPER-mediated transactivation of the epidermal growth factor receptor (EGFR) by heparin-bound EGF (HB-EGF) [75]. Further elucidation revealed that activation of GPER leads to the activation of Src and matrix metalloproteases, which subsequently cleave HB-EGF and induce the activation of EGFR and the resulting downstream ERK1/2 and PI3K pathways [82].

Aside from inducing rapid non-genomic signaling, GPER (following E2-activation) also induces genomic signaling, however not to the same extent as ER\(\alpha\) and ER\(\beta\). Genes that are regulated by GPER include \(c\)-\textit{fos}, which was shown to be upregulated in an endometrial cancer cell line following stimulation with E2 [83]. Transcriptional regulation by GPER could occur via the cAMP/CREB signaling axis (cAMP is a product of GPER activation), which is known to induce transcription of \(c\)-\textit{fos} [84, 85]. GPER has been reported to be present in the nucleus of cancer-associated fibroblasts [81, 86, 87], where it is hypothesized to act as a transcription factor, possibly in a complex with EGFR [87]. However, the mechanism through which GPER could potentially act as a transcription factor is not clearly understood. A comprehensive list of GPER-regulated genes can be found here [82].
Thus, E2-induced activation of GPER results in the activation of a range of rapid, non-genomic pathways, that lead to proliferative, migratory and pro-survival cellular responses. Furthermore, GPER can regulate some genes, however it is mainly associated with E2-induced rapid, non-genomic signaling.

**Figure 1.3.** Pathways activated by GPER and the classical ERs. Estrogen, environmental estrogens and tamoxifen are hydrophobic compounds that can readily cross the membranes and gain access to the classical ERs and GPER. Activation of ER by estrogen or environmental estrogens leads to modulation of target gene transcription, which leads to cell proliferation and other pro-survival cellular responses. Activation of GPER by estrogen or other estrogenic compounds (e.g. tamoxifen) leads to the activation of heterotrimeric G proteins, which subsequently activate multiple
downstream rapid signaling cascades including cAMP production, MAPK and PI3K activation (through Src-dependent transactivation of EGFR). These rapid actions lead to several cellular responses including cell proliferation and cell survival.

1.4.3 GPER-selective ligands

GPER and the classical ERs have overlapping signaling pathways. Therefore, given their co-expression in many tissues, distinguishing which receptor is mediating certain cellular responses within a given tissue has been challenging. Selective ligands to the individual receptors (or to GPER versus the classical ERs) would aid in better understanding the roles of the individual receptors. Ligand specificity between estrogen receptor subtypes has been difficult to achieve with regard to the classical estrogen receptors given their high degree of homology within the ligand-binding pocket (previously discussed). Surprisingly, given the large structural differences between the classical ERs (transcription factors) and GPER (a GPCR), all currently evaluated ligands of the classical ERs, even PPT (which was originally considered an ERα-selective compound), have been shown to bind and either activate or inactivate GPER [23, 88]. Therefore, obtaining a selective ligand to GPER or the ERs seemed unlikely. However, in 2006 Bologa et al. [89] discovered a small GPER-selective ligand, termed G-1, which selectively binds to GPER over the classical ERs, showing negligible activity towards the classical ERs (Fig. 1.4). They showed that G-1 acts as a selective agonist of GPER. A few years later Dennis et al. reported the successful conversion of G-1 into a GPER-selective antagonist, termed G15 [90], followed by the identification of the even more selective GPER antagonist G36 [91], two years later. Lappano
et al. later reported the identification of two GPER-selective agonists, termed GPER-L1 and GPER-L2 [92], which have ~10-fold lower affinity for GPER versus G-1 (Binding affinity of G-1 is 7-10 nM versus ~100 nM of GPER-L1 and GPER-L2). Identification of GPER-selective compounds has made a great impact on better understanding the role of GPER in various systems. To date G-1, G15 and G36 remain the most popular GPER-selective ligands, while truly ER-selective ligands remain elusive.

Figure 1.4. Structures of the GPER-selective agonist G-1 and the GPER-selective antagonists G15 and G36.

1.5 Estrogen and its role in breast cancer

Aside from its physiological role, estrogen also plays an important role in disease, the most prominent being its role in breast cancer [10, 93]. Breast cancer is the most commonly diagnosed cancer amongst women, with more than an estimated 250,000 newly diagnosed cases of invasive breast cancer each year in the United States [94]. There are four main subtypes of breast cancers: Luminal A (characterized as being ERα+ and/or PR+ and negative for the human epidermal growth factor receptor 2 (HER-2)), Luminal B (ERα+ and/or PR+, HER-2+/− with high Ki-67 positivity), HER-2-enriched (ERα−, PR− and HER-2+) and triple negative breast cancers (TNBCs, ERα−, PR− and HER-2−). Luminal A is the main type of
clinically-observed breast cancer, with more than 70% of cases being classified as this subtype [94]. Luminal A, Luminal B and HER-2-enriched subtypes can be treated with targeted therapy against ERα (for Luminal A and Luminal B cases) or HER-2 (for HER-2-enriched cases). TNBCs, which lack any of these molecular targets are currently not treatable with targeted therapies and patients presenting with this subtype have the worst prognosis (compared to the other 3 subtypes).

ERα is an important initiator and driver of breast cancer [95]. Due to its role (exerting e.g. proliferative and survival signaling) and prevalence in the majority of breast cancers (~70%), ERα has been an important molecular target for the treatment of ERα-positive breast cancers for over 40 years [96, 97]. Inhibition of ERα-signaling by endocrine therapy (also called anti-hormone therapy), using selective estrogen receptor modulators (SERMs, such as tamoxifen), the selective estrogen receptor downregulator (SERD) fulvestrant, and aromatase inhibitors (AIs, such as letrozole and exemestane), has shown great clinical success in treating ERα-positive breast cancers (discussed in the next section) [98, 99].

1.6 Breast cancer endocrine therapy

Endocrine therapy is effective at treating ERα-positive breast cancers and has prolonged the lives of millions of women [98, 99]. It focuses on inhibiting the growth of ERα-positive breast cancers by blocking the activation of the ERα, and thus its downstream proliferative and pro-survival signaling. Endocrine therapy achieves this either by directly blocking estrogen binding to ERα (using SERMs or SERDs) or by decreasing the plasma levels of circulating estrogen by inhibiting its
production (using AlS).

### 1.6.1 SERMs and SERDs

SERMs are a class of small molecules that act as antagonists of ERα by competing with estrogen for binding to the receptor, thereby blocking the recruitment of co-regulators to the receptor (Fig. 1.5) [100]. Their activity is tissue specific, acting as ERα antagonists in the breast, while functioning as ERα agonists in the bone and uterus [101, 102]. Currently Food and Drug Administration (FDA)-approved SERMs include tamoxifen, raloxifene and toremifene.

![Structures of SERMs](image)

**Figure 1.5.** Structures of the SERMs tamoxifen and its active metabolite 4-hydroxytamoxifen, raloxifene, toremifene and the SERD fulvestrant (ICI182,780).

Tamoxifen (Nolvadex®, Soltamox®), the most prescribed SERM, has shown great success in the clinic for both the treatment and prevention of ERα-positive breast cancers in pre- and post-menopausal women, showing a significant decrease in ERα-positive breast cancer-related mortality and recurrence rates.
when taken over a 5 (and up to 10) year period [103, 104]. Unfortunately, due to its agonist activity in the endometrium, women taking tamoxifen experience up to a 7.5-fold increased risk of developing endometrial cancer [101, 105, 106]. Patients taking tamoxifen also have an increased risk of developing thromboembolisms [107, 108]. However, these risks do not tend to outweigh the benefits of the drug. Compared to tamoxifen, raloxifene (Evista®) carries a lower risk of developing thromboembolisms, but is less effective at preventing invasive breast cancer [109]. Nevertheless, raloxifene significantly decreases the risk of developing invasive breast cancer in post-menopausal women with osteoporosis [110]. Raloxifene is currently approved for the prevention of invasive breast cancer in post-menopausal women with osteoporosis. The third FDA-approved SERM, toremifene (Fareston®), also increases the risk of developing thromboembolisms, but data regarding its causal role in developing endometrial cancer is limited [111]. Toremifene is approved for the treatment of advanced ERα-positive breast cancers in post-menopausal women. To date, tamoxifen is the only SERM approved for the treatment of ERα-positive breast cancers in pre-menopausal women.

SERDs are a class of small molecules that, like SERMs, act as antagonists of the ERα (Fig. 1.5). However, unlike SERMs, SERDs not only antagonize the receptor, but also induce its degradation, due to destabilization of helix 12 in the ER-LBD, resulting in destabilization of the entire protein and a decrease in ERα protein levels [112]. Furthermore, SERDs are “pure” antagonists of the ERα, in that they do not show agonist properties in other tissues [113]. Currently, fulvestrant (Faslodex®) is the only FDA-approved SERD and is used for the treatment of
metastatic ERα-positive breast cancers in post-menopausal women with disease progression following prior endocrine therapies.

### 1.6.2 Aromatase inhibitors (AIs)

AIs are a class of small molecules that block the production of estrogen by inhibiting the enzyme aromatase (CYP19A1) ([Fig. 1.6](#)) [114]. They have been highly effective in the clinic for the treatment of ERα-positive breast cancers in post-menopausal women [115]. In fact, they are more efficacious than tamoxifen in this setting [99, 116]. However, this class of drugs has significant side-effects, including muscle/joint pain, bone loss, hot flashes and insomnia, causing over 30% of patients to discontinue treatment [117-119]. Nevertheless, AIs are highly effective in the treatment of ERα-positive breast cancers and are becoming a more popular standard first-line endocrine therapy option for post-menopausal women. Currently FDA-approved AIs include the reversible AIs anastrozole (Arimidex®) and letrozole (Femara®) and the irreversible AI, exemestane (Aromasin®).

![Figure 1.6. Structures of the aromatase inhibitors exemestane, anastrozole and letrozole.](#)
1.7 Endocrine resistance

Endocrine therapy is the standard of care for the treatment of ERα-positive breast cancers and has been highly effective in the clinic. Unfortunately, some 30% of patients eventually develop acquired endocrine resistance to the initial therapy, with reports suggesting that this number may be as high as 40-50% [98, 120]. Several mechanisms have been proposed to account for the development of endocrine resistance, including alterations in growth factor signaling (e.g. overexpression of the EGFR or HER-2), changes in the expression of ERα or its co-activators/co-repressors, mutations in ERα, changes in the metabolism of tamoxifen and tamoxifen-induced GPER signaling (discussed later) [121-125].

In the late 1990s, Zhang et al. reported the identification of three ESR1 (the gene encoding ERα) mutations in metastatic breast cancer samples [126]. One of the mutations, Y537N, was found to cause constitutive activation of the receptor, even in the absence of estrogen. More importantly, this mutation made the receptor less sensitive to inhibition by tamoxifen (the maximum tested dose was 100 nM). This led to the proposal that mutations of this site could contribute to breast cancer progression and endocrine resistance. Similar findings regarding mutations at the Y537 codon were previously reported by Weis et al. in a structure-activity relationship study [127]. More recently, studies identified several ESR1 mutations in up to 55% of metastatic breast cancer samples of patients who relapsed while on endocrine therapy [128-131]. Importantly, these mutations were absent in the corresponding primary breast tumors and primary breast tumors in general, pointing to a possible role in endocrine resistance. The most commonly
occurring mutations were Y537S and D538G located in the ligand-binding domain (LBD) of ERα. These mutations result in constitutive activation of the receptor by inducing a ligand-bound confirmation of the receptor in the absence of estrogen. More specifically, they stabilize helix 12, an important structural component in the LBD of ERα, in a closed agonist-bound confirmation, thereby exposing a co-regulator binding site on the receptor that is normally only revealed following the binding of estrogen. This stabilized ligand-bound confirmation also makes the mutant receptor less susceptible to inhibition by tamoxifen and fulvestrant, by impairing access to the ligand binding pocket of the receptor [20, 128, 129]. These observations support the role of these mutations in endocrine resistance in response to not only AIs, but also SERM- and SERD-based therapies. Interestingly, the Y537S mutation has been reported to be more resistant than the D538G mutation, to in vivo inhibition by fulvestrant [132].

Clinical data of patients that have relapsed while on an AI therapy have shown that the Y537S and D538G mutants are associated with more aggressive relapse tumors [133]. This aggressive phenotype could be due to the unique transcriptome induced by the Y537S and D538G mutants (compared to that of wildtype ERα) that includes the modulation of pro-metastatic genes [134]. This merits further research into genes that are specific targets of the mutant forms of ERα, as potentially new therapeutic targets for the treatment of relapsed breast tumors expressing mutant ERα. Interestingly, in a retrospective analysis of the FERGI trial (a comparison of treatment with a PI3K inhibitor + fulvestrant versus placebo + fulvestrant, in ERα-positive breast cancer patients with locally advanced
or metastatic disease), *Spoerke et al.* did not observe a difference in the control placebo + fulvestrant arm of the trial with respect to progression-free survival (PFS) between patients harboring wildtype *ESR1* versus patients harboring mutated *ESR1*, as measured using circulating tumor DNA [135]. This underlines the complexity of tumor heterogeneity and how the presence of other mutations alongside *ESR1* mutations could potentially influence ERα-targeted endocrine therapies. Furthermore, it could imply that *ESR1* mutations are more important in endocrine resistance in a hormone-deprived setting (e.g. in patients on AI therapies) than in tumors that relapse on SERM/SERD therapies. Nevertheless, the presence and activity of mutant forms of ERα in the metastatic relapse setting have become a highly studied resistance mechanism and are now strongly considered in the development of next-generation SERMs and SERDs (discussed in the next section).

Upregulation of positive regulators of the cell cycle (e.g. cyclins E1 and D1) is another example of a mechanism involved in endocrine resistance [125]. This mechanism leads to e.g. the activation of cyclin-dependent kinases (CDKs) that counteract the anti-proliferative role of endocrine therapy by driving the cell through *G1* phase. This mechanism is heavily studied and is exemplified by the recent FDA approval of three CDK4/6 inhibitors (palbociclib, ribociclib and abemaciclib) for the treatment of metastatic breast cancer (in combination with endocrine therapies such AIs or fulvestrant) [136]. For other resistance mechanisms, readers are directed to comprehensive reviews on other mechanisms cited earlier in this section.
1.8 Next generation SERMs and SERDs

Acquired endocrine resistance is a major clinical problem in treating ERα-positive breast cancers. The presence of the Y537S and D538G ERα mutants in many relapse tumors highlights the need for the development of improved SERMs and SERDs that can effectively inhibit both the wildtype and clinically-observed mutant forms of ERα [128-130]. Such new drugs could be effective in treating relapse tumors harboring the mutant receptor and improve disease stabilization, thereby prolonging the lives of patients.

Currently, for women with advanced metastatic ERα-positive breast cancer that has relapsed while on endocrine therapy, therapeutic options are limited. In this setting, the SERD fulvestrant has shown the most promising results in extending PFS, either as a monotherapy or in combination with targeted therapies like cyclin-dependent kinase 4/6 (CDK4/6) inhibitors [137, 138]. With regard to the presence of ESR1 mutations, Fribbens et al. analyzed ESR1 mutations in baseline plasma from the SOFEA trial (comparing exemestane versus fulvestrant-containing therapies) and the PALOMA3 trial (comparing fulvestrant + placebo versus fulvestrant + the CDK4/6 inhibitor palbociclib) [139]. They found that fulvestrant provided a significant benefit over exemestane in patients with tumors harboring ESR1 mutations. However, treatment with fulvestrant had a modestly worse PFS outcome for patients with ESR1 mutations when compared to patients with wildtype ESR1. The latter result is not surprising given that mutant forms of ERα are less sensitive to fulvestrant and require higher drug concentrations to be fully inhibited [128, 131, 132]. The currently approved dosage of fulvestrant (500
mg) has been shown to be ineffective in fully blocking ERα in tumors, and in turn associated with early disease progression [140]. Taken together, the aforementioned observations imply that achieving higher plasma levels of fulvestrant could be of great therapeutic benefit, especially with regard to relapse tumors harboring ESR1 mutations. Unfortunately, fulvestrant has poor pharmacological properties, requiring it to be administered through painful, monthly intramuscular injections, thereby limiting the maximum achievable dose of the drug. As a consequence, the development of new, more bioavailable SERMs/SERDs that possess better pharmacological properties, while also efficaciously inhibiting ERα mutants, is the goal of much current research.

The small molecule ZB716, a boronic acid-modified version of fulvestrant with high oral bioavailability, is an example of how improving the bioavailability of a drug can lead to higher plasma levels (Fig. 1.7) [141-143]. Liu et al. showed that ZB716 achieved over 10 times higher plasma levels than fulvestrant when orally administered to mice and that ZB716 has similar levels of efficacy (compared to fulvestrant) in vitro [141]. Similarly, Guo et al. showed that ZB716 achieved higher plasma levels in both the blood and tumors in xenograft models [143]. The latter study also demonstrated that ZB716 is more efficacious than fulvestrant in tumor growth inhibition in a cell line-derived xenograft model. Interestingly, ZB716 displayed no observed benefit (versus fulvestrant) in inhibiting tumor growth in a patient-derived xenograft (PDX) model.

Several new SERDs are currently being developed and assessed in clinical trials to treat advanced endocrine-resistant breast cancers. AZD9496, an orally
bioavailable SERD developed by AstraZeneca (Fig. 1.7), has been shown to be highly effective at inhibiting ERα-positive breast cancer cells \textit{in vitro} and \textit{in vivo}, while also being highly effective at binding and degrading wildtype and mutant forms of ERα (the binding IC$_{50}$ values for AZD9496 are >6-fold improved \textit{versus} those of fulvestrant) [144, 145]. Furthermore, Weir \textit{et al.} showed that AZD9496 inhibited the growth of a PDX tumor harboring the D538G ERα mutant [145]. A phase 1 clinical trial of AZD9496 was recently completed and indicated that the drug is tolerated at the maximum tested dose (600 mg), even showing some early signs of disease stabilization over a 12 month period [146].

\textbf{Figure 1.7.} Structures of next generation SERMs and SERDs.
Another orally bioavailable SERD, G1T48, developed by G1 Therapeutics Inc., is effective at inhibiting ERα-positive tumor growth in in vivo models of tamoxifen and aromatase resistance [147]. Furthermore, G1T48 inhibits the growth of ERα-Y537S- and ERα-D538G-expressing breast cancer cells. G1T48 also showed synergistic inhibition in a tamoxifen-resistant xenograft model when used in combination with the novel CDK4/6 inhibitor, G1T38. G1T48 recently entered a Phase 1 clinical trial (NCT03455270) for initial in-human safety testing in women with advanced ERα-positive breast cancer.

Other orally bioavailable SERDs, including LSZ102 (Novartis Pharmaceuticals, NCT02734615), SAR439859 (Sanofi Inc., NCT03284957) and GDC-0927 (Genentech Inc., NCT02316509) are also currently being evaluated both in the lab and in clinical trials as potentially new therapeutic agents for the treatment of advanced ERα-positive breast cancers [148-152]. Newly emerging SERDs show promising results as potential ERα-targeting breast cancer therapies, but only time will tell if any of these compounds are efficacious in patients and gain clinical approval.

Aside from orally bioavailable SERDs, the already clinically-approved SERM/SERD hybrid, bazedoxifene (Fig. 1.7), is also gaining more interest as a potential therapeutic agent for treating ERα-positive advanced breast cancers. Bazedoxifene, which is already used in hormone replacement therapy and for the prevention of post-menopausal osteoporosis, exhibits favorable efficacy in various endocrine-resistant breast cancer models [153-156]. Furthermore, Fanning et al. reported that bazedoxifene inhibits the activity of the Y537S and D538G mutant
forms of ERα, offering structural insights into its inhibitory mechanism [157]. Its activity in models of endocrine resistance and its well tolerated drug profile, make bazedoxifene a candidate that could prove efficacious in treating ERα-positive breast cancers.

1.9 GPER: a culprit in endocrine resistance?

1.9.1 Implications for a role of GPER in endocrine resistance

The non-classical estrogen receptor, GPER, has gained attention as a possible player in the development of endocrine resistance in breast cancer. This theory stems from multiple clinical observations. Firstly, Filardo et al. reported that GPER is expressed in over 60% of invasive breast cancers, with GPER positivity correlating with a larger tumor size [158]. In the same study, they also observed that GPER was co-expressed with ERα in ~40% of ER-positive invasive breast tumors. GPER expression has also been found to be significantly higher in relapse tumors of patients that had undergone prior tamoxifen therapy (compared to matched primary tumors) [159, 160]. Furthermore, GPER expression has been shown to correlate negatively with relapse-free survival, further indicating an unfavorable role of the receptor in endocrine resistance [159]. Recently, Ignatov et al. reported the opposite, finding a positive correlation between GPER-expression and disease-free survival. However, this correlation was not statistically significant [161]. Interestingly, they did report that treatment with tamoxifen was less beneficial than an AI-based therapy in patients with GPER-positive breast tumors.
*In vitro* data also supports the clinical observations that point to a negative role for GPER in endocrine resistance. *Ignatov et al.* showed that tamoxifen-induced proliferation of tamoxifen-resistant MCF-7 cells could be abrogated by downregulation of GPER [124]. Similar findings were reported by *Mo et al.* who showed that GPER-inhibition, using the GPER-selective antagonist G15 [90], could “re-sensitize” tamoxifen-resistant cells to tamoxifen [160]. Tamoxifen-induced proliferation, via a GPER-mediated pathway, has also been observed in other cell types [83, 162].

Some elucidation for a role of GPER in endocrine resistance came from *Catalano et al.* who showed that tamoxifen-resistant MCF-7 cells expressed higher levels of aromatase [163]. Increased aromatase expression could potentially create a local increase in estrogen levels, thereby allowing tumors to overcome the inhibitory effects of tamoxifen and AI therapies. Importantly, the increased aromatase expression could be reverted back to baseline levels by reducing GPER expression or inhibiting its function using G15.

*Zekas et al.* also provided a potential mechanism for GPER in endocrine resistance [164]. They observed that tamoxifen and fulvestrant, through a GPER-mediated pathway, induce the translocation of the pro-apoptotic transcription factor Forkhead box protein O3a (FOXO3a) out of the nucleus. Nuclear expulsion of FOXO3a is a phenotype that is associated with its inactivation and promotes a pro-survival state [165-167]. Importantly, knockdown of GPER abrogated the observed ligand-induced translocation of FOXO3a. It was suggested that tamoxifen-induced inactivation of FOXO3a could provide a fraction of tumor cells
with a survival advantage to overcome inhibitory effects of tamoxifen mediated through ERα, eventually leading to the acquisition of endocrine resistance through mutations.

An important observation linking GPER to endocrine resistance was provided by Mo et al. [160]. They reported that a tamoxifen-resistant MCF-7 tumor could be “re-sensitized” to tamoxifen treatment of mice with a combination of tamoxifen and G15. Importantly, mono-therapy with either tamoxifen or G15 had no effect on tumor growth. This implies that pro-survival signaling by GPER (through its downstream target Akt) is possibly overcoming the tamoxifen-induced apoptotic signaling (via ERα), a consequence of tamoxifen-mediated activation of GPER (discussed in the next section).

The body of scientific data pointing to a role for GPER in endocrine resistance has been met with some contradictory reports. A small number of publications have reported that activation of GPER, using the GPER-selective agonist G-1, induces cell death of breast cancer cells [89, 168, 169]. Whether this cell death is caused by hyperstimulation of GPER or is a possible off-target effect due to the high doses of G-1 tested, remains unclear and warrants further investigation. Nevertheless, these findings do not diminish the numerous observations implying a pro-survival role for GPER in endocrine resistance.

### 1.9.2 A proposed role for GPER in endocrine resistance and how to circumvent it

SERMs and SERDs are widely used to inhibit ERα signaling in breast
cancers. However, contrary to their antagonism towards ERα, currently approved SERMs (tamoxifen and raloxifene) and SERD (fulvestrant) have been shown to act as agonists of GPER in breast cancer cell models, resulting in the activation of the ERK and PI3K pathway [72, 75, 88]. Similarly, many other estrogenic compounds (e.g. xenoestrogens and phytoestrogens) that act on the classical ERs have also been found to act as agonists of GPER [23].

This cross-activation of GPER, particularly by tamoxifen, is a common element seen in all of the observations discussed in the previous section and is a potential clue into the role of GPER in the development of endocrine resistance [124, 160, 163]. In the case of tamoxifen, patients are treated for a minimum of 5 years with cancer relapse often occurring after completion of this 5 year period [98]. It is possible that chronic activation of GPER by tamoxifen, promotes long-term survival of a fraction of primary tumor cells or alternatively breast cancer stem cell. This prolonged survival could grant this subset of tumor cells sufficient time to acquire additional mutations, resulting in resistance to the primary therapy (e.g. acquiring endocrine-resistant ESR1 mutations) and leading to the development of endocrine-resistant relapse tumors (Fig. 1.8). This possible role highlights the potentially significant benefit of developing truly ERα-selective therapeutic antagonists that do not cross-activate GPER. A truly ERα-selective antagonist would lack cross-activation of GPER and its resulting pro-survival signaling, thus potentially delaying or decreasing the development of endocrine-resistant relapse tumors.

At this time, no truly ERα-selective compounds (agonists or antagonists)
have been identified in the literature. The current development of next-generation SERDs as focused on the compound’s growth inhibitory potential in models of endocrine resistance, yet none have been assessed for their potential cross-activity towards GPER. Although these new compounds look promising, harboring cross-activity towards GPER could limit their long-term efficacy in treating ERα-positive advanced breast cancers. This emphasizes the potential benefit of truly ERα-selective antagonists. It is worth noting that to date one compound, termed MIBE, has been reported to act as an antagonist towards both the ERα and GPER [170]. However, its efficacy has not yet been assessed in models of endocrine resistance.

**Figure 1.8.** Proposed role of GPER in the development of endocrine resistance. Tamoxifen (as well as other SERMs and SERDs) inhibits its molecular target (ERα) *(red line)*, but simultaneously cross-activates GPER *(green arrow)*. This cross-activation induces the downstream activation of Akt and other survival signals. Chronic cross-activation of GPER by tamoxifen (over the course of the average 5 year tamoxifen regimen) provides a subset of primary tumor cells with prolonged survival signaling that opposes the inhibitory cell death induced through the tamoxifen-mediated inhibition of ERα. Prolonged survival thus provides this subset of surviving primary tumor cells
sufficient time to acquire additional mutations that lead to resistance to the primary therapy, resulting in the development of endocrine-resistant relapse tumors.

1.10 Summary

Estrogen plays a role in many physiological processes and it exerts its effect through three known estrogen receptors: ERα, ERβ and GPER. While it is known that the receptors share overlapping signaling pathways, it has been challenging to understand the roles of the individual receptors, in a given system co-expressing GPER and ERα or ERβ, due to a lack of selective ligands to the individual receptors. The majority of currently evaluated ER ligands, including clinically used ER antagonists (such as tamoxifen and fulvestrant), have been shown to be cross-selective towards GPER, acting as either an agonist of GPER \[23\]. To date, selective agonists and antagonists of GPER, lacking cross-selectivity towards the ERs have been identified, but truly ER-selective ligands have remained elusive.

In breast cancer, GPER is hypothesized to contribute to the development of and endocrine resistance due to its cross-activation by SERMs and SERDs. This cross-reactivity of clinically used ER antagonists (in particular tamoxifen) towards GPER, and the resulting activation of downstream pro-survival signals, highlights the potential benefit of truly ER-selective antagonists for the treatment of ER-positive breast cancers.

In this dissertation work we address these dilemmas by firstly searching for a truly ER-selective ligand that lacks GPER selectivity in Chapter 2. We identify a small molecule, termed AB-1 that acts as an ER-selective agonist, activating only ERα/β transcriptional activity while acting as an antagonist of rapid ERα/β
signaling. Importantly, we show that AB-1 does not bind or activate GPER. In Chapter 3 we identify a truly ER-selective antagonist, termed AB-82P, following structural modification of AB-1. We show that AB-82P inhibits ER activity in vitro and is effective in multiple cell line models of endocrine resistance.

Taken together, this dissertation work identifies a novel class of ER-selective ligands that lack cross-selectivity towards GPER. These newly identified small molecules could be utilized to gain a better understanding of the roles of the classical ERs (versus that of GPER) in health and disease. More importantly, AB-82P could serve as the structural basis for the development of new, truly ERα-selective antagonists for the treatment of ERα-positive breast cancers and potentially decrease or delay the development of endocrine resistance.
CHAPTER 2

A Selective Ligand for Estrogen Receptor Proteins
Discriminates Rapid and Genomic Signaling

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2.1 Abstract

Estrogen exerts extensive and diverse effects throughout the body of women. In addition to the classical nuclear estrogen receptors (ERα and ERβ), the G protein-coupled estrogen receptor GPER is an important mediator of estrogen action. Existing ER-targeted therapeutic agents act as GPER agonists. Here, we report the identification of a small molecule, named AB-1, with the novel activity of high selectivity for binding classical ERs over GPER. AB-1 also possesses a unique functional activity profile as an agonist of transcriptional activity but an antagonist of rapid signaling through ERα. Our results define a novel class of small molecules that discriminate between the classical ERs and GPER, as well as between modes of signaling within the classical ERs. Such an activity profile if developed into an ER antagonist could represent an opportunity for the development of first-in-class nuclear hormone receptor-targeted therapeutics for breast cancer exhibiting reduced acquired and de novo resistance.
2.2 Introduction

Estrogens (predominantly 17β-estradiol, E2) regulate multiple diverse aspects of physiology throughout the body, particularly during development, puberty and reproduction, but also in metabolic, endocrine, cardiovascular, nervous, musculoskeletal and immune functions\[5\]. Although many of these effects are traditionally associated with women, E2 also has important roles in male physiology \[4\]. As a result of these varied actions, targeting E2 pathways has been exploited extensively in the development of therapeutic and preventative approaches \[171\]. For example, E2 and its derivatives have been used for over a half a century as the primary constituent of contraceptive pills \[172\].

E2 and its receptors also play important roles in both health and disease, particularly breast cancer development and treatment. In addition to the classical nuclear estrogen receptors (ERα and ERβ), the 7-transmembrane spanning G protein-coupled estrogen receptor GPER (previously GPR30) has become recognized as an important mediator of E2 action \[23, 173-177\]. Although many of the effects of E2 are mediated by ERα and ERβ through transcriptional regulation, rapid signaling pathways (e.g. kinase activation, such as ERK1/2 and Akt, cAMP production and ion fluxes) that occur in the time frame of seconds to minutes are now understood to be activated by both ERα \[178\] and GPER \[173\]. Pharmacological approaches have identified families of compounds for breast cancer therapy as well as for managing symptoms of menopause (including osteoporosis) termed selective estrogen receptor modulators and downregulators (SERMs, such as tamoxifen and raloxifene, and SERDs, such as fulvestrant,
respectively) [56, 179], based on their (tissue-dependent, in the case of SERMs) transcriptional activities assumed to occur exclusively through ERα; however, tested SERMs and SERDs lack selectivity with respect to GPER, functioning as GPER agonists [72, 75, 88]. In fact, a broad array of xenoestrogens, including synthetic (industrial, agricultural and pharmacological) and natural (phyto- and myco-estrogens), have been shown not only to bind GPER but also to function as GPER agonists [23, 71, 74]. This lack of ER/GPER pharmacological discrimination led us and others to seek novel compounds with the ability to selectively modulate GPER activity, in the absence of ERα/β activity [89-92]. The most widely used GPER-selective ligands are the tetrahydroquinolines G-1 [89] (an agonist), G15 [90] and G36 [91] (both antagonists). G-1 mediates or reproduces many of the salutary effects of E2, particularly those associated with rapid signaling, in rodent models of multiple sclerosis [180, 181], stroke [182, 183], cerebral ischemia following cardiac arrest [184], traumatic brain and spinal cord injury [185, 186], myocardial infarction [187], atherosclerosis [188], obesity [175], diabetes [189], pancreatic islet survival [190] and transplantation [191], hypertension [192, 193], and diastolic dysfunction [194], among others [23, 174]. In contrast, the GPER antagonists G15 and G36 have been shown to have important applications in carcinogenesis [88, 160, 195] and cardiovascular aging [196], the latter through the regulation of NADPH oxidase-mediated superoxide production [197].

A similar lack of pharmacological selectivity towards the classical estrogen receptors ERα/β and against GPER has resulted in important experimental and clinical challenges. This is evident as the result of unexpected agonist activities of
both SERMs and SERDs via GPER in both experimental systems [75, 88, 164, 198, 199] and clinical use of the SERD ICI182,780 (fulvestrant) as an anti-hormone therapy for advanced breast cancer in women where, for example, symptomatic hypotension is a common side effect [200], consistent with the GPER-mediated vasodilatory activity of ICI182,780 observed ex vivo [201]. There is also evidence suggesting that the acquired resistance observed in women treated with anti-estrogens (SERMs and SERDs) for prolonged periods may result in part from chronic activation of GPER [159, 160, 202], potentially through the inactivation of the pro-apoptotic transcription factor Foxo3a [164].

Here we present the discovery of the first truly ER-selective ligand that lacks binding and activity towards GPER, defined as a selective ligand for estrogen receptor proteins (SLERP). We employed a combination of computational and biomolecular screening to identify AB-1, an oxabicyclic compound that binds both ERα and ERβ with similarly high affinity but lacks binding to GPER. Due to the absence of binding to GPER, rapid signaling via GPER is expectedly absent; however, although transcriptional activity via ERα is virtually identical to that of E2, AB-1 surprisingly lacks the ability to initiate multiple rapid signaling events via ERα. Thus, in addition to discriminating between ERα and GPER, AB-1 also discriminates between the transcriptional (genomic) and signaling (non-genomic) activities of ERα, providing the complementary activity profile to compounds that elicit extra-nuclear signaling but not transcriptional activity through ERα [203].
2.3 Results

Employing computational and virtual screening of a 10,000 compound GPCR-optimized library, we previously identified the GPER-selective agonist G-1 [89], the GPER-selective antagonist G15 [90] and subsequently optimized the even more selective antagonist, G36 [91], as compounds that lack ERα/β-binding (Fig. 2.1). The discovery of these GPER-selective compounds has facilitated a better understanding of the physiological roles of GPER in E2 signaling [23, 204]. To further distinguish the roles of ERs and GPER in E2 signaling, we sought to expand our repertoire of selective compounds, this time screening for compounds harboring an inverse profile to that of our GPER-selective compounds (i.e. high selectivity for binding ERα/β over GPER). Employing high-throughput flow cytometry-based biomolecular screening with ERα-GFP- and ERβ-GFP-expressing COS7 cells and the fluorescently labeled E2 derivative E2-Alexa633 as previously described [89], we screened the top 100 virtual hits of our compound library for selective binding activity towards ERα and ERβ. We identified one compound, a phenol-substituted oxabicyclo[3.3.1]nonene, hereafter termed AB-1 (Fig. 2.1), that competed with E2-Alexa633 binding to ERα and ERβ.

Figure 2.1. Chemical structure of estrogen (17β-estradiol, E2), GPER-selective ligand G-1 and ER-selective ligand AB-1.
To validate the activity and confirm the chemical identity of our primary hit, we synthesized AB-1 (4-(5-(hydroxymethyl)-8-methyl-3-oxabicyclo[3.3.1]non-7-en-2-yl))-phenol) [205, 206], following a modified procedure employing a hafnium(IV) triflate catalyzed Prins cyclization [207] (see Supplemental Methods). The compound was fully characterized and was identical to previously reported NMR spectra with characteristic $^1$H NMR signals observed for the C8-methyl (δ 1.01, 3H) and benzylic hydrogen at C2 (δ 4.50, 1H) [206] (Supplementary Fig. 1).

To confirm our findings and examine selectivity with respect to GPER, we co-expressed ERα-GFP or ERβ-GFP with GPER-mRFP1 in COS7 cells, incubated the cells with E2-Alexa633, and imaged by confocal microscopy. Because ERα/β and GPER localization is mutually exclusive, with ERα/β in the nucleus and GPER in the endoplasmic reticulum (i.e. cytosolic), selectivity of E2-Alexa633 binding can be assessed through spatial co-localization with each receptor (Fig. 2.2a). In cells expressing ERα-GFP and GPER-mRFP1, E2-Alexa633 is localized to both ERα and GPER (Fig. 2.2a, top row). Addition of E2 blocked binding of E2-Alexa633 to ERα-GFP (and ERβ-GFP, not shown) as well as GPER (GPER-mRFP1) (Fig. 2.2a, second row), whereas addition of AB-1 blocked the binding of E2-Alexa633 to both ERα-GFP and ERβ-GFP, but not to GPER-mRFP1 (Fig. 2.2a, third and fourth rows). To characterize the binding properties of AB-1 in greater detail, we determined its binding affinity to the individual ERs (Table 1.1). Using a flow cytometry-based competitive binding assay with transiently transfected COS7 cells, we determined that AB-1 blocked E2-Alexa633 binding to ERα and ERβ with
IC$_{50}$ values of 3 nM and 26 nM, respectively (Fig. 2.2b-c). IC$_{50}$ values for E2 were 0.3 nM and 0.6 nM for ER$\alpha$ and ER$\beta$, respectively. Importantly, AB-1 did not significantly block E2-Alexa633 binding to GPER at concentrations up to 10 μM.

![Image](image.png)

**Figure 2.2.** Ligand-binding properties of AB-1. (a) COS7 cells co-expressing ER$\alpha$-GFP or ER$\beta$-GFP with GPER-mRFP1 were stained with E2-Alexa633 in the presence or absence of unlabeled E2 (100 nM) or AB-1 (1 μM). AB-1 blocks the binding of E2-Alexa633 to ER$\alpha$ and ER$\beta$, but not to GPER. Confocal images are representative of three independent experiments. White scale bar
represents 10 μm. Data are from three independent experiments. (b-d) Binding affinities of E2 and AB-1 for ERα, ERβ and GPER. Competitive ligand binding assays were performed using 2 nM E2-Alexa633 and the indicated concentrations of unlabeled E2 (■) or AB-1 (▲) in COS7 cells transfected with either ERα-GFP (b), ERβ-GFP (c) or GPER-GFP (d). Data are mean ± s.e.m. from three independent experiments.

(Fig. 2.2d). Binding affinities to the purified ligand binding domain (LBD) of ERα and ERβ were also determined employing a TR-FRET-based competitive binding assay, revealing IC₅₀ values for ERα and ERβ LBDs of 38 nM and 24 nM, respectively (with IC₅₀ values for E2 of 0.26 nM and 0.47 nM for ERα and ERβ, respectively) (Supplementary Fig. 2.2 and Table 2.1). Taken together, these results show that AB-1 selectively binds to ERα and ERβ, but not GPER.

To assess the functional properties of AB-1, we first examined its effect on ER-mediated transcription in MCF-7 cells stably expressing an ERE-GFP reporter gene [208]. Like E2, AB-1 dose-dependently induced ERE activation with an EC₅₀ value of ~15 nM (vs. ~0.08 nM for E2) (Fig. 2.3a). To expand upon its transcriptional activity, we also assessed the effect of AB-1 on global ER-mediated gene transcription compared to that of E2 in MCF-7 cells. Interestingly, AB-1 induced an almost identical transcription profile (both in terms of activation and inhibition) to that of E2 (Fig. 2.3b, r=0.94, p<0.0001), with two of the best characterized E2/ER-stimulated genes (Progesterone Receptor and GREB1) showing virtually identical levels of upregulation, implying that AB-1 functions as an ER transcriptional regulator that activates and inhibits expression of ER-target genes similar to that of E2. Interestingly, a small number of the most E2/ER-repressed genes (e.g. PSCA, MYCN and FAM65C) were repressed to a lesser
extent by AB-1 compared to E2, although other genes repressed by about 8-10-fold by E2 were similarly repressed by AB-1.

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<th>E2</th>
<th>AB-1</th>
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<td>ERα cell binding (IC₅₀)</td>
<td>0.30 nM</td>
<td>3 nM</td>
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<td>ERβ cell binding (IC₅₀)</td>
<td>0.65 nM</td>
<td>26 nM</td>
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<td>ERα LBD binding (IC₅₀)</td>
<td>0.26 nM</td>
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<td>ERβ LBD binding (IC₅₀)</td>
<td>0.47 nM</td>
<td>24 nM</td>
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<tr>
<td>GPER cell binding</td>
<td>~8 nM</td>
<td>&gt;&gt; 10 μM</td>
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<td>ERE expression (EC₅₀)</td>
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<td>15 nM</td>
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<tr>
<td>MCF-7 proliferation (EC₅₀)</td>
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<td>ERα protein degradation (%)</td>
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<td>52 %</td>
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<td>Calcium signaling ERα (IC₅₀)</td>
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<td>Uterine Proliferation (EC₅₀)</td>
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**Table 2.1.** Summary of AB-1 properties

To further confirm the agonist nature of AB-1, we tested its ability to induce MCF-7 cell growth, which is not only induced by ER activation, but also dependent on it. AB-1 stimulated cell growth to a similar (in fact, slightly greater) maximal extent compared to E2, with an EC₅₀ of ~0.5 nM (vs. ~0.3 pM for E2) (Fig. 2.3c). Upon binding of both agonists and antagonists (classical SERDs), ERα protein undergoes degradation and ultimately downregulation of its steady state levels [209]. Therefore, to determine whether AB-1 exerts the same effect as E2 on ERα stability and protein levels, we treated MCF-7 cells with E2, AB-1, the SERM 4-hydroxytamoxifen (4-OHT), which stabilizes ERα, or the SERD fulvestrant (ICI182,780), which potently downregulates ERα. AB-1 induced a ~50% decrease in ERα levels, similar to that of E2, whereas 4-OHT and ICI182,780, as expected,
moderately increased and potently decreased ERα levels, respectively (Fig. 2.3d and Supplementary Fig. 2.3) [209]. Together, these data demonstrate that AB-1 acts as an agonist of ERα/β transcriptional activity, stimulating MCF-7 cell growth and inducing ERα degradation.

Figure 2.3. Transcriptional activity of AB-1. (a) Ligand-induced expression of GFP in MCF-7 cells. MCF-7 cells stably expressing an ERE-GFP reporter were stimulated with the indicated concentrations of E2 (■) or AB-1 (▲) and GFP expression was measured by flow cytometry. Data indicate mean ± s.e.m. of four independent experiments. (b) Ligand-induced global ER-mediated gene transcription profile. MCF-7/WS8 cells were stimulated with 1 nM E2 or 1 μM AB-1 and gene expression was assessed in duplicate. Gene expression changes of 1231 genes (greater than 1.5 fold) are shown as average log₂ fold-change compared to vehicle-treated cells for E2 (x-axis) and AB-1 (y-axis). Expression of GREB1 and PGR are shown with arrows. Correlation factor (R)
was 0.94 with a $p$-value <0.0001. (c) Effect of AB-1 on MCF-7 cell growth. MCF-7 cells were stimulated with the indicated concentrations of E2 (■) or AB-1 (▲) and total cell numbers were analyzed after 5 days. Cell numbers are shown as percentages relative to E2-treated cells (100%). Data points are mean ± s.e.m. of three independent experiments each performed in triplicate. (d) Ligand-induced protein degradation of ERα. MCF-7 cells were cultured with the indicated compounds and ERα levels determined by Western blot. Data is normalized to DMSO-treated samples and is shown as mean ± s.e.m. of at least 4 independent experiments. ***$p$<0.001 vs. DMSO by one-sample t-test.

To determine whether AB-1 mediates rapid signaling as observed for E2, we examined the PI3K/Akt-mediated inactivation of Foxo3a in MCF-7 cells [164]. Foxo3a is a forkhead box transcriptional activator of pro-apoptotic genes in the absence of survival factors. Growth factors (e.g. EGF) that stimulate the PI3K pathway lead to the Akt-mediated phosphorylation of Foxo3a, which in turn leads to its translocation to the cytoplasm and subsequent proteasomal degradation. To evaluate Foxo3a localization, we employed a Foxo3a-GFP construct that was transiently expressed in MCF-7 cells. Following EGF stimulation, Foxo3a translocated from the nucleus to the cytoplasm (Fig. 2.4a). E2 and the GPER-selective agonist G-1 also stimulated cytosolic translocation, although in a lower percentage of cells (Fig. 2.4b). In contrast, AB-1 had no effect on Foxo3a translocation, nor did it alter the extent of E2- or G-1-mediated translocation (Fig. 2.4b). This result is in fact consistent with our previous observations that the E2-mediated activation of PI3K and Akt, leading to Foxo3a inactivation, is mediated by GPER.

In order to determine whether AB-1 can also mediate E2-dependent rapid signaling specifically via the classical estrogen receptors, we employed COS7 cells
Figure 2.4. Ligand-induced intracellular translocation of FOXO3a. (a) Intracellular localization of FOXO3a-GFP. MCF-7 cells transiently expressing FOXO3a-GFP were treated with vehicle (Ctl), E2 (10 nM), G-1 (100 nM), AB-1 (1 μM), EGF (50 ng/mL) or a combination of AB-1 + E2 or AB-1 + G-1 and FOXO3a-GFP localization determined by confocal microscopy. Data are representative of three independent experiments. (b) Quantification of data in (a) and represent the mean ± s.e.m. of at least 3 independent experiments. *p<0.05 vs. vehicle (Ctl) by one-way ANOVA with Bonferroni post-hoc test.

expressing either ERα, ERβ or GPER. We first examined the ability of AB-1 to induce calcium mobilization. Surprisingly, unlike E2, which induced rapid calcium
mobilization in COS7 cells expressing ERα, ERβ or GPER (Fig. 2.5a), AB-1 did not induce calcium mobilization in any of these cells (Fig. 2.5b). More importantly, AB-1 dose-dependently inhibited E2-mediated calcium mobilization in COS7 cells expressing either ERα (IC$_{50} =$ 33 nM) or ERβ (IC$_{50} =$ 75 nM) (Fig. 2.5c), but did not block E2-mediated calcium mobilization in GPER-expressing COS7 cells (Fig. 2.5b). This result suggests that despite acting as an agonist of transcriptional activation via ERα, AB-1 acts as an antagonist or inverse agonist of ER-mediated rapid calcium signaling.

Despite E2-dependent PI3K/Akt activation in MCF-7 cells being mediated by GPER, we have previously shown that E2 can mediate PI3K activation by both classical estrogen receptors (ERα and ERβ) and GPER in transfected COS7 cells [72]. Thus, to determine whether the inhibitory effect of AB-1 on rapid calcium signaling extends to other rapid signaling pathways, we next examined whether AB-1 could regulate PI3K activation in COS7 cells transfected with either ERα-GFP, ERβ-GFP or GPER-GFP. Cells were co-transfected with the PH-mRFP1 reporter, which contains the PIP$_3$-binding pleckstrin homology (PH) domain of Akt fused to a red fluorescent protein and thus translocates to sites of PI3K activity and PIP$_3$ accumulation [72]. Employing this system, we observed that E2 induced strong nuclear localization of the PH-mRFP1 reporter in COS7 cells expressing ERα, ERβ or GPER (Fig. 2.5d, second row), indicative of PI3K activation, as previously reported [72]. However, unlike E2, AB-1 did not induce nuclear translocation of the PH-mRFP1 reporter in COS7 cells expressing ERα, ERβ or GPER (Fig. 2.5d, third row). Furthermore, AB-1 was again able to block
E2-mediated signaling via ERα and ERβ, but not through GPER (Fig. 2.5d, bottom row). Together, the calcium and PI3K signaling results not only further confirm the binding selectivity of AB-1 for ERα and ERβ vs. GPER, but more importantly and surprisingly, they reveal that AB-1 acts as an antagonist of rapid signaling via the classical estrogen receptors ERα and ERβ.
Figure 2.5. AB-1 antagonizes classical ER-mediated rapid signaling. (a-c) Ligand-induced effect on intracellular calcium mobilization through individual ERs. COS7 cells transiently expressing ERα-GFP (red curve), ERβ-GFP (blue curve) or GPER-GFP (green curve) were stimulated with either 1 nM E2 (a) or 1 μM AB-1 followed by 1 nM E2 (b). Intracellular calcium mobilization was evaluated using indo1-AM and ligands were added at 20 s or 80 s as indicated. Data is shown as the relative 490nm/400nm ratio change (y-axis) compared to mock-transfected COS7 cells (black curve) and re representative of three independent experiments. (c) Intracellular calcium mobilization dose-response curves for E2-stimulated COS7 cells expressing ERα-GFP (▲) or ERβ-GFP (■), treated with the indicated concentrations of AB-1. Data indicate mean ± s.e.m. of three independent experiments. (d) AB-1 antagonism of PI3K activation through ERα and ERβ. COS7 cells co-expressing PH-mRFP1 and either ERα-GFP (left panel), ERβ-GFP (middle panel) or GPER-GFP (right panel) were stimulated with vehicle (DMSO), 1 nM E2, 1 μM AB-1 or a combination of E2 + AB-1. PI3K activation was assessed by the translocation of the PH-mRFP1 reporter from the cytoplasm to the nucleus as exemplified by E2 treatment of ERα and ERβ-expressing cells. Confocal images are representative of three independent experiments.

In vivo assessment of compound estrogenicity has traditionally been carried out employing the uterotrophic assay, based on highly E2-dependent actions in the uterus. Upon E2 depletion in mice, typically through ovariectomy, the uterus regresses with the epithelium entering a non-proliferative state and the uterine losing electrolytes and water, resulting in substantial weight reduction. Treatment with E2 for 1-3 days leads to an acute stimulation of proliferation within the uterine epithelium and an increase in overall weight due to water uptake, termed imbibition. To investigate the estrogenic effects of AB-1 in vivo, we evaluated the uterotrophic effects of AB-1 compared to E2. Whereas E2 yielded a strong imbibition response at a dose of 10 ng (with an EC₅₀ estimated between 2 and 10 ng), AB-1 displayed imbibition only at a dose of 91 μg (with an almost 2-fold increase in uterine wet weight over that of sham-treated mice), with no effect at
doses of 2 and 10 μg, suggesting an EC$_{50}$ in the 50-90 μg range (Fig. 2.6a). We also examined the effect of AB-1 on the proliferative response of uterine epithelial cells in the same mice used for the uterotrophic assay. AB-1, at the highest dose tested, induced an almost 12-fold increase in epithelial proliferation (measured as Ki-67 positive staining) vs. sham-treated mice, similar to the response observed with 10 ng E2 (Fig. 2.6b). Together, these results demonstrate that AB-1 stimulates multiple murine uterine effects associated with the activities of ERα, though with less potency compared to E2.

**Figure 2.6.** Estrogenic effects of AB-1 in the mouse uterus. (a) Ligand-induced effect on mouse uterine weight. Ovariectomized mice were treated with vehicle (sham) or the indicated amounts of E2 or AB-1 for 18 h and body weights and uterine wet weights determined. Uterine weights are shown as ratios to total body weights (mean ± s.e.m.). (b) Uterine epithelial cell proliferation. Fixed uterine sections from samples in (a) were assessed for epithelial cell proliferation by staining for Ki-67 expression. Data are the mean ± s.e.m. of 3 mice per group; *p < 0.05 vs. sham by one-way ANOVA with Bonferroni post-hoc test.
2.4 Discussion

Our understanding of E2 signaling has evolved over the last half century, from the earliest cellular studies of rapid signaling responses [210, 211], to the subsequent appreciation of its transcriptional regulation through ERα and later ERβ. With the discovery of GPR30 as an additional estrogen receptor (leading to its designation as GPER) that mediates many of the rapid signaling events in response to E2, the landscape of E2 signaling mediators became more complicated. Pharmacological approaches have traditionally been critical in unravelling the roles of individual receptor subtypes within a family. In the case of the classical ERs and GPER, this approach has been complicated by the high degree of overlap in ligand specificity [17]. Not only are the ligand binding pockets of ERα and ERβ highly homologous, but to date all tested ER-binding compounds exhibit binding and/or activity towards GPER [23]. This is particularly true of the family of SERMs and SERDs, which despite generally inhibiting activity of the classical ERs, act as agonists of GPER. Studies of GPER were facilitated with the identification of the highly selective GPER agonist G-1 [89] and soon thereafter GPER antagonists (G15 and G36) [90, 91], all of which exhibit little to no activity towards the classical ERs. Unfortunately, compounds with the inverse selectivity, i.e. binding to ERs but not GPER, have to date not been identified. In this report, we described the identification of the first such compound AB-1, that binds with high affinity to both ERα (and ERβ) but not to GPER, defining AB-1 as a novel SLERP.

Pharmacological selectivity between the two classical estrogen receptors
(ERα and ERβ) has been difficult to achieve, largely due to the extremely high sequence and structural conservation of the ligand binding pockets of these two receptors. Following decades of optimization, the most highly ERα-selective compound PPT (PPT) exhibits only about 400-fold selectivity for ERα over ERβ [65]. Despite this, PPT has been shown to lack selectivity for ERα against GPER, where it acts as an agonist [88]. Thus, based on the fact that to date all tested ERα ligands bind to or activate GPER [23], one might speculate that achieving ERα selectivity vs. GPER might be extremely difficult. This is in contrast to the high selectivity (>10^5 fold) of the GPER-selective agonist G-1 for GPER over ERα [91], which is believed to be due to the fact that G-1 is slightly larger than E2 [89], precluding its occupancy of the ligand binding pocket of ERα or ERβ while allowing its binding to the presumably slightly larger or conformationally more accommodating ligand binding pocket of GPER.

Although “bulky” bicyclic compounds may seem like a poor substitute for the planar E2 molecule, the ability of bicyclic compounds, such as bicyclo[3.3.1]nonanes, to function as ER ligands was reported by Katzenellenbogen in 2003 [212]. Compounds of the oxabicyclo[3.3.1]nonene structural class were first identified as ER ligands through screening campaigns carried out by multiple independent groups in the mid-2000s. In 2003, Sibley et al. at Bayer AG, identified AB-1 (termed compound 2) in a primary screen as an ER ligand [205]. In 2005, Hamann et al. at Ligand Pharmaceutical again identified AB-1 (compound 3) in a primary screen [206] and in 2006 Hsieh et al. reported the characterization of AB-1 (termed OBCP-1M) identified from a high-throughput
functional screen of the ChemBridge 10,000-compound chemical library (San Diego, CA) [213]. Thus, the inclusion of compounds with the oxabicyclo[3.3.1]nonene scaffold has been a recurring occurrence in the design of chemical libraries, perhaps due to its structural rigidity. Interestingly, our virtual screen for similarity to E2 ranked AB-1 as 53rd, whereas G-1 was ranked as 92nd of the 10,000 compounds in our GPCR-optimized library. Thus, both compounds fell within the top 1% of the library in terms of E2 “similarity”, despite that fact that they display inverse properties with respect to ER and GPER selectivity.

The previous three reports identifying AB-1 as an ER ligand were published prior to the wide acceptance of GPER as an E2 receptor; as a consequence, no evaluation of GPER selectivity, either in terms of binding or function, was performed. Furthermore, none of the reports examined rapid signaling mechanisms such as those observed for E2. Selectivity of ERα vs. ERβ was however examined. Hamann et al., reported, based on transcriptional reporter assays, a 2-fold difference in EC\(_{50}\) of racemic AB-1, favoring ERβ over ERα [206]. Whereas the (+) and (-) isomer displayed similar EC\(_{50}\) values for ERβ, similar to the (-) isomer for ERα, the (+) isomer displayed a 20-fold worse EC\(_{50}\) for ERα. Hsieh et al. also observed a selectivity for ERβ employing racemic AB-1, both in terms of binding to purified ER ligand binding domain (~10-fold selectivity) and function (transcriptional reporter assays, ~60-fold) [213]. Interestingly, in permeabilized whole cell ligand binding assays, we observed comparable binding of AB-1 to ERα and ERβ (Table 2.1).

Our results demonstrate an exceptionally high correlation between the gene
expression profiles of E2 and AB-1 in MCF-7 cells. Given the lack of rapid signaling observed for AB-1, this would suggest that rapid signaling has a minimal impact on ERα-mediated transcriptional activity. In contrast, downregulation of ERK2 (via siRNA) has been shown to alter the gene expression profile of E2 in MCF-7 cells [214], suggesting a role for MAPK signaling in transcriptional activity of ERα. In the same study, ERK2 expression was also shown to be critical for E2-mediated MCF-7 cell proliferation. In our gene expression study, MCF-7 cells were deprived of E2 for a total of 4 days prior to stimulation with either E2 or AB-1 for 24 hours. Under these conditions, basal levels of ERK2 activity are expected to be decreased but perhaps not to the same extent as in the presence of ERK2 knockdown, suggesting that basal ERK2 activity may be sufficient to support E2-mediated regulation of transcription. Finally, the high concordance between E2- and AB-1-mediated transcriptional regulation suggests that the conformation of ERα induced by AB-1 is very similar to that of E2, resulting in the similar recruitment of co-activators and co-repressors.

The ability of E2 to mediate rapid (i.e. non-genomic) signaling has been known for over 50 years, from early studies of E2-mediated cAMP production and calcium (45Ca) mobilization [210, 211], to the resurgence of interest in such pathways in the 1990s [215, 216]. Multiple approaches have been employed over the years to investigate mechanisms of rapid E2-mediated signaling, including the generation of mutant forms of ERα (e.g. membrane- or nuclear-targeted forms of the receptor) [178] and pharmacological approaches employing novel ligands, such as large E2-dendrimers (that cannot pass the plasma membrane) [217] and
small molecule PaPEs (pathway preferential estrogens) that exhibit exceptionally low affinity for ERα, resulting in activation of non-genomic signaling but not transcriptional activity [203]. The advent of GPER-selective ligands further enhanced our understanding of rapid E2-mediated signaling events in multiple cell types and tissues by selectively activating or inhibiting GPER in the absence of ER activity [23]. Now, for the first time, we have identified a truly ER-selective compound that displays no binding affinity or activity towards GPER, enabling studies of ER-specific activities in the absence of GPER signaling. Furthermore, the selective profile of AB-1 with respect to ER activity, activating transcription while precluding ER-mediated rapid signaling, provides additional selectivity that will further our understanding of ER function. It should be noted that we only examined two aspects of ER-specific rapid signaling, namely calcium mobilization and PI3K activation, limiting our conclusions to these pathways. Because the mechanisms of ER-mediated signaling are in general poorly understood, it is possible that other aspects of rapid signaling may be preserved. Nevertheless, the ability of AB-1 to regulate ER-mediated gene expression in an almost identical manner to E2, while having no effect on GPER-mediated signaling, represents a novel pharmacological profile.

There has been mounting evidence that GPER expression and activation by currently employed anti-estrogens, particularly tamoxifen, play an important role in resistance to these drugs, as suggested by the poor prognosis of breast cancer patients treated only with tamoxifen [159], increased GPER expression in breast cancer patient biopsies following tamoxifen treatment [159], enhanced GPER
signaling in tamoxifen-resistant MCF-7 cells [202], inhibition of tamoxifen-resistant breast cancer cell growth by GPER antagonists [160] and improved survival of MCF-7 cells in the presence of G-1 [164]. Based on such results, the development of highly ER-selective antagonists that lack GPER cross-reactivity could be of significant clinical benefit, lowering the occurrence of resistance seen with current anti-estrogen therapies.
2.5 Materials and methods

**Cell culture and transfection.** Cell lines were obtained from the American Type Culture Collection. Cells were cultured in the stated medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Transient transfection experiments were performed 24 h after seeding cells using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. The expression plasmids have been previously described [72]. For E2 deprivation, cells were grown for 24-48 h (with intermediate changes of medium) in phenol red-free medium lacking serum or supplemented with 10% charcoal-stripped FBS, both of which were further supplemented with 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin.

**Virtual Screening.** A database containing structures of 10,000 molecules (CDLDB) provided by Chemical Diversity Labs Inc (San Diego, CA), to which 17β-estradiol was added, was processed as described previously [218]. Briefly, using 17β-estradiol as reference point, 2D-based similarity coefficients were computed employing both Daylight and MDL fingerprints using Tanimoto’s symmetric distance-between-patterns [219] and Tversky’s asymmetric contrast model [220]. We also obtained 3D shape similarity coefficients using the Tanimoto [219] and Tversky [220] formulae using Rapid Overlay of Chemical Structures [221]. An additional pharmacophore-based 3D similarity metric was derived from ALMOND descriptors [222]. The combined similarity score attributed 40% weighting to 2D fingerprints, 40% to the shape-based similarities and 20% to pharmacophore-
based similarity. Given this composite score, the top 100 ranked molecules were selected for physical screening employing a fluorescent whole cell ligand-binding assay.

**Chemical Synthesis.** G-1 was synthesized as previously described [223]. The compound **AB-1** (4-(5-(hydroxymethyl)-8-methyl-3-oxabicyclo[3.3.1]non-7-en-2-yl)-phenol) has been reported previously [205, 206], and was synthesized by a modified procedure [207] and obtained as a diastereomerically pure, racemic mixture of enantiomers. Compound identity was verified by comparison of high field $^1$H NMR (500 MHz) spectra to published values [206], and purity was demonstrated by quantitative analytical HPLC chromatography to be >98%. Full experimental details and spectroscopic data confirming the identification of AB-1 are provided in the supplementary section (**Appendices A and B**).

**Ligand-binding assays.** Binding assays for ERα, ERβ and GPER were performed as previously described [72]. Briefly, COS7 cells were transiently transfected with ERα-GFP, ERβ-GFP or GPER-GFP. Following serum starvation for 24 h, cells (~5x10⁴) were incubated with competitor for 20 min prior to addition of an equal volume of 4 nM E2-Alexa633 in saponin-based permeabilization buffer. Following 10 min at 25 °C, cells were washed once with PBS/2%BSA. For flow cytometric analysis, cells were resuspended in 20 µL and 2 µL samples were analyzed on a DAKO Cyan flow cytometer using HyperCyt™ as described [224]. For confocal microscopy, cells were stained as above and fixed with 2% PFA in PBS containing
1 mM CaCl₂ and 1 mM MgCl₂ for 15 min, washed, mounted in Vectashield and analyzed immediately by confocal microscopy using a Zeiss LSM510 confocal fluorescent microscope.

**Intracellular calcium mobilization.** COS7 cells transfected with ERα-GFP, ERβ-GFP or GPER-GFP (5 x 10⁶ cells) were incubated at room temperature in HBSS containing 5 μM indo1-AM and 0.05% pluronic acid for 30 min. Cells were then washed once with HBSS and resuspended in HBSS at a density of 10⁷ cells/mL. Ca²⁺ mobilization was determined ratiometrically using λ<sub>ex</sub> 340 nm and λ<sub>em</sub> 400/490 nm at 37°C in a spectrofluorometer (QM-2000-2, Photon Technology International) equipped with a magnetic stirrer and heated sample chamber. The relative 490nm/400nm ratio is plotted as a function of time.

**PI3K activation.** The PIP3-binding domain of Akt fused to mRFP1 (PH-mRFP1) was employed to assess cellular PIP3 production and localization as described [72]. Briefly, COS7 cells (co-transfected with PH-mRFP1 and either ERα-GFP, ERβ-GFP or GPER-GFP) were plated on coverslips and serum starved for 24 h followed by stimulation with ligands as indicated for 15 min. The cells were fixed with 2% PFA in PBS, washed, mounted in Vectashield and analyzed by confocal microscopy using a Zeiss LSM510 confocal fluorescent microscope.

**ER-ERE transcription.** ERα activity via EREs was determined using MCF-7 cells stably transfected with an ERE-GFP reporter construct [208] as previously
described [91]. Briefly, cells were deprived of E2 for 4 days (with one intermediate medium change) in phenol red-free DMEM/F12 supplemented with 10% charcoal-stripped FBS. Cells (~80,000) were seeded in 24 well plates, and 24 hours later treated with the indicated compounds (dissolved in DMSO, 0.1% final) for 24 hours in triplicate, trypsinized, washed and analyzed for green fluorescence by flow cytometry. Mean fluorescence intensities of gated live cells were determined and normalized to E2 values following subtraction of vehicle control values.

**Gene expression analysis.** MCF-7/WS8 cells, provided by Craig Jordan (MD Anderson), were cultured in RPMI supplemented with 10% FBS, 2 mM L-glutamine, non-essential amino acids, antibiotic/antimycotic and 6 ng/ml of insulin. E2 depletion was carried out by culturing cells in E2-depleted medium with daily medium changes for three days. Cells were seeded sparsely (2x10⁶ cells per 15 cm dish) in E2-depleted medium and treated the following day with 1 nM E2, 1 μM AB-1 or DMSO (vehicle control) for 24 hours. Final DMSO concentrations were 0.01%. Total RNA was isolated using QIAGEN RNeasy minikits following homogenization using QIAshredders and employing the direct lysis protocol for cell monolayers. Total RNA (500ng) was reverse transcribed using a T7 Oligo(dT) primer, followed by second strand synthesis and purification of the double stranded cDNA. In vitro transcription was performed on this product using a mix of biotinylated nucleotides to generate biotin labeled cRNA as described (Ambion/Applied Biosystems Illumina Total Prep RNA Amplification Kit). cRNA samples were hybridized to the BeadChip array, washed, stained with
C3-strepavidin following the manufacturer’s protocols (Illumina). The BeadChip was scanned and data analyzed using the Genome Studio Gene Expression Module (Illumina). Samples were normalized using a rank invariant normalization. Missing data were imputed, and Benjamini and Hochberg false discovery rate calculations were applied. The DMSO controls were used as reference samples and the Illumina custom error model was employed.

**Cell proliferation.** MCF-7 cells were grown in E2-depleted medium for 4 days (with one intermediate medium change) in phenol red free DMEM/F12 supplemented with 10% charcoal-stripped FBS. Cells were seeded in 96 well plates at low density, and 24 hours later treated with the indicated concentrations of compounds (dissolved in DMSO, 0.1% final) for 3-5 days in triplicate. Cell growth was determined by Alamar Blue staining.

**ERα degradation.** MCF-7 cells were seeded (500,000 cells/well) in 6-well plates in complete culture medium. The following day, cells were transferred to medium containing charcoal-stripped serum for 48 h (with one intermediate change of medium) and subsequently treated with the indicated compounds (0.01% DMSO final) for 24 h. Cells were washed once with ice-cold PBS, lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% Na-deoxycholate and 0.1% SDS) containing 50 mM NaF, 1 mM Na₃VO₄ and protease cocktail (1x) and passed through a 20G needle (10-16 times). Lysates were cleared by centrifugation (13,000 rpm for 15 min at 4°C) and protein concentrations
determined using the Pierce™ BCA Protein Assay Kit. Samples (20 μg) were resolved by SDS-PAGE (4-12% Bis-Tris gel), transferred to nitrocellulose membranes and subjected to Western blot analysis. Membranes were probed overnight with a rabbit anti-ERα antibody (Cell Signaling, 1:1000) in 4% BSA-TBST at 4°C followed by a secondary HRP-linked goat anti-rabbit antibody (1:5000) for 1 h at RT. Bands were visualized by chemiluminescence. To detect actin, membranes were stripped (30 min at RT) and probed with a mouse anti-actin antibody (Millipore, 1:5000) for 1 h at RT followed by a secondary HRP-linked goat anti-mouse antibody (1:2500) for 1 h at RT. Bands were quantified using ImageJ software (NIH).

**FOXO3a translocation.** FOXO3a localization assays were performed as described [164]. Briefly, MCF-7 cells were seeded on 12 mm coverslips in a 24-well plate one day before transfection. Cells were transfected with 0.3 μg FOXO3a-GFP using the Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol. Twenty-four hours post-transfection, cells were serum starved for 24 h prior to treatment. Cells were fixed in 2 % PFA, washed with PBS and mounted in Vectashield on coverslips. Coverslips were imaged on a Zeiss LSM800 microscope and localization determined from 10 fields per condition.

**Mouse uterine estrogenicity.** C57Bl6 female mice (Harlan) were ovariectomized at 10 weeks of age. E2 and AB-1 were dissolved in absolute ethanol at 1 mg/mL and diluted in ethanol. For treatment, 10 μL of diluted E2 or AB-1 was added to
90 μL aqueous vehicle (0.9% NaCl with 0.1% albumin and 0.1% Tween-20). Ethanol alone (10 µL) was added to 90 µL aqueous vehicle as control (sham). Twelve days post-ovariectomy, mice were injected subcutaneously at 5:00 pm with 100 µL sham, E2 or AB-1. Eighteen hours after injection, mice were killed, weighed and uteri removed and weighed (normalizing to body weight) after the mesometrium and any attached adipose tissue was trimmed away. Uteri were then fixed in 4% paraformaldehyde, and embedded in paraffin. Five-micron sections were placed on slides, and proliferation in uterine epithelia was quantitated by immunofluorescence using anti-Ki-67 antibody (LabVision) followed by goat anti-mouse IgG conjugated to Alexa488 (Invitrogen). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). At least 4 animals per treatment were analyzed, and the Ki-67 immunodetection was repeated three times per mouse. Percent Ki-67 positive cells = (number of Ki-67 positive cells / total number of DAPI-stained luminal epithelial cells) x 100 for three different fields per sample.

**Statistical analyses.** Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test, by two-tailed, unpaired Student’s t-test or by one-sample t-test as appropriate. Non-linear regression curves were determined using a variable slope fit. Values are expressed as mean ± s.e.m.; n equals the number of assay replicates or animals used. Statistical significance was accepted at a P value < 0.05. All analyses were carried out using Prism version 5.0 for Macintosh, GraphPad Software.
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CHAPTER 3
Identification of an ER-selective antagonist lacking GPER activity

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3.1 Abstract

Endocrine therapy has shown tremendous success for the treatment of estrogen receptor (ER)-positive breast cancers for over 40 years, with tamoxifen being a widely used first-line therapeutic drug option. Unfortunately, many women on tamoxifen and similar anti-hormone therapies develop resistance to these agents. Cross-reactivity of these drugs to the G protein-coupled estrogen receptor (GPER), is thought to contribute to this clinically observed resistance. This highlights the need for truly ER-selective antagonists that lack cross-reactivity to GPER. Here, we describe the identification of a small molecule, termed AB-82P, that has the unique property of acting as an ER antagonist while lacking activity towards GPER. AB-82P defines a new class of ER antagonists and could serve as the structural basis for the development of truly ER-selective antagonists for the treatment of ER-positive breast cancers; potentially improving patient outcomes by decreasing the overall development of endocrine resistance in breast cancer in patients.
3.2 Introduction

Estrogen (mainly E2) plays an important role in many physiological systems including the female reproductive, the cardiovascular, and the immune systems [5, 19, 175]. It exerts its function through the classical estrogen receptors, ERα and ERβ, and the 7-transmembrane spanning G protein-coupled estrogen receptor (GPER, formerly GPR30) [16, 72, 225]. The classical ERs are mainly responsible for E2-mediated transcriptional signaling, whereas GPER mainly exerts E2-mediated rapid, non-genomic signaling, such as ERK1/2 and Akt activation [17, 226]. Estrogen also plays a role in disease, exemplified by its role in breast cancer development and progression [95]. In many breast cancers (~70%), estrogen exerts proliferative and survival signaling through ERα, making it critical in the growth of ERα-positive tumors [96]. This has made ERα an important therapeutic target in breast cancer therapy for over 40 years [97].

Endocrine therapy, using selective estrogen receptor modulators (SERMs, such as tamoxifen), downregulators (SERDs, such as fulvestrant) and aromatase inhibitors which block the synthesis of E2, has shown great success in the clinic for the treatment of ERα-positive breast tumors, having prolonged the lives of millions of breast cancer patients [98, 99]. Until recently, SERMs, tamoxifen in particular, have been a popular first-line therapy, alongside aromatase inhibitors (in post-menopausal women), in treating ERα-positive breast cancers, but the SERD fulvestrant (ICI182,780, typically used in metastatic relapse cases) has been gaining traction as a first-line therapy due to the potent therapeutic efficacy of SERDs [227, 228].
Tamoxifen is one of the most widely used endocrine therapies for treating ER-positive breast cancers and is the most prescribed SERM. Unfortunately, patients taking tamoxifen often relapse with tamoxifen-resistant tumors during or following the completion of the standard 5-year regimen [98, 120]. Moreover, about a third of patients do not respond initially to the therapy due to de novo resistance. Several mechanisms have been described to contribute to the development of acquired endocrine resistance, including the development of endocrine-resistant ERα mutants (e.g. the Y537S and D538G mutants) [121-123]. One mechanism, in particular, that has been proposed to contribute to the development of tamoxifen resistance is thought to be chronic tamoxifen-induced activation of GPER [124, 160]. Tamoxifen, an ERα antagonist, has been shown to cross-activate GPER in breast and other cell types, leading to the activation of the proliferative and pro-survival factors ERK1/2 and Akt [72, 75, 88, 162]. In ERα-positive breast tumors, this cross-activation of GPER (and subsequent activation of survival pathways), is hypothesized to oppose the pro-apoptotic signals induced by the inhibitory effects of tamoxifen (via ERα), thereby potentially prolonging the survival of a subset of breast cancer cells. This prolonged survival may lead to the eventual acquisition of tamoxifen-resistant mutations (including the ERα Y537S) and development of tamoxifen-resistant relapse tumors (Pepermans and Prossnitz in review). Importantly, many other SERMs and SERDs, including raloxifene and fulvestrant, have also been shown to cross-activate GPER in breast cancer cells [23]. Clinical data also supports the role of GPER as a contributor to the development of endocrine resistance. Ignatov et al. reported higher levels of GPER
expression in relapse tumors (compared to matched primary tumors) of patients that had undergone prior treatment with tamoxifen versus GPER expression levels observed in relapse tumors of patients that had not undergone prior tamoxifen therapy [159]. Together these observations highlight the potential need for the development of new SERMs and SERDs that inhibit ERα yet do not cross-activate GPER.

Here we describe the identification of a small ligand, termed AB-82P, that acts as a truly selective antagonist of the classical ERs (ERα and ERβ), while not showing any cross-activity towards GPER. Employing molecular modeling of the previously identified ER-selective transcriptional agonist, AB-1 (Revankar, Bologa, Pepermans, Sharma et al. in review) in the ligand binding domain (LBD) of ERα and its X-ray structure [213], we identified a site on AB-1 that, following the addition of an alkyl chain, converted the ligand to an antagonist (AB-82P) of the classical ERs, while maintaining its non-reactivity towards GPER. We show that AB-82P binds to the classical ERs while not inducing rapid GPER-mediated signaling. Furthermore, AB-82P blocks E2-mediated ERα transcriptional activity and induces degradation of both the wildtype and the Y537S mutant form of ERα. Lastly, we show that AB-82P exhibits growth inhibitory properties in wildtype MCF-7 cells and two cell line models of endocrine resistance.
3.3 Results

GPER is implicated in the development of tamoxifen resistance in breast cancer, possibly through its cross-activation by tamoxifen. This highlights a therapeutic benefit for truly ER-selective antagonists. Currently, no ER-selective antagonists exist that lack cross-reactivity towards GPER. Therefore, we sought to identify compounds that harbor such properties. We previously identified the ER-selective agonist, AB-1, by high-throughput screening (manuscript under review). Employing molecular modeling, using the crystal structure of AB-1 bound to the ERα-LBD (PDB 2B1V, termed OBCP-1M) and the crystal structure of the ERα antagonist ICI164,384 bound to rat ERβ-LBD (PDB 1HJ1), we observed that the methyl group at carbon-8 of AB-1 protruded out of the ERα-LBD at a site similar to the carbon-tail of ICI164,384 (Fig. 3.1, red arrow) [213, 229]. We hypothesized that the addition of an alkyl chain at this position would convert AB-1 into an ER antagonist while maintaining its selectivity for the classical ERs over GPER. To test this, we synthesized an AB-1 derivative, termed AB-82P, with an alkyl chain attached at the carbon-8 position of AB-1 (Fig. 3.1 and Supplementary Fig. 3.1).

![Figure 3.1](image)

**Figure 3.1.** Chemical structure of estrogen (E2), the ER-selective agonist AB-1 and the ER-selective ligand AB-82P. Red arrow indicates carbon-8 position of AB-1.
Figure 3.2. Ligand-binding properties of AB-82P. (a) Binding affinities of AB-82P for ERα and ERβ. GST-tagged ERα-LBD (●) or ERβ-LBD (■) was incubated with 3 nM fluorescently-labeled E2 analogue in the presence of the indicated concentrations of AB-82P. Competitive binding was assessed by TR-FRET by labeling the ER-LBDs with an electron donor, terbium-labeled antibody. (b) Ligand-induced activation of ERK. Hec50 cells were treated with the indicated compounds for 15 min and phosphorylated ERK levels were assessed by Western blot analysis and normalized to the DMSO-treated samples. Data are mean ± s.e.m. of three independent experiments.

To determine whether the addition of the alkyl chain resulted in AB-82P becoming a selective ER antagonist, we first assessed whether it still bound to the classical ERs. Employing a TR-FRET-based competitive binding assay, purified of ERα-LBD and ERβ-LBD were incubated with a fluorescent E2 analogue and increasing concentrations of AB-82P. AB-82P dose-dependently blocked binding of the fluorescent E2 analogue to both the ERα-LBD and the ERβ-LBD with IC50 values of 0.22 μM and 1.8 μM, respectively (Fig. 3.2a). Next, we tested whether AB-82P shows any activity towards GPER by assessing its ability to induce GPER-mediated ERK activation. We treated Hec50 cells, which express GPER but neither ERα or ERβ, with E2, the GPER-selective agonist G-1, AB-82P or a
combination of E2 or G-1 with AB-82P [89]. While both E2 and G-1 increased ERK activity in the cells, AB-82P had no effect on ERK activity. More, importantly, AB-82P did not affect E2- or G-1-induced activation of ERK, implying that AB-82P does not bind or have activity towards GPER (Fig. 3.2b). Together these data show that the addition of the alkyl chain to AB-1 did not alter the ER-binding selectivity of the molecule and that AB-82P selectively binds to the classical ERs, but not to GPER.

Next, we assessed the functional properties of AB-82P. First, we determined its ability to induce ER-mediated ERE activation in MCF-7 cells stably expressing an ERE-GFP reporter gene [230]. Whereas E2 dose-dependently induced ER-mediated ERE activation (EC$_{50}$≈ 30 pM), AB-82P did not induce ERE activation up to the maximum tested dose (5 μM) (Fig. 3.3a). Similarly, the ERα antagonists, 4-hydroxytamoxifen (4-OHT, the active form of tamoxifen) and fulvestrant (ICI), at 1 μM, did not induce ERE activation. To examine whether AB-82P could block E2-induced ERE activation, we treated the cells with increasing doses of AB-82P in the presence of E2. AB-82P dose-dependently blocked E2-mediated ERE activation with an IC$_{50}$ of 2 μM, which is ≈1000-fold higher than that of 4-OHT and ICI (IC$_{50}$ values for 4-OHT and ICI were both 3 nM) (Fig. 3.3b). These data indicate that AB-82P acts as an antagonist of the classical ERs. To expand upon its transcriptional reporter activity, we tested whether AB-82P could block E2-mediated transcription of known ERα target gene by qRT-PCR. MCF-7 cells were treated with E2, AB-82P or a combination of the two and RNA levels of TFF1, GREB1 and progesterone receptor (PGR) were
measured. AB-82P alone did not induce gene transcription of \textit{TFF1}, \textit{GREB1} or \textit{PGR}. In fact, AB-82P induced a slight decrease in \textit{TFF1} and \textit{GREB1} transcript levels, possibly due to residual E2 in the charcoal-stripped medium (Fig. 3.3c). Importantly, AB-82P blocked E2-induced transcription of these target genes. ICI was used as a control inhibitor and showed strong inhibition of E2-induced gene transcription at a 5-fold lower concentration than AB-82P.

\textbf{Figure 3.3.} Transcriptional activity of AB-82P. (a-b) Ligand-induced expression of GFP in MCF-7 cells. MCF-7 cells stably expressing an \textit{ERE-GFP} reporter were treated with the indicated concentrations of E2 (●), AB-82P (■), ICI (▲) or 4-OHT (▼) and in the absence (a) or presence (b) of 100 pM E2 and GFP expression was assessed by flow cytometry. Data are mean ± s.e.m. of three independent experiments. (c) Ligand-induced expression of ERα target genes. MCF-7 cells were treated with the indicated compounds and gene expression of \textit{TFF1}, \textit{GREB1} and \textit{PGR} were
assessed. Data are mean ± s.e.m. of at least three independent experiments. *p<0.05 and **p<0.01 vs. DMSO by one-sample t-test. *p<0.0001 vs. E2 by one-way ANOVA with a Bonferroni’s post-test. (d) Effect of AB-82P on MCF-7 cell growth. MCF-7 cells were treated with the indicated concentrations of AB-82P in the absence (●) or presence (■) of 100 pM E2. Cell viability was assessed after 5 days. Cell viability was normalized to corresponding DMSO-treated cells. Dashed lines indicate inhibitory levels of 1 μM 4-OHT and 1 μM ICI in the presence of 100 pM E2.

Transcriptional signaling by ERα is crucial for MCF-7 cell growth. Therefore, we next determined whether AB-82P could inhibit AB-82P cell growth. MCF-7 cells were treated with increasing concentrations of AB-82P and cell viability was assessed. AB-82P dose-dependently inhibited growth of MCF-7 cells with an IC₅₀ value of ≈8 μM (Fig. 3.3d, black curve). AB-82P also inhibited E2-mediated cell growth of MCF-7 cells (IC₅₀ value ≈4.2 μM) (Fig. 3.3d, red curve). 4-OHT and ICI were used as control inhibitors in the presence of E2 (Fig. 3.3d, dotted lines). Together, these data reveal that AB-82P acts as a selective antagonist of the classical ERs and inhibits MCF-7 cell growth. Thus, addition of the alkyl chain to the carbon-8 position of AB-1 converted the molecule from an ER-selective transcriptional agonist to an ER antagonist, while maintaining reactivity to the classical ERs over GPER.

The field of ERα antagonists has recently been largely focused on developing new SERDs, rather than SERMs, for the treatment of ER-positive breast cancers due to the greater efficacy of SERDs over SERMs in inhibiting ERα activation. This is primarily due to ligand-induced degradation of ERα by SERDs, but not by SERMs. Therefore, we examined whether AB-82P acts as a SERD or a SERM. MCF-7 cells were treated with E2, AB-82P, or ICI and ERα protein levels were measured. E2 induced >40% decrease in ERα levels, which is a known
mechanism responsible for terminating ERα signaling following its activation by E2 (Fig. 3.4a) [209]. Like ICI, AB-82P induced strong ERα degradation (>80% for Figure 3.4a). AB-82P induces degradation of ERα. (a) Ligand-induced degradation of ERα. MCF-7 were treated with the indicated compounds and ERα protein levels were assessed by Western blot (top panel). Actin served as a loading control (bottom panel). Quantification of the western blot is

**Figure 3.4.** AB-82P induces degradation of ERα. (a) Ligand-induced degradation of ERα. MCF-7 were treated with the indicated compounds and ERα protein levels were assessed by Western blot (top panel). Actin served as a loading control (bottom panel). Quantification of the western blot is
shown on the right. Data are mean ± s.e.m. of at least three independent experiments. **p<0.01, ***p<0.001 and ****p<0.0001 vs. DMSO. (b) Dose-dependent degradation of ERα. MCF-7 cells were treated with the indicated concentrations of AB-82P or ICI (top panel) and ERα protein levels were assessed by Western blot. Quantifications of the corresponding blots (bottom panel) are mean ± s.e.m. of three independent experiments. (c) Ligand-induced proteasomal degradation of ERα. MCF-7 cells were treated with the indicated compounds in the absence (top panel) or presence (bottom panel) of the proteasome inhibitor MG-132 and ERα protein levels were assessed by Western blot. Cells were either pre-treated with DMSO or MG-132 for 1 h before ligand stimulation (in the presence of DMSO or MG-132). Blots are representative of three independent experiments.

AB-82P and ≈70% for ICI), albeit at a 10-fold higher concentration than ICI. To better compare the potency of AB-82P versus ICI, at inducing ERα degradation, cells were treated with increasing concentrations of either compound. We found an EC₅₀ value of 1.9 μM for AB-82P versus 0.6 nM for ICI, which was expected given the higher affinity of ICI for the receptor (Fig. 3.4b).

Degradation of ERα occurs through a proteasome-mediated pathway. To examine the ERα degradation pathway mediated by AB-82P, we treated MCF-7 cells with E2, AB-82P or ICI in the presence or absence of the proteasome inhibitor, MG-132. Whereas, AB-82P induced strong ERα degradation in the absence of MG-132, MG-132 completely blocked the observed ligand-induced degradation of the receptor (Fig. 3.4c). Furthermore, MG-132 similarly blocked both E2- and ICI-induced degradation of the receptor. Together, these data indicate that AB-82P acts as a SERD and induces strong ERα degradation through a proteasome-mediated pathway.

Endocrine resistance in breast cancer is a serious clinical issue and understanding the mechanisms responsible for its development is an intensely studied area of research. Several resistance mechanisms, including the
emergence of constitutively active, endocrine-resistant ERα mutants (e.g. Y537S), have been described [121, 130]. Therefore, new SERMs and SERDs need to be efficacious against endocrine-resistant tumors, in general, but also specifically against clinically relevant mutant forms of ERα. We therefore sought to assess the efficacy of AB-82P in cell line models of endocrine-resistance. First, we examined whether AB-82P could degrade the clinically relevant ERα-Y537S mutant. HEK293 cells, transiently expressing a HA-tagged ERα-Y537S construct, were treated with AB-82P or ICI and protein levels of the ERα mutant were assessed. AB-82P reduced HA-ERα-Y537S levels by \( \approx 40\% \), which is about half the efficiency observed for degrading wildtype ERα (compared to \( >80\% \) degradation for wildtype ERα) (Fig. 3.5a). ICI, showed a comparable decrease in mutant ERα protein levels to those of AB-82P, albeit at a 10-fold lower concentration. Next, we tested whether AB-82P could inhibit cell growth of tamoxifen-resistant MCF-7 cells. Tamoxifen-resistant MCF-7 cells (TamR) were treated with increasing concentrations of AB-82P or 4-OHT and cell viability was assessed. AB-82P dose-dependently inhibited TamR cell growth, although maximum inhibition was not reached at the highest tested dose (Fig. 3.5b, black curve). As expected, 4-OHT did not inhibit TamR cell growth (Fig. 3.5b, red curve). However, slight inhibition was observed at 5 μM 4-OHT. Lastly, we tested the ability of AB-82P to inhibit growth of a long-term estrogen-deprived (LTED) MCF-7 cell line (a model for aromatase inhibitor resistance). MCF-7 LTED cells were treated with increasing concentrations of AB-82P or ICI. Both AB-82P and ICI dose-dependently inhibited the growth of MCF-7 LTED cells with IC\(_{50}\) values of 2.3 μM and 0.3 nM,
respectively (Fig. 3.5b, green and blue curves). Importantly, AB-82P was able to achieve a greater overall level of growth inhibition than ICI, though at much higher concentrations. Together these data demonstrate that AB-82P, at sufficiently high concentrations, can degrade the ERα-Y537S, albeit to a lesser extent than that observed for degradation of wildtype ERα and is effective at inhibiting cell growth in different cell line models of endocrine resistance.

**Figure 3.5.** Inhibitory effects of AB-82P in cell line models of endocrine resistance. (a) AB-82P-induced degradation of ERα-Y537S. HEK293 cells transfected with the HA-ERα-Y537S mutant were stimulated with the indicated compounds and HA-ERα-Y537S protein levels were assessed by Western blot. Blots are representative of three independent experiments. Quantification of the blots are shown to the right panel. *p<0.05 vs. DMSO. (b) TamR (●) and MCF-7 LTED (▲) cells were treated with the indicated concentrations of AB-82P, 4-OHT (for TamR) (■) or ICI182,780 (for MCF-7 LTED) (▼) and cell viability was assessed after 5 days. Data are mean ± s.e.m. of three independent experiments. Cell viability was normalized to corresponding DMSO-treated cells.
3.4 Discussion

Breast cancer is the most common type of cancer among women, with over 250,000 new cases per year in the United States alone [94]. The majority (>70%) of these cancers are driven by ERα, which has led to the development of potent ERα-targeted endocrine therapies. Tamoxifen, which was originally developed as a female contraceptive, has been one of the most utilized forms of endocrine therapy since the late 1970s [231]. It has maintained its popularity despite the development of potent aromatase inhibitors, one reason being its potent efficacy accompanied by well-tolerated side-effects [104]. Although tamoxifen represents an effective endocrine therapy for primary ER-positive breast cancers, women often develop recurrent disease, presenting at relapse with tamoxifen-resistant tumors. Ignatov et al. showed that relapse tumors in women that were treated with tamoxifen have a statistically significant increase in GPER expression compared to their matched primary breast tumors [159]. Similar observations were made by Mo et al. in a separate cohort of women [160]. This observed increase in GPER expression in relapse tumors is consistent with multiple findings that tamoxifen acts as an agonist of GPER and therefore may contribute to the development of tamoxifen resistance [88, 124, 160, 162]. Women are usually prescribed tamoxifen for a minimum of 5 years, thus chronic activation of GPER by tamoxifen could provide a subset of primary tumor cells with sufficient Akt signaling (a downstream target of GPER) to survive the tamoxifen regimen. This idea is supported by observations of Mo et al., who showed that tamoxifen-resistant tumors were “re-sensitized” to tamoxifen when co-treated with the GPER-selective antagonist,
G15 [90, 160]. Together, these observations point to a potential benefit of truly selective ERα antagonist that do not activate GPER. To date, all of the tested ERα antagonists, including fulvestrant, raloxifene and even the presumed ERα-selective agonist, PPT, have been shown to act as agonists of GPER [23]. Therefore, this makes AB-82P the first molecule of its class to show no activity towards GPER, making it the first, truly ER-selective antagonist. We acknowledge that we did not directly test AB-82P binding to GPER, but since AB-82P was derived from the truly ER-selective agonist, AB-1, it is likely that AB-82P maintained its non-selectivity toward GPER. Receptor selectivity was previously maintained when the selective GPER agonist, G-1 was converted to the GPER-selective antagonists G15 and G36 by modifying the agonist [90, 91]. Therefore, we expect receptor selectivity to be maintained for AB-82P. However, further testing is required to confirm this. It is also worth mentioning that while our claim that AB-82P is the first, truly ER-selective antagonist, several new SERDs (e.g. AZD9496), have been developed, but have not yet been tested for their selectivity towards GPER [145].

The ability to clinically inhibit ERα in the absence of GPER activation potentially has significant clinical benefits, including decreasing and/or delaying the development of endocrine resistance. Although AB-82P is currently the only compound with this ability, its current therapeutic value is limited due to its affinity for ERα. In our binding assay, using purified ERα-LBD, we measured an IC₅₀ value of 0.22 μM. More importantly, in functional assays, we measured IC₅₀ and EC₅₀ values ranging between 2-8 μM, consistent with the need to have ~90% receptor
occupancy to effectively inhibit E2 action. These results also reveal that addition of an alkyl chain to the carbon-8 position of AB-1 is detrimental to the affinity of the molecule (cf. IC$_{50}$ value for ER$\alpha$ of AB-1, 38 nM vs. that of AB-82P, 0.22 μM). The addition of the alkyl chain could affect how well AB-82P fits into the binding pocket of ER$\alpha$, which would account for the loss in affinity. How this reduced affinity of AB-82P translates to in vivo efficacy is still unknown and merits further testing. However, AB-82P has the potential of being improved by modifying its molecular structure. Hamann et al., showed that modifying AB-1 (termed compound 3), by addition of a gem-dimethyl and fluorine-group, improved the affinity of AB-1 by >200-fold [206]. One could speculate, that the addition of these groups to AB-82P could result in a similar improvement in ER$\alpha$ binding affinity (possibly as low as 1-10 nM) and functionality, thereby making it a better therapeutic candidate.

Endocrine resistance is a major clinical problem. The presence of ER$\alpha$ mutants that are less sensitive to tamoxifen and fulvestrant, in relapse tumors, is one mechanism by which tumors evade killing by current endocrine therapies [123, 125]. Therefore, new SERMs and SERDs have to be effective at inhibiting (and degrading, in the case of SERDs) clinically relevant mutant forms of ER$\alpha$. We have shown that AB-82P is effective at degrading (∼40% decrease) one of the most commonly found ER$\alpha$ mutants in relapse tumors (ER$\alpha$-Y537S). Furthermore, we have shown that AB-82P can inhibit cell growth of both a tamoxifen-resistant cell line and an MCF-7 LTED cell line (a model for aromatase inhibitor resistance [232, 233]). Thus, despite AB-82P’s lower affinity for ER$\alpha$ (vs. fulvestrant), it inhibits the growth of relevant models of endocrine resistance, albeit at a clinically unfavorable
concentration for a therapeutic candidate drug.

Tamoxifen has proven to be an effective ERα antagonist, albeit at the expense of cross-activating GPER. While developing a truly selective ERα antagonist would circumvent the cross-activation of GPER, this undesirability can also be avoided through a combinatorial drug approach. Therefore instead of replacing tamoxifen (and its favored efficacy), adding an additional therapy, which would block its cross-activation of GPER, might also be beneficial in decreasing the likelihood of patients developing endocrine resistance. A combination therapy of tamoxifen and one of the well-known GPER-selective antagonists, G15 or G36, could be a valid option [90, 91]. One downside to this would be that the increased risk for developing endometrial cancer (due to the tamoxifen treatment) would still be present. Furthermore, G15 or G36 would have to outcompete tamoxifen for GPER binding. Therefore, completely blocking tamoxifen binding to GPER might not be achieved and the resulting pro-survival signaling would not be fully prevented. Thus, although a combinatorial approach is feasible, treating with a single, truly ERα-selective antagonist, like AB-82P would eliminate this undesirable risk. Furthermore, a single agent therapy would eliminate any potential side-effects related to the second drug therapy. It should be noted that we have not tested AB-82P for potential agonist activity in the endometrium, but given its SERD properties (i.e. inducing ERα degradation), it is likely that AB-82P, like fulvestrant, is a “pure” ERα antagonist [113].

Aside from its potential therapeutic benefits, AB-82P could also serve as a tool to better study the intricate signaling pathways of estrogen. Our lab previously
identified the GPER-selective agonist G-1, and the selective antagonists, G15 and G36. These ligands have provided researchers with the tools to study the role of GPER in systems (e.g. MCF-7 cells and in vivo murine studies) where the classical estrogen receptors are also present. Now with the addition of AB-82P (and AB-1), researchers can utilize the complementary approach to study the selective roles of classical estrogen receptors in systems that co-express GPER.

In this report we have identified the first truly ER-selective antagonist that was derived from our previously identified, ER-selective transcriptional agonist, AB-1. With mounting evidence that cross-activation of GPER by tamoxifen and other clinically-used ERα antagonists contributes to the development of endocrine resistance, the benefits of a truly ER-selective antagonist could be of great significance. Although AB-82P has poor pharmacological properties (e.g. low binding affinity for ERα), it could serves as a lead compound presenting the structural basis for future ER-selective antagonists that could prolong the lives the breast cancer patients by leading to a decrease and/or delay in the development of endocrine resistance.
3.5 Materials and methods

**Reagents.** E2 (17β-estradiol), 4-hydroxytamoxifen and MG-132 were purchased from Sigma-Aldrich and ICI182,780 was purchased from Selleckchem. All stock compounds were dissolved in DMSO unless specified. G-1 was synthesized as previously described [234]. AB-82P was synthesized by Jeffrey Arterburn at New Mexico State University (Supplementary Fig. 3.1, detailed chemical synthesis not shown).

**Cell culture.** Cell lines were obtained from the American Type Culture Collection. MCF-7, Hec50 and HEK293 cells were cultured in the DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Tamoxifen resistant MCF-7 (TamR), derived from a tamoxifen-resistant tumor [235], were a gift from Dr. Donald McDonnell (Duke University) and were maintained in DMEM/F-12 supplemented with 8% FBS and 100 nM 4-OHT. Long-term estrogen-deprived cells (MCF-7 LTED), derived from an estrogen-deprived MCF-7 tumor, were also provided by Dr. Donald McDonnell and were maintained in phenol red-free DMEM/F-12 supplemented with 8% charcoal-stripped FBS. For E2 deprivation, cells were grown for the indicated times (with intermediate changes of medium) in phenol red-free DMEM/F-12 medium supplemented with 10% (or 8%) charcoal-stripped FBS supplemented with 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. All cell lines were cultured at 37°C in a humidified 5% CO₂ chamber.
**Ligand-binding assays.** Binding assays for ERα-LBD and ERβ-LBD were performed using the LanthaScreen TR-FRET Competitive Binding Assay (ThermoFisher Scientific) by the SelectScreen Biochemical Nuclear Receptor Profiling Service (ThermoFisher Scientific). AB-82P was tested at a 10 μM dose and subsequent 3-fold dilutions for a final 10-point dilution curve.

**ERE transcriptional activity.** MCF-7 cells stably expressing an ERE-GFP reporter construct (a kind gift from Dr. Yuri Yamaguchi [230]) were plated in phenol red-free DMEM/F-12 supplemented with 5% charcoal-stripped FBS in 6-well plates (150,000 cells/well). After 5 days (with intermediate medium changes on days 2 and 4), cells were treated with the indicated compounds (dissolved in DMSO, 0.1% final) in the presence or absence of 100 pM E2 for 24 h. Following stimulation, cells were washed once with PBS and trypsinized with phenol red-free trypsin. Trypsin was deactivated by adding an equal volume of ice-cold medium and cells were spun down for 5 min (2,000 rpm) at 4°C. Cells were resuspended in 400 μL ice-cold PBS, passed through a 0.22 μm filter and analyzed on a BD LSRFortessa (BD Biosciences) for GFP expression. DMSO-treated cells were used to acquire background GFP signal. Per experiment, values were normalized to the maximum value for E2-stimulated cells. This value was the “Top” value obtained when fitting the E2 curve in Prism GraphPad and set to 100%.

**RNA isolation and quantitative real-time PCR.** MCF-7 cells were plated in 6-well plates in DMEM supplemented with 10% FBS (300,000 cells/well). The next day,
media was changed to phenol red-free DMEM/F-12 supplemented with 5% charcoal-stripped FBS. Twenty four hours later, cells were stimulated with the indicated compounds (dissolved in DMSO, 0.1% final) for 16 h and subsequently washed twice with ice-cold PBS and RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA (100 ng) was reverse transcribed using the ImProm-II Reverse Transcription System (Promega) with random hexamer primers according to the manufacturer’s protocol. qPCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems), SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA) and 2 μL of cDNA. Gene expression levels were calculated using the ΔΔCt method and normalized to that of 18S. Primer sequences used were as follows: 18S Fw 5’-TTTTCGGAACTGAGGCATG, Rv 5’-TGGCAAATGCTTTCGCTCTG. GREB1, TFF1 and PGR primer sequences were previously published [236, 237].

**ERα degradation.** MCF-7 cells were plated in DMEM supplemented with 10% FBS in 6-wells plates (500,000 cells/well). The next day, the media was changed to phenol red-free DMEM/F-12 supplemented with 10% charcoal-stripped FBS. Following 48 h of E2 deprivation (one intermediate medium change), cells were treated for 24 h with the indicated compounds in E2-deprived medium. At the end of the treatment, cells were washed twice with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% Na-deoxycholate and 0.1% SDS) supplemented with protease/phosphatase cocktail (ThermoFisher Scientific), passed through a 20G needle (12-15 times) and protein
concentrations determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Samples (20 μg) were resolved by SDS-PAGE (4-12% Bis-Tris gel), transferred to nitrocellulose membranes and subjected to Western blot analysis. Membranes were probed overnight with a rabbit anti-ERα antibody (Cell Signaling, 1:1000) or mouse anti-Actin antibody (Millipore, 1:5000) in 4% BSA-TBST at 4˚C followed by a secondary HRP-linked goat anti-rabbit antibody (1:5000) or HRP-linked goat anti-mouse antibody (1:2500) for 1 h at RT. Bands were visualized by chemiluminescence and quantified using ImageJ software (NIH).

For experiments with the MG-132 inhibitor, cells were pre-treated for 1 h with 0.1% DMSO or 10 μM MG-132 prior to stimulation with the indicated compounds for 6 h (in the presence of DMSO or inhibitor). For experiments with the ERα-Y537S construct, HEK293 cells were seeded (400,000 cells/well) in 6-well plates in phenol red-free DMEM/F-12 supplemented with 10% charcoal-stripped FBS. The following day, cells were transfected with 1.5 μg pcDNA-HA-ER Y537S (a gift from Sarat Chandarlapaty, Addgene plasmid # 49499) using Lipofectamine 3000 (ThermoFisher Scientific) according to the manufacturer’s protocol in medium lacking pen/strep. Twenty four hours post-transfection, cells were treated with the indicated compounds for 48 h. Blotting for HA-tag was performed as described above using a rabbit anti-HA antibody (Cell Signaling, 1:1000) O/N at 4˚C.

**Cell viability assay.** MCF-7 (and MCF-7 variants) cells were plated (2,500 cells/well) in 96-well plates phenol red-free DMEM/F-12 supplemented with 10%
charcoal-stripped FBS (8% charcoal-stripped FBS for TamR and MCF-7 LTED cells). Three days later, cells were treated with the indicated compounds for 5 days in the presence or absence of 100 pM E2 (one intermediate change of stimulation medium on day 3). Following stimulation, cell viability was assessed using the CellTiter-Glo kit (Promega) according to the manufacturer’s protocol and luminescence was measured on a Biotek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek) using a 1 s integration time.

**Statistical analysis.** Data was analyzed by one-sample *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test. Non-linear regression curves were determined using a variable slope fit. Values are expressed as mean ± s.e.m.; *n* equals the number of assay replicates. Statistical significance was accepted at a *P* value <0.05. All analyses were carried out using Prism version 5.0 for Windows, GraphPad Software.

**3.6 Acknowledgments**

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Chapter 4
Discussion

4.1 Overview and significance

Estrogen plays a role in many physiological processes [5]. Its physiological role is complicated by the existence of three different estrogen receptors (ERα, ERβ and GPER). Although each of these receptors have been studied for over a decade, deciphering the role of the individual receptors in a cell type or tissue expressing both classical ERs and GPER has been challenging due to overlapping signaling pathways between the classical ERs and GPER.

Selective ligands (agonists and/or antagonists) to an individual receptor within a receptor family have generally aided in better understanding the roles of the individual receptors within that family. Identification of GPER-selective ligands, e.g. G-1 [89], G15 [90] and G36 [91], which show no activity (or binding) towards ERα and ERβ (G15 shows a low level of reactivity towards ERα at high (>1 μM) concentrations), have greatly improved our knowledge on the role of GPER in estrogen biology. However, selective ligands to the classical ERs, lacking cross selectivity towards GPER have been elusive. The majority of molecules that bind the classical ERs have been shown to generally activate (or inactivate) GPER [23]. Similarly, obtaining ligand selectivity between ERα and ERβ has also been challenging due to the high degree of homology between their ligand-binding pockets [17]. To date, only a few ERα and ERβ subtype-biased ligands have been identified. The small molecule PPT [65] shows ~400-fold binding preference towards ERα over ERβ, while the ligand DPN [66] shows ~70-fold binding
preference towards ERβ over ERα. Importantly, PPT has been shown to activate GPER [88].

In Chapter 2 we identified the small molecule, AB-1, as a highly selective ERα/β agonist that showed no binding or activity towards GPER in cell-based assays (negligible binding was observed at 10 μM AB-1). This marks the identification of the first truly ERα-selective ligand that shows no binding to GPER. Interestingly, AB-1 acts not only as an ER-selective ligand, but also as a functionally selective agonist of the classical ERs, activating only their transcriptional activity while inhibiting non-genomic ER signaling. Functional selectivity of an ER ligand has been previously reported with the identification of PaPEs (pathways preferential estrogens) [203]. PaPEs are ER ligands with very low affinities for ERα and ERβ (K_i values >10 μM) that have been shown to induce rapid ERα signaling, but not ERα-mediated transcriptional signaling. It is hypothesized that the functional selectivity of PaPEs is due to its low affinity for ERs. This low affinity (which corresponds to a high dissociation rate) is thought to be sufficient for the activation of rapid, non-genomic signaling (which occurs on a timescale of seconds to minutes), but not for the activation of genomic signaling (which occurs on a timescale of hours). This hypothesis would theoretically not hold true for AB-1, which has a high affinity for the ERs (ERα IC_{50}= 3 nM and ERβ IC_{50}= 26 nM), implying a potentially novel (and unknown) mechanism through which AB-1 is regulating its functionally-selective signaling by ERs. Importantly, it is currently unknown whether PaPEs bind to GPER.

Identification of AB-1 is a significant contribution to the field of estrogen
biology due to the novelty of its receptor-binding properties (i.e. highly selective towards both ERs, but not GPER). Being the first ERα/β ligand of its kind will allow the field to utilize its selective binding properties to study the role of the classical ERs in the absence of GPER activation. Selective activation of ERs (particularly ERα) over GPER could generally only occur with a combinatorial approach employing GPER-targeting small-interfering RNAs (siRNAs) or G15/G36. However, even then complete prevention of GPER activation is not ensured. Due to the absence of its binding to GPER, the use of AB-1 will avoid potential cross-activation of GPER, making scientific observations more conclusive than those made using GPER-targeting siRNAs or G15/G36. A drawback to using AB-1 would be its inability to induce rapid, non-genomic ER-mediated signaling. Therefore, it would be limited in its use and could only be used to study the transcriptional role of ERs in a given system (in the absence of GPER-induced transcriptional activity). However, since ER signaling consists mainly of transcriptional activity, AB-1 will still be useful for a wide range of applications.

As the first truly ER-selective (importantly the ERα aspect) agonist, AB-1 will serve as a beneficial tool to selectively study the physiological roles of the classical ERs; building upon the current set of truly selective ligands (such as G-1, G15 and G36) to the three known estrogen receptors. To further expand the collection of truly estrogen receptor-selective ligands, we sought to identify a small molecule that harbored the same receptor selectivity as AB-1, but having an opposite functional profile (i.e. being an ER-selective antagonist). Based on molecular modeling of AB-1 in the ERα-LBD, we identified AB-82P (in Chapter 3),
which acts as a truly ER-selective antagonist. Its ability to selectively block E2-induced transcriptional activity of the classical ERs, while not blocking E2-induced GPER-mediated rapid signaling makes it a valuable and truly ER-selective antagonist to study estrogen biology. ER antagonists, such as tamoxifen and fulvestrant, which inhibit both ERα and ERβ, have been previously identified. A subtype-selective ERα antagonist, termed MPP (methyl-piperidino-pyrazole), has also been identified [238]. Although these small molecules can be used to study the role of classical ERs in estrogen biology, they lack (in the case of e.g. tamoxifen and fulvestrant) true selectivity towards the classical ERs, acting generally as GPER agonists [23]. Therefore, conclusions drawn from experiments using these compounds need to be carefully assessed since GPER could potentially be activated in the experiment. This potential ligand-induced cross-activation of GPER is absent in the case of AB-82P, making it a more useful ER-selective ligand. Whether MPP cross-reacts to GPER is currently unknown. Thus with the identification of AB-82P, the current set of truly selective ligands to the estrogen receptors consists of a) the truly GPER-selective ligands G-1 (agonist), G15 (antagonist), G36 (antagonist), GPER-L1 (agonist) [92] and GPER-L2 (agonist) [92] and b) the truly ER-selective ligands AB-1 (agonist) and AB-82P (antagonist).

Aside from its role as an ER-selective tool to study the roles of the classical ERs in estrogen biology, the identification of AB-82P potentially holds significant clinical value. Estrogen, through ERα, plays an important role in disease, particularly in breast cancer [93]. Inhibiting ERα signaling is an effective strategy
in breast cancer therapy due to the pro-tumorigenic role of the receptor. As such, tamoxifen has been indispensable in treating ERα-positive breast cancers. Unfortunately, tamoxifen and other clinical SERMs/SERDs, that act as ER antagonists, cross-activate GPER [23]. This cross-activation of GPER (in particular by tamoxifen) has been hypothesized to contribute to clinically-observed endocrine resistance [124, 159, 160, 163]. Therefore, a truly ERα-selective antagonist, lacking cross-activation of GPER, could potentially delay or reduce the development of endocrine resistance of ERα-positive breast cancers. With the identification of AB-82P, it is currently the only known truly ER-selective antagonist that lacks cross-reactivity towards GPER, making it a prime candidate molecule for the potential development of new ERα antagonists with favorable clinical properties (i.e. lacking GPER activity). Newly developing SERDs, such as AZD9496 [145] have shown great pre-clinical efficacy in treating models of ERα-positive breast cancers, with some even showing promising preliminary results in phase I trials [146]. However, their cross-activity towards GPER has not been assessed. Therefore, it is unknown if their long-term clinical efficacy would be hindered by their potential cross-activation of GPER and possibly the resulting contribution to the development of endocrine therapy-resistant relapse tumors.

The potential benefit of a truly ERα-selective antagonist has, to a certain extent, been indirectly assessed in clinical trials. Gefitinib, an EGFR tyrosine kinase inhibitor, has been assessed in a phase II clinical trial in combination with tamoxifen for the treatment of metastatic ERα-positive breast cancer [239]. EGFR is activated downstream of GPER and is critical for GPER-mediated activation of
the MAPK and PI3K pathways [72, 75, 82]. Thus, inhibiting EGFR is, to an extent, similar yet not identical to inhibiting GPER and its downstream pro-survival signaling pathways. In the gefitinib/tamoxifen phase II trial, Osborne et al. stratified patients into two groups: patients that had endocrine therapy-naïve metastatic tumors or who had relapsed while on/after tamoxifen therapy (group 1) and patients that had relapsed while on/after an AI therapy (group 2) [239]. Each group was then split into two treatment arms (gefitinib plus tamoxifen or placebo plus tamoxifen). For group 1, the PFS of the gefitinib plus tamoxifen arm versus placebo plus tamoxifen arm was 10.9 and 8.8 months, respectively. Although the difference in PFS was not statistically significant, the authors deemed the treatment strategy (gefitinib plus tamoxifen) worthy of further investigation. It should be noted that in the gefinitib plus tamoxifen arm, tamoxifen-mediated GPER cross-activation (and its resulting activation of EGFR and subsequent pro-survival signaling pathways) could still occur due to the presence of tamoxifen. Therefore, the extent of EGFR inhibition (by gefitinib) in the treatment arm is unknown. Nonetheless, the phase II data, reported by Osborne and colleagues [239], shows a benefit of inhibiting EGFR signaling in combination with tamoxifen, which is a scenario that could potentially be achieved using a single, truly ERα-selective antagonist (such as AB-82P). Thus, a truly ERα-selective antagonist could potentially achieve similar or even further improved PFS data than that reported in the gefitinib/tamoxifen phase II trial [239]. Although AB-82P is currently the only known truly ER-selective antagonist, its current therapeutic value is limited by its poor affinity for ERα, but this factor has the potential to be improved (discussed in the next section).
4.2 Future directions

As an ER-selective tool, AB-1 has great scientific value due to its high affinity for the classical ERs ($\text{ER}\alpha \text{IC}_{50} = 3$-$38$ nM, $\text{ER}\beta \text{IC}_{50} = 24$-$26$ nM), however its affinity for the receptors could be further improved. With regards to affinity, an improved version of AB-1 has been previously been reported by Hamann et al., who showed that modifying AB-1 (termed compound 3), by addition of a gem-dimethyl and fluorine-group, improved the affinity of the compound by $>200$-fold [206]. However, whether this improved compound 3 retains its selectivity for the classical ERs over GPER is unknown.

Whether AB-1 has any clinical applications is unknown. In the bone, activation of ER$\alpha$, by SERMs such as raloxifene, has been shown to be beneficial for treatment and prevention of postmenopausal osteoporosis [240]. However, these SERMs act as ER$\alpha$ antagonists in the breast. AB-1, could be used to in the treatment of osteoporosis, however a potential risk would be an increased risk of developing breast cancer due to its agonist properties in the breast. Therefore, whether AB-1 has any clinical application is still unknown, but it is highly applicable for research purposes.

As previously discussed, the selective properties of AB-82P are potentially of great clinical significance, however, in its current form, AB-82P does not harbor much clinical value due to its poor affinity for ER$\alpha$. AB-1 was employed as the structural basis for the development of AB-82P. Therefore, the previously discussed modifications to AB-1, which have been shown to improve its affinity [206], could be incorporated into AB-82P, potentially improving its affinity for ER$\alpha$. 
and increasing its clinical value. An important aspect of AB-82P that needs to be assessed is its in vivo activity. AB-82P should be assessed for its antagonist properties in the classical uterotrophic assay [9], but more importantly, whether it can inhibit tumor growth in xenograft models of ERα-positive breast cancers (e.g. MCF-7 xenograft model). Given its poor affinity for ERα, we hypothesize that AB-82P, in its current form, harbors poor in vivo efficacy. However, this could potentially be improved by improving the overall affinity of AB-82P for ERα.

Oral bioavailability is a highly desired property in newly developing SERDs [147-152]. Therefore AB-82P should also be assessed for its oral bioavailability. If it lacks this property, it could potentially be molecularly altered to achieve this desired property. It is important to note that all modified versions of AB-82P will have to be assessed for their activity towards GPER, since modification of AB-82P could potentially result in loss of its highly desired selectivity towards the classical ERs over GPER.

4.3 Conclusions

True ligand selectivity for the classical ERs over GPER has, to date, been an elusive molecular property. In this dissertation we have identified two small molecules, AB-1 and AB-82P, which harbor this elusive molecular property. Importantly, they act as an agonist (AB-1) and antagonist (AB-82P) of the classical ERs. Due to the hypothesized contribution of GPER to the development of endocrine resistance to ERα-positive breast cancer therapies, AB-82P has potentially significant clinical value and merits further investigating either in its
current form or as the structural basis for future ERα-selective antagonist, which could potentially reduce or delay the development of clinically observed endocrine resistance to breast cancer therapies.
APPENDICES

Appendix A: Chapter 2 supplementary methods

Chemical synthesis.

All compounds were synthesized in an efficient fume-hood. All other commercially available solvents and reagents were purchased and used without further purification. Preparative chromatography was performed by medium pressure column chromatography using AnaLogix SuperFlash pre-packed columns. $^1$H NMR spectra were acquired using Varian Oxford 300 MHz, Varian Unity 400 MHz, and 500 MHz spectrometers and $^{13}$C NMR were acquired using Varian Oxford 75 MHz, Varian Unity 100 MHz and 125 MHz spectrometers at ambient temperatures (20±2 °C). $^1$H NMR spectra in CDCl$_3$ and acetone-d$_6$ were referred to TMS. Mass spectra were obtained using an Orbitrap Fusion Mass Spectrometer (Thermo Fisher, San Jose, CA) acquired with funding from NSF MRI #1626468.

Diethyl 4-methylcyclohex-3-ene-1,1-dicarboxylate.

\[
\text{EtO}_2\text{C} \xrightleftharpoons{\text{ZnCl}_2} \xrightarrow{\text{THF}} \text{CO}_2\text{Et} \quad \text{CO}_2\text{Et}
\]

A sealed tube containing a diethylmalonate (0.800 g, 5.0 mmol), paraformaldehyde (0.450 g, 15.0 mmol), 2-methyl-1,3-butadiene (0.408 g, 6.0 mmol) and zinc chloride (0.09 g, 0.66 mmol, 7.5 mol %) in dry tetrahydrofuran (2.5 mL) was stirred at 70 °C for 24 h. The reaction mixture was concentrated under reduced pressure, diluted with dichloromethane (45 mL) and washed successively with saturated
aqueous NaHCO₃, and H₂O (25 mL each), dried over Na₂SO₄, evaporated in vacuo, and purified by silica gel column chromatography eluting with ethyl acetate/hexanes (1:99) to obtain the pure product as a colorless oil (0.668 g, 57%).

¹H NMR (300 MHz, CDCl₃) δ 5.37-5.35 (m, 1H), 4.18 (q, J = 7.23 Hz, 4H), 2.53-2.51 (m, 2H), 2.16-2.12 (m, 2H), 2.02-1.94 (m, 2H), 1.63 (bs, 3H), 1.23 (t, J = 7.40 Hz, 6H); FT-IR (Neat), 2960, 1731, 1210, 1151, 503 cm⁻¹).

(4-Methylcyclohex-3-ene-1,1-diyl)dimethanol.

\[
\begin{align*}
\text{CO₂Et} & \quad \text{LiAlH₄} \\
\text{Et₂O} & \quad \text{OH} \\
\text{CO₂Et} & \quad \text{OH}
\end{align*}
\]

A solution of diethyl 4-methylcyclohex-3-ene-1,1-dicarboxylate (0.68 g, 2.83 mmol) in dry diethylether (5 mL) was added dropwise to a cooled (0°C) suspension of lithium aluminum hydride (0.240 g, 6.32 mmol) in dry diethylether (1 mL) and allowed to warm to ambient temperature with magnetic stirring under a nitrogen atmosphere for 3 h. The reaction mixture was cooled in an ice-bath, and worked up by successive slow addition of water, 10% sodium hydroxide, and three additional portions of water (240 µL each) to yield tractable aluminum salt precipitates that were filtered, and the filtrate was concentrated and dried under vacuum to provide the product (0.327 g, 74 % mp 103-108 °C). ¹H NMR (300 MHz, CDCl₃) δ 5.30-5.27 (m, 1H), 3.61 (d, J = 5.47 Hz, 4H), 2.13 (t, J = 5.47Hz, 2H), 1.96-1.91 (m, 2H), 1.81-1.77 (m, 2H), 1.66-1.64 (bs, 3H), 1.60 (t, J = 6.64 Hz, 2H); FT-IR (Neat) 3300, 1610, 1518, 1269, 1071 cm⁻¹.

4-(5-(hydroxymethyl)-8-methyl-3-oxabicyclo[3.3.1]non-7-en-2-yl))-phenol [AB-1].

To a solution of the (4-methylcyclohex-3-ene-1,1-diyldimethanol (0.161 g, 1.032 mmol) and 4-hydroxybenzaldehyde (0.15 g, 1.23 mmol) in anhydrous acetonitrile (4 mL) was added 5 mol% hafnium(IV) trifluoromethanesulfonate monohydrate (0.040 g, 0.051 mmol). The reaction mixture was stirred at ambient temperature under a nitrogen atmosphere for 18 h. The reaction mixture was quenched with sat. NaHCO₃ (10 mL), diluted with water (25 mL) and the product was extracted using CH₂Cl₂ (3x10 mL), dried over Na₂SO₄, and evaporated in vacuo. The product was purified by silica gel column chromatography eluted with EtOAc/hexanes (45:55) to isolate the product as white solid (0.23 g, 86%; mp 164-168 °C) (Rᵣ = 0.3). ¹H NMR (500 MHz, acetone-d₆) δ 8.03 (bs, 1H), 7.10 (d, J = 8.85 Hz, 2H), 6.71 (d, J = 8.85 Hz, 2H), 5.45-5.46 (m, 1H), 4.42 (d, J = 1.83Hz, 1H), 3.83 (dd, J = 10.99, 2.83 Hz, 1H), 3.65 (bs, 1H), 3.53 (d, J = 10.99 Hz, 1H), 3.26 (s, 2H), 2.28-2.26 (m, 1H), 2.22-2.03 (m, 2H), 1.8 (dd, J = 11.6, 2.75 Hz, 1H), 1.65 (m, 1H), 1.01 (dd, J = 3.97, 2.14 Hz, 3H) (Supplementary Fig. 2.1a); ¹³C NMR (125 MHz, CD₃COCD₃) δ 157.03, 134.54, 134.16, 127.43, 124.75, 115.36, 80.51, 78.74, 69.90, 44.05, 35.62, 34.85, 34.68, 30.67, 24.4 (Supplementary Fig. 2.1b); FT-IR
(Neat) 3300, 2975, 1610, 1092, 1051 cm\(^{-1}\). HRMS (m/z) calcd for C\(_{16}\)H\(_{21}\)O\(_{3}\), 261.1485 [M+ H\(^+\)]; found, 261.1484 (data not shown). The UV absorbance peak areas in the HPLC chromatogram of the AB-1 sample (data not shown) were integrated and demonstrated compound purity of 98.6%.
Supplementary Figure 2.1. Spectroscopic data of AB-1. (a) $^1$H NMR (500 MHz) and (b) $^{13}$C NMR (125 MHz) of AB-1.
**Supplementary Figure 2.2.** Competitive ligand-binding assay of the ligand-binding domain (LBD) of ERα and ERβ. Data are averaged from 2 independent experiments, each performed in duplicate.

**Supplementary Figure 2.3.** Ligand-induced protein degradation of ERα. MCF-7 cells were cultured with the indicated compounds and ERα levels were determined by Western blot. Blots are representative of at least 4 independent experiments.
Supplementary Figure 3.1. AB-82P synthesis scheme.
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