Role of Environmental Estrogens and Acquired Endocrine Resistance in Breast Cancer and Implications for Treatment with Novel Antiestrogens

by

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Dedication

To my grandparents

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List of Abbreviations

2OH	2-hydroxy *iso*-butyl 4-hydroxybenzoate
3OH	3-hydroxy *n*-butyl 4-hydroxybenzoate
ACTH	Adrenocorticotropic hormone
AIs	Aromatase inhibitors
AR	Androgen receptor
CRH	Corticotropin-releasing hormone
CCS	Charcoal stripped calf serum
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone-3-sulfate
DHT	Dihydrotestosterone
E1	Estrone
E2	17ß-Estradiol
ER	Estrogen receptor
EDC	Endocrine disrupting chemicals
ER	Estrogen receptor
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GREB1	Gene regulated by estrogen in breast cancer 1
HPGA	Hypothalamic-pituitary gonadal axis
HPLC	High-performance liquid chromatography
ICI	ICI 182,780; fulvestrant
RMSD	root-mean-square deviation
IMEM	Improved minimum essential medium
LH	Luteinizing hormone
LBD	Ligand binding domain
PCPs	Personal Care Products
PR	Progesterone receptor
PROTAC	Proteolysis targeting chimera
RMSD	Root-mean-square deviation
TAM	Tamoxifen
ZG	Zona glomerulosa
ZF	Zona fasiculata
ZR	Zona reticularis

Abstract

Breast cancer is the most diagnosed malignancy among women in the United States. Approximately 70% of breast tumors express estrogen receptor (ER)-alpha and are deemed ER-positive. ER-positive breast tumors depend upon endogenous estrogens to promote ER-mediated cellular proliferation. Although adjuvant endocrine therapy is an effective treatment option for ER-positive breast cancer, recurrence remains an unresolved issue. Studies suggest that *ESR1* ligand binding domain (ESR1-LBD) mutations and exposure to alternative estrogens may serve as potential mechanisms of resistance to endocrine therapy.

Chapter II of this dissertation examined whether two oxidized metabolites of *n*-butylparaben and *iso*-butylparaben, discovered in human urine, bind to ER-alpha and promote estrogen signaling. The estrogenic properties of metabolites 3-hydroxy *n*-butyl 4-hydroxybenzoate (3OH) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate (2OH) were determined using the ER-positive, estrogen-dependent breast cancer cell line MCF-7. The 3OH and 2OH metabolites induced cellular proliferation with EC50 of 8.2 and 2.2 µM, respectively. The expression of a pro-proliferative, estrogen-inducible gene (*GREB1*) was induced by these compounds and blocked by co-administration of an ER antagonist. The metabolites promoted ER-dependent transcriptional activity of an ERE-luciferase reporter construct.

Crystal structures exist for human, but not rodent, ER-alpha-LBD. Consequently, rodent studies involving binding of compounds to ER-alpha-LBD are limited in their molecular-level

interpretation and extrapolation to humans. In chapter III, we used the human ER-alpha-LBD structure (PDB 3UUD) as a template to produce rat and mouse homology models which were employed to generate docking poses of 23 Group A ligands (estradiol, diethylstilbestrol, and 21 paraben analogs) in AutoDock Vina. Numbers of interspecies ligand-receptor residue contacts were highly similar: Sorensen Sc = 93.1 ± 7.5% (human-mouse) and 92.5 ± 7.1% (human-rat). Pyramid plots of numbers of ligand receptor atom contacts by residue exhibited high interspecies symmetry. Group B ligands were 15 3,5-disubstituted parabens shown to exhibit decreased binding to human ER-alpha and increased antimicrobial activity. Ligand efficiencies calculated from docking of Group B ligands into human ER-alpha-LBD were highly correlated with those derived from published experimental data.

The most common recurring ESR1-LBD mutations, D538G and Y537S, are detected in ~30% of patients with metastatic breast cancer who are resistant to endocrine therapy. In chapter IV, we used the MCF-7 cells to develop in vitro models that express the Y537S and D538G mutants using CRISPR knock-in and screened novel compounds that target ER-alpha for degradation. Results show that compound ERD-148 attenuated ER-dependent growth with IC50 values of 0.8, 10.5, and 6.1 nM in wildtype MCF-7, Y537S, and D538G cells respectively. MCF-7 cells treated with ERD-148 for 24 hours show lower levels of ER-alpha protein expression compared to mutant cells treated at 1 nM for 24 hours. *GREB1* gene expression was downregulated at nanomolar concentrations in MCF-7 and the mutant cell lines treated with ERD-148 for 24 hours.

In summary, our results indicate that oxidized paraben metabolites exhibit behavior akin to weak estrogens. Our constructed rodent ER-alpha-LBD receptors interact with ligands in like manner to the human receptor, thus providing a high level of confidence in extrapolations of rodent to human ligand-receptor interactions. ERD-148 was shown to inhibit the growth of ER-positive breast cancer via antagonism of ER-alpha. Future studies are needed to determine whether exposure to estrogenic EDCs or the presence of *ESR1* mutations contribute to a greater risk of recurrence or poorer clinical outcomes specifically among patients with advanced disease.

Chapter I.

Introduction

Estrogen and other steroid hormones are lipophilic small molecules that are primarily produced in the gonads and adrenal glands [1, 2]. Steroid hormones are well known to play prominent roles in reproduction, organ development, and the regulation of normal physiological processes [3-7]. Organs and tissues such as the brain [8], lungs [1], gonads [9, 10], and breast [11] are just a few examples of targets which steroid hormones act upon. Some of these same hormones have also been shown to be critically involved in the development or progression of steroid hormone-dependent diseases such as prostate [12] and breast cancer [13]. For example, cumulative lifetime exposure to estrogens has been associated with an increased risk of breast cancer in postmenopausal women [13]. The importance of estrogens in breast cancer progression is highlighted by the fact that approximately 70% of all breast tumors express the estrogen receptor and proliferate in the presence of estrogenic hormones [14]. There has been increasing interest and debate as to whether environmental exposure to endocrine disrupting chemicals (EDCs) can alter normal steroid hormone regulated processes and contribute to estrogen-dependent diseases such as breast cancer. Here, I will review the current understanding of the biosynthetic pathway for estrogen, the implications of suspected EDCs on altering normal human physiology and estrogen regulated pathways, the relationship between estrogen and breast cancer, acquired mechanisms of resistance to endocrine therapy, the potential role of estrogen mimics in breast cancer progression, and the rational for my thesis.

Human Physiology of Steroid Hormones

Located at the base of the brain, neurons within the hypothalamus are responsible for secreting hormones, commonly referred to as hypophysiotropic or hypothalamic hormones, which regulate the function of the pituitary gland [15, 16]. Upon receiving input from the hypothalamus, the pituitary gland will then control the production and secretion of numerous peptide and steroid hormones [17], with secretion occurring specifically from anterior pituitary gland [16]. Hypothalamic hormones secreted from neurons of the hypothalamus first enter capillaries located at the junction between the hypothalamus and pituitary gland, known as the median eminence, and are carried to the anterior pituitary gland via the hypothalamo-hypophyseal portal vessels [18, 19]. Upon diffusing out of the anterior pituitary gland capillaries and into the interstitial fluid, hypothalamic hormones bind to specific membrane receptors to either stimulate or inhibit the release of anterior pituitary gland peptide and steroid hormones [20]. Some of these hormone pathways are directly involved in controlling the production of gonadal steroids and their precursors through gonadal and adrenal gland control systems [21, 22].

Adrenal Control Systems

Upon receiving synaptic input from the central nervous system, the hypothalamus will secrete corticotropin-releasing hormone (CRH) to simulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland [22]. ACTH secretion then acts on the adrenal cortex to produce cortisol which exerts negative feedback on CRH and ACTH secretion to prevent the overproduction of cortisol [23]. Cortisol secretion is primarily responsible for regulating metabolism and stress response and is a critical component of the hypothalamic-pituitary adrenal axis [22, 24]. In addition to cortisol production, the adrenal cortex is also responsible for

secreting four other major hormones all derived from cholesterol which are known as aldosterone, androstenedione, corticosterone, and dehydroepiandrosterone (DHEA) [25].

Adrenal Steroidogenesis

The adrenal cortex is comprised of three main cell layers with the outer layer being the zona glomerulosa (ZG), the middle layer zona fasciculata (ZF), and the interior layer called the zona reticularis (ZR) [25]. The ZG cells express the enzymes needed to synthesize corticosterone which is converted to aldosterone under the control of the hormone angiotensin II [25]. The ZF and ZR cells both produce cortisol and androgens, however, cortisol is primarily secreted from ZF cells while the ZR cells primarily secrete androgens such as DHEA and androstenedione [25]. Although DHEA and androstenedione are both considered weak androgens, they are not nearly as potent as the major male androgen hormone, testosterone, that is primarily produced in the testes from androstenedione [2, 26]. Similarly, estrogen is the predominant female steroid hormone that is produced in ovarian endocrine cells that express aromatase, the cytochrome P450 enzyme that converts precursor androgens to estrogens [2, 27].

Gonadal Control Systems

The proper control of gonadal steroid secretion from either the testes or ovarian endocrine cells is essential for regulating biological process such as germ cell development, reproduction, and sexual differentiation [28-30]. For example, the presence or absence of gonadal hormones can influence the formation of accessory reproductive organs in females which include breast development and ductal formation [11]. These gonadal hormones can also influence the development of secondary sex characteristics, and the external differences between males and

females, which consist of differences in hair distribution, body shape, and relative adult height [31-33]. Gonadal hormone secretion and reproductive function in both male and females is largely dependent upon peptide hormone signaling by gonadotropins released under the control of hypothalamic-pituitary gonadal axis (HPGA) [34]. To promote the synthesis of gonadal sex hormones, the HPGA first begins with the secretion of a hypothalamic peptide hormone known as gonadotropin-releasing hormone (GnRH) from the hypothalamus [35]. Secreted GnRH then travels to the anterior pituitary gland via the hypothalamo-hypophyseal portal vessels to induce the release of two gonadotropins referred to as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [18, 34]. Both FSH and LH stimulate the gonads to produce and secrete sex hormones that also stimulate the release of GnRH from the hypothalamus and gonadotropins secreted from the anterior pituitary gland via a negative feedback loop [34, 36].

Gonadal Steroidogenesis

Testosterone and estrogen production are not unique to each biological sex. Instead, it is the concentration ratio of these two hormones that are very different and unique between the sexes. Both male and female gonadal sex hormones, similar to the steroid hormones produced in the adrenal cortex, are derived from the same precursor molecule, cholesterol [2]. The first step in gonadal steroidogenesis begins with the conversion of cholesterol to pregnenolone via the cholesterol side-chain cleavage enzyme (P450scc) (Figure 1.1) [2]. ZR cells of the adrenal cortex are able to produce large quantities of DHEA from pregnenolone and 17-hydroxypregenolone via the catalytic activity of the 17α-hydroxylase (P450c17) [37]. DHEA produced in the adrenal cortex is mainly sulfated and exists as the most abundant circulating steroid in humans, dehydroepiandrosterone-3-sulfate (DHEAS), sulfated by sulphotransferase 2A1 (SULT2A1)

[38]. Although males can produce DHEA in the testis as well adrenal cortex [39], the formation of DHEAS is bypassed due to the lack of SULT2A1 expression in this reproductive organ. Male gonadal DHEA is then converted to androstenedione by type 2 3β-hydroxysteroid dehydrogenase (3ßHSD2) and subsequently to testosterone by type 3 17β-hydroxysteroid dehydrogenase (17ßHSD3) [40] within the Leydig cells under the stimulation of LH [41]. Testosterone will then act on the Sertoli cells of the testis to facilitate spermatogenesis and the secretion of a peptide hormone, inhibin, that regulates FSH release from the anterior pituitary gland [42]. The prostate gland in males will also convert some testosterone to a more potent androgen, dihydrotestosterone (DHT), via the catalytic activity of the enzyme 5α-reductase [41]. In target tissues, such as the brain and adipose, testosterone may undergo irreversible aromatization to 17ß-estradiol (E2) by the cytochrome P450 enzyme aromatase [41].

In females, gonadal steroidogenesis is highly dependent upon the stage of the ovarian cycle as well as the cooperation of cell specific steroidogenic enzymes expressed in granulosa and theca cells surrounding the oocyte which together form a follicle [2]. With respect to ovarian stage, E2 is the primary steroid hormone produced during the follicular phase while progesterone is primarily synthesized during the luteal phase. In order to enzymatically synthesize E2, secreted LH stimulates the expression of P450scc within granulosa cells to produce pregnenolone which diffuses across the cellular membranes into the adjacent theca cells (Figure 1.1) [43]. Unlike Leydig cells in males, granulosa cells do not express P450c17 and are unable to convert pregnenolone to DHEA and therefore must rely on the cooperation of theca cells to produce E2 [41]. Once pregnenolone has diffused into the theca cells, it is converted to 17-hydroxypregenolone by P450c17 and then to DHEA by the same enzyme [41]. The newly formed DHEA is converted to androstenedione by 3ßHSD2 and a portion of the androstenedione

will be converted to testosterone. However, most synthesized androstenedione will return to the granulosa cells [44]. Under the control of FSH, androstenedione is converted to estrone by aromatase which is then converted to E2 by type 1 17β-hydroxysteroid dehydrogenase (17ßHSD1) within the granulosa cells [41, 44]. During the luteal phase, greater enzymatic activity of 3ßHSD2 in the corpus luteum leads to the increased production of progesterone from pregnenolone [2]. Progesterone and E2, as well as inhibin produced by the granulosa cells, exert negative feedback on the HPGA to decrease GnRH pulses and regulate FSH and LH secretion [45, 46].

Transport of E2 in the Blood

Due to the lipophilic nature of steroid hormones, they can readily diffuse across cellular membranes and into systemic circulation [47]. Unlike peptide hormones, which are generally highly water-soluble, steroid hormones are poorly soluble in blood due to their lipophilic nature. Therefore, most steroid hormones are reversibly bound to carrier proteins in the plasma which are in equilibrium with the free and bound state of a given hormone [48]. Albumin, sex hormone-binding globulin, and corticosteroid-binding globulin are examples of carrier proteins that transport steroid hormones in the blood [49]. However, it is the unbound, or free steroid, that diffuse out of the capillaries to elicit their biological effects on target cells expressing their cognate receptors, including sex steroid nuclear receptors.

In women, the concentration of circulating E2 varies greatly depending on the stage of the ovarian cycle, pregnancy, and whether normal ovarian function has ceased after menopause [50]. The combined concentration of bound and free circulating E2 in premenopausal woman with normal ovarian function can vary considerably from 73pM – 1600pM and as high as 26nM

during pregnancy [51-53] while in men the standard range of E2 is 36pM – 305pM [54]. During menopause, the ovaries no longer respond to gonadotropins released by the HGPA. As a result, the production of E2 and inhibin from the ovaries decreases and these hormones are less able to exert negative feedback on FSH and LH secretion. However, the peripheral aromatization of adrenal androgens by aromatase maintain circulating concentration of E2 in postmenopausal women albeit at much lower concentrations (<47pM) compared to premenopausal women [55]. Yet, this low E2 concentration is not able to sustain regular physiologic function of estrogen-dependent tissues such as the breasts and genital organs. Atrophy of these tissues is common during menopause and is associated with an increased risk of coronary artery disease and osteoporosis, a decline in bone mineral density [56]. These physiological phenomena observed in postmenopausal women highlight the apparent protective effect of E2 and is the reason why hormone replacement therapy (HRT) is prescribed to many women [57]. Despite the potential health benefits of HRT in women, its use is limited due to the fact that long-term exposure of E2 is associated with an increased risk for endometrial and breast cancer [13, 55, 58]. Tumor cell proliferation in these two cancer types is generally maintained from the estrogen binding to and activating its cognate nuclear receptor.

Nuclear receptors

Nuclear receptors are a class of transcription factors that regulate gene expression and modulate various physiological properties upon binding to their specific receptor ligands [59]. Ligand-binding to their nuclear receptor induces a conformational change that leads to transcriptional activation in order to achieve a biochemical response [60]. Sex hormones are examples of ligands known to promote both genomic or non-genomic actions upon binding to their cognate

receptor [61]. For example, the estrogen receptor (ER) and the androgen receptor (AR) are two nuclear receptors that have been reported to utilize genomic or non-genomic signaling pathways [60, 62-64]. There are at least 48 known genes encoding for nuclear receptors in humans [59]. The remainder of this chapter, however, will focus primarily on estrogen signaling and its relationship to the concept of endocrine disruption and ER-dependent breast cancer.

The Estrogen Receptor

ERß

There are two main subtypes of ER known as estrogen receptor α (ERα) and estrogen receptor ß (ERß) (Figure 1.2) [65]. The first reports of the cloning and sequencing of ERα occurred in 1986 by two separate groups (Green *et al.*, 1986; Greene *et al*., 1986) [66, 67], while Kuiper *et al*. were the first to clone and identify ERß from rat prostate several years later in 1996 [68]. Recent reports have identified at least five isoforms of ERß that further add to the complexity of the possible regulatory roles of ERß [69]. However, most research groups do agree that ERß primarily exhibits antiproliferative, pro-apoptotic, and tumor suppressive functions by opposing and antagonizing ERα mediated pathways [70-73]. It has been suggested that ERß achieves these opposing actions by sequestering E2 or by forming heterodimers with ERα to exert a negative regulatory effect on ERα function [74, 75]. Despite these findings, major issues with the cell lines and inadequately validated antibodies for ERß were recently described [76]. Nelson *et al*. showed that one of the main antibodies used to detect ERß, NCL-ER-BETA, is non-specific for this receptor [76]. Using antibodies verified by liquid chromatography–mass spectrometry, Nelson *et al*. also showed that ERß was not expressed in either MCF-7 or LNCaP cell lines which have been commonly used to study ERß and potentially calls into question some of the

conclusions made in studies using NCL-ER-BETA antibody [76]. The findings by Nelson *et al*. likely explain much of the uncertainty and inconsistencies regarding the role of ERß in breast and prostate cancer [77] and contributes to the rationale for this chapter focusing on ERα in the context of EDCs instead.

ERα66

For ERα, at least three isoforms have been reported with molecular weights of 36 (ERα36), 46 (ERα46), and 66 kDa (ERα66) [66, 67, 78, 79]. ERα66 is a nuclear receptor and transcription factor that is primarily localized to the cellular nucleus and is comprised of six unique domains that serve separate functions [66, 67, 80] (Figure 1.2). The A/B domain includes the region of the receptor known as activation function–1 (AF-1) that contains a serine residue at position 118 (Ser^{118}). Phosphorylation of Ser^{118} (ERα-P-Ser^{118}) has been shown to be critical for proper function of ER where this receptor modification is likely controlled in part by the mitogen-activated protein kinase pathway, with CDK7 or IKK-α [81-83]. Weitsman *et al*. used chromatin immunoprecipitation to show that ERα-P-Ser^{118} binds to known ERα coactivators proteins, specifically p300 and steroid receptor coactivator-3 [84], to provide evidence of the functional involvement of ERα-P-Ser^{118} in estrogen regulated pathways [82]. The DNA binding domain (DBD) is in the C domain of ERα66 and is responsible for binding to the estrogen response element (ERE) located at the promotor region of estrogen regulated genes. The interaction of the DBD with an ERE occurs through recognition of the consensus palindromic sequence identified as GGTCAnnnTGACC [85, 86]. Additionally, the D domain hinge region has been implicated in the recruitment of transcription factors such as c-Jun and contains the nuclear localization sequence which aids in the translocation of ERα to the nucleus [87].

The ligand binding domain (LBD) and activation function-2 (AF-2) region of ERα66 are both located in the E domain of this receptor (Figure 1.2). However, the functions of the LBD and AF-2 of ERα66 are primarily activated upon binding of endogenous estrogens [80, 88]. Brzozowski *et al*. were the first to report the crystal structure for the LBD of ERα66 in complex with E2 using x-ray diffraction [89]. They noted that the helical arrangement of ERα66 formed a hydrophobic ligand binding cavity that complemented the lipophilic characteristic of E2. The shape of this binding site cavity appeared to favor the formation of specific hydrogen bonds with E2 which are key to orienting the bound hormone [80, 89]. The binding of a high affinity agonist such as E2 induces a conformational change in ER that stabilizes the positioning of helix 12 (H12) which has been shown to be a critical LBD helix whose positioning directly influences the transcriptional activity of ERα66 [88]. The stabilization of H12 exposes a hydrophobic grove between helices H3, H4, and H12 that recognizes and recruits coactivators that contain LxxLL helical motifs to the AF-2 region [88, 90, 91]. In contrast, the binding of antagonists destabilizes the positioning of H12 which prevents LxxLL coactivator association with ER and favors the recruitment of transcriptional corepressors as observed with the ER antagonist tamoxifen and its more potent metabolite, 4-hydroxytamoxifen [92, 93]. The final domain of ERα66 is known as the F domain which has been suggested to be involved in modulating the positioning of H12 and activity of ERα66 (Figure 1.2) [94, 95]. Targeted mutations of the F domain have been shown to alter the affinity of E2 and ER antagonists such as tamoxifen or even preventing the interaction of some coactivator proteins [96-99].

Other ERα Isoforms and GPR30

The ERα46 isoform was first cloned by Flouriot *et al*. in 2000 with ERα36 first identified by Wang *et al*. in 2005 [79, 100]. Alternative splicing of the *ESR1* gene likely explains why ERα46 isoform completely lacks the AF-1 domain while the rest of the receptor is mostly identical to ERα66 [101]. However, ERα36 is a truncated isoform that lacks both the AF-1 and AF-2 domain observed in ERα66 [101] (Figure 1.2). Interestingly, Flouriot *et al*. reported that ERα46 may form heterodimers with ERα66 to regulate genomic estrogen signaling mediated by the AF-1 domain of ERα66 [79]. In contrast to ERα46, ERα36 has been described as being predominantly a membrane associated isoform of ER that may function to inhibit the estrogen-dependent transactivation of ERα66 [102]. However, the exact mechanism by which ERα36 inhibits genomic estrogen signaling remains unclear. Both ERα36 and ERα46 retain the ability to dimerize and contain intact DBD which allows these isoforms to interact with and compete for same EREs that ERα66 normally associates with [103]. Much of the published work studying the shorter ERα isoforms relied on immunohistochemistry techniques that have limited ability to effectively distinguish between different ERα isoforms due to the lack of isoform specific ERα antibodies [75]. To date, the specific interaction and roles of each of these ERα isoforms in the context of E2-induced cell proliferation remains unclear and controversial.

GPR30 is a G-protein-coupled membrane receptor that was first reported by Owman *et al*. in 1996 and was named GPR30 in 1997 [104, 105]. The exact cellular localization of GPR30 has been controversial due to multiple reports stating that GPR30 is only localized to the plasma membrane while others have claimed that it is an intracellular membrane protein located on the endoplasmic reticulum [106, 107]. The role of GPR30 as an estrogen receptor has also received skepticism due to conflicting reports on whether or not E2 binds to GPR30 [106-109]. Otto *et al*. conducted an *in vivo* study using GPR30 deficient mice and concluded that GPR30 was not

required for mediating the effect of E2 on reproductive target organs in murine models [110]. Pedram *et al*. used endothelial cells from ERα/ERß deficient mice (DERKO mice) and showed that E2 did not bind to these cells nor was E2 signaling pathways activated in these cells as determined by extracellular-signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) expression [111]. In contrast to endothelial cells from DERKO mice, wild type endothelial cells readily bind to E2 with and exhibit increased ERK and PI3K stimulation even though both cell types expressed similar levels of GPR30 [111]. Pedram *et al*. also used small interfering RNA (siRNA) directed to GPR30 and showed that rapid E2 signaling was not affected in MCF-7 cells whereas siRNA to ERα prevented these effects [111]. The findings by Otto *et al*. and Pedram *et al*. are just a few examples that highlight the importance of classical ERs as well as the need to clarify the potential role of GPR30 in the context of estrogen signaling.

Due to the significant knowledge gaps with the shorter ERα isoforms and inconsistencies regarding the GPR30 membrane receptor [112], the remainder of this chapter will focus on the ERα66 isoform. All following discussions of ERα will refer to the ERα66 isoform which is an important biological target in the context of endocrine disruption including its well-established role in breast cancer as described in the following sections**.**

Environmental Estrogens and Anti-androgenic Endocrine Disrupting Compounds

The field of endocrine disruption and the concern that environmental exposures may contribute to adverse health effects was pioneered by the American marine biologist, author, and conservationist Rachel Carson (1907 – 1964). In 1962, Carson's book *Silent Spring* raised concerns about the widespread use of the pesticide dichlorodiphenyltrichloroethane (DDT) in the late 1950s and its potential harm to ecosystems and human health [113, 114]. Carson's book

immediately gained national attention both from the public and the US government. In 1970, the US government established the United States Environmental Protection Agency (US EPA) with the authority to implement regulations designed to protect the environment and human health [113]. In 1972, the US banned the use of DDT over concerns that it was associated with an increase in reproductive abnormalities observed in fish and wildlife as well as its ability to biomagnify within adipose tissue of higher species of the ecosystem [115]. Although the widespread environmental contamination of DDT helped give rise to the US EPA, it took the medical tragedy of diethylstilbestrol (DES) to highlight the need for thorough screening and testing programs to identify compounds that may alter normal hormone regulated processes.

DES is a nonsteroidal synthetic estrogen that was given to women in the US between 1940 to 1970 with the intent of preventing miscarriages [113]. However, in the late 1960s, several young women between the ages of 15-22, who were seen at the Massachusetts General Hospital, were found to have clear cell adenocarcinoma of the vagina which was previously not observed in patients under the age of 30 [116, 117]. It was later determined that the mothers of these young women had previously all received DES during the first trimester of pregnancy. Gestational exposure to DES is associated with a range of health issues in both sons and daughters of women exposed to DES [113]. Among the daughters, infertility and reproductive tract abnormalities, such as a T-shaped uterus, were associated with maternal DES exposure [118, 119]. Among the sons, DES exposure was associated with an increased likelihood for the formation of non-cancerous epididymal cysts with some inconsistent findings as whether DES exposure was associated with infertility and genital abnormalities (cryptorchidism and hypospadias) [120-122]. The medical problems associated with DES exposure is just one

example that led to an increase in environmental research and awareness concerning the potential effect of hormone mimics on human health [123].

In 1996, the US Congress issued a legislative mandate to the US EPA to establish an endocrine screening and testing program which culminated in the formation of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) [113]. In that same year, the US EPA provided the following definition of an "endocrine disruptor" [124]:

> "An endocrine disruptor is an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock, 1996).

Similar definitions of an endocrine disruptor were later put forth by EDSTAC [125], the European Union [126], and the World Health Organization [127]. A more recent definition of an endocrine disruptor was proposed by the Society of Endocrinology as "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" [125]. Today, the field of endocrine disruption has significantly expanded to investigate compounds such as phthalates [128], DDT and its analogs [129], methoxychlor [130], ultraviolet filters (UV-filters) [131], bisphenols [132, 133], and parabens [134] for their potential adverse effects on hormone regulated processes in humans and animals. Although many of these compounds have been determined to have binding affinities several order of magnitude weaker than endogenous hormones such as estrogens and androgens, it is the ubiquitous and chronic exposure to environmental EDCs that guides much of this research.

Phthalates

Phthalates are plasticizer compounds that are commonly used in polyvinyl chloride and medical tubing as well in numerous consumer products such as cosmetics, paints, and lubricants [128]. Previous studies have indicated that phthalates display evidence of anti-androgenic behavior with weak estrogen effects [135]. For example, di-n-butyl phthalate (DBP) does not induce an increase in uterine weight or uterotrophic response in ovariectomized female rats exposed to this compound [135]. Furthermore, DBP, di-(2-ethylhexyl) phthalate (DEHP), and their metabolites do not appear to compete for or bind to AR in transcriptional activation assays [136, 137]. However, numerous studies have provided evidence suggesting that phthalates primarily behave as environmental antiandrogens [136, 138]. Prenatal exposure to DBP, DEHP, benzyl butyl phthalate (BBP), and di-isononyl phthalate (DINP) have been shown to induce testicular abnormalities and other reproductive malformation in androgen-dependent tissues in male rats [136, 138]. Similarly, male rabbits exposed to DBP at a dose of 400 mg/kg/day *in utero* (gestation days 15 – 29) had a reduction in numbers of ejaculated sperm, testis weight, and accessory sex gland weight relative to control animals [139]. A recent report detected the presence of two metabolites of diisopentyl phthalate (DiPeP), one of the most potent anti-androgenic phthalates, in 98% of all early pregnancy (<16 weeks) urine samples from women in Brazil [140]. It has been shown that DiPeP is more potent than DBP for inhibiting fetal testosterone production among pregnant rats exposed to either of these compounds (gestation days 14 – 18) [140]. Further research is needed to assess the potential risk from exposure to DiPeP, and similar phthalates, on male reproductive development.

DDT and its Analogs

DDT and it analogs are organochlorine insecticides which were widely used from the 1940s to late 1970s for insect and malaria control [129]. Today, most countries have banned the use of DDT primarily over ecological concerns [129]. Although its common trade name is DDT, technical grade DDT typically contains a mixture of several isomers with the largest percentage of the mixture being attributed to *p,p′*-DDT [141]. Some reports have found that chronic exposure to DDT has been associated with tumor formation in the liver and adverse reproductive effects in wildlife [142, 143].

In the late 1960s, Derek Ratcliffe reported on an association between the increased use of DDT and the observed eggshell thinning contributing to the population decline in several species of birds [144, 145]. Experimental evidence has shown that a metabolite of *p,p′*-DDT, known as *p,p′*-DDE, is responsible for the eggshell thinning effect observed in some species of bids and this has been reviewed extensively [146]. Kelce *et al*. showed that *p,p′*-DDE is a potent androgen receptor antagonist *in vitro* and this metabolite exhibits antiandrogenic effects in male mice [147]. Treating male mice with 100 mg/kg *p,p′*-DDE led to delayed onset of puberty and a reduction in prostate and seminal vesicle weight suggesting that exposure to this metabolite may induce abnormalities in male sex development [147]. Technical grade DDT also contains ~15% *o,p′*-DDT, an isomer of *p,p′*-DDT, which has been shown to act as a potent ERα agonist in competitive receptor binding assays [147]. Data showing that *o,p′*-DDT is an ERα agonist suggests that it is a component of technical grade DDT contributing to endocrine disrupting effects in observed in fish and wildlife [141, 143]. Due to their lipophilic nature, environmental persistence, and the reintroduction of these compounds in some parts of the world for malaria control, human exposure to DDT and its analogs remains as a topic of relevance and concern among environmental researchers [129, 141].

Methoxychlor

Methoxychlor is the *p,p'*-dimethoxy analog of *p,p'*-DDT that was originally intended to be a replacement for DDT due to its low acute toxicity, short biological half-life, and decreased potential for bioaccumulation [148]. Several studies have determined that metabolites of methoxychlor are ERα agonists and likely provided a basis for its ban when it was denied reregistration by the US EPA in 2004 [148]. Wilson *et al*. used a luciferase reporter assay to show evidence that HPTE, a metabolite of methoxchlor, binds to ERα and promotes estrogen-dependent gene activation that could be inhibited by co-treating with a potent antiestrogen [149]. Further i*n vivo* data has shown that the male pups of female rats exposed to methoxychlor exhibit reduced weight of the testis, epididymis, seminal vesicles, and prostate [150]. In female pups, methoxychlor treatment lead to a mixture of estrogenic and antiestrogenic effects which included accelerated vaginal opening, constant estrus, and atrophy of the uterus and ovaries in normal intact females [130, 150, 151]. In contrast, methoxychlor induced an estrogenic effect in ovariectomized female rats that was determined by an increase of uterine weight [130].

UV-filters

UV-filters are a class of chemicals that absorb a broad spectrum of UV radiation which is why they are widely used in personal care products (PCPs) such as sunscreens, lotions, lipsticks, and creams [131, 152]. The lipophilic properties of UV-filter compounds allow to them to be ready absorbed across the skin and serves as a major route exposure in humans [131, 153]. Several UV-filter compounds have been shown to exhibit weak estrogenic behavior, including benzophenone-3 (BP3) which is one of most common UV-filter compounds found in PCPs [154,

155]. *In vitro* studies have shown that UV-filter compounds BP-3, 4-methyl-benzylidene camphor (4-MBC), and octyl-methoxycinnamate (OMC) can promote increased cell proliferation of estrogen-dependent MCF-7 cells and induce a uterotrophic effect in immature rats [154]. Wielogórska *et al*. characterized BP-3, 4-MBC, and OMC using an ERE-luciferase reporter assay and also showed evidence of weak estrogenic behavior for these three UV-filter compounds [156]. Interestingly, an epidemiological study reported a possible association between a metabolite of BP-3, benzophone-1, and an increased risk of endometriosis in women [157]. The potential relationship between UV-filter compounds with estrogen-dependent diseases such as endometriosis requires further mechanistic studies to elucidate the possible mechanisms underlying this observation.

Bisphenols

Bisphenol-A (BPA) is widely used in the manufacture of polycarbonate plastics used in bottles and toys, epoxy resins used in the lining of metal cans, and in numerous other plastic consumer products [132, 158]. Several studies have suggested that BPA is a thyroid hormone disrupting chemical whereas some *in vivo* studies suggest that BPA does not affect thyroid hormone levels [159, 160]. Aung *et al*. conducted a case-control study of pregnant women and observed an inverse association between urinary BPA levels with thyroid stimulating hormone measured in plasma [161]. These findings suggest that exposure to BPA during vulnerable periods of gestation may influence fetal development, however, further studies are needed to understand the mechanism behind the inhibition of thyroid stimulating hormone and BPA in humans. Additional studies have provided evidence that BPA behaves as a weak estrogen and is able to bind to ERα [133, 162]. Naciff *et al*. studied pregnant rats and compared changes in global gene expression

profiles from tissues of pups that were exposed in utero to BPA and two other ERα agonists 17 α-ethynyl estradiol (EE) and the phytoestrogen genistein [163]. They found that all three compounds significantly upregulated the same 50 ER-regulated genes which suggests BPA has a common mode of action with known ERα agonists such as EE and genistein [163].

Murray *et al*. previously studied the relationship between the weak estrogenic behavior of BPA and its effect on mammary gland development by exposing rats to 2.5 µg/kg BW/day of BPA during gestation [164]. They found that adult rats (postnatal day 95) exposed to BPA during gestation were observed with an increase of intraductal hyperplasias in the mammary glands. Tissue sections from epithelial cells in the intraductal hyperplasias were determined by immunostaining to have significantly higher expression of ERα compared to normal mammary glands with additional evidence of increased proliferative activity. They concluded that the increased expression of ERα in the hyperplastic tissues suggest that the proliferative activity among these cells might be driven by estrogen and that these tissues are more likely to be stimulated by estrogens later in life [164]. Murray *et al.* also speculated that their findings support the hypothesis that fetal exposure to BPA and estrogen mimics may contribute to an increased risk of breast cancer later in life that might be attributed to altered mammary gland morphology. This same group conducted a similar study in nonhuman primates which were exposed to BPA during gestation and observed subtle but significant differences in morphological parameters in mammary gland density versus unexposed controls [165]. Although morphometric analysis indicated that the overall development of the mammary gland tissue was more advanced in the BPA treatment groups, immunostaining did not show evidence of increased ERα expression as reported in their earlier rodent study. Taken together, these *in vivo* findings are relevant in the context of breast cancer development in humans in light of evidence

that high mammographic density is associated with an increased risk of developing breast cancer [166].

The concern over the potential endocrine disrupting effects attributed to BPA has led to a reduction in its commercial use and the recent implementation of BPA substitutes bisphenol-S (BPS) and bisphenol-F (BPF) in numerous consumer goods [167]. Interestingly, comprehensive reviews of the reported literature suggests that both BPS and BPF exhibit similar hormonal activity as BPA where they have been found to induce similar *in vivo* effects such as promoting an increase in uterine weight in rats [167]. The widespread exposure of bisphenols and their potential endocrine disrupting effect on humans remains as a current issue that warrants further investigation [161, 168, 169].

Parabens

Parabens, and their analogs, are alkyl esters of 4-hydroxybenzoic acid that are commonly used as preservatives in numerous consumer products including lotions, creams, cosmetics, pharmaceuticals, shampoos, sunscreens, and several other types of PCPs [152, 170, 171]. The widespread use of parabens in PCPs has led to research studies to determine the extent of human exposure and investigate whether there is evidence for endocrine disrupting behavior [131]. *In vitro* studies have shown that parabens exhibit weak estrogenic behavior, bind to ERα to promote ER-dependent gene transcription, and induce increased cellular proliferation of ER-dependent breast cancer cells [156, 162, 172-174]. Evidence of estrogenic behavior for *n*-butylparaben *in vivo* was observed when Hu *et al*. treated immature Sprague Dawley rats at a dose of 0.16 mg/kg/day for three days via intragastric administration [175]. Hu *et al*. found that *n*-

butylparaben was able to induce an estrogenic response in the immature rats which was assessed by an increase of uterine weight [175].

Although parabens are rapidly metabolized in humans, studies have detected their presence in adipose, placental, and breast tissue, as well as breast milk, serum, seminal fluid, and urine [134, 176-183]. A common finding among many biomonitoring studies is that urinary concentrations of parabens in women tends to be several-fold higher than in samples from men [182-185]. Other reports suggest differential exposure among women exposed to parabens and related environmental phenols and these appear to be associated with race/ethnicity and geographic location [185-187]. In men, *n*-butylparaben has been associated with markers of DNA damage in sperm [178]. Similarly, reduced sperm production in male rats has been shown when they are exposed to either propylparaben or *n*-butylparaben in their diet [188-190]. Despite these findings, Nishihama *et al*. did not detect an association between male urinary paraben exposure and semen parameters, however, the study's small sample size may have limited the power to detect a true association [191].

Among third trimester pregnant women, higher concentrations of parabens measured in maternal urine and cord blood were associated with an increased risk of pre-term birth, reduced birth weight, and decreased birth length [192]. Nishihama *et al*. provided evidence that parabens exhibit endocrine disrupting behavior when they reported a dose dependent association between paraben exposure and shorter self-reported menstrual cycle lengths among female Japanese university students [170]. Interestingly, Pollock *et al*. treated female mice with 3 mg of *n*-butylparaben by subcutaneous injection and found that urinary E2 concentrations were elevated in the mice 6 hours after the initial treatment [193]. These *in vivo* findings support the human data presented by Nishihama *et al*. which suggests that parabens may influence normal

reproductive function. Moreover, these data suggest paraben exposure may have additional implications in estrogen-dependent diseases such as breast cancer. For example, breast tissue concentrations of *iso*-butylparaben and *n*-butylparaben measured in patients with ER+ progesterone receptor (PR)+ primary breast tumors have been detected at relevant effect concentrations determined in *in vitro* studies [176]. For example, *iso*-butylparaben and *n*-butylparaben were measured in breast tissue samples at concentrations near their experimentally determined half maximal effective concentration (EC_{50}) or EC_{30} respectively [174]. However, control breast tissue samples from healthy women were not included in this study which makes it difficult to determine if weakly estrogenic compounds like parabens have a role in breast cancer or not. The ubiquitous human and wildlife [194, 195] exposure to estrogenic paraben compounds and their associations with potentially altering estrogen regulated processes warrants further investigation.

As will be discussed in greater detail in the following sections, most diagnosed breast tumors express ER and cellular proliferation can be induced by endogenous estrogens. EDCs that exhibit estrogenic behavior, including some of the compounds described above, are suspected to play role in ER-dependent tumor progression. Alternatively, EDCs may also contribute to breast cancer recurrence and contralateral breast cancer in a subset of women receiving adjuvant endocrine therapy. A discussion on the prominent relationship between estrogen and ER pathway in breast cancer follows.

The Role of Estrogen and the ER Pathway in Breast Cancer

Breast cancer is currently the most commonly diagnosed malignancy among women in the United States (US) with an estimated 266,000 new diagnoses and 41,000 deaths in 2018 alone

[196]. Despite these numbers, the mortality rate for breast cancer has been steadily declining over the last three decades [196]. The reported decline in the mortality rate has largely been attributed to early detection, significant advancements in patient treatment, and a greater understanding of the biological mechanisms driving different breast tumor types [197].

Tumor Cell Subtypes in Breast Cancer

There are at least four different molecular subtypes in breast cancer which are characterized by the expression of biological markers that include ER, PR, and the receptor tyrosine-protein kinase epidermal growth factor receptor 2 (HER2) [198, 199]. Luminal A, or hormone-receptor positive (HR+/HER2-) breast cancer, accounts for approximately 70% of all diagnosed breast tumors and are more likely to produce a favorable response to endocrine therapy [14]. The Luminal A tumor subtype is characteristically identified by the prominent expression of ER and/or PR but not HER2 with a tendency to be less aggressive than the other breast cancer subtypes. The Luminal B, another HR+ subtype, comprises 10% of diagnosed tumors and is identified by the expression of ER and/or PR as well as HER2 expression. In contrast, triple negative breast cancer does not express ER, PR, or HER2 and accounts for approximately 12% of breast cancer diagnoses. Triple negative breast cancer is generally more aggressive than the other subtypes and is twice as common in African American women compared to Caucasian women in the US [200]. Patients with triple negative breast cancer also tend to have poorer prognosis' due to the lack of targeted therapies for this subtype. The last major molecular subtype is referred to as HER2-enriched (HR-/HER2+) and makes up 20% of all breast cancer diagnoses. Although the HR-/HER2+ subtype may spread more aggressively than HR+/HER2- breast cancer, developments in targeted therapy for HR-/HER2+ has led to improvements in

reported patient outcomes for this molecular subtype [201]. Since HR+/HER2- breast cancer accounts for most breast cancer diagnoses, the following sections will provide a historical account on discovering the relationship between estrogen and breast cancer as well as a brief commentary on the critical issue of breast tumor recurrence.

Discovering the Relationship Between Breast Cancer and Estrogen

The first documentation that endogenous ovarian hormones may be associated with breast cancer was noted by a surgeon at Middlesex Hospital by the name of Thomas William Nunn [202]. In 1882, Nunn recorded the case history of a perimenopausal woman with breast cancer, whose disease regressed 6 months after her menstruation ceased [202]. Although Nunn's observations were an important first step in connecting ovarian function and its production of estrogen with breast cancer, his findings largely went unnoticed at the time. Later in 1889, Albert Schinzinger proposed oophorectomy, the surgical removal of the ovaries, as a treatment for breast cancer but it appears that he was not able to fully convince his colleagues to use this procedure [203]. Soon after in 1896, a British physician named Sir George Thomas Beatson described a bilateral oophorectomy that he performed on a patient with metastatic breast cancer who showed signs of improvement and remission of her tumor for at least eight months after the operation [204]. In 1900, Stanly Boyd would later report that 35% of breast cancer patients who received an oophorectomy would exhibit some form of benefit and their tumors temporarily regressed after the procedure [205]. Although Beatson would be recognized for establishing oophorectomy as a treatment for breast cancer, a full understanding of the biological mechanism by which the procedure works in a subset of patients would not emerge until many years later.

In 1960, Jensen and Jacobson showed that radiolabeled E2 injected into immature female rats would localize and be retained in specific target tissues that included the uterus and vagina but not in others such as the kidneys or liver [206]. This fundamental discovery led to subsequent reports describing the biochemical response and nuclear localization of an estrogen receptor that was bound to radiolabeled E2 in cell extracts from rat uterus [207]. A later study also used radiolabeled E2 and determined that the antiuterotropic effect of ethamoxytriphetol, the first non-steroidal antiestrogen, mechanistically worked by preventing binding and localization of E2 in rat uterus [208]. The discovery of the biological mechanism behind ethamoxytriphetol's antiestrogenic behavior was influential in providing insight as to why endocrine therapy would show clinical benefit in some patients and not others. In 1974, potential benefit of antiestrogens were illuminated when the National Cancer Institute sponsored an international workshop that examined data from 436 clinical trials to evaluate whether ER expression could be used as a prognostic marker to predict response to endocrine therapy [209]. The findings from the international workshop were reported in 1975 where it was determined that 55 – 60% of ER expressing tumors responded to endocrine therapy while ER-negative tumors showed little to no tumor regression following treatment. That same year, Lippman *et al*. used the ER-positive, E2-dependent MCF-7 breast cancer cell line to show that the antiestrogen tamoxifen could inhibit MCF-7 cell proliferation and E2-induced DNA synthesis and that these inhibitory effects could be reversed in the presence of E2 [210, 211]. These early clinical and mechanistic studies in the 1960s to late 1970s significantly increased our understanding of the importance of estrogen and ERα in breast cancer and would help pave the way for the continued development of novel antiestrogens.

Types of Antiestrogens

Although antiestrogens including tamoxifen have been used clinically to treat both pre- and postmenopausal patients with breast cancer, a monohydroxylated metabolite of tamoxifen has been found to be a more potent antiestrogen than its parent compound [212]. Mechanistically, tamoxifen and its metabolite work by inhibiting agonist binding and prevent ER from adopting a stable and active conformation [88]. The instability of ERα is a direct result of a redirection in the positioning of Helix 12 which interrupts the recruitment of key coactivator proteins. Alternatively, potent pure antiestrogens such as Fulvestrant have been developed that not only compete for the active site of ER, but they also induce degradation of the receptor via the ubiquitin proteasomal pathway in cytosol [213, 214]. Despite Fulvestrant's clinical use to treat patients with advanced breast cancer [215], this compound's effectiveness is inherently limited to due to its poor bioavailability [213] and must be administered by intramuscular injection. In contrast, aromatase inhibitors (AIs) do not compete with E2 for binding to ER and are designed to inhibit the enzymatic conversion of androgens to estrogens in the peripheral tissues of postmenopausal women [216]. Although the long-term use of third-generation AIs to treat postmenopausal women with breast has been shown to be more effective than tamoxifen [217], recurrence is common and attributed to *de novo* and acquired resistance to AIs [174, 218, 219]. It has been suggested that exposure to estrogen mimicking compounds, as well as the emergence of LBD mutations in advanced breast cancer [220, 221], may explain why a significant number of patients will recur after and while receiving endocrine therapy [222].

Endocrine Therapy Resistance and Recurrence in Breast Cancer

Although ERα-P-Ser118 is an example of a prognostic marker that has been reported to predict which breast cancer patients might benefit from endocrine therapy at diagnosis [223], additional biological markers are needed to identify which patients are more likely to experience a recurrence of their cancer. For instance, Pan *et al*. showed that there is a persistent risk of recurrence and death from breast cancer for at least 20 years after receiving 5 years of adjuvant endocrine therapy [222]. This persistent risk of breast recurrence was determined to increase at a rate of approximately 1 – 2% every year [217] irrespective of a patient's nodal status and stage [222]. For breast cancer patients on AIs, it has been suggested that non-classical estrogens arising from androgen metabolism [218], might explain why upwards of 20% of these patients will recur within 10 years of receiving endocrine therapy [217, 222].

Mechanistically, AIs do not prevent the binding of estrogens to ER and instead work by minimizing their biological synthesis. Therefore, low concentrations of residual circulating estrogens and/or exposure to environmental estrogenic EDCs may offer another potential explanation as to why endocrine therapy fails in some breast cancer patients. For instance, López-Carrillo *et al*. conducted a case-control study among women living in the northern states of Mexico and showed that exposure to diethyl phthalate was associated with an increased risk of breast cancer [224]. Likewise, Cohn *et al*. conducted as prospective nested case-control study in Alameda County, California, and showed that maternal exposure to the potent ERα agonist, *o,p'*-DDT, was associated with a increased risk of breast cancer among the daughters of the exposed women [225]. Palmer *et al*. conducted a follow-up cohort study and reported that prenatal exposure to DES, a potent nonsteroidal ERα agonist, was associated with an increased risk of breast cancer among women greater than 40 years of age [226]. Additionally, adipose tissue concentrations of polychlorinated biphenyls (PCBs) from nonmetastatic breast cancer patients in

New York were shown to be associated with an increased risk of disease recurrence [227]. Another study conducted a meta-analysis of 16 studies and reported a pooled odds ratio that indicated a greater risk of breast cancer from several different PCBs [228]. Despite the detection of numerous congeners of PCBs in breast tissue, human exposure to these compounds are not always associated with clinical and pathological characteristics in breast cancer [229]. Currently, there are a lack of studies demonstrating that paraben exposure is associated with an increased risk of breast cancer despite their detection in breast tissue [176, 177] at biologically relevant concentrations [174]. Future studies are needed to clarify whether parabens, or unidentified environmental estrogen mimics, have a role in breast cancer progression or disease recurrence.

Although some EDCs have been associated with an increased risk of breast cancer, another explanation for why patients recur while on endocrine therapy is due to presence of LBD domain mutations discovered in ERα [221, 230, 231]. Interestingly, these LBD mutations are rare in primary breast cancer tissue and have been reported to be present at very low mutant allele frequencies [232]. However, emerging evidence suggests that these mutations evolve under the selective pressure of aromatase inhibitor therapy and likely are a mechanism of acquired resistance to AIs [233-235]. Two of the most common LBD mutations are found in Helix 12 of ERα, Y537S and D538G, and are frequently detected in 30 – 40% of ER-positive metastatic breast cancer patients. Chandarlapaty *et al*. evaluated the relationship between the presence of LBD mutations and clinical outcomes using baseline plasma samples from 541 postmenopausal women with metastatic breast cancer enrolled in the BOLERO-2 double-blind phase 3 study [236]. They analyzed cell-free DNA from the baseline plasma samples and showed that the Y537S and D538G mutations were associated with worse clinical outcomes including shorter median overall survival. *In vitro* data has indicated that the LBD mutations appear to

confer ligand-independent receptor activation of ERα and promote constitutive activity of ER mediated cell proliferation [220, 221]. Furthermore, *in vitro* studies have indicated that some antiestrogens used to treat breast cancer, (ie: tamoxifen and Fulvestrant), are less potent in cell models expressing these ERα LBD mutations [220, 221, 230]. In summary, these studies highlight critical need to develop more potent and selective antiestrogens to effectively treat breast cancer patients who may be harboring ERα LBD mutations in their tumors.

Rationale for Thesis

Although endocrine therapy for ER-positive breast cancer has been shown to be effective, disease recurrence is a prominent and unresolved issue. The characterization of recently identified metabolites of paraben compounds used in numerous consumer goods will be explored. The data presented in Chapter II suggest that the oxidized paraben metabolites represent an equally important component of paraben exposure. Therefore, these metabolites should be accounted for in studies seeking to determine an association between parabens and endocrine disruption or breast cancer progression. Novel approaches to identify environmental estrogens and those that may be used in manufacturing of consumer items are currently lacking. In Chapter III, I will demonstrate the utility of using an *in silico* approach to identify ERα agonists and show how this methodology can be applied in the development of potentially safer and less estrogenic paraben compounds that could be used in the manufacture of consumer products. Regardless of whether exposure to estrogenic EDCs are conclusively determined to have a role in breast cancer or not, more potent and orally bioavailable antiestrogens are currently needed to effectively treat ER-positive breast cancer. In Chapter IV I will present *in vitro* data on the characterization and development of novel degraders of ERα for applications in targeted therapy for ER-positive metastatic breast cancer patients.

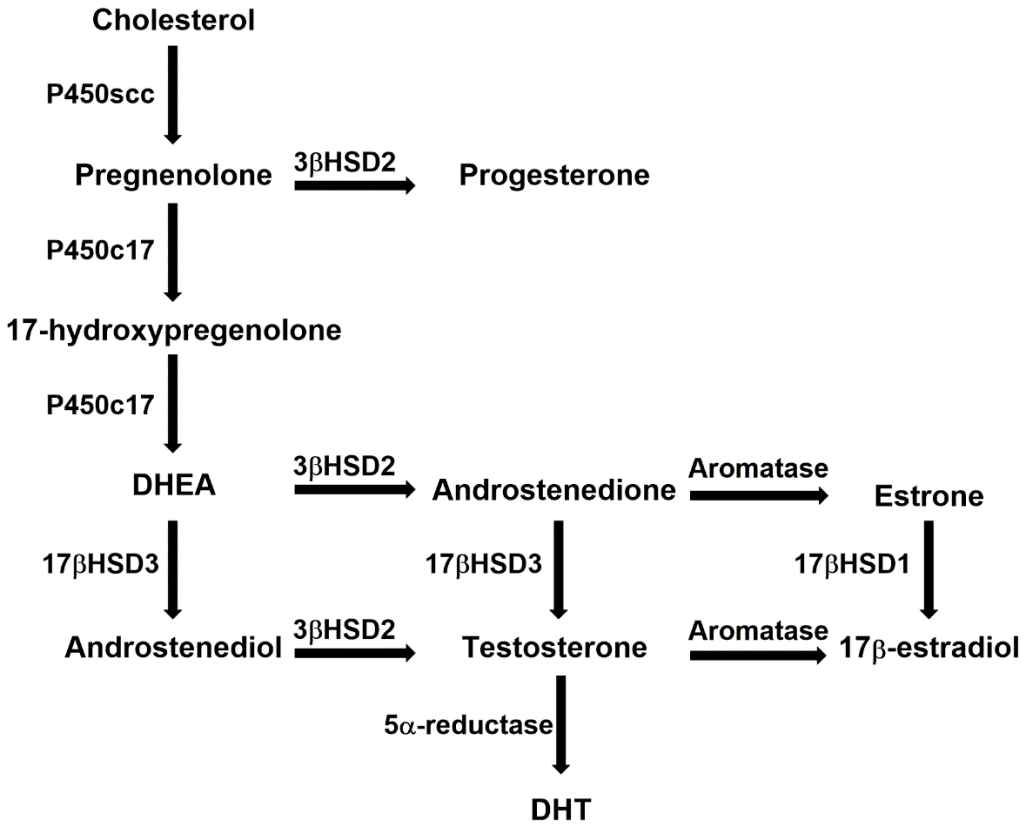

Figure 1.1. Gonadal Steroidogenesis. Depiction of major biosynthetic pathways in the gonads. Key enzymes are shown near arrows indicating biochemical reactions. Modified from Miller and Auchus, 2011.

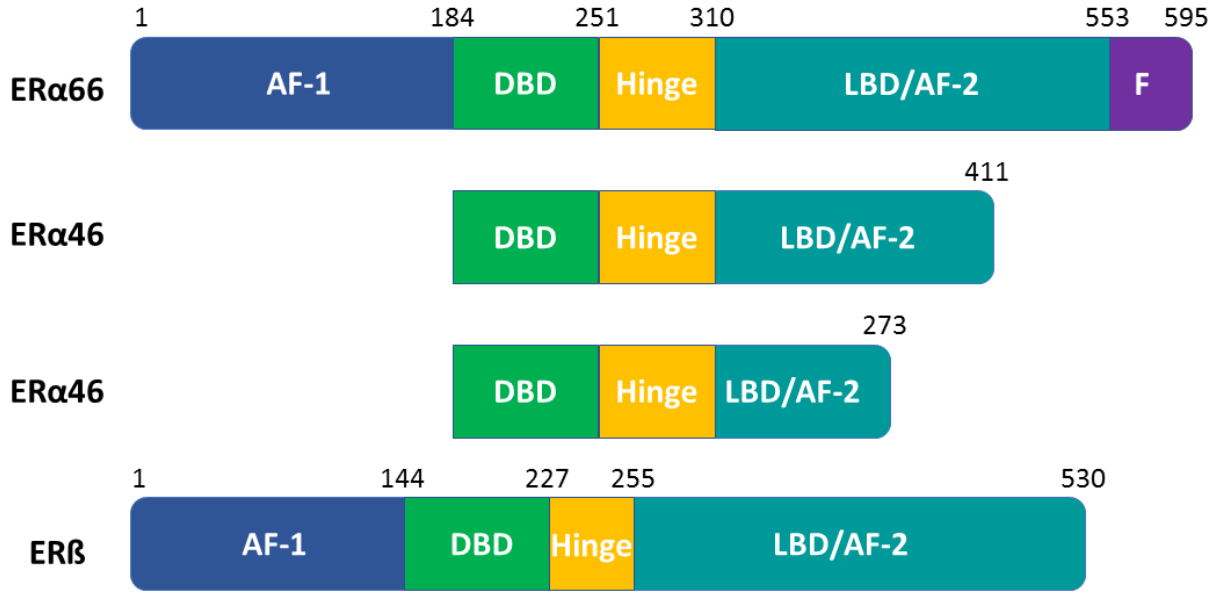

Figure 1.2. Schematic representation of Estrogen receptor domains. The structural domains comprising full length ERα (ERα66), its isoforms (ERα46 and ERα36), and ERβ. The ER domains include activation function-1 domain (AF-1), the DNA-binding domain (DBD), the hinge domain, and the ligand-binding domain/activation function-2 domain (LBD/AF-2), F-domin (F). Modified from Lipovka *et al*. 2016.

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Chapter II.

Metabolites of *n*-Butylparaben and *iso*-Butylparaben Exhibit Estrogenic Properties in MCF-7 and T47D Human Breast Cancer Cell Lines

Introduction

Breast cancer is currently the most commonly diagnosed malignancy among women in the United States (US) with an estimated 266,000 new diagnoses and 41,000 deaths in 2018 alone [1]. An estimated 7% of breast cancer diagnoses are attributed to an inherited genetic predisposition with the remaining ~93% being attributed to other likely risk factors such as lifestyle choices, obesity, or exposure to environmental carcinogens [2]. In addition, lifetime exposure to estrogens has been shown to be correlated with breast cancer risk [3]. Both preclinical and clinical studies have shown that estrogens, specifically 17ß-estradiol (E2), can induce breast cancer pathogenesis [4, 5]. The ability of estrogen to induce breast cancer cellular proliferation is concerning because it is estimated that approximately 70% of all diagnosed breast cancers express the estrogen receptor (ER) and are deemed ER-positive [6]. Pharmacological approaches have been developed to block ER signaling using ER antagonists such as tamoxifen, and more recently aromatase inhibitors (AIs), which inhibit the synthesis of estrogens [7], have been used. While highly effective, *de novo* and acquired resistance to AIs is common, and a possible mechanism of resistance is attributed to exposure to environmental estrogen mimicking chemicals, or xenoestrogens [8, 9] including the ones we chose to study in this report [10].

Recently, there has been increasing public awareness and research into environmental chemicals that may have biologically relevant, endocrine disrupting properties [11].

Xenoestrogens include many of these suspected endocrine disrupting compounds that have previously been shown to display agonistic behavior toward estrogen receptor-α (ERα) [12, 13]. Alkyl esters of *p*-hydroxybenzoic acid (parabens) are one type of xenoestrogen that have been investigated for whether current human exposure levels are a cause for concern [14]. Given their antimicrobial properties, parabens are frequently used as a preservative in numerous pharmaceuticals, food products, and personal care products [15, 16]. Compared to oral ingestion, dermal contact encompasses a broader range of paraben exposure sources that are found in numerous cosmetics and personal care products [17]. As an example, daily paraben exposure for a 63 kg average adult in Korea is estimated to be a sum total of 18,960 µg/day for methyl and ethylparaben combined, and 1,580 µg/day for propyl paraben alone [18]. Similarly, dermal intake of parabens has been estimated to be 31 µg/kg-bw/day for adult females and range between 58.6 to 766 µg/kg-bw/day among infants and toddlers within United States in 2012 [15]. Exposure estimates for parabens in Belgium, Germany, and several other nations have been summarized and reported elsewhere [19-22]. The ubiquitous exposure to parabens has led to reports of their detection in breast tissue, breast milk, placental tissue, serum, seminal fluid, and urine samples from numerous general populations around the world [19, 23-27]. In spite of evidence showing the presence of parabens in various human tissues such as adipose [28] and breast tissue [29], there is still significant debate over their current risk to the general population.

To date, studies have focused on the estrogenic properties of paraben parent compounds [12, 30-32]. Two oxidized paraben metabolites, 3-hydroxy *n*-butyl 4-hydroxybenzoate (3OH) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate (2OH) (Figure 2.1), have recently been discovered, but their potential estrogenic properties have not yet been studied [10]. Unlike E2, which forms three hydrogen bonds in the ligand binding pocket of human ER according to X-ray

crystallography structures [33], BuP is unable to hydrogen bond with His524 [11]. The lack of a second hydroxyl group on BuP likely contributes to its relatively weak ER binding affinity. We hypothesized that the metabolites' additional hydroxyl group might enable binding interactions similar to that of 17ß-estradiol and confer increased estrogenic potency compared to their respective parent compounds. To test this, we used pre-clinical *in vitro* models of ER-positive breast cancer and demonstrated that the oxidized metabolites promote cell proliferation in an ER-dependent manner comparable to their respective parent compounds. Computational docking studies indicated that the metabolites display hydrogen bonding capabilities similar to 17ß-estradiol within the human ERα ligand-binding domain in support of our *in vitro* data.

Materials and Methods

Reagents

17ß-estradiol (E2), ICI 182,780 (ICI), and *n*-butylparaben (BuP) were purchased from Sigma-Aldrich Inc. (St. Louis, MO) with purity ≥ 98% determined by HPLC. 5α-Androstan-17β-ol-3-one (DHT) was purchased from Sigma-Aldrich Inc. (St. Louis, MO) with purity ≥ 97 % determined by TLC. E2, DHT, and ICI were dissolved to 10 mM in absolute ethanol and stored at -20 °C. *Iso*-butylparaben (IsoBuP) was purchased from TCI America (Portland, OR) with purity ≥ 98% determined by HPLC. 3-hydroxy *n*-butyl 4-hydroxybenzoate (3OH) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate (2OH) were kindly provided as a gift by Dr. Vladimir Belov, Max Planck Institute for Biophysical Chemistry in Germany. The 3OH and 2OH compounds had a purity ≥ 95 % determined by HPLC with UV (254 nm) detection. BuP, IsoBuP, 3OH, and 2OH were dissolved to 100 mM in DMSO stored at -20 °C. The final concentration of ethanol or DMSO did not exceed 0.1 % (v/v) in culture media. All compounds were stored protected from light.

Cell lines, culture conditions, and proliferation assays

MCF-7, T47D, and MDA-MB-231 cells were obtained from the Tissue Culture Shared Resource (TCSR) at the Lombardi Comprehensive Cancer Center (Georgetown University, Washington, DC). LNCaP cells were obtained from the laboratory of Ken Pienta at the University of Michigan. Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Life Technologies, Grand Island, NY) supplemented with 10 % (v/v) fetal calf serum (Valley Biomedical Inc., Winchester, VA), at 37 °C in a humidified 5 % (v/v) CO_2 atmosphere. The identity of the cells was confirmed by short tandem repeat profiling by the TCSR and shown to be free of mycoplasma contamination. For assays in defined steroid deplete conditions, cells were repeatedly washed and grown in steroid depleted media over 3 days before proliferation assays (phenol red-free IMEM supplemented with 10 % (v/v) charcoal stripped bovine calf serum – CCS) (Valley Biomedical Inc., Winchester, VA) based on a previously described method [34]. For growth assays, MCF-7, T47D, MDA-MB-231, and LNCaP cells were withdrawn from steroids as previously described [35] and plated in steroid-free media at 1000 cells / well, 2000 cells / well, 500 cells / well, and 7000 cells / well respectively in 96-well plates and allowed to attach overnight. LNCaP cells were plated in 96-well plates coated with poly-D-lysine to improve cell adhesion. LNCaP cells were treated with indicated compound within 1 hr of plating.

PrestoBlue® cell viability assay

MCF-7 and T47D cells were plated in steroid-free media at 1000 cells / well and 2000 cells / well, respectively, in 96-well plates and cultured overnight. The following day, cells were treated

with the specified compounds at the indicated concentrations and the vehicle controls (ethanol or DMSO) diluted in IMEM supplemented with 10 % (v/v) CCS. Six days after treatment, cell viability was assessed using Presto Blue reagent (ThermoFisher Scientific, Waltham, MA) according to manufacturer's instructions. Briefly, cells were incubated for 3 hrs in the presence of 10 % (v/v) Presto Blue and fluorescence was measured using a POLARstar Omega plate reader with the excitation/emission wavelengths set at 544/590 nm. Cell viability data was confirmed using the crystal violet assay based on a previously described method [36].

RNA Expression Assay

MCF-7 cells were repeatedly washed and grown in steroid depleted media over 3 days as described above and plated in steroid depleted media at 400,000 cells/well in 6-well plates at least 10 hours before treatment with parabens for 2, 4, and 6 hour durations. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Yield and quantity were determined by spectrophotometry (NanoDrop ND-1000). All samples were stored at -80 °C. Total RNA (1 µg) was reverse transcribed (RT) using Reverse Transcription System (Promega, Madison, WI) and the cDNA amplified in a 25 µl reaction containing Gene Expression Master Mix and gene specific primers both from Thermo Fisher Scientific (Waltham, MA). *GREB1* mRNA expression was measured using a TaqMan RT-PCR assay as described previously [34]. *GREB1* expression was normalized against GAPDH with relative expression being calculated using the $\Delta\Delta C_T$ method [37].

Estrogen-response element (ERE)-luciferase reporter assay

The ERE-luciferase reporter plasmid (a gift from Dr. Anna T. Riegel, Georgetown University) was co-transfected into MCF-7 cells with Fugene 6 transfection reagent (Promega, Madison, WI) according to manufacturer's instructions with the renilla plasmid (100:1 v:v luciferase to renilla). After 18 hrs, transfected cells were repeatedly washed with phenol red-free 10 % (v/v) CCS I-MEM (Valley Biomedical Inc., Winchester, VA) to produce a steroid-free environment. The following day cells were plated in a 24-well plate (Corning/Costar) in 10 % (v/v) CCS I-MEM (Valley Biomedical Inc., Winchester, VA). After 6 hrs, cells were treated with either 2OH, 3OH, BuP, IsoBuP, E2, or a vehicle control (ethanol or DMSO). After 18 hrs, cell lysates were processed and relative light unit (RLU) reading were measured according to manufacturer's recommendations on a Leader 50 luminometer (Gen-Probe).

Molecular Docking

The crystal structure of the human ERα ligand-binding domain in complex with 17ß-estradiol was downloaded from the protein data bank PDB ID: 3UUD and prepared for docking with the YASARA molecular modelling suite [38]. The PDB entry 3UUD was selected for docking due to its high resolution (1.6 Å). The presence of an endogenous ligand was another factor considered when selecting this structure to ensure that the ligand binding domain was a suitable representation of the agonist conformation. All crystallographic waters were removed, hydrogen atoms were added, bond orders were corrected for S-hydroxycysteine residues, missing loops were repaired [39], and only the A-chain was retained. Docking was performed with VINA [40] using default parameters, and setup was conducted with YASARA as the graphical front-end [41]. A grid size of 25 Å × 25 Å × 25 Å was centered on the C9 carbon of E2 before removing the native ligand and set as the search space for the paraben compounds treated as flexible

ligands. The best pose of 25 runs was defined as producing the smallest root-mean-square deviation (RMSD) between the benzoyl group of the paraben compounds and comparable carbon atoms (1-5, 9, & 10) and phenol oxygen atom of the phenolic-A ring of E2. Hydrogen bond networks of the docked structures were optimized with YASARA before determining RMSD values [42].

2D Interaction Diagrams

The 2D ligand interaction diagrams were drawn using BIOVIA Discovery Studio Visualizer [43]

Statistical Analysis

The curve-fitting program GraphPad Prism 7.0 was used to plot all data. The time and relative fold expression of *GREB1* within each treatment group was processed by one-way ANOVA ($P < 0.005$) followed by a Bonferroni post hoc analysis to determine which time points differed from each other. Technical replicates were derived and analyzed from the same sample source across the indicated treatment conditions. Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions.

Results

3OH and 2OH butyl paraben metabolites induce the proliferation of estrogen-dependent human breast cancer cells grown in steroid-free conditions

To determine the whether the paraben metabolites could induce human breast cancer growth, estrogen-dependent MCF-7 and T47D cell lines were treated with BuP, IsoBuP, and their respective metabolites. The calculated effective concentrations inducing 50% growth (EC_{50}) for

BuP and IsoBuP (1.2 μM & 0.30 μM respectively) were similar to those reported previously [11, 29, 44, 45] (Figures 2.2A – 2.2D). MCF-7 cells treated with the 3OH (Figure 2.3A) and 2OH (Figure 2.3B) paraben metabolites induced cellular proliferation in a concentration-dependent manner. T47D cells treated with 3OH (Figure 2.3C) or 2OH (Figure 2.3D) required slightly higher concentrations than MCF-7 cells to induce proliferation but they did not achieve a sigmoidal concentration dependence. Higher concentrations of the 3OH metabolite would exceed 0.1% DMSO vehicle concentration which prevented an EC_{50} from being determined in the T47D cell line. The EC_{50} for 3OH and 2OH in MCF-7 and T47D cells was calculated (3OH**:** EC_{50} 8.0 μM in MCF-7; 2OH**:** EC_{50} 2.2 μM in MCF-7 and 43.0 μM in T47D). All PrestoBlue® cell viability assay results were confirmed with the crystal violet growth assay as described in Materials and Methods (data not shown). Previous reports have investigated the androgenic and anti-androgenic behavior of parabens [46, 47] prior to the discovery of the 3OH and 2OH paraben metabolites. Therefore, we also examined whether the 3OH and 2OH paraben metabolites exhibited androgenic properties by treating androgen-dependent LNCaP cells and we found that the paraben metabolites do not exhibit androgenic behavior at concentration ranges of 10 pM – 10 μM (Figure 2.4A and 2.4B). Non-estrogen receptor α expressing MDA-MB-231 cells were treated with 3OH, 2OH, and their respective parent compounds to show no effect on cell proliferation. (Figure 2.5A – 2.5D).

The pure anti-estrogen receptor antagonist (ICI 182,780) inhibits 3OH and 2OH metabolite induced proliferation of estrogen-dependent human breast cancer

To confirm that the cell proliferation induced by 3OH and 2OH paraben metabolites was due to ERα signaling, we assessed whether their proliferative effects could be blocked by the pure anti-

estrogen ICI 182,780 (Fulvestrant). Estrogen-dependent MCF-7 cells were treated at the identified EC_{50} concentrations of BuP (1.2 µM), IsoBuP (0.3 µM), and their metabolites either alone or in the presence of increasing concentrations of ICI. BuP and IsoBuP induced proliferation was inhibited by ICI at 0.8 nM and 1.4 nM, respectively, comparable to previous reports [32] (Figure 2.6A and 2.6B). Cell proliferation induced by the 3OH and 2OH paraben metabolites was inhibited in a concentration-dependent manner by ICI with an IC_{50} of 0.7 nM and 1.2 nM respectively (Figure 2.6A, and 2.6B).

3OH and 2OH metabolites induce expression of the estrogen regulated gene, *GREB1*

To confirm the estrogen agonist action of the paraben metabolites we tested the ability of these compounds to induce the expression of an estrogen responsive gene, *GREB1*. We measured the effects of paraben-treated ER-positive MCF-7 cell lines on *GREB1* mRNA levels. *GREB1* is a critical downstream target of ERα signaling the expression of which is induced by exposure to E2, which can be suppressed by ICI 182,780 as previously described [34]. GREB1 has also been described as a critical estrogen-specific ER-interacting protein, an interaction that has been shown to be highly enriched upon estrogen exposure [48]. ER-positive MCF-7 cells were treated at 10 µM with BuP, IsoBuP, 3OH and 2OH as described in Materials and Methods. E2 was used as a positive control; it induced GREB1 expression (~29-fold at 6 hrs; $P < 0.001$) at all time-points compared with the vehicle control (Figure 2.7). Treatment with BuP, IsoBuP, 2OH, and 3OH induced GREB1 expression ~30, ~36, ~20, and ~10-fold at 6 hrs respectively ($P < 0.001$) compared to vehicle control. Neither ethanol nor DMSO significantly affected expression levels compared to media (CCS) alone (data not shown). Co-treatment with the paraben compounds and the pure anti-estrogen ICI 182,780 at 6 hrs blocked these effects (Figure 2.8).

2OH metabolite induces luciferase expression in MCF-7 cells transfected with an estrogen-responsive luciferase reporter construct

To further confirm that the estrogenic activity of the paraben metabolites is mediated by classical ER mediated signaling, we tested the ability of the compounds to induce ER-dependent transcription using an ERE-luciferase reporter construct [49]. The 2OH metabolite significantly ($P < 0.05$) induced transcriptional activity of the ERE-luciferase reporter construct at 10 and 20 µM (Figure 2.9). Transcriptional activity by the 3OH metabolite was found to be non-significant at 10 and 20 µM. BuP and IsoBuP demonstrated increased ERE transcriptional activity consistent with previous reports [12, 50] (Data not shown).

3OH and 2OH metabolites can be docked within the ligand binding pocket of estrogen receptor α

To provide additional support that the hydroxylated metabolites were promoting ERE mediated transcription and stimulated cellular proliferation, molecular docking was used to characterize the interactions of the metabolites as flexible ligands within the known active conformation of the ligand binding site of human ERα. The E2 ligand in the crystal structure of ERα (PDB ID: 3UUD) was removed and re-docked into the active site. The RMSD among all carbon and oxygen atoms for the binding pose of the docked E2 ligand and the pose identified in the crystal structure of ERα was 0.819 Å, indicating that our prepared ERα structure could accurately reproduce experimentally determined binding poses. The high resolution of the 3UUD ERα structure (1.6 Å), compared to PDB entries 4TV1 (1.85 Å) and 4MG9 (2.0 Å) ERα structures complexed with either propyl- or *n*-butylparaben respectively, was a significant contributing

factor in choosing this structure for docking the hydroxylated paraben metabolites. Docking of either propyl- and or *n*-butylparaben to 4TV1 and 4MG9 resulted in relatively poorer RMSD values compared to 3UUD which was another consideration for proceeding with the 3UUD structure for this study. The binding poses of the hydroxylated paraben metabolites and their respective parent compound were analyzed, and each of them displayed a preference to dock to the reported active site of ERα [11].

Hydrogen bonding interactions of the paraben compounds with key side-chain residues in the active site of ERα are shown in Figure 2.10A-2.10C and Figures 2.11A-2.11C. The 2OH compound displayed hydrogen bonding capabilities with amino acid residues Arg394 and Glu353 (Figure 2.10A) which have been shown to hydrogen bond to the phenol ring of E2 [13, 33]. 2D interaction diagrams generated with Discovery Studio Visualizer indicated the potential for two additional hydrogen bonds to be formed with L387 and G521 (Figure 2.11A). The calculated RMSD for E2 and the 2OH metabolite was determined as described in the Materials and Methods and indicated a high degree of binding similarity with E2 (0.658 Å). Due to the presence of a chiral center located at the secondary alcohol group of the 3OH compound and unknown proportions of a potential racemic mixture of our 3OH test substance, it was necessary to dock both the *R* and *S* isomer of the 3OH compound. The identified binding poses of the *R* & *S* isomers both suggested favorable hydrogen bonding interactions with amino acids Arg394 and Glu353, but the *S* isomer was additionally capable of forming hydrogen bonds with His524 and Gly521 (Figure 2.10B, 2.10C). 2D interaction diagrams showed that the *R* & *S* isomer may both form additional hydrogen bonds with L387 but only the *S* isomer may form an additional hydrogen bond with L525 (Figure 2.11B, 2.11C). The calculated RMSD for E2 compared with the 3OH *R* & *S* isomers both indicated a high degree of binding similarity with E2 (0.905 Å and

0.562 Å respectively) and determined as described in the Materials and methods. The calculated RMSD for E2 between each of the parent compounds BuP and IsoBuP demonstrated comparable binding similarity as the metabolites (1.001 Å and 0.976 Å respectively). The calculated RMSD for each of the metabolite and parent compound pairs also indicated; high binding similarity with each other (2OH & IsoBuP: 0.123 Å; 3OH *R* & BuP: 0.293 Å; 3OH *S* & BuP: 0.555 Å).

Discussion

In addition to the numerous urine biomonitoring studies of parabens in diverse populations around the world [18-20, 28], *in vivo* studies using male rats suggest potential disruption of testosterone and sperm production from propyl- and *n*-butylparaben (BuP) exposures [51, 52]. In contrast, human epidemiological studies have not conclusively demonstrated that paraben concentrations in urine are associated with altered male steroid hormone levels or with indicators of sperm DNA damage [53, 54]. However, a common finding among studies measuring paraben compounds in urine is the several fold higher paraben exposure among females compared to males [15, 19, 53]. Urine studies have reported median concentrations of paraben compounds in urine of females generally at low nanomolar concentrations including 1.0 – 1.9 μM for methylparaben, 41.0 – 250.0 nM for propylparaben, and 3.1 – 3.6 nM for butylparaben [55, 56]. One report found significantly higher urine BuP levels (as a marker of higher exposure) being associated with shorter menstrual cycle length among female Japanese women age 19 to 22 despite only reporting a median concentration of 3.6 nM for this compound [56] which is 2 to 3 orders of magnitude lower than the EC_{50} for butylparaben as determined in our study. A larger study showed an association between higher levels of estrogen metabolites in urine, such as estrone sulfate and estrone glucuronide, and shorter menstrual cycles in a group of women that

were part of the Women's Reproductive Health Study [57]. Therefore, the presence of estrogen mimicking compounds in systemic circulation, such as parabens, may have the potential to alter normal hormone or estrogen regulated processes such as those observed in ER-positive breast cancer.

Similarly, it has been well documented that the paraben parent compounds, BuP and *iso*-butylparaben (IsoBuP), induce estrogen-dependent breast cancer cell proliferation by ERα signaling and behave as ER agonists as observed in reporter gene and ER binding assays [12, 32, 44, 50]. Two novel oxidized paraben metabolites were recently shown to be present in human urine samples which structurally appeared to meet the key amino acid hydrogen bonding requirements for ERα in complex with E2 [11, 13]. We also determined the chemical similarity of the oxidized metabolites, indicated by their Tanimoto coefficient (*Tc*) (Table 2.1), which were found to be comparable to known agonists, BuP and IsoBuP, relative to E2. Given these structural similarities of the oxidized metabolites and their parent compounds with E2, we characterized the potential estrogenic behavior of these novel paraben metabolites and provide evidence as to whether they can promote cell proliferation by an estrogen signaling mechanism.

Using *in vitro* growth assays, we were able to demonstrate that the novel paraben metabolites 2OH and 3OH display characteristics of estrogenic behavior in ER-positive, estrogen-dependent human breast cancer cell lines. Although the 2OH and 3OH metabolites induce the proliferation of MCF-7 breast cancer cells, they appear to be less potent in T47D cells, particularly 3OH which required concentrations above 10 µM to induce significant cell proliferation. The proliferative ability of ER-negative MDA-MB-231 cells treated with the hydroxylated metabolites was not affected further suggesting that the metabolites induce cell proliferation via an ER signaling mechanism. Despite the paraben metabolite induced growth of

MCF-7 cells, 2OH and 3OH were found to exhibit estrogenic behavior that was less potent in comparison to their respective parent compounds. A possible explanation for the observed lower potency of the metabolites may be the result of lower membrane permeability and increased hydrophilic characteristics among the metabolites compared to BuP and IsoBuP indicated by their computationally derived log *P* values (Table 2.1). The presence of the second hydroxyl group on the 2OH and 3OH metabolites likely contributes to an increase in polarity making it more difficult to diffuse across the cell membrane compared their more lipophilic parent compounds. The correlation between membrane permeability and physiochemical properties of small molecules has been widely discussed elsewhere [58, 59].

We also investigated whether the cell proliferation of the estrogen-dependent breast cancer cells induced by the 3OH and 2OH paraben metabolites was due to ERα signaling. MCF-7 cell proliferation induced by each of the paraben compounds was blocked in the presence of a pure-antiestrogen suggesting that the metabolites promote cell proliferation via ERα. We also examined the effect of the 3OH and 2OH metabolites on the transcriptional activity of an estrogen-inducible promoter by ERE-luciferase assay. We found that 2OH could significantly promote increased transcriptional activity at the concentrations tested; however, the transcriptional activity induced by 3OH was not statistically significant only at the concentrations tested. In addition, we observed significant metabolite-induced expression of the estrogen regulated gene, *GREB1,* which is a well characterized downstream target of ERα signaling. Increased expression of *GREB1* was found to be time-dependent upon exposure to the metabolites as observed with the E2 positive-control. *GREB1* expression induced by the compounds was blocked in the presence of a pure anti-estrogen for all tested paraben compounds which is consistent with the results from the growth assays as previously discussed. Lastly, our

in silico modeling data suggest that the paraben metabolites display a preference for docking to the ligand binding domain of ERα and demonstrate favorable interactions with key amino acid residues as seen in reported crystal structures of ERα with either E2, propylparaben, or BuP [11, 13, 33]. Despite the favorable ligand binding domain interactions predicted from the docking experiments, the computationally derived partition coefficients and greater hydrophilic characteristics of the metabolites suggest that poor bioavailability might explain why the metabolites were not more potent than the parent compounds. It is important to note that our *in silico* approach cannot be used to make a distinction of whether the oxidized metabolites are indeed true agonists or antagonists. However, future work will focus on elucidating other potential binding modes or allosteric interactions among other hormone receptors and the oxidized metabolites. Collectively, these data, suggest that the novel 3OH and 2OH metabolites demonstrate behavior consistent with their being weak estrogens.

Although the paraben metabolites were found to be generally less potent than their parent compounds, their calculated EC_{50} values were still within a similar order of magnitude as their parent compounds according to previous reports. Furthermore, the extent of oxidative modification for the oxidized metabolites have been shown to be present ~2.3 fold or ~1.1 fold higher than their parent compounds in human urine for the 2OH and 3OH metabolites respectively [10]. This is especially important due to the presence of parabens in breast tissue [29] where the tissue concentrations of the oxidized metabolites is currently unknown. Charles and Darbre (2013) have previously shown that 27 % of breast tissue samples taken from patients with ER+PR+ primary tumors contained at least one measurable paraben compound that was above its lowest-observed-effect concentration in MCF-7 cells [29]. Although the breast tissue concentrations analyzed by Charles and Darbre were found to have median concentrations in the

low nanomolar range, some of the tissue samples were observed to have measurable paraben compounds in the micromolar range within 1 to 3 orders of magnitude of their experimental EC_{50} values [29]. This same study also reported a few breast tissue concentrations that were at or above our experimentally determined EC_{50} for IsoBuP or near the EC_{30} for BuP [23, 29]. Despite these findings, healthy control tissue was not examined for paraben content making it difficult to interpret what potential biological effect the presence of these paraben compounds in breast tissue might have on ER-positive breast cancer. Furthermore, it is not well understood what potential effect these estrogen mimicking compounds might have in the context of ER-positive breast cancer patients who are on antiestrogen therapy, such as aromatase inhibitors (AIs), and whether total paraben exposure could contribute as possible mechanism of resistance for these patients. Therefore, combined exposure from the metabolites and their parent compounds should not be overlooked when being assessed in biomonitoring studies due to the risk of underestimating human exposure. Future work is needed to establish whether the metabolites would have a combined estrogenic effect in the presence of relevant concentrations of other paraben compounds that have been previously measured in tissue samples from patients with ER+ PR+ primary breast cancer [23, 29].

To our knowledge, this is the first report to characterize the estrogenic behavior of the novel paraben metabolites, 3-hydroxy *n*-butyl 4-hydroxybenzoate (3OH) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate (2OH). We have demonstrated that the oxidized 3OH and 2OH paraben metabolites induce breast cancer cell proliferation by estrogen signaling on the same order of magnitude as their parent compounds. However, the derived EC_{50} for the metabolites suggest that they are relatively less potent than their parent compounds. Given the lower potency of the metabolites relative to their respective parent compounds, existing regulatory standards and

industry trends toward safer alternatives may be adequate in limiting human exposure to paraben compounds. However, future biomonitoring studies should attempt to account for the metabolites when determining total daily intake averages to prevent an underestimation of an equally important component of potential endocrine disrupting effects upon paraben exposure.

This chapter has been published in the July 2018 issue of Toxicological Sciences as an original research article entitled: Metabolites of *n*-Butylparaben and *iso*-Butylparaben Exhibit Estrogenic Properties in MCF-7 and T47D Human Breast Cancer Cell Lines. Authors: Thomas L. Gonzalez, Rebecca K. Moos, Christina L. Gersch, Michael D. Johnson, Rudy J Richardson, Holger M. Koch, James M. Rae. PMID 29945225.

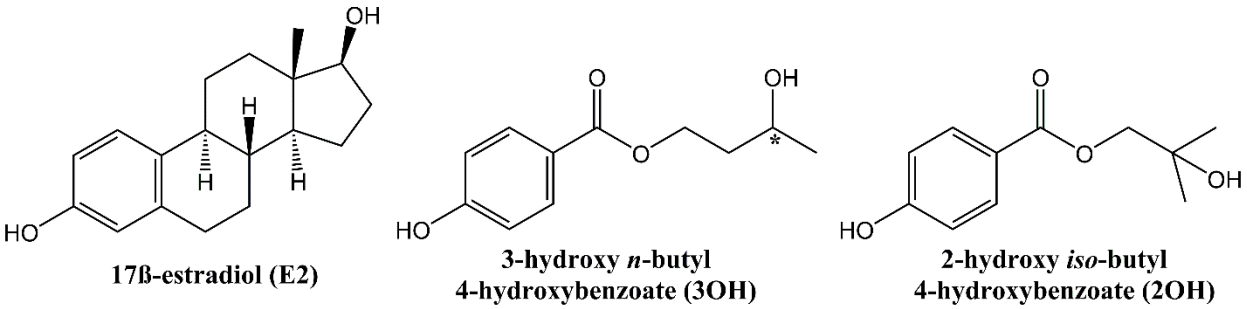

Figure 2.1. Chemical structures of 17ß-estradiol, 3OH, and 2OH paraben metabolites. * - Indicates chiral center.

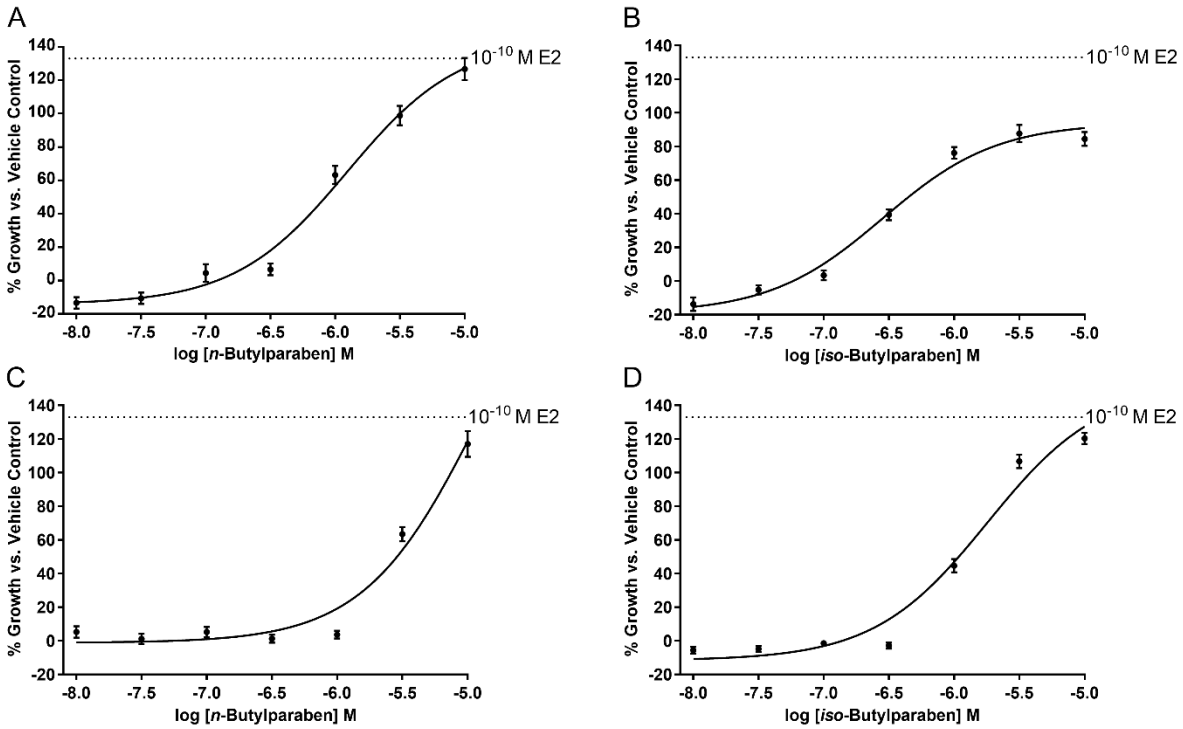

Figure 2.2. *n*-Butylparaben and *iso*-Butylparaben induce breast cancer cell proliferation. MCF-7 and T47D cells were grown in steroid-deplete conditions as described in Methods section. PrestoBlue® cell viability assay was used as a surrogate to determine relative cell number. Estrogen dependent MCF-7 cells were treated with either *n*-butylparaben (BuP) (A) or *iso*-butylparaben (IsoBuP) (B) and T47D cells were treated with BuP (C) or IsoBuP (D) at the indicated concentrations. *Growth curves* represents percentage of cell growth compared to DMSO (vehicle) control (0 %). Points on dose response *curve* represent 6-day growth vs. vehicle treated control ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown). Dotted line indicates growth induced by 100 pM E2.

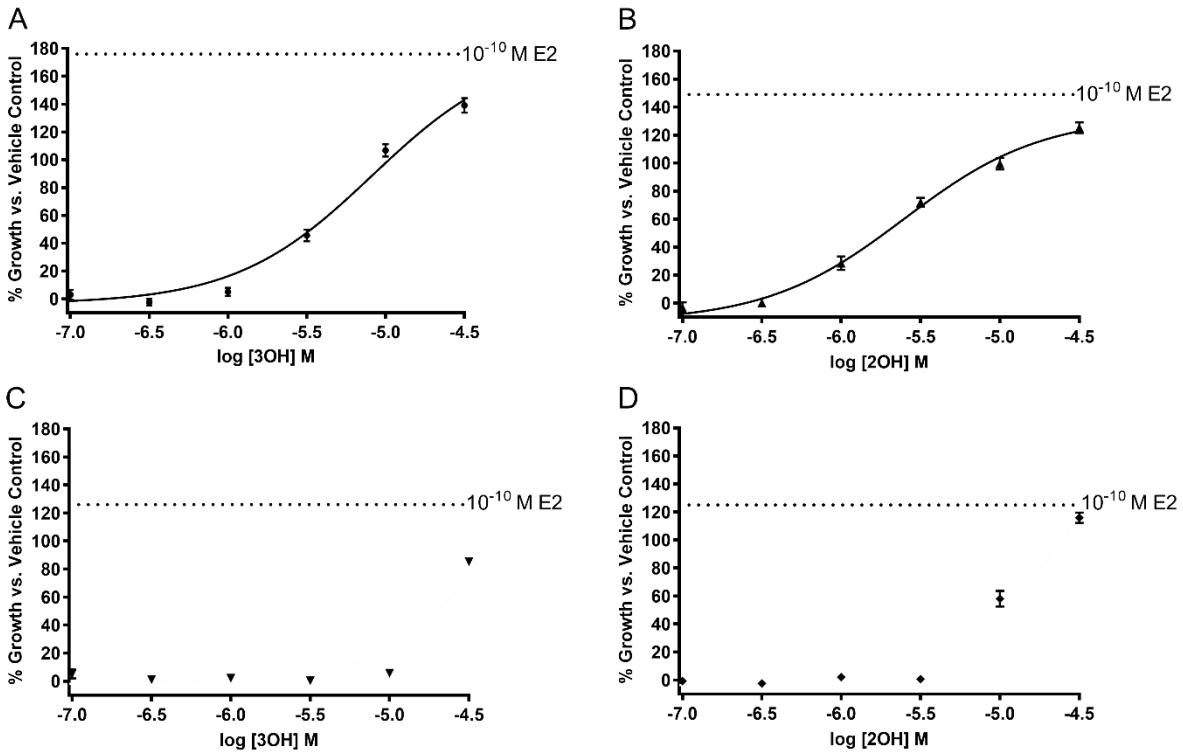

Figure 2.3. 3OH and 2OH paraben metabolites induce breast cancer cell proliferation. MCF-7 and T47D cells were grown in steroid-deplete conditions as described in Methods section. PrestoBlue® cell viability assay was used as a surrogate to determine relative cell number. **(A)** MCF-7 cells were treated with either 3OH or **(B)** 2OH and **(C)** T47D cells were treated with 3OH or **(D)** 2OH at the indicated concentrations. *Growth curves* for A – D represent percentage of cell growth compared to DMSO (vehicle) control (0 %). Points on dose response *curve* represent 6-day growth vs. vehicle treated control ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown). Dotted line indicates growth induced by 100 pM E2.

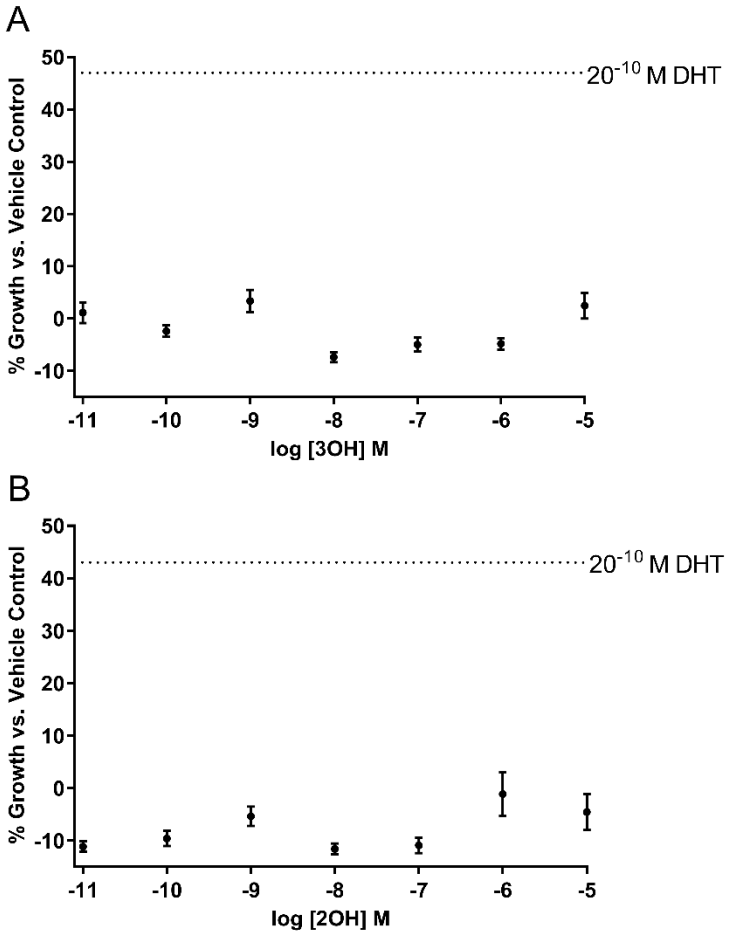

Figure 2.4. 3OH and 2OH paraben metabolites do not induce androgen dependent cancer growth. LNCaP cells were grown in steroid-deplete conditions as described in Methods section. Crystal violet growth assay was used as a surrogate to determine relative cell number. LNCaP cells were treated with **(A)** 3OH or **(B)** 2OH and assayed for growth 6 days after treatment. Growth curves represents percentage of cell growth compared to DMSO (vehicle) control (0 %). Points indicate average growth of 6 replicates versus vehicle control ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown). Dotted line indicates growth induced by 200 pM DHT.

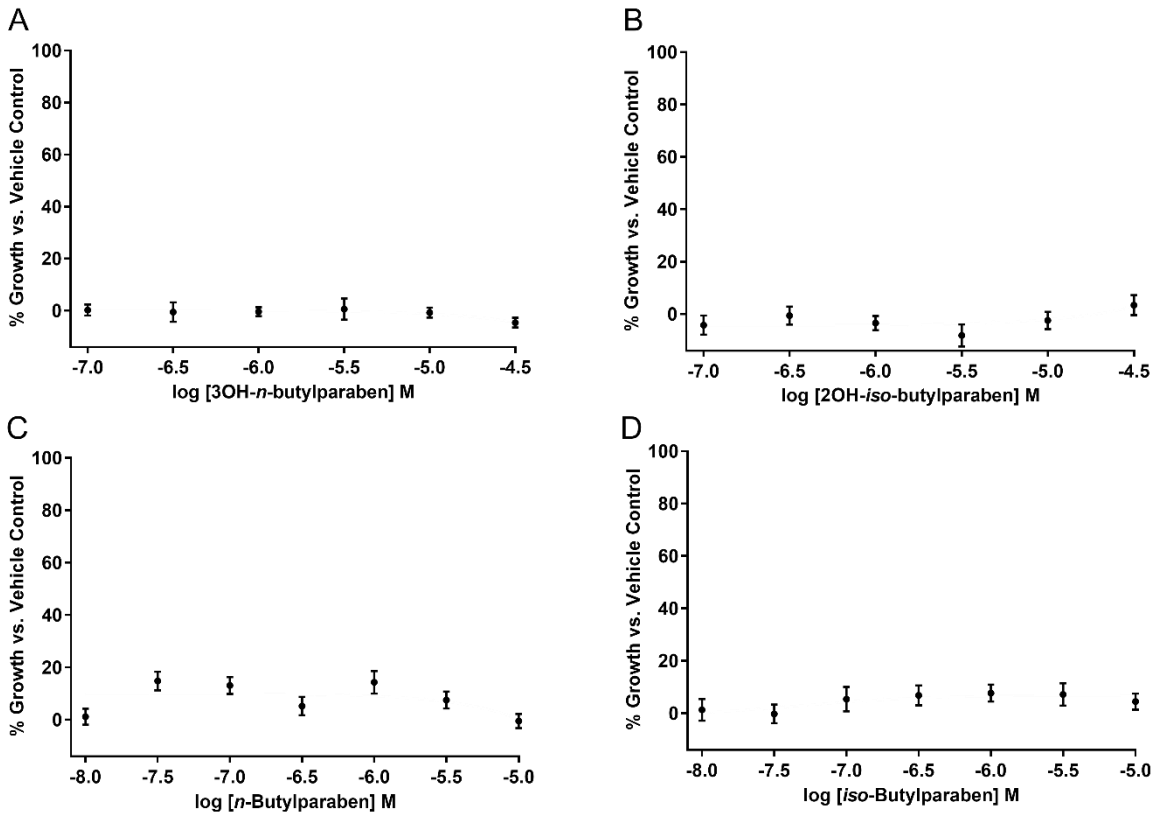

Figure 2.5. 3OH, 2OH, *n*-Butylparaben and *iso*-Butylparaben do not induce a proliferative effect on ERα negative MDA-MB-231 breast cancer cells. MDA-MB-231 cells were grown in steroid-deplete conditions as described in Methods section. PrestoBlue® cell viability assay was used as a surrogate to determine relative cell number. MDA-MB-231 cells were treated with either **(A)** 3OH, **(B)** 2OH, **(C)** *n*-butylparaben (BuP), or **(D)** *iso*-butylparaben (IsoBuP) at the indicated concentrations. *Growth curves* represents percentage of cell growth compared to DMSO (vehicle) control (0 %). Points on dose response *curve* represent 4-day growth vs. vehicle treated control ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown).

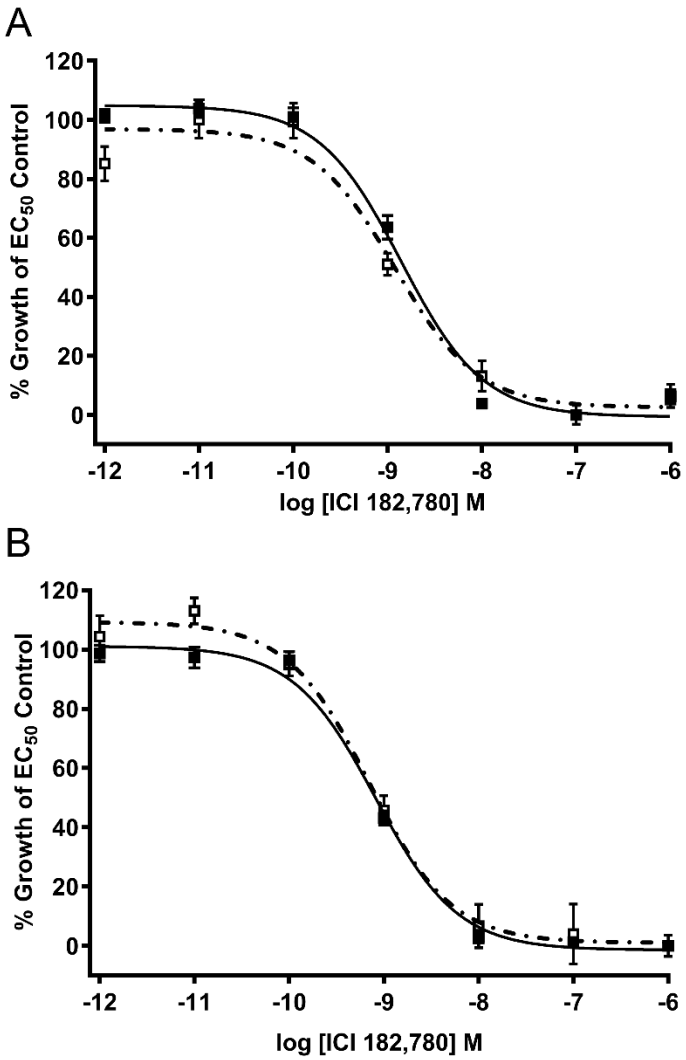

Figure 2.6. Pure anti-estrogen blocks 3OH, 2OH, respective parent compound induced breast cancer cell proliferation. MCF-7 cells were grown in steroid-deplete conditions as described in Methods section. PrestoBlue® cell viability assay was used as a surrogate to determine relative cell number. Growth induction by a fixed dose of either **(A)** (▪) 0.3 µM *Iso*-butylparaben (solid line) or (▫) 2.15 µM 2OH (dashed line) or **(B)** by (▪) 1.2 µM Butylparaben or (▫) 8.22 µM 3OH was antagonized by the pure anti-estrogen, ICI 182,780 (ICI). ICI was added to final concentrations ranging from 1 pM – 1µM at log intervals. *Growth curves* represents percentage of cell growth compared to 2OH or *Iso*-butylparaben at the fixed concentrations indicated above. Data are normalized from maximum growth (100 %) to minimum growth (0 %) for each treatment. Points on dose response curve represent Points represent 6-day growth vs. proliferation with EC_{50} of indicated paraben without ICI ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown).

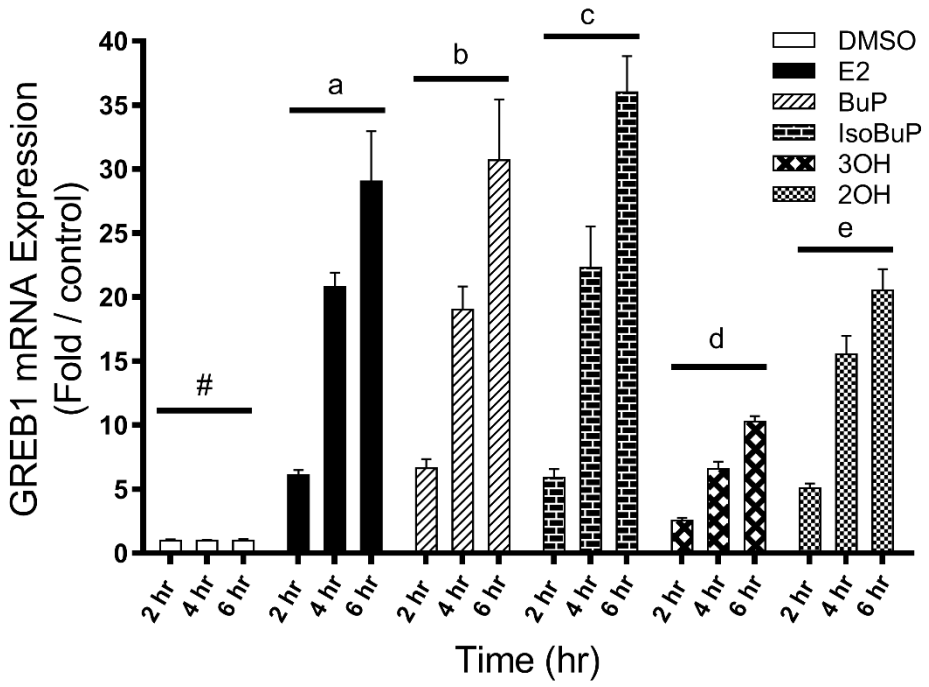

Figure 2.7. Time course induction of *GREB1* expression in MCF-7 cells by the 3OH and 2OH paraben metabolites. MCF-7 cells were assayed in steroid-deplete conditions. Cells were treated with a vehicle control (0.001% ethanol or 0.1% DMSO), or 100 pM E2, for 2, 4, and 6 hrs. Neither ethanol or DMSO significantly affected expression levels compared to media (CCS) alone (data not shown). Cells were also treated at 10 µM with butylparaben (BuP), *Iso*-butylparaben (IsoBuP), 3OH, and 2OH. *Bars* represent *GREB1* expression versus vehicle-treated control using the *ΔΔCT* method. Bars represent the mean from 3 technical replicates ± SE. Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown). Statistical significance within each treatment group was determined by one-way ANOVA followed by Bonferroni posthoc analysis. # = not significant; a,b,c,d,e = $P \leq 0.005$.

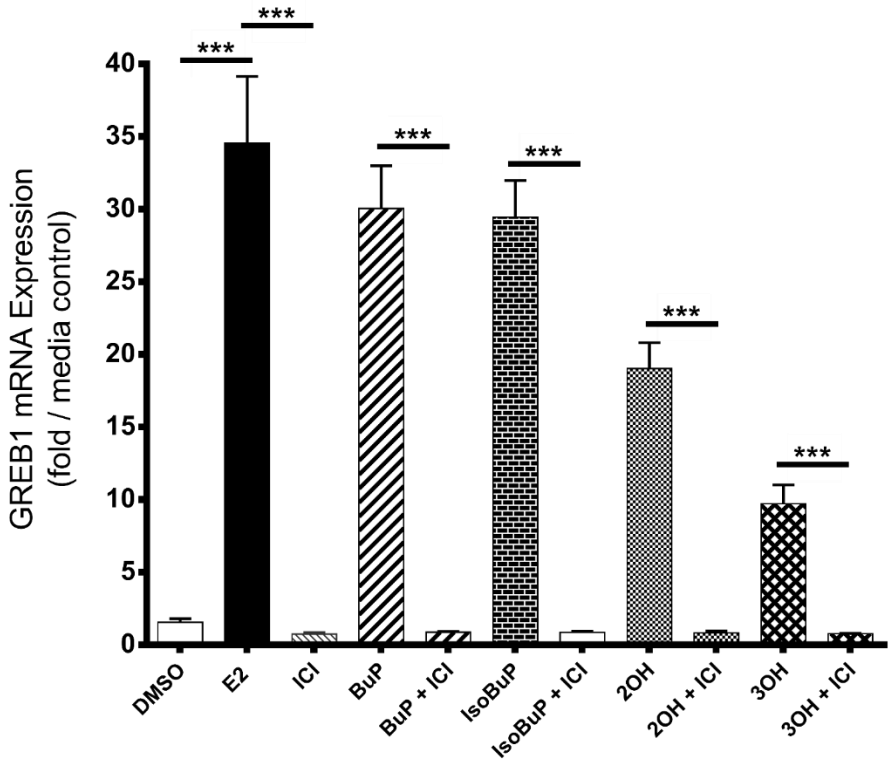

Figure 2.8. Induction of *GREB1* expression in MCF-7 cells by the 3OH and 2OH paraben metabolites is blocked in the presence of ICI 182, 780. MCF-7 cells were assayed in steroid-deplete conditions. Cells were treated with a vehicle control (0.001% ethanol or 0.1% DMSO), 100 pM E2, or 100 nM ICI for 6 hrs. Neither ethanol or DMSO significantly affected expression levels compared to media (CCS) alone (data not shown). Cells were also treated at 10 µM with *n*-butylparaben (BuP), *Iso*-butylparaben (IsoBuP), 3OH, and 2OH alone or in combination with 100 nM ICI. *Bars* represent *GREB1* expression versus vehicle-treated control using the *ΔΔCT* method. Bars represent the mean from 3 technical replicates ± SE. Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown). Statistical significance between for treatments with and without ICI were determined by one-way ANOVA followed by Tukey posthoc analysis. *** = $P \leq 0.0001$.

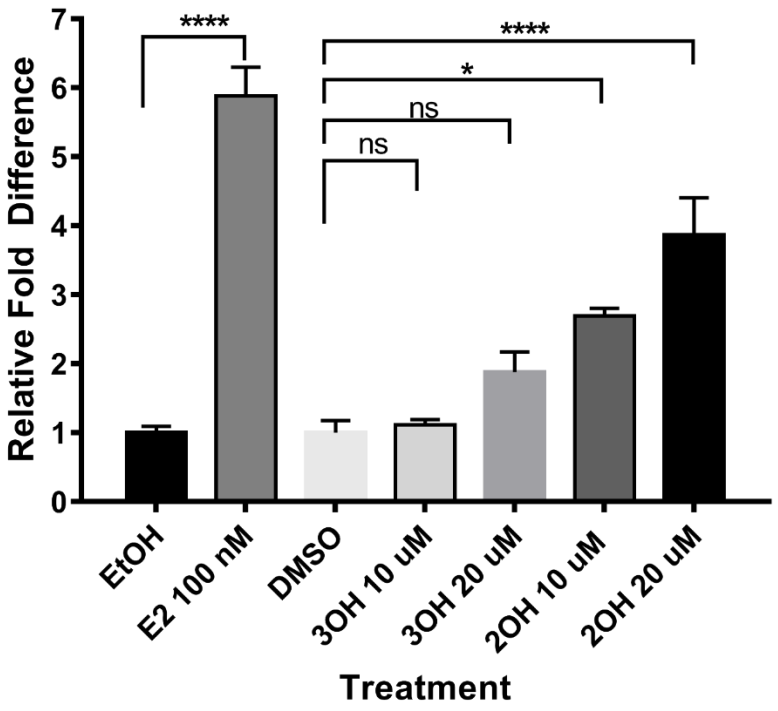

Figure 2.9. The 2OH paraben metabolite promotes significant ERE-luciferase transcriptional activity. The 2OH and 3OH paraben metabolites significantly induce ERE-luciferase activity. MCF-7 cells were co-transfected with the ERE-luciferase construct and renilla reporter plasmid in steroid-deplete conditions and treated at the indicated concentrations for each compound. Relative firefly luciferase activity was plotted over renilla luciferase activity induced by treatment from each specified compound versus ethanol vehicle control for E2 and DMSO vehicle control for each paraben compound. Statistical significance between each indicated pair was determined by one-way ANOVA followed by Tukey posthoc analysis. Bars represent the mean from 3 technical replicates ± SE. Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown). ns = not significant; *$P < 0.05$; **, **** = $P < 0.0001$.

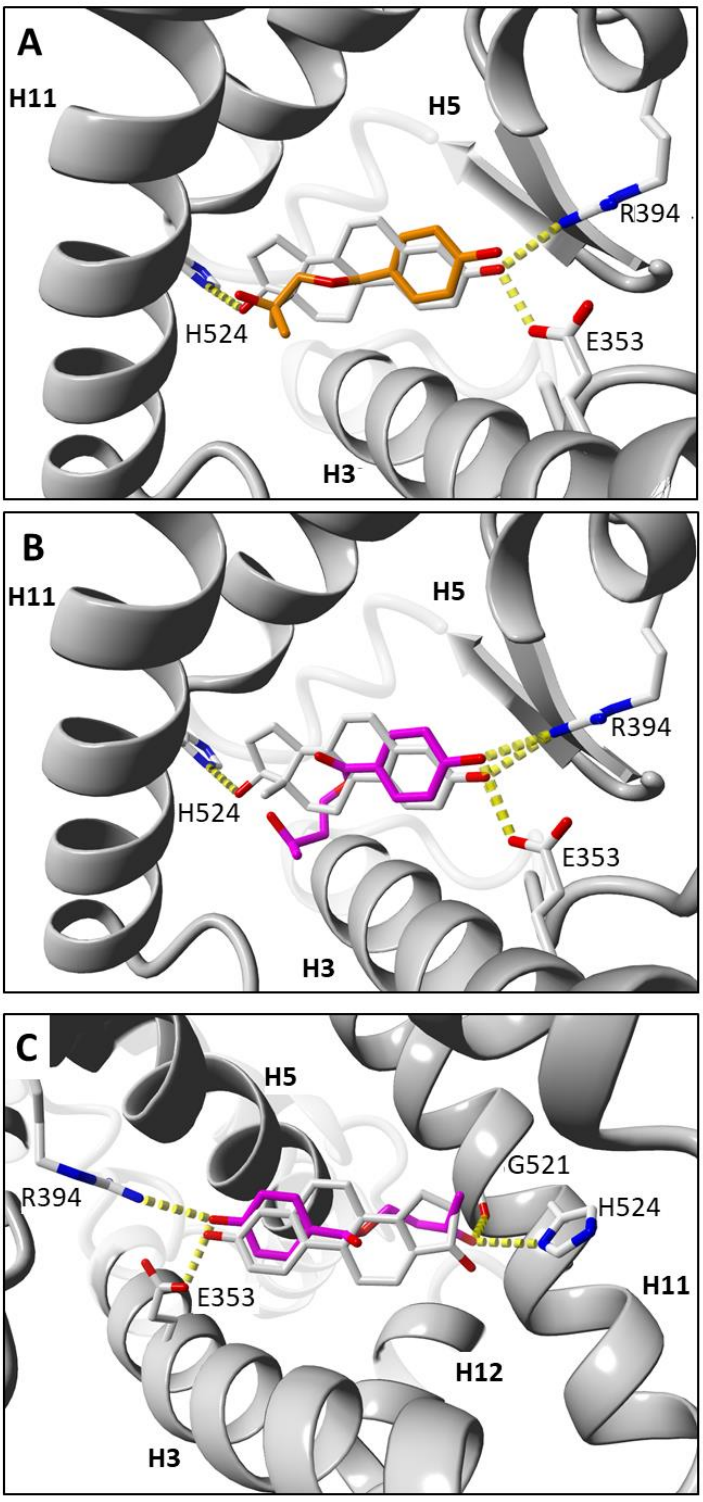

Figure 2.10. 3OH and 2OH paraben metabolites dock to estrogen receptor α. Human estrogen receptor α ligand binding domain docked with **(A)** 2OH isomer **(B)** 3OH *R* isomer or **(C)** 3OH *S* isomer. 2OH and 3OH are colored orange and magenta respectively. 17ß-estradiol has been overlaid with each ligand in grey for comparison. Hydrogen bonds are represented as yellow dashes. Oxygen and nitrogen atoms are colored in red and blue respectively. Active site helices (H3, H5, H11, & H12) are displayed in bold for clarity and hydrogen bonding residues are displayed and labeled.

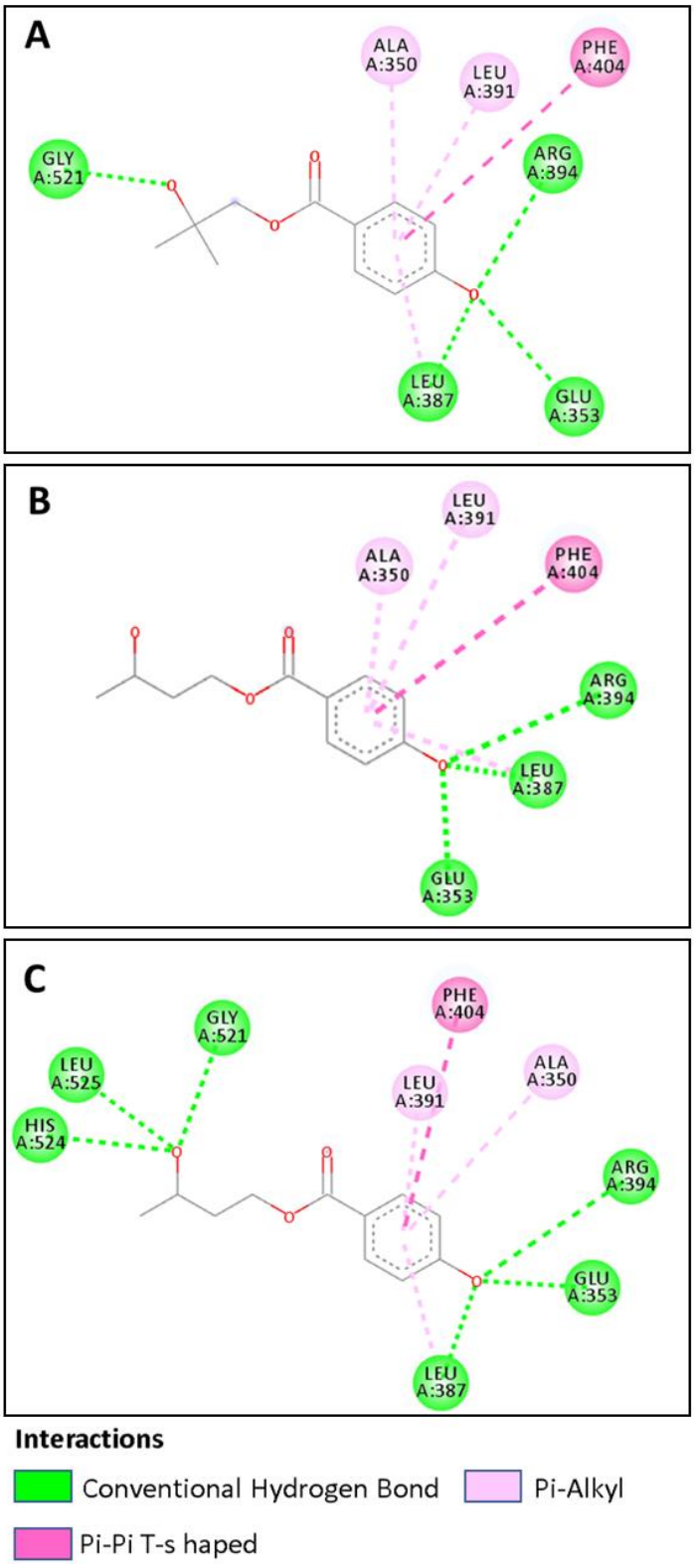

Figure 2.11. 2D interaction diagrams for the 3OH and 2OH paraben metabolites. 2D ligand interaction networks for **(A)** 2OH **(B)** 3OH *R* and **(C)** 3OH *S* isomers. Dashed green lines indicate hydrogen bonds. All other relevant interactions are outlined in each figure legend.

Table 2.1. Calculated partition coefficients and Tanimoto similarity metrics.

Compounds	Log *P* (Octanol/water)	Tanimoto Coefficient (Tc) (Relative to E2)
17ß-estradiol (E2)	3.71	1.000
n-Butylparaben	3.12	0.524
Iso-Butylparaben	3.12	0.527
(3*S*)-3-hydroxy n-butyl 4-hydroxybenzoate (3OH)	1.63	0.549
(3*R*)-3-hydroxy n-butyl 4-hydroxybenzoate (3OH)	1.63	0.556
2-hydroxy iso-butyl 4-hydroxybenzoate (2OH)	1.66	0.530

Partition coefficients and Tanimoto similarity scores were determined for each isomer of the parent and hydroxylated metabolites butylparaben. Calculator Plugins were used for structure property prediction and calculation [60].

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Chapter III.

Homology Models of Mouse and Rat Estrogen Receptor-α Ligand-Binding Domain Created by *In Silico* Mutagenesis of a Human Template: Molecular Docking with 17ß-Estradiol, Diethylstilbestrol, and Paraben Analogs

Introduction

Numerous toxicology studies depend on rodent *in vivo* models [1, 2] as well as *in vitro* screening assays [3-5] to identify and characterize suspected endocrine disrupting compounds (EDCs). Traditional methods of generating toxicity data for risk assessment that rely on animal models or even *in vitro* assays can quickly become too costly or time-consuming to adequately screen and establish toxicological profiles for the tens of thousands of chemicals cataloged by the United States Environmental Protection Agency [6]. Efforts are underway to minimize the use of rodent models and establish new approach methodologies [7] that could be used more routinely to screen and identify suspected EDCs via *in silico* approaches [8, 9]. However, attempts to establish *in silico* protocols for identifying EDCs that act on estrogen signaling pathways are hampered due to the lack of reported structural data for mouse or rat estrogen receptor-α (ERα) within the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) [10, 11].

Interspecies sequence identities for the entire ERα receptor are 88.5% (human-mouse), 87.5% (human-rat), and 97.5% (mouse-rat). For the ligand binding domain (ERα-LBD) alone, the interspecies sequence identities are 95.5% (human-mouse), 95.1% (human-rat), and 99.2% (mouse-rat) [12] (Figure 3.1). We therefore hypothesized that these receptor species should

display similar 3D structures and exhibit comparable ligand binding interactions with known agonists. Although this contention appears logical and likely, subtle differences in the protein sequence could affect tertiary structure and/or ligand binding to a given receptor [13]. For example, several clinically relevant mutations resulting in single amino acid substitutions in the human ERα-LBD have been shown to confer a ligand-independent phenotype compared to the wild type ERα-LBD [14-16], which further demonstrates the apparent structural and functional sensitivity of the ERα-LBD to small differences in its primary sequence.

To determine whether the differing residues among the rodent and human ERα-LBD receptors could be considered neutral substitutions, we created ERα-LBD structures for the unreported rodent species via *in silico* mutagenesis of a human ERα-LBD template. Structural improvement of these *in silico* models was achieved by energy minimization and molecular dynamics refinement in the YASARA molecular modeling suite [17]. The construction of these *in silico* receptors allowed us to address the lack of reported mouse and rat ERα-LBD structures and compare how each of these ERα-LBDs might interact with known or postulated ERα agonists using molecular docking simulations. Comparative docking into rodent and human ERα-LBDs was carried out with 23 compounds designated Group A ligands. These compounds included 17β-estradiol (**E2**), diethylstilbestrol (**DES**), 17 paraben analogs and 4 paraben metabolites (Figure 3.2, Table 3.1).

Molecular docking of **E2**, the most potent endogenous estrogen in both humans and rodents [18, 19], with the human and rodent ERα-LBDs provided us with a point of comparison for analyzing ligand-residue interactions among different ERα-LBDs agonists. For further calibration, we included **DES**, a potent synthetic nonsteroidal ERα agonist [20] that has been used as a model compound in the characterization of EDCs with estrogenic activity [21].

Parabens (esters of *p*-hydroxybenzoic acid) constitute a class of chemicals that have received considerable [3, 22] and controversial [23, 24] attention as suspected EDCs. Although they are found in relatively low concentrations in human tissues and possess only weak estrogenic and antiandrogenic activity [25, 26], parabens are ubiquitous in the environment owing to their widespread use as preservatives in a variety of foods and personal care products [27]. However, apart from concerns about potential adverse health impacts of parabens, the main purpose of the present study was to use members of a homologous series of paraben compounds, paraben metabolites, **E2**, and **DES** as tools in molecular docking simulations to assess the degree of similarity of ligand binding between human and rodent ERα-LBDs. A set of experimental estrogenic activity data for the human ERα was also available for 13 of the parabens and **E2** [28], enabling us to compare these results with corresponding potencies derived from docking.

Recently, it has been found that various substitutions in the benzene ring of *n*-butyl and *n*-octyl parabens, especially in the 3,5-position, result in decreased binding to human ERα with concomitant enhancement of antimicrobial activity [29]. This discovery opens up the possibility of replacing existing parabens with analogs that are more effective preservatives and even less likely to act as EDCs. Moreover, this publication provided us with an additional consistent set of experimental structure-activity data on paraben analogs for assessing the effectiveness of our docking protocols. Accordingly, we carried out docking of these 15 compounds (designated Group B ligands, Figure 3.3, Table 3.2) into the human ERα-LBD to determine the extent to which computationally predicted affinities agreed with experimentally determined binding potencies.

A preliminary version of this work was presented at the 57th annual meeting of the Society of Toxicology [30].

Materials and Methods

Receptor preparation with YASARA

The crystal structure of the human ERα-LBD (Y537S) in complex with 17β-estradiol (PDB 3UUD) [31] was downloaded and prepared for docking with the YASARA molecular modelling suite (YASARA-Structure version 17.4.17 for Windows) [17]. This structure was selected as a template for creating homology models of mouse and rat ERα-LBD via *in silico* mutagenesis due to its high resolution (1.6 Å) and the presence of the Y537S mutated residue, which stabilizes the agonist-binding conformation of the protein without compromising the overall structure or agonist ligand-binding properties of the protein [31]. The Y537S residue in the human ERα-LBD template receptor was retained for the mouse and rat structures created via *in silico* mutagenesis. All crystallographic waters were removed, hydrogen atoms were added, bond orders were corrected for S-hydroxycysteine residues, missing loops were repaired [32], and only chain-A in complex with its 17ß-estradiol ligand (**E2**) was retained. Differing residues between protein sequences for human [33], mouse [34], and rat [35] ERα-LBD were identified by protein sequence alignments (Figure 3.1) performed with Geneious bioinformatics software (version 11.1.5 for Windows) [36, 37]. These residues in the human 3D structure were then mutated *in silico* using YASARA to create separate mouse and rat ERα-LBD receptors. The residue mutations for mouse were L306P, I326M, L327I, T334S, V368G, T371N, Q502R, and S527N. The residue mutations for rat were L306P, I326L, L327I, T334S, V368G, T371N, T483N, Q502R, and S527N. Side-chains of mutated residues were optimized with YASARA using the SCWALL method, which combines semi-empirical quantum mechanics, rotamer library, and steepest-descent algorithms [38].

For the human and rodent ERα-LBD receptors created via *in silico* mutagenesis, a cubic simulation cell was set to automatically encompass the entire receptor in YASARA plus an additional 2.5 Å margin in the x, y, and z directions. With the **E2** ligand in the active site, each prepared receptor was separately subjected to energy minimization using the YASARA2 force field, which is based on AMBER14 with the addition of knowledge-based dihedral and interaction potentials [38]. The energy-minimized structure was subjected to a 500 ps molecular dynamics refinement in explicit water solvent using YASARA and the YASARA2 force field [39]. Each receptor and refinement step were analyzed for structural errors and scored using MolProbity [40, 41]. Based on their MolProbity scores, the best ERα-LBD receptor for human, mouse, and rat was chosen for docking comparisons among all three receptor species.

Receptor preparation with UCSF Chimera

All crystallographic waters were removed, hydrogen atoms were added, and missing loops were repaired with UCSF Chimera (version 1.11 for Windows) [42] as a graphical user interface for MODELLER using default settings. Only chain-A with its native **E2** ligand was retained for further refinement. Differing residues between human, mouse, and rat ERα-LBD were identified as described in the previous section. Residue differences observed in either mouse or rat ERα-LBD were swapped using the Dunbrack rotamer library via UCSF Chimera to create separate receptors using the prepared human ERα-LBD structure as a template. All observed clashes among swapped residues were optimized by subjecting them to energy minimization in UCSF Chimera using default settings for each ERα-LBD receptor. Energy minimization of the entire receptor was performed for comparison using 100 steepest descent minimization steps and 10 conjugate gradient steps. Molecular dynamics refinement in explicit

solvent was performed with the YASARA molecular modeling suite with default settings using a 500 ps simulation as described in the previous section. Each receptor and refinement step were analyzed for structural errors and scored using MolProbity [40, 41].

Receptor preparation with I-TASSER

The protein sequence for mouse [34] and rat [35] ERα were uploaded to the I-TASSER online server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) for receptor assembly by iterative threading using default parameters [43]. I-TASSER does not produce a receptor with a ligand bound to it. Therefore, a cubic simulation cell was fitted around the entire mouse or rat ERα-LBD structure produced by I-TASSER plus an additional 5 Å margin in the x, y, and z directions. A docking simulation was performed with AutoDock Vina [44] for 100 runs using default parameters, and setup was conducted with YASARA as the graphical front-end [45] using the **E2** ligand extracted from the crystal structure of human ERα-LBD (PDB 3UUD). The top pose for each docking run in the rodent receptors were selected for further structural refinement via energy minimization using the YASARA2 force field as described above. The energy-minimized structure was subjected to 500 ps molecular dynamics refinement in explicit solvent using YASARA with the YASARA2 force field as described above [39]. Each receptor and refinement step were analyzed for structural errors and scored using MolProbity [40, 41].

Selection and preparation of Group A and Group B ligands

Group A ligands are shown in Figure 3.2. These 23 compounds included **E2**, **DES**, and 21 paraben analogs. Many of the parabens in this group have been previously characterized *in vitro* in estrogen-dependent breast cancer cell lines or reporter assays, which have demonstrated

relatively weak estrogenic behavior for these compounds [3, 5, 22, 28]. We also included (*R*)- and (*S*)-3-hydroxybutyl 4-hydroxybenzoate (**3OHR** and **3OHS**) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate (**2OH**) [46], metabolites of *n*-butylparaben (**BuP**) and *iso*-butylparaben (**iBuP**), which we recently characterized *in vitro* as weak estrogenic compounds in human ERα-expressing breast cancer cell lines [47].

Group B ligands are shown in Figure 3.3. These 17 compounds consisted of **BuP** and **E2** along with 15 parabens containing various substituents in the benzene ring [29].

Initial structures for Group A and Group B ligands were generated using ChemDraw Professional version 17.1.0.105 for Windows and saved as CDX structure files. Each CDX file was imported into Chem3D Ultra version 17.1.0.105 for Windows and energy-minimized using the MMFF94 functionality in Chem3D Ultra to ensure that the structures had the correct orders, lengths, and angles for all bonds. The minimized structures were saved as PDB files for use in the docking simulations.

Ligand PDB files were converted to SDF files using OpenBabel version 2.4.1 64-bit for Windows [48, 49] for calculation of pKa and solvent-accessible surface area (SASA) using SimulationsPlus ADMET_Predictor version 9.0.0.10 64-bit for Windows [50]. These values are listed in Table 3.1 (Group A ligands) and Table 3.2 (Group B ligands).

Docking simulations

Docking was performed with AutoDock Vina [44] using default parameters, and setup was conducted with YASARA as the graphical front-end [45, 51]. A cubic simulation cell (25 Å × 25 Å × 25 Å) was centered on the C9 carbon of **E2** before removing the native ligand from each receptor and set as the search space for all test compounds, which were treated as flexible

ligands. The best pose of 25 runs was selected based on the most favorable free energy of binding (ΔG) among poses in which the 4-hydroxy group of the ligand benzene ring was oriented toward the R394 and E353 residues in the receptor binding site for potential hydrogen-bond formation, as seen in crystal structures of **PrP**, **BuP**, and **E2** [31, 52, 53]. Hydrogen-bond networks of the docked structures were optimized with YASARA [54], and protein structures of docking complexes were aligned with MUSTANG [55] before determining interspecies root-mean-square deviation (RMSD) values for docked ligands. Throughout this paper, ligand RMSDs are reported as heavy-atom (all atoms except hydrogen) values that have been symmetry-corrected to reduce false negative docking results [56]. Protein RMSDs are reported as CA backbone values [57, 58].

Because ΔG for docking tends to be biased in favor of larger molecules [59], we have expressed potency derived from docking in terms of ligand efficiency (LE). Thus, $LE_{dock} = -\Delta G/N_h$, where N_h = the number of heavy (non-hydrogen) atoms in the ligand) [60, 61].

In order to compare LE_{dock} values with experimental data, we calculated experimental ligand efficiencies using the general relationship, $LE_{exp} = p(Activity)/N_h$, where LE_{exp} = experimental ligand efficiency, p(Activity) = -log(Activity), and N_h = the number of heavy (non-hydrogen) atoms in the ligand. This comparison requires self-consistent sets of experimental data obtained with a given method under the same assay conditions [60]. For Group A ligands, experimental activity data meeting these criteria were available for 14 of the 23 compounds in terms of EC20 values for transcriptional activation in an estrogen response element (ERE) luciferase reporter assay for human ERα [28]. For Group B ligands, experimental activity data were available for all 17 compounds (15 ring-substituted paraben analogs along with **E2** and **BuP**) as IC50 values for binding to the human ERα receptor [29].

Receptor screening and assessment of unknown rodent structures

Prepared mouse and rat ERα-LBD receptors created with YASARA [17], UCSF Chimera [42], and I-TASSER [43] were scored and compared using MolProbity (Tables 3.4 and 3.5). Side-chain optimization and energy minimization of the entire ERα-LBD structure in YASARA produced the highest-scoring receptors with the fewest structural errors for ERα-LBD among all three receptor preparation methods. The highest-scoring rodent receptors prepared with YASARA were selected to determine ligand-binding similarity among human and rodent ERα-LBD and to carry out the remainder of the docking simulations conducted in this study.

Structural similarity of the highest scoring ERα-LBD receptors were determined by the template-modeling score (TM-score) [58]. The TM-align web server (https://zhanglab.ccmb.med.umich.edu/TM-align/) was used to perform a structural alignment and generate TM-scores among the three species of ERα-LBD used for calibration. The TM-scores and receptor CA backbone RMSDs between human and mouse (TM-score: 0.99; RMSD: 0.16 Å) or rat (TM-score: 0.99; RMSD: 0.17 Å) were determined. The TM-score between mouse and rat (TM-score: 0.99; RMSD: 0.03 Å) was also calculated. A TM-score of 1.00 indicates a perfect match [58], and a CA backbone RMSD < 1 Å is within the generally accepted range for equivalent protein structures [62]. Thus, our three prepared *in silico* ERα-LBD models displayed a high degree of structural similarity.

The docking results for **E2** were subjected to 3D protein alignment via MUSTANG in YASARA for human and mouse (Figure 3.4A) or human and rat (Figure 3.4B) ERα-LBD, which indicated a docking preference for the known active site of human ERα. Further receptor calibration was carried out by aligning the proteins associated with the **DES** docking poses in

YASARA with MUSTANG for human and mouse (Figure 3.4C) or human and rat (Figure 3.4D) ERα-LBD. Protein-aligned mouse and rat docking poses for **E2** (Figure 3.4E) or **DES** (Figure 3.4F) are likewise shown. Hydrogen-bonding interactions of **E2** and **DES** with key side-chain residues in the ERα-LBD receptors are shown in Figure 3.4. The ligand RMSD values for the aligned docking poses of **E2** and **DES** in Figure 3.4 are summarized in Table 3.3. Aligned mouse and rat ERα-LBD poses for docked **E2** and **DES** were determined to have ligand RMSD values of 0.18 Å and 0.05 Å, respectively. Given that ligand RMSD values of 2.0 to 2.5 A are the traditional cutoff range for equivalent poses [63, 64], the extremely small values we obtained for **E2** and **DES** to calibrate the prepared receptors indicate that our *in silico* mouse and rat ERα-LBD models produced virtually identical docking results among the human and rodent ERα-LBD structures. These calibrated structures were therefore selected for further analysis of ligand binding similarity.

Statistical analyses

GraphPad Prism 7.04 for Windows was used to create correlation and pyramid plots of ligand-receptor residue or atom contacts obtained from the docking results; it was also used to determine Pearson (r) or Spearman (r_s) correlation coefficients (GraphPad Software, La Jolla California USA, www.graphpad.com). The Pearson r was used for normally distributed data, and the Spearman r_s was used for non-normally distributed data as determined by the D'Agostino & Pearson and Shapiro-Wilk normality tests in Prism. Data sets were treated as non-normally distributed if they failed to pass one or both normality tests (alpha = 0.05). Summary statistics of non-normally distributed data are presented as median values with 95% confidence intervals (CI). Correlations of LE_{exp} vs. LE_{dock} were obtained using Pearson partial correlation (r_p) computed with OriginPro 2018b 64-bit for Windows to correct for the N_h covariate (OriginLab,

Northampton, Massachusetts USA, www.originlab.com). The Sorensen similarity coefficient (Sc, expressed as a percentage; also known as the Dice or Sorensen-Dice coefficient) for each docking comparison of ligand-receptor atom or residue contacts was obtained with the following equation: $Sc = [2(N_{AB})/(N_A + N_B)] \times 100]$, where N_{AB} = number of contacts for both species, N_A = number of contacts for species A, and N_B = number of contacts for species B [65, 66]. Sc was calculated using the Python "distance" package version 0.1.3 in Anaconda Python 3.6.5 for 64-bit Linux.

Results

Group A ligand RMSD values indicate overall agreement of docking poses between species

Upon calibration of the prepared ERα-LBD structures shown in Figure 3.4, we used the Group A ligands shown in Figure 3.2 and Table 3.1 to determine ligand-receptor binding similarities among the refined human structure and *in silico* rodent models of the ERα-LBD.

Our first comparison was an examination of the interspecies ligand RMSD values for each Group A compound (Table 3.3). Overall group RMSD values (median, 95% CI, $n = 23$) were 0.49 (0.21-1.82) Å (human-mouse), 1.19 (0.22-1.83) Å (human-rat), and 0.18 (0.12-0.34) Å (mouse-rat). By definition, RMSD (human-human) = 0.00 Å. Because the generally accepted RMSD range for equivalent docking poses is 2.0-2.5 Å [63, 64], the small values we obtained indicate excellent overall agreement in docking members of this set of ligands into human, mouse, and rat ERα-LBD receptors. In general, the calibration ligands, **DES** and **E2,** showed the most favorable ligand RMSD values among the compounds tested. Among the paraben compounds, *n*-pentyl through *n*-nonyl and *iso*-alkyl analogs generally displayed more favorable RMSD values than those with shorter or longer *n*-alkyl chains. This optimal behavior is likely due to a combination of factors, including an overall reduction and constriction of possible

translational motion of the relatively larger compounds within the active site, as well as their ability to fully occupy the available space in this binding pocket in a manner similar to endogenous ERα agonists such as **E2**. Experimental data on relative estrogenic potency of paraben analogs also suggest that optimal activity reflects an ideal juxtaposition of molecular size and hydrophobicity [28].

An example of a relatively poor docking result is shown with *n*-butylparaben (**BuP**) docked to human ERα-LBD and aligned with the top docking pose for mouse or rat (Figure 3.5A, 3.5B). The rather large ligand RMSD values for the top docking poses of **BuP** in Figure 3.5 indicate comparatively incongruent ligand alignment in human vs. rodent ERα-LBDs.

In contrast, the top docking pose for *iso*-butylparaben (**iBuP**), a structural isomer of **BuP**, was found to display a nearly perfect alignment between human vs. mouse or rat receptors as evidenced by visual inspection (Figure 3.5C, 3.5D) and by the ligand RMSDs (Table 3.3). The alignment for **iBuP** docked to mouse vs. rat was also found to have an exceptional ligand RMSD as likewise observed in the case of **BuP** docked to mouse vs. rat receptors (Figure 3.5E, 3.5F, Table 3.3).

Numbers of interspecies ligand-receptor contacts are highly correlated

Our next comparison was an exploration of the degree of interspecies correlations of the numbers of residue and atom receptor contacts for each compound in Group A. The numbers of ligand-receptor residue or atom contacts between human and mouse ERα-LBD among all Group A ligands tested were found to have Pearson $r = 0.913$ and 0.978, respectively (Figure 3.6A, 3.6B). Even higher correlations were obtained with the numbers of ligand-receptor receptor or atom contacts between human and rat ERα-LBD ($r = 0.925$ and 0.986, respectively,

Figure 3.6C, 3.6D). Finally, the numbers of ligand-receptor residue or atom contacts between mouse and rat ERα-LBD gave the highest correlations of all (r = 0.945 and 0.990, respectively, Figure 3.6E, 3.6F). The extraordinary correspondence between the numbers of ligand-receptor interactions in mouse and rat ERα-LBDs agrees with the high level of sequence identity between the rodent species (Figure 3.1). Overall, with respect to the numbers of residue or atom ligand-receptor contacts, the mouse and rat ERα-LBDs were found to interact with the series of paraben analogs and known ERα agonists from Group A ligands in a manner highly similar to that of the human ERα-LBD.

Interspecies ligand-receptor contacts arising from specific residues are highly similar

To assess how similarly Group A compounds interacted with specific residues within the active site of ERα, we calculated the interspecies Sorensen similarity coefficients (Sc) for the numbers of residue contacts for each compound docked into human, rat, or mouse ERα-LBD (Table 3.3). Rather than simply correlating the number of ligand-receptor residue contacts for a given ligand between two receptors, the Sc takes into account the specific residues in each receptor making contacts with the ligand. This analysis yielded an interspecies Sc value for each compound. As can be seen in Table 3.3, low RMSD values for a given compound tended to be reflected by correspondingly high Sc values.

The group Sc coefficients (median, 95% CI) for all residue contacts among all Group A ligands for each pair of species were 96.8 (90.0-100)% (human-mouse), 97.7 (89.5-100)% (human-rat), and 100 (97.8-100)% (mouse-rat). These Sc values indicate an overall high degree of similarity between human and rodent ERα-LBDs as well as between mouse and rat

ERα-LBDs with respect to the numbers of residue contacts between ligands and receptors arising from specific residues.

In order to gain a clearer picture of interspecies similarity of ligand-receptor atom contacts by residue for all Group A compounds, we displayed the data in the form of "pyramid plots", in like manner to the classic "population pyramids" used to visually categorize demographic information by comparing the numbers of people in different age groups by gender [67]. Here, we replaced age groups with protein residue sequence numbers, and we replaced genders with species. As can be seen in Figure 3.7, the remarkable interspecies similarity in ligand-receptor atom contacts by residue is readily apparent from the symmetry of the pyramid plots. Moreover, on a quantitative basis, Sc values were 96.9% (human-mouse), 93.5% (human-rat), and 96.9% (mouse-rat), and r_s values were 0.977 (human-mouse), 0.966 (human-rat), and 0.991 (mouse-rat). These plots were also useful for assessing the relative prevalence of ligand-receptor interactions in a set of ligands. For example, among Group A ligands, there were highly frequent hydrophobic interactions with L346, L387, and F404 and less frequent hydrogen bonding interactions with R394, E353, and H524.

Overall, the Sc values for numbers of residue contacts along with the pyramid plots and their associated Sc values and r_s coefficients demonstrate that the Group A ligands give rise to sets of specific active site contacts that are highly similar and consistent across human and rodent ERα-LBDs.

Group A LE_{dock} values are highly correlated between species

Interspecies correlation plots of LE_{dock} values for Group A ligands are shown in Figure 3.8A,B (human-rodent) and Figure 3.8E (mouse-rat). Each plot also shows the partial Pearson

correlation coefficients (r_p) to determine the degree of correlation corrected for the covariate, N_h (number of heavy atoms). These values were 0.958 (human-mouse), 0.981 (human-rat), and 0.960 (mouse-rat), demonstrating excellent interspecies agreement in the predicted strength of ligand interactions with the ERα-LBD receptors.

It is also noteworthy that the three longest-chain parabens, **DeP**, **UnDeP**, and **DoDeP**, were clustered at the low end of the potency scale, below LE_{dock} values of 0.35 kcal/mol/N_h. This result agrees with published *in vitro* data showing that these three compounds exhibited little or no estrogenic activity as assessed by transcriptional activation in an estrogen response element (ERE) luciferase reporter assay [28]. However, the LE_{dock} scores for 4OH and MeP calculated from our docking results were near the top end of the scale, whereas these compounds were negative in the ERE assay. Explanations of the anomalous results for these low molecular weight compounds are provided below in the Discussion section.

Group B LE_{dock} values are highly correlated between species and decreased by ring substitution

Interspecies correlation plots of LE_{dock} values for Group B ligands are shown in Figure 3.8C,D (human-rodent) and Figure 3.8F (mouse-rat). In these cases, values for **OcP** from Group A ligands were included along with values for **BuP** that were already part of Group B in order to enable direct assessment of the effect of ring substitutions on predicted ligand binding of both *n*-butyl- and *n*-octylparabens. Values of r_p were 0.957 (human-mouse), 0.964 (human-rat), and 0.990 (mouse-rat), indicating strong interspecies correlations.

Moreover, it was readily apparent from inspection of the plots in Figure 3.8C,D and 3.8F that the predicted efficiencies of ligand binding of all ring-substituted *n*-butyl- and *n*-

octylparabens (**2a** through **2k**) were less than that of **BuP**. Furthermore, the LE_{dock} values of all of the ring-substituted *n*-octylparabens (**3e** through **3k**) were less than that of **OcP**. Thus, molecular docking predicts that adding ring substituents to *n*-butyl- or *n*-octylparaben as shown in Figure 3.3 will decrease the avidity of binding of these ligands to human, mouse, and rat ERα-LBD receptors.

Human LE_{dock} and LE_{exp} values display good agreement for both Group A and Group B ligands

Figure 3.9 shows correlation plots for human LE_{exp} vs. LE_{dock} for Group A and Group B ligands. Self-consistent experimental data sets were available for 14 of the 23 ligands in Group A [28] and all 17 of the ligands in Group B [29]. The r_p values were 0.894 for Group A and 0.918 for Group B, indicating good agreement between ligand efficiencies computed from molecular docking and those derived from experimental data. In addition, Figure 3.9B shows that both LE_{dock} and LE_{exp} values for all of the ring-substituted parabens fall below these values for **BuP**, which has no substituents in its benzene ring.

Discussion

The identification and characterization of suspected endocrine disrupting compounds (EDCs) using both *in vitro* and *in vivo* methods has been considered one of the major fields of toxicology research for several decades [68]. Parabens represent one category of suspected EDCs that have been investigated for their potential action on ERα and related hormone signaling pathways [28, 47]. Although paraben compounds are generally considered weak agonists of ERα, they are still being investigated to determine whether current exposures may lead to

adverse impacts on human health [24, 27, 69]. For example, an epidemiological study reported a possible association between increased **BuP** exposure and markers of sperm DNA damage in men [70]. Another study showed a possible dose-response relationship between higher paraben exposure and shorter self-reported menstrual cycle length among female Japanese university students [69]. In addition, the detection of parabens in numerous human tissues [27, 71-77] and their associations with possible endocrine disruption in humans further demonstrate the toxicological relevance of these compounds as test ligands in our study. These findings also highlight the need for the further development of *in vitro* and *in silico* screening methods for recognizing and categorizing EDCs.

Among computational approaches, there have been other reports on molecular docking of parabens. While these studies differed from our investigation in several respects (e.g., fewer paraben compounds and/or targets other than ERα), they also provided an important degree of corroboration and additional insight into our results. For example, in a study of five *n*-alkyl parabens [78], docking of **BuP** into the human ERα-LBD was shown to have a comparatively high RMSD from the crystal structure owing to the relatively unconstrained *n*-butyl group being able to adopt multiple conformations, as noted in our present work. In other reports [79, 80], **MeP**, **EtP**, and **BzP** were found to exert estrogenic effects in a uterotrophic assay in rats, and docking was used to show that these compounds adopted apparent bioactive conformations in the human ERα-LBD, similar to our findings. In a study of interactions of parabens with the human androgen receptor [25], docking scores for *n*-alkyl parabens were found to be inversely correlated with chain length, as found in our study with estrogen receptors. Moreover, dividing their $-\Delta G$ values by N_h to yield LE_{dock} scores resulted in changing their relative ranking of **4OH**, **PhP**, and **BzP** from 7, 2, and 1 to 1, 4, and 6, compared to our relative ranking of the same nine

compounds as 1, 5, and 6, respectively. Finally, a docking study has been conducted on the inverse antagonistic activity of five parabens against the estrogen-related receptor gamma (ERR-γ) [26], which has recently been shown to function as a tumor suppressor in gastric cancer [81]. The binding pocket of ERR-γ is similar to that of ERα, and parabens were found to dock in the known agonist site in like manner to our docking poses of parabens in the ERα-LBD structures.

Although ERß might have been examined in the present study, ERα would be expected to contribute to a greater proportion of effects observed by binding of an ER agonist. For example, only the expression of ERα, and not ERß, is currently used to make clinical decisions regarding ER-positive breast cancer primarily due to the relatively poor understanding of the role of ERß within this tumor subtype [82]. Further study is needed to determine the extent to which structural differences or differential expression of these estrogen receptor subtypes might elicit an estrogenic response from exposures to exogenous ER agonists.

The data presented in the present report demonstrate that our *in silico* mutagenesis modeling of mouse and rat ERα-LBD was effective in producing structures with minimal errors and show that paraben analogs, **E2**, and **DES** interact similarly with the human receptor relative to rodent receptors. We have also shown that our *in silico* receptor structures are capable of recognizing a series of weak ERα agonists and producing docking results that agree with *in vitro* data regarding their ability to bind to ERα and modulate estrogen signaling pathways [3, 5, 28, 47, 53, 83]. Furthermore, our docking results indicate that rodent and human ERα would likely experience similar LBD interactions with ERα agonists. Accordingly, the sequence differences in the rodent LBDs could be considered neutral substitutions. Additionally, our *in silico* models were shown to be effective in corroborating previously reported relative potencies of

unsubstituted parabens [28] as well as ring-substituted parabens [29]. These findings support the validity of using *in vitro* and *in vivo* rodent models to characterize suspected human ERα agonists and highlight the valuable alternative methodology of pre-screening and identifying these compounds *in silico* prior to conducting *in vivo* studies. Lastly, the use of the Sorensen coefficient generated useful quantitative measures for assessing the similarity of ligand interactions with different species of receptors, and our adaptation of pyramid plots provides a graphical method for assessing the similarity or dissimilarity of ligand-receptor interactions between species.

Nevertheless, there were some apparent anomalous findings among our results. In an experimental study comparing estrogenic activities of paraben analogs [28], MeP and the common paraben metabolite, **4OH**, failed to elicit an estrogenic response, yet we obtained a positive docking result with this compound in the three species of ERα-LBD. The negative experimental result with **4OH** is not surprising, given the fact that parabens are neutral esters, whereas **4OH** is a carboxylic acid with an experimental pKa of 4.54 [84] and calculated pKa of 4.01 (Table 3.1). Therefore, this compound would be ionized at a physiological pH of 7.4. Because of its negative charge, **4OH** would be expected to encounter difficulty gaining access to the hydrophobic interior of the ERα; however, when docked in the active site, hydrogen bonds to the phenol group and hydrophobic interactions with the benzene ring would serve to stabilize the complex [78].

As shown in Figure 3.10A, the mean LE_{dock} values obtained from the docking results of all three species of ERα-LBD displayed a strong negative correlation with the solvent-accessible surface area (SASA) of the Group A parabens analogs, including the metabolite, **4OH**. In this plot, **4OH** aligns with the paraben analogs as the compound with the highest LE_{dock} value and the

lowest SASA. At the same time, a plot of mean LE_{dock} vs. pKa (Figure 3.10B) shows that **4OH** clearly stands apart from the paraben analogs, which span the full range of LE_{dock} values with little change in pKa. However, it is important to note that AutoDock Vina does not make use of partial atomic charges [85], and the same result was obtained whether **4OH** was docked as a neutral molecule or as an ionized species (data not shown).

In comparison, Figure 3.10C shows a negative correlation between the mean LE_{dock} values of the Group B paraben analogs with SASA, similar to what was observed with the Group A ligands. Note that in Figure 3.10D, there is no statistically significant correlation between mean LE_{dock} values and pKa, yet this representation and the data in Table 3.2 show that eight of the 17 compounds would be ionized to some extent at physiological pH. In the case of Group B compounds, the ionized group would be a phenolic oxygen. Here again, whereas docking scores were inversely related to molecular size, they were indifferent to the potential of a given compound to ionize.

Moreover, the discrepant relative ranking of **4OH** potency came about from the simple calculation of dividing $-\Delta G$ by the number of heavy atoms in the molecule (N_h) to generate LE_{dock} values. The motivation for this calculation arises from the fact that docking scores tend to favor larger molecules, and the LE metric provides an expedient way to correct this bias [60, 61]. If the Group A ligands were ranked by $-\Delta G$ rather than LE_{dock}, **4OH** would move from first to last place. Nevertheless, regardless of its relative ranking, **4OH** would retain a docking score. Therefore, the compound would not be deemed completely inactive, in keeping with its weak estrogenic activity in mouse bioassays [86] and human breast cancer cell lines [87]. Moreover, the general notion of **4OH** as a ligand in complex with proteins should not be surprising, given that it has been docked into human cyclooxygenase-2 (COX-2) [88], despite negative results in a

COX-2 dependent human smooth muscle cell assay [89]. Furthermore, **4OH** is found as a bound ligand in a variety of enzymes, including carbonic anhydrase [90], *p*-hydroxybenzoate hydroxylase [91], and 4-hydroxybenzoate octaprenyltransferase [92]. Lastly, **4OH** was found to have intermediate antagonistic activity among paraben analogs against the human androgen receptor [25], and when its reported docking score was converted to an LE_{dock} value, its relative rank increased from 7th to 1st place out of nine compounds, similar to the results we obtained in the present study.

Although LE values (LE_{dock} and/or LE_{exp}) should be used and interpreted with due caution [93, 94], their validity and utility have been well established and widely accepted [60, 61, 95]. Nevertheless, when LE values are employed to select optimally binding ligands for a given receptor, it is important to recognize that, in general, LE is not a linear function of N_h [59]. In particular, based on compilations from large databases of ligand-receptor complexes, LE values decrease markedly within an N_h range of 10 to 20 [94]. Thus, the use of LE values for compound selection can be problematic, especially for small molecules such as **4OH** and paraben analogs with relatively short alkyl chains, such as **MeP**. The problem of smaller ligands having disproportionately large LE values has given rise to a variety of compensatory methods with varying degrees of success [96]. The more effective methods for correcting LE according to molecular size depend on deriving parameters from curve-fitting plots of LE vs. Nh. Obtaining meaningful values for such parameters would require much larger data sets than the ones described in the present study. Moreover, it must also be borne in mind that the main purpose of our investigation was not to determine the receptor-binding capabilities of individual parabens or to assess their potential human health impacts, but to use these compounds as tools to evaluate the similarity of the human ERα-LBD structure to our homology models of mouse and rat ERα-

LBDs. In this way, our use of LE correlations along with those of other variables helped to demonstrate the strong structural and implied functional correspondence between human and rodent estrogen receptors.

In summary, we have demonstrated that *in silico* mutagenesis of a template receptor with high sequence similarity to a target receptor of unknown tertiary structure is an acceptable method that produces models with minimal structural errors and highly similar docking interactions to those observed in the template. In addition, we have made a novel application of the Sorensen similarity coefficient (Sc) to compare LBD interactions of docking targets. Our data suggest that the Sc could adopted as an additional screening metric with potential applications in toxicology as well as drug discovery and development. In addition, our adaptation of pyramid plots affords a new and useful graphical method for visualizing similarities in docking results. Whereas LE values can provide a basis for useful correlations, they should be interpreted with caution, especially for low molecular weight ligands. Future studies investigating suspected environmental ERα agonists should consider screening such compounds using *in silico* methodologies, akin to those reported here, prior to conducting *in vivo* studies.

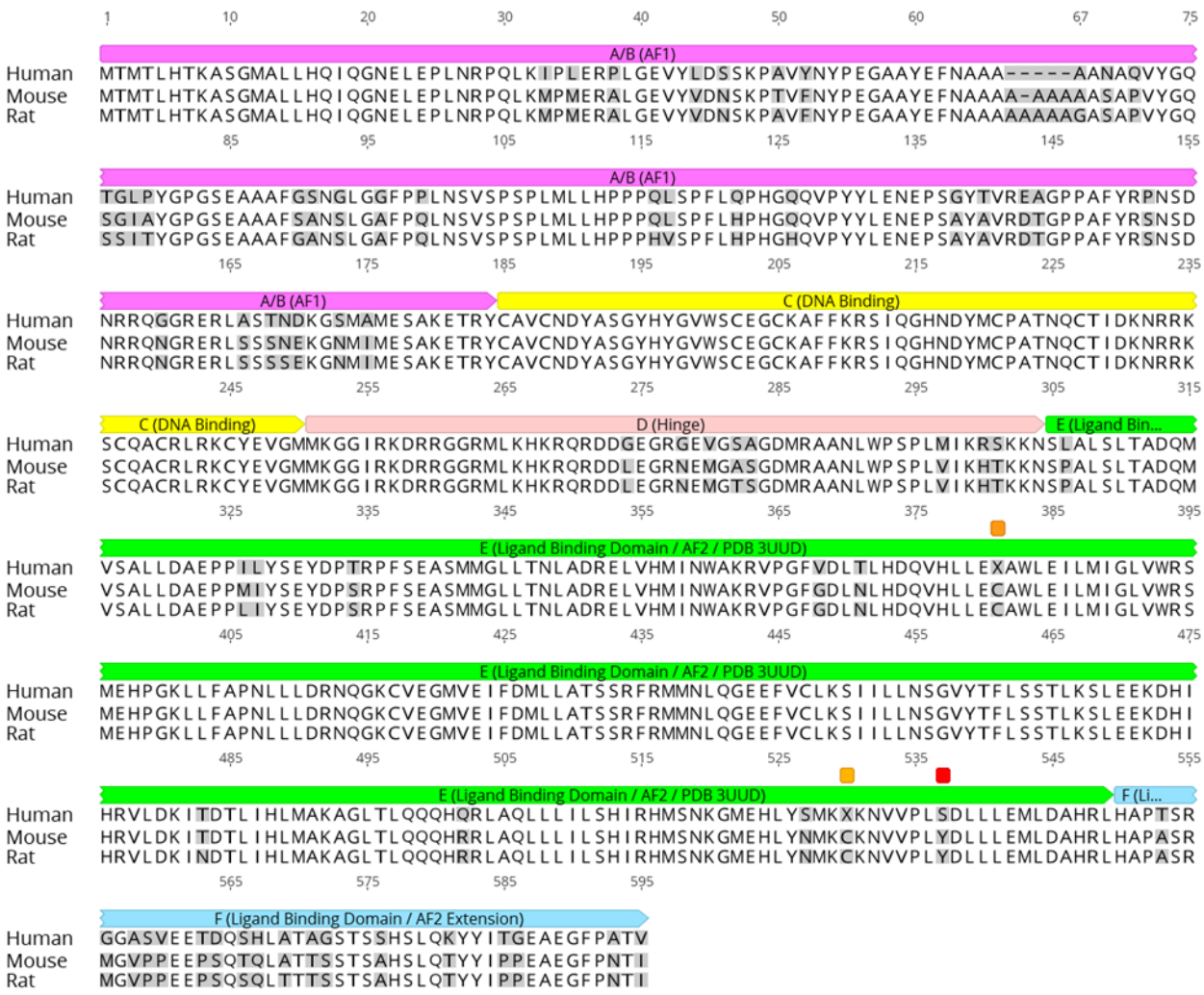
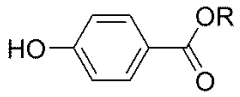
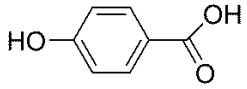


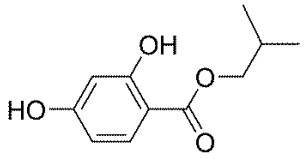
Figure 3.1. Multiple protein sequence alignment of human, mouse, and rat ERα. Residues differing from the human in any sequence are shaded gray. Domains: A/B (AF1) = magenta; C (DNA binding) = yellow; D (Hinge region) = light red; E (Ligand Binding Domain /AF2; region encompassed by PDB 3UUD) = green; F (C-terminal extension of Ligand Binding Domain/AF2) = light blue. The "X" residues 381 and 530 marked with orange annotations in the human sequence are hydroxyCys that were kept as Cys in the mouse and rat homology models. S537 marked with a red annotation in the human sequence is the Y537S mutation that was introduced to stabilize the agonist conformation in PDB 3UUD; the mouse and rat homology models were likewise mutated to serine residues at this site. Sequences were downloaded from Uniprot (www.uniprot.com). Alignment was carried out by Geneious 11.1.5 (Geneious, 2018; Kearse *et al*., 2012) using the Clustal-Omega algorithm. Domain assignments were adapted from Uniprot (2018a-c) and Sanchez *et al*. (2002).



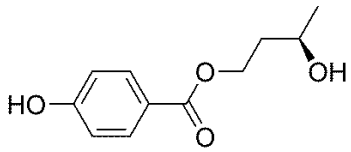
R-paraben (RP)



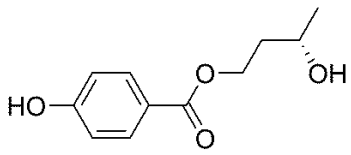
***p*-hydroxybenzoic acid (4OH)**



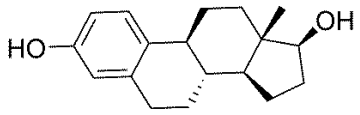
isobutyl 2,4-dihydroxybenzoate (2OH)



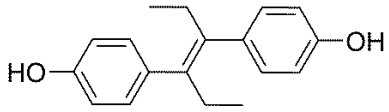
(*R*)-3-hydroxybutyl 4-hydroxybenzoate (3OHR)



(*S*)-3-hydroxybutyl 4-hydroxybenzoate (3OHS)



17β-estradiol (E2)



diethylstilbestrol (DES)

Figure 3.2. Structures of Group A ligands used for docking studies. R-groups for parabens are listed in Table 3.1.

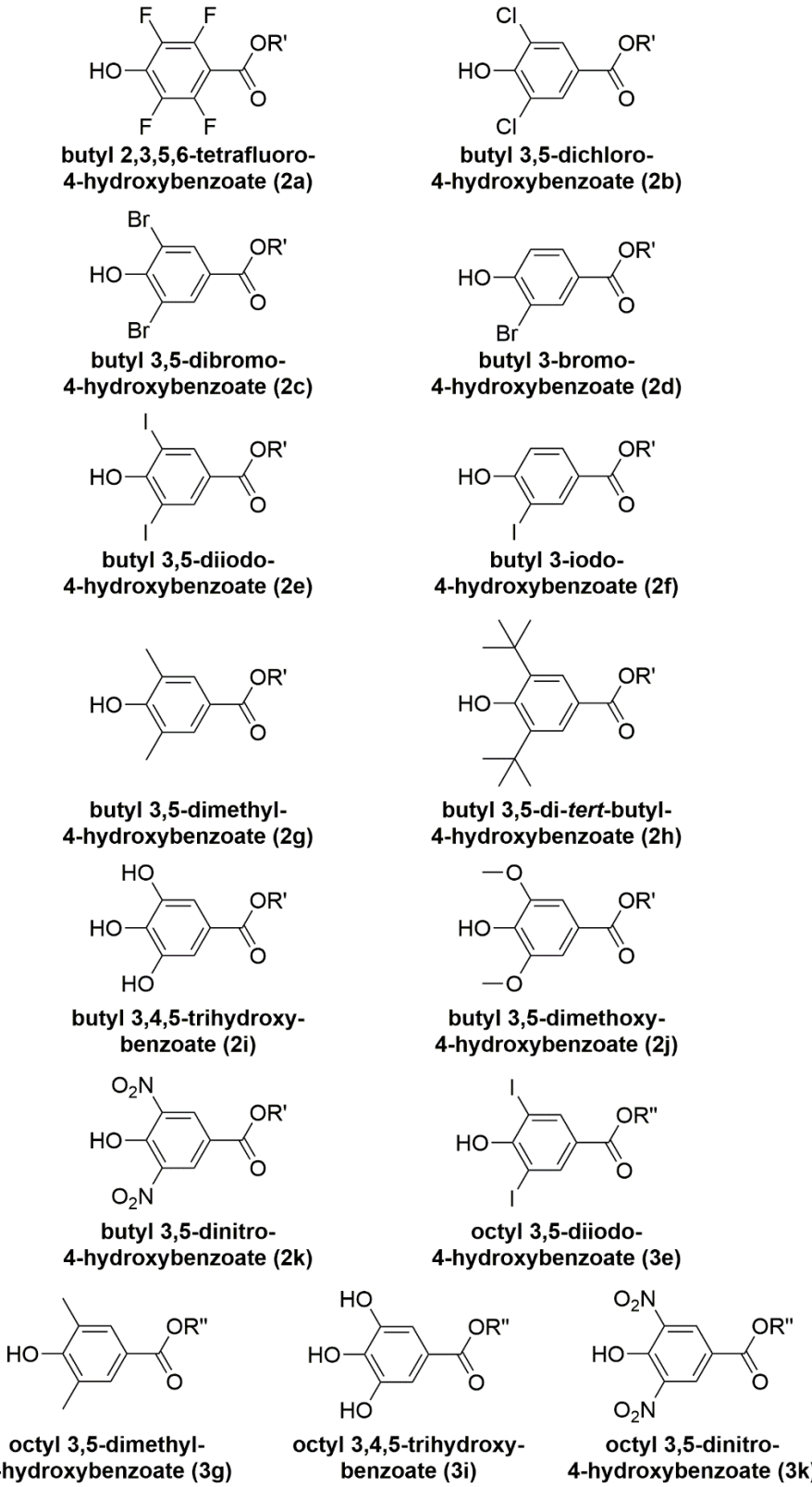


Figure 3.3. Structures of Group B ligands used for docking studies. Compounds are parabens with various substituents in the benzene ring. Compound designations are the same as in (Bergquist *et al*., 2018) as listed in Table 3.2. R′ = butyl; R″ = octyl.

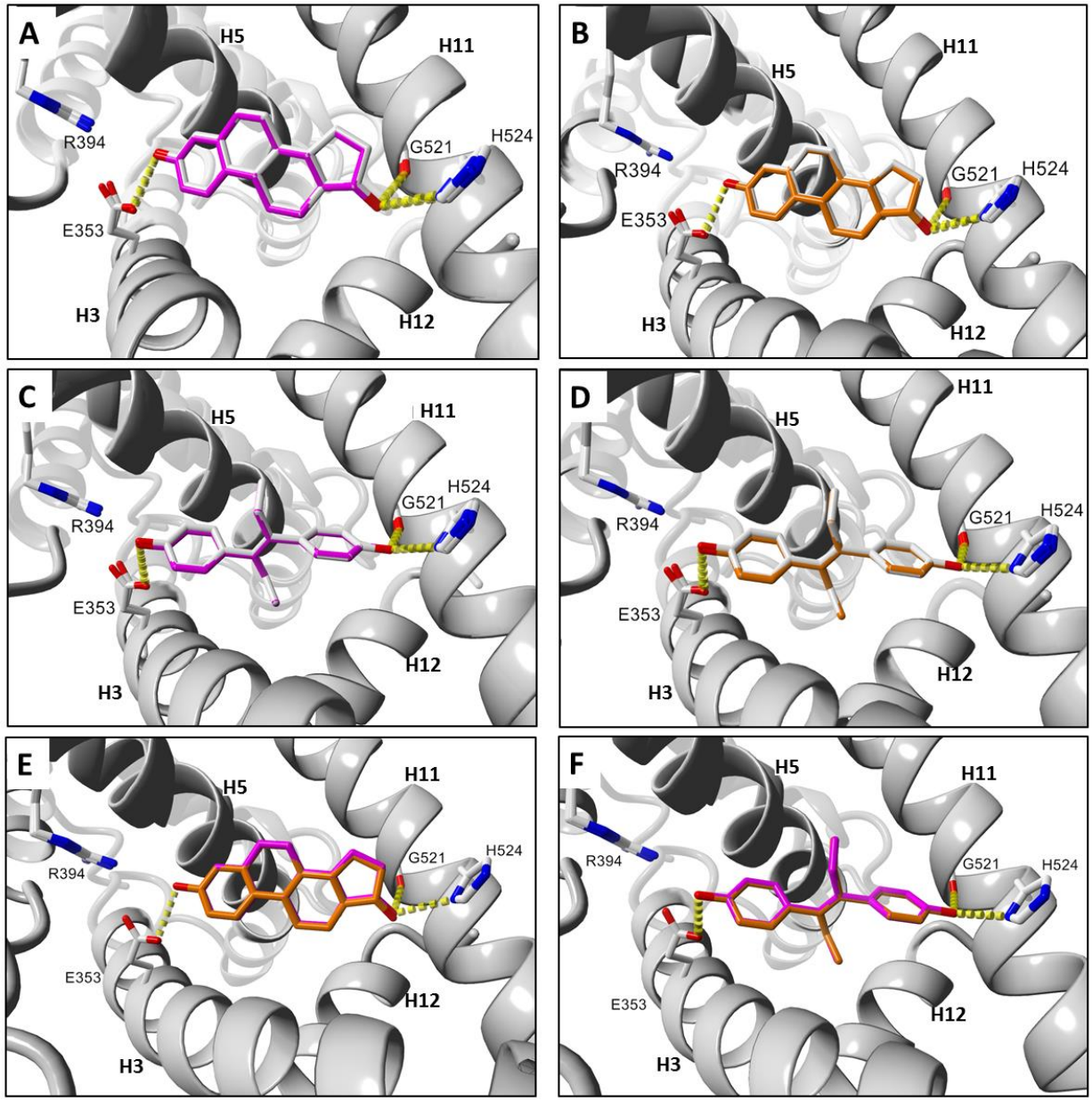


Figure 3.4. Docking comparisons of known ERα agonist ligands in human and rodent ERα-LBD receptors. (A) E2 human and mouse. (**B**) **E2** human and rat. (**C**) **DES** human and mouse. (**D**) **DES** human and rat. (**E**) **E2** docked into mouse vs. rat. (**F**) **DES** docked into mouse vs. rat. Ligand colors: gray = human, magenta = mouse, orange = rat. Hydrogen bonds are represented as yellow dashes. Oxygen and nitrogen atoms are colored red and blue, respectively. Active site helix labels (**H5**, **H11**, and **H12**) are displayed in bold face. Hydrogen-bonding residues are displayed and labeled.

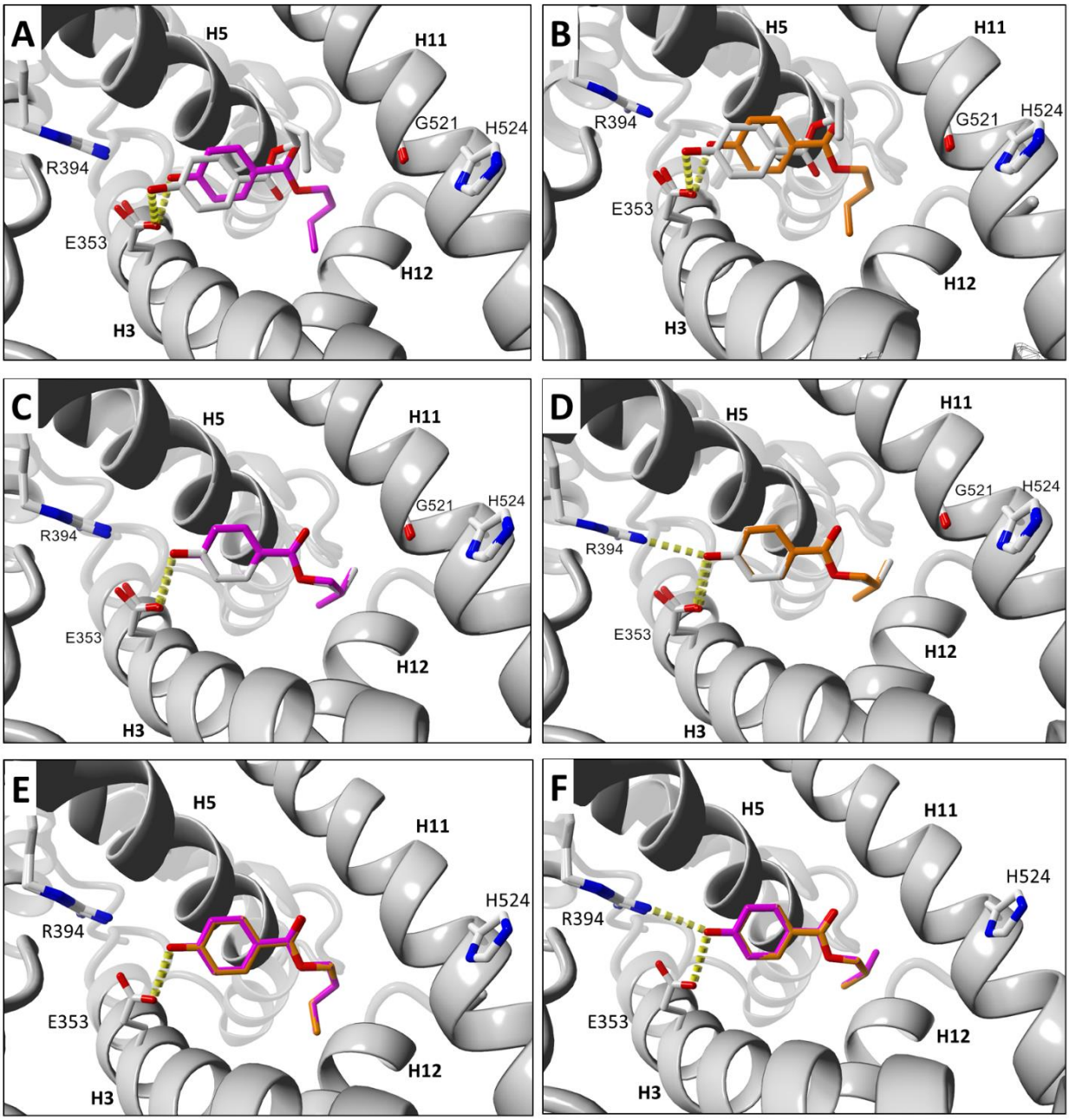


Figure 3.5. Docking comparisons of Group A paraben ligands in human and rodent ERα-LBD receptors. (**A**) **BuP** human and mouse. (**B**) **BuP** human and rat. (**C**) **iBuP** human and mouse. (**D**) **iBuP** human and rat. (**E**) **BuP** mouse and rat. (**F**) **iBuP** mouse and rat. Ligand colors: gray = human, magenta = mouse, orange = rat. Hydrogen bonds are represented as yellow dashes. Oxygen and nitrogen atoms are colored red and blue, respectively. Active site helix labels (**H5**, **H11**, and **H12**) are displayed in bold face. Hydrogen-bonding residues are displayed and labeled.

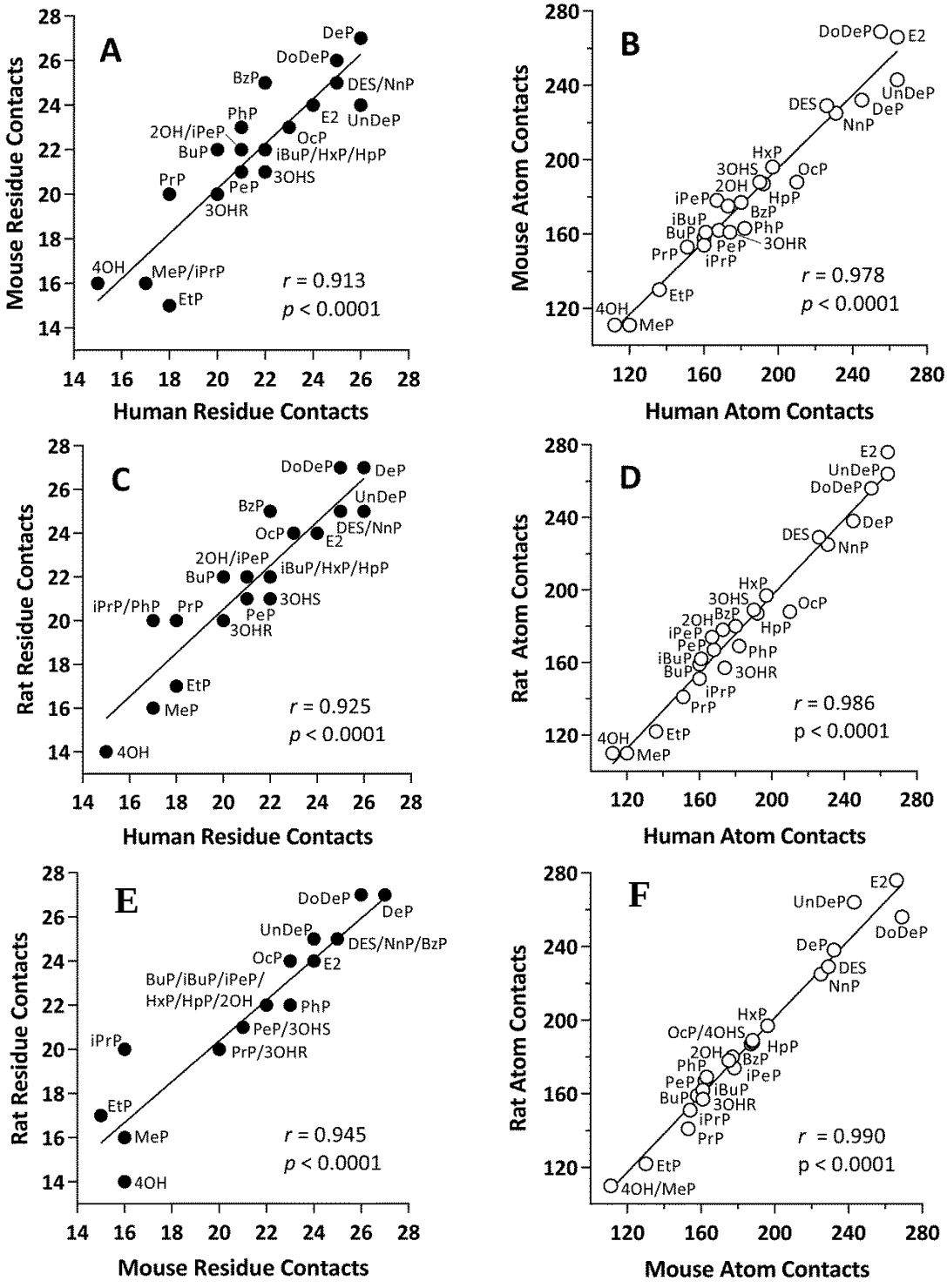


Figure 3.6. Correlations of numbers of contacts for Group A ligands docked into human and rodent ERα-LBD receptors. (**A**) Human-mouse residue contacts. (**B**) Human-mouse atom contacts. (**C**) Human-rat residue contacts. (**D**) Human-rat atom contacts. (**E**) Rat-mouse residue contacts. (**F**) Rat-mouse atom contacts. Residue contacts = filled circles, atom contacts = open circles. Each data point is labeled with the ligand name (See Figure 3.2 and Table 3.1). The Pearson correlation coefficient (*r*) and associated *p*-value are shown in each panel.

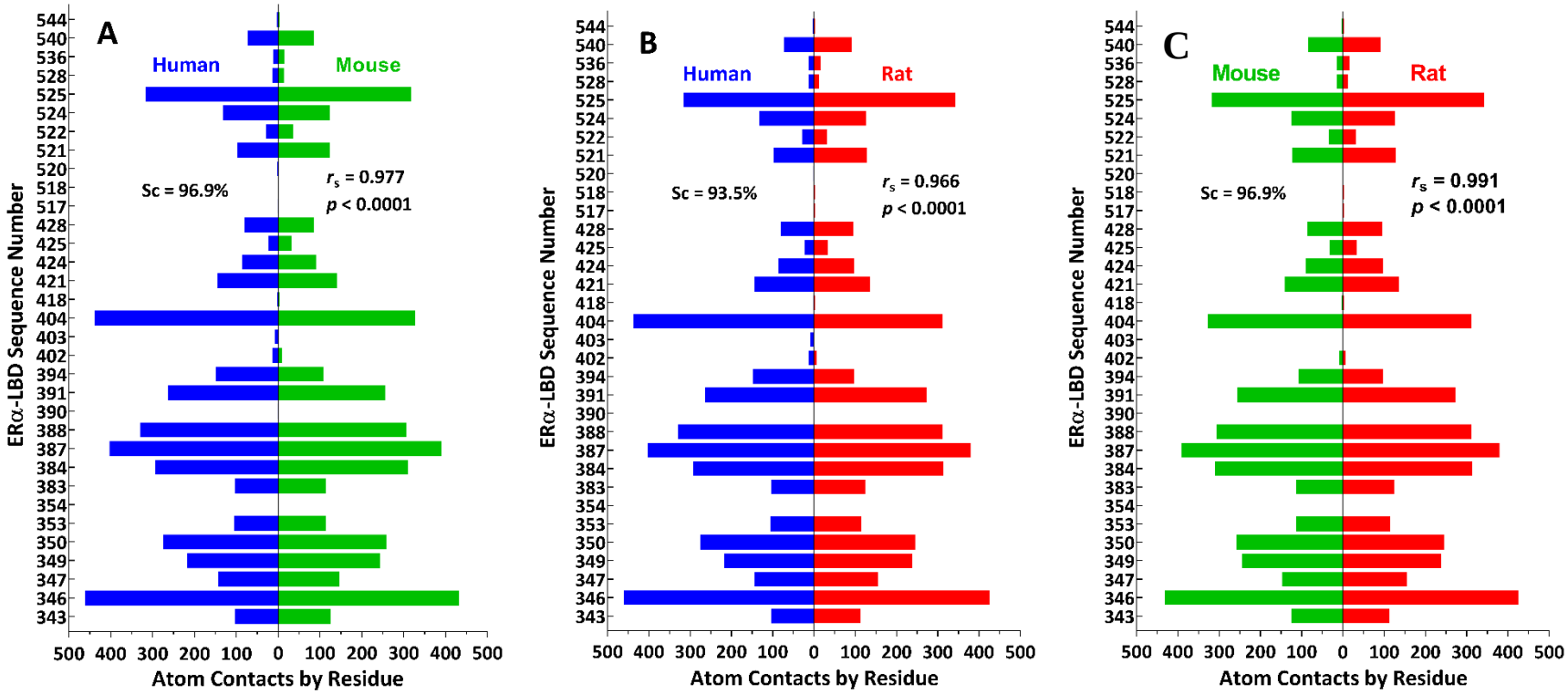


Figure 3.7. Numbers of ligand-receptor atom contacts by residue for Group A ligands docked into human and rodent ERα-LBD receptors. (**A**) Human (blue bars on left) and mouse (green bars on right) receptors. (**B**) Human (blue bars on left) and rat (red bars on right) receptors. (**C**) Mouse (green bars on left) and rat (red bars on the right). The Sorensen similarity coefficient (Sc, expressed as a percentage) along with the Spearman correlation coefficient (r_s) and associated *p*-value are shown in each panel. Vertical axis = ERα-LBD sequence number; horizontal axis = number of atom contacts by residue.

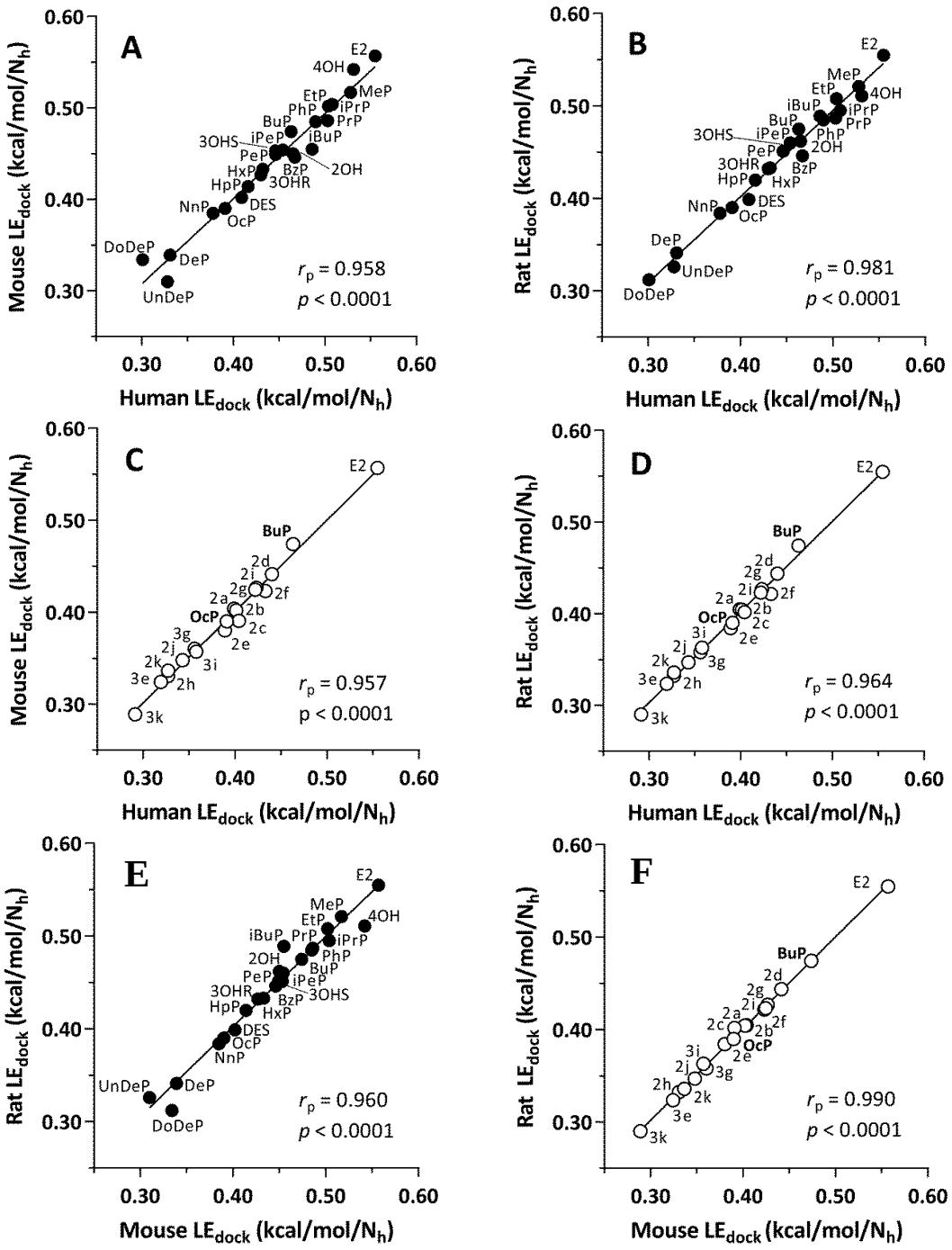


Figure 3.8. Human-rodent LE$_{dock}$ correlations for Group A and Group B ligands. (**A**) Human-mouse, Group A ligands. (**B**) Human-rat, Group A ligands. (**C**) Human-mouse, Group B ligands. (**D**) Human-rat, Group B ligands. (**E**) Mouse-rat, Group A ligands. (**F**) Mouse-rat, Group B ligands. LE$_{dock}$ = -ΔG/N$_h$, where LE$_{dock}$ = ligand efficiency for docking, ΔG = free energy of ligand-receptor binding from docking results, and N$_h$ = number of heavy (non-hydrogen) atoms in the ligand. Each point is labeled with the name of the ligand (Group A ligands = closed circles, see Figure 3.2 and Table 3.1; Group B ligands = open circles, see Figure 3.3 and Table 3.2). The Pearson partial correlation coefficient (r_p, to correct for the covariate, N$_h$) and associated *p*-value are shown in each panel.

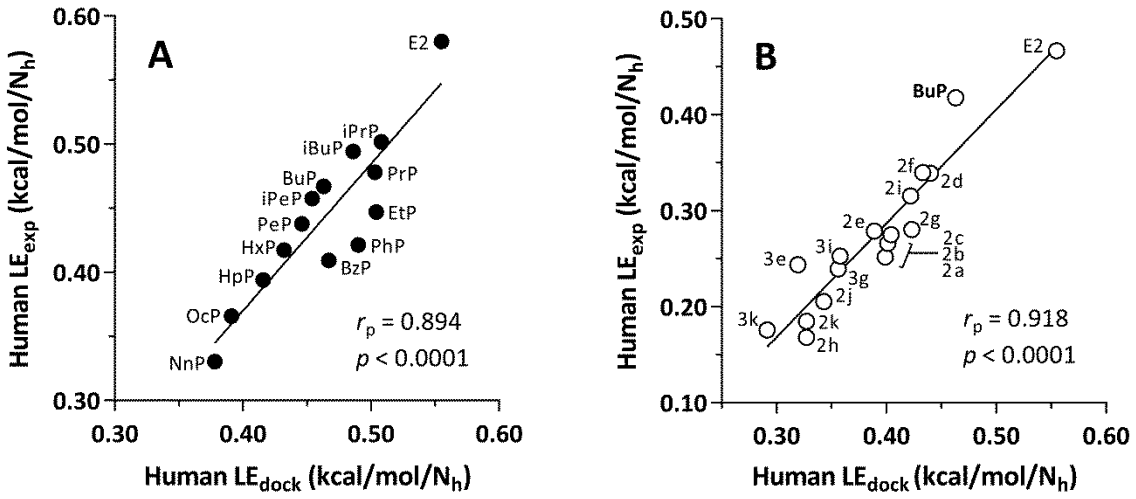


Figure 3.9. LE_{dock} and LE_{exp} correlations for Group A and B ligands with human ERa-LBD. (**A**) Group A ligands (filled circles) with names (Figure 3.2, Table 3.1). (**B**) Group B ligands (open circles) with names according to Bergquist *et al*. (2018) (Figure 3.3, Table 3.2). $LE_{dock} = -\Delta G/N_h$, where LE_{dock} = ligand efficiency for docking, ΔG = free energy of ligand-receptor binding from docking results, and N_h = number of heavy (non-hydrogen) atoms in the ligand. The Pearson partial correlation coefficient (r_p, to correct for the covariate, N_h) and associated *p*-value are shown in each panel.

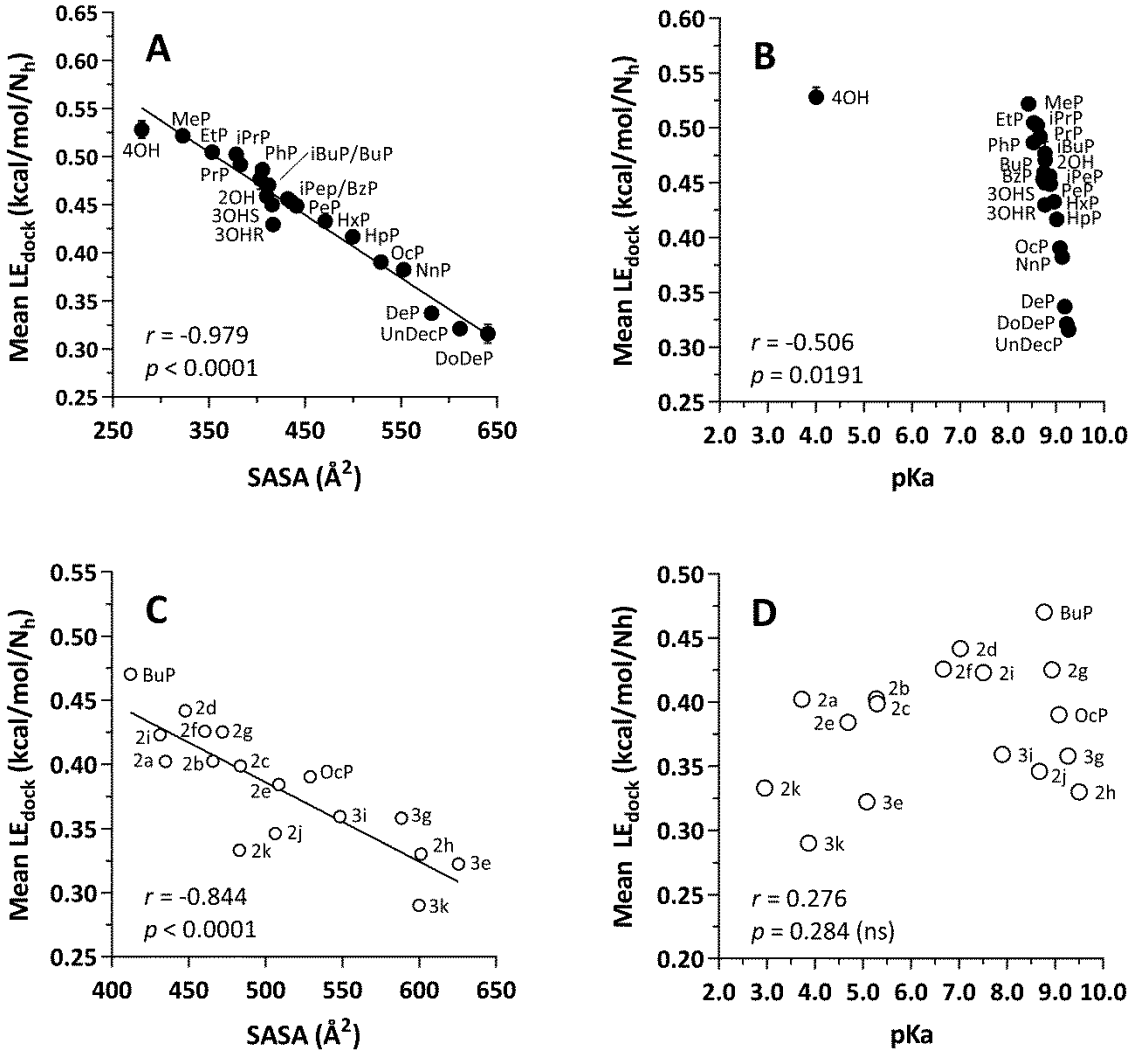


Figure 3.10. Correlations for Group A and Group B ligands with physiochemical parameters. (**A**) LE_{dock} vs. SASA for Group A ligands. (**B**) LE_{dock} vs. pKa for Group A ligands. (**C**) LE_{dock} vs, SASA) for Group B ligands. (**D**) LE_{dock} vs. pKa for Group B ligands. Group A = closed circles; see Figure 3.2 and Table 3.1 for names. Group B = open circles, see Figure 3.3 and Table 3.2 for names. Each point = mean ± SEM, n = 3 for human, mouse, and rat. In most cases, the error bars fall inside the diameter of the data markers. $LE_{dock} = -\Delta G/N_h$, where LE_{dock} = ligand efficiency for docking, ΔG = free energy of ligand-receptor binding from docking results, and N_h = number of heavy (non-hydrogen) atoms in the ligand. SASA = solvent-accessible surface area of the ligand (Å^2). The Pearson correlation coefficient (r) and associated p-value are shown in each panel. In panel (**B**), note that **4OH** is a carboxylic acid, pKa = 4.01, that would be ionized at pH 7.4. The other Group A ligands are neutral esters with pKa values for their phenol groups within a narrow range, whereas their LE_{dock} values span a wide range. In panel (**D**), note that some of the ring substitutions in the Group B ligands result in considerable lowering of the pKa of the phenol group, yet a strong correlation between LE_{dock} and SASA is maintained (panel **C**).

Table 3.1. Group A ligands and their computed pKa and SASA values.

Compound	Paraben R-group[a] or Chemical Name	pKa[b]	SASA (Å^2)[c]
Parabens			
MP	Methyl	8.43	322.94
EP	Ethyl	8.54	353.49
PrP	Propyl	8.67	382.68
BuP	Butyl	8.78	412.14
PeP	Pentyl	8.88	441.39
HxP	Hexyl	8.96	471.17
HpP	Heptyl	9.02	499.83
OcP	Octyl	9.08	529.01
NnP	Nonyl	9.13	552.71
DecP	Decyl	9.18	581.77
UnDecP	Undecyl	9.22	611.46
DoDecP	Dodecyl	9.26	640.59
iPrP	*Iso*-propyl	8.61	378.45
iBuP	*Iso*-butyl	8.77	403.42
iPeP	*Iso*-pentyl	8.87	432.28
PhP	Phenyl	8.53	405.94
BzP	Benzyl	8.73	435.85
Paraben metabolites			
4OH	4-hydroxybenzoic acid	4.01	280.28
2OH	2-hydroxy-*iso*-butyl 4-hydroxybenzoate	8.75	410.28
3OHR	(R)-3-hydroxy *n*-butyl 4-hydroxybenzoate	8.77	416.90
3OHS	(S)-3-hydroxy *n*-butyl 4-hydroxybenzoate	8.77	415.76
Established ERα agonists			
E2	17β-estradiol	10.06	457.02
DES	diethylstilbestrol	10.31	489.45

[a] Unless designated otherwise, all alkyl groups are normal (*n*) straight chains.
[b] Most acidic pKa computed with ADMET_Predictor 9.0.
[c] Solvent-accessible surface area computed with ADMET_Predictor 9.0.

Table 3.2. Group B ligands and their computed pKa and SASA values.

Compound[a]	Added Ring Substituents[b]	pKa[c]	SASA (Å^2)[d]
n-Butyl parabens			
BuP	None	8.78	412.14
2a	2,3,5,6-tetrafluoro	3.73	434.90
2b	3,5-dichloro	5.28	465.74
2c	3,5-dibromo	5.30	483.57
2d	3-bromo	7.03	447.79
2e	3,5-diiodo	4.69	508.60
2f	3-iodo	6.68	460.43
2g	3,5-dimethyl	8.94	471.89
2h	3,5-di-*tert*-butyl	9.50	601.17
2i	3,5-dihydroxy	7.51	431.45
2j	3,5-dimethoxy	8.68	506.35
2k	3,5-dinitro	2.96	482.96
n-Octyl parabens			
OcP	None	9.08	529.01
3e	3,5-diiodo	5.08	625.49
3g	3,5-dimethyl	9.27	588.38
3i	3,5-dihydroxy	7.90	548.40
3k	3,5-dinitro	3.87	599.88
Established ERa agonist			
E2	None	10.31	457.02

[a] Parent *n*-butyl and *n*-octyl paraben and 17β-estradiol (**E2**) names from Group A (Figure 3.2; Table 3.1). Ring-substituted paraben names from Figure 3.3 (Bergquist *et al*., 2018).
[b] 4-position in each case occupied by a hydroxyl group.
[c] Most acidic pKa computed with ADMET_Predictor 9.0.d
[d] Solvent-accessible surface area computed with ADMET_Predictor 9.0.

Table 3.3. Interspecies ligand RMSD values and Sc indices for ligand-receptor residue contacts of Group A ligands docked into human, mouse, and rat ERα-LBD receptors.

Compound	RMSD (Å)[a]			Sc index (%)[b]		
	Human/ Mouse	Human/ Rat	Mouse/ Rat	Human/ Mouse	Human/ Rat	Mouse/ Rat
Parabens						
MeP	1.82	1.83	0.08	84.8	84.8	100
EtP	2.51	2.96	1.05	72.7	74.3	93.8
PrP	1.20	1.33	0.47	84.2	89.5	95
BuP	3.22	3.30	0.10	81.0	81.0	100
PeP	0.31	0.21	0.19	100	100	100
HxP	0.18	0.16	0.12	100	100	100
HpP	0.21	0.13	0.13	100	100	100
OcP	0.49	0.47	0.15	100	97.9	97.9
NnP	0.23	0.27	0.23	100	100	100
DecP	3.60	3.57	0.12	90.6	90.6	100
UnDecP	3.45	1.19	3.52	96.0	98.0	93.9
DoDecP	0.66	3.62	3.64	94.1	92.3	96.3
iPrP	0.36	1.74	1.54	97.0	86.5	88.9
iBuP	0.14	0.18	0.09	100	100	100
iPeP	0.46	0.50	0.24	97.7	97.7	100
PhP	1.79	1.72	0.13	95.5	97.7	97.8
BzP	2.09	2.11	0.09	89.4	89.4	100
Parabens metabolites						
4OH	0.15	0.27	0.34	96.8	89.7	93.3
2OH	2.09	2.08	1.02	88.4	88.4	100
3OHR	1.79	1.73	0.18	90.0	90.0	100
3OHS	0.15	0.13	0.16	97.7	97.7	100
Established ERα agonists						
E2	0.08	0.22	0.18	100	100	100
DES	0.11	0.14	0.05	100	100	100

[a] Symmetry-corrected heavy-atom interspecies ligand RMSD values for Group A ligands docked into mouse and rat ERα-LBD receptors. Overall group RMSD values (median, 95% CI, n = 23) were 0.49 (0.21, 1.82) Å (human-mouse), 1.19 (0.22, 1.83) Å (human-rat), and 0.18 (0.12, 0.34) Å (mouse-rat). By definition, RMSD (human-human) = 0.00 Å.

[b] Sorenson similarity coefficient (Sc) = $[2(N_{AB})/(N_A + N_B)] \times 100$, where N_A = number of contacts in receptor A, N_B = number of contacts in receptor B, and N_{AB} = number of contacts shared by receptors A and B. In this case, contacts are ligand-receptor residue contacts. Sc values (median, 95% CI, n = 23) for all ligands were 96.8 (90.0, 100)% (human-mouse), 97.7 (89.5, 100)% (human-rat), and 100 (97.8, 100)% (mouse-rat). By definition, Sc (human-human) = 100%.

Table 3.4. Comparison of MolProbity receptor quality assessment of refined mouse ERα-LBD structures.

Mouse ERα-LBD Molprobity Structure Analysis[a]									
MolProbity Analysis Statistics[b]	Receptor Prep Method								
	YASARA			UCSF Chimera			I-TASSER		
	Mouse *In Silico*	Mouse EM	Mouse MD Refined	Mouse *In Silico*	Mouse EM	Mouse MD Refined[c]	Mouse Threading	Mouse EM[d]	Mouse MD Refined[c]
MolProbity Score (percentile)	1.48 (90th)	0.80 (100th)	0.50 (100th)	2.15 (68th)	1.14 (99th)	0.50 (100th)	1.76 (87th)	1.41 (97th)	0.60 (100th)
Clashscore, all atoms (percentile)	9.11 (71st)	1.01 (99th)	0 (100th)	24.98 (22nd)	1.77 (99th)	0 (100th)	0.51 (99th)	1.77 (99th)	0 (100th)
Poor Rotamers Goal: <0.3%	0 (0.0%)	0 (0.0%)	1 (0.45%)	3 (1.36%)	2 (0.91%)	1 (0.45%)	18 (8.18%)	9 (4.09%)	3 (1.36%)
Favored Rotamers Goal: >98%	219 (99.55%)	217 (98.64%)	219 (99.55%)	215 (97.73%)	215 (97.73%)	217 (98.64%)	175 (79.55%)	198 (90.00%)	214 (97.27%)
Ramachandran Outliers Goal: <0.05%	0 (0.00%)	0 (0.00%)	0 (0.00%)	3 (1.23%)	1 (0.41%)	0 (0.00%)	2 (0.82%)	0 (0.00%)	0 (0.00%)
Ramachandran Favored Goal: >98%	242 (99.59%)	241 (98.18%)	241 (99.18%)	236 (97.12%)	235 (96.71%)	240 (98.77%)	229 (94.24%)	238 (97.94%)	240 (98.77%)
C β deviations > 0.25 Å Goal: 0	0 0 (0.0%)	0 0 (0.0%)	0 0 (0.0%)	3 (1.28%)	3 (1.28%)	0 0 (0.0%)	6 (2.56%)	0 0 (0.0%)	0 0 (0.0%)
Bad Bonds Goal: 0 %	1/1988 (0.05 %)	0/1988 0 (0.0%)	2/1988 (0.10 %)	14/1987 (0.70 %)	0/1987 (0.0%)	9/1988 (0.45 %)	0/1988 (0.0%)	22/1988 (1.11 %)	10/1988 (0.50 %)
Bad Angles Goal: <0.1%	2/2685 (0.07 %)	0/2685 (0.00 %)	3/2685 (0.11 %)	33/2683 (1.23 %)	26/2683 (0.97 %)	10/2685 (0.37 %)	20/2683 (0.75 %)	11/2685 (0.41 %)	12/2685 (0.45 %)
Cis Prolines Expected: ≤1 per chain, or ≤5 %	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)
Cis non Prolines: Goal: <0.05%	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)
Twisted Peptides Goal: 0	0/244 (0.00 %)	0/244 (0.00 %)	0/244 (0.00 %)	0/244 (0.00 %)	3 / 244 (1.23%)	0/244 (0.00 %)	4 / 244 (1.64%)	1/244 (0.41 %)	0/244 (0.00 %)
CaBLAM outliers Goal: <1.0%	1 (0.41 %)	0 (0.00 %)	0 (0.00 %)	2 (0.83 %)	2 (0.83 %)	1 (0.41 %)	5 (2.07 %)	2 (0.83 %)	2 (0.83 %)
CA Geometry outliers Goal: <0.5%	0 (0.00 %)	0 (0.00 %)	0 (0.00 %)	3 (1.24%)	3 (1.24%)	1 (0.41 %)	0 (0.00 %)	0 (0.00 %)	0 (0.00 %)

[a] Molprobity analysis carried out by the Molprobity web server (MolProbity, 2018; Chen *et al*., 2010).
[b] MolProbity analysis statistics as defined by the Molprobity web server (MolProbity, 2018; Chen *et al*., 2010).
[c] Molecular dynamics refinement performed with YASARA.
[d] Energy minimization performed with YASARA.

Table 3.5. Comparison of MolProbity receptor quality assessment of refined rat ERα-LBD structures.

Rat ERα-LBD Molprobity Structure Analysis[a]									
MolProbity Analysis Statistics[b]	Receptor Prep Method								
	YASARA			UCSF Chimera			I-TASSER		
	Rat *In Silico*	Rat EM	Rat MD Refined	Rat *In Silico*	Rat EM	Rat MD Refined[c]	Rat Threading	Rat EM[d]	Rat MD Refined[c]
MolProbity Score (percentile)	1.48 (90th)	0.74 (100th)	0.60 (100th)	1.89 (81st)	0.90 (100th)	0.50 (100th)	1.41 (97th)	1.19 (99th)	0.68 (100th)
Clashscore, all atoms (percentile)	9.1 (71st)	0.76 (99th)	0 (100th)	25 (22nd)	0.76 (99th)	0 (100th)	1.52 (99th)	1.26 (99th)	0.51 (99th)
Poor Rotamers Goal: <0.3%	0 (0.0%)	0 (0.0%)	3 (1.36%)	1 (0.45%)	1 (0.45%)	1 (0.45%)	10 (4.55%)	6 (2.73%)	2 (0.91%)
Favored Rotamers Goal: >98%	219 (99.55%)	217 (98.64%)	214 (97.27%)	216 (98.18%)	211 (95.91%)	218 (99.09%)	195 (88.64%)	197 (89.55%)	213 (96.62%)
Ramachandran Outliers Goal: <0.05%	0 (0.00%)	0 (0.00%)	0 (0.00%)	2 (0.82%)	2 (0.82%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Ramachandran Favored Goal: >98%	242 (99.59%)	240 (98.77%)	241 (99.18%)	239 (98.35%)	236 (97.12%)	239 (98.35%)	238 (97.94%)	238 (97.94%)	240 (98.77%)
Cß deviations > 0.25 Å Goal: 0	0 0 (0.0%)	0 0 (0.0%)	0 0 (0.0%)	3 (1.28%)	2 (0.85%)	0 0 (0.0%)	0 0 (0.0%)	0 0 (0.0%)	0 0 (0.0%)
Bad Bonds Goal: 0 %	1/1989 (0.05 %)	0/1989 0 (0.0%)	3/1989 (0.15 %)	14/1988 (0.70 %)	0/1989 (0.0%)	2/1989 (0.10 %)	12/1988 (0.60 %)	12/1988 (0.60 %)	6/1988 (0.30 %)
Bad Angles Goal: <0.1%	2/2687 (0.07 %)	0/2687 (0.00 %)	4/2687 (0.15 %)	28/2685 (1.03 %)	23/2687 (0.86 %)	3/2687 (0.11 %)	6/2685 (0.20 %)	6/2685 (0.22 %)	8/2685 (0.30 %)
Cis Prolines Expected: ≤1 per chain, or ≤5 %	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)	0 / 9 (0.0%)
Cis non Prolines: Goal: <0.05%	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)	0/235 0 (0.0%)
Twisted Peptides Goal: 0	0/244 (0.00 %)	0/244 (0.00 %)	0/244 (0.00 %)	0/244 (0.00 %)	3 / 244 (1.23%)	0/244 (0.00 %)	1 / 244 (0.41%)	1/244 (0.41 %)	0/244 (0.00 %)
CaBLAM outliers Goal: <1.0%	1 (0.41 %)	0 (0.00 %)	0 (0.00 %)	2 (0.83 %)	1 (0.41 %)	2 (0.83 %)	2 (0.83 %)	2 (0.83 %)	3 (1.24%)
CA Geometry outliers Goal: <0.5%	0 (0.00 %)	0 (0.00 %)	0 (0.00 %)	2 (0.83%)	3 (1.24%)	1 (0.41 %)	0 (0.00 %)	0 (0.00 %)	0 (0.00 %)

[a] Molprobity analysis carried out by the Molprobity web server (MolProbity, 2018; Chen *et al*., 2010).
[b] MolProbity analysis statistics as defined by the Molprobity web server (MolProbity, 2018; Chen *et al*., 2010).
[c] Molecular dynamics refinement performed with YASARA.
[d] Energy minimization performed with YASARA.

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Chapter IV.

Targeted Degradation of Activating Estrogen Receptor α Ligand-Binding Domain Mutations in Cell Models of Human Breast Cancer

Introduction

Breast cancer is currently the most commonly diagnosed malignancy among women in the United States (US) with an estimated 266,000 new diagnoses and 41,000 deaths in 2018 alone [1]. Patients with estrogen receptor positive (ER-positive) breast cancer make up approximately 70% of all breast cancer diagnoses and are often treated with tamoxifen or an aromatase inhibitor (AI) during the first 5 years of adjuvant endocrine therapy using [2]. Although adjuvant endocrine therapy is effective in lowering the rate of recurrence and disease-free survival among ER-positive patients, ~20 % of patients treated with either tamoxifen, an AI, or both will still recur within 10 years of treatment [2, 3]. Interestingly, during the period of 5 to 20 years following the cessation of endocrine therapy, ER-positive patients exhibit a persistent risk of recurrence that increases at a rate of ~1-2% every year irrespective of their nodal status and stage [4]. *In vitro* studies investigating the potential mechanisms of AI resistance have observed adaptation and maintained cellular proliferation of estrogen receptor α (ERα) expressing MCF-7 cells in long-term estrogen-deprived (LTED) conditions [5], and others have suggested that non-classical estrogens resulting from androgen metabolism could contribute to AI resistance [6].

However, the re-discovery of constitutively active *ESR1* ligand-binding domain (LBD) somatic mutations in endocrine therapy resistant, metastatic ER-positive breast cancers proved to

be another likely mechanism of acquired resistance to endocrine therapy [7-9]. Two of the most common LBD mutations, Asp538Gly (D538G) and Tyr537Ser (Y537S), are detected at a frequency of ~30-40 % among patients resistant to endocrine therapy [10]. Given the rarity of these mutations in primary breast cancer [11] and their absence in matched primary samples from patients with metastatic disease, it has been proposed that the acquisition of *ESR1* mutants may be a result of the selective-pressure of endocrine therapy that allows for tumors cells already harboring these mutations to survive [12]. Some *ESR1* mutations have recently been reported in PDX models [13] and were found to occur naturally in LTED ER-positive cell lines [10] where the phenotype of maintained cell proliferation was observed in the absence of estrogen. Functionally, the LBD mutations were shown *in vitro* to induce the agonist conformation of ERα and promote constitutive activity of the receptor in the absence of a ligand as well as confer resistance to known antiestrogens [7, 8, 14, 15]. Fulvestrant is a common antiestrogen that is given to patients with advanced disease by intramuscular injection and has been designed to induce degradation of ERα via the ubiquitin-proteasome pathway [16]. Although Fulvestrant has displayed clinical benefit in patients with advanced stage breast cancer [17], the drug resistant phenotype of the *ESR1* mutants demonstrate the need to develop more potent and selective downregulators of ERα that are also orally bioavailable. AZD9496 is a recent example of a compound that is an oral, non-steroidal ERα downregulator that has undergone a recent Phase I clinical trial where four patients were reported to have stable disease at 12 months of treatment [18, 19]. However, recent advancements in the Proteolysis Targeting Chimeras (PROTAC) method for developing small-molecules for targeted degradation of specific proteins [20] is a novel approach that may lead to the improvement of compounds like AZD9496 as well as the discovery new drug agents with greater potency such as the ones described in this report.

Since the introduction of the PROTAC method in 2001 to induce efficient and targeted degradation of proteins [21], significant progress has been made towards developing similar PROTAC molecules for potential applications in leukemia and triple negative breast cancer [22-24]. PROTACs achieve degradation by hijacking the ubiquitin-proteasome pathway where these bifunctional small molecules recruit a target protein and bring it near an E3 ubiquitin ligase to initiate the degradation process [25]. Although the *ESR1* mutants are constitutively active in the absence of endogenous estrogens, they still are dependent on the presence of ERα to promote ER mediated cell proliferation. Therefore, the use of the PROTAC method to develop bifunctional molecules with a ligand specific for ERα linked to a ligand for Cullin-4A ligase complex should lead to a reduction in ER mediated cell proliferation.

In this chapter, we report the development ERD-148 as a novel degrader of ERα that is a more potent downregulator of ERα protein expression compared to Fulvestrant in both WT and monoclonal MCF-7s cell lines engineered to express the Y537S and D538G mutants using CRISPR-cas9 genome editing (cY537S and cD538G).

Materials and Methods

Chemicals

17ß-estradiol (E2), tamoxifen (TAM), and Fulvestrant (ICI 182,780 - (ICI)) were purchased from Sigma-Aldrich Inc. (St. Louis, MO) with purity ≥ 98% determined by HPLC. E2 and ICI were dissolved to 10 mM in absolute ethanol and stored at -20 °C. The identity and synthetic procedures for compounds ERD-32, ERD-56, and ERD-148 are pending patent approval and will be released at a later date. Each PROTAC compound was dissolved to 10 mM in DMSO stored

at -20 °C. The final concentration of ethanol or DMSO did not exceed 0.1 % (v/v) in culture media. All compounds were stored protected from light.

Cell lines and culture conditions

MCF-7, T47D, MDA-MB-231, and MDA-MB-468 cells were obtained from the Tissue Culture Shared Resource (TCSR) at the Lombardi Comprehensive Cancer Center (Georgetown University, Washington, DC). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Life Technologies, Grand Island, NY) supplemented with 5% (v/v) fetal bovine serum (FBS) (Valley Biomedical Inc., Winchester, VA) for MCF-7 and MDA-MB-231 cells, or 10% FBS medium for T47D and MDA-MB-468 cells, at 37 °C in a humidified 5 % (v/v) CO_2 atmosphere. The identity of the cells was confirmed by short tandem repeat profiling by the TCSR and shown to be free of mycoplasma contamination. For assays in defined steroid deplete conditions, cells were repeatedly washed and grown in steroid-depleted media before proliferation assays (phenol red-free IMEM supplemented with charcoal stripped bovine serum – CBS) (Atlanta Biologicals Inc., Flowery Branch, GA) based on a previously described method [26].

Creation of LBD mutation homology directed repair templates

A 2,629 base pair fragment of the *ESR1* gene containing exon 8, the ligand binding domain, was amplified using the HDR primers outlined in Table 4.1 from genomic DNA harvested from MCF-7 cells and cloned in pUC19 (Thermo Fisher Scientific, Waltham, MA). The forward and reverse primers also contained a 5'-KpnI and 5'-SalI sites respectively. Once the *ESR1* gene fragment was cloned into pUC19, the wild-type sequence was verified by Sanger sequencing at

the University of Michigan Sequence Core using the above primers with M13 tails added, as well as with an internal sequencing primer. The ligand binding domain mutations Y537S and D538G in the pUC19-*ESR1* exon8 plasmid were induced by site directed mutagenesis using the QuikChange II kit (Agilent technologies, Santa Clara, CA). The site directed mutagenesis primers were designed using their online mutagenesis sequencing tool (https://www.genomics.agilent.com/primerDesignProgram.jsp). The Y537S and D538G mutations were created using the primers listed in Table 4.1. The sequence of pUC19-wtESR1, pUC19-ESR1Y537S, and pUC19-ESR1D538G plasmids were verified by Sanger sequencing. Each pUC19-ESR1 plasmids was purified and isolated with Qiaprep Spin miniprep kit (Qiagen, Germantown, MD). Prior to transfection of the HDR template in MCF-7 cells, pUC19-ESR1Y537S and pUC19-ESR1D538G plasmids were digested with KpnI/SalI and treated with Antarctic phosphatase (New England Biolabs, Ipswich, MA) and purified with QIAquick PCR Purification Kit (Qiagen, Germantown, MD).

Creation of sgDNA targeting exon 8 of the *ESR1* gene

To target exon 8 of the *ESR1* gene for CRISPR/Cas9-induced DNA mutation, we used the sequence CRISPR058819 as previously described [12]. This sequence was cloned into the plasmid pSpCas9(BB)-2A-GFP (Adgene plasmid 48140), a kind gift from the Zheng lab as previously described [27].

CRISPR/Cas9-induced *ESR1* LBD mutation

Using the mutated HDR templates and sgDNA plasmid, WT MCF-7 cells were transfected with 2.5ug of sgDNA plasmid, and with either the digested pUC19-ESR1Y537S or the pUC19-

ESR1D538G HDR templates using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Transfected cells harboring the Y537S (cY537S) or D538G (cD538G) mutations were selected for by maintaining them in hormone deplete medium for four weeks before seeding them at a density of 1 cell / well in 96-well plates containing hormone deplete medium supplemented with 5% CBS using an MoFlo Astrios cell sorter at the University of Michigan Flow Cytometry Core. Surviving colonies were expanded in hormone deplete media for 3 months and screened by PCR amplifying and sequencing a 417 bp DNA sequence encompassing exon 8 at the University of Michigan Sequence Core using M13 primers with the sequences 5'-AAGTGGGTCTTTAAACAGGA-3' and 5'-AGGAGACAGAATTTGGCTAA -3' to identify the cells harboring the Y537S or D538G mutations (Figure 4.1). Verified cY537S and cD538G cells were maintained in steroid deplete IMEM/5% CBS media before treatments.

PrestoBlue® cell viability assay

For proliferation assays, MCF-7 and T47D cells were withdrawn from steroids as previously described [28] and plated in 5% CBS at 1000 cells / well and 2000 cells / well respectively in 96-well plates and cultured overnight. cY537S and cD538G cells were seeded into 96-well plates at a density of 1000 cells / well in 5% CBS and allowed to attach overnight. MBA-MB-231 and MDA-MB-468 cells were plated in 5% FBS at 600 cells / well and 10% FBS at 1500 cells / well respectively in 96-well plates and cultured overnight. Cells were treated with the specified compounds at the indicated concentrations or the vehicle controls (ethanol or DMSO) diluted in IMEM supplemented with the CBS concentration for each cell line described above. After specified duration of treatment, cell viability was assessed using Presto Blue reagent

(ThermoFisher Scientific, Waltham, MA) according to manufacturer's specifications. Plated cells were incubated for 3 hrs in the presence of 10 % (v/v) Presto Blue and absorbance was measured using a POLARstar Omega plate reader with the excitation/emission wavelengths set at 544/590 nm. Relative cell number was confirmed by crystal violet stain immediately after Presto Blue measurement as previously described [28]. Technical replicates were derived and analyzed from the same sample source across the indicated treatment conditions. Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions.

Western Blotting

MCF-7, cY537S, or cD538G cells were plated in steroid-deplete media at a density of 2 x 10^6 cells in 10cm dishes. Whole cell lysates were isolated from each specified cell line 24 hrs after treatment with the indicated compounds. Cells were lysed using RIPA buffer (ThermoFisher Scientific, Waltham, MA) supplemented with Protease Inhibitor Cocktail Tablets and Phosphatase Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN). Total protein from the lysates was quantified by the Bradford Assay (Bio-Rad, Hercules, CA). Twenty micrograms of reduced protein per lane was assayed on 4–20% Tris-glycine gradient polyacrylamide gels (Thermo Fisher Scientific, Waltham, MA), and transferred to a PVDF membrane according to manufacturer's specifications. The PVDF membrane was blocked for 1 hr with Blocking-Grade Blocker (Bio-Rad, Hercules, CA) and then incubated at 4°C overnight with primary antibody for ERα (sc-56836, Santa Cruz Biotechnology, 1:500). PVDF membranes were washed 3x over a period of 30 min with TBS containing 0.1% Tween 20 before incubating

the PVDF membrane with anti-mouse HRP-linked secondary antibody (7076S, Cell Signaling Technology, 1:10000) for 1 hr at room temperature. HRP-linked ß-Actin (12262, Cell Signaling Technology, 1:10000) was used a loading control. Blots were developed with Supersignal WestDura Extended Duration (ThermoFisher Scientific, Waltham, MA) on a Konica SRX-101A developer according to manufacturer's specifications.

RNA Expression Assay

MCF-7, cY537S, or cD538G cells were plated in steroid depleted media at 350,000 cells / well in 6-well plates and cultured overnight before treatment with each specific compound. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Yield and quantity were determined by spectrophotometry (NanoDrop ND-1000). All samples were stored at -80 °C. Total RNA (1 µg) was reverse transcribed (RT) using Reverse Transcription System (Promega, Madison, WI) and the cDNA amplified in a 25 µl reaction containing Gene Expression Master Mix and gene specific primers (Thermo Fisher Scientific, Waltham, MA). *GREB1* mRNA expression was measured using a TaqMan RT-PCR assay as previously described [26]. *GREB1* expression was normalized against *GAPDH* with relative expression determined using the $\Delta\Delta C_T$ method [29].

Results

ERD-148 inhibits the proliferative ability of estrogen-dependent WT MCF-7 and cY537S and cD538G human breast cancer cells in steroid-free conditions

To determine whether our series of PROTAC ERα degraders could inhibit the proliferative ability of human breast cancer growth, estrogen-dependent MCF-7 and T47D cell lines were

treated with either Fulvestrant (ICI) or the specified ERα degrader (Figures 4.2A & 4.2B, Table 4.2). After a five-day treatment, cell viability data -confirmed by crystal violet staining- indicated that PROTAC degrader ERD-148 displayed comparable potency as ICI with an IC_{50} of 0.8 nM and 0.5 nM in WT MCF-7 and T47D cells respectively. The IC_{50} for ICI was determined to be 1.3 nM and 0.6 nM MCF-7 and T47D cells respectively. ERD-56 and 32 were determined to be relatively less potent PROTAC degraders compared to ERD-148 with their IC_{50} values shown in Table 4.1. The observation of inhibited cell proliferation in both ER-dependent MCF-7 and T47D cell lines upon treatment with the PROTAC degraders suggests that this effect is not cell line specific.

To confirm the estrogen independent and drug resistant phenotype of our CRISPR cY537S and cD538G cell lines, we conducted a five-day growth assay and treated each of these mutant cell lines with either E2, TAM, or ICI and compared their proliferative potential to WT MCF-7 cells (Figure 4.3A–4.3C). The proliferative ability of the cY537S and cD538G mutant cells did not appear to be affected by the addition of E2 at the indicated concentrations suggesting that they exhibited an E2 independent phenotype compared to WT MCF-7s and could proliferate in steroid-deplete media (Figure 4.3A). The IC_{50} for WT MCF-7, cY537S and cD538G cells treated with TAM was 0.33, 6.80, and 2.50 μM respectively (Figure 4.3B). The IC_{50} for WT MCF-7, cY537S and cD538G cells treated with ICI was 0.85, 5.78, and 4.30 nM in respectively (Figure 4.3C). The relatively lower potency of the antiestrogens, TAM and ICI, in mutant cY537S and cD538G treated cells suggest that they display a drug resistant phenotype compared to WT MCF-7 cells which was consistent with previous reports [15].

Once we determined that our mutant cY537S and cD538G cells exhibited an E2 independent phenotype, we attempted to evaluate the relative potency of the PROTAC degraders

compared to WT MCF-7 cells (Figures 4.2C, 4.2D, and Table 4.2). Compound ERD-148 was found to have comparable potency as ICI in cY537S cells with IC_{50} values of 10.5 and 4.2 nM respectively. A similar trend was also observed cD538G cells between compound ERD-148 and ICI with their IC_{50} shown in Table 4.2. As expected from the growth assay data seen in Figures 4.3B and 4.3C, there appears to be evidence of resistance to ERD-148 in the mutant cells versus WT MCF-7 cells indicated by the shift in the IC_{50} to higher concentrations. This apparent resistant phenotype was also observed for PROTAC degraders ERD-56 and 32 with their IC_{50} values summarized for each cell line in Table 4.2.

ERD-148 shows evidence of marginal non-specific toxicity in non-estrogen receptor α expressing cell lines

To investigate potential signs of non-specific toxicity and whether ERD-148 was inhibiting cell proliferation due to antagonism of ER-mediated cell growth, we treated non-ERα expressing MDA-MB-468 and MDA-MB-231 cells with each PROTAC degrader and ICI (Figure 4.4A & 4.4B). As seen in Table 4.2, the IC_{50} for ERD-148 and ICI are log shifted ~1-3 orders of magnitude to higher concentrations in the non-ERα expressing cells compared to the IC_{50} values as determined in WT MCF-7 cells. This data suggests that off-target effects may be occurring at doses greater than 100 nM for ERD-148 and that concentrations near 1 nM may inhibit WT MCF-7 cell proliferation via antagonism of ERα. There appears to be some separation of the IC_{50} values for ERD-56 and 32 between the non-ERα and ERα expressing cells, however, cell proliferation that is inhibited near these IC_{50} concentrations may due to a combination of both non-specific toxicity and ERα antagonism for these two compounds.

PROTAC degraders induce downregulation of ERα protein expression in WT MCF-7, cY537S, and cD538G human breast cancer cells lines

To determine if our series of PROTAC degraders were inhibiting the proliferative ability of ER-positive cell lines by downregulating the expression of ERα, we treated WT MCF-7 and the cells expressing mutant cY537S and cD538G for 24 hrs with each PROTAC degrader and performed a western blot assay (Figure 4.5A & 4.5B). Treatment with compounds ERD-56, 32, and ICI at 300 nM resulted in relatively lower levels of ERα expression in WT MCF-7 cells versus DMSO control (Figure 4.5A). These same compounds did not appear to be as effective in downregulating ERα expression at 300 nM in the mutant cell lines as indicating by the comparable protein bands for the DMSO control and the treatments (Figure 4.5A). PROTAC degrader ERD-148 downregulated ERα expression at concentrations as low as 1nM in WT MCF-7 cells and appeared to do so more effectively than ICI (Figure 4.5B). Comparable ERα protein bands were seen among the DMSO control and the indicated treatment conditions in the mutant cell lines (Figure 4.5B) suggesting that there is some resistance to ERD-148 which is consistent with the growth assay data shown in Figure 4.2. Of the PROTAC compounds tested, ERD-148 showed the most effective downregulation of ERα and was superior to ICI.

PROTAC degraders downregulate mRNA expression of an ERα regulated gene, *GREB1,* in WT MCF-7, cY537S, and cD538G cells lines

To determine if the PROTAC degraders were inhibiting the proliferative ability of the ER-positive cell lines by antagonizing ERα, we performed RT-PCR to measure the relative expression of a downstream target of ER signaling, *GREB1*, in ER-positive treated cells (Figure 4.6A – 4.6F). WT MCF-7 cells treated with ERD-56 and 32 at 100 nM show significant

downregulation of the ER regulated gene, *GREB1*, versus the 50 pM E2 positive control at 24 hrs (Figure 4.5A). ERD-148 was able to achieve significant downregulation of *GREB1* expression at concentrations as low 10 nM in WT MCF-7 cells versus 50 pM E2 treated cells (Figure 4.6B). Consistent with our previous observations, there appears to be evidence of resistance to ERD-56 and 32 in the cY537S cells but significant downregulation of *GREB1* was observed at 300 nM in both mutant cell lines treated with ERD-56 (Figure 4.6C & 4.6E). Similarly, evidence of resistance was seen in cY537S treated with ERD-148 at 10 nM but significant downregulation of GREB1 was reached at 100 nM treatment with ERD-148 in both mutant cell lines (Figure 4.6D & 4.6F). WT MCF-7 and mutant cells treated with ICI showed significant downregulation of *GREB1* at all concentrations tested ($p<0.0001$) (Figure 4.6A – 4.6F). Of the PROTAC compounds tested, ERD-148 was found to downregulate the expression of *GREB1* most similar to ICI.

Addition of 17ß-estradiol competes with the PROTAC Degraders

To provide additional evidence that ERα antagonism was the primary mechanism of these PROTAC degraders, we co-treated each ER-positive cell line at a fixed dose with each indicated degrader and increased the dose of E2 logarithmically (Figure 4.7A – 4.7C). WT MCF-7 and the mutant cell lines all responded with increased cell proliferation upon exposure to higher doses of E2 while co-treated with each PROTAC compound. The observation of increased cell proliferation suggests that E2 competes with the PROTAC compounds for ERα and that cell inhibition achieved by these degraders is reversible in the presence of a pure agonist given that the mutants do not respond to E2 treatment alone (Figure 4.3A).

Discussion

In this chapter, we investigated the mechanism by which a series of PROTAC degraders inhibit the proliferative ability of ERα expressing breast cancer cell lines. ERD-148 was determined to be the most potent in our series of PROTAC compounds and downregulated the expression of ERα superior to Fulvestrant in WT MCF-7 and MCF-7 cells harboring the CRISPR/cas9 knock-in LBD mutations. The expression of a critical ER-regulated gene, *GREB1* [26, 30], was also found to be significantly downregulated in ER-positive cell lines upon exposure to ERD-148 in a manner comparable to Fulvestrant. Increased cell proliferation was observed in ER-positive cells co-treated with ERD-148 and E2 indicating that estrogen competes for the ERα receptor and suggests that the primary mechanism of these PROTAC compounds is ER antagonism. The ER-negative, estrogen-independent cell lines treated with ERD-148 showed evidence of minimal non-specific toxicity at concentrations above its ~IC_{90} which highlight the therapeutic potential for ERD-148.

Breast cancer cells which are ER-positive and HER2-negative generally rely on the expression of the hormone receptor, ERα, to promote ER-mediated cell proliferation [26, 30]. Pharmacological interference of ERα signaling or the inhibition of E2 hormone synthesis *in vivo* are recognized as effective methods to treat ER-positive breast cancer, however, acquired resistance to endocrine therapy is a common occurrence [2-4]. Previous publications have indicated the potential for ER-positive breast cancer cell lines to adapt to estrogen deprived conditions [5, 10, 31] and a significant number of patients with advanced disease have developed LBD mutations in ERα that may allow ER-positive tumor cells to proliferative in the absence of endogenous estrogens [7-9, 12]. Previous studies have also suggested that these LBD mutants

reduce the efficacy of ER antagonists [15] and highlight the need to develop more potent and selective ER antagonists to improve overall patient outcomes.

Although patients with advanced ER-positive breast cancer initially respond to endocrine therapy with drugs such as Fulvestrant [17], many of these patients will ultimately progress while on treatment [32-34] potentially through heterogeneous resistance mechanisms [34-36]. The absence of *ESR1* LBD mutations detected in primary breast cancers has led to the theory that the selective pressure of endocrine therapy may promote the outgrowth of low-frequency sub-clones already harboring these mutations, and these cells exhibit reduced sensitivity to ERα antagonists [12, 37]. If these mutations are a result of clonal evolution, then more potent and orally bioavailable ER antagonists that are superior to Fulvestrant [18], or alternative combination therapy options need to be explored. Combination therapy of small molecule inhibitors of essential coactivators of ER-mediated cell proliferation, such as SRC-3 which is recruited by ERα [38], with ERα downregulators is one approach that has been proposed that may offer therapeutic potential for patients with tumors expressing *ESR1* mutants [39]. Similarly, other reports have shown some therapeutic potential in models of endocrine therapy resistant breast cancer co-treated with ER antagonists and inhibitors of cyclin dependent kinases 4 and 6 [40]. Further *in vivo* testing of ERD-148 and the development of more potent iterations of this compound will be needed before clinical trials can be conducted to determine the clinical benefit of ERD-148 alone or in combination with drugs inhibiting other oncogenic targets.

In conclusion, we have presented preclinical data characterizing a series of synthetic small-molecules designed via the PROTAC method for targeted chemical knockdown of ERα. Overall, compound ERD-148 demonstrated the greatest therapeutic potential in ER-positive breast cancer cell lines which was achieved by specifically antagonizing the ERα receptor.

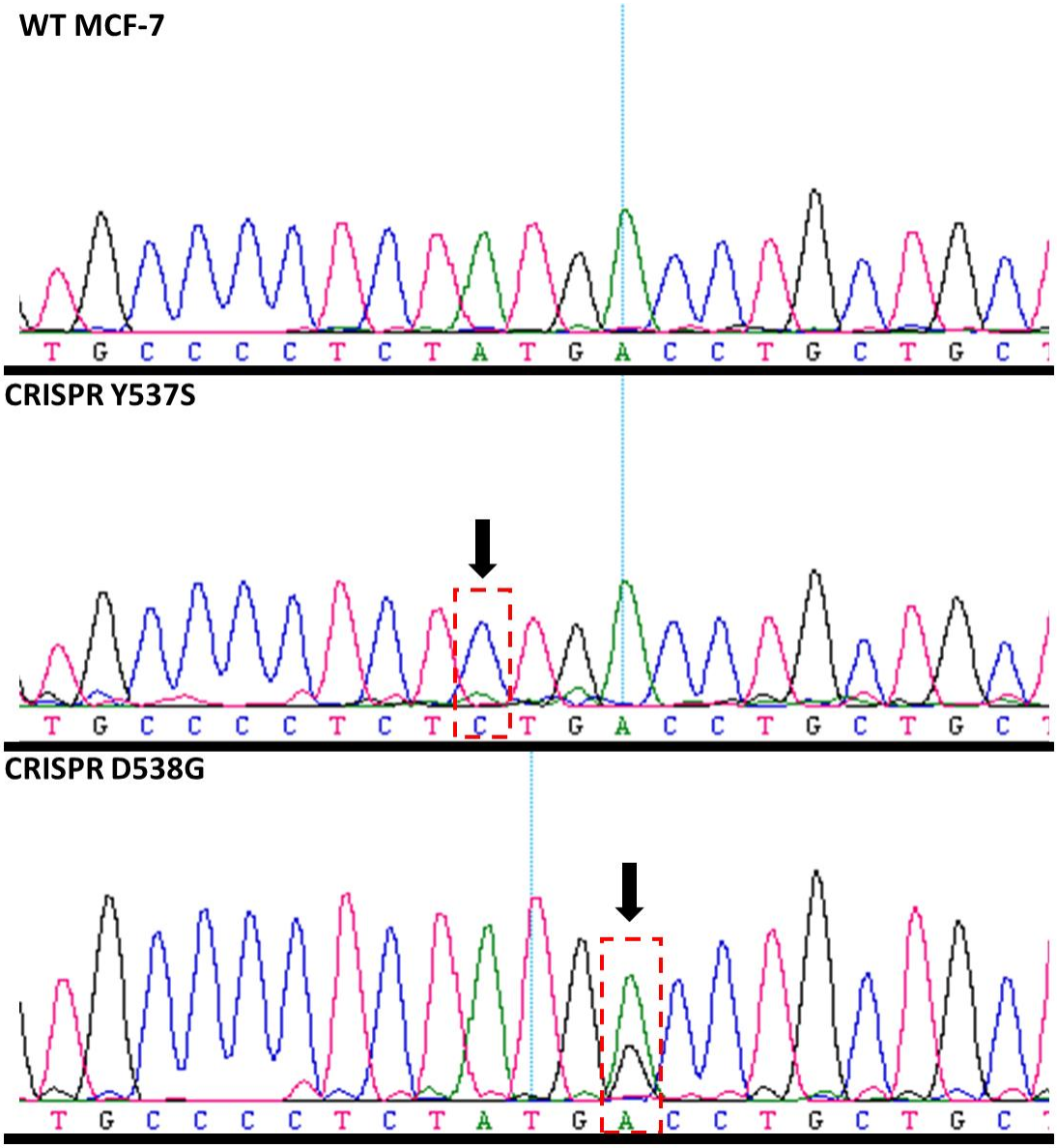

Figure 4.1. *ESR1* sequence alignment of exon 8 in WT MCF-7 and mutant cell lines. Sanger sequencing was performed on genomic DNA isolated from indicated cell lines as described in the Materials and Methods. Dashed red boxes and black arrows indicate location of mutated nucleotides in each labeled cell line. Alignment and visualization of Sanger sequencing results was performed using Lasergene SeqMan Pro version 14.0 for Windows (DNASTAR, Madison, WI).

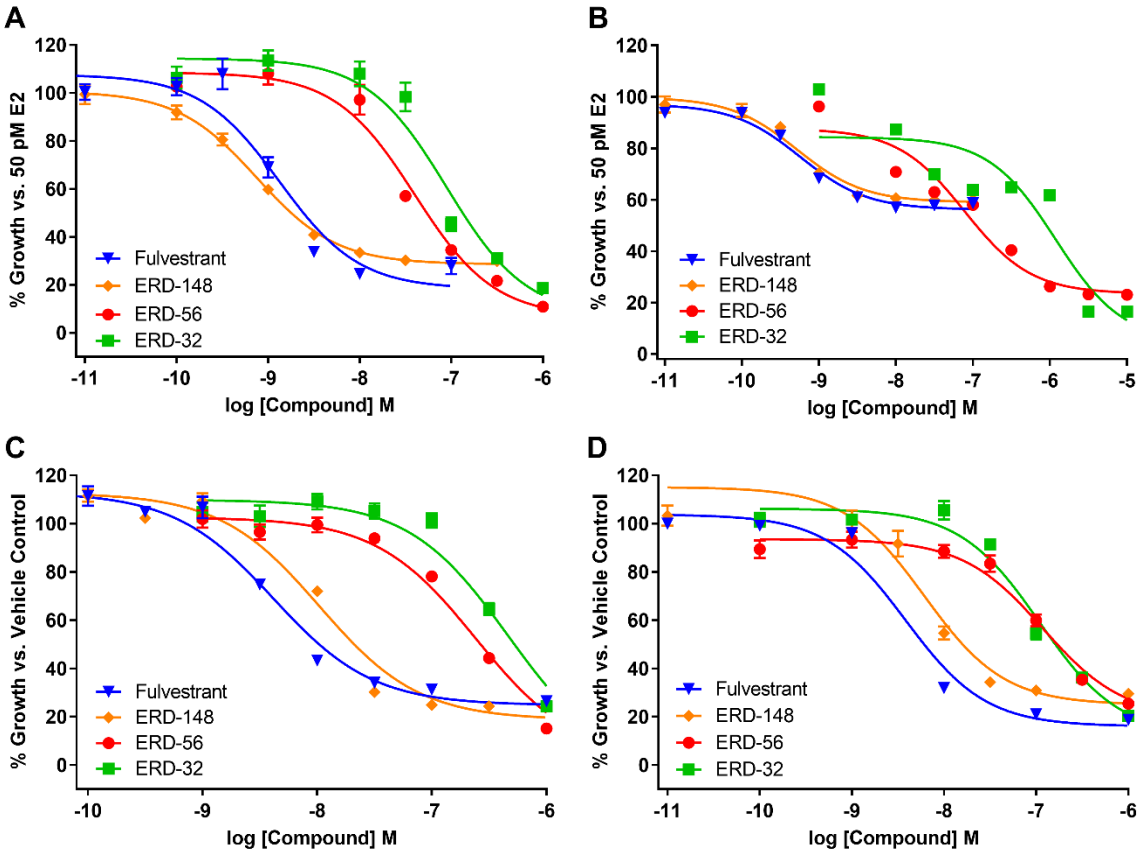

Figure 4.2. PROTAC ERα degraders inhibit the proliferative ability of ER-dependent breast cancer cell lines. (A) WT MCF-7, **(B)** T47D, **(C)** cY537S and **(D)** cD538G cells were grown in steroid-deplete media as described in the Methods section and treated with the indicated PROTAC degraders. *Growth curves* for A – D represent percentage of cell growth compared to 50 pM E2 treated WT MCF-7 or DMSO (vehicle) treated mutant MCF-7 as control (100 %). Points on dose response curve represent 5-day growth vs. control ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown).

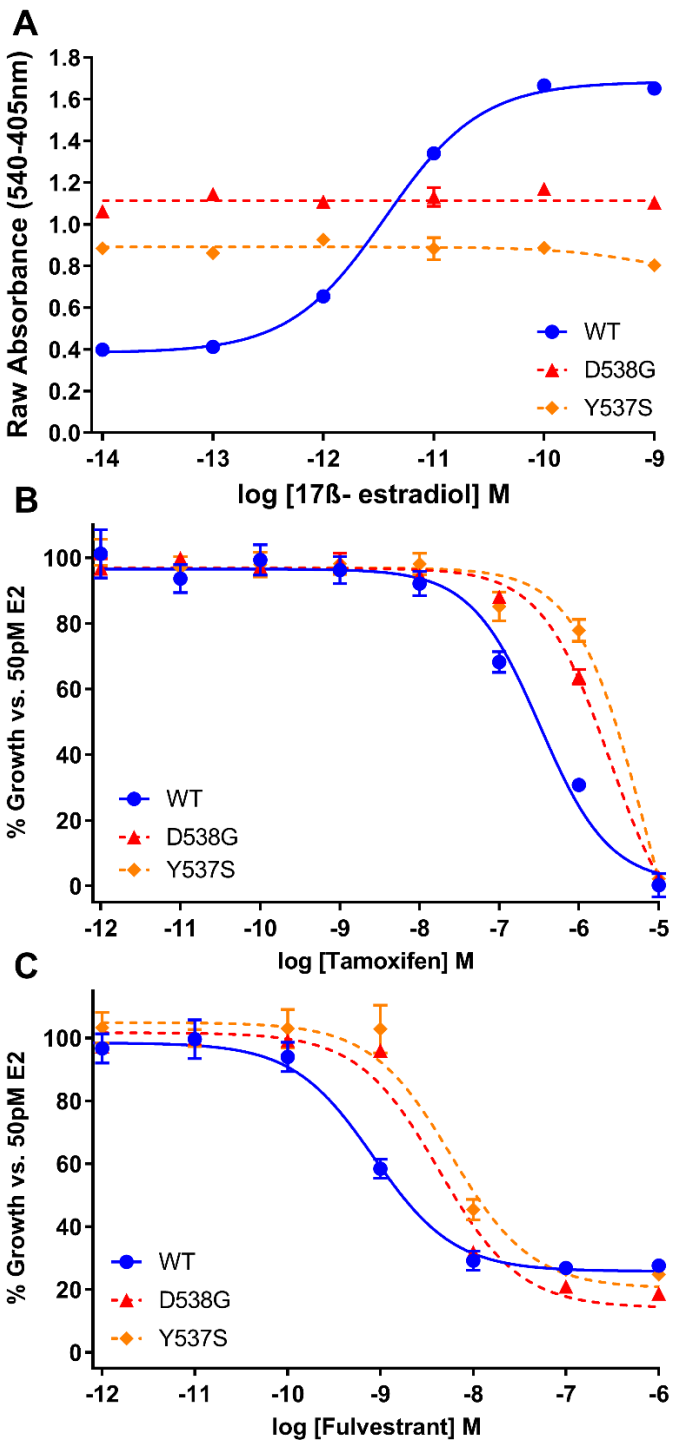

Figure 4.3. ERα mutants show evidence of estrogen independent growth and resistance to antiestrogens. WT MCF-7, cY537S, and cD538G cells treated with either **(A)** 17ß-estradiol, **(B)** tamoxifen, or **(C)** Fulvestrant. Cells were grown in steroid-deplete media as described in the Methods section. *Growth curves* for B and C represent percentage of cell growth compared to 50 pM E2 treated cells control (100 %). Points on dose response curve represent 5-day growth vs. control ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown).

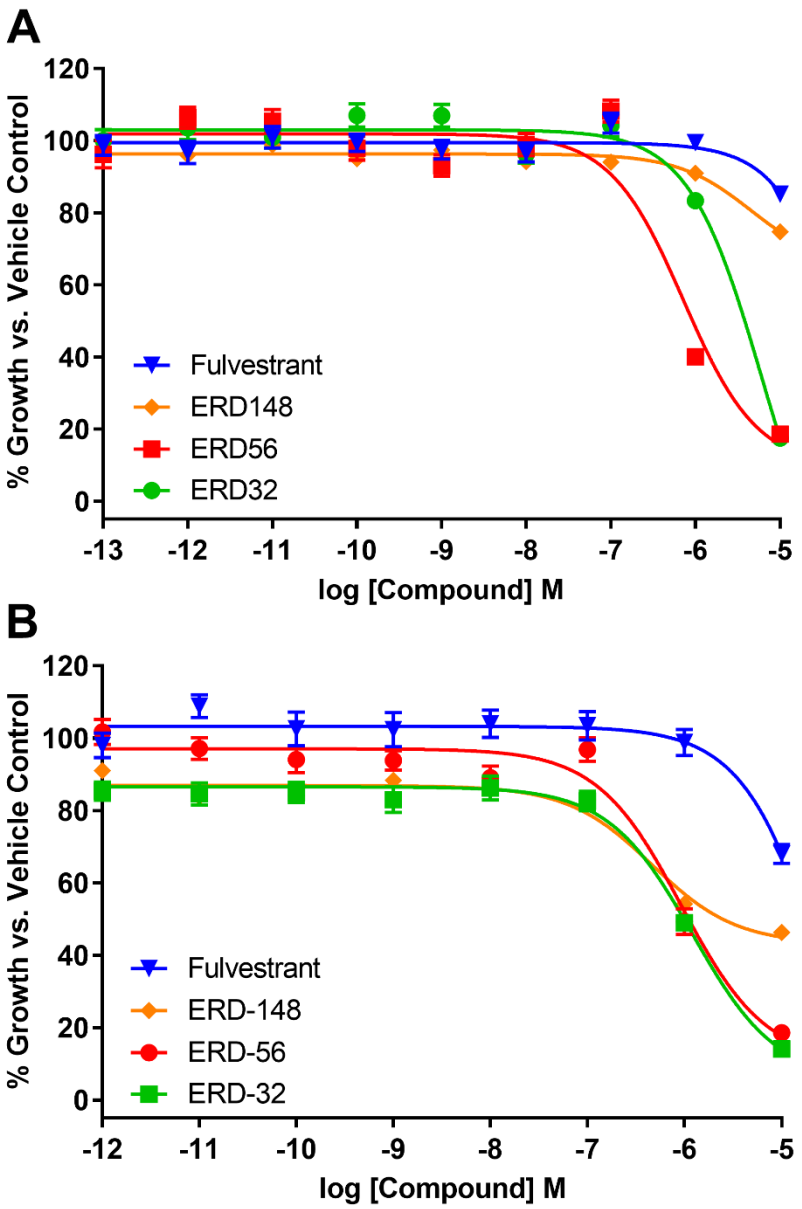

Figure 4.4. PROTAC ERα degraders demonstrate evidence of marginal non-specific toxicity in non-ERα expressing cell lines. (A) MDA-MB-468 and **(B)** MDA-MB-231 cells were grown in DMEM/5% FBS medium as described in the Methods section and treated with the indicated PROTAC degraders. *Growth curves* for A and B represent percentage of cell growth compared to DMSO (vehicle) control (100 %). Points on dose response curve represent 4-day growth vs. control ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown).

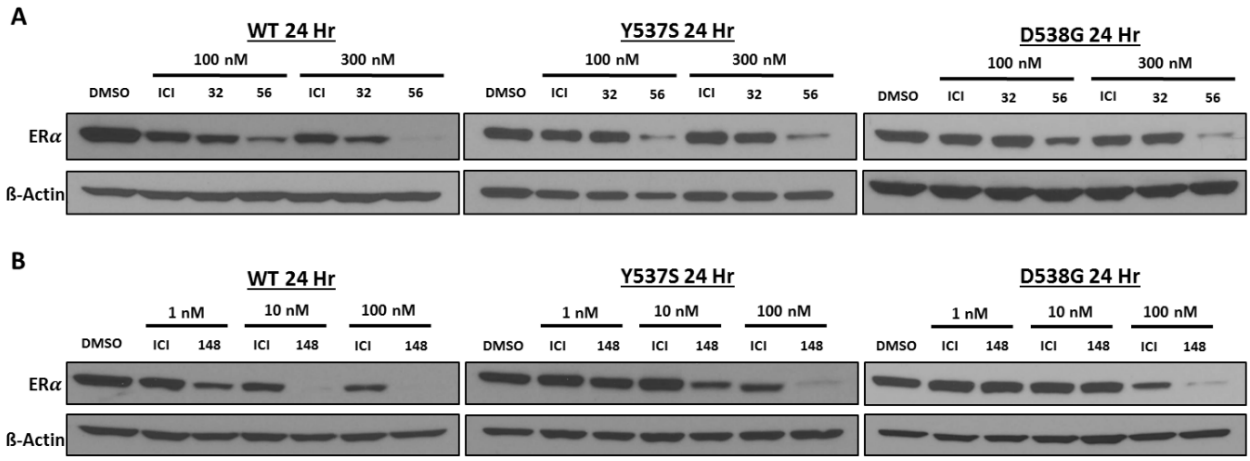

Figure 4.5. PROTAC ERα degraders downregulate the expression of ERα in WT MCF-7 and mutant cell lines. WT MCF-7, cY537S, and cD538G cells were grown in steroid-deplete media as described in Methods section. Relative ERα expression was determined by western blot assay and ß-actin was used as the loading control. **(A)** Cells were treated with either Fulvestrant (ICI), ERD-56, or ERD-32 for 24 hrs at the specified concentrations. **(B)** Cells treated with either ICI or ERD-148 for 24 hrs at the indicated concentrations. Representative data sets are shown.

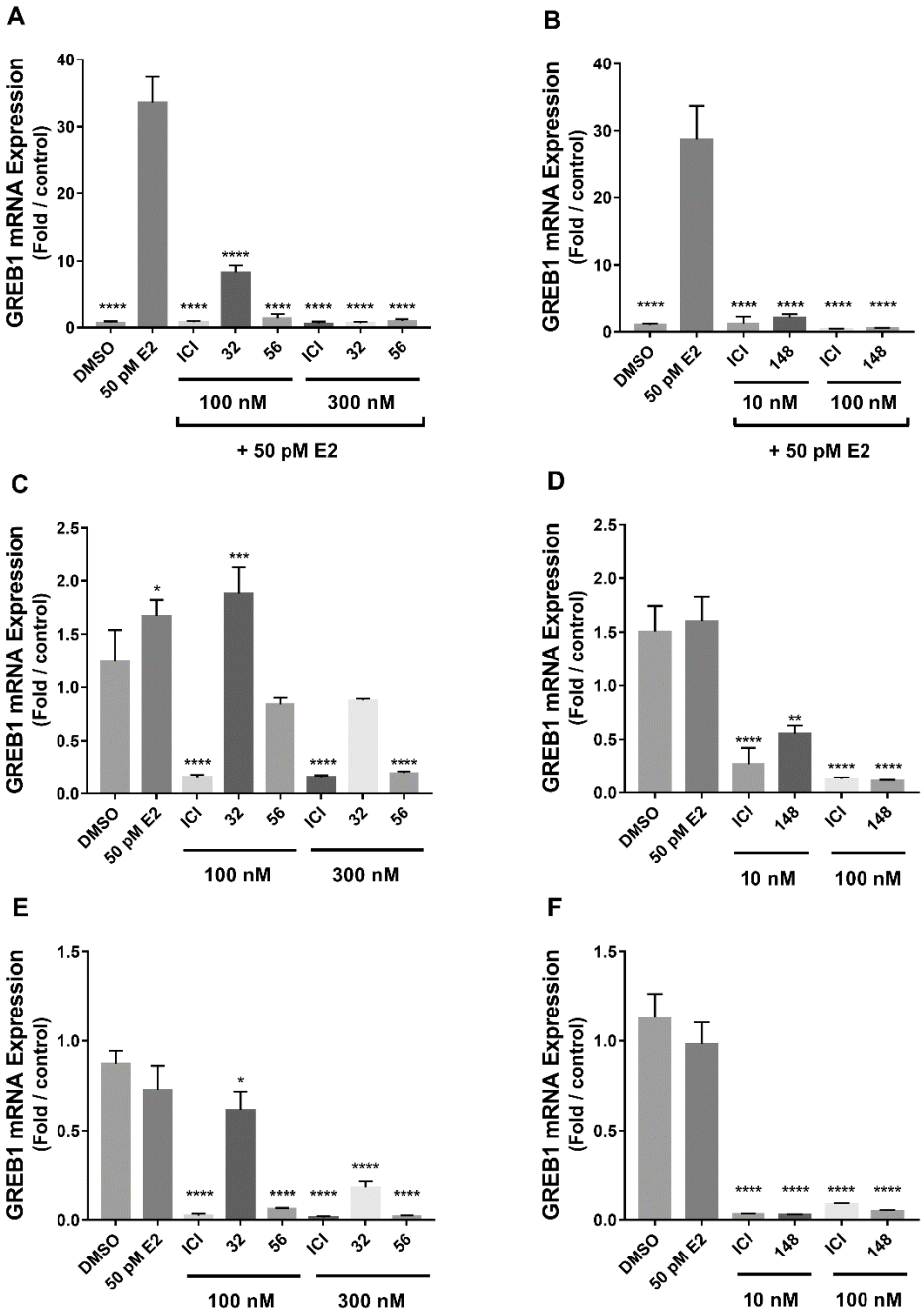

Figure 4.6. PROTAC degraders inhibit *GREB1* mRNA expression in WT MCF-7 and mutant cell lines. (A-B) *GREB1* mRNA expression for WT MCF-7, **(C-D)** cY537S, and **(E-F)** cD538G cells were assayed in steroid-deplete media after a 24 hr exposure to each specified compound. Cells were treated with a vehicle control (DMSO), 50 pM E2, Fulvestrant (ICI), or the indicated PROTAC degrader. *Bars* represent *GREB1* expression versus DMSO control for WT MCF-7, cY537S, and cD538G cells using the *ΔΔCT* method. Bars represent the mean from 3 technical replicates ± SE. Statistical significance was determined by one-way ANOVA followed by Tukey post-hoc analysis. Reference for significance is 50 pM E2 for panels *A – B* and DMSO for *C – F*. E2 (50pM) is required to stimulate the ER pathway in MCF-7 cells under steroid-deplete conditions. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

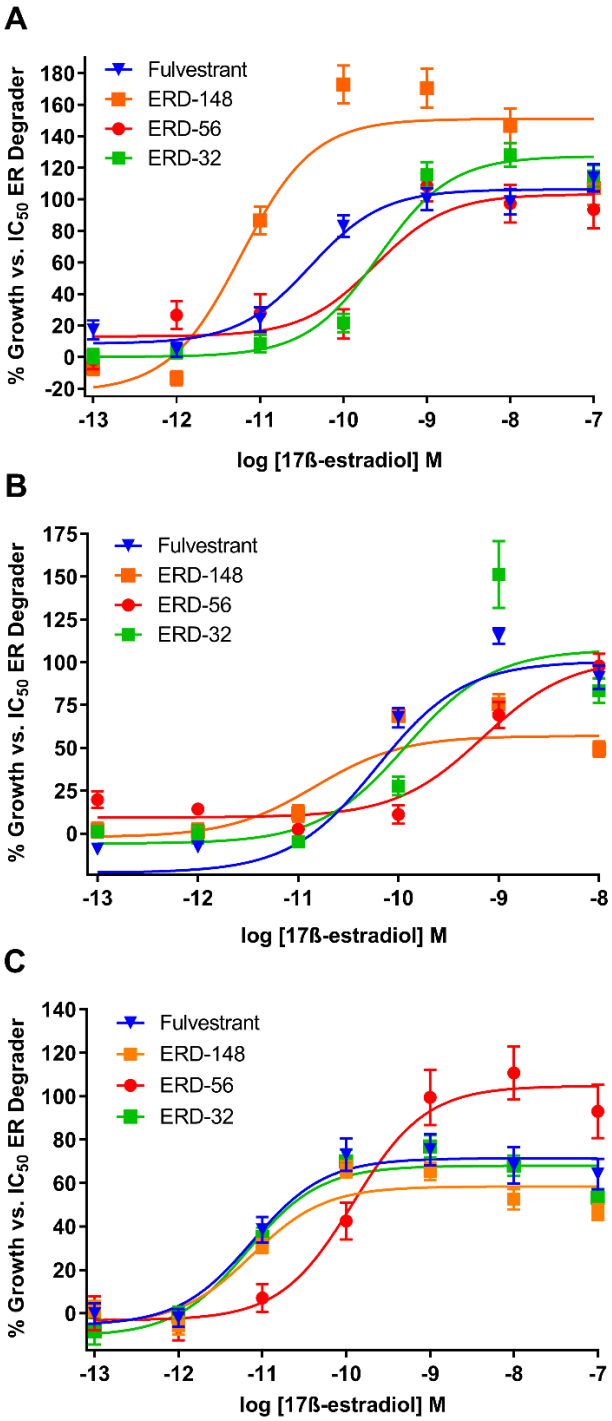

Figure 4.7. Addition of 17ß-estradiol competes with the PROTAC Degraders. (A) WT MCF-7, **(B)** cY537S, and **(C)** cD538G cells were grown in steroid-deplete medium as described in the Methods section. Each cell line was co-treated at a fixed dose of the identified IC_{50} for each PROTAC degrader and with the indicated concentrations for E2. *Growth curves* for A – C represent percentage of cell growth compared to IC_{50} of each degrader alone control (0 %). Points on dose response curve represent 5-day growth vs. control ± SE (n = 6 technical replicates). Each set of technical replicates were independently validated with a secondary experiment under identical treatment conditions (not shown).

Table 4.1 List of primer sequences for CRISPR-cas9 generation of *ESR1* LBD mutant cell lines.

Primers	**Sequences**
HDR forward cloning	5'-ATTGGTACCGGCAGCAGAGTTGTGGCTAGTGGAG-3'
HDR reverse cloning	5'-AAGTGTCGACCAGGGTGCTGGGCCAATTGTAGGAAC-3'
HDR internal sequencing primer	5'-TGAATGCATTTAGGTCCTAT-3'
Y537S mut Top	5'-GTGGTGCCCCTCTCTGACCTGCTGCTG-3'
Y537S mut Bottom	5'-CAGCAGCAGGTCAGAGAGGGGCACCAC-3'
D538G mut Top	5'-GTGGTGCCCCTCTATGGCCTGCTGCTG-3'
D538G mut Bottom	5'-CAGCAGCAGGTCAGAGAGGGGCACCAC-3'

Table 4.2 List of IC_{50} values for PROTAC degraders cell line.

IC_{50} (nM)	WT MCF-7	T47D	cY537S	cD538G	MDA-MB-231	MDA-MB-468
Fulvestrant	1.3	0.6	4.2	3.8	3667.0	NC
ERD-148	0.8	0.5	10.5	6.1	462.2	4630.0
ERD-56	39.9	77.8	242.4	118.0	928.0	724.0
ERD-32	89.0	1200.0	467.3	109.0	1208.0	6183.0

NC: Not calculable

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Chapter V.

Conclusions and Future Directions

Although adjuvant endocrine therapy is an effective treatment option for most ER-positive breast cancer patients, breast cancer recurrence is still an ongoing clinical problem [1]. Sikora *et al*. suggested that alternative estrogens, arising from androgen metabolism, may contribute to AI resistance and partially explain why a significant number of patients will experience a recurrence within 10 years of receiving adjuvant endocrine therapy [2]. The data presented by Sikora *et al*. is just one example that highlights the importance of identifying related estrogen mimics in the context of ER-positive breast cancer recurrence and AI resistance. Similarly, determining what role suspected EDCs may have on AI resistance and breast cancer progression is relevant to elucidating alternative mechanisms of breast cancer recurrence. For example, the detection of weak ER agonists in breast tissue, such as parabens [3, 4], has contributed to much of the controversy and debate regarding the suspected role of EDCs in breast cancer and endocrine disruption. Additionally, the discovery of commonly reoccurring *ESR1* LBD mutations in metastatic breast tumors may explain why endocrine therapy fails numerous patients and why they are associated with poor clinical outcomes [5].

To date, most toxicology studies have focused primarily on characterizing the estrogenic behavior of unmetabolized short-chain paraben compounds [6-8]. The research presented in this dissertation tested the hypothesis that recently identified urinary human metabolites of *n*-butylparaben (nBuP) and *iso*-butylparaben (iBuP) may also exhibit weak estrogenic behavior. We report that oxidized metabolites of nBuP and iBuP, 2-hydroxy *iso*-butyl 4-hydroxybenzoate

(2OH) and 3-hydroxy *n*-butyl 4-hydroxybenzoate (3OH), were found to exhibit weak estrogenic behavior *in vitro* [9]. The discovery of weak estrogenic behavior among these paraben metabolites are relevant in the context of risk assessment for several reasons. First, the 2OH and 3OH metabolites were originally detected in human urine samples at concentrations which were ~2.3 or 1.1 fold higher than their parent compounds for the metabolites, respectively [10]. Higher urinary concentrations of these metabolites are likely due to increased water solubility that would be attributed to their oxidative modifications as compared to the parent compounds which are relatively more lipophilic. Given that our data has shown that these metabolites are weak estrogens, these compounds might contribute to an equal or greater proportion of observed associations between other parabens and their associated adverse health outcomes [11-13]. Furthermore, the recent discovery of these metabolites implies that previous biomonitoring and exposure studies were unknowingly underreporting measured concentrations of nBuP and iBuP in humans. Therefore, future studies should attempt to account for the existence of these metabolites, either by direct measurement or the inclusion of a correction factor [10], when making conclusions about the extent of human exposure to nBuP and iBuP.

Likewise, it is unknown whether these urinary paraben metabolites are present in human adipose or breast tissue where their parent compounds have been detected [3, 14]. If it is determined that the 2OH and 3OH metabolites are also present in human tissues, such as the breast, *in vitro* studies should examine whether there is evidence of estrogenic additivity or synergism between the metabolites and the parent compounds. The need to investigate the potential combined estrogenic effect attributed to these metabolites is relevant considering the findings from a prior report that conducted mixture studies of paraben compounds in ER-dependent MCF-7 cells. This *in vitro* mixture study found that MCF-7 cells exposed to

representative breast tissue concentrations of parabens near their no observed effect concentrations could promote increased cell proliferation more effectively in combination with several parabens rather than as single compound exposures [15]. Thus, the potential combined exposure among the paraben metabolites and other parabens commonly detected in humans should not be overlooked in the context of risk assessment. Although it is possible that the influence of estrogenic EDCs is dependent upon the stage of ER-positive breast cancer and they may have a limited role in advanced disease. Further research is needed to conclusively demonstrate that local tissue concentrations of parabens are mechanistically involved in progression and recurrence of ER-positive breast cancer beyond their mere presence in these tissues.

Whether parabens are involved in disease progression of ER-positive breast cancer or not, further work is needed to identify uncharacterized EDCs and steroid hormone mimics for evidence of estrogenic behavior. Although most toxicology studies often rely on *in vitro* or rodent models to characterize and identify estrogenic compounds, this approach is inherently too time consuming and costly to screen the tens of thousands of compounds currently cataloged by the US EPA [16]. Recently, there has been considerable effort to minimize the use the rodents and animals and utilize *in silico*-based approaches for toxicological profiling. Although there is increasing acceptance of *in silico*-based approaches for compound screening and identifying estrogenic EDCs, efforts to develop *in silico* protocols are hampered due to the lack of reported structural data for mouse or rat ERα. To address this knowledge gap, we have demonstrated that *in silico* mutagenesis of a human template ERα-LBD is a valid approach to develop *in silico* models for unsolved structures for mouse and rat ERα-LBD.

Using known ERα agonists and a homologous series of paraben compounds, our *in silico* models of ERα-LBD produced molecular docking data that recognized both potent and weak ER agonists. These *in silico* models were also able to produce docking results that were used to sort the test compounds according to their predicted affinity for ERα, which agreed with their reported *in vitro* potencies towards ERα. Additionally, our novel application of the Sorenson similarity index suggested that these same ER agonists were predicted to make similar ligand-receptor residue contacts in both human and rodent ERα-LBDs. These findings not only validate the use of rodent models as a tissue source for identify estrogenic EDCs, but they also highlight the effectiveness of an *in silico* approach to characterize compounds for their predicted affinity towards ERα.

The use of *in silico* models, such as the ones reported here, demonstrate the potential to be applied in the development of safer and less estrogenic paraben compounds commonly used in the manufacture of consumer goods. For example, Bergquist *et al*. recently reported that 3,5-disubstituion of nBuP and octylparaben can simultaneously increase the antimicrobial potency of parabens while decreasing their affinity towards ERα [17]. To test the utility of our *in silico* models beyond our initial series of test ligands, we conducted additional docking studies with 15 3,5-disubstituted paraben compounds and compared their predicted affinity towards ERα with their *in vitro* potencies presented by Bergquist *et al*. The use of ligand efficiency metrics allowed us to directly compare the relative strength of ligand-receptor interactions between the Bergquist *et al. in vitro* data with our *in silico* docking results. Strikingly, we found a high correlation between the ligand efficiency data between the Bergquist *et al* experimental data and our *in silico* molecular docking results. These data suggest that our *in silico* models were not only

effective in predicting the relative affinity 3,5-disubstituted paraben compounds, but our models demonstrate the potential to applied in design of potentially safer preservative compounds.

In contrast to EDCs, the detection of commonly reoccurring *ESR1* LBD somatic mutations found in metastatic breast cancer patients may serve as another explanation for the issue of recurrence observed in ER-positive breast cancer. It has been reported that these LBD mutations are frequently detected in 30-40% of patients with advanced disease who have become resistant to endocrine therapy [18]. *In vitro* characterization has shown that cells expressing these LBD mutations appear to confer a drug resistant phenotype to compounds commonly used in the adjuvant setting to treat ER-positive breast cancer [19, 20]. This drug resistant phenotype is especially problematic for patients with advanced disease who are often treated with the selective estrogen receptor degrader (SERD) Fulvestrant. Currently, the effectiveness of Fulvestrant is limited to due to poor bioavailability and that it must be administered intramuscularly. Therefore, the presence of LBD mutations necessitates the development of orally bioavailable and potent SERDs to inhibit ER-dependent tumor cell proliferation. Fortunately, significant progress in synthetic chemistry has led to improvements in the Proteolysis Targeting Chimera (PROTAC) method to induce targeted degradation of specific proteins or receptors [21]. The PROTAC method works by allowing for the development of bifunctional small molecules designed to target and degrade proteins by hijacking the ubiquitin-proteasome pathway.

Thus, we tested the hypothesis that novel agents designed with the PROTAC method could achieve superior degradation of ERα as compared to current compounds used in endocrine therapy. We found that PROTAC degrader ERD-148 was able to achieve superior downregulation of ERα as compared to Fulvestrant in WT and mutant cells expressing LBD mutations. Overall, we have provided evidence which suggests ERD-148 inhibits the

proliferative ability of ER-dependent breast cancer cells by downregulating ERα and suppressing ER-dependent gene activation. Although ERD-148 has been designed to be an oral SERD, animal models are needed to establish the therapeutic potential of this compound relative to Fulvestrant or similar ERα degraders. Furthermore, additional studies are needed to develop more potent iterations of ERD-148 and evaluate their therapeutic potential for use in clinical trials of metastatic ER-positive breast cancer patients. Patients with advanced breast cancer, as well as those harboring *ESR1* mutations, would likely benefit the most from treatments with a compound like ERD-148 if it is shown to be more effective than Fulvestrant in *in vivo* models. However, a thorough toxicological profile for ERD-148 and safety assessment is needed prior to its use in clinical trials.

In summary, the findings presented in this dissertation have shown that: 1) human metabolites of nBuP and iBuP exhibit weak estrogenic behavior and should be considered as equally important components of total environmental exposure from paraben compounds; 2) *in silico* mutagenesis of a template receptor with high sequence similarity is an effective approach to model unknown receptors which also has substantial utility in manufacturing or drug development applications when comparing ligand-receptor interactions; and 3) inhibiting ER-dependent cell proliferation in breast cancer with novel agents designed via the PROTAC method demonstrate potential for effective treatment of patients with acquired resistance to endocrine therapy (Figure 5.1). Overall, these findings contribute to our understanding of EDCs that may be associated with breast cancer recurrence and highlights the utility of *in silico* models in identifying estrogen mimics prior to conducting and *in vitro* or *in vivo* study. We have also shown that novel agents designed with the PROTAC method could be used to develop more effective SERDs used in to treat patients with advanced breast cancer. Future studies will be

needed to determine whether exposure to estrogenic EDCs or the presence of *ESR1* mutations contribute to a greater risk of recurrence or poorer clinical outcomes specifically among patients with advanced disease.

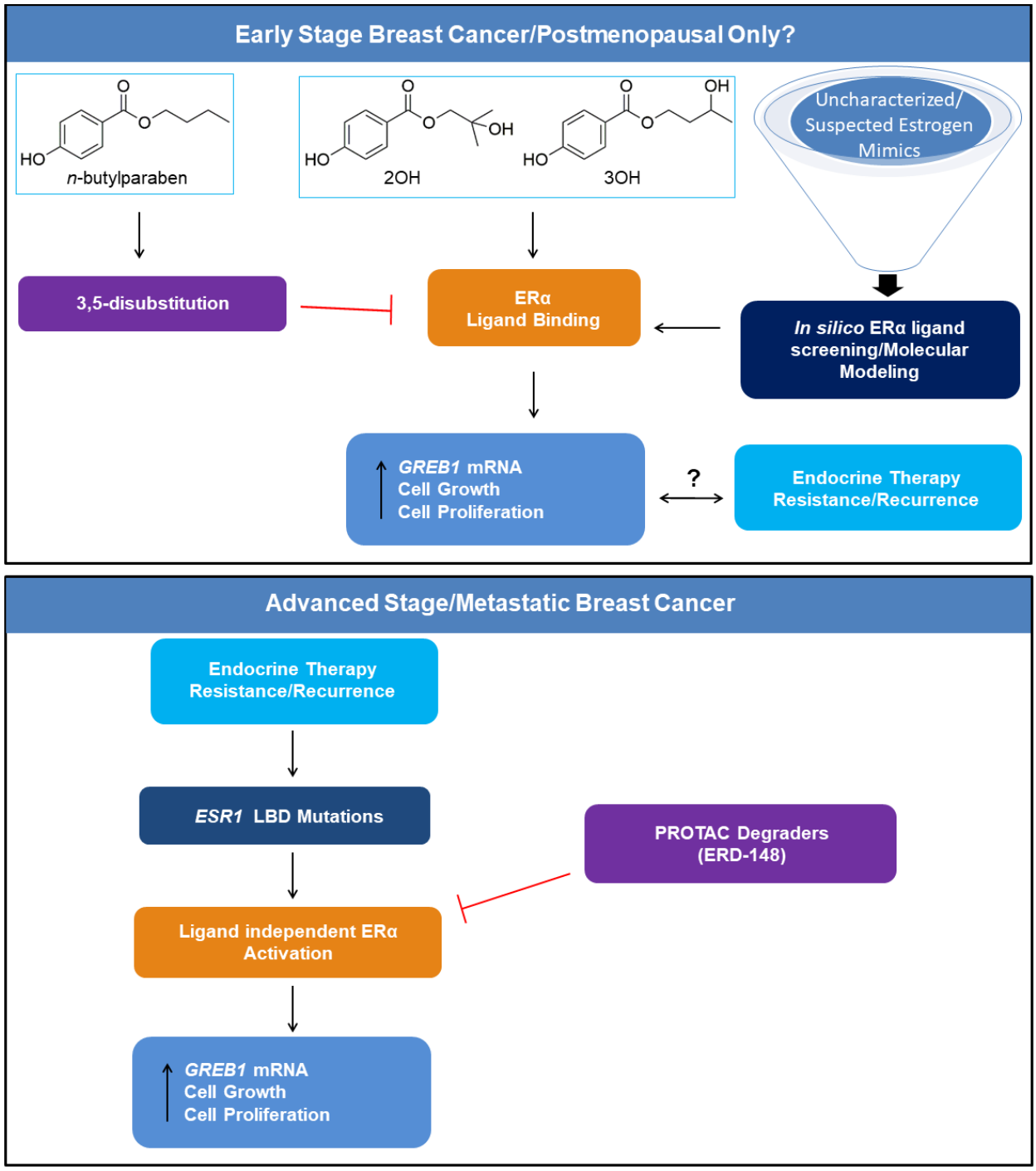

Figure 5.1. Conceptual Model of the role suspected of estrogen mimics and acquired resistance in ER-positive breast cancer. Paraben metabolites, 2OH and 3OH, bind to ERα and promote an increase in ER-dependent gene expression and cell proliferation. *In silico* experiments supported literature data regarding the use of 3,5-substitution to decrease the predicted binding affinity of n-butylparaben towards ERα. Similar suspected estrogen mimics can be prescreened with *in silico* models of human and rodent ERα-LBD with relatively high confidence. Targeted degradation of *ESR1* mutants inhibits ERα signaling and cell proliferation in models of ER-dependent breast cancer.

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Appendix

Evaluating the Association between Biomarkers of Parabens and Phenol Exposure and Recurrence in ER-Positive Breast Cancer Patients

Introduction

Breast cancer is currently the most commonly diagnosed malignancy among women in the United States (US) with an estimated 266,000 new diagnoses and 41,000 deaths in 2018 alone [1]. The ability of estrogen to induce breast cancer cellular proliferation is concerning because it is estimated that approximately 70% of all diagnosed breast cancers express the estrogen receptor (ER) and are deemed ER-positive [2]. Pharmacological approaches have been developed to block ER signaling using ER antagonists such as tamoxifen, and more recently aromatase inhibitors (AIs), which inhibit the synthesis of estrogens [3], have been used. Postmenopausal women with ER-positive breast cancer are typically given AIs in the adjuvant setting to inhibit the synthesis of estrogens produced in the peripheral tissues and adrenal glands [4]. This pharmacological approach works mechanistically by blocking the action of CYP19 aromatase which minimizes the stimulation of estrogen dependent breast tumors from endogenous estrogens [5]. Nevertheless, many women still experience disease recurrence for unknown reasons despite evidence of improvement in disease-free survival rates among postmenopausal women taking AIs with ER-positive breast cancer [6, 7]. For example, after five years of endocrine therapy, the recurrence rate among postmenopausal women with ER-positive breast cancer has been reported to continuously increase ~1-2% every year among those who have not recurred [7-9]. More recently, a meta-analysis of 88 clinical trials showed that the rate

of recurrence still increases ~1-2% every year for at least 20 years post treatment among ER-positive patients even when adjusting for several tumor characteristics [10]. It has been suggested that non-classical estrogens resulting from androgen metabolism could contribute as potential mechanism of AI resistance and as a potential explanation for recurrence in ER-positive patients [5]. Alternatively, resistance to antiestrogen therapy for patients on AIs could also be attributed to exposure from environmental estrogen mimicking chemicals, or xenoestrogens [5, 11].

Recently, there has been increasing public awareness and research into environmental chemicals that may have biologically relevant, endocrine disrupting properties [12]. Xenoestrogens include many of these suspected endocrine disrupting compounds that have previously been shown to display agonistic behavior toward estrogen receptor-α (ERα) *in vitro* [13, 14]. Parabens are one class of xenoestrogen that have been investigated for whether current human exposure levels are a cause for concern [15]. Given their antimicrobial properties, parabens are frequently used as a preservative in numerous pharmaceuticals, food products, and personal care products [16, 17]. One report has measured five different paraben compounds in breast mastectomy samples taken from patients with ER+PR+ primary tumors where it was shown that 27% of breast tissue samples contained at least one measurable paraben compound that was above its lowest-observed-effect concentration as determined in ER-positive MCF-7 cells [18]. The same report also showed that representative concentrations of paraben compounds measured in breast tissue could promote cellular proliferation of ER-positive MCF-7 cells in combination whereas many these same compounds could not do so when tested alone [18].

Due to ubiquitous exposure of the public to xenoestrogens like parabens, it is possible that exposure to these compounds could explain part of the reason why adjuvant chemotherapy

can fail certain breast cancer patients taking drugs such as aromatase inhibitors. Although AIs have been proven to be effective in treating ER-positive breast cancer, their inherent design and mechanism of action cannot inhibit the weak agonist behavior from exposure to environmental sources of estrogen mimicking compounds, or xenoestrogens. Postmenopausal women taking AIs with ER-positive breast cancer could be susceptible to further disease progression and recurrence due to their individual exposure level of xenoestrogens such as parabens. It is possible that the combined exposure of parabens and some of their metabolites may be a contributing factor for disease recurrence in ER-positive breast cancer patients on anti-estrogen therapy, such as AIs.

Despite evidence demonstrating the presence of parabens in several human tissues, there is still significant debate over their current risk to the general population [15]. To provide insight into whether weak ERα agonists like parabens may contribute to a greater risk for recurrence among ER-positive patients on anti-estrogen therapy, we conducted a pilot study comparing urinary paraben and phenol exposure between non-metastatic, ER-positive patients currently on anti-estrogen therapy with samples from metastatic ER-positive patients the moment disease progression was determined. Our hypothesis is that ER-positive breast cancer patients who have recurred will have significantly higher levels of paraben or phenol exposure compared to breast cancer patients who have not recurred. The concentration of several parabens and phenols was determined in these samples and we evaluated whether there was an association between urinary paraben and phenol exposure with breast cancer progression.

Materials and Methods

Study participants

All participants were patients with a diagnosis of ER-positive and HER2-negative (ER+/HER2-) breast cancer after 2005 who were enrolled into an ongoing prospective study at the University of Michigan Cancer Center. All participants were given informed consent and urine samples were de-identified prior to analysis. Cohort 1 consisted of patients (n=20) with stage II or III hormone receptor positive breast cancer (ER+ and/or PR > 1%) and HER2-negative tumor type who were diagnosed after 2005 who are at a high-risk for recurrence, have completed surgery, chemotherapy, radiation therapy, and show no indication of disease recurrence while on anti-estrogen therapy for at least 2-3 years. Patients in cohort 1 who progressed to metastatic disease since enrollment or had another systemic malignancy within the previous five years of recruitment were excluded from analysis. Participants in cohort 1 were asked to provide a baseline urine sample at the time of enrollment. Each patient in cohort 1 was repeatedly followed up with in 2-3 month intervals for a maximum of 36 months to determine whether there was evidence of tumor progression. Cohort 2 consisted of patients (n=11) who were diagnosed with ER+/HER2- metastatic breast cancer with high disease burden who have documented disease progression. Patients in cohort 2 who were diagnosed with another active systemic malignancy within the last five years were excluded. Urine samples were collected from patients in cohort 2 at the time progression was determined by their medical oncologist.

Urinary paraben and phenol measurement

All urine samples were collected and aliquoted into 1.5 mL Eppendorf tubes with EDTA preservative solution and stored at –80 °C until analysis by NSF International (Ann Arbor, MI). Twelve phenols and parabens, comprising bisphenol-A (BPA), bisphenol-S (BPS), bisphenol-F (BPF), benzophenone-3 (BP3), triclosan (TCS), triclocarban (TCC), 2,4-dichlorophenol (2,4-

DCP), 2,5-dichlorophenol (2,5-DCP), *n*-butylparaben (BuP), ethylparaben (EP), methylparaben (MP), and propylparaben (PrP), were measured in urine at NSF International (Ann Arbor, MI) using isotope dilution–liquid chromatography–tandem mass spectrometry (ID–LC–MS/MS) as described previously [19]. Sample concentrations with a zero value were substituted with the second lowest concentration measured for that analyte. Urinary creatinine was measured at the Michigan Diabetes Research Center (MDRC) (Ann Arbor, MI) on a Randox RX Series Daytona chemistry analyzer. The instrument was calibrated using 0.9% NaCl as zero calibrator, Randox Calibration Serum Level 2, and Randox Calibration Serum Level 3. The Randox Calibration Serum Level 2 and 3 are traceable to creatinine reference materials NIST 909b and NIST 967. Cliniqa Bi-Level Liquid QC Urine Chemistry Controls are also analyzed with each run for quality control. The Intra assay precision (%CV) was determined at three levels (n=88): 2.1% (51 mg/dL), 2.1% (102 mg/dL), and 1.5% (203 mg/dL). The Inter assay precision (%CV) was determined at three levels (n=88): 3.0% (51 mg/dL), 3.0% (102 mg/dL), and 2.8% (203 mg/dL).

Data Analysis

All models were adjusted for with available demographic or tumor characteristics. Covariates that were considered included: recurrence status (binary), creatinine concentration (continuous, mg/dL); age (continuous, years); and body mass index (BMI, kg/m2). Multiple linear regression models were fit using the following format: Concentration ~Recurrence Status + Age + BMI. For all models, the concentration outcome variable was defined as log (analyte concentration). A total of 31 participants were included in final regression analyses. Statistical significance was assumed at $p < 0.05$ level. All analyses were performed using R Studio Version 1.1.383.

Results

Population characteristics and exposure

Available demographic and tumor characteristics of the breast cancer patients in the pilot study are presented in Table A1. The average age for all patients was 58 years, all determined to be ER+HER2-, most determined to be node positive (90%), and identifying as white or Caucasian (94%). A total of 31 urine samples were analyzed for twelve different parabens and phenols as described in the *Methods*. Concentrations of each paraben or phenol measured from urine samples are shown as per *g* of urinary creatinine with the medians and means for each compound displayed in Table A2. BPS was not detectable in any of the urine samples analyzed. TCC concentrations were found to be below the LOD for all analyzed samples. Parabens and phenols that were detected in less than 80% of samples included BPF (29% detection), TCS (48% detection), EP (48% detection), and BuP (29% detection) as summarized in Table A2.

Paraben phenol exposure and breast cancer progression

Patients with clinically determined disease progression were observed with 3 to 6 fold higher, but non-significant, BPA median and mean concentrations respectively as compared to non-progressing patients after adjusting for urinary creatinine, age, and BMI ($p = 0.498$) (Table A3). Patients who were progressing were also found to have significantly lower urinary BP3 concentrations than the non-progressing group after adjusting for urinary creatinine only (Table A3). Disease progression was not determined to show a meaningful statistical association with any of the other urinary parabens or phenols listed in Table A3.

Discussion

Exposure from suspected endocrine disrupting compounds, such as phthalates [20] and polychlorinated biphenyls (PCBs) [21, 22], have been associated with an increased risk for breast cancer with limited reports examining the same relationship for paraben compounds. Additionally, paraben exposure has not been reported in the context of breast cancer recurrence whereas exposure to PCB118 and total PCB exposure, measured in adipose tissue, have previously been shown to be associated with an increased risk for recurrence [23]. To examine the potential association between paraben exposure and breast cancer recurrence, we measured the concentration of several parabens and phenols in banked urine samples from non-metastatic breast cancer patients and samples from metastatic patients at time disease progression was determined. Several of the analytes measured were determined to show concentration ranges that were fairly consistent with prior publications with the exception of BPA being nearly 10 to 100 fold higher than female non-cancer patients [19, 24]. However, multiple linear regression analysis did not produce any meaningful statistical associations for any of the parabens or phenols included in urine analysis. BPA median urine concentrations were found to be several fold higher in patients with disease progression but this observation was still not found to be significant after adjusting for urinary creatinine, age, and BMI.

There are several limitations and issues with the original urine sample collection which likely explain the non-statistically significant associations found in this pilot study as well as why many samples were determined to be below the LOD. Since these banked urine samples were initially collected for a separate ongoing clinical study with alternative biomarkers of interest, we did not have control over how these urine samples were collected where the urine was stored in large volumes of EDTA upon collection. Furthermore, logistical issues prevented

onsite adjustment in the EDTA to urine volume ratio during sample collection which prevented us from knowing the exact dilution for each sample and subsequent non-uniform EDTA to urine ratios across all the samples analyzed. This processing step ultimately resulted in highly dilute samples among the metastatic patients and with generally more analyte measurements with concentrations below the LOD relative to the non-metastatic patients. Another unavoidable factor was that these samples underwent several freeze-thaw cycles by the time urine concentrations were determined and likely explain why BPS could not be detected in any of the provided samples. Of the four paraben compounds measured, EP and BuP were detected less frequently in our samples which is consistent with prior publications [25] but the EDTA dilution may be a greater contributing factor as to why many of these measurements were found to be below the LOD. Parabens are also known to be metabolized and excreted within several hours [26] and may be subject to temporal variability [27, 28] which could not be accounted for during initial urine collection.

Due to the density of EDTA and the large volume of it used in the sample collection, we could not measure the specific gravity of our samples for normalization. Therefore, we had to rely on urinary creatinine as a means to standardize our samples before determining any statistical association. For the statistical analysis, we only had access to limited demographic information about each of the enrolled patients which hampers our ability to adjust for other variables such as income, level of education, socioeconomic status or similar characteristics during the analysis stage. Given the issue of several measurements being below the LOD for some of our analytes and the limited number of samples from patients with the outcome of disease progression, it is difficult to make meaningful conclusions about this data set. However, the observation of BPA exposure being several fold higher in the patients with progression is a

phenomenon worth exploring in a larger prospective study that is not underpowered which is the case of our pilot study reported here.

Table A1. Population characteristics of ER+/HER2- breast cancer patients in study.

Characteristic	All	Non-metastatic	Metastatic Progressions
Number	31	20	11
Age at enrollment (years) [mean ± SD (range)]	57.5 ± 10.1 (40-85)	54.0 ± 10.4 (40-85)	64.1 ± 5.2 (55-75)
Race [*n*(%)]			
White or Caucasian	29 (93.5)	18 (90)	11 (100)
Asian	1(3.2)	1(5)	0 (0)
Missing	1(3.2)	1 (5)	0 (0)
BMI [mean ± SD (range)]	28.4 ± 7.7 (15.8-48.3)	29.1 ± 8.4 (18.5-48.3)	27.1 ± 6.3 (15.8-37.1)
ER+/HER2- [n(%)]	31 (100)	20 (100)	11 (100)
Tumor size [n(%)]			
≤ 2 cm	8 (25.8)	5 (25)	3 (27.2)
≥ 2 cm	20 (64.5)	15 (75)	5 (45.5)
Missing	3 (9.6)	0 (0)	3 (27.2)
Axillary nodal status [n(%)]			
Node positive	28 (90.3)	20 (100)	8 (72.7)
Node negative	1 (3.2)	0 (0)	1 (9.1)
Missing	2 (6.4)	0 (0)	2 (18.2)

Table A2. Creatinine-corrected urinary paraben and phenols concentrations (µg/g Creatinine).

Urinary Paraben/Phenol Concentrations		**All Patients**	**Non-Metastatic** (n=20)				**Metastatic Progressions** (n=11)			
Compounds (µg/g Creatinine)	LOD	% ≥ LOD (All)	% ≥ LOD	Mean	SEM	Median	% ≥ LOD	Mean	SEM	Median
BPA	0.4	94	100	152.36	32.99	124.03	82	971.89	576.22	467.79
BPF	0.4	29	30	0.80	0.09	0.72	27	3.38	1.81	1.28
BP3	0.4	84	100	85.02	63.51	4.57	55	5.52	1.42	3.84
TCS	2.0	48	60	25.02	12.28	3.96	27	46.91	43.98	2.60
TCC	2.0	0	0	0.24	0.04	0.16	0	0.55	0.12	0.43
24-DCP	0.2	81	90	0.70	0.08	0.64	64	1.62	0.50	0.74
25-DCP	0.2	87	90	1.20	0.23	0.97	82	1.75	0.43	1.22
MP	1.0	100	100	248.47	85.75	125.09	100	85.20	23.23	51.80
EP	1.0	48	65	100.37	37.17	4.64	18	21.25	13.86	1.36
PrP	0.2	100	100	48.08	16.44	13.59	100	25.73	10.86	9.85
BuP	0.2	29	35	0.91	0.34	0.29	18	1.02	0.50	0.34

Table A3. Associations between urinary paraben or phenol concentrations of ER+/HER2- breast cancer patients and progression status. Multiple linear regression models adjusting for log(creatinine concentrations), age and BMI are shown.

ß (Status)	**Model 1:** Log(Phenol)=Status			**Model 2:** Log(Phenol)=Status+ log(CR)			**Model 3:** Log(Phenol)=Status+ log(CR)+ Age			**Model 4:** Log(Phenol)=Status+ log(CR)+ Age+ BMI		
Compound	**ß**	**95% CI**	**p-value**	**ß**	**95% CI**	**p-value**	**ß**	**95% CI**	**p-value**	**ß**	**95% CI**	**p-value**
MP	-0.606	-1.123, -0.083	**0.025**	-0.188	-0.730, 0.353	0.482	-0.116	-0.736, 0.503	0.704	-0.115	-0.749, 0.518	0.711
PrP	-0.462	-1.107, 0.184	0.154	-0.054	-0.764, 0.654	0.876	0.068	-0.741, 0.877	0.865	0.060	-0.762, 0.881	0.882
BPA	-0.017	-0.848, 0.816	0.969	0.082	-0.909, 1.073	0.866	0.381	-0.729, 1.492	0.487	0.379	-0.755, 1.513	0.498
25-DCP	-0.181	-0.389, 0.026	0.084	-0.052	-0.280, 0.176	0.647	-0.129	-0.383, 0.125	0.306	-0.129	-0.388, 0.130	0.317
24-DCP	-0.135	-0.308, 0.038	0.121	-0.067	-0.267, 0.133	0.497	-0.049	-0.278, 0.181	0.666	-0.053	-0.283, 0.176	0.642
BP3	-0.799	-1.361, -0.237	**0.007**	-0.734	-1.403, -0.064	**0.033**	-0.514	-1.261, 0.233	0.170	-0.512	-1.275, 0.250	0.179
BPF	0.036	-0.230, 0.303	0.782	0.235	-0.048, 0.517	0.100	0.222	-0.103, 0.546	0.172	0.225	-0.104, 0.554	0.171
BuP	-0.243	-0.620, 0.135	0.199	-0.092	-0.529, 0.344	0.667	-0.174	-0.671, 0.324	0.480	-0.184	-0.679, 0.311	0.451
EP	-0.925	-1.743, -0.107	**0.028**	-0.709	-1.673, 0.255	0.143	-0.659	-1.767, 0.449	0.233	-0.669	-1.795, 0.457	0.233
TCS	-0.524	-1.093, 0.045	**0.070**	-0.171	-0.798, 0.456	0.580	-0.063	-0.778, 0.652	0.859	-0.072	-0.795, 0.650	0.838
TCC	0.012	-0.163, 0.187	0.892	0.052	-0.155, 0.258	0.614	0.056	-0.181, 0.294	0.630	0.062	-0.172, 0.297	0.590

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