Functional Characterization of Cytochrome P450 17A1 (CYP17A1) Gene Variants for their Steroidogenic Enzymatic Activities

by

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Dedication

To my Grandpa Phil

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ACTH	Adrenocorticotropin
AI	Aromatase inhibitor
AR	Androgen receptor
CAH	Congenital adrenal hyperplasia
CRH	Corticotropin releasing hormone
CCS	Charcoal stripped calf serum
CRPC	Castrate resistant prostate cancer
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DOC	Deoxycorticosterone
E1	Estrone
E1S	Estrone sulfate
E2	Estradiol
ER	Estrogen receptor
FBS	Fetal bovine serum
GnRH	Gonadotropin-releasing hormone
GREB1	Gene regulated by estrogen in breast cancer 1
HPLC	High-performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
ICI	ICI 182,780; fulvestrant
LC-MS/MS	Liquid chromatography/tandem mass spectrometry
P4	Progesterone
PCOS	Polycystic ovary syndrome
SNP	Single nucleotide polymorphism
STS	Steroid sulfatase
SULT	Sulfotransferase
ZG	Zona glomerulosa
ZF	Zona fasiculata
ZR	Zona reticularis

Chapter I.

Introduction

Steroid hormones and their precursors are synthesized and extensively metabolized primarily in the adrenals and gonads of healthy men and women [1]. These steroid products are secreted into the systemic circulation and exert their physiological effects by: 1) binding to their cognate receptors in target tissues and initiating signaling pathways required for cellular growth and sexual maturation, and 2) acting as substrates for further metabolism to active hormones, which then act on target tissues. The testes and the ovaries primarily synthesize testosterone or estradiol, respectively, which promote the development of secondary sexual characteristics, enable reproduction, and serve additional functions in the skeleton, brain, and other organs.

Among the most common malignancies in humans are prostate cancer in men and breast cancer in women, neoplasias of epithelial cells in glands whose development is driven by sex-specific gonadal steroids [2]. In many cases, these gonadal steroids fuel the growth and progression of these tumors, and hormone-deprivation therapies are used with or without surgery as first-line treatments. Unfortunately, these cancers often demonstrate either *de novo* resistance to hormonal therapies or subsequently acquire compensatory mechanisms to proliferate despite castrate concentrations of androgens and estrogens in the circulation. Here, I will review the current state of knowledge on how tumors obtain and synthesize these steroids, approaches to study the acquisition of resistance to treatment, and the rational for my thesis.

Normal Physiology

The Hypothalamic-Pituitary Adrenal Axis

Under the regulation of higher brain centers, neurons in the paraventricular nucleus of the hypothalamus release corticotropin-releasing hormone (CRH) [3] into the portal circulation, which stimulates adrenocorticotropin (ACTH) secretion from the corticotrope cells in the anterior pituitary [4]. ACTH binds to its extracellular receptor on cells of the adrenal cortex to stimulate the synthesis of cortisol and androgen precursors [5], which are not stored but are continuously released in the systemic circulation. Cortisol exerts negative feedback on CRH and ACTH production, achieving homeostasis. Aldosterone production is primarily under the control of a separate axis, renin-angiotensin-aldosterone system.

Adrenal Steroidogenesis

The adrenal glands are responsible for the synthesis of mineralocorticoids, glucocorticoids, and small amounts of androgens but relatively large amounts of androgen precursors. Specifically, within the adrenal gland, the adrenal cortex cells express steroidogenic enzymes and cofactor proteins in a zone-specific manner (Figure 1). The adrenal cortex is comprised of the three zones, each expressing their own complement of proteins necessary for efficient synthesis of a dominant steroid product. The zona glomerulosa (ZG) expresses the enzymes necessary for aldosterone synthesis, while the zona fasiculata (ZF) primarily synthesizes cortisol. The zona reticularis (ZR) is the adrenal zone responsible for the production of androgens and estrogens under the stimulation of ACTH, but these cells primarily synthesize androgen precursors. The ZR is characterized by very little 3β-hydroxysteroid dehydrogenase/isomerase (3βHSD)

expression in the adult human. Consequently, steroid synthesis mostly follows the Δ^5-pathway from pregnenolone to dehydroepiandrosterone (DHEA) [6,7], which is sulfated and exported as dehydroepiandrosterone sulfate (DHEAS). DHEAS is the predominant circulating 19-carbon androgen precursor steroid, with a plasma concentration of about 10 μmol/L throughout most of adult life but declining progressively after about age 60 [8].

Cholesterol is the sole precursor for all steroid hormone synthesis. Steroid synthesis begins with the steroidogenic acute regulatory (StAR) protein aiding in the translocation of cholesterol from a pool in the outer mitochondrial membrane to the inner mitochrondrial membrane. The mitochondrial cytochrome P450 (CYP) cholesterol side chain cleavage enzyme (P450scc, CYP11A1) cleaves the bond between the 20-22 carbons of cholesterol through a series of 3 oxygenation reactions. The final product of this reaction is the 21-carbon, Δ^5-steroid pregnenolone, which is the common initial precursor for downstream synthesis of mineralocorticoids, glucocorticoids, and sex steroids. Pregnenolone is a substrate for both 3βHSD and steroid 17-hydroxylase/17,20-lyase (P450c17, CYP17A1). 3βHSD is the enzyme responsible for converting pregnenolone to its 21-carbon, Δ^4-steroid congener, progesterone [9]. CYP17A1 is a bifunctional P450 that catalyzes two major reactions within the endoplasmic reticulum of steroidogenic cells. CYP17A1 can hydroxylate the 17-carbon of progesterone and pregnenolone to form their 17-hydroxy products, 17OH-pregnenolone or 17OH-progesterone [10-12]. These 17-hydroxy products are substrates for other enzymes in their further metabolism to cortisol, or they are further metabolized by CYP17A1's second function, which is the 17,20-lyase activity. This 17,20-lyase activity cleaves the C-C bond between carbons 17 and 20 of the aforementioned 17-hydroxy substrates to form the 19-carbon androgen precursors DHEA (major

Δ^5-pathway) or androstenedione (minor Δ^4 pathway). CYP17A1's 17,20-lyase activity is enhanced by the coexpression of cytochrome b_5 (CYB5A), which allosterically stimulates this reaction [13,14]. While the ZR expresses both CYB5A and CYP17A1, the ZF expresses only CYP17A1 [15,16]. This zone-specific expression of CYB5A helps to explain why the ZF primarily synthesizes the 21-carbon steroid cortisol, while the ZR synthesizes large amounts of 19-carbon androgens and their precursors.

Using human adrenal vein samples, Nakamura et al. showed that testosterone is synthesized in small amounts in the human adrenal [17]. Type 5 17β-hydroxysteroid dehydrogenase (17βHSD5 or AKR1C3) has been implicated as the steroidogenic enzyme responsible for catalyzing the limited conversion of androstenedione to testosterone in the ZR. Microarray analysis and qPCR studies confirmed that the ZR expresses AKR1C3 mRNA and protein. Knockdown of AKR1C3 via siRNA in the human adrenal H295R cell line reduced testosterone production by 40% compared to scrambled control siRNA [17]. These data highlight the potential for direct adrenal testosterone synthesis beyond the well-known production of 19-carbon androgen precursors, which are metabolized to active androgens in peripheral organs and target tissues.

The Hypothalamic-Pituitary Gonadal Axis

With the onset of puberty, loss of repression from higher brain centers allows neurons in the arcuate nucleus of the hypothalamus to resume the pulsatile secretion of gonadotropin-releasing hormone (GnRH) every 90-120 minutes. This GnRH enters the portal circulation and stimulates pulsatile release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotropes in the anterior pituitary. Pulsatile secretion is critical for

reproductive function, because constant exposure to GnRH down-regulates is receptor on gonadotropes and thwarts axis function [18]. In males, LH acts on the testicular Leydig cells to stimulate testosterone synthesis, and in peripheral tissues, steroid 5α-reductases convert testosterone to the more potent androgen, 5α-dihydrotestosterone (DHT). In women, LH acts on the ovarian theca cells and, to a lesser extent, the granulosa cells, to drive androgen synthesis, but the ovary lacks 17βHSD type 3, the enzyme that most efficiently converts androstenedione into testosterone. In the ovary, FSH induces the expression of the aromatase (P450aro, CYP19A1) enzyme, which converts androstenedione and testosterone from the theca cells to the estrogens estrone (E1) and estradiol (E2) [19], as well as 17βHSD type 1, the specific 17βHSD isoform that efficiently converts E1 to E2 [20]. In men, FSH acts on the Sertoli cells to facilitate spermatogenesis. In both males and females, androgens and estrogens exert negative feedback on GnRH and LH production [21]. FSH production is primarily under the tonic negative feedback of inhibin B, a protein produced in the Sertoli and granulosa cells [22].

Gonadal Steroidogenesis

Although the ZR in the adrenal cortex produces less sex steroids than the gonads and large amounts of their precursors, the primary site of sex steroid synthesis is the gonads, using the same enzymes and pathways to get as far as DHEA. Similar to the adrenal cortex, the theca and granulosa cells of the ovaries express their own host of different steroid-metabolizing enzymes that orchestrate the synthesis of specific steroids (Figure 2A). Immunohistochemical (IHC) studies in human ovaries by Sasano and colleagues revealed high CYP11A1 and CYP17A1 in the theca interna cells adjacent to the developing follicles but found CYP19A1 expression confined to the granulosa cells [23]. Therefore, the granulosa cells are responsible for estrogen

synthesis and secretion by way of aromatizing the androgens produced in the ovarian theca cells. In the testes, only the Leydig cells express CYP17A1 (Figure 2B), and Leydig cells are the only human cells that normally express the androgenic 17βHSD3, which efficiently converts 19-carbon, 17-ketosteroids to active androgens, such as androstenedione to testosterone.

Peripheral Steroid Metabolism

Despite interventions that prevent gonadal hormone secretion, sufficient amounts of androgens and estrogens may remain in the circulation to activate their respective receptors [24,25]. As discussed above, the adrenal glands produce very small amounts of testosterone and estradiol directly, yet the adrenal is a source of abundant 19-carbon androgen precursors such as DHEAS. Even a small portion of orally administered DHEA is converted to testosterone, indicating that tissues other than the adrenals and gonads possess the enzymatic machinery to complete the pathways to androgens and estrogens. One major reason for this capacity for extra-gonadal hormone generation is the redundancy of key enzyme activities. While the gonads and adrenals primarily express 3βHSD type 2 [9], the liver, skin, and other tissues contain a second, highly homologous isoenzyme, 3βHSD type 1 [26]. The human 17βHSD family includes at least 14 isoenzymes, each with its characteristic spectrum of activities and tissue-specific expression patterns [27]. Even steroid 5α-reductase activity derives from the type 1 and type 2 isoenzymes, with 2 genes bearing different ontogenies [28]. Among the AKR1C enzymes, all isoforms possess both 17βHSD and 3αHSD activities, which interconvert active and inactive hormones in peripheral and target tissues. The complexity of peripheral steroid metabolism provides a conduit for active hormones, and these hormones can drive breast and prostate cancer progression despite strategies to suppress gonadal steroid synthesis.

An early demonstration of the importance of peripheral hormone synthesis is in the estrogen dependence of most breast cancers in postmenopausal women, who lack ovarian-derived estrogens [29]. In an attempt to pinpoint the source of postmenopausal estrogen production, Grodin et al. analyzed plasma samples from 6 postmenopausal women and measured the conversion of androstenedione to E1, the predominant estrogen in postmenopausal women. [30] Patients were administered [^{14}C]-androstenedione, and subsequent conversion to E1 was measured in urine samples. Because the investigators were able to attribute nearly all of the measured E1 to the administered [^{14}C]-androstenedione, they concluded that peripheral aromatization of androstenedione is the primary source of postmenopausal E1, as opposed to being of ovarian or gonadal origin.

Nuclear Hormone Receptor Signaling

Androgen receptor (AR) and estrogen receptor (ER) are steroid receptors and members of the protein superfamily known as nuclear hormone receptors [31]. These steroid receptors exist most commonly as unbound monomers in a dynamic equilibrium between the nucleus and cytoplasm under the regulation of heat-shock and other chaperone proteins [32]. The receptors possess a unique ligand-binding domain (LBD) [33], and upon ligand binding, these receptors dissociate from the chaperone complex and undergo characteristic conformational changes that promote receptor homodimerization [34]. These ligand-activated receptor dimers translocate to the nucleus, where they bind to cognate response elements on DNA and initiate the transcription or repression of genes involved in growth and development. The recruitment of accessory

proteins known as coactivators and corepressors to the transcription start site aid in the determination of which genes are expressed or repressed [35]. These coregulator proteins represent a potential strategy for further modulation of nuclear-hormone receptor signaling.

Estrogens and Breast Cancer

Estrogen Receptor and Breast Cancer

In the later part of the 19th century, surgeons began performing bilateral oophorectomies to treat women with breast cancer, and Dr. George Beatson was a prominent early investigator [36]. Although there were differing opinions on the rationale for why this treatment was successful, the general consensus was that the ovaries secreted factors that promoted tumor growth. Over the next 100 years, our knowledge of these factors—primarily E2, its cognate receptor (ER), and ER signaling—has led to considerable advancements in treating women with ER-positive breast cancers [37].

Over 231,000 new cases of invasive breast cancer will be diagnosed this year in the United States [2]. Over two-thirds of these cases will express ER ("ER-positive tumors"), and for these patients with ER-positive cancers, hormonal manipulation reduces the risk of recurrence or death, particularly in postmenopausal women [38,39]. Drugs that antagonize estrogen action are effective treatments for patients with metastatic disease and clearly reduce breast cancer mortality when given in the adjuvant setting [37,40-42]. These data are consistent with Beatson's success performing oophorectomies in premenopausal women, which led to research over the following 40 years exploring the sources of estrogens in postmenopausal women and subsequent strategies to block estrogen synthesis and ER signaling in breast cancer.

Lippmann and colleagues first reported the importance of ER in breast cancer *in vitro* in the early 1970s. Using breast cancer cell culture models, specifically the ER-positive, E2-dependent MCF-7 cells, they demonstrated increased cellular proliferation by measuring DNA, RNA, and protein synthesis after E2 treatment. In addition, they showed that competitive inhibition of E2 binding to ER using the antiestrogen tamoxifen blocked the E2-induced effects [43,44].

Pathways of Estrogen Synthesis in Breast Cancer

In the absence of functional ovaries, the adrenals were suspected as the source of estrogens in postmenopausal women. Adrenalectomy or hypophysectomy were modestly successful in these patients with remission rates between 25-50%, and "medical adrenalectomy" with aminoglutethimide showed similar efficacy [45]. Nevertheless, the adrenal gland is known to produce abundant DHEAS, but not E2. It is now recognized that peripheral adipose tissue expresses CYP19A1 and contributes to circulating estrogens in the postmenopausal setting [46]. A study using reverse transcription polymerase chain reaction (RT-PCR) showed that *CYP19A1* mRNA expression levels in fat from the buttocks, thighs, and abdomens of postmenopausal women were 2-4 times higher than those observed in young women [47]. Indeed, *CYP19A1* mRNA is highly expressed in breast adipose and breast epithelial tissues, and tissue concentrations of E2 are approximately twice as high in breast tumor tissue compared to normal tissue [48], consistent with the local aromatization of adrenal-derived precursors.

In addition to the CYP19A1-mediated aromatization of androgens in peripheral tissues, a sulfatase enzyme has also been implicated in contributing to the delivery of E2 precursors to tumors [49] (Figure 3). The steroid sulfatase (STS) enzyme removes the sulfate group of estrone

sulfate (E1S) to yield E1. E1 can then be converted to E2 via 17βHSD1, which is also expressed in many of the same peripheral tissues as CYP19A1 [50]. An analysis of STS expression and function in breast cancer revealed that STS activity is higher in breast tumor tissue compared to healthy controls and that E1S and E2 were also elevated in breast tumor tissue [48]. The hydrolysis of the sulfate group is reversible, as local expression of sulfotransferases (known as SULTs) can repeat the sulfonation reaction. Over 44 SULT isoforms have been discovered, but only a handful of these sulfonate steroids. Notable SULTs include SULT1E1 (estrogens) and SULT2A1 (nonaromatic steroids.) Given that the risk of developing breast cancer is highly associated with endogenous sex hormone levels, particularly E2, E1, and E1S [51], this pathway represents a source of estrogens contributing to breast cancer progression.

Estrogen Receptor Antagonists

Two major pharmacological approaches have been developed to block the action of estrogen: 1) direct competition with estrogen for ER binding (e.g. tamoxifen and fulvestrant), and 2) blocking the production of estrogen in post-menopausal women (e.g. letrozole, anastrozole, and exemestane). Both of these approaches have been shown to reduce disease recurrence and prolong survival in postmenopausal breast cancer patients with ER-positive disease [37,42]. Although the use of ER expression in breast cancers is essential to determine if a patient should receive any form of endocrine therapy, there is no other biomarker to further personalize the type of endocrine therapy that should be administered.

The first successful approach to targeting estrogen's action in breast cancer was the development of antiestrogens [52]. Tamoxifen is an ER antagonist, or more precisely, a selective estrogen-

receptor modulator (SERM), because it has tissue-specific estrogenic and anti-estrogenic effects. SERMs, including tamoxifen, can be ER agonists or ER antagonists depending on tissue expression of the nuclear regulatory proteins (coactivators and corepressors) that regulate the expression of estrogen receptor-regulated genes [35]. Therefore, it is an effect of the recruited coregulatory proteins that mediates a SERM's pharmacologic activity. The composition of coregulatory proteins in complex with ligand-bound ER appears to be ligand specific and determined by receptor conformation [53]. Endogenous ligands such as E2 induce a different conformational change [54] than a SERM like tamoxifen [55], and therefore, recruit different coregulatory proteins to the site of DNA binding within the nucleus. Tamoxifen has been shown to reduce disease recurrence and to prolong survival in both premenopausal and postmenopausal women with ER-positive breast cancer as well prevent breast cancer in high-risk women [56]. Several of its metabolites, including 4-hydroxytamoxifen [57] and endoxifen [57,58], are also ER antagonists, which are even more potent than tamoxifen itself. However, one of the major drawbacks of SERMs is that their tissue-specific properties can lead to off-target effects by acting as ER agonists in other tissues. For example, tamoxifen acts as an agonist in bone [59] as well as in the uterus and endometrium [60], where ER agonism by SERMs can lead to endometrial hyperplasia and cancer [60]. In addition, tamoxifen therapy carries a similar risk of venous thrombosis as other estrogen therapies [56].

A second class of estrogen antagonists is the selective estrogen-receptor downregulators (SERDs). SERDs differ in their mechanism of action from SERMs in that they promote the degradation of ER protein [61], whereas SERMs like tamoxifen still allow for ligand-bound receptor to bind to DNA within the nucleus. Fulvestrant is the only FDA-approved SERD that is

used clinically; however, its clinical use is limited because it must be administered via intramuscular injection as opposed to an orally administered antiestrogen like tamoxifen. This drawback has led to the development of newer orally bioavailable SERDs, some of which are currently being tested in early Phase I and II clinical trials for the management of ER-positive breast cancer [62].

Aromatase Inhibitors

A third approach to treating ER-positive breast cancer is to block the production of E2 by inhibiting CYP19A1 [63]. Pharmacological inhibition of CYP19A1 was first achieved with aminoglutethimide (AG) [25]. Trials comparing AG to tamoxifen demonstrated similar efficacy in each treatment arm, but AG therapy was associated with worse side effects [64]. Despite its ability to inhibit estrogen synthesis, AG lacks selectivity for CYP19A1 and requires hydrocortisone replacement. These properties limited the use of AG for the treatment of ER-positive breast cancer and illustrated the need for more selective aromatase inhibitors (AIs). The first rationally designed AIs were mechanism-based substrate analogs, including 4-hydroxyandrostenedione, testolactone, 10-propargylestr-4-ene-3,17-dione, and exemestane [65-67]. Second- and third-generation inhibitors are azole-based non-steroidal compounds with high affinity and irreversible binding to the heme iron of the enzyme, including fadrozole, anastrozole, and letrozole. Of these, exemestane, anastrozole, and letrozole are used clinically in the adjuvant setting to treat ER-positive breast cancer.

Two large Phase III clinical trials compared the efficacy of tamoxifen to an AI, alone or in combination. The ATAC (Arimidex Tamoxifen Alone or in Combination) trial showed that in

postmenopausal women with localized breast cancer, AI therapy was superior to tamoxifen over the course of 5 years of treatment. Anastrazole (trade name Arimidex) significantly prolonged disease-free survival and significantly reduced distant metastases compared to tamoxifen [68]. The Breast International Group (BIG) 1-98 trial compared the efficacy of the AI letrozole to tamoxifen and again showed that AI therapy is superior to tamoxifen in postmenopausal women with ER-positive breast cancer [69]. The letrozole-treatment arm showed significantly increased progression-free survival and also a reduced incidence of distant metastases compared to tamoxifen [69]. The ATAC and BIG 1-98 trial data resulted in the adoption of AIs as the standard of care for postmenopausal women with ER-positive tumors.

Androgens and Prostate Cancer

Androgen Receptor and Prostate Cancer

The observation that prostate gland development is absent in 46,XY individuals with complete androgen insensitivity and steroid 5α-reductase type 2 deficiency firmly established the dependence of prostate growth on androgens [70]. Nearly all prostate cancers express AR, and prostate hyperplasia is androgen-dependent. Androgen deprivation therapy (ADT) was first described as a viable treatment option for prostate cancer in the early 1940s. Huggins and Hodges reported that removal of the testes (orchiectomy) promoted prostate tumor regression [71,72]. ADT causes tumor regression or stabilization in the majority of patients; however, a substantial number on of patients experience disease relapse months to years later. Originally, prostate-cancer recurrences during ADT were assumed to be "androgen-independent," but several groups have shown that androgen-dependent genes are expressed in relapsing tumors and their metastases [73]. Hence, this clinical condition has been renamed "castration-resistant

prostate cancer" (CRPC), and most prostate cancer deaths are due to CRPC [74]. Among the possible mechanisms of resistance include amplification or over-expression of AR, which makes the receptor more sensitive to lower levels of circulating androgens [75]; gain-of-function mutations in AR, which render the receptor "promiscuous" and activated by host of other steroids including AR antagonists [76]; and the acquisition of mechanisms to either produce androgens *de novo* or by limited metabolism of circulating precursor steroids [77].

Pathways of Androgen Synthesis in Prostate Cancer

Belanger et al. observed that castration in adult males reduced circulating testosterone and DHT levels to approximately 5-10% of their pre-castration values. Of note, however, is that castration had no effect on adrenal 19-carbon androgen precursors such as DHEA, DHEAS, and androstenedione [78]. A more recent study by Titus et al. examined patient samples to better understand androgen signaling in recurrent prostate cancers upon progression during ADT. By comparing recurrent prostate tumor tissue to androgen-stimulated benign prostate tissue, they noted similar concentrations of testosterone but 91% lower amounts of DHT in the recurrent tumor tissue compared to control [24]. It is believed that these remaining concentrations of DHT are still sufficient to activate AR and induce cancer growth.

Given the abundance of DHEAS in the circulation and the limited number of steps to testosterone (3) or DHT (4) via redundant pathways, adrenal-derived 19-carbon steroids and their metabolism have received considerable study as a mechanism driving CRPC. In some prostate cancer cell lines and tumor xenografts, DHEA stimulates growth similar to that of testosterone, but only if converted to Δ^4-metabolites. The limiting enzyme in this conversion to active

androgens is 3βHSD [79], but in prostate cancers, the major species is generally the type 1 isoenzyme rather than the type 2 found in the adrenal and testis. Inhibitors of 3βHSD shift the dose-response curve for DHEA in proportion to the enzymatic blockade [80]. In 2013, a common allelic variant of the *HSD3B1* gene was reported to increase enzyme stability and to prevent proteasomal degradation. The prolonged half-life of the 3βHSD1-N367T variant results in greater amounts of DHT synthesis from DHEA compared to wild-type enzyme [77]. In human CRPC metastases, the selection pressure leads to over-representation of this allele, and the presence of this variant portends poor prognosis. The 3βHSD1-N367T variant has major implications for prostate cancer, as its increased expression can promote increased androgen synthesis from adrenal-derived precursors.

While the conversion of testosterone to the more potent androgen DHT is required for normal prostate development and prostate hyperplasia, the importance of DHT in prostate cancer is not as clear. Of the two 5α-reductase isoenzymes, the type 2 (SRD5A2) is the principal enzyme expressed in the normal or hyperplastic prostate tissue as well as genital skin, where it catalyzes the synthesis of DHT in the fetus during male sexual development. The type 1 isoenzyme (SRD5A1) is normally expressed in the liver and all other skin; however, SRD5A1 is also the predominant isoenzyme in prostate cancers [28]. While both isoenzymes have broad substrate specificity for most 21-carbon and 19-carbon Δ^4-steroids, their relative efficiencies for various substrates varies somewhat, particularly under castrate conditions when circulating testosterone concentrations are low. In prostate cancer cell lines and tumor xenografts, Chang et al. demonstrated that SRD5A1 converts androstenedione—derived from DHEA via 3βHSD—to 5α-androstanedione, which is then converted to DHT via 17βHSD-mediated catalysis. This

alternative pathway to DHT, which bypasses testosterone as an intermediate, appears to be the dominant route to DHT from circulating adrenal-derived 19-carbon steroids in CRPC [81]. More recent studies using metastatic tumor samples from patients have confirmed this pathway to DHT and characterized its impact in men with prostate cancer, who had stopped responding to traditional AR antagonists. In addition to this pathway, another alternative or "backdoor pathway" to DHT involves the SRD5A1-catalyzed 5α- and subsequent 3α-reduction of 21-carbon steroids, which then undergo cleavage via the 17,20-lyase activity of CYP17A1 to androsterone [82,83]. Androsterone undergoes 17βHSD-catalyzed reduction to 5α-androstane-3α,17β-diol and then 3αHSD-catalyzed oxidation to DHT. Evidence for contributions from these alternate pathways to DHT, neither of which use testosterone as an intermediate, in the progression of CRPC derive from several laboratories and independent studies.

In addition to further metabolism of gonadal and adrenal precursors, other studies show that androgens can derive *de novo* from the CRPC tumor itself (Figure 4). Dillard et al. showed that, in cell culture models of prostate cancer that have been passaged to mimic an androgen-deprived state, the expression of steroidogenic enzymes necessary for intracine testosterone synthesis are increased [7]. Thin-layer chromatography (TLC) analysis suggested that these cells could convert radiolabeled cholesterol into testosterone, presumably due to higher expression of steroidogenic enzymes not present in the parental prostate cancer cells. Montgomery and colleagues confirmed these findings by extensively characterizing which androgen signaling mechanisms are still present in human tissues of those with CRPC. They also identified several steroidogenic enzymes that are upregulated in CRPC tumor metastases compared to the primary tumor tissue, including CYP17A1, 3βHSD1, 17βHSD3, and CYP19A1 [84].

Targeting of Androgen Synthesis and Action in Prostate Cancer

Long-acting gonadotropin-releasing hormone (GnRH) agonists and antagonists achieve medical castration by suppressing LH release and thus ablating testicular androgen synthesis. Long-acting GnRH agonists such as leuprolide acetate produce an initial surge in LH and testosterone, then disrupt the pulsatile stimulation of pituitary gonadotropin receptors, resulting in receptor desensitization. GnRH antagonists such as degarelix competitively inhibit GnRH binding and do not produce an initial hormone surge; chronically, both treatments decrease LH and testosterone concentrations to castrate levels. GnRH analogs are the cornerstone of ADT in prostate cancer, and these drugs have been used also for ovarian estrogen suppression in premenopausal women with breast cancer [85-87]. Although GnRH agonists and antagonists effectively ablate most androgen and estrogen production in the tissues primarily responsible for sex steroid production, these drugs do not block adrenal steroid synthesis or intracrine steroid production.

Androgen-receptor antagonists directly inhibit ligand binding to AR [88,89]. Because testosterone and DHT have such high affinity (~1 nM) for AR, the early generations of anti-androgens were not sufficiently potent to block all androgen action and showed limited efficacy in men with CRPC. Flutamide and bicalutamide bind to AR with affinities approximately 30-fold less than DHT. These drugs bind AR in the cytoplasm and inhibit ligand binding but still permit nuclear translocation. A next-generation, more potent AR antagonist is enzalutamide, which has a much higher affinity for AR compared to older drugs and also prevents nuclear translocation [90]. Enzalutamide treatment after chemotherapy in men with CRPC resulted in a 4.8-month increase in overall survival and 37% reduction in risk of death compared to placebo

[91]. In chemotherapy naïve men with metastatic prostate cancer, enzalutamide decreased the risk of death by 29% and delayed chemotherapy initiation by a median of 17 months compared to placebo [92].

CYP17A1 inhibition

Beyond suppressing LH secretion and blocking AR, a third strategy to treat CRPC is to inhibit the synthesis of testosterone. Ketoconazole is an azole drug commonly used to treat fungal infections by inhibiting lanosterol demethylase (CYP51A1) [93] and thus ergosterol production, which is essential for fungal cell membrane integrity. Ketoconazole gained traction as a viable treatment option for CRPC, because ketoconazole demonstrates clinically relevant off-target inhibition of several human cytochrome P450s, including CYP11A1, CYP11B1, and CYP17A1 [94,95]. Unfortunately, ketoconazole is a weak CYP17A1 inhibitor (Ki ~130 nM) [96], and it strongly inhibits the important drug-metabolizing enzyme CYP3A4, thus limiting its clinical use. Consequently, considerable effort has been expended to develop selective CYP17A1 inhibitors to treat CRPC, and the "holy grail" of these efforts is the development of a drug the specifically inhibits only the 17,20-lyase activity.

Abiraterone is a potent (~3 nM) [96], functionally irreversible inhibitor of both the 17α-hydroxylase and 17,20-lyase activities of CYP17A1. Inhibition of 17,20-lyase activity with abiraterone significantly reduces circulating concentrations of all 19-carbon steroids, including DHEA, androstenedione, and testosterone. Simultaneous inhibition of 17α-hydroxylase activity prevents the conversion of pregnenolone into cortisol, relieves cortisol negative feedback, allows ACTH to rise, and drives the accumulation of cortisol precursors with mineralocorticoid activity,

primarily 11-deoxycorticosterone (DOC) and corticosterone [97]. DOC accumulation causes hypertension and hypokalemia similar to genetic 17-hydroxylase deficiency [98], and administration of mineralocorticoid antagonist or glucocorticoid normalizes these side effects [97]. Consequently, abiraterone treatment requires concomitant administration of a glucocorticoid (such as prednisolone 5 mg BID) to avoid these side effects. An improved CYP17A1 inhibitor that only blocks the 17,20-lyase activity could have a profound impact on the clinical care of CRPC patients, allowing early stage treatment without chronic glucocorticoid co-administration.

Early clinical trials demonstrated abiraterone's ability to completely suppress testosterone, DHEA, and androstenedione synthesis in men with CRPC to below the limits of detection within twenty days of starting treatment [99]. In the first randomized phase III trial, de Bono and colleagues showed that abiraterone prolongs overall survival in men with CRPC, who had been previously treated with docetaxel, a commonly used chemotherapeutic agent. Overall survival increased by 3.9 months in the abiraterone-treatment group compared to placebo [100]. A subsequent study in docetaxel-naïve patients with CRPC demonstrated that abiraterone plus prednisone prolonged radiographic-free survival by 8.2 months over placebo plus prednisone and showed a trend toward improved survival [101].

Some of the CYP17A1 inhibitors under current development also bind directly to AR and antagonize its activity. In vitro binding studies have shown that abiraterone binds to AR with rather weak affinity in the high micromolar range compared to 1 nM for T and DHT [102]. In contrast, the Δ^4-metabolite of abiraterone is a more potent AR antagonist than enzalutamide, and

this compound also inhibits 3βHSD and 5α-reductase [103]. Galeterone represents another drug that has exhibited preclinical success with respect to androgen synthesis and androgen signaling blockade. Galeterone has the same chemical Δ^5-background structure as DHEA and abiraterone with the Δ^{16}-modification of abiraterone but a benzimidazole moiety to bind the heme iron rather than the 3′-pyridyl group of abiraterone. Galeterone shows some preferential inhibition of 17,20-lyase activity and also antagonizes AR in the 1-10 μM range [104]. Galeterone not only antagonizes AR activity but it also promotes AR protein degradation, representing a novel antiandrogen mechanism of action [105]. In phase I and II trials of men with CRPC, galeterone was well tolerated at 2,550 mg/d administered orally. Galeterone treatment decreased serum testosterone without an increase in DOC or hypertension and hypokalemia characteristic of abiraterone treatment, suggesting preferential inhibition of 17,20-lyase activity [106].

VT-464 is a CYP17A1 inhibitor that has been show to preferentially inhibit 17,20-lyase activity in preclinical models. VT-464 was rationally designed to both inhibit CYP17A1 and to also antagonize AR [107]. Orteronel (TAK-700) is another purported 17,20-lyase-specific CYP17A1 inhibitor that underwent clinical testing. Preclinical studies of orteronel demonstrated a 5.4-times greater potency for 17,20-lyase activity compared to the 17α-hydroxylase activity in cell-free assays; however, circulating progesterone concentrations rose in monkeys treated with orteronel, consistent with significant 17α-hydroxylase inhibitory activity [108]. In Phase III clinical testing, orteronel plus prednisone failed to prolong overall survival compared to placebo plus prednisone in men with CRPC who failed docetaxel chemotherapy [109].

The 5α-reductase inhibitors finasteride and dutasteride reduce the conversion of testosterone to the DHT, which is 5 times more potent than testosterone as an AR agonist. Finasteride is selective for SRD5A2, but dutasteride inhibits both SRD5A1 and SRD5A2. The Prostate Cancer Prevention Trial (PCPT) aimed to determine the effectiveness of prophylactic SRD5A2 inhibition at preventing or delaying the onset of prostate cancer [110]. The study results showed that finasteride blocked DHT synthesis and demonstrated a 24.8% reduction in prostate cancer prevalence over the seven years of treatment; however, the risk for high-grade tumors increased to 6.4% in finasteride treated men compared to 5.1% in the placebo group [110]. These risks have outweighed any potential benefit of using 5α-reductase inhibitors for prostate cancer prevention or treatment.

Alternative Pathways to Steroid Receptor Activation

Despite the high initial response rate to tamoxifen and AI therapies, breast cancer recurrence still poses a major treatment hurdle for women already treated with hormonal therapy. One possible mechanism of tumor recurrence and drug resistance is alternative pathways to steroid synthesis and non-canonical endogenous ER ligands. One example of such a ligand is the androgen metabolite 5α-androstane-3β,17β-diol (3βAdiol). Sikora et al. showed that 3βAdiol binds to and activates ER, and this binding can be blocked with the pure antiestrogen fulvestrant [111].

Another example of an endogenous ligand with estrogenic properties is 27-hydroxycholesterol (27HC). The oxysterol 27HC is synthesized from cholesterol by the cytochrome P450 27A1 (CYP27A1) enzyme [112]. 27HC was first shown to exhibit SERM properties in the cardiovascular system where it antagonized the cardioprotective effects of estrogen in smooth muscle and endothelial cells using mouse and rat models [113]. Dusell et al. later characterized

27HC's agonist activity in the ER-positive breast cancer cell line MCF-7 and showed that 1 μM 27HC induced expression of ER-regulated genes, while 100 nM fulvestrant blocked this induction. Additionally, 27HC treatment in MCF-7 cells resulted in a dose-dependent increase in cell number [114]. Such findings illustrate the potential impact alternative endogenous steroid-receptor ligands can have on disease progression and therapy response.

Mutations in the ligand-binding domain of ER have also been recently identified [115,116]. Of interest, these mutations seem to be significantly more frequent in women that have been treated with AIs, suggesting that estrogen deprivation selected for cells bearing these mutations. Consistent with this model, preclinical data suggest that these patients might still respond to direct ER antagonists [116]; however, this strategy has not been validated in appropriate clinical trials.

CYP17A1 Inhibitors in Breast Cancer

During AI treatment, local conversion of androgens to estrogens is impaired, therefore leading to accumulation of androgens. Consequently, another plausible mechanism of resistance to AI therapy is the acquired expression of AR and an active signaling pathway. Indeed, AR expression in breast cancers has been recognized for some time, and recent evidence suggests that AR expression is increased during AI treatment [117], with increases in circulating androgens also detected [118]. Because abiraterone acts upstream of aromatase and blocks the production of androgen precursors, CYP17A1 inhibition has been tested for the management of ER-positive breast cancer. The first clinical trial testing abiraterone in breast-cancer patients compared the efficacy of abiraterone plus prednisone to the AI exemestane, alone or in

combination [119]. The patient population for this study was women with metastatic, ER-positive breast cancer, who had failed previous endocrine therapies. The trial's pharmacodynamics endpoints showed that abiraterone use successfully suppressed both circulating androgen and estrogen concentrations; however, this reduction in circulating sex steroids did not translate into significant clinical benefit. Progression-free survival in the three treatment arms was similar, 3.7 months, 3.7 months, and 4.5 months in exemestate, abiraterone, and abiraterone-plus-exemestane arms, respectively [119]. A limitation of this study is that only heavily pre-treated patients with advanced tumors were randomized, raising the possibility that they were unlikely to respond to any form of treatment. Indeed, these data are consistent with studies on alternative growth signaling pathways beyond AR and ER, which are not targeted with a CYP17A1 inhibitor like abiraterone and might be active in some breast cancers [120].

Androgen Receptor-Positive Triple-Negative Breast Cancer

A subset of triple-negative breast cancers (TNBCs) expresses AR, and these tumors are believed to be androgen-dependent [121]. TNBCs, which account for approximately 10% of all breast cancers, are characterized by lacking expression of ER, progesterone receptor, and the receptor tyrosine-protein kinase erbB-2, also known as HER2 [122,123]. These tumors have historically been harder to treat, due to limited options for targeted treatment [124]. Antiandrogen therapies commonly used to treat CRPC have been investigated recently in AR-positive TNBC, based on the hypothesis that these tumors are dependent upon AR for cellular growth. One study using a mouse xenograft model of AR-positive TNBC cells demonstrated that these tumors were sensitive to bicalutamide treatment [121]. A clinical case study highlighted the potential success of bicalutamide as an option for TNBC with intact AR signaling pathways. A 55-year old

women with metastatic AR-positive TNBC exhibited a complete response to bicalutamide despite disease progression on all previous forms of chemotherapy [125]. A phase II trial testing bicalutamide in women with AR-positive, ER-negative metastatic breast cancer showed promising but modest activity. Of the 26 patients evaluated for the primary endpoint, 5 exhibited evidence of stable disease translating to a clinical benefit rate of 19% [126]. Similar preclinical data have been reported using the more potent antiandrogen, enzalutamide [127]. Preliminary analysis from a Phase II trial assessing enzalutamide therapy in advanced AR-positive breast cancer suggests that patients with tumors characterized by androgen-driven gene signatures display a robust response to enzalutamide, as evidenced by a significant increase in progression-free survival compared to patients with tumors lacking this gene signature [128]. These studies demonstrate that AR is a viable target in AR-positive breast cancers that rely on AR-mediated signaling for growth.

Rationale for Thesis

Inhibition of CYP17A1 has become a viable strategy for prostate cancer, but trials of CYP17A1 inhibition in breast cancer remain in their infancy. The results of the data reported in Chapter II are potentially clinically meaningful as they provide a novel explanation for the lack of efficacy seen when CYP17A1 is inhibited in women with breast cancer. I further aimed to better understand the implications of CYP17A1 expression in breast cancer by developing a preclinical model system that stably expresses CYP17A1 and allows for characterizing enzyme activity. In Chapter III I report for the first time that ER-positive breast cancer cells expressing CYP17A1 are a valuable tool to model steroidogenesis, and these cells can be used to test inhibitors of androgen and estrogen synthesis. Finally, functional characterization of *de novo* CYP17A1

mutations identified in humans greatly contributed to our current understanding of CYP17A1 activity and function. In Chapter IV I will discuss how these mutations elucidated the residues mediating interactions between CYP17A1 and redox partners. I hypothesized that characterizing previously unreported CYP17A1 variants would provide novel insights into enzyme activity and stability. Chapter IV highlights my successful characterization of two CYP17A1 variants outside of the active site that affect both enzyme stability and activity.

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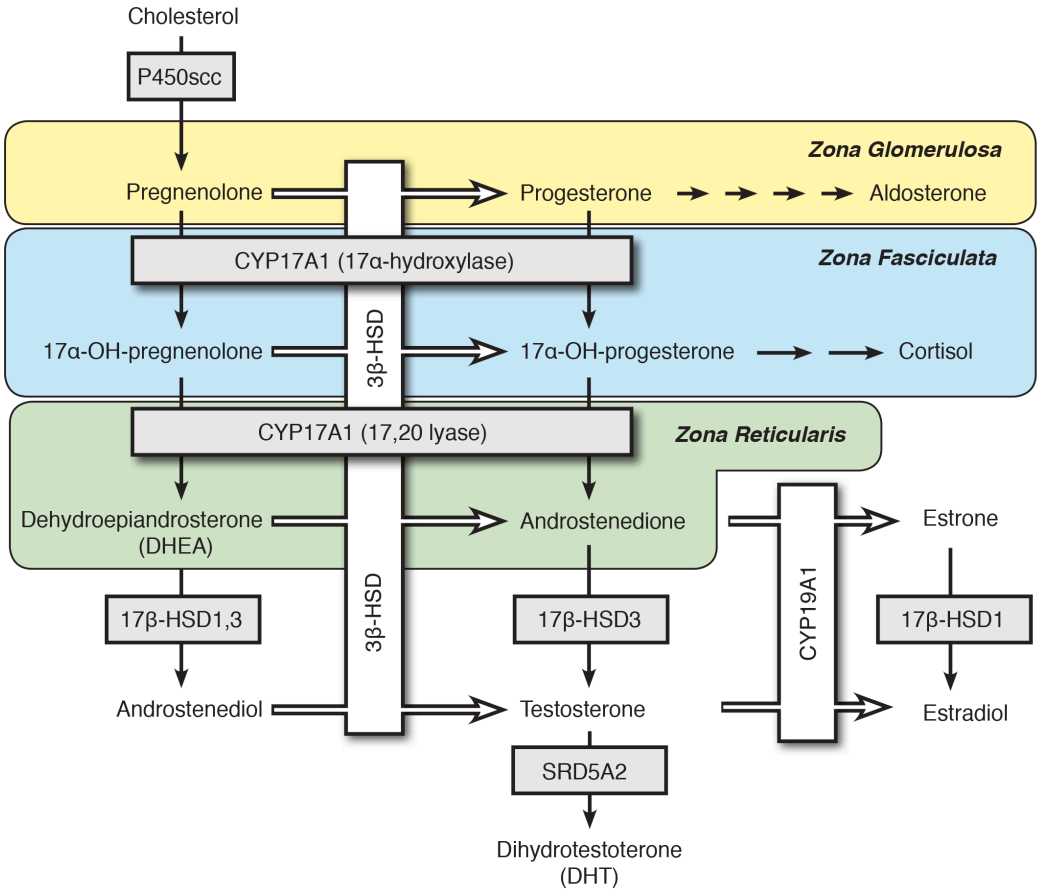

Figure 1.1. Adrenal Steroidogenesis. This schematic illustrates the biosynthesis pathways of mineralocorticoids, glucocorticoids, and sex steroids in the adrenal cortex by highlighting the predominant substrates and products within each zone. The three zones: zona glomerulosa (ZG), zona fasiculata (ZF) and zona reticularis (ZR) are labeled and designated with different background colors. Boxes denote steroidogenic enzymes, and arrows represent directionality of the enzymatic reactions. The pathway begins in the upper left hand corner with the conversion of cholesterol to pregnenolone. Multi-step conversions are indicated with multiple arrows when the enzymes are not specified.

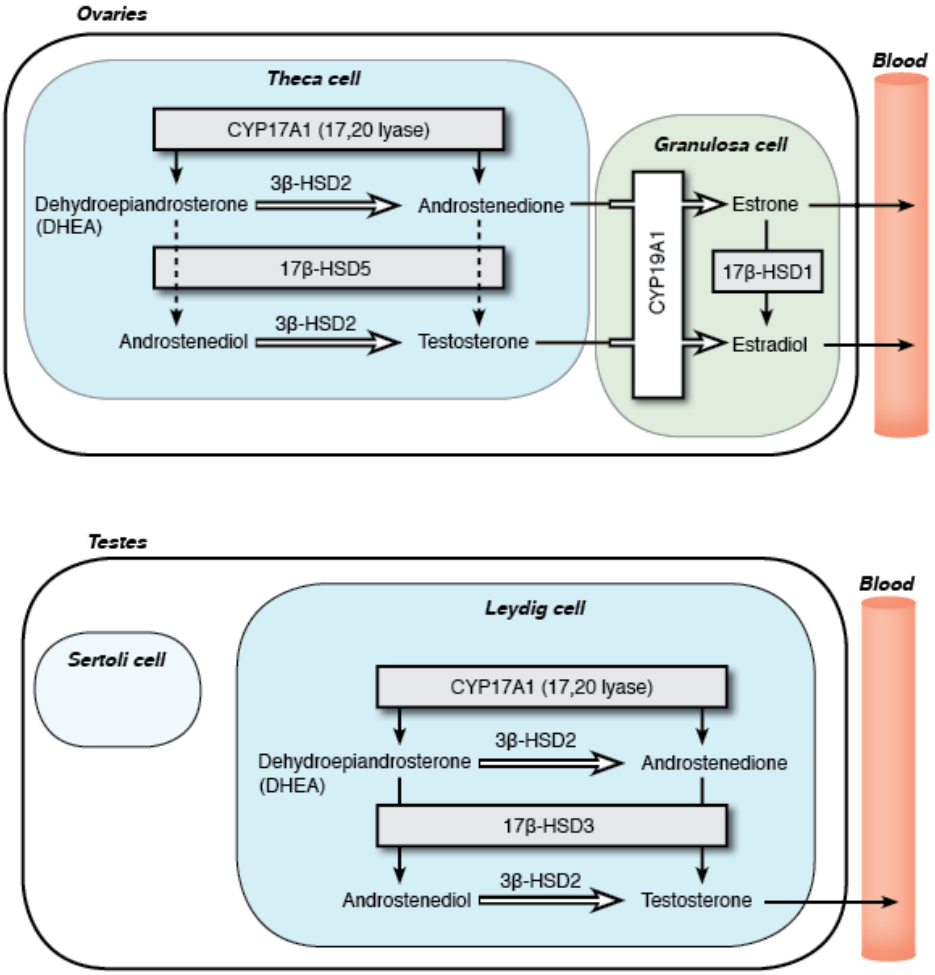

Figure 1.2. Gonadal Steroidogenesis. This figure depicts the enzymes expressed in the cells that comprise the gonads of females and males. **A.** Ovarian theca cells express CYP17A1 to produce androstenedione and a small amount of testosterone, and these androgens are further aromatized into estrogens in ovarian granulosa cells before entering the circulation. **B.** The testicular Leydig cells are the major steroidogenic cells in the male gonads, and these cells express CYP17A1 to convert androgen precursors into testosterone. Note the different 17βHSD isoenzymes present in the ovary and testis, which afford the major products E2 and testosterone, respectively.

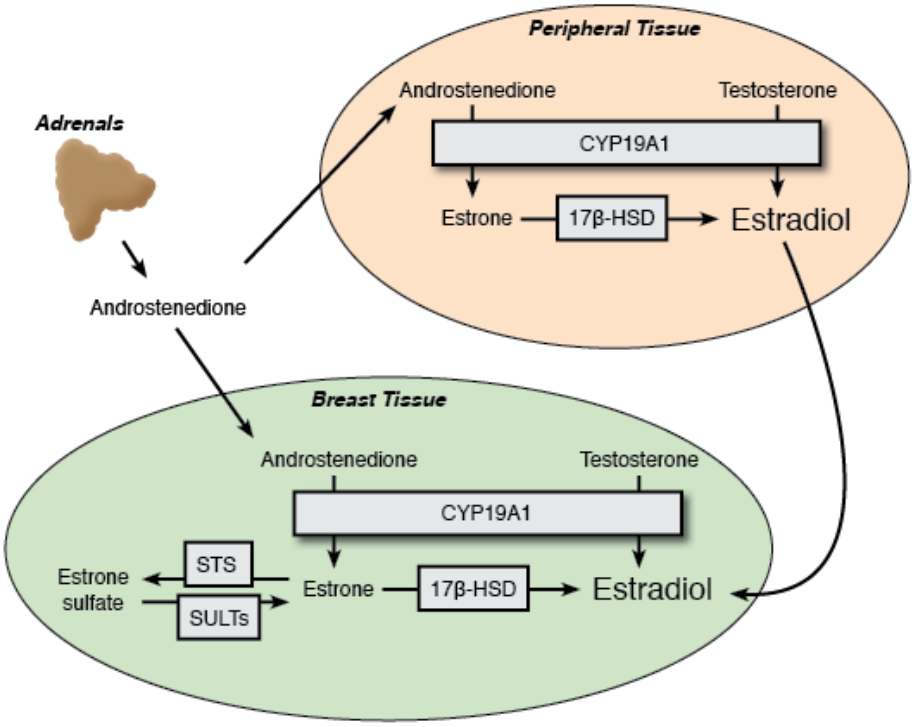

Figure 1.3. Sources of estrogen in postmenopausal women. Extragonadal estrogen secretion via intracrine and paracrine pathways significantly contributes to breast cancer progression. Breast tissue, in addition to other peripheral tissues, expresses CYP19A1 that mediates the conversion of circulating androgen precursors originating primarily in the adrenal, into E2. Following menopause, E2 remains a potent growth stimulus to the roughly 70% of breast cancer cells expressing ER. AIs work by blocking the local conversion of androgens into estrogens in these extragonadal tissues and significantly reducing circulating E2 levels to prevent E2-induced tumor growth. E1S is an additional source of E2, as the local expression of estrone sulfatase (STS) is able to convert E1S back to the E2 precursor, E1.

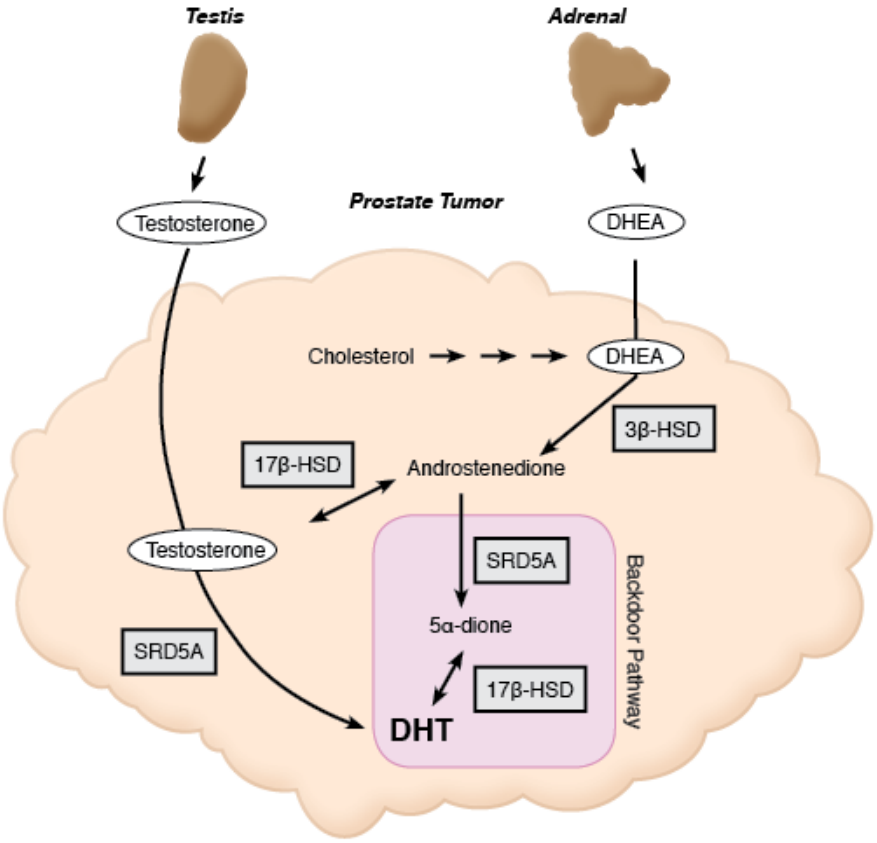

Figure 1.4. Pathways of androgen synthesis in CRPC. Shown in this figure are the pathways contributing to androgen synthesis that occurs in the testes, adrenals, and prostate tumor itself in men with CRPC. Expression of CYP17A1 in all three tissues produces androgens, and further metabolism of these androgenic substrates results in the synthesis of the potent androgen receptor agonists, testosterone and DHT. Highlighted in purple is the backdoor pathway to DHT synthesis that bypasses testosterone as an intermediate. Independent studies have shown evidence to support this particular pathway being intact in CRPC.

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Chapter II.

The CYP17A1 inhibitor abiraterone exhibits estrogen receptor agonist activity in breast cancer

Introduction

Abiraterone received FDA approval in 2011 for the treatment of metastatic castration-resistant prostate cancer (mCRPC) [1] and subsequently in chemotherapy naïve men [2]. Though resistant to most androgen receptor (AR) antagonists, mCRPCs still rely on androgens for growth [3]. Androgens remain a growth stimulus for prostate cancer cells, and so in the metastatic setting, chemical castration focuses on preventing androgen synthesis in the testes, the primary site of androgen production. The adrenal gland [4] and the prostate tumor itself [5] are, however, also capable of synthesizing androgens and current CRPC therapies like abiraterone target extra gonadal androgen synthesis to combat this issue.

Abiraterone acetate is a prodrug that gets metabolized by esterases to form abiraterone, a potent, steroidal inhibitor of CYP17A1. CYP17A1 is a bifunctional cytochrome P450 enzyme with both 17α-hydroxylase activity that leads to cortisol synthesis and C17,20 lyase activity that is responsible for androgen production [6-8]. Androgens synthesized in this pathway can be further metabolized into more potent androgens and also into estrogens—the two major contributors to prostate and breast cancer etiology, respectively. Irreversible inhibition of the C17,20 lyase activity with abiraterone results in a significant reduction of dehydroepiandrosterone (DHEA),

androstenedione, and testosterone [9, 10]. Blocking of CYP17A1's 17α-hydroxylase activity decreases cortisol production, and patients prescribed abiraterone are, therefore, also prescribed prednisone to offset unwanted mineralocorticoid excess that can lead to hypertension, hyperkalemia, and/or edema [11].

It has been proposed that pharmacologic inhibition of CYP17A1 could be an effective therapy for ER-positive breast cancer because it would block androgen production required for estrogen synthesis. Over 231,000 new cases of invasive breast cancer will be diagnosed this year in the United States and over two thirds of these cases will be ER-positive [12, 13]. ER is a nuclear receptor to which the steroid hormone estrogens bind and induce cancer cell proliferation in women with ER-positive breast cancers. The aromatization of androgens into estrogens by CYP19A1 (aromatase) is the primary source of estrogen synthesis both in the ovaries and in peripheral tissues [14, 15]. For postmenopausal women with ER-positive breast cancer, aromatase inhibitors (AIs) are first line hormonal therapy because of their ability to block estrogen production[15-18]. In a similar vein, inhibiting androgen production would also lead to a decrease in estrogen production because it decreases the amount of substrate available for CYP19A1. In fact, Phase I pharmacokinetic data from a prostate cancer trial revealed that abiraterone reduced circulating estradiol (E2) levels from median baseline concentrations of 196 pg/dL to less than 80 pg/dL in all 28 trial participants [9].

Based on abiraterone's structural similarity to sex steroid hormones, we hypothesized that abiraterone may be able to bind to ER. Using two different estrogen receptor positive cell lines, MCF-7 and T47D, we show that abiraterone is an ER agonist. This agonist activity promotes

breast cancer cell proliferation in both ER-positive cell lines and induces the expression of an estrogen response gene, *GREB1*. In addition, abiraterone induces ERE-luciferase activity in MCF-7 cells. Lastly, abiraterone's ER actions can be blocked using the selective ER down-regulator (SERD) fulvestrant (ICI 182,780) in a dose-dependent manner. Our results may help explain why some women with breast cancer may not favorably respond to abiraterone treatment as seen in the first trial of abiraterone in postmenoapausal women with metastatic ER-positive tumors [19]. These data will also help to identify which women and what drug combinations with abiraterone might be the most efficacious in a breast cancer setting.

Methods

Reagents: 17β-estradiol (E2) and ICI 182,780 were purchased from Sigma-Aldrich Inc. (St. Louis, MO), and abiraterone from Toronto Research Chemicals (TRC) (Toronto, Ontario, Canada). All compounds were dissolved in ethanol at 10mM and stored at -20°C protected from light.

Cell Lines, Culture Conditions, and Growth Assay: MCF-7, T47D, and BT-474 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) Gibco/Life Technologies, Grand Island, NY supplemented with 10% fetal bovine serum (FBS) (Valley Biomedical Inc., Winchester, VA). For assays requiring defined steroid hormone conditions, cells were cultured in phenol red-free IMEM (Gibco/Life Technologies, Grand Island, NY) supplemented with 10% charcoal stripped calf bovine serum (Valley Biomedical Inc., Winchester, VA)) as previously described [20]. For growth assays, cells were plated in 96 well plates at a density of 10^3 cells/well ($2x10^3$ for BT-474) and treated the following day with vehicle control (ethanol) or varying concentrations of E2, abiraterone, and ICI 182,780. Five days after treatment, cell

number was determined using the crystal violet assay, and absorbance was measured using a POLARstar Omega plate reader as previously described [21].

Measuring *GREB1* expression by Real-time PCR: 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^{o}C. *GREB1* mRNA expression was measured using a TaqMan real-time PCR assay as described previously [20]. Briefly, 1µg total RNA was reverse transcribed using Reverse Transcription System (Promega, Madison, WI) and the cDNA amplified in a 25µl reaction containing Gene Expression Master Mix and gene specific primers both from Thermo Fisher Scientific (Waltham, MA). *GREB1* expression was normalized against GAPDH with relative expression being calculated using the $\Delta\Delta C_T$ method [22].

Estrogen Response Element (ERE)-luciferase reporter assay: The ERE-luciferase reporter plasmid, (a gift of Dr Dorraya El-Ashry, University of Miami) was co-transfected into cells with FuGene 6 reagent at 3:1 ratio of reagent to DNA along with renilla plasmid (100:1 ratio of luciferase to renilla.) The following day cells were washed and medium was replaced with phenol-red free 10% CCS IMEM 5 times to ensure depletion of steroids. Cells were then trypsinized (with phenol red-free trypsin) and plated in 24 well plates. Cells were treated with ethanol, abiraterone, and E2 in triplicate for 24 hours. Lysates were prepared and luminescence levels were measured with the Dual-Luciferase Assay System following the manufacturers recommendations (Promega) using a luminometer.

Results

Abiraterone stimulates the proliferation of estrogen deprived ER-positive breast cancer cells

Previous work in our lab identified an androgen derivative, 5α-androstane-3β,17β-diol (3βAdiol), that induced breast cancer cell growth by exhibiting ER agonist activity [23]. Given the core steroid structure of abiraterone, we hypothesized that abiraterone might also bind to and activate ER. We therefore tested the ability of abiraterone to induce growth in two different ER-positive, estrogen-dependent breast cancer cell lines, MCF-7 and T47D. Cells were cultured under estrogen-free conditions, treated with increasing concentrations of abiraterone or E2, and cellular proliferation was measured after five days of treatment. We observed that abiraterone induced proliferation in a dose dependent manner in both cell lines (Figure 2.1, *A-D*). E2 is more potent than abiraterone with EC50 values of 5.5 pM in MCF-7 cells and 8.3 pM in T47D cells. Panels B and D of Figure 1 illustrate abiraterone-induced growth as a percent of vehicle control after cells were treated with abiraterone concentrations ranging from 100 nM to 10 μM. Maximal growth induction was observed at 8 μM in both cell lines and this maximal growth is 2.5 fold and 2.8 fold over vehicle control in MCF-7 and T47D cells, respectively. As a control, ER-negative cell line MDA-MB-231 was used in parallel studies and showed no proliferative response to treatment with abiraterone suggesting that these growth effects are mediated through ER (data not shown).

Abiraterone–stimulated proliferation is inhibited by an ER antagonist

To further test the hypothesis that abiraterone-induced growth is mediated by ER signaling, we tested the pure ER antagonist ICI 182,780 for its ability to inhibit the effects of abiraterone

induced cellular growth. MCF-7 and T47D cells were treated with maximally growth-stimulating dose of 8μM abiraterone and increasing concentrations of ICI 182,780 ranging from 10pM to 100nM. After 5 days of treatment cell proliferation was measured using the crystal violet assay. ICI 182,780 is equally potent at inhibiting E2 and abiraterone induced growth in both ER-positive cell lines (Figure 2.2). ICI 182,780 has calculated IC_{50} inhibitory values of 2.3nM (E2) and 5.6nM (abiraterone) in MCF-7 cells and 4.9 nM (E2) and 2.4 nM (abiraterone) in T47D cells. The ability of the ER antagonist ICI 182,780 to inhibit abiraterone-induced growth further suggests that abiraterone is acting as an agonist at the ER.

Abiraterone antagonizes E2-induced growth in MCF-7 cells

We reasoned that if abiraterone is acting as a weak ER agonist, it should, by definition, competitively reduce E2-stimulated proliferation. To test this, MCF-7 cells were treated with a fixed dose of E2 (100pM) in combination with increasing concentrations of abiraterone or ICI 182,780. The results show that 10μM abiraterone inhibits E2 stimulated growth by 20% in MCF-7 cells (Figure 2.3). In comparison, ICI 182,780 inhibits E2 induced growth with an IC_{50} of 4.6nM. These results are consistent with the growth data suggesting that abiraterone's effects are most robust at low micromolar concentrations.

Abiraterone induces the expression of the ER-responsive gene *GREB1* in ER-positive cell lines and this induction is blocked by the ER antagonist, ICI 182,780

To further characterize the apparent estrogenic activity of abiraterone, we determined the effects of abiraterone treatment on the mRNA expression of the ER regulated gene, *GREB1*. *GREB1* expression is markedly increased in ER-positive breast cancer cells upon E2 treatment, and this

increase can be blocked by treatment with ICI 182,780 as described previously [20]. We hypothesized that if abiraterone was indeed an ER agonist it would induce *GREB1* expression, and that ICI 182,780 should inhibit this ER-mediated *GREB1* induction. We treated estrogen-deprived MCF-7 and T47D cells with E2 and abiraterone along with ICI 182,780, alone and in combination. As shown previously, 100pM E2 induces *GREB1* expression in both of the ER-positive cell lines. *GREB1* induction following E2 treatment was most robust in the MCF-7 treated cells where *GREB1* mRNA expression increased by more than 30 fold compared to vehicle. E2 treatment in T47D cells induced *GREB1* mRNA expression by 4 fold over vehicle. (Figure 4, A and B). Similarly, 10μM abiraterone induces *GREB1* expression in both cell lines. Abiraterone induces *GREB1* expression by 10 fold over vehicle in MCF-7 cells (Figure 2.4, *A*) and 2.5 fold over vehicle in T47D cells (Figure 2,4, *B*). These increases in *GREB1* expression over vehicle control can be significantly inhibited by treatment with 10nM ICI 182,780 (Figure 2.4, *A* and *B*). These data further support the hypothesis that abiraterone is an ER agonist.

Abiraterone induces dose-dependent luciferase expression in MCF-7 cells transfected with an estrogen responsive luciferase reporter construct

An estrogen responsive luciferase reporter based on the estrogen-responsive TGF-alpha promoter [24] was used to further characterize the interaction between abiraterone and the ER. Estrogen-responsive reporter constructs have proven useful in the past for characterizing the estrogenic and antiestrogenic activity of different compounds [25]. MCF-7 cells were transiently transfected with the ERE-luciferase construct and then cultured in the absence of estrogen as described in the methods section. We hypothesized that the binding of abiraterone to the ER would permit interaction with the ERE construct and induce measureable luminescence in the same way that

E2 binds ER and initiates gene transcription. We observed that treatment with either E2 or abiraterone induce luciferase expression (Figure 2.5). In a dose dependent manner, abiraterone increases luciferase activity up to nearly 6 fold over vehicle at the highest concentration, 8 μM. E2 increases luciferase activity over 30 fold more than vehicle. These data are again consistent with our observation that abiraterone appears to be a weak ER agonist and provide further evidence that abiraterone can promote a direct interaction between the ER and an ERE.

Discussion

These data show that abiraterone exhibits weak estrogenic activity in preclinical breast cancer models, and we propose that these findings may be of clinical relevance. The micromolar concentrations of abiraterone required to induce proliferation do, however, raise the question of whether this is a pharmacologically relevant phenomenon. Clinical pharmacokinetic studies in thirty-three men with chemotherapy naïve CRPC showed that the C_{max} in circulation can reach up to 1 μM in the fasted state when taking the recommended 1,000 mg dose of abiraterone [10]. Moreover, abiraterone concentration in circulation increases when taken with a high fat meal [10]. Specifically, abiraterone serum concentrations can reach as much as 3μM when taken with food as compared to taken in a fasted state [9, 10] Additionally, it is well known that the concentration of a drug in a tissue can be many times higher than what is observed in circulation. For example, pharmacokinetic studies of the antiestrogen tamoxifen, used for the treatment of ER-positive breast cancer, and its metabolites reveal that drug and metabolite concentrations can be 5-11 times higher in the cancer tissue compared to serum [26]. Preapproval observations suggest that abiraterone tissue concentrations can be 10-20 fold higher compared to what is

observed in circulation [27]. Overall, we conclude that the high concentrations of abiraterone necessary for growth in our assays may still be clinically relevant.

Given that the CYP17A1 enzyme is required for androgen synthesis and estrogen production, researchers have hypothesized that drugs like abiraterone may be clinically useful in women with ER-positive breast cancer. The first clinical trial testing abiraterone in women with metastatic, ER-positive breast cancer that had failed previous endocrine therapies, compared the efficacy of abiraterone plus prednisone to the AI exemestane, alone or in combination. Pharmacodynamic endpoints demonstrated that abiraterone suppressed both circulating androgen and estrogen concentrations; however, this reduction in circulating sex steroids did not translate into significant clinical benefit with progression-free survival in the three treatment arms being comparable, 3.7 months, 3.7 months, and 4.5 months in exemestane, abiraterone, abiraterone plus exemestane, respectively [19].

Despite the aforementioned results, there remains a subset of breast cancer patients that may derive even more benefit from CYP17A1 inhibition. In contrast to ER-positive tumors, triple negative breast cancers (TNBCs) are aggressive tumors with a poor prognosis as they do not respond to hormone therapy or HER-2 targeted therapy due to their lack of ER, progesterone receptor (PR), and HER-2 expression [28]. A subset of TNBCs do, however, express AR and early studies suggest that these tumors respond to antiandrogen therapies that are conventionally used to treat prostate cancers [29-31]. In addition to AR antagonism, these patients may also benefit from CYP17A1 inhibition with a drug like abiraterone to block androgen synthesis. However, very little preclinical data exists concerning the use of CYP17A1 inhibitors in breast

cancer models. Although the results of early clinical studies testing abiraterone in ER-positive breast cancer demonstrated that abiraterone plus exemestane treatment did not significantly improve progression-free survival compared to exemestane alone, abiraterone treatment was able to significantly decrease circulating levels of estrogens and androgens in these women [19]. These data would suggest that inhibition of CYP17A1 in women can block E2 production and may have benefit for the treatment of ER-positive breast cancers. Indeed, a possible explanation for the lack of survival benefit in the abiraterone arm could be related to the clinical trial design. Subjects enrolled in this study had received prior endocrine therapy, and this could allow for the selection of ESR1 ligand binding domain (LBD) mutations that confer constitutively active ER signaling and promote resistance to endocrine therapy [32, 33]. Since it is very likely a significant portion of these women's tumors harbored various mechanisms of resistance, especially ESR1 LBD mutations, it is possible these confounding variables masked abiraterone's potential effectiveness. We do not feel that this trial data preclude the use of abiraterone in ER-positive tumors, however. Instead, these results highlight the importance of understanding all viable pathways of ER-stimulated tumor growth in the metastatic setting—including the possibility that abiraterone itself is promoting growth.

Further clinical implications revolve around our knowledge that 15-20% of women recur following AI therapy [34], and one possible explanation that has been proposed is that circulating androgen metabolites may also be estrogenic and can contribute to AI resistance. Previously we showed that the androgen metabolite 3βAdiol may act as a weak estrogen to promote therapy resistance by acting at the ER to continue growth signaling [23]. It is therefore possible that abiraterone therapy, at carefully monitored doses, represents a potential strategy to

inhibit resistant pathways of ER-positive tumor growth by not only suppressing canonical ER and AR ligands, but also various metabolites that possess agonist activity.

Our results are not that surprising since other research demonstrates that abiraterone can also interact with the AR and another steroidogenic enzyme, 3β-HSD [35, 36]. Abiraterone's steroidal based structure which was modeled after the structure of the CYP17A1 substrate pregnenolone, further lends support to the observed results [37]. Abiraterone shares the distinctive carbon based steroidal backbone with other nuclear steroid hormones, including the sex steroids. Ekena et al. highlighted the importance of the A-ring phenolic hydroxyl group of estrogens and determined this unique characteristic distinguishes these steroids from other classes of hormones [38]. This hydroxyl group attached to the 3 carbon that abiraterone shares with the potent ER ligand E2 may give abiraterone its affinity for ER. Abiraterone derives its specificity for irreversible CYP17A1 binding from a 3-pyridyl substituent attached to the 17 carbon and a double bond between the 16 and 17 carbon [37, 39-41]. Due to the profound structural similarities between abiraterone and endogenous steroid receptor ligands, it appears that abiraterone is likely capable of interacting with the ER.

In conclusion, abiraterone may be a viable treatment option in women with metastatic ER-positive breast cancer but also in women with triple-negative disease that are AR positive [42]. Studies have shown that a subset of these women have tumors that initially respond to anti-androgen therapy with drugs like bicalutamide and enzalutamide [29-31, 43, 44]. Abiraterone's ability to block androgen synthesis suggests it might also have clinical utility with AR-positive disease in the triple negative setting. A clinical trial studying abiraterone in women with AR-

positive, ER-negative tumors is currently underway [19]. Further studies are still necessary to better characterize which breast cancer patients would derive the most benefit and how to best monitor drug distribution and absorption to avoid off-target effects.

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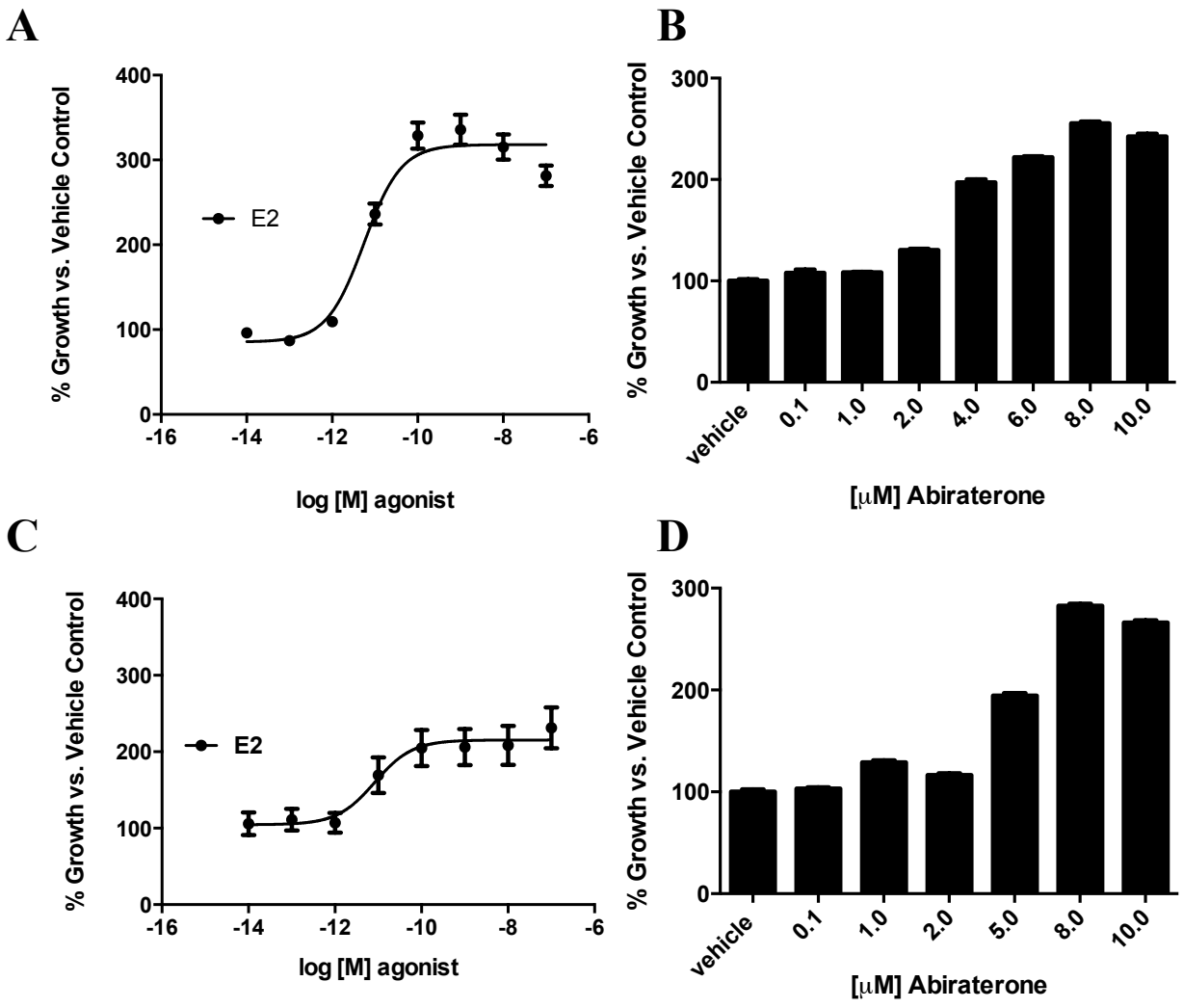

Figure 2.1. Abiraterone induces the proliferation of ER-positive breast cancer cells. MCF-7 cells (Panels A & B) and T47D cells (Panels C & D) were grown in steroid-free media for 2 days prior to treatment. Cells were plated in 96 well plates and treated with increasing concentrations of E2 or abiraterone dissolved in ethanol vehicle. Crystal violet staining was used as a measure of cell numer and raw absorbance values were measured using a plate reader. Growth curves represent percentage of cell growth compared to ethanol (vehicle) control (100%). Points on dose response curve and bar graphs represent the mean of 6 replicates ± SEM

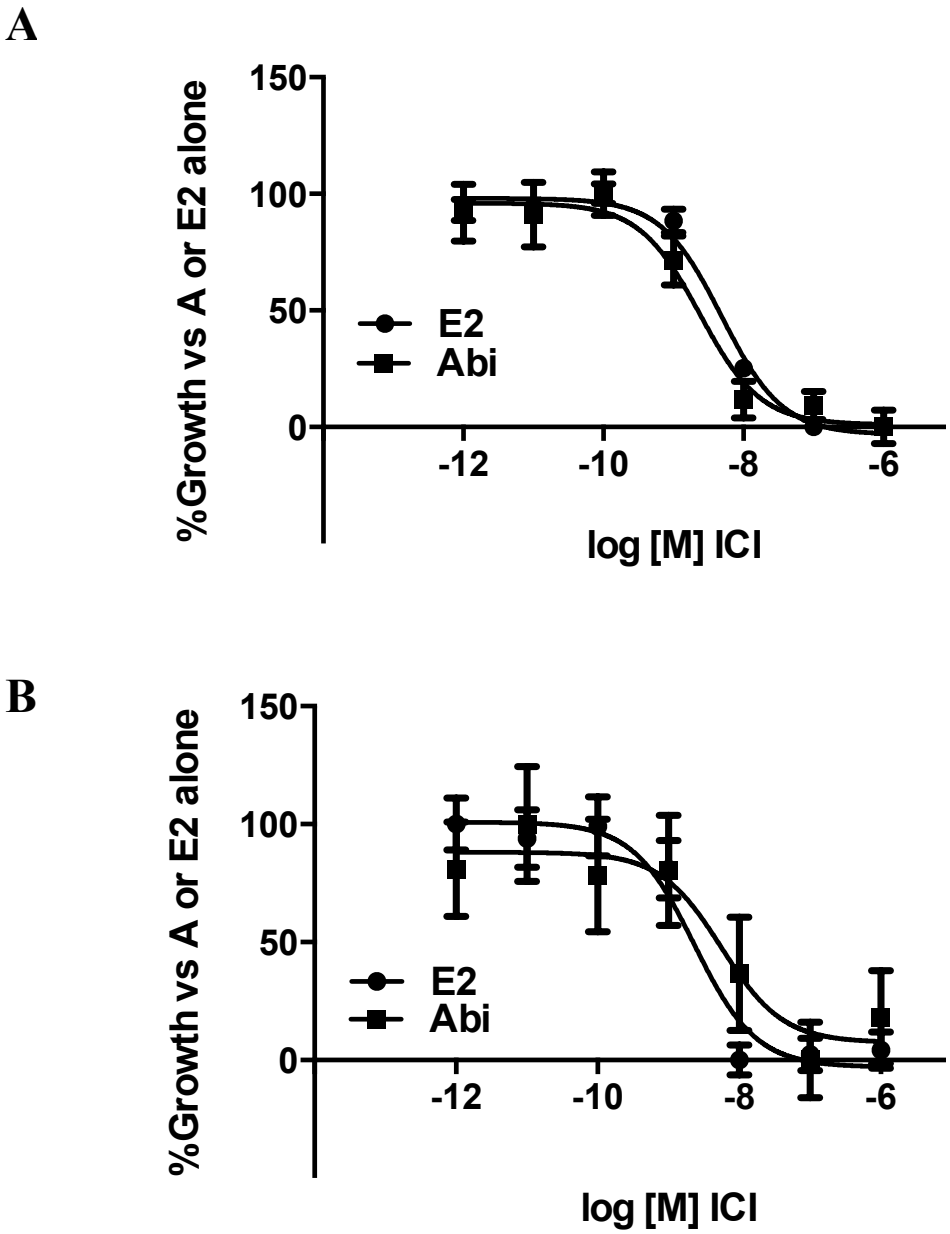

Figure 2.2. Fulvestrant (ICI 182,780) antagonizes abiraterone induced MCF-7 and T47D proliferation. MCF-7 (A) and T47D (B) cells were grown in steroid depleted media as described previously. Cells were plated in 96 well plates and treated with increasing concentrations of ICI 182,780 (ICI) dissolved in ethanol vehicle and a fixed dose of 8 μM abiraterone or 100 pM E2. Crystal violet staining was used to assay growth and raw absorbance values were measured using a plate reader. Data is normalized from maximum growth (100%) to minimum growth (0%) for each treatment. Points represent the mean of 6 replicates ± SEM.

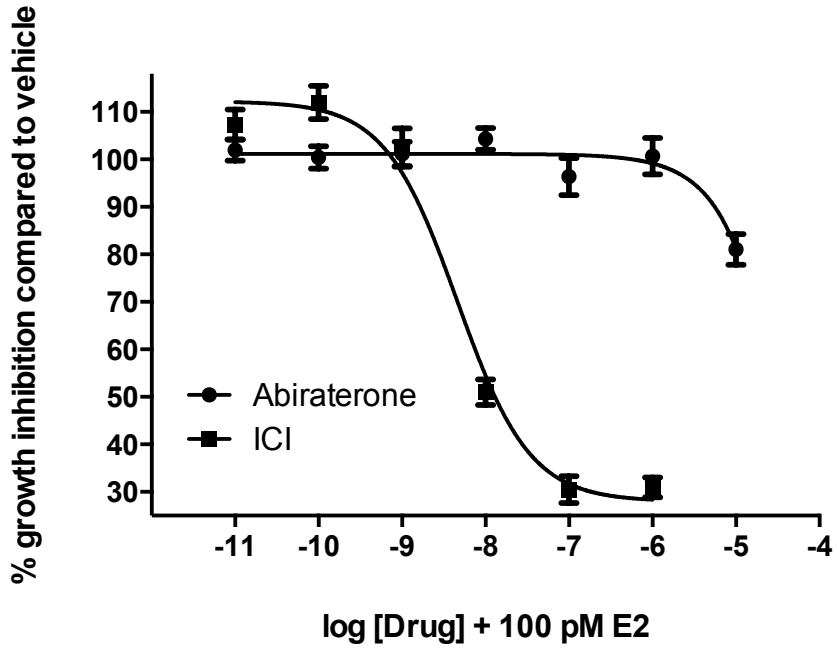

Figure 2.3. Abiraterone antagonizes E2 induced MCF-7 proliferation. MCF-7 cells were grown in steroid depleted media as described previously. Cells were plated in 96 well plates and treated with increasing concentrations of abiraterone or ICI 182, 780 (ICI) dissolved in ethanol vehicle and a fixed dose of 100pM E2. Crystal violet staining was used to assay growth and raw absorbance values were measured using plate reader. Growth curve represents percentage of cell growth compared to 100pM E2 + ethanol (vehicle) control. Data is normalized to 100pM E2 growth at 100%. Points represent the mean of 6 replicates ± SEM.

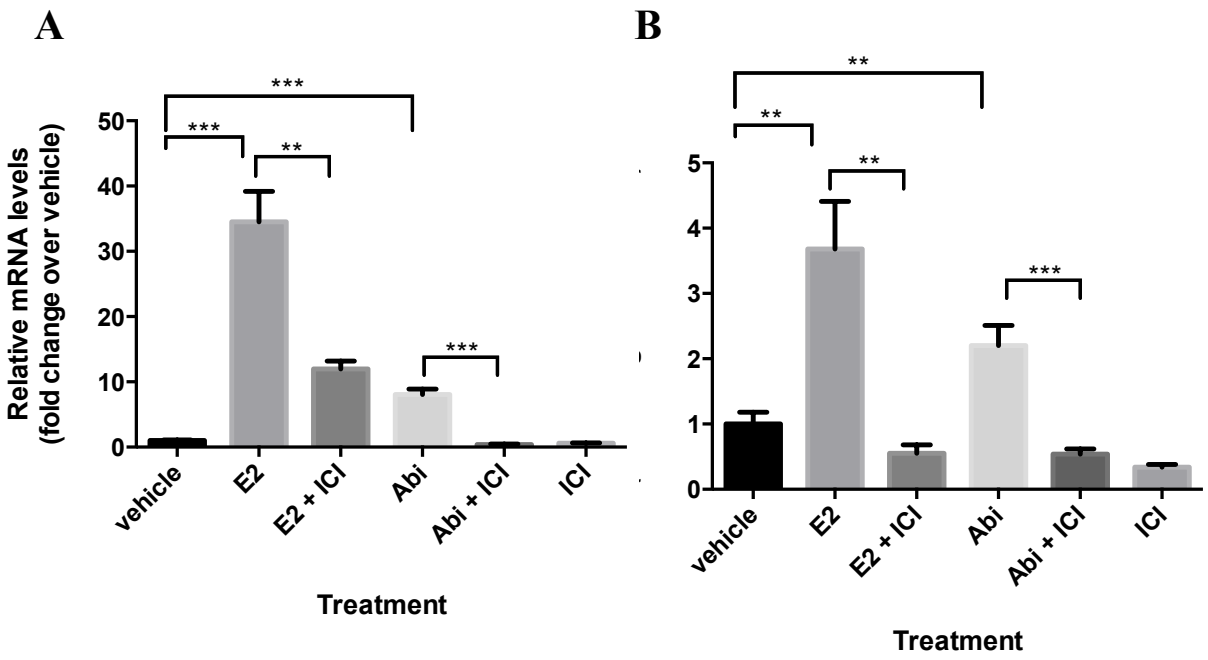

Figure 2.4. Abiraterone induces *GREB1* expression in MCF-7 and T47D cells. MCF-7 cells (Panel A) and T47D cells (Panel B) were grown in steroid-free media as described previously. Cells were plated in 6 well plates and treated for 48 hours as follows: 100pM E2, 10μM abiraterone, and 10nM ICI 182,780 (ICI). Bars represent *GREB1* expression vs. vehicle treated control using the ΔΔCt method. Bars represent mean from 3 replicates ± SEM. * = $P \leq 0.05$ **= $P \leq 0.01$ *** = $P \leq 0.001$

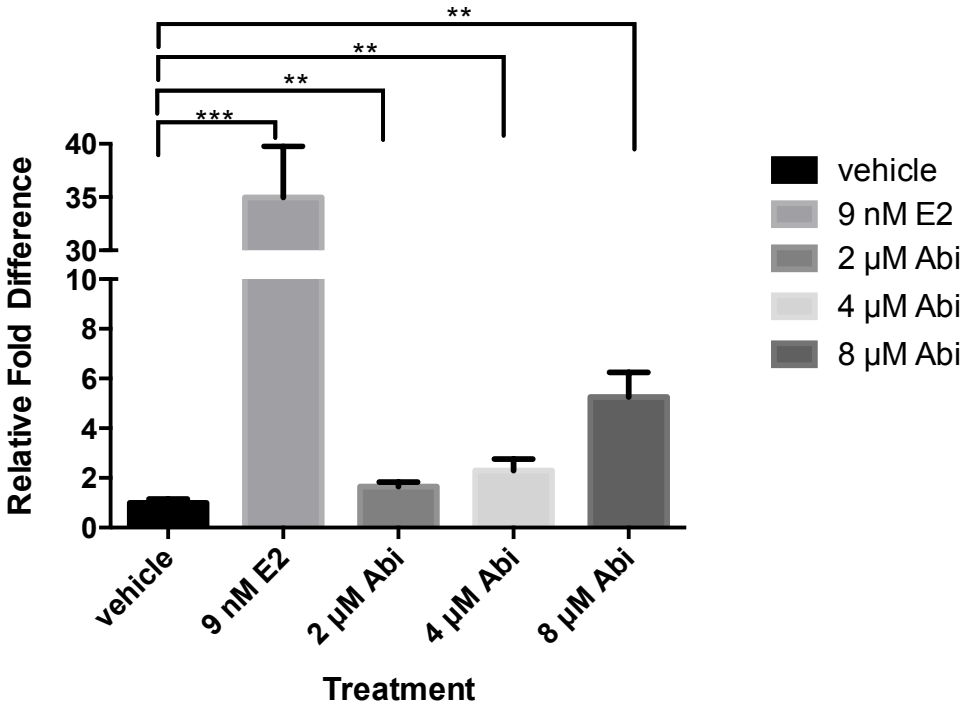

Figure 2.5. Abiraterone induces ERE-luciferase activity. MCF-7 cells were transiently co-transfected with the ERE luciferase construct and renilla reporter plasmid, grown in steroid-free media, and then treated with the indicated concentrations of E2 or abiraterone. Luminescence was measured using the dual luciferase method and a luminometer. Graph depicts the renilla-normalized relative fold difference in luciferase activity induced by increasing concentrations of abiraterone vs ethanol (vehicle) control. E2 is used as positive control. Bars represent the mean from 3 replicates ± SEM. * = $P \leq 0.05$ **= $P \leq 0.01$ *** = $P \leq 0.001$

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Chapter III.

CYP17A1 expression in MCF-7 breast cancer cells accurately models steroidogenesis and can be used as a tool to identify inhibitors of sex steroid synthesis

Introduction

Cytochrome P450 17A1, an enzyme required for sex steroid synthesis, has been mostly studied for its role in androgen production [1] and has emerged as a clinically validated drug target for prostate cancer [2]. Dysregulated CYP17A1 activity is associated with disease states characterized by excess androgen production such as polycystic ovary syndrome (PCOS). PCOS is a genetic disorder with common symptoms including hyperandrogenism and ovulatory dysfunction [3]. Hyperactive CYP17A1 activity has been implicated in contributing to excess androgen levels in women with PCOS [4, 5]. Alternatively, deficiencies in CYP17A1 can manifest as rare endocrinopathies known as 17-hydroxylase deficiency, 17,20-lyase deficiency, or combined 17-hydroxlase/17,20-lyase deficiency depending on which activities of the protein are impaired [6-10]. These genetic aberrations in the *CYP17A1* gene can result in decreased production of CYP17A1 products including cortisol, androgens, and estrogens. Patients generally present with decreased levels of the aforementioned hormones measured in circulation, sexual infantilism, as well as mild hypertension due to mineralocorticoid excess [10]. Adrenal insufficiency is avoided, however, due to excess production of corticosterone which has moderate glucocorticoid activity and is able to adequately suppress ACTH feedback [11].

CYP17A1 expression in adrenal insufficiencies and prostate cancer has been studied extensively; however, the literature is not as complete concerning CYP17A1 expression in breast cancer.

In vitro studies of enzyme catalytic activity, including studies of P450s such as CYP17A1, utilize reconstituted system requiring purified protein [11]. The concentrations of enzyme, substrate, and cofactors can all be precisely measured and therefore, these *in vitro* or "test tube" assays can be carried out under carefully defined conditions. This can prove especially helpful when determining the kinetics of enzyme activity such as the on-off rate, V_{max} (maximum reaction rate when enzyme is saturated with substrate), or K_M (Michaelis constant denoting substrate concentration at ½ V_{max}.) However, expression and purification of relatively large quantities of certain enzymes, especially microsomal P450s, P450s situated in the endoplasmic reticulum membrane of expressing cells, required for *in vitro* functional characterization studies can often prove to be difficult [12, 13]. As an example, purification strategies for CYP17A1 were still being improved in 2010 [14] despite a purification system for CYP17A1 being described decades earlier [15]. Because of these challenges, I aimed to establish a cell culture model system expressing CYP17A1 that better represented its physiological environment in a breast cancer model and obviated the need for purified protein. In addition to being able to model CYP17A1 activity and inhibition, we are also able to screen for off-target effects of CYP17A1 inhibitors, such as abiraterone's ER agonist activity described in Chapter II.

The estrogen receptor-positive, estrogen-dependent MCF-7 breast cancer cells express appreciable levels of endogenous CYP19A1 as well as 3βHSD and 17βHSD when grown under estrogen-free conditions[15]. These cells were stably transfected with CYP17A1 and treated

with progesterone. We hypothesized this would permit the sequential transformation of progesterone into downstream steroid hormones including the 17β-estradiol (E2). Given that MCF-7 cells are estrogen-dependent, cellular growth can be used as a readout for E2 production in this model system. Additionally, we used this model system to examine the extent of the effects of genetic variants in CYP17A1 on estradiol synthesis by measuring growth and *GREB1* (ER-response gene) expression by quantitative real-time PCR.

Methods

Cloning of CYP17A1 Expression Constructs

A pCMV6-XL4 plasmid expressing full-length human CYP17A1 cDNA was obtained from Origene (SC102224, Rockville, MD). NotI restriction enzyme sites were used to digest the CYP17A1 cDNA. Following insertion and ligation of the CYP17A1 cDNA into a pCMV6-Neo mammalian expression vector, diagnostic digests with SpeI and XbaI along with sequencing identified a plasmid with the insert in the correct orientation.

CYP17A1 variant constructs were generated using Agilent Technologies (Santa Clara, CA) QuikChange Lightning site directed mutagenesis kit. Site directed mutagenesis primers were designed using Agilent QuikChange primer design tool software. Oligonucleotides from primer sequences were synthesized by IDT technologies (Coralville, USA). Large quantities of wild type and variant plasmid DNA were generated using a Qiagen (Valencia, CA) maxi prep kit. All resultant plasmid were sequence verified to ensure site-specific mutagenesis and to ensure no off target effects.

Stable Transfection of MCF-7 Breast Cancer Cells

The wild-type CYP17A1 cDNA was stably transfected into MCF-7 cells using the pCMV6-Neo plasmid expressing a neomycin mammalian selection marker. FuGENE HD transfection reagent (Promega, Inc.) was used for the transfection reaction using a 3:1 ratio of μl transfection reagent to μg plasmid DNA. Cells were passaged in G418 containing medium to select for pooled clones stably expressing CYP17A1. Western blot analysis confirmed stable expression of CYP17A1 protein in these cells.

Western blot analysis

Whole cell protein extracts were collected from parental and stably transfected MCF-7 cells using RIPA buffer (Sigma-Aldrich) with protease inhibitor cocktail (Roche). Protein concentration was determined by Protein Assay Dye (Bio-Rad), and 40 μg protein was resolved on a 4-20% SDS-PAGE gel (Pierce). Protein was transferred to PVDF membrane and CYP17A1 expression was detected with mouse anti-CYP17A1 monoclonal antibody (Origene) while β-actin (Cell Signaling Technology, Danvers, MA) served as a loading control.

Cell Lines, Culture Conditions, Reagents and Growth Assay

MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Valley Biomedical Inc., Winchester, VA). For assays requiring defined steroid hormone conditions, cells were cultured in phenol red-free IMEM (Gibco/Life Technologies, Grand Island, NY) supplemented with 10% charcoal stripped calf bovine serum (Valley Biomedical Inc., Winchester, VA)) as previously described [16]. Abiraterone (Selleckchem, Houston, TX),

progesterone (Sigma Aldrich, St. Louis, MO) and letrozole (Sigma Aldrich) were dissolved in ethanol at 10mM stock concentrations and stored at -20°C protected from light. For growth assays, cells were plated in 96 well plates at a density of 10^3 cells/well and treated the following day with vehicle control (ethanol) or varying concentrations of progesterone. Five days after treatment, cells number was determined using a crystal violet stain and absorbance was measured using a POLARstar Omega plate reader as previously described [17].

Measuring *GREB1* mRNA expression by Real-time PCR

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. *GREB1* mRNA expression was measured using a TaqMan real-time PCR assay as described previously [16]. Briefly, 1µg total RNA was reverse transcribed using Reverse Transcription System (Promega, Madison, WI) and the cDNA amplified in a 25µl reaction containing Gene Expression Master Mix and gene specific primers both from Thermo Fisher Scientific (Waltham, MA). *GREB1* expression was normalized against GAPDH with relative expression being calculated using the $\Delta\Delta C_T$ method [18].

Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) to quantify steroids

Parental MCF-7 cells and MCF-7/CYP17A1 cells were grown in steroid-free medium for 3 days and then treated with 1 µM progesterone for 48 hours. Conditioned medium was collected from these cells and prepared for LC-MS/MS analysis. Samples were injected via autosampler and resolved with an Agilent 1290 binary pump HPLC on a Kinetex 50 × 2.1 mm, 3 µm particle size biphenyl column (Phenomenex). The column effluent was sent to the source of an Agilent 6490

triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode. Data were analyzed using multiple reaction monitoring mode.

Results

Progesterone induces growth in MCF-7 cells stably expressing WT CYP17A1

To study the effects of CYP17A1 expression in ER-positive breast cancers, we stably transfected a plasmid expressing full length WT CYP17A1 in MCF-7 cells (denoted as MCF-7/CYP17A1 cells), an estrogen dependent, estrogen receptor-positive (ER-positive) breast cancer cell line. Figure 3.1 *B* is a western blot confirming stable expression of the 57 kDa WT CYP17A1 protein in MCF-7 cells (note the lack of expression of CYP17A1 protein in parental cells.) These MCF-7 cells stably transfected with CYP17A1 combined with their endogenous expression of 17βHSD and CYP19A1 express all the required enzymes for estradiol synthesis using progesterone as the initial precursor [19]. We hypothesized that the MCF-7/CYP17A1 cells growing in estrogen-free conditions would be capable of synthesizing estradiol and thereby induce their own growth when treated with progesterone. To test this hypothesis, cells were grown under steroid hormone-free conditions for 3 days, plated in 96-well dishes, and then treated with increasing concentrations of progesterone. Untransfected MCF-7 cells were used as negative controls. Figure 3.1 *A* shows that progesterone induces growth in MCF-7/CYP17A1 cells with a calculated EC_{50} value of 6.83 nM whereas the apparent EC_{50} value in parental cells is nearly 2 logs greater at 488 nM. An F-test of the EC_{50} values determined them to be statistically significant by using nonlinear regression to curve fit the dose responses ($p = 0.0069$). Maximal progesterone induced growth was more pronounced in the stably transfected cells which exhibited maximal growth nearly 2-fold over vehicle control. Progesterone's effects were more

subdued in the parental cells as maximal growth was observed at 1.5 fold over vehicle. The modest increase in growth seen in the parental MCF-7 cells is most likely a result of residual estrogens that remained following the rigorous cell washing procedure to remove hormones in the culture media. This result highlights the necessity of extensive hormone depletion prior to studies on sex hormone signaling to ensure accurate results.

Progesterone treatment induces *GREB1* expression in MCF-7 cells stably expressing WT CYP17A1

To determine if the ultimate conversion of progesterone into estradiol was the growth stimulus driving MCF-7/CYP17A1 growth induction, we measured the ability of progesterone treatment to induce *GREB1* (gene regulated by estrogen in breast cancer 1) mRNA expression in these cells. As discussed in Chapter II, *GREB1* is an ER responsive gene positively regulated by activation of ER [16]. While estradiol should induce *GREB1* expression by binding to ER, progesterone is not an ER ligand and therefore, *GREB1* induction should be absent. To test this parental MCF-7 and MCF-7/CYP17A1 cells were grown under steroid hormone-free conditions and then treated with a dose range of progesterone. Total RNA was isolated and *GREB1* expression levels determined by RT-PCR. MCF-7 cells stably expressing CYP17A1 show increased *GREB1* expression that was dose dependent which further supports our theory that the added progesterone is being converted to E2 (Figure 3.2 *A*). E2 treatment was used as a positive control for ER signaling and the results show that E2 induced *GREB1* expression 30 fold over vehicle control treated cells. We observed statistically significant increases in *GREB1* expression starting at 1 nM progesterone (p values ≤ 0.5) with maximal expression noted at approximately 25 fold over vehicle control. In contrast, parental MCF-7 cells, which lack

endogenous CYP17A1 expression, showed no dose response to progesterone and no significant increases in *GREB1* expression at all progesterone concentrations tested (Figure 3.2 *B*).

CYP17A1 and CYP19A1 inhibition completely ablates progesterone metabolite-induced GREB1 expression in MCF-7 cells stably expressing WT CYP17A1

We next tested the hypothesis that inhibitors of the CYP17A1 and CYP19A1 enzymes would block estradiol synthesis and inhibit progesterone metabolite-induced *GREB1* expression in MCF-7/CYP17A1 cells. To test this, the MCF-7/CYP17A1 cells were treated with increasing concentrations of the CYP17A1 inhibitor, abiraterone, and the CYP19A1 inhibitor, letrozole in combination with the maximally stimulating *GREB1* expression dose of 10^{-7} M progesterone. Figure 3.3 *A*,*B* shows that both abiraterone and letrozole inhibit *GREB1* expression in a dose-dependent manner, suggesting successful blockade of CYP17A1 and CYP19A1 activity, respectively. These data also demonstrate that these enzymes are required for the synthesis of estradiol from progesterone and support our hypothesis that progesterone is ultimately converted to estradiol in this cell culture model system.

Steroid quantification using LC-MS/MS

To quantitatively assess the amount of each intermediate steroid synthesized by MCF-7/CYP17A1 cells, conditioned medium from MCF-7/CYP17A1 cells and parental MCF-7 cells was subjected to LC-MS/MS analysis. MCF-7/CYP17A1 cells were treated with 100 nM progesterone for 48 hours, and steroids were extracted from the conditioned medium. Representative steroid peaks were identified using internal standards for progesterone, 17OH-progesterone, androstenedione, testosterone, and estradiol. Figure 3.4 shows that both cell lines

were treated with equal concentrations of progesterone, but only the cells stably expressing CYP17A1 are able to convert progesterone into measurable levels of 17OH-progesterone (806 pg/mL, androstenedione (1433 pg/mL), and testosterone (214 pg/mL). Although E2 concentrations were below the limit of detection for this assay (~5 nM), we and others have shown previously that concentrations below the level of detection in this assay are capable of inducing growth [20] Another explanation for these results is that much of the synthesized E2 remains bound to ER inside the cells and does not distribute into the conditioned media therefore what little may get out falls below the limit of detection.

Using a novel model system to characterize CYP17A1 genetic variants

Previous retrospective genotyping analyses and functional characterization of CYP17A1 gene variants have focused on the more common promoter and intronic SNPs of CYP17A1. We therefore focused our studies on variants in CYP17A1's coding region. The 11 SNPs outlined in Table 3.1 were identified using the dbSNP online database [21]. Using knowledge of the CYP17A1 protein crystal structure, we chose these variants based on their structural location in addition to the predicted significance of the amino acid substitution using some of the guidelines outlined in the cited review article [22].

Using our established MCF-7 cell culture model system, we hypothesized that MCF-7 cells transfected with CYP17A1 genetic variants will have altered responses to progesterone treatment. Therefore, if a genetic variant were to adversely affect substrate binding, we would expect to see a decrease in progesterone metabolite-induced growth as well as a decrease in progesterone metabolite-induced *GREB1* expression. Figure 3.5 depicts a representative *GREB1* expression profile of one variant of CYP17A1, in this case the T152R (rs58822002) amino acid substitution

in which a tyrosine is substituted for an arginine at residue 152. The T152R construct was made using site-directed mutagenesis and then stably transfected into MCF-7 cells using the established method described earlier. Compared to WT CYP17A1, E2 induces a higher maximal response in the T152R expressing cells, however, *GREB1* expression induced by progesterone treatment is markedly reduced at all concentrations in the variant compared to WT. Maximal *GREB1* induction is 4-fold less than WT in the variant expressing cells. The induction of *GREB1* by the T152R construct, albeit reduced compared to WT CYP17A1, indicates that this variant results in a functional enzyme. One explanation for this phenotype is that the enzymatic activity of CYP17A1 is altered in such a way that the T152R variant is less efficient at metabolizing progesterone into downstream products, which results in decreased induction compared to WT. Other possibilities will be considered in detail in the discussion section of this chapter.

Identifying CYP17A1 inhibitors using MCF-7/CYP17A1 model system

One of the additional uses of our cell model system is that it can be quickly used to screen new therapeutic compounds for CYP17A1 inhibitory activity. As proof of principal, in collaboration with Dr. Hollenberg, we tested three different compounds for lyase specific CYP17A1 inhibition. All three compounds were identified using a high-throughput screen of the NIH clinical compound library. One of these compounds, UMPHZ523, demonstrated a significant inhibitory preference for the hydroxylase activity compared to the lyase activity with a hydroxylase to lyase IC_{50} ratio of 30:1 (Data generated in lab of Dr. Hollenberg and Dr. Haoming Zhang). In the MCF-7/CYP17A1 model system, this compound showed very modest inhibition, an IC_{50} in the micromolar range, compared to abiraterone that has an IC_{50} in the low nanomolar range (3 nM).

The structure may be useful, however, as a scaffold for the development of novel drugs targeting CYP17A1.

Discussion

One of the first established cell lines to model steroid biosynthesis was the National Cancer Institute (NCI) H295 cells that originated from a primary adrenocortical carcinoma [23]. Over 30 different steroids could be detected in the culture medium from these cells and mRNA levels for many of the steroid metabolizing enzymes such as P450scc, CYP17A1, and CYP21A2 were abundantly expressed [24]. NCI-H295 cells endogenously express CYP17A1, and treating these cells with ACTH, forskolin, and dbcAMP further increased CYP17A1 expression and activity [25]. H295 cells are known to express ER [26], but unlike MCF-7 cells they predominantly express ERβ, an ER isoform with an unclear role in breast cancer [27]. Other models of steroidogenesis exist [28], but none combine a model of complete sex steroid biosynthesis with cancer cell proliferation. The purported contribution of CYP17A1 to intratumoral sex steroid synthesis in cancer and thus cancer progression underscored a need for a breast cancer specific model system.

One intronic CYP17A1 SNP of extensive interest is rs743572 located in the 5' UTR of the *CYP17A1* gene. Carey et al. first identified this SNP in 1994 and postulated that this base change results in an additional binding site for the transcription factor SP-1 leading to increased *CYP17A1* expression [29]. This hypothesis was unable to be corroborated experimentally as the polymorphic allele motifs failed to interact with SP-1 in contrast to the strong SP-1 binding

observed when using the consensus sequence [30]. The primary driving force behind this observed increase in expression remains unclear.

In this study we report a novel cell culture model system of CYP17A1 expression in breast cancer. Despite CYP17A1's requisite role in estrogen synthesis, previous studies neglect in depth investigations of CYP17A1 expression in breast cancer. Prior work in the 1990s by Feigelson et al. suggested that the aforementioned -34t>a SNP (rs743572) within CYP17A1 contributed to excess circulating hormone levels [31], including estrogen, and that individuals expressing this polymorphic variant were at an increased risk for breast cancer [32]. Further analysis by Haiman and colleagues studied this association in a case-control study as part of the Nurses' Health Study cohort. In contrast to Feigelson's findings, they reported that this CYP17A1 SNP was not an independent risk factor for breast cancer [33]. Later studies have attempted to validate Feigelson's early findings, with the majority of them finding borderline or no significant correlations [34]. A comprehensive study by Dunning et al. in 1975 normal postmenopausal women revealed that some SNPs in steroidogenic enzymes, such as CYP19A1, significantly contribute to circulating estradiol levels however no significant association could be attributed to *CYP17A1* variation [35]. A more recently characterized SNP, rs17115149, is also upstream of the *CYP17A1* coding region and is purported to be associated with male infertility and decreased testosterone levels by acting as a CpG methylation site [36].

Western blot data (not shown) demonstrating CYP17A1 variant specific protein expression in these stably transfected MCF-7/CYP17A1 cells suggests that even though these variants result in functional protein, differences in enzyme activity illustrated in Figure 3.5 may be simply due to

variable protein expression. To address this issue, a different model system that can either directly quantify steroid levels or control for differences in protein expression should be used. One such model system will be discussed in detail in Chapter IV of this thesis.

This model system of CYP17A1 expression in breast cancer required transfecting CYP17A1, as MCF-7 cells do not demonstrate endogenous expression at either the mRNA or protein level. Still, some evidence exists that P450s not expressed in cell culture can be measured in patient samples of the same tissue type or in mouse xenografts using the cultured cells. For example, one major limitation of studying drug metabolizing P450s in cultured hepatic cells is the pronounced decrease in P450 expression [37]. Similarly, CYP17A1 expression in breast and prostate cancer lines is mostly non-existent, with very little mRNA detected and no measurable protein expression. Investigators in a recent study compared steroidogenic enzyme expression in normal human prostate cell lines (WPMY-1 and WPE1-NA22) as well as commonly studied prostate cancer cell lines (PC-3, LNCaP, VCaP) [38]. They compared mRNA and protein expression in the cell lines to biopsy tissue samples. Whereas none of the prostate cancer cell lines showed significant CYP17A1 gene expression, 83% of prostate cancer tissue samples demonstrated measurable CYP17A1 mRNA levels. An earlier study by Cai and colleagues reported that despite low expression of CYP17A1 in VCaP cells, VCaP xenografts show an increase in CYP17A1 expression in the presence of a CYP17A1 inhibitor such as abiraterone [39]. The authors postulated that this could be one of the many pathways leading to abiraterone and CYP17A1 inhibitor resistance.

By applying the previously discussed results in prostate cancer cell lines, it appears plausible that the lack of CYP17A1 expression observed in breast cancer cell lines does not preclude the possibility that breast tumors in patients express significant levels of CYP17A1 protein. Thus, the MCF-7/CYP17A1 cell culture model may be physiologically and clinically relevant. The data presented in this chapter suggests that breast cancer cells expressing CYP17A1 would be capable of synthesizing estradiol from circulating progesterone, and progesterone is primarily synthesized in the adrenals which would still be intact and functioning in post-menopausal women who are devoid of ovarian estrogen production.

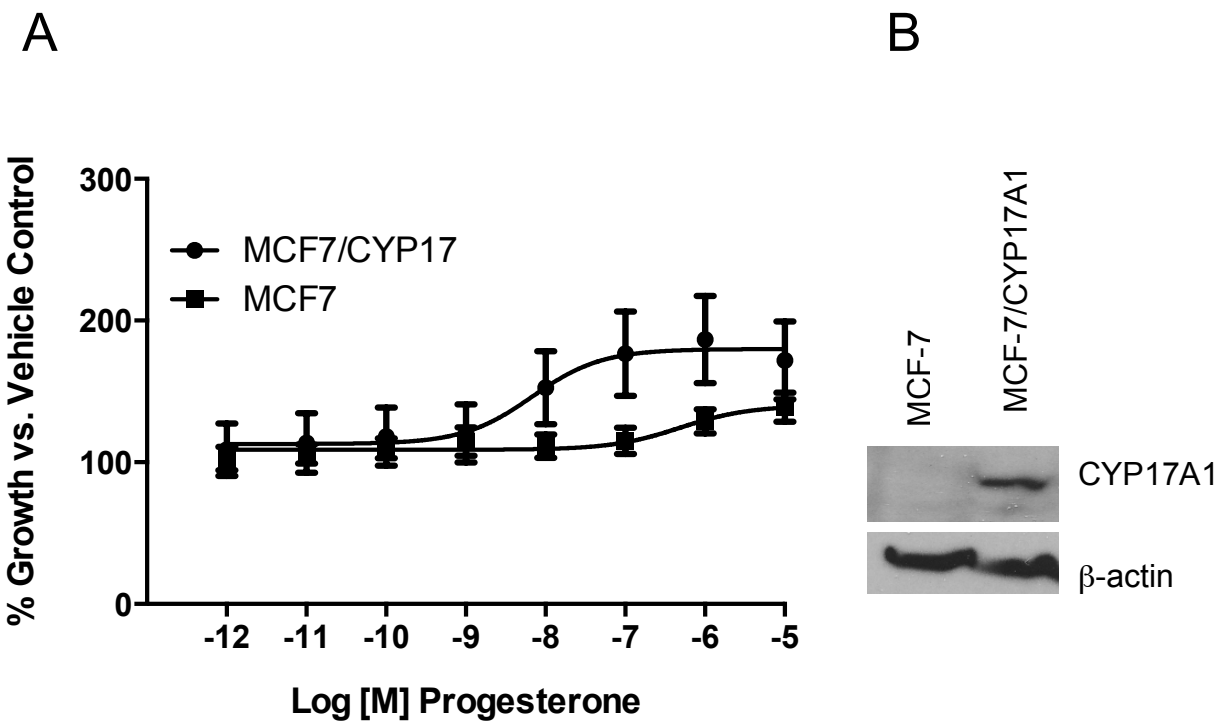

Figure 3.1. Progesterone treatment induces growth in MCF-7 cells stably expressing WT CYP17A1. (A) Parental MCF-7 cells (closed squares) and MCF-7 (closed circles) cells transfected with WT CYP17A1 were grown in steroid-free media as described in material and methods. Cells were plated in 96 well plates and treated with increasing concentrations of progesterone. Growth was assessed 6 days following treatment. Growth curves represent percentage of cell growth compared to ethanol (vehicle) control normalized to 100%. Points represent the mean of 6 replicates ± SEM. **(B)** Western blot illustrating successful expression of WT CYP17A1 protein in MCF-7 cells following stable transfection. CYP17A1 is not expressed in parental MCF-7 cells.

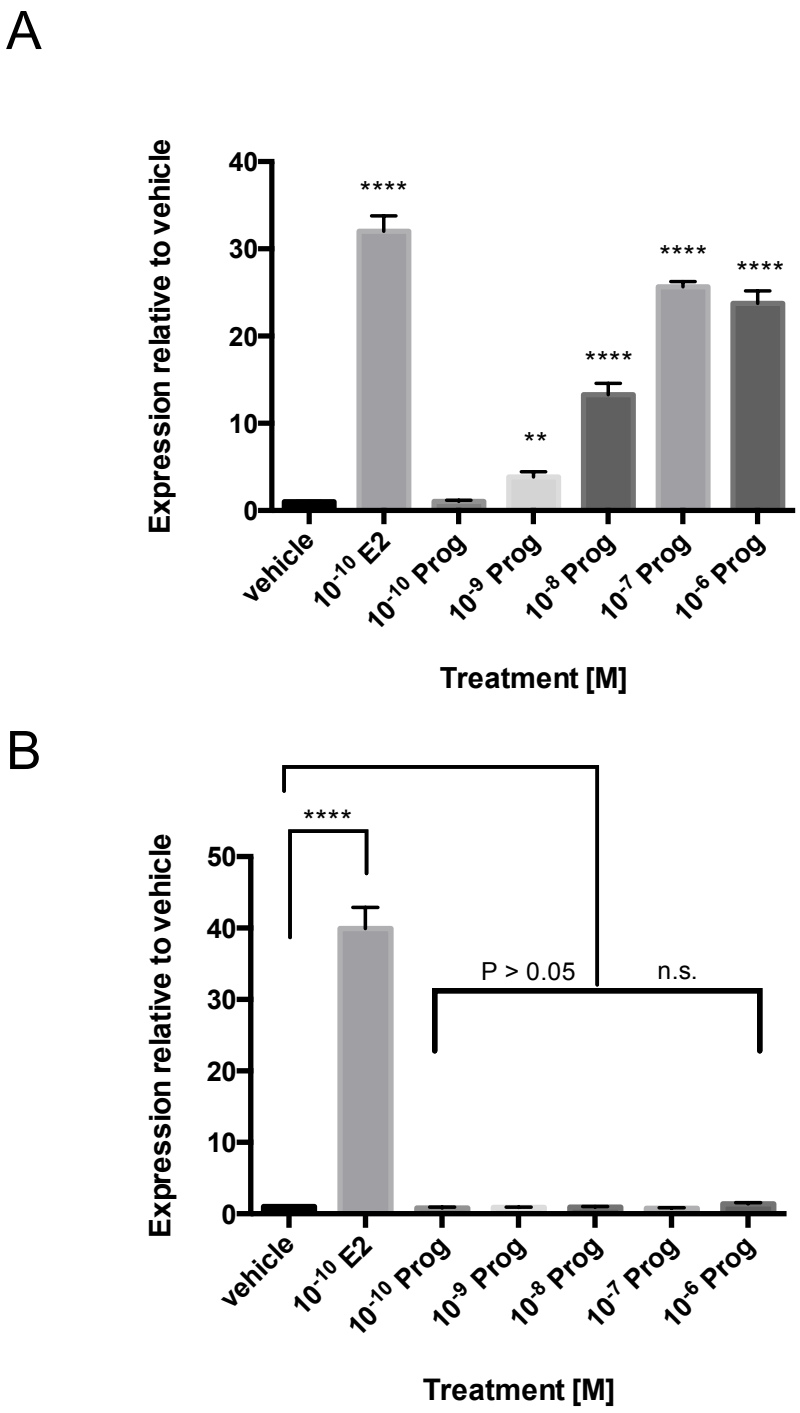

Figure 3.2. Progesterone treatment induces *GREB1* expression in MCF-7/CYP17A1 cells. (A), MCF-7 cells stably expressing WT CYP17A1 and (B) parental MCF-7 cells were grown in steroid-free media and plated in 6 well plates. Cells were treated with increasing concentrations of progesterone as indicated for 48 hours. *GREB1* mRNA expression was measured using RT-PCR. Expression levels are normalized to GAPDH and above bars represent *GREB1* expression relative to vehicle. Student's T-tests were used to compare expression differences. Bars represent the mean from 3 replicates ± SD. **= $P \leq 0.01$ *** = $P \leq 0.001$ **** = $P \leq 0.0001$

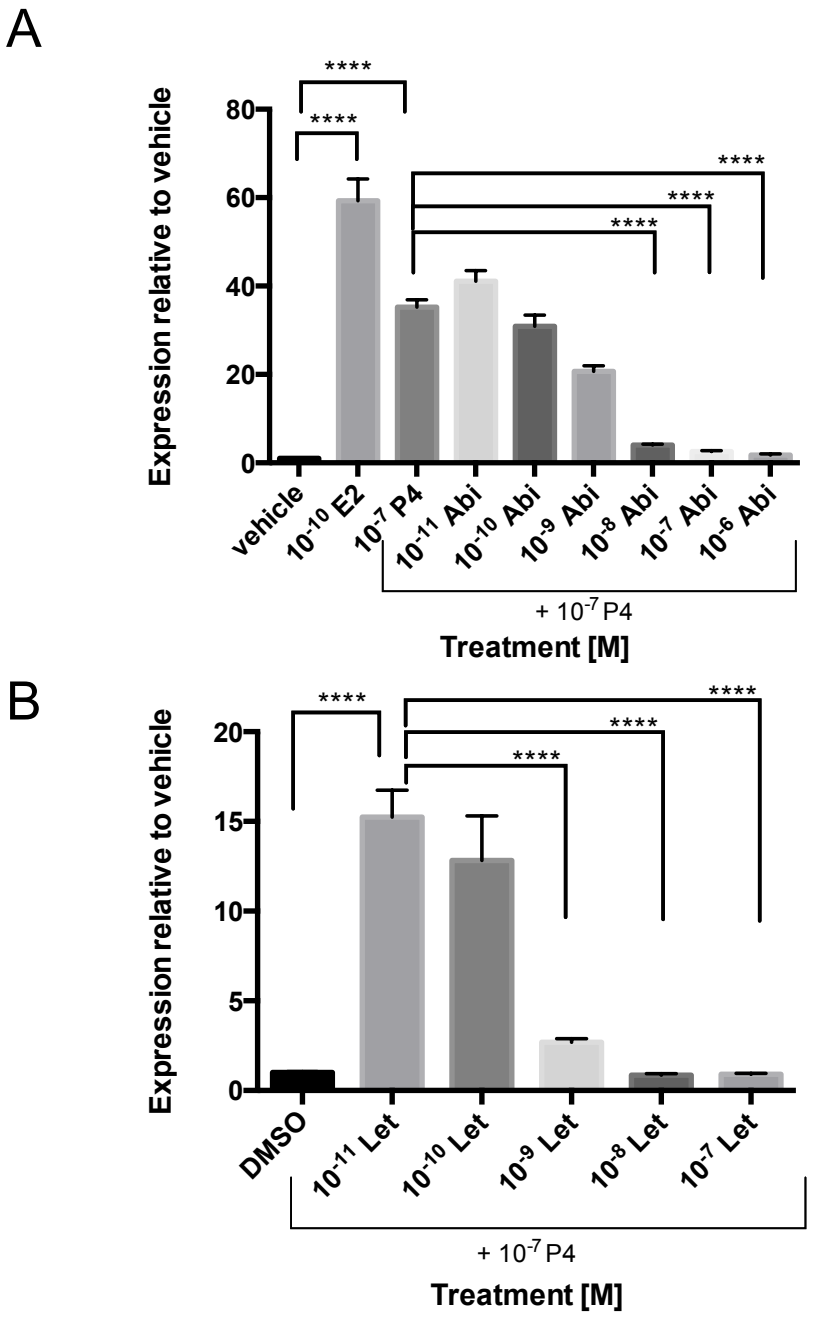

Figure 3.3. CYP17A1 and CYP19A1 inhibition in MCF-7/CYP17A1 cells blocks progesterone induced growth. MCF-7/CYP17A1 cells were grown in steroid-free media and then treated with increasing concentrations of **(**A) the CYP17A1 inhibitor abiraterone or (B) the CYP19A1 inhibitor letrozole. Cells were co-treated with 100 nM progesterone to induce *GREB1* expression. *GREB1* mRNA expression was measured using RT-PCR. Expression levels are normalized to GAPDH and above bars represent GREB1 expression relative to vehicle. Student's T-tests were used to compare expression differences. Bars represent the mean from 3 replicates ± SD. **= $P \leq 0.01$ *** = $P \leq 0.001$ **** = $P \leq 0.0001$

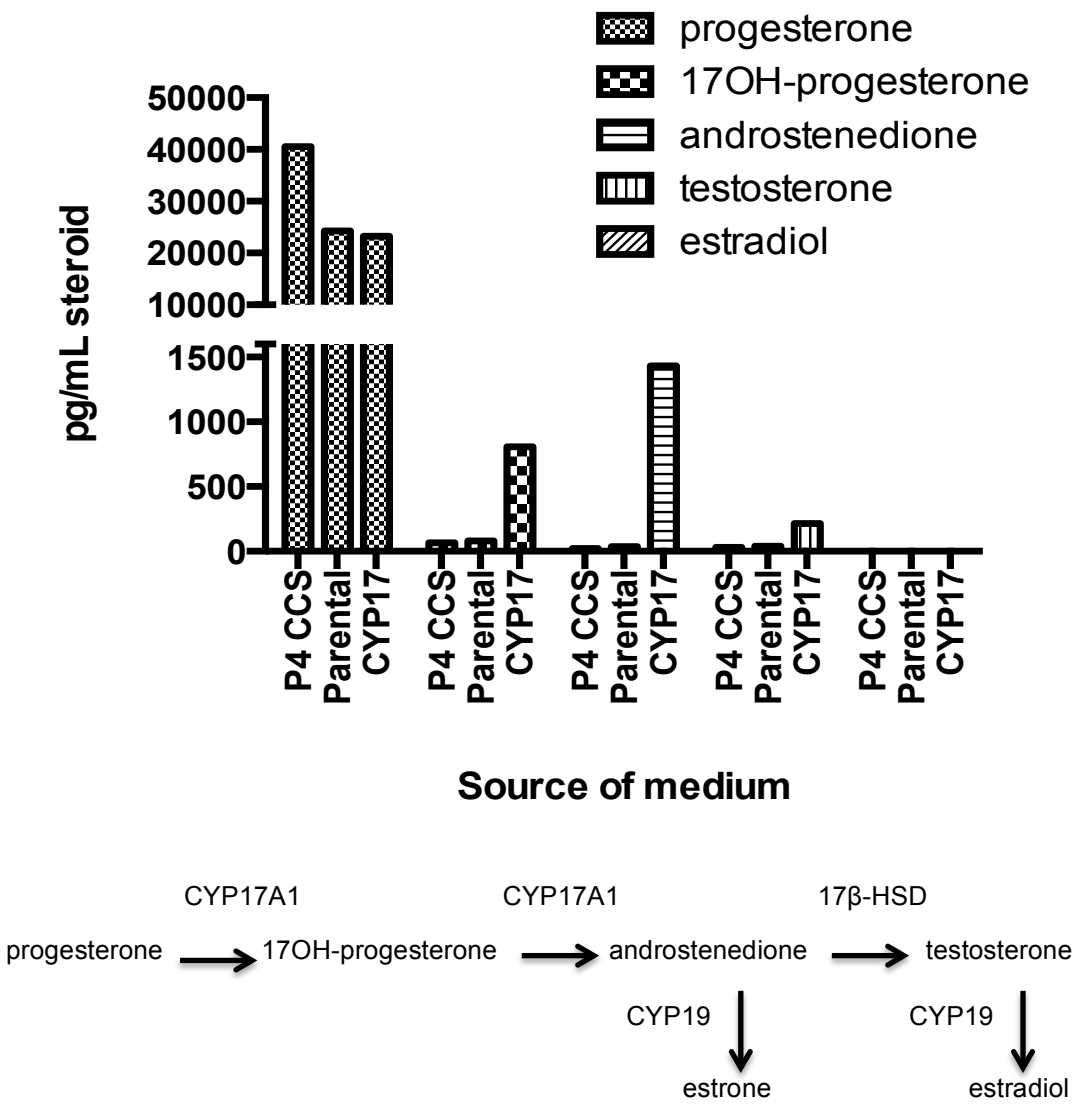

Figure 3.4. LC-MS/MS analysis measures progesterone metabolism to steroid intermediates in MCF-7/CYP17A1 cells. MCF-7 (parental) and MCF-7/CYP17A1 (CYP17) cells were grown in steroid-free media and incubated in 100 nM progesterone for 48 hours. The conditioned medium was collected and subjected to LC-MS/MS analysis. Prior to cell treatment, an aliquot of steroid-free media containing 100 nM progesterone (P4 CCS) was taken to use as the baseline controls. Steroids were measured in pg/mL by using known standards, and the above plot compares the measured steroid quantities for all three conditions.

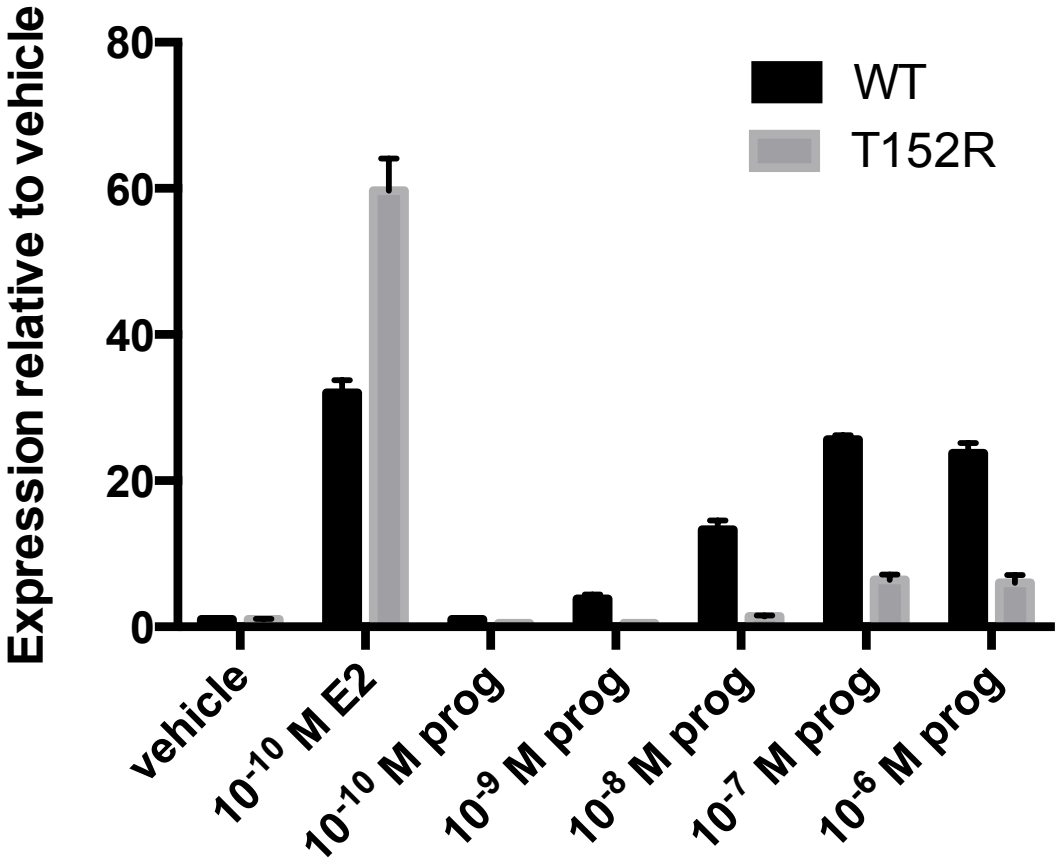

Figure 3.5. CYP17A1 variants expressed in MCF-7 cells demonstrate diminished activity compared to WT. This plot illustrates a representative *GREB1* expression profile from the various variants tested. Briefly, the T152R construct was made using site directed mutagenesis and stably transfected into MCF-7 cells. A pooled clone was generated and these cells were grown in steroid-free media and then treated with increasing concentrations of progesterone for 48 hours. E2 was used as a positive control.

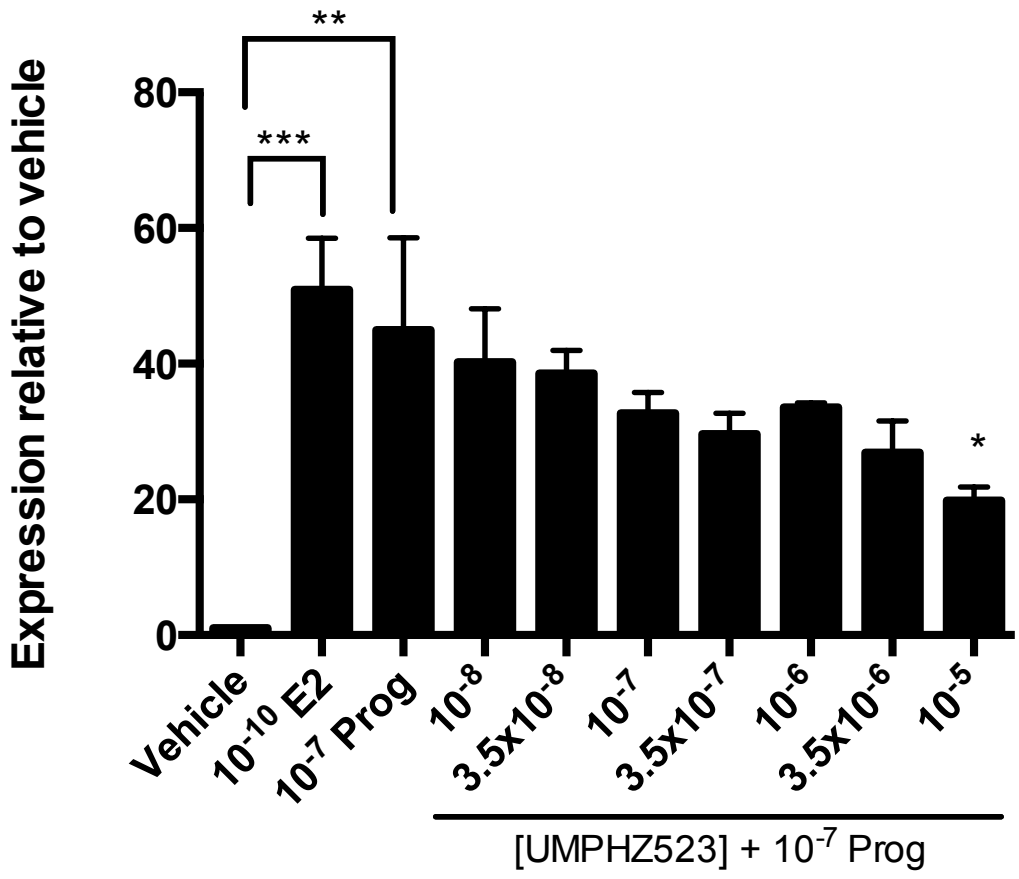

Figure 3.6. Identification of a novel, lyase-specific, CYP17A1 inhibitor using the MCF-7/CYP17A1 model system. MCF-7/CYP17A1 cells were grown in steroid-free media and then treated with increasing concentrations of UMPHZ523. 100 pM E2 was used as a positive control. Bars represent *GREB1* mRNA expression vs. vehicle treated control using the ΔΔCt method. Bars represent mean from 3 replicates ± SD. * = $P \leq 0.05$ **= $P \leq 0.01$ *** = $P \leq 0.001$

Variant	Nucleotide	Sense (5'-3')	Antisense (5'-3')
G471E	g1412a	gtgccagatgatgagcagctgccctcc	ggagggcagctgctcatcatctggcac
R349C	c1045t	atcagtgaccgtaactgtctcctcctgctgg	ccagcaggaggagacagttacggtcactgat
E330K	g988a	gtgaagaagaagctctacaaggagattgaccagaatg	cattctggtcaatctccttgtagagcttcttcttcac
E248A	a743c	tgatctgctgaataaaatacttgcaaattacaaggagaaattccgga	tccggaatttctccttgtaatttgcaagtattttattcagcagatca
D241Y	g721t	ctggaaaaattaaagagccatgttaaaatacgaaattatctgctgaataaaatacttg	caagtattttattcagcagataatttcgtattttaacatggctctttaatttttccag
S234R	c702g	caaaaccctggaaaaattaaagaggcatgttaaaatacgaaatgatctg	cagatcatttcgtattttaacatgcctctttaatttttccagggttttg
D216H	g646c	agacagcctggtgcacctagtcccctg	caggggactaggtgcaccaggctgtct
V197D	t590a	ggggaccctgagttgaatgacatacagaattacaatgaa	ttcattgtaattctgtatgtcattcaactcagggtcccc
G162R	g484a	ctggccacccacaacagacagtccatagaca	tgtctatggactgtctgttgtgggtggccag
T152R	c455g	atttgtcaggaaatcagtagattgtgtgatatgctggcc	ggccagcatatcacacaatctactgatttcctgacaaat
A105V	c314t	ggcaactctagacatcgtgtccaacaaccgtaagg	ccttacggttgttggacacgatgtctagagttgcc
A105L	g313t c314t g315a	tcaaatggcaactctagacatcttatccaacaaccgtaagggtatcg	cgataccttacggttgttggataagatgtctagagttgccatttga

Variant	RS number
G471E	rs140903153
R349C	rs138905928
E330K	rs142037395
E248A	rs201002001
D241Y	rs61754275
S234R	rs146311005
D216H	rs200063521
V197D	rs61754273
G162R	rs141821705
T152R	rs58822002
A105V	rs139936404

Table 3.1. Site-directed mutagenesis primers for CYP17A1 variants of interest. The A105L is a previously reported artificial substitution requiring more than one nucleotide alteration [40].

Table 3.2. RS numbers for variants identified on dbSNP.

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Chapter IV.

Functional characterization of the D216H and G162R CYP17A1 genetic variants

Introduction

CYP17A1 is a dual-function steroidogenic enzyme that catalyzes the two reactions necessary for cortisol and androgen biosynthesis. Expression of bovine CYP17A1 cDNA in nonsteroidogenic COS-1 cells displayed products of two separate enzymatic reactions confirming that CYP17A1 is a single steroidogenic enzyme with two key functions [1]. CYP17A1 hydroxylates the 17 carbon of pregnenolone and progesterone to form their 17α-hydroxylated products. A minor metabolite, 16OH-progesterone, can also be formed [2, 3]. The two major products of CYP17A1 hydroxylase activity, 17OH-progesterone and 17OH-pregnenolone, can act as substrates for cortisol biosynthesis or further metabolism into sex steroids. CYP17A1's second function is to cleave the C-C bond between the 17 and 20 carbon of the hydroxylated forms of pregnenolone and progesterone. This 17,20 lyase reaction gives rise to dehydroepiandrosterone (DHEA) and androstenedione and makes CYP17A1 a critical enzyme for androgen synthesis. These androgenic steroids can be further metabolized into the more potent androgens, testosterone (T) and dihydrotestosterone (DHT), or eventually into estrogens. CYP17A1's contribution to sex steroid synthesis makes it a target for hormone-dependent cancers that rely on androgens and estrogens for growth. Inhibition of CYP17A1 activity with abiraterone has proven to be clinically successful by prolonging overall survival in men with CRPC

pretreated with chemotherapy [4] as well as prolonging the time to chemotherapy in chemotherapy naïve men with prostate cancer [5]. CYP17A1 catalysis of hydroxylated substrates is more efficient with the Δ5 steroid, 17OH-pregnenolone, compared to the Δ4 substrate, 17OH-progesterone [6]. The enzymatic reactions of CYP17A1 requires electron donation via reduced nicotinamide adenine dinucleotide phosphate (NADPH) from P450 oxidoreductase (POR) [7], and the 17,20 lyase activity is enhanced nearly 10-fold in the presence of cytochrome b5. Cytochrome b5 acts as an allosteric modulator of lyase activity by helping to indirectly facilitate electron transfer [8]. CYP17A1 is oriented in the membrane of the endoplasmic reticulum where it interacts with its redox partners POR and b_5 [9]. The cytochrome b_5 and POR binding site residues were determined from the functional characterization of CYP17A1 mutations discovered in humans that resulted in amino acid changes, R347H and 358Q [10]. These substitutions selectively impaired CYP17A1 lyase activity while sparing any detrimental effect on hydroxylase activity underscoring the preferential influence these redox partners have on mediating the lyase reaction.

Germline *CYP17A1* genetic variants are extremely rare; however, characterization of these variants provides critical insight into CYP17A1 protein structure and function. By querying the dbSNP online database and publically available data from the 1000 genomes project we identified two CYP17A1 nonsynonymous genetic variants with unclear consequences on enzymatic activity and stability. The dbSNP database [11] aggregates uploads from sequencing efforts that included CYP17A1, while the 1000 genomes project was an initiative to sequence the genome of 1000 individuals from various ethnic backgrounds to use as a reference tool for genetic variants and their relative frequency [12]. The associated PolyPhen (Polymorphism

Phenotyping) scores of these variants, an *in silico* tool that predicts possible impact of amino acid substitutions on the structure and function of human proteins [13], suggested a high likelihood of these variants adversely affecting CYP17A1 protein, which warranted further investigation. We set out to functionally characterize the two variants with PolyPhen scores suggesting that substitutions would be probably damaging, G162R and D216H.

Amino acid substitutions at important positions in a protein can have a profound impact on secondary structure, enzyme activity, and protein stability [14]. Unstable proteins are commonly turned over or degraded much more rapidly than their wild type counterparts. In addition to protein stability, nonsynonymous coding SNPs can also have a significant impact on enzyme activity [15]. As an alternative to using purified CYP17A1 protein, we focused our efforts on optimizing a cell based assay for measuring CYP17A1 activity in variants of interest. Using transient transfections in HEK-293T cells, we treated cells with radiolabeled substrates and measured the conversion of these substrates into their products via HPLC. We hypothesized that these CYP17A1 variants would either impair catalysis of the aforementioned reactions or compromise CYP17A1 protein stability.

Methods

CYP17A1 Expression Construct

A pCMV6-XL4 plasmid expressing full-length human CYP17A1 cDNA was obtained from Origene (SC102224, Rockville, MD). NotI restriction enzyme sites were used to digest the CYP17A1 cDNA. Following insertion and ligation of the CYP17A1 cDNA into a pCMV6-Neo mammalian expression vector, diagnostic digests with SpeI and XbaI along with sequencing

identified a plasmid with the insert in the correct orientation. CYP17A1 variant constructs were generated using Agilent Technologies (Santa Clara, CA) QuikChange Lightning site directed mutagenesis kit. Site directed mutagenesis primers were designed using Agilent QuikChange primer design tool software. Oligonucleotides from primer sequences were synthesized by IDT technologies (Coralville, USA). Large quantities of wild type and variant plasmid DNA were generated using a Qiagen (Valencia, CA) maxi prep kit. All resultant plasmids were sequence verified to ensure site-specific mutagenesis and to ensure no off target effects. Primer sequences are as follows: G162R (sense) 5'-ctggccacccacaacagacagtccatagaca-3' G162R(antisense) 5'-tgtctatggactgtctgttgtgggtggccag-3'. D216G mutagenesis primer sequences D216H (sense) 5'-agacagcctggtgcacctagtcccctg-3'. D216H (antisense) 5'-caggggactaggtgcaccaggctgtct-3'.

Cell culture and reagents

HEK-293T cells were routinely cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Valley Biomedical). MG132 and Cycloheximide (CHX) were purchased from Sigma Aldrich (St. Louis, MO). Abiraterone was purchased from Selleckchem (Houston, TX). [^{3}H]progesterone was purchased from PerkinElmer (Waltham, MA).

Western Blotting

Western blot analysis was performed using whole cell lysates from HEK-293T cells. Cells were plated in 6 well plates and harvested 48 hours after transfection. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0 supplemented with

mini protease inhibitor tablet (Roche) and 5mM N-ethylmaleimide (Sigma) added immediately prior to lysis. Lysates were sonicated and centrifuged for 20 minutes at 14,000 x *g*. Supernatant was collected, and protein concentration was determined using the Bradford protein assay (BioRad, Hercules, CA). Absorbance was measured using PolarStar Omega plate reader. 30-50 μg of protein was loaded per lane and resolved on a 10% SDS-polyacrylamide gel (ThermoScientific) and transferred onto PVDF membrane. Protein levels were determined by blotting with a monoclonal anti-CYP17A1 antibody (Origene) and an HRP-conjugated mouse secondary antibody (Cell Signaling). Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagent (Super Signal, Pierce) on film. Transfected cells were treated with MG 132 (5 uM) or abiraterone (10 uM) for the specified amount of time where indicated.

CYP17A1 hydroxylase activity

HEK293T cells were plated in 12 well plates at ~80% confluency. Cells were transiently transfected with WT CYP17A1 constructs or variant CYP17A1 constructs using FuGENE HD transfection reagent (Promega). 24 hours post transfection cells were incubated with a mixture of radioactive (^{3}H-labelled) progesterone and non-radioactive progesterone for 3 hours. Steroids were extracted with 1 mL 1:1 ethyl acetate:isooctane and concentrated under nitrogen gas. HPLC analysis was performed using an Agilent 1260 HPLC. Dried samples were reconstituted in 20μl methanol, and 7 μl was injected into the HPLC. Injections were resolved with a 100 × 2.1 mm, 2.6 μm, C_8 Kinetex column (Phenomenex, Torrance, CA) and methanol/water gradients at 30°C. The column effluent was analyzed using a β-RAM4 in-line radioactivity detector

(LabLogic) using Liquiscint scintillation cocktail (National Diagnostics). Steroid products were quantitated by integration of radioactivity peaks using Laura4 software (LabLogic).

Results

Variant Protein Expression

Table 4.1 describes five of the variants narrowed down from our initial search. The putative damaging effect of these substitutions is converted to a PolyPhen score from 0 to 1. Scores above 0.85 are confidently predicted to have a damaging effect on protein function or stability. Accelerated degradation has been one of the most common causes of protein instability during previous studies of the functional effects of genetic variants [16]. Figure 4.1 illustrates the location of variants of interest on the crystal structure of CYP17A1. We first assessed protein stability by immunoblotting for CYP17A1 following a transient transfection of WT and variant CYP17A1 constructs in HEK-293T cells. Figure 4.2, *A* shows a western blot demonstrating the differences in protein expression of 3 different variants compared to wild type (WT) CYP17A1. It is apparent that there is less protein being expressed in the lane loaded with the G162R variant with β-actin serving as a loading control. These data support the PolyPhen score that the G162R variant in particular has a high probability of affecting enzyme stability or function.

Proteasome inhibition with MG132 recovers G162R

Decreased G162R variant protein expression suggests this variant is less stable and more susceptible to accelerated proteasomal degradation. Therefore, we hypothesized that we should be able to restore G162R protein expression to wild type expression levels by blocking the proteasome with the proteasome inhibitor, MG132. MG132 should prevent the degradation of the G162R variant, and this recovered protein can be visualized via immunoblot as only

degradation is being blocked, not protein synthesis. We again transiently transfected HEK293T cells with wild type CYP17A1 and the G162R variant. 12 hours prior to protein collection, we treated both WT and G162R transfected cells with 5 μM MG132. Figure 4.2, *B* indicates that there is significantly more WT CYP17A1 protein compared to G162R when the proteasome is not blocked. However, blocking proteasomal degradation of the unstable G162R variant restores its expression to WT levels. There is a minimal effect of MG132 on WT protein levels because of its prolonged stability.

G162R variant exhibits preferential ubiquitination

Because the degradation of the G162R variant appears to be mediated by the proteasome, we aimed to assess ubiquitination of the variant and how variant ubiquitination compares to WT. Misfolded proteins can be labeled with ubiquitin (Ub) to signal the cell to transport this protein to the proteasome [17]. To measure protein ubiquitination, HEK-293T cells were transiently transfected with WT and G162R constructs for 48 hours. The endogenous ubiquitin pool in HEK-293T cells is large enough that co-transfection of Ub is unnecessary [18]. The cells were treated with the proteasome inhibitor MG132 for 4, 2, and 1 hours prior to collection. Figure 4.3 illustrates CYP17A1 protein expression measured via western blot. CYP17A1 protein is approximately 57 kDa, and this band can be seen both during a short exposure and a long exposure. Higher mass bands appearing above 57 kDa are poly-ubiquitinated CYP17A1 products. Cells transfected with the WT construct show little evidence of ubiquitination regardless of a longer exposure. Conversely, cells expressing the G162R variant demonstrate a series of higher molecular weight bands. This “smear” is characteristic of poly-ubiquitinated

protein and is convincingly evident in a longer exposure. The intensity of the smear increases in a time-dependent manner with MG132 exposure.

Abiraterone stabilizes CYP17A1 protein expression

The CYP17A1 inhibitor abiraterone is known to be potent and slowly reversible due to the strong C-N bond between the drug and the heme iron of the P450 [19]. It is important to note, however, that this tight binding affinity of abiraterone is not mechanism-based inactivation. Instead, evidence suggests that most abiraterone remains bound for the life of the protein, and enzymatic activity is inhibited until new protein is translated and synthesized [20]. We hypothesized that abiraterone may show similar results to MG132 proteasome inhibition by binding to CYP17A1 and stabilizing both the WT protein and the G162 variant protein. HEK293T cells were transiently transfected with the WT and G162R variant constructs for 48 hours, and 10μM abiraterone was added to the cells for the specified times in Figure 4.5 prior to protein collection. Figure 4.4 demonstrates that abiraterone does indeed stabilize CYP17A1 protein expression as evidenced by the time-dependent increase in protein levels for both WT and the G162R variant. WT protein levels increase 2-fold following 12 hours of abiraterone treatment while G162R variant levels increase by nearly 10-fold. The 5-fold difference between the two constructs results from significantly less G162R protein at time zero due to its instability and premature degradation.

CYP17A1 variants affect CYP17A1 hydroxylase activity

It was observed that replacing alanine with a leucine at position 105 of CYP17A1 selectively impairs metabolism of progesterone to its minor metabolite, 16α-hydroxyprogesterone [20].

Alanine105 also faces the active site, and an updated crystal structure with this mutation in the protein suggests that this position is normally involved in a hydrogen-bonding network with bound substrate [21]. Substituting leucine abolishes this binding and disfavors the orientation in the active site for the 16-carbon of progesterone to be hydroxylated. To determine if the G162R or D216H (the other variant with a PolyPhen score above 0.85) variants had any effect on CYP17A1 hydroxylase activity, we measured the ability of this variant to convert progesterone into its major and minor hydroxylation products, 17OH-progesterone and 16OH-progesterone, respectively. We transfected HEK293T cells for 24 hours with WT, G162R, and D216H constructs. Cells were incubated with [3H]progesterone at a concentration of 1μM cells for 3 hours. Next, steroids were extracted and run through a HPLC with a scintillation counter to measure radioactivity. To compare the differences in hydroxylase activity, we determined the ratio of 17OH-progesterone that is formed compared to the amount of 16OH progesterone formed.

Figure 4.5 shows the HPLC chromatograms for 4 different conditions (empty vector, WT, D216H and G162R.) The top panel, empty vector, confirms that HEK293T cells are not steroidogenic and do not express CYP17A1. In cells transfected with WT CYP17A1, 8.11% and 55.5% of the [3H]-progesterone was recovered as 16-OH progesterone and 17-OH progesterone, respectively. Shown in the bar graph in Figure 4.6 is a comparison of WT CYP17A1 to D216H and the A105L mutations. Using the ratio of 17-OH progesterone to 16-OH progesterone as a quantitative measure of CYP17A1 enzyme activity, wild-type CYP17A1 yields a ratio of 6.9+/-0.05. In contrast, the SNP (rs200063521) encoding the D216H variant, results in a 2.2 fold decrease in 16OH-progesterone synthesis compared to wild-type suggesting altered hydroxylase

function. Whereas WT CYP17A1 hydroxylase activity in this assay results in a 5:1 ratio of major to minor product, in both variants this ratio is significantly increased to approximately 18:1 for A105L and 13:1 for D216H. The G162R substitution described earlier does not appear to have an effect on 16-OH progesterone formation, but it does appear to result in overall diminished activity. Quantification of the chromatogram peaks revealed that WT CYP17A1 converted 4 fold more progesterone into downstream products compared to the G162R variant (Figure 4.5). This result would be consistent with an unstable protein that retained activity, albeit reduced.

Discussion

Although the substitutions described herein were identified using SNP databases, their rarity and relative infrequency suggests they should more appropriately be considered genetic variants. Importantly, all genetic variants were identified in humans and therefore, we believe they can still provide insight into human disease processes and a better understanding of CYP17A1 structure and stability with regards to specific amino acid substitutions. This reasoning is further supported by Ng and Henikoff who reported most methods predict that 25-30% of nonsynonymous variants in a gene's coding region will have a negative effect on protein function [22]. Variants within the coding sequence that affect enzyme activity are often located in or near the active site. There do exist examples, however, of P450 variants situated on the periphery of P450s protein known to affect either enzyme activity or stability as is seen in the D216H and G162R variants of CYP17A1 [23, 24].

Various CYP17A1 mutations contribute to CYP17A1 deficiencies that may result in rare forms of CAH. Individuals expressing these mutations suffer from decreased levels of circulating sex

steroids and consequently delayed or absent puberty onset and various other disorders of sexual development. They often present with mild hypertension as a result of the excess mineralocorticoids in the circulation and a noticeable lack of distinguishing secondary sexual characteristics. These deficiencies can often be successfully managed with glucocorticoid replacement therapy to suppress ACTH feedback and prevent mineralocorticoid excess as well as sex hormone supplementation to promote pubertal and adult sexual progression.

The successful crystallization of CYP17A1 in 2012 has helped to explain how these genetic variants affect enzyme structure and function [25]. Before a crystal structure, we relied heavily on *in silico* modeling and borrowing from our collective knowledge of other members in the P450 family [9]. The CYP17A1 crystal structure highlighted the importance of several key residues necessary for substrate binding, both with respect to endogenous steroid hormones as well as inhibitors such as abiraterone. One of the residues most responsible for substrate positioning in the active site appears to be residue E305 that forms a hydrogen bond with N202 located in the F helix. Abiraterone binds strongly to the heme iron via its C17 pyridyl ring, and the 3β-OH group of abiraterone forms a hydrogen bond with N202.

One mutation observed in a kindred, the E305G mutation, results in isolated 17,20 lyase deficiency [26]. Residue 305 is a conserved residue amongst many of the cytochrome P450s, and it lies in the active site. This missense mutation in CYP17A1 is peculiar because it preferentially alters substrate binding. Whereas substrates prior to hydroxylation bind with similar affinities, 17OH-pregnenolone binding is impaired and DHEA synthesis is reduced due

to this substitution. It is now thought that this phenotype results from a disruption of the hydrogen bonding that normally occurs between E305 and N202 [25]

Most of the functional characterization studies of CYP17A1 variants have revealed adverse effects on hydroxylase activity, lyase activity, or both. In this study we report a variant that is less stable and more susceptible to protein turnover and degradation compared to wild-type protein. Previously, two mutations, Y329D and P428L, were identified in patients and characterized as having decreased activity and being unstable and degraded prematurely compared to WT [27, 28]. A portion of the enzyme must be properly folded for the minimal activity to remain; however, these data suggest that a majority of the protein is not folded correctly, which targets these variant proteins for proteasomal degradation leading to clinically relevant CYP17A1 deficiencies.

Dhir et al. described similar findings in 2013 in a study that functionally characterized four amino acid substitutions in CYP17A1 protein, A174E, V178D, R440C, and L465P identified in three different patients [29]. All four variants showed decreased expression on a western blot compared to WT. The authors explain these findings by suggesting each of these variants results in protein instability. The patients with these variants all presented with mild hypertension and significantly delayed sexual development. This study was published prior to the resolution of a CYP17A1 crystal structure, so predictions of impact were based on a computer model of CYP17A1.

Crystal structures with abiraterone or endogenous substrates bound to CYP17A1 quantifies the impact of the A105L substitution on active site volume due to the addition of a larger amino acid side chain (leucine > alanine) [21]. The A105L mutant reduces the active site volume from 677 $Å^3$ to 642 $Å^3$. We hypothesize that the D216H variant may also indirectly affect active site volume. The histidine, a positively charged, aromatic amino acid, replaces an aspartic acid, a negatively charged aliphatic amino acid. As described herein, the 17α-hydroxylation of progesterone is not impaired by this missense mutation, but there is very little 16α-hydroxyprogesterone synthesized with this substitution. We ascribe this phenotype to an orientation effect. There is no evidence of differences in binding affinity with this variant, only a difference in minor metabolite formation, which suggests that progesterone is not in the correct orientation to be hydroxylated at the 16 carbon. Although D216 does not directly face the active site, we hypothesize that this substitution still hinders active site orientation of substrates by means of steric hindrance. The biological significance, if any, of 16OH-progesterone remains largely unknown [2, 3].

Very little is known about CYP17A1 ubiquitination, and only one study appears to address this pathway of regulation [30]. In the ubiquitination-proteasome pathway, an 8.5 kDa ubiquitin molecule is covalently attached to a residue on an altered or unstable protein in a sequential pattern mediated by an E1 ubiquitin-activating enzyme, and E2 ubiquitin conjugating enzyme, and E3 ubiquitin ligase. Repetition of these aforementioned steps results in chains of ubiquitin attached to the protein, known as polyubiquitination. The 26S proteasome recognizes ubiquitinated substrates and proceeds to hydrolyze the protein into smaller peptides, thus degrading the protein [31]. Ubiquitination of P450s has been extensively studied, yet our

understanding appears to be much more complete for hepatic P450s [32]. A literature search for examples of CYP11A1, CYP17A1, CYP21A2, and CYP19A1 ubiquitination returned very little. Polyubiquitination, seen in Figure 4.4 is most likely acting as a signal for proteasomal degradation [33]. Further studies are necessary to better understand post-translational modifications such as ubiquitination of CYP17A1 and their implications.

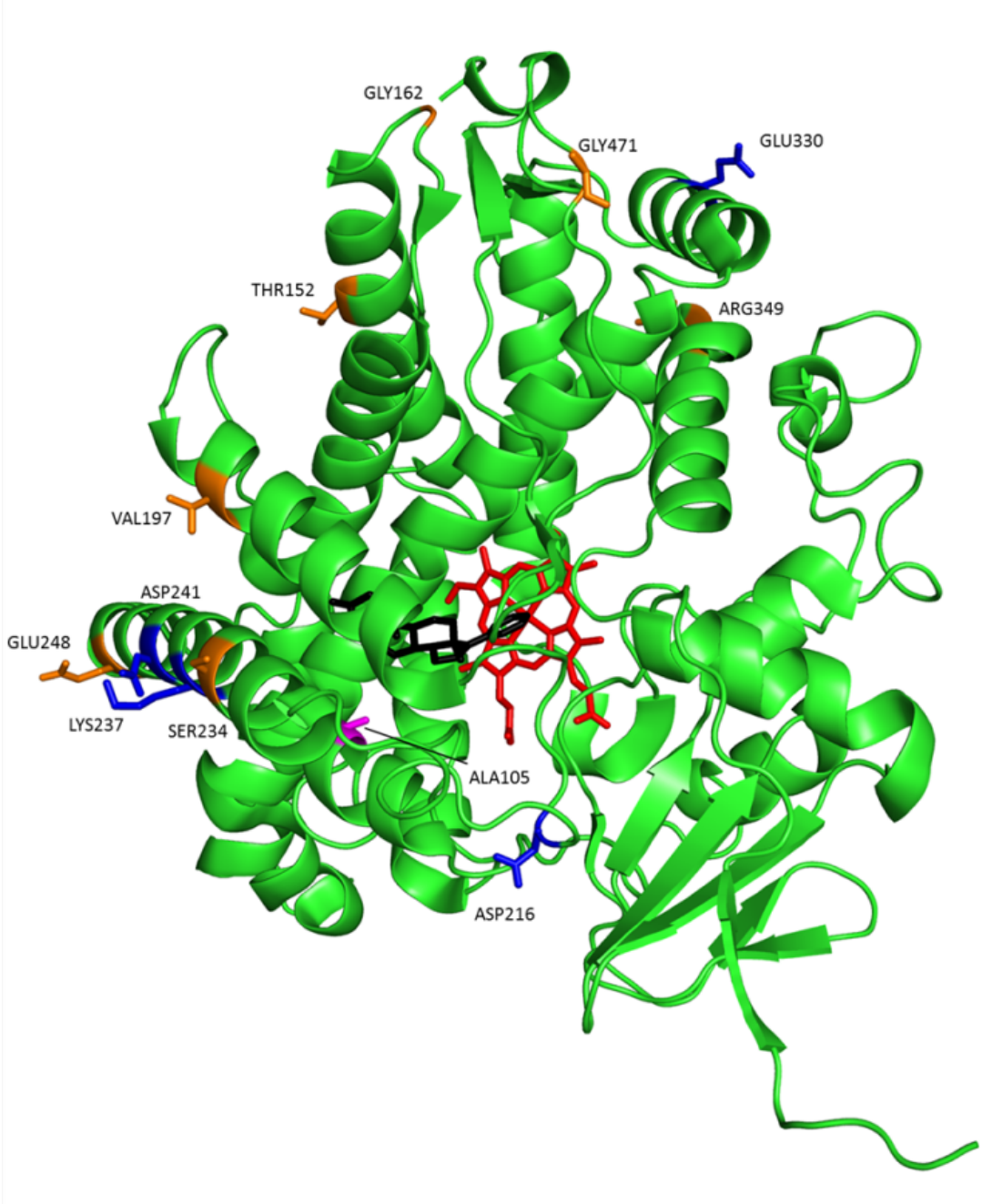

Figure 4.1. Crystal structure of CYP17A1 with variants of interest highlighted. This figure shows the location of our initial 12 variants of interest using the crystal structure of CYP17A1 in complex with the inhibitor abiraterone (shown in black) (PDBID 3RUK). The heme (shown in red) sits in the center of the P450. Image was generated using PyMol software.

Type	Alleles	AA	AA coord	PolyPhen score
missense variant	C/G	D/H	216	1
missense variant	C/T	G/R	162	0.962
missense variant	C/A	D/Y	241	0.663
missense variant	A/T	V/D	197	0.588
missense variant	C/T	G/E	471	0.452

Table 4.1. Characteristics and PolyPhen scores of variants of interest. This table shows the 5 variants most likely to have an effect on the structure and function of CYP17A1 based on their PolyPhen scores. PolyPhen scores > 0.85 = probably damaging.

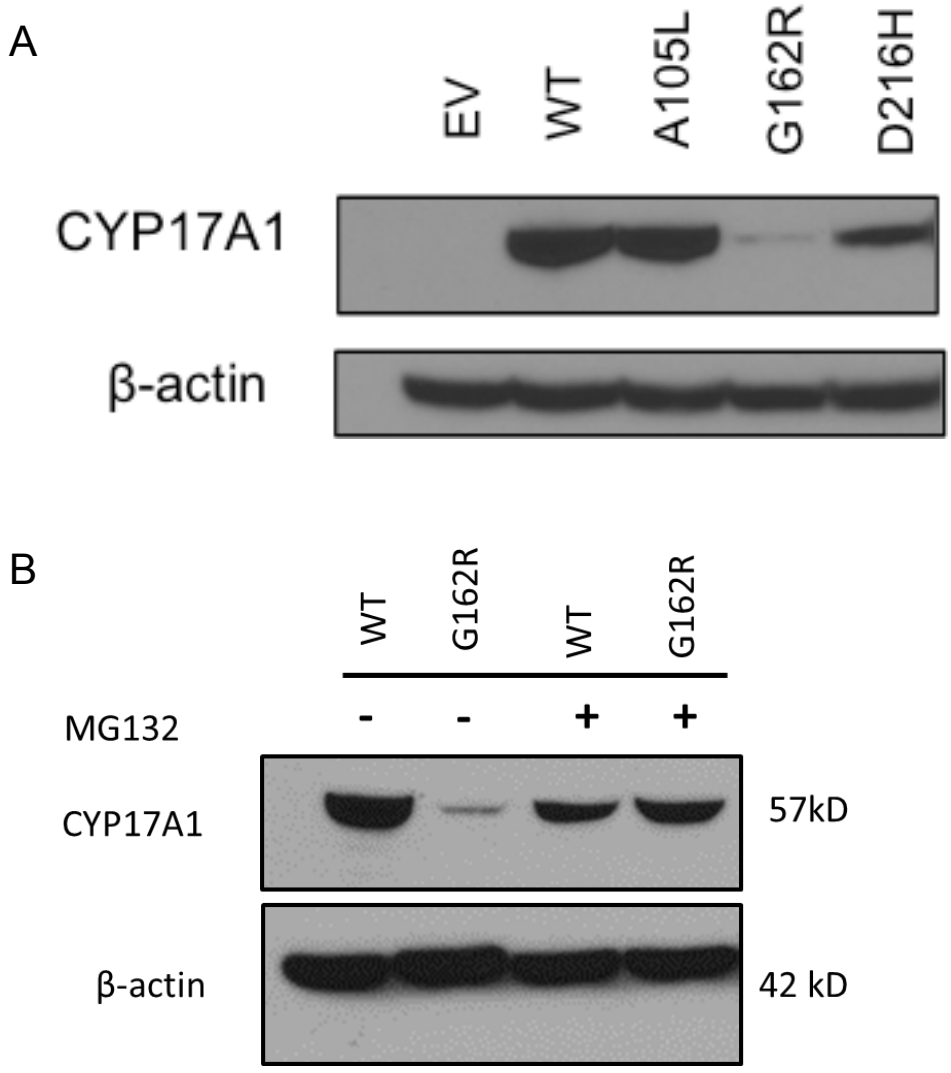

Figure 4.2. (A) Decreased G162R protein expression compared to wild-type. Western blot showing the differences in protein expression of variant CYP17A1 constructs compared to wild type. Protein was collected following a 48hr transfection. An empty vector was transfected and used a negative control. β-actin was used as a loading control. **(B) MG132 treatment blocks proteasome mediated degradation of the G162R variant.** Western blot showing that MG132 treatment for 12 hours recovers variant protein expression and restores it to WT levels. β-actin was used as a loading control.

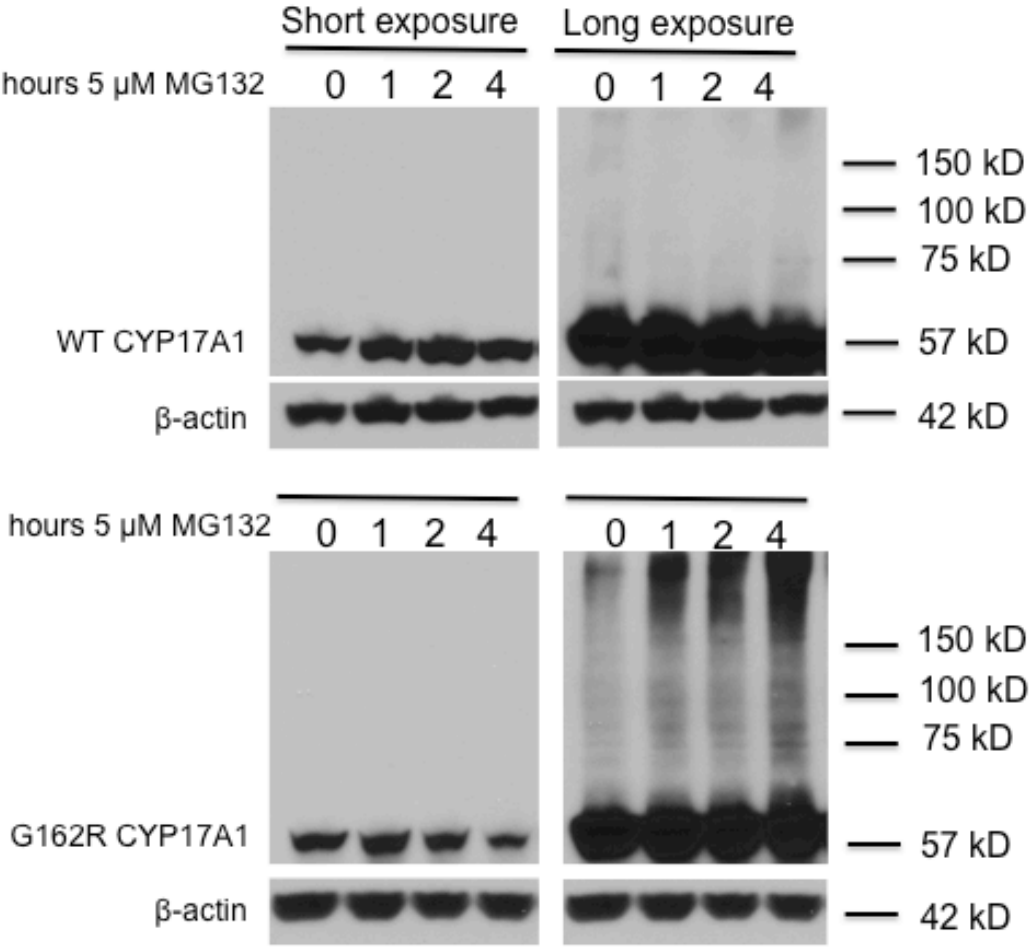

Figure 4.3. The G162R variant is polyubiquitinated when expressed in HEK293T cells. HEK293T cells were transiently transfected with either WT CYP17A1 or G162R constructs for 48 hours. For the indicated times prior to protein collection, the cells were treated with 5 μM MG132. Protein was collected using RIPA lysis buffer supplemented with a protease inhibitor tablet and 5 mM NEM to prevent deubiquitination. Whole cells lysates were subjected to western blot analysis on 10% SDS-PAGE gels and transferred to PVDF membrane. Blots were probed with anti CYP17A1 antibody and imaged on film using a HRP conjugated secondary antibody and chemiluminescent substrate.

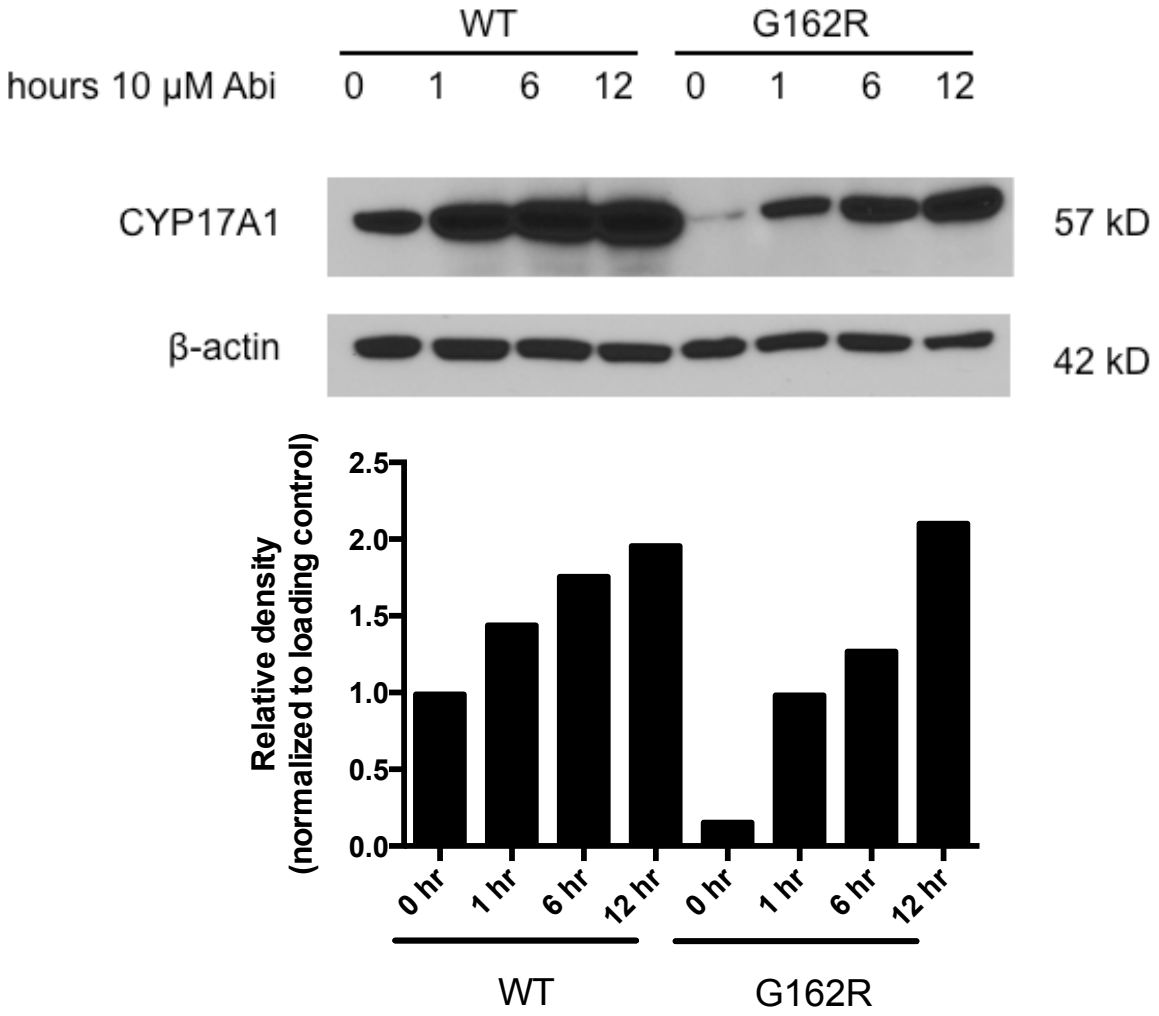

Figure 4.4. Abiraterone stabilizes CYP17A1 protein expression in HEK293T cells. HEK293T cells were transiently transfected with either WT CYP17A1 or G162R constructs for 48 hours. For the indicated times prior to protein collection, the cells were treated with 10 μM abiraterone (Abi). The bar graph depicts the relative density of the bands measured by ImageJ software and calculated as a ratio of CYP17A1 over actin.

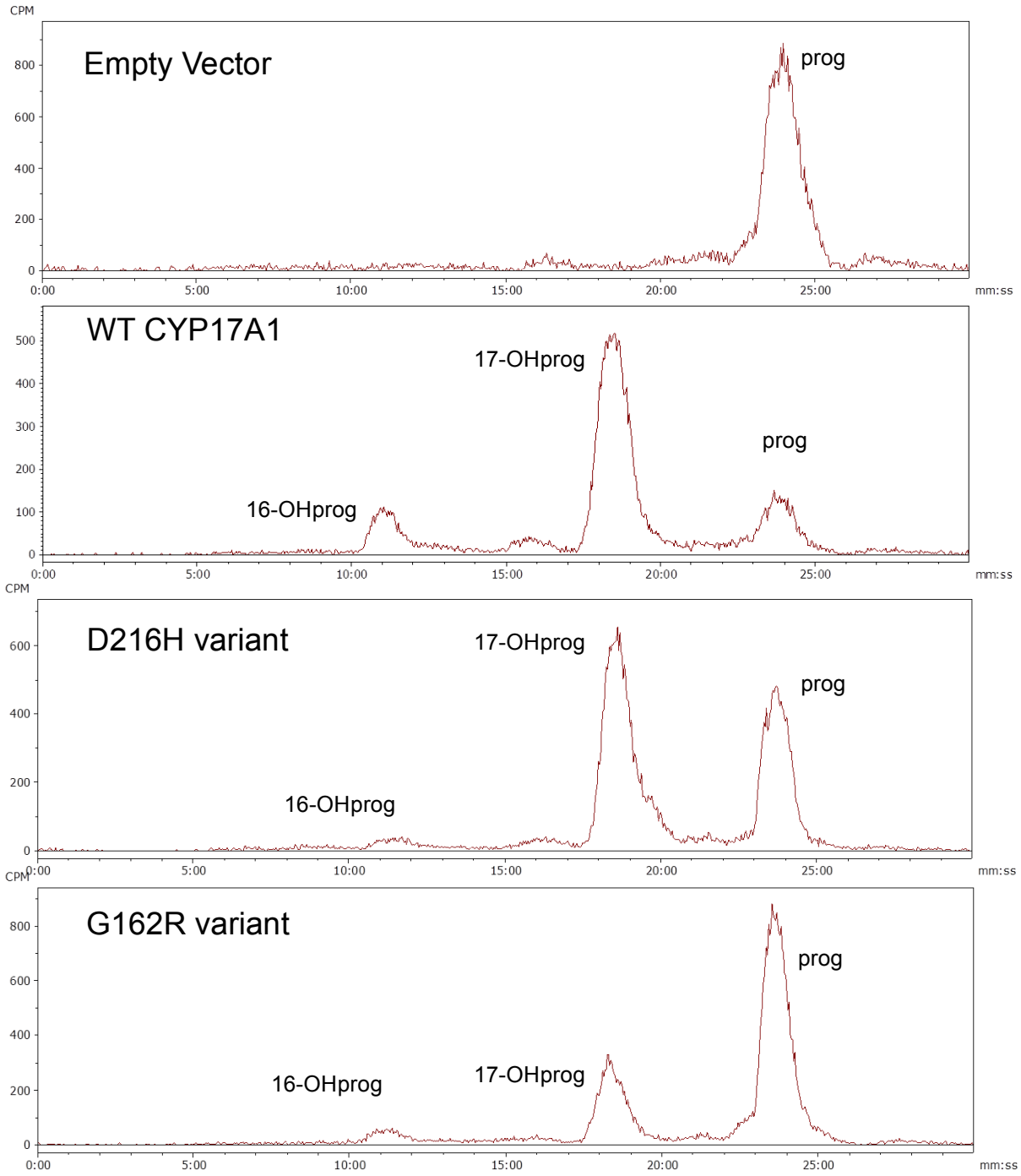

Figure 4.5. HPLC chromatograms showing the peaks representative of the labeled substrate (progesterone) and products (17 and 16 OH progesterone). HEK-293T cells were transiently transfected with WT or variant constructs in duplicate for 24 hours. Cells were then treated with 1 μM [^{3}H] progesterone for 3 hours and conversion of substrate to downstream products was measured by HPLC and an in-line scintillation counter. Retention times are consistent with values obtained from standards run prior to each experiment.

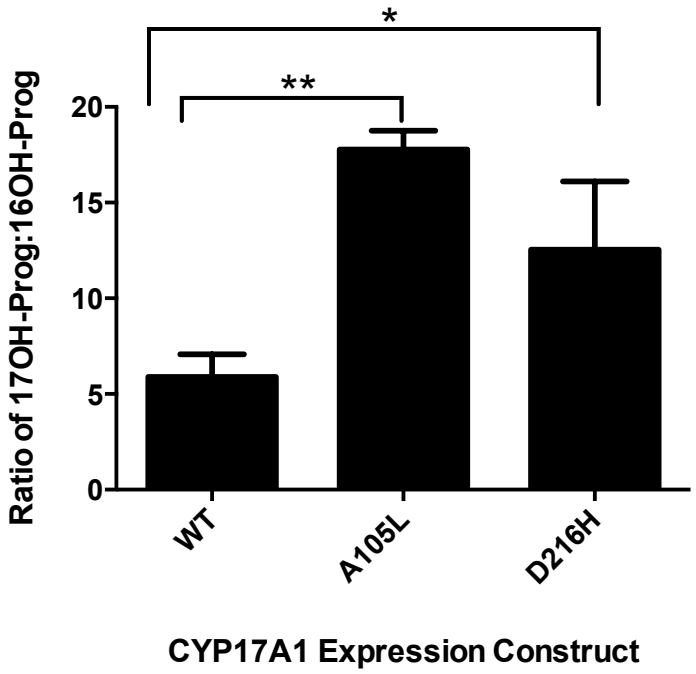

Figure 4.5 D216H variant shares phenotype with artificial A105L mutant. HEK-293T cells were transiently transfected with WT, A105L, or D216H CYP17A1 constructs in duplicate. 24 hours later the transfected cells were treated with 1 μM [^{3}H] progesterone for 3 hours and conversion of substrate to downstream products was measured by HPLC and an in-line scintillation counter. Bar graph depicts the ratio of 17OH progesterone to 16OH progesterone as determined by calculating the relative area under the peak on their respective HPLC chromatogram. Error bars represent the mean ± SEM of 2 independent experiments performed in duplicate. * = $P \leq 0.05$ ** = $P \leq 0.01$

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Chapter V.

Conclusions and Future Directions

Discussion

CYP17A1 inhibition in CRPC tumors significantly reduces extragonadal sources of androgen synthesis [1, 2]. The only currently FDA-approved CYP17A1 inhibitor, abiraterone, binds to CYP17A1 with a low nM affinity; however, it lacks selectivity for just the lyase activity and also inhibits cortisol synthesis by blocking the enzyme's hydroxylase activity. Cortisol synthesis blockade prevents the pituitary from receiving an ACTH negative feedback signal. Without ACTH signaling suppression, mineralocorticoid synthesis persists leading to mineralocorticoid excess and characteristic clinical symptoms such as hypertension, hypernatremia, and hypokalemia [3]. For this reason, men receiving abiraterone are concomitantly administered a glucocorticoid like prednisone to restore the ACTH negative feedback loop.

A lyase selective CYP17A1 inhibitor would circumvent the need for co-treatment with a glucocorticoid and also prevent the side effects associated with hydroxylase activity inhibition. Rationally designed inhibitors with preferential affinity for the lyase reaction have been generated; however, clinical success has been limited or absent compared to abiraterone. One novel approach of current investigation to achieve lyase reaction selectivity is to block the cytochrome b_5 interaction site on CYP17A1. One of the ways CYP17A1 activity is regulated from birth through puberty and into adulthood is by the tissue specific-expression of CYP17A1

redox partners that increase the efficiency of certain reactions [4, 5]. For example, the CYP17A1 lyase activity is ten times more efficient when cytochrome b_5 is bound to an allosteric site on the CYP17A1 protein [6-8]. This appears to occur without direct electron transfer as apo-b_5, b_5 lacking a heme group, is still able to stimulate lyase activity [7]. Multiple studies have aided in the identification of CYP17A1, cytochrome b_5, and POR residues that are required for coordinating the protein-protein interactions with CYP17A1 and these redox partners. The anionic E48 and E49 residues on cytochrome b_5 appear to be critical for b_5 interaction with the cationic R347, R358, and R449 residues of CYP17A1 [9-11]. Substituting the E48 and E49 residues of cytochrome b_5 with Ala, Gly, Cys, or Gln completely ablates enhancement of 17,20 lyase activity [12]. Mutations in POR have also been shown clinically to affect CYP17A1 activity. For example, the POR mutation G539R results in a phenotype similar to isolated 17,20 lyase deficiency while the hydroxylase activity remains intact [13]. These data highlight the necessity of POR and b_5 for efficient CYP17A1 lyase activity and identify areas of CYP17A1 to therapeutically target. Although disrupting protein-protein interactions pharmacologically is considered more difficult than inhibiting substrate binding directly in the active site, the undesirable clinical consequences of total CYP17A1 blockade requires novel strategies of inhibition. Some groups have focused their efforts on synthesizing new structure-based ligands that bind to CYP17A1, either in a lyase selective manner [14, 15] or perhaps another binding site exists on the periphery of the protein [16]. One of the newer, lyase selective CYP17A1 inhibitors is VT-464 [15]. While abiraterone is steroidal based, VT-464 is nonsteroidal small-molecule CYP17A1 inhibitor. VT-464 has been confirmed to be selective *in vivo* when administered to male, chemically castrate rhesus monkeys. Whereas abiraterone treatment increased the concentrations of CYP17A1 hydroxylase substrates such as progesterone, VT-464

administration resulted in no significant increase in upstream hormones suggesting successful selectivity of CYP17A1 lyase activity [17].

Another new compound showing preclinical promise is BMS-351. BMS-351 appears to be 10 times more specific for the lyase reaction with low nanomolar affinity (19 nM) and shows little affinity for the off target CYP1A1 (9 μM). When compared to abiraterone (42 mg/kg BID) in a cynomolgus monkey model, BMS-351 (1.5 mg/kg BID) showed similar efficacy by reducing T levels below the level of detection. Furthermore, BMS-351 did appear to be a more specific lyase inhibitor as evidenced by a smaller increase in progesterone concentrations compared to abiraterone [18]. Still, one common theme with inhibitors that attempt to selectively block the lyase activity by binding to the active site is that they sacrifice binding affinity for selectivity. Abiraterone is a potent, irreversible CYP17A1 inhibitor that covalently bonds to the heme iron. Selectivity seems to be a challenge that will not be overcome with other active site inhibitors. However, being able to abolish the b_5 interaction site on CYP17A1 appears to be an attractive targeting strategy.

Because CYP17A1 variants are so rare, it does not seem likely that they are contributing to prostate cancer resistance mechanisms. Abiraterone therapy does seem to promote AR mutations and AR splice variants, however, which contribute to disease progression in the metastatic castrate resistant setting. Chen et al. published in 2015 the finding that CYP17A1 inhibition with abiraterone appeared to select for an androgen receptor mutation, T878A, which recognizes progesterone as an agonist [19]. Romanel et al. optimized an assay testing plasma DNA from patients with CRPC treated with abiraterone for AR copy number gain and AR gene

aberrations. The investigators concluded that the 45% of patients with AR copy number gain or AR mutations, T878A or L702H, prior to abiraterone therapy, were nearly 5 times less likely to see a ≥ 50% reduction in prostate-specific antigen (PSA) levels. In this same investigation, targeted sequence analysis revealed that 13% of tumors showed emergence of the T878 and L702H amino acid substitutions at the time of progression on abiraterone that were not present in baseline plasma samples [20]. Further analysis is necessary to conclusively elucidate whether these are *de novo* mutations or mutations that arise only after treatment.

One particular AR splice variant that appears to be a major contributor to therapy resistance in CRPC is the AR-V7 splice variant [21]. This mutated form of AR lacks a ligand-binding domain (LBD) but remains constitutively active. This splice variant is resistant to antiandrogen therapy, as antiandrogens like bicalutamide and enzalutamide work by competing with androgens for the LBD. Moreover, this AR variant also represents a potential resistance mechanism to CYP17A1 inhibition as this variant would remain active in spite of ligand depletion. Preclinical studies support this hypothesis [22] as well as a prospective study that assessed AR-V7 mRNA in circulating tumor cells of men with CRPC [21].

While phosphorylation and other post-translational modifications on CYP17A1 have been reported [23-27], the overall regulation of CYP17A1 stability and steps involved in its degradation remain mostly unclear. In terms of ubiquitination, hepatic P450 ubiquitination appears to be much more well characterized [28] than steroidogenic P450s.

In Chapter II, we reported the novel observation that the CYP17A1 inhibitor, abiraterone, is able to interact with ER and induce growth in ER-postive breast cancer cell lines as well as induce the expression of ER responsive genes. This growth and gene induction can be blocked by directly inhibiting ER with the ER antagonist ICI 182,780 further suggesting that abiraterone's effects in these cell lines are ER-dependent. Analysis of various ligands for ER suggests the A ring 3β-OH group present on estrogens as well as abiraterone gives abiraterone its estrogenic properties. A newer CYP17A1 inhibitor, galeterone, which also possesses an A ring 3β-OH group shows a similar ability to induce GREB1 expression in two different ER-positive cell lines (Figure 5.1). A phase II trial of abiraterone in women with metastatic ER-positive breast cancer was unsuccessful in that abiraterone was not superior to AI therapy in these patients. We propose that these women harbored various other resistance mechanisms, as they were heavily pretreated prior to trial enrollment. We also propose that the estrogenic potential of abiraterone detailed in Chapter II as well as the relatively high concentrations of abiraterone found in circulation could be an explanation of the trial results.

Chapter III outlines a novel model system of CYP17A1 expression in breast cancer cells to characterize inhibitors of steroidogenic enzymes. As these cells are dependent on estrogens for growth, we leveraged this characteristic to allow us to use growth as a readout of enzyme activity and enzyme inhibition. We hypothesized that ER-positive breast cancer cells stably expressing CYP17A1, a protein they do not express to appreciable levels endogenously, would metabolize progesterone into the growth stimulus E2 due to the expression of all enzymes necessary for E2 synthesis. We validated this hypothesis by showing progesterone-induced growth in MCF-7 cells stably expressing WT CYP17A1, while this growth was absent in untransfected cells.

Furthermore, progesterone treatment induced expression of *GREB1* mRNA in a dose-dependent manner in MCF-7/CYP17A1 cells, whereas progesterone had no effect on *GREB1* induction in parental MCF-7 cells. Additionally, *GREB1* induction in MCF-7/CYP17A1 cells was blocked by pharmacological inhibition of CYP17A1 and CYP19A1 independently. We attempted to use this model system to assess the functional activity of CYP17A1 genetic variants but concluded that differences in protein expression would confound our conclusions.

Finally, in Chapter IV two CYP17A1 genetic variants identified in humans were functionally characterized for the first time. Using transiently transfected HEK-293T cells treated with radiolabeled substrates, we were able to compare the activity profiles of WT and variant CYP17A1 with regards to product formation. We determined the D216H variant to have decreased 16-hydroxylase activity as evidenced by the 5-fold decrease in 16OH-progesterone formed compared to WT. We hypothesize that this phenotype is due to altered active-site substrate orientation that restricts progesterone hydroxylation on the 16 carbon. This observed phenotype was similar to the previously studied artificial (not identified in humans) substitution, A105L. We next characterized the G162R variant of CYP17A1 and determined this amino acid substitution resulted in a less stable protein compared to WT. Inhibition of the proteasome recovered G162R variant to WT levels and abiraterone treatment appeared to stabilize the variant protein and prolong its half-life.

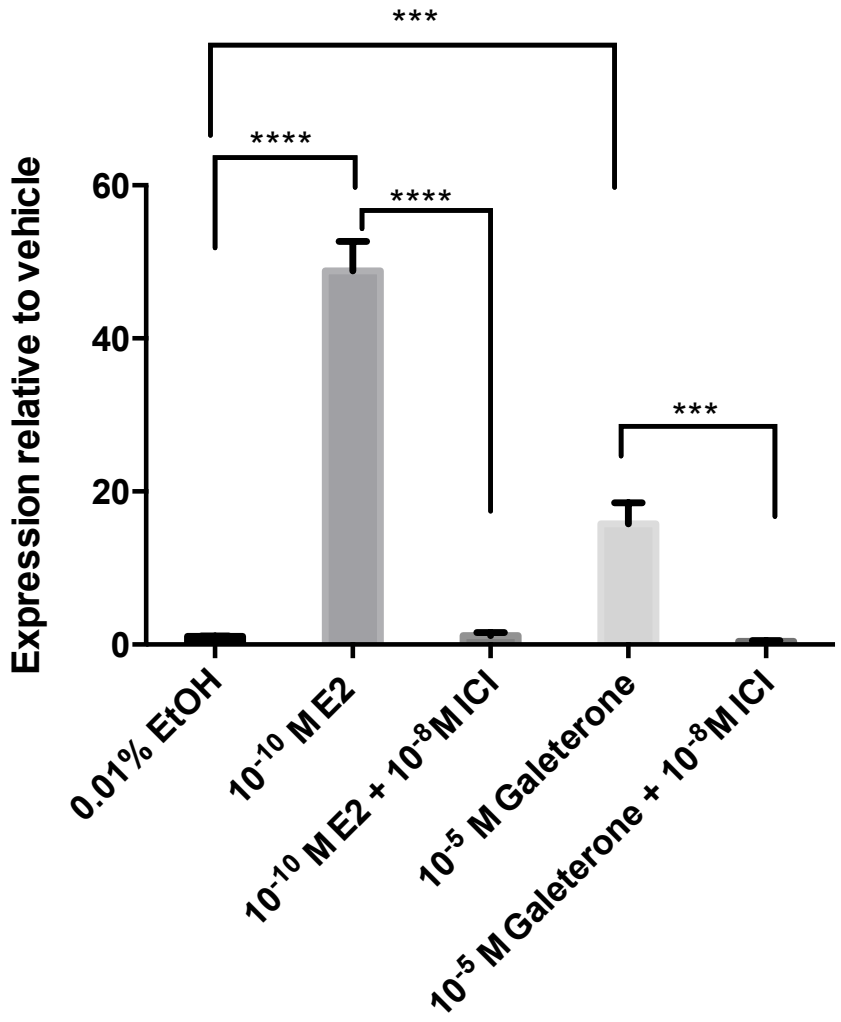

Figure 5.1. Galeterone induces *GREB1* expression in MCF-7 is inhibited by ICI 182,780. MCF-7 cells were grown in steroid-free media as described previously. Cells were plated in 6 well plates and treated for 48 hours as follows: 100pM E2, 10μM galeterone (Selleckchem, Houston, TX), and 10nM ICI 182,780 (ICI). Bars represent *GREB1* expression vs. vehicle treated control using the ΔΔCt method. Bars represent mean from 3 replicates ± SEM. *** = $P \leq 0.001$ **** = $P \leq 0.0001$

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