A Dissertation entitled

Drug Targets to Improve the Outcome of Hormonal Adjuvant Therapy in Breast and Prostate Cancers

By

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Science

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An Abstract of

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Tamoxifen and aromatase inhibitors (AI) are a mainstay in the treatment of estrogen receptor (ER)-positive breast cancer. However, long-term treatment of women at high risk for breast cancer leads to development of resistance against the drug and reduces the incidence of both invasive and non-invasive ER-positive breast cancer in only approximately 50% of cases. For resistance to develop during either hormone depletion (AI) treatment or tamoxifen adjuvant therapy, there must be a fraction of cells that must maintain a basal level of cell cycling. Growth and proliferation of these latent tumor cells later lead to the development of resistance. Our lab has shown that: “Under conditions of hormonal adjuvant treatment the estrogen receptor apoprotein supports breast cancer cell cycling through the retinoic acid receptor (RAR) -α1 apoprotein”. RARα1 is the only isoform expressed in breast tumor cells and it being genetically redundant, targeting it specifically may lead to improved outcome of current hormonal adjuvant therapies with minimal side effects. We have synthesized a high affinity RARα-specific antagonist, AGN194301, as a carrier molecule to selectively target RARα. AGN194301 by itself did
not affect the ability of RARα1 to support basal cell proliferation and did not alter the level of RARα1 protein. Structure-activity-relationship (SAR) studies showed the functional group(s) of the compound that could be modified without abrogating its ability to bind to RARα. Further studies and strategies for attaining downregulation of RARα1 at the protein level using modified versions of this compound are discussed.

The androgen receptor (AR) is essential for diverse aspects of prostate development and function. Molecular mechanisms by which prostate cancer (PC) cells redirect AR signaling to genes that primarily support growth are unclear. A systematic search for critical AR-tethering proteins led to ELK1, an ETS transcription factor of the ternary complex factor subfamily. Although genetically redundant, ELK1 was obligatory for AR-dependent growth and clonogenic survival in both hormone-dependent PC and castration-recurrent PC cells but not for AR-negative cell growth. This study discusses detailed mapping of the peptide sequences within AR and Elk1 that are required for the interaction between them. We found that the N-terminal A/B domain of AR is alone sufficient for its interaction with Elk1 and for Elk1-dependent transactivation by AR. We further mapped the AR-A/B domain using deletion analysis and demonstrated that aa 156-329 within the A/B domain selectively inhibits Elk1-dependent gene activation by androgen as well as androgen-dependent growth of LNCaP cells. Synthetic peptides corresponding to sequence aa 156-206 and aa 181-231 inhibited the binding of the AR A/B domain to Elk1 and also inhibited the androgen-dependent growth of LNCaP cells. Simultaneously, deletion mapping of Elk1 and the use of mammalian two-hybrid assays identified the sequence aa 287-317 of the N-terminus and aa 387-428 of C-terminus as
essential for the binding of Elk1 to AR. We also tested a phosphorylation mutant of Elk1 which was found to retain its ability to associate with AR. The results suggest that major splice variants of AR that lack the C-terminal ligand binding domain and that are known to support hormone-independent growth of prostate cancer cells may utilize Elk1 for growth signaling. Further, these results also confirm that phosphorylation of Elk1 is not involved in the Elk1-AR synergy. Finally, the results from this study led to identification of peptides that may be used to selectively disrupt the synergy between Elk1 and AR without interfering with other aspects of AR signaling.
I dedicate my dissertation to my undergraduate Professor and Head of the Department - Dr. Tara Menon, for always supporting me, believing in me, teaching me the importance of perseverance and will and for always being a source of inspiration and encouragement which has helped me become the man I am today.
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Chapter 1

Literature Review

1.1 Breast Cancer

Breast cancer is the most common malignancy and is the leading cause of cancer death after lung and bronchus cancer in women. More than two-thirds of breast cancer cases are estrogen receptor (ER) selective – positive. Tamoxifen, a selective estrogen receptor modulator (SERM), is a mainstay in the treatment and prevention of ER-positive breast cancer in both pre and postmenopausal women. However, long-term treatment of women at high risk for breast cancer leads to development of resistance against the drug and reduces the incidence of both invasive and non-invasive ER-positive breast cancer by only about 50 percent. Additionally, it also increases the risk of endometrial cancer. Long-term treatment with aromatase inhibitors (AIs) which have been considered as a suitable alternative to tamoxifen treatment also leads to development of resistance. Moreover, AI can be used only to treat postmenopausal women suffering from ER-positive breast cancer.
1.1.1 Breast Cancer etiology and epidemiology

Breast cancer is the most common type of cancer diagnosed and the second leading cause of death in American women, affecting 1 in every 8 women, according to the U.S. Cancer Statistics Working Group and the Center for Disease Control and Prevention (www.cdc.gov/uscs). Endocrine ablation by surgery or by radiation-induced oophorectomy was the main treatment option for patients with advanced breast cancer for the first 60 years in the 20th century; however, it had a success rate of only 30%. The estrogen receptor (ER) was discovered in 1962 and this led to development of better targeted treatment options. The expression of ER in the breast tissue was also established as a predictive marker for ablative surgery (Jensen, Block et al. 1971); and was reported to predict the outcome of breast cancer treatment in 1974 (Jordan 2003) This led to molecular basis of classification of breast cancer based on the presence of hormone receptors – estrogen receptor (ER) and progesterone receptor (PR). Hormone receptor positive breast cancer accounts for about 60% of the premenopausal and 80% of the postmenopausal breast cancers (Clark, Osborne et al. 1984). In this case, anti-estrogen adjuvant therapy is the mainstay in the treatment of ER positive tumors. HER2/neu (human epidermal growth factor 2), a member of the tyrosine kinase family (Coussens, Yang-Feng et al. 1985) was found to be amplified in 30% of breast cancers (Slamon, Clark et al. 1987, Slamon, Godolphin et al. 1989) and was correlated to be a negative prognosticator of breast cancer (Ross and Fletcher 1998, Schnitt 2001). Triple negative breast cancer (TNBC) is described as a subtype of breast cancer that lacks expression of the hormone receptors ER and PR and does not overexpress HER2 receptor protein and accounts for about 15% of the breast cancers (Hudis and Gianni 2011) TNBC has poor
prognosis as treatment of it solely depends on surgery, chemotherapy and radiation lacking an effective targeted treatment strategy. Molecular subtypes of breast cancer, classified on the basis of distinct gene expression patterns, include luminal A (ER, PR positive, HER2 negative), luminal B (ER, PR, HER2 positive), basal (ER, PR, HER2 negative) and HER2 (ER, PR negative and HER2 positive) (Perou, Sorlie et al. 2000). The luminal subtype expresses high amounts of luminal cytokeratins and genetic markers of luminal and normal breast epithelial cells, whereas, the basal subtype, arising from the outer basal layer expresses cytokeratins associated with basal types of cancers and is typically high-grade and poorly differentiated (Rakha, El-Sayed et al. 2007, Sotiriou and Pusztai 2009)(Rakha et al., 2007; Sotiriou et al., 2009).

Most breast cancer malignancies are lesions occurring in the epithelial lining of the ductal-lobular system involved in breast milk production (Yoder, Wilkinson et al. 2007). Ductal carcinomas account for approximately 75-80% of all cases of breast carcinomas and are divided into two subtypes: ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC). Ductal carcinoma in situ or intraductal carcinoma usually characterizes early stages of the disease, when tumor cells are not invasive and proliferation is still confined to the mammary ducts (Tamimi, Baer et al. 2008); however, patients with DCIS are at a greater risk of developing IDC, characterized by an aggressive metastatic phenotype and poorer prognosis (Lagios 1995).

Unlike DCIS, the contribution of lobular carcinoma in situ lesions (LCIS), also known as lobular neoplasia to the invasive phenotype (Carolin, Tekyi-Mensah et al.
2002), as well as the incidence of LCIS in the general population is still unclear (Simpson, Gale et al. 2003). Invasive lobular carcinomas (ILC) arise in the milk producing glands at the terminal ends of the ducts, representing around 10% of all breast cancer cases. Importantly, ILC and IDC have distinct metastatic patterns; metastasis to the peritoneum, ovary and gastrointestinal systems are associated with ILC whereas IDC are more likely to metastasize to the lungs and CNS (Arpino, Bardou et al. 2004). Other types of breast cancer, including tubular, invasive cribriform, invasive solid papillary, pure mucinous and apocrine carcinomas are rare (Yerushalmi, Hayes et al. 2009).

Even though breast cancer etiology is poorly understood, several risk factors have been identified and altogether, are important to help to prevent and reduce breast cancer incidence in women at high risk. Family history, hormonal and reproductive factors, as well as exposure to environmental carcinogens have been shown to greatly increase the risk of developing breast cancer (DeBruin and Josephy 2002, Hankinson, Colditz et al. 2004). Among women with a first degree relative who had breast cancer, inheritance of mutant BRCA1 or BRCA2 genes at least doubles the risk of developing sporadic breast tumors when compared with the general population (Colditz, Willett et al. 1993, Hedenfalk, Duggan et al. 2001); however the prognostic value of family history for screening purposes remains controversial (Phillips, Andrulis et al. 1999, Thalib, Wedren et al. 2004) and genetic predisposition only accounts for up to 10% of all cases of breast cancer (McPherson, Steel et al. 2000).
Regardless of family history of breast cancer and genetic predisposition, breast cancer incidence in humans increases with aging (Benz and Yau 2008). While hormonal replacement therapy (HRT) attenuates menopausal symptoms and helps to prevent osteoporosis and other chronic conditions in women (Nelson, Humphrey et al. 2002), studies have demonstrated a direct association between HRT using estrogen-progestin regimen with increased breast cancer risk during the course of treatment (Schairer, Lubin et al. 2000, Chlebowski, Kuller et al. 2009). Further, postmenopausal women undergoing HRT using estrogen plus progestin that developed breast cancer were more likely to be diagnosed with advanced stage invasive breast cancer than the placebo group (Chlebowski, Hendrix et al. 2003).

Acquired mutations due to occupational exposure to hazardous agents, chemical pollutants and lifestyle factors like smoking and diet are known to represent potential risk factors for development of cancer in general, but a direct relationship between exposure to environmental xenoestrogens or synthetic chemicals and risk of developing breast cancer may be difficult to establish due to the lack of proper controlled experimental populations (Weyandt, Ellsworth et al. 2008).

1.2 Estrogen (E$_2$) Biology

Among the different forms of estrogen, 17β-estradiol (E$_2$) is the major form and is essential in development, maintenance and function of the female reproductive system. It is also required for the health and maintenance of various other tissues like the cardiovascular system, bone, brain centers that maintain body temperature and the
vaginal lining (Russo and Russo 1998, Riggs, Khosla et al. 2002, Huang and Kaley 2004, Maggi, Ciana et al. 2004). Apart from the functions mentioned above, E2 secreted by the ovaries stimulates breast tumor growth. Time duration and level of exposure to estrogen correlates with breast cancer risk (Safe 1998, Clemons and Goss 2001, Ali and Coombes 2002). In postmenopausal women, although the source of estrogen is non-ovarian tissues like the adipose, muscle, liver or brain; breast tumor growth is supported by estrogen produced within the tumor whose levels are >20 times of that in plasma (Ali and Coombes 2002). This is due to increased local synthesis of E2 (Castagnetta, Lo Casto et al. 1996, Pasqualini, Chetrite et al. 1996). Most of the breast epithelial cells are ER– negative, however, two-thirds of the breast tumor cells are ER–positive and their growth and progression is stimulated by E2.

1.3  **Estrogen Receptor (ER)**

ER has two known sub-types: ERα and ERβ. ERα has a clear role in stimulating breast cancer growth and is a marker for prognosis, whereas the role of ERβ is less known (Pearce and Jordan 2004). ERβ may modulate E2 signaling by heterodimerizing with ERα, however, majority of ER in breast tumors is ER type α. Also, during progression to malignancy, expression of ERα increases while that of ERβ decreases, thus questioning the role of ERβ in breast cancer. In most of the cases, tumors that were initially hormone responsive but later developed endocrine resistance, ER persists and continues to support tumor growth by alternate mechanisms, which will be discussed further.
1.3.1 Estrogen Receptor Structure:

The human estrogen receptor (ER) has a structure that is shared by all members of the steroid receptor family. It consists of six domains namely A-F (Kumar, Green et al. 1987). The N-terminal A/B domain consists of the hormone-independent activation function (AF-1) domain. The middle C-domain that consists of the DNA-binding domain is made up of two zinc finger motifs, which are responsible for ER binding to estrogen response elements (EREs) (Klein-Hitpass, Ryffel et al. 1988). The C-domain also consists of a D-box which is responsible for dimerization of ER on the EREs. The D-domain is the hinge region and is involved in binding of co-regulatory proteins (Jackson, Richer et al. 1997). Lastly, the C-terminal domain consists of the ligand binding domain (LBD) which is a region implicated in modulating agonist activity of non-steroidal antiestrogens (Montano, Muller et al. 1995). The LBD comprises of the ligand-dependent transactivation functions AF-2 and AF-2a (Norris, Fan et al. 1997), hsp binding region (Chambraud, Berry et al. 1990), a nuclear localization signal (NLS) to localize into the nucleus following ligand binding (Picard and Yamamoto 1987) and another dimerization domain (Peters and Khan 1999). The crystal structure of the LBD revealed 12 α-helices, of which five (helices 3, 6, 8, 11 and 12), form a hydrophobic ligand-binding pocket. On ligand binding, helix 12 undergoes repositioning to a great extent, which depends upon the type of ligand binding to ER (Pike, Brzozowski et al. 1999). These data in combination with functional studies of ER suggest that ligand-induced conformational changes in steroid receptors affect co-factor recruitment and modulate the receptors transcriptional activity (Sommer and Fuqua 2001).
1.3.2 ER Activation and Signaling:

The discovery of such a large number of nuclear receptor co-factors and the evidence of alternative ER activation via signaling pathways etc., other than the so-called ‘classical’ ligand-dependent ER activation has complicated the understanding of the simple model of ER activation. The ligand-dependent modes of actions of estrogen receptor (ER) broadly include (i) genomic action of ER and (ii) non-genomic action of ER.

1.3.2.1 Ligand dependent modes of action of ER:

Classical Genomic action of ER: Among the large superfamily of nuclear receptors, the estrogen receptor (ER) is one of the most well characterized. Steroid hormone receptors including ER, have been grouped as Class I nuclear receptors (Mangelsdorf, Thummel et al. 1995). ER shares a typical domain structure with the other steroid hormone receptors. This includes a ligand-independent activation function 1 (AF1) in the N-terminal domain (NTD), a ligand-dependent activation function 2 (AF2), a DNA binding domain, and a C-terminal ligand binding domain (LBD). All steroid receptors exist in the cytosol typically bound in a complex with molecular chaperonins. The hormone [estrogen (E₂) in this case], binds to the AF2 domain and activates both AF1 and AF2 domain. However, in certain cell and promoter contexts AF1 could be independent and dominant (Tora, White et al. 1989, Berry, Metzger et al. 1990, Tzukerman, Esty et al. 1994).

E₂ on binding of to AF1 causes ER to dissociate from the molecular chaperonins
followed by nuclear localization, where it binds to its response element, via its DBD. These response elements occur as inverted repeat half-sites within the promoter on the DNA. ER agonists such as estradiol (E$_2$) induce preferential recruitment of co-activators vs. co-repressors by the DNA-bound receptor resulting in transactivation of the target promoter. Selective estrogen receptor modulators (SERMs) such as Tamoxifen may bind to ER and act as antagonists by recruiting co-repressors (Glass and Rosenfeld 2000, Smith and O'Malley 2004, McDonnell 2005).

**Non-Classical Genomic action of ER:** Genes that are to be activated by the E$_2$/ER pathway may lack the classical estrogen response element (ERE). In such instances, ER may be tethered to the target promoter by DNA-bound transcription factors like Sp1 (Safe and Kim 2004), AP-1 (DeNardo, Kim et al. 2005), C/EBP and Oct (Carroll, Meyer et al. 2006). The binding of ER to chromatin may also be facilitated by FoxA1 (Carroll, Liu et al. 2005, Laganiere, Deblois et al. 2005, Carroll, Meyer et al. 2006). This is followed by recruitment of co-activators and activation of transcription leading eventual gene expression.

**Non-genomic actions of ER:** ER can regulate cell signaling pathways through protein-protein interactions in the cytosol. The non-genomic mechanisms include interaction of ER with SH2 domain of Src, the p85α subunit of PI3 kinase etc. (Sanchez, Nguyen et al. 2002). These actions of ER are rapid and transient. In contrast to transcriptional activity of ER, ligand specificities of its non-genomic actions are broad and are not affected by subtle changes in conformation induced by various SERMs.
1.3.2.2 **Ligand-independent modes of action of ER:**

ER is activated independent of its ligand via phosphorylation at AF-1 and AF-2 sites. This phosphorylation is mediated by cell signaling pathways downstream of growth factor receptors such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), insulin receptor and Her2/neu (Sommer and Fuqua 2001). This phosphorylation of ER causes activation of ER, regulates dimerization, and regulates association of ER with co-regulatory proteins depending on the site of phosphorylation (Chen, Pace et al. 1999). As a result of this, ER regulates transcription and expression of genes required for growth factor activity that results in complex interaction between all these pathways. Ligand-independent ER activation has also been demonstrated by other molecules such as cyclic AMP (cAMP) (Aronica and Katzenellenbogen 1993), dopamine (Gangolli, Conneely et al. 1997) and vandate (Auricchio, Di Domenico et al. 1995), a phosphatase inhibitor.

Apart from growth factor receptors, the cell cycle protein cyclin D1 is also capable of activating ER via a mechanism that does not involve phosphorylation (Zwijsen, Wientjens et al. 1997). However, all these ligand-independent effects of ER that are mentioned above are based on *in vitro* experiments and thus it remains uncertain whether any of these pathways are actually significant *in vivo*.

1.3.3 **Current Treatments for Breast Cancer:**

The primary mode of treatment on detection of breast tumor is surgery, which is followed by tamoxifen treatment or aromatase inhibitor treatment or both and/or in
combination with chemotherapy.

1.3.3.1 Clinical status of tamoxifen treatment:

Tamoxifen is a mainstay in the treatment of estrogen receptor (ER) –positive breast cancer. It is that is recommended for adjuvant therapy for ER-positive breast cancer patients and also is used as a chemopreventive agent in women at risk for breast cancer (1998, Fisher, Costantino et al. 1998, Osborne 1998, Cuzick, Powles et al. 2003, Jordan 2004). Tamoxifen has been equally effective in treatment and prevention of ER-positive breast cancer in both, pre- and postmenopausal women. Tamoxifen treatment has a beneficial effect for up to 5 years of treatment and for an additional 5 years after treatment cessation (1998). More than 3 years of this treatment, only results in approx. 50% reduction in the incidence of invasive breast cancer in women at high risk, whereas about a third of ER- positive breast tumors are intrinsically resistant to tamoxifen (Dunn and Ford 2001, Cuzick, Powles et al. 2003). In earlier studies it was demonstrated that the frequency of endometrial cancer doubled as a side effect of tamoxifen in postmenopausal women (Cuzick, Powles et al. 2003). Recent studies have revealed this effect in case of premenopausal women as well (Swerdlow, Jones et al. 2005). Also, the risk of endometrial cancer was reported to increase with duration of tamoxifen treatment and this risk did not reduce up to 5 years after stopping the treatment (Swerdlow, Jones et al. 2005). On the other hand, SERM raloxifene has been proved to be as effective as tamoxifen with respect to prevention/treatment of breast cancer but with lower additional risk of endometrial cancer (Vogel, Costantino et al. 2006). Raloxifene was previously used as treatment measure to increase bone density in postmenopausal women (Delmas,
Bjarnason et al. (1997) and it was only recently approved by the FDA as an alternative to tamoxifen for breast cancer treatment in certain groups of postmenopausal women. Use of aromatase inhibitors in the treatment of breast cancer in postmenopausal women particularly when used in sequence with tamoxifen was found to be very effective (Goss and von Eichel 2007). Pure anti-estrogens like fulvestrant are an undesirable choice as a primary treatment in breast cancer as they cause ER degradation, thus abrogating the beneficial effects of ER in other tissues. Raloxifene cannot be used in breast cancer treatment due to its effect on bone density. Also, aromatase inhibitors, if used for the same, will lead to stimulation of estrogen production in the ovary due to feed-back mechanism.

*Limitations of tamoxifen in the prevention/treatment of breast cancer:* Tamoxifen treatment for approximately > 3 years leads to development of resistance. This treatment is also associated with a 2-4 fold increased risk of endometrial cancer (1998). Resistance developed against tamoxifen could be intrinsic (de novo) or acquired. In intrinsic tamoxifen resistance, ER-positive tumors may simply fail to respond to the drug, whereas in acquired resistance resulting from long term adjuvant treatment, the tumors acquire a dependence on tamoxifen for growth (Osborne 1998, Fisher, Jeong et al. 2001, Ali and Coombes 2002, Lewis and Jordan 2005). Human tumor xenograft models also show the same kind of clinical observation of both, tamoxifen-dependent endometrial cancer and acquired tamoxifen-resistance or tamoxifen-dependence of breast cancer growth (Osborne, Hobbs et al. 1985, Osborne, Coronado et al. 1987, Gottardis and Jordan 1988, Wolf and Jordan 1994).
There are multiple potential mechanisms of tamoxifen resistance, but those that depend on receptor dimerization and translocation are probably of greatest importance (Howell and Dowsett 2004). Resistance may be caused by increased growth factor activity via AF1 or alteration of the co-activator/co-repressor ratio (Howell and Dowsett 2004). An example of the former mechanism is development of tamoxifen resistance in MCF-7 cells by transfection with the gene for ErbB2 receptor, which can be reversed by blocking the activity of the receptor (Kurokawa and Arteaga 2003). Examples of the latter mechanism are the demonstrations that increased expression of the co-activator AIB1 (also known as SRC3) (Osborne, Bardou et al. 2003) and decreased expression of the co-repressor (Girault, Lerebours et al. 2003) are associated with tamoxifen resistance in women with breast cancer. Other potential mechanisms of tamoxifen resistance, such as altered pharmacokinetics, differential cell uptake and receptor mutation appear to be less important than it was previously thought (Schiff, Massarweh et al. 2003).

In patients with advanced breast cancer, in whom cancer progresses after a response to tamoxifen, stopping tamoxifen leads to tumor remission (Howell and Dowsett 2004). This suggests that tamoxifen may be acting as an agonist (Howell, Dodwell et al. 1992). Classically both estradiol and tamoxifen bind to ER and cause dimerization and translocation of the receptor to the promoter region of estrogen-regulated genes. However, whereas estradiol activates two regions of the ER molecule, that is the AF1 and AF2 regions, tamoxifen inhibits the AF2 but not the AF1. AF1 remains active in the presence of tamoxifen and thus could be responsible for the partial agonistic activity of tamoxifen (Howell and Dowsett 2004). Tamoxifen may become an agonist for MCF-7
cells growing in nude mice (Gottardis, Jiang et al. 1989, Osborne, Coronado-Heinsohn et al. 1995). Initially, in this model tamoxifen inhibits growth. However, when these tumors are re-transplanted into new mice, tamoxifen treatment causes growth stimulation. In vitro, cells from tamoxifen-resistant human pleural effusions have been shown to be growth stimulated by tamoxifen. Inhibition of growth can be demonstrated by treating with fulvestrant, an estrogen receptor (ER) downregulator, suggesting that the agonistic activity of tamoxifen occurs via ER pathways, as expected (DeFriend, Anderson et al. 1994).

1.3.3.2 Aromatase inhibitors (AI):

Endocrine therapies act by either blocking or downregulating the estrogen receptor or by reducing estrogen concentrations around and within the cancer cell. In postmenopausal women, estrogen suppression is achieved by inhibition of the enzyme aromatase by aromatase inhibitors (Howell and Dowsett 2004). Aromatase is an enzyme that is responsible for a key step in the biosynthesis of estrogen. It is involved in converting androgens to estrogens. In premenopausal women, estrogen produced by the ovaries accounts for its major source, whereas in case of postmenopausal women, estrogen produced by the peripheral tissues like the adipose tissue due to the action of the enzyme aromatase, is the primary source of estrogen. In premenopausal women suffering from breast cancer, aromatase inhibitors (AIs) cannot be used as it would lead to a feedback loop and lead to increased production of estrogen. AIs are used for treating postmenopausal women suffering from breast cancer. Modern AIs (anastrozole, letrozole and exemestane) are more potent than those used earlier and also suppress estradiol levels
to virtually undetectable levels (Howell and Dowsett 2004). Earlier studies also show that second generation AIs like formestane and fadrozole, and third generation AIs like letrozole and anastrozole, both, are more potent than antiestrogen tamoxifen (Perez Carrion, Alberola Candel et al. 1994, Thurlimann, Beretta et al. 1996, Gershanovich, Chaudri et al. 1998). The action of AIs is relatively simple. It is presumed that estradiol levels are reduced to such an extent that receptor dimerization and translocation into the nucleus do not take place to any appreciable extent. Thus, in estrogen responsive tumor cell, growth is abrogated.

**Limitations of aromatase inhibitors in the treatment of breast cancer:** Like in the case of tamoxifen treatment, breast cancer treatment with aromatase inhibitors also leads to development of resistance in about 3 to 5 years of treatment. This is then followed by chemotherapy. Also, the use of AIs is limited only to postmenopausal women suffering from breast cancer, as treating premenopausal women would lead to a feedback loop and increase the estrogen production in the ovaries (Howell and Dowsett 2004).

Studies performed earlier have indicated that, breast tumor cells when treated with AIs for about 3-6 months led to the development of resistant tumor cells; that is, these cells were sensitive to very low concentrations of estrogen. Proliferation at such low concentrations of estrogen can be inhibited by fulvestrant (a pure anti-estrogen), suggesting that hypersensitivity occurs via an estrogen receptor (ER) -dependent mechanism (Masamura, Santner et al. 1995, Martin, Farmer et al. 2003). Resistance to low estradiol concentrations is associated with several cellular changes, including
enhanced HER2 receptor expression, elevated levels of insulin like growth factor-1 receptor and ER and increased signal transduction via mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways.

1.3.3.3 Fulvestrant – a selective estrogen receptor (ER) down-regulator (SERD):

New hormonal therapies with novel mechanisms of action that are not cross-resistant with the existing treatments make important additions to the repertoire of treatments for breast cancer. Fulvestrant (‘Faslodex’) is the first new type of endocrine treatment – an estrogen receptor antagonist that downregulates ER and has no agonistic effects (Osborne, Wakeling et al. 2004). Fulvestrant is a 7α-alkylsulphinyl analogue of 17-β-estradiol, which is distinctly different in chemical structure from the non-steroidal structures of tamoxifen, raloxifene and other SERMs. Fulvestrant completely inhibits binding of estradiol to ER, with a binding affinity of 89% of that of estradiol (Wakeling and Bowler 1987). This is markedly greater affinity than the affinity of tamoxifen for the ER (which is 2.5% that of estradiol) (Wakeling and Bowler 1987, Wakeling, Dukes et al. 1991). Fulvestrant-ER binding impairs receptor dimerization and energy-dependent nucleo-cytoplasmic shuttling, thereby blocking nuclear localization of the receptor (Fawell, White et al. 1990, Dauvois, White et al. 1993). Additionally, any fulvestrant-ER complex that enters the nucleus is transcriptionally inactive because both AF1 and AF2 are disabled. Finally, this complex is highly unstable resulting in accelerated degradation of the ER protein (Nicholson, Ali et al. 1995). This downregulation of ER occurs without a reduction in ER mRNA. Thus fulvestrant binds, blocks and accelerates degradation of

Use of fulvestrant in breast cancer therapy has many advantages, a few of them include: (i) fulvestrant has no demonstrable agonistic activity (Osborne, Wakeling et al. 2004), (ii) it also blocks ER-mediated effects in MCF-7 cells by decreasing the levels of transforming growth factor α (TGFα), thereby reducing crosstalk between these pathways (Hutcheson, Knowlden et al. 2003), (iii) fulvestrant is a more potent inhibitor of ER-positive MCF-7 human breast cancer cells than tamoxifen and (iv) it has also demonstrated anti-tumor activity in tamoxifen-resistant MCF-7 cells, confirming a lack of cross-resistance between tamoxifen and fulvestrant (Hu, Veroni et al. 1993, Lykkesfeldt, Madsen et al. 1994).

Limitations of Fulvestrant in breast cancer therapy: Estrogen receptor is essential in many developmental processes like the development and function of the female reproductive system, health and maintenance of cardiovascular system, bone and brain centers and lastly to maintain body temperature and the vaginal lining (Russo and Russo 1998, Riggs, Khosla et al. 2002, Huang and Kaley 2004, Maggi, Ciana et al. 2004). Thus, ER deprivation caused by treatment with pure anti-estrogens like fulvestrant may lead to harmful effects with respect to normal physiology. Due to this reason, the use of anti-estrogens like fulvestrant is less desirable and restricted to postmenopausal women. Additionally, it has also been observed that treating breast cancer in postmenopausal
women with fulvestrant also leads to development of resistance over a period of time (Nicholson, Gee et al. 2003).

In an earlier study (Hutcheson, Knowlden et al. 2003), it was shown that MCF-7 cells express only modest levels of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), whereas tamoxifen resistant cells show increased expression of EGFR and HER2. This same study also showed that EGFR and HER2, immunoprecipitated as heterodimers, had increased basal activation and were associated with increased downstream mitogen-activated protein kinase (MAPK) activation versus parental MCF-7 cells. Results obtained from fulvestrant-resistant cells are similar to data obtained in tamoxifen-resistant cells, in that the transition from hormone-sensitive to endocrine-resistant states is associated with increased EGFR levels and enhancement of its signaling (Hutcheson, Knowlden et al. 2003).

Due to all the limitations described above, with respect to estrogen receptor (ER), there is room for improvement in endocrine therapy for breast cancer treatment.

1.4 Mechanisms of Endocrine Resistance

Several mechanisms of resistance to endocrine therapy have been proposed and include loss of ERs, different signaling pathways, altered expression of coregulators, as well as tamoxifen metabolism. In addition, both genomic and non-genomic crosstalk and the complex interrelation between the estrogen receptors subtypes and growth factors contribute to development of endocrine resistance (Chang and Fan 2013).
1.4.1 Loss or Modification in the ER Expression

ER expression is the main predictor of response to endocrine therapy, and lack of expression of ER is the principal mechanism of de novo resistance to hormonal therapy. Several mechanisms have been proposed to explain the absence of ER expression. These mechanisms involve epigenetic changes such as aberrant methylation of the ER promoter and histone deacetylation, resulting in a compact nucleosome structure that limits transcripption (Weigel and deConinck 1993, Ottaviano, Issa et al. 1994, Yang, Phillips et al. 2001, Parl 2003). In vitro and in vivo studies showed that the treatment with the histone deacetylase inhibitor entinostat (ENT) increased the expression of ERα and aromatase. Notably, ERα and aromatase upregulation resulted in sensitization of breast cancer cells to estrogen and letrozole (Sabnis, Goloubeva et al. 2011). Moreover, Scriptaid (a novel HDAC inhibitor) has also shown to cause ER upregulation, which causes inhibition of tumor growth thereby sensitizing hormone-resistant breast cancer cells to tamoxifen (Keen, Yan et al. 2003, Giacinti, Giacinti et al. 2012).

Other mechanisms proposed in the loss of ER expression include hypoxia, overexpression of EGFR or HER2, MAPKs hyperactivation, and involvement of p53 and pRb2/p130. Hypoxia induces proteasome-dependent degradation of ER in ZR-75 breast cancer cells, leading to decreased protein levels (Stoner, Saville et al. 2002). High expression of EGFR and HER2 in ER-negative breast cancers were found, suggesting that activation of growth factor signaling and consequently, the activation of MAPK might contribute to transcriptional repression of ER gene, resulting in endocrine resistance (Stoica, Saceda et al. 2000, Creighton, Hilger et al. 2006). Recent results
showed that p53 upregulates ER gene expression through elements located upstream of the ER promoter. Interestingly, a high percentage of breast tumors with p53 mutations are ER-negative. These studies suggest that specific p53 mutations in breast tumors may contribute not only to oncogenesis but also to hormonal resistance. Furthermore, the treatment of MCF-7 cells with paclitaxel resulted in the induction of ER gene transcription, which may be mediated through the induction of p53 (Angeloni, Martin et al. 2004, Martin, Angeloni et al. 2004). Modifications in ER expression such as mutations might affect the response to anti-estrogens. For example, substitution of aspartate by tyrosine at position 537 in the ligand-binding domain causes ER activation in the absence of the ligand (Zhang, Borg et al. 1997). The change of aspartate to tyrosine at amino acid 351 in the ER has been identified in a tamoxifen-stimulated cell line (Wolf and Jordan 1994), and modification of lysine 303 to arginine results in increased ER sensitivity to estrogens (Fuqua, Wiltschke et al. 2000). Another mechanism that is associated with SERM resistance is the altered expression of ERβ. High levels of ERβ are found in pre-invasive mammary tumors of tamoxifen-resistant patients (Speirs, Parkes et al. 1999). However, other studies have reported that ERβ has a negative effect on ERα promoted transcription and that low levels of ERβ may contribute to endocrine resistance (Hopp, Weiss et al. 2004, Borgquist, Holm et al. 2008).

1.4.2 Regulation of Signal Transduction Pathways

Crosstalk between ER and different signaling pathways, such as growth factor receptor, cell survival (PI3K/AKT), stress and/or cytokine signaling pathway, have been implicated in acquired and intrinsic resistance to endocrine therapy in breast cancer.
1.4.2.1 Growth Factor Receptor Signaling Pathways

The growth factor receptor signaling pathways are capable of stimulating cancer growth either in concert with ER signaling or bypassing it. Preclinical and clinical evidence has suggested that growth factor receptor signaling play a role in development of endocrine resistance. The membrane ER can activate growth factor receptor signaling, which in turn phosphorylates ER and its coregulator proteins (Shou, Massarweh et al. 2004). Phosphorylation of serine residues within the activation function-1 (AF-1) domain of ER promotes re-activation of ER function in a ligand-independent manner and contributes to transcription of estrogen-sensitive genes in the presence of antiestrogen agents (Bunone, Briand et al. 1996, Joel, Smith et al. 1998, Joel, Traish et al. 1998). Recent studies in tamoxifen resistant cells have also indicated that EGFR/MAPK-promotes ER AF-1 phosphorylation which enhances the agonistic behavior of tamoxifen, resulting in the expression of estrogen regulated genes (Britton, Hutcheson et al. 2006). Overexpression and activation of growth factor receptors, such as EGFR, HER2 and IGF1R, drive the proliferation and survival through activation of MAPK and PI3K/AKT signaling pathways in endocrine-resistant breast cancer (Nicholson, Hutcheson et al. 2007). In MCF-7/HER2-18 cells manipulated to overexpress HER2 as well as ER, tamoxifen has been shown to behave as an estrogen agonist, stimulating their growth. Phosphorylation and activation of both ER and EGFR/HER2 receptors as well as MAPK and AKT signaling pathways, and recruitment of AIB1 coactivator was increased in these cells compared with wild-type MCF-7 cells. In tumors that acquired resistance to fulvestrant, there was a marked upregulation of HER-2 and its downstream target,
MAPK; suggesting that this pathway may mediate the resistant phenotype (Massarweh, Osborne et al. 2006).

Therefore, the use of either specific inhibitors or blockers of growth factor receptors has been one of the most promising therapeutic approaches in breast cancer. Gefitinib, a selective inhibitor of EGFR, restores the effects of tamoxifen in HER2-overexpressing tamoxifen-resistant MCF-7 cells, while trastuzumab, a monoclonal antibody that blocks HER2, can inhibit proliferation of endocrine-resistant ZR-75-1 cells (Shou, Massarweh et al. 2004). Furthermore, the HER2 tyrosine kinase inhibitor AG1478 and trastuzumab have also proved efficacy in MCF-7 models of de novo tamoxifen resistance and in BT-474 cells that overexpress HER2.

As previously mentioned, the IGF1R pathway has a close bidirectional crosstalk with ER. Some studies have established that elevated IGF1R-promoted kinases contribute to the phosphorylation of ER. In turn, estrogens enhance expression of several IGF1R pathway components, like insulin-like growth factor II (IGF2), to reinforce IGF1R signaling (Surmacz 2000). ER-positive MCF7 cells with ectopic expression of IGF1R, are highly resistant to tamoxifen and fulvestrant. Response to IGF1 ligand stimulation was found to stimulate enhanced activation of MAPK and PI3K signaling pathways; this was independent of ER signaling. However, while inhibitors of the IGF1R pathway are currently in clinical trials, early reports have shown that IGF1R-specific inhibitors (like AG1024 and AEW541) or an IGF2 neutralizing antibody inhibited basal IGF1R, c-SRC,
AKT and EGFR phosphorylation, and significantly reduced tamoxifen-resistant basal cell growth.

1.4.2.2 PI3K Cell Survival Pathways

ER activity is also associated to the PI3K pathway, which is activated by tyrosine kinase receptors in response to growth factors. The PI3K/AKT signaling pathway has been extensively investigated for its role in oncogenic transformation. AKT is one of the downstream targets of PI3K, promotes cellular proliferation and anti-apoptotic responses (Datta, Brunet et al. 1999). Recent studies have indicated that estrogen stimulates association of ERα with IGF1R and p85 regulatory subunit of PI3K in the plasma membrane (Simoncini, Hafezi-Moghadam et al. 2000, Clark, West et al. 2002), which leads to AKT activation and its subsequent downstream effects. In turn, AKT phosphorylates nuclear ERα at serine 167, resulting in ligand-independent activation (Martin, Franke et al. 2000, Campbell, Bhat-Nakshatri et al. 2001). In various experimental models it has been demonstrated that PI3K pathway activation confers antiestrogen resistance. Knockdown of the suppressor phosphatase and tensin homolog (PTEN, a negative regulator of AKT), increased PI3K and AKT phosphorylation in ER-positive breast cancer cell lines, resulting in hormone-independent growth and resistance to tamoxifen and fulvestrant (Li, Yen et al. 1997, Miller, Perez-Torres et al. 2009). Treatment with PI3K pathway inhibitors increases the pro-apoptotic effects of tamoxifen, supporting the notion that either high expression of AKT or altered activity of the PI3K/AKT pathway could be associated with endocrine resistance (Campbell, Bhat-Nakshatri et al. 2001, Clark, West et al. 2002). The combination of BEZ235
(PI3K/mTOR inhibitor) and tamoxifen has been shown to inhibit growth more effectively than either tamoxifen or BEZ235 alone (Creighton, Fu et al. 2010).

### 1.4.3 Coregulatory Proteins

Tamoxifen acts as an ER antagonist in breast cancer but as an agonist in other tissues such as uterus, cardiovascular system, and bone. These differences in tamoxifen activity could be explained by several mechanisms. One of these mechanisms involves changes in the level of expression of coregulatory proteins (coactivators and corepressors) that can influence regulation of ER transcriptional activity (Smith, Nawaz et al. 1997, Jordan and O'Malley 2007).

The ER coactivator AIB1 (also known as SRC-3) is considered to be a proto-oncogene, which is overexpressed in more than 30%, and is genetically amplified in 5%–10%, of breast tumors (Anzick, Kononen et al. 1997, Murphy, Simon et al. 2000, List, Reiter et al. 2001, Lydon and O'Malley 2011). High levels of ER coactivators may enhance the estrogen-agonist activity of tamoxifen and contribute to tamoxifen resistance (Tzukerman, Esty et al. 1994, Smith, Nawaz et al. 1997, Kressler, Hock et al. 2007). High SRC-3 expression has been associated with poor overall breast cancer patient survival (Harigopal, Heymann et al. 2009). Moreover, overexpression of SRC-3 along with HER2 converts tamoxifen into an agonist with increase in the molecular crosstalk between the ER and HER2 pathway (Shou, Massarweh et al. 2004). Further, it has been shown that tamoxifen induces ERα-SRC-3 interaction in HER2-positive human breast cancer (Mc...
Ilroy, Fleming et al. 2006). In contrast, dissociation of SRC-3 from ER has been shown to restore sensitivity in tamoxifen resistant cells (Wang, Yang et al. 2006).

In surgical specimens from ER-positive breast cancer patients who received neoadjuvant tamoxifen therapy, tamoxifen significantly increased the expression of SRC-1, SRC-2 and SRC-3 (Haugan Moi, Hauglid Flageng et al. 2010). SRC-1 and SRC-3 protein levels were higher in an endocrine resistant cell lines in comparison with endocrine sensitive cells. Knockdown of SRC-1 and SRC-3 resensitized endocrine resistant cells to tamoxifen treatment. Also, colocalization of SRC-1 and SRC-3 with ERα was increased in endocrine resistant cells following treatment with tamoxifen in comparison with endocrine sensitive cells. These data implicate the involvement of inappropriate interactions between ER and its coactivator in the endocrine resistance (Redmond, Bane et al. 2009).

Paired Box 2 (Pax2) has been shown to be an important mediator of repression of HER2 in ER-positive breast cancer cells. After tamoxifen treatment, Pax2 is recruited to the ER functioning as an ER-associated transcriptional repressor. It was observed that knockdown of Pax2 led to increased HER2 transcript and protein levels in the presence of both estrogen and tamoxifen and reversed the growth inhibitory effects of tamoxifen (Hurtado, Holmes et al. 2008). Overexpression of SRC-3 has been shown to block association of Pax2 with ER, resulting in increased HER2 transcription and cell proliferation in the presence of tamoxifen. These data confirm an essential role for SRC-3
and HER2 in tamoxifen resistance in ER positive breast cancer cells (McCartan, Bolger et al. 2012).

The role of coactivators in resistance to AI therapy has also been documented. Flageng et al. demonstrated that co-activators and HER2 are upregulated in the tumors from breast cancer patients during neo-adjuvant treatment with aromatase inhibitors (Flageng, Moi et al. 2009). Moreover, expression of AIB1 was associated with disease recurrence and reduced disease-free survival time in patients treated with an AI as first-line therapy (O'Hara, Vareslija et al. 2012).

The nuclear receptor corepressor (NCoR) has been considered as an important predictor of the tamoxifen response (Cottone, Orso et al. 2001, Girault, Lerebours et al. 2003). In a xenograft model for human breast cancer, NCoR levels decreased in the tumors that acquired resistance to tamoxifen (Lavinsky, Jepsen et al. 1998). In a recent clinical study it was shown that patients with the best prognosis had high NCoR expression levels and normal HER2 expression (Kurokawa, Lenferink et al. 2000). Another study also showed that in ERα-positive breast tumors from postmenopausal patients treated with tamoxifen after surgery demonstrated that low NCoR expression was associated with shorter relapse-free survival (Girault, Lerebours et al. 2003). The Tab2 protein is a facultative component of the NCoR complex (Baek, Ohgi et al. 2002) and was found as an important mediator of resistance to endocrine therapy (Cutrupi, Reineri et al. 2012). Interaction between Tab2 and ERα was observed in tamoxifen resistance cells and interfering with this binding restored tamoxifen response.
Furthermore, downregulation of Tab2 increased the antiproliferative response to tamoxifen (Cutrupi, Reineri et al. 2012). Tab2 interacts with ERα/NCoR and causes dismissal of NCoR, leading to loss of the antiproliferative response in prostate cancer cells (Zhu, Baek et al. 2006). Tamoxifen-sensitive cells showed high NCoR binding to the ER in comparison with tamoxifen-resistant cells. Combination treatment with either tamoxifen and IKK inhibitor parthenoide (PA), or tamoxifen and proteasome inhibitor bortezomib (PS341), which were capable of suppressing NFkB and AP-1, regulates gene expression. These combinations were able to restore the levels of NCoR binding to tamoxifen-liganded ER in the tamoxifen-resistant cells (Zhou, Yau et al. 2007). Taken together, these data indicate the possibility that reduced corepressor activity during tamoxifen therapy may enhance the agonist effects of tamoxifen on the ER contributing to tamoxifen resistance.

1.5 Retinoids & Retinoic Acid Receptors

Retinoids are vitamin A-related compounds that have been found to prevent cancer in animals and humans. The retinoid receptors are expressed in normal as well as in malignant breast cells, and are critical for normal development. In breast cells, when bound by retinoids, these proteins regulate proliferation, apoptosis, and differentiation. The mechanism by which retinoids inhibit breast cell growth has not been completely elucidated; however, retinoids have been shown to affect multiple signal transduction pathways, including IGF-, TGFβ-, and AP-1-dependent pathways. Retinoids have also been shown to suppress the growth and prevent the development of breast cancer in animals (Yang, Tin et al. 1999). The physiological actions of retinoids are mediated
through two distinct NR families: the retinoic acid receptors (RARs) which include RARα, RARβ, and RARγ subtypes, each of which bind to both all-trans-retinoic acid (ATRA) or 9-cis-retinoic acid (9-cis-RA), and the retinoid X receptors (RXRs) which include the RXRα, RXRβ and RXRγ subtypes, which preferentially bind 9-cis-RA (Germain, Staels et al. 2006). Our main focus in this proposal is on retinoic acid receptor α (RARα).

1.5.1 Retinoic acid receptors (RARs)

Retinoic acid receptors are class II type of nuclear receptors (Germain, Staels et al. 2006) that are activated by all-trans retinoic (ATRA) acid and 9-cis retinoic acid (9cRA) (Allenby, Bocquel et al. 1993). There are 3 subtypes of RARs: RARα, RARβ and RARγ which are encoded by RARA, RARB and RARG genes respectively. Each receptor isoform has several splice variants: 2 - for alpha, 4 - for beta, and 2 - for gamma.

1.5.2 Mechanism of action of retinoic acid receptors (RARs)

Like other class II nuclear receptors, RARs once produced in the cytosol, translocate into the nucleus where they heterodimerize with retinoid X receptors (RXRs). The RAR/RXR heterodimer interacts with specific DNA response elements of target genes and its effect on transcription is mediated also through recruitment of coregulators (corepressors and coactivators). RAR/RXR heterodimers bind to specific DNA sequence – retinoic acid response elements (RAREs), characterized by direct repeats of two hexamers (A/G)GGTCA separated predominantly by five nucleotides (DR+5) or two nucleotides (DR+2) (Chambon 1996, Crowe 2002, Sun and Lotan 2002). In the absence
of ligand like ATRA, the RAR/RXR heterodimer recruits nuclear receptor corepressor proteins, e.g. nuclear receptor corepressor (N-CoR) or silencing mediator of retinoid and thyroid hormone receptor (SMRT) (Freedman 1999). All-trans retinoic acid (ATRA) binding leads to the dissociation of corepressor proteins and enables association of coactivator proteins with liganded receptor complex yielding in chromatin decondensation and activation of gene transcription (Brtko 2007).

Retinoids have been shown to inhibit cell growth through several mechanisms. In breast cancer cells, retinoids inhibit cell proliferation by decreasing the expression or activity of growth stimulators, or by increasing the expression or activity of growth inhibitors. Alternatively, retinoids like ATRA can induce differentiation or apoptosis. Several investigators have proposed that retinoids suppress breast cell growth by inhibiting mitogenic signaling pathways (Brtko 2007). One such signaling complex inhibited by retinoids is the AP-1 transcription factor. AP-1 transcription factors are composed of heterodimers of Jun and Fos proto-oncoproteins or homodimers of Jun proteins. These complexes are activated in breast cells by critical growth factors such as estrogen, EGF, TGFα, and IGFs (Chen, Smith et al. 1996, Smith, Birrer et al. 1997), and transduce mitogenic signals in these cells. Retinoids inhibit AP-1 activity in breast cancer cells (Pfahl 1993, Yang, Kim et al. 1997), and thus block growth factor-induced mitogenesis.

1.5.3 Retinoic acid receptor and breast cancer

ER is known to regulate genes in a ligand-independent manner (Cvoro,
Tzagarakis-Foster et al. 2006, Cardamone, Bardella et al. 2009). Hormone-independent actions of ER play an important role in supporting the growth of hormone-refractory breast tumors (Musgrove and Sutherland 2009). On the other hand, studies of gene regulation by ER in estrogen-sensitive breast cancer cells have mostly focused on estrogen-responsive genes that have profound roles in tumor growth and development and the effects of tamoxifen on gene regulation by estrogen (Welboren, Sweep et al. 2009). In contrast, recent studies from our lab (Salazar, Ratnam et al. 2011) have highlighted a potentially significant mechanism of hormone-independent transcriptional action of ER in hormone-sensitive breast cancer cells.

Hormonal adjuvant therapy of breast cancer is overall tumoristatic where cell death balances a basal rate of cell proliferation (Millar and Lynch 2003). From a mechanistic point of view, for resistance to develop in the long term during either hormone depletion or tamoxifen adjuvant therapy, these latent tumors must maintain a low level of cell growth (basal level of cell cycling) to enable the generation and/or progression of genetic or epigenetic changes (Badia, Oliva et al. 2007) leading to resistance. Our studies have shown that - 

**During hormone depletion or tamoxifen treatment of breast cancer cells the estrogen receptor apoprotein supports cell cycling through the retinoic acid receptor a1 apoprotein (Salazar, Ratnam et al. 2011)**

In hormone-sensitive breast cancer cells, the well-established ER-RAR axis has been best characterized in the context of ligand effects (estrogen, retinoids, tamoxifen and retinoids in combination) (Wang, He et al. 2007, Ross-Innes, Stark et al. 2010). Studies
form our lab have shown that in hormone-sensitive cells that are depleted of hormone or treated with tamoxifen, a major mechanism by which ER supports the cell cycle is by supporting the basal expression of RARα1. The role of RARα1 in mediating the action of apo-ER was strongly evident from the following observations: (i) In hormone-depleted cells, apo-ER maintained the basal expression level of RARα1 but was not itself regulated by RARα1; (ii) The regulation of RARα1 by apo-ER was insensitive to tamoxifen; (iii) Knocking down RARα1 negatively impacted the basal cell cycle progression and restoring basal apo-RARα1 levels rescued basal level cell division following depletion of ER; (iv) Apo-RARα1 independently regulated a complement of genes in a manner that strongly favored cell division similar to their regulation by apo-ER (Salazar, Ratnam et al. 2011). This mechanism was remarkable for the following reasons. Firstly, apo-ER regulated the α1 sub-type of RAR but not RARs -β or -γ. Secondly, most of the common target genes of apo-ER and apo-RARα1 including all of the genes involved in the cell division cycle were insensitive to ATRA. These findings suggested that a major molecular mechanism by which apo-ER supports basal cell division in hormone-sensitive breast cancer cells may not be sensitive to conventional RAR ligands (agonists), but would be predictably opposed by specific inactivators or down-regulators of RARα1.

Studies from our lab also indicated that multiple molecular mechanisms must underlie the downstream action of apo-RARα1 on target genes in the context of mediating the effects of apo-ER. The apo-ER/apo-RARα1 axis were shown to regulate genes in both a positive and a negative manner to support cell division; both sets of target
genes were enriched for associated chromatin sites of RAR binding, suggesting that RARα1 must act on these target genes by direct as well as indirect mechanisms. RAR belongs to the Class II subfamily of nuclear receptors, which typically, in their ligand-free (apoprotein) form, maintain a transcriptionally repressed state of target genes activated by the corresponding agonists (Altucci and Gronemeyer 2001). However, only a small fraction of genes regulated by the apo-ER-RARα1 axis were found to be regulated by this classical mechanism of action of RARα1, since (i) the genes repressed by apo-RARα1 were largely insensitive to ATRA and (ii) most genes activated by apo-RARα1 were ATRA-insensitive. Therefore, apo-RARα1 must act by non-classical mechanisms on most of the target genes, including those with associated RAR binding sites.

RARα is consistently present in the nucleus in breast tumors and its expression levels correlate with that of the proliferation marker, ki-67 (van der Leede, Geertzema et al. 1996). The functional RARα isoform in different hormone-sensitive breast cancer cell lines and that identified in a limited number of breast tumors was almost exclusively of type 1, an isoform that is believed to be genetically redundant (Lufkin, Lohnes et al. 1993). Thus we hypothesize that targeted inactivation or downregulation of RARα in combination with current available hormonal adjuvants would increase the time taken to develop resistance to hormonal adjuvant therapy.

1.5.4 Limitations of Using Retinoids in Breast Cancer Treatment

ATRA was found to cause cell cycle arrest of normal human mammary epithelial
cells expressing endogenous RARβ receptor (Seewaldt, Kim et al. 1997). In vivo work demonstrated that 9-cis-RA suppresses mammary tumor development in the C3(1)-simian virus 40 large T-antigen mouse model of breast cancer, in which mice display a loss of estrogen receptor (ER)-α expression during tumor progression (Wu, Kim et al. 2000). However, when tested in patients in Phase I clinical trials, 9-cis-RA was poorly tolerated due to elevated triglyceride levels and moderate to severe liver and skin toxicity (Miller, Rigas et al. 1996). Because of the potential for retinoids to block cellular division, ATRA has also been tested in patients with advanced breast cancer; however, ATRA does not have significant activity in patients with hormone refractory, metastatic breast cancer (Sutton, Warmuth et al. 1997). Also, there are many reports with regard to the cytotoxic nature of ATRA. In the setting of chemoprevention, the synthetic amide of retinoic acid, N-(4-hydroxyphenyl) retinamide, or fenretinide, has been widely been studied because of its favorable toxicity profile. Studies that were carried out later revealed that fenretinide was only modestly effective in premenopausal women and had no effect in postmenopausal women suffering from breast cancer (Chambon 1996, Brtko 2007).

In summary, all treatment options are either limited to premenopausal women (fenretinide) or postmenopausal women (aromatase inhibitors, fulvestrant), or they lead to the development of resistance over a period of time (tamoxifen and aromatase inhibitors). Development of resistance to hormonal adjuvants in breast cancer requires a basal level of cell cycling during the treatment for which the underlying causes are unclear. Our lab has shown that: “Under Conditions of Hormonal Adjuvant Treatment the
Estrogen Receptor Apoprotein Supports Breast Cancer Cell Cycling through the RARα1 Apoprotein”. Thus we hypothesize that: Targeted downregulation of RARα1 will lead to improved outcome of hormonal adjuvant therapy in breast cancer.

2.1 Prostate Cancer

Prostate cancer is the leading cause of illness and death, after lung and bronchus cancer, in men, in the United States of America and Western Europe. Autopsy studies have shown that approx. 29 percent of men aged between 30 and 40 years and 64 percent of men aged between 60 and 70 years revealed small prostatic carcinomas. According to the American Cancer Society (ACS) statistics of 2002, there were approximately 189,000 cases diagnosed with prostate cancer and approximately 30,200 deaths due to prostate cancer. However, with diagnostic tools like screening for levels of prostate specific antigen (PSA) and digital rectal examination, as well as treatment of localized prostate cancer that is detected at an earlier stage, the death rate due to prostate cancer is decreasing at a slow, but steady pace.

Prostate cancer risk has been associated with factors such as, age, hormonal levels (androgens), diet, race/ethnicity etc. Over the past few decades, several studies have revealed the different mechanisms/factors associated with development and progression of prostate cancer. One of the key players in prostate cancer development and progression is the androgen receptor (AR). Several studies have shown that alterations in AR or AR regulation plays a key role, not only in the development of androgen-dependent prostate
cancer, but also in development of androgen-independent prostate cancer, which is commonly referred to as castrate recurrent prostate cancer (CRPC).

2.1.1 Prostate Cancer Management and Therapeutic Targets

Prostate cancer incidence is very rare in men less than 50 years of age. In a recent study it was shown that 27 % of men in their forties and 34 % of men in their fifth decades of life show sub-clinical prostate cancer lesion in their prostate (Shaffer, Jivan et al. 2004). Since all localized prostate cancer may or may not progress to show clinical presentation, the management of prostate cancer depends on: the clinical stage of the cancer, pathological stage, Gleason sum, PSA level, the underlying medical condition and age of the patient.

2.1.1.1 Localized prostate cancer:

Currently there is no optimal treatment available for localized prostate cancer and this remains to be controversial (Schwartz 2009); hence the clinical decision is largely based on informed discussion between the patient and physician on weighing the benefits and side effects of each approaches. The available approaches are active surveillance, radical prostatectomy and radiotherapy. Also, the clinical stage of the tumor, histological grade, life expectancy of the patients, underlying health conditions are important considerations in deciding the treatment modalities (Singh, Trabulsi et al. 2010).
2.1.1.2 Advanced (metastatic) prostate cancer:

Hormone deprivation therapy or androgen deprivation therapy (ADT) is the mainstay in the management of locally advanced prostate cancer. Androgen ablation therapy is aimed at lowering the serum testosterone level. One of the means of androgen ablation therapy includes medical castration using luteinizing-hormone releasing hormone (LHRH) analogues, which are used to lower the androgen level (Labrie, Dupont et al. 1983). Medical castration is usually used in combination with antiandrogen therapy to achieve total androgen blockade. However, the majority of patients on androgen ablation therapy invariably progress into a castration recurrent stage (Gelmann 2002).

2.1.1.3 Castration recurrent prostate cancer:

Majority of patients respond favorably to androgen ablation therapy with substantial tumor remission and decline of PSA level. However, most patients develop resistance to therapy characterized by tumor recurrence and biochemical relapse. Such tumors are refractory for further hormonal manipulation and progress into metastatic stage which is referred to as hormone refractory prostate cancer or castration recurrent prostate cancer (CRPC) or androgen ablation insensitive prostate cancer (AIPC) (Gelmann 2002). CRPC has limited treatment options and grim prognosis and the median survival time for CRPC patients is 16-18 months (Gelmann 2002, Sun, Yang et al. 2003). Three treatment approaches are used to manage patients that progress to CRPC: additional hormone manipulation, chemotherapy and bisphosphonate therapy (Lipton, Small et al. 2002, Tannock, de Wit et al. 2004).
2.1.2 Molecularly Targeted Therapies in Prostate Cancer

Signaling pathways that support the growth and progression of prostate cancer are often perturbed and can be used as a target in the development of new treatment strategy. For example, over-expression of the androgen receptor (AR), abnormal activation of PI3K/Akt pathway, overexpression of insulin like growth factor-1 (IGF-1), HER-2/neu (Cilig, Hobisch et al. 1994, Berrevoets, Doesburg et al. 1998, Ittmann 1998) have been reported to drive development and progression of prostate cancer. A new generation anti-AR, MDV3100, has been developed for the treatment of CRPC (Scher, Beer et al. 2010). This experimental drug targets AR and inhibits its nuclear translocation and prevents co-activators from being recruited to the transcriptional machinery (Scher, Beer et al. 2010).

To block the ligand dependent action of AR, the androgen synthesis pathway has been explored as therapeutic target. Experimental molecules specifically inhibiting CYP17, the rate-limiting enzyme in steroid hormone biosynthesis, are under investigation to achieve complete androgen suppression in the treatment of CRPC (Attard, Reid et al. 2008).

Several small molecule inhibitors that target PI3K/Akt/mTOR pathway have been developed. Pre-clinical studies have shown that inhibition of mTOR induces apoptosis of the prostate epithelial cells and reversal of neoplastic phenotype in PTEN-null murine model of prostate cancer (Majumder, Febbo et al. 2004, Cao, Subhawong et al. 2006). Antiangiogenic agents targeting vascular endothelial growth factor (VEGF) such as sunitinib and bevacizumab have shown significant activity in other tumors. Inhibition of angiogenesis by bevacizumab in combination with docetaxel has shown PSA response and has been observed to be well tolerated in phase II study of docetaxel pretreated
CRPC patients (Picus, Halabi et al. 2011).

A monoclonal antibody against RANKL, denosumab, has shown to inhibit RANKL that mediates osteoclastic bone resorption. A phase II clinical trial of prostate cancer patients with bone metastasis, have shown reduction of skeletal related events and normalization of resorption in patients treated with denosumab (Fizazi, Bosserman et al. 2009, Fizazi, Lipton et al. 2009).

2.2 Androgen Receptor (AR)

The androgen receptor (AR) is a member of the steroid and nuclear receptor super family consisting of mineralocorticoid, glucocorticoid, estrogen and progesterone receptors (MR, GR, ER and PR, respectively) (Montgomery, Price et al. 2001, Heinlein and Chang 2002). All these receptors are classified as ‘Class I’ type of nuclear receptors, which include, thyroid hormone receptors, peroxisome proliferator-activated receptors and also vitamin D receptors (Detera-Wadleigh and Fanning 1994). AR is involved in cell proliferation, differentiation, apoptosis and metabolism in many different tissues, and also plays an important role in secretory activity of proteins such as prostate specific antigen (PSA) within the prostatic tissue, thereby playing a pivotal role in tissue maintenance and homeostasis (Nelson, Clegg et al. 2002). However, AR can play a pivotal role in development of diseases such as hypogonadism (Montgomery, Price et al. 2001), benign prostatic hyperplasia (BPH) (Koivisto, Schleutker et al. 1999, Zitzmann, Depenbusch et al. 2003), male pattern baldness (Loprinzi, Michalak et al. 1994, Strum,
McDermid et al. 1997), androgen insensitivity syndrome (AIS) (Brinkmann 2001), and later in life, development and progression of prostate cancer (Han, Buchanan et al. 2005).

2.2.1 The Androgen Receptor Gene Structure and Function

The human AR gene is located on the X chromosome (q11-12) and consists of eight exons. It is transcribed to form a cDNA, which is 2760 nucleotides in length, which in turn is translated to an AR protein that is 920 amino acids in length. The AR protein, like other members of the nuclear receptor superfamily, consists of four structurally and functionally distinct domains: N-terminal domain (NTD), a highly conserved DNA binding domain (DBD), a small hinge region and a moderately conserved C-terminal ligand-binding domain (LBD) (Rahman, Miyamoto et al. 2004).

The N-terminal domain (aa 1-558) consists of regions that are important for AR conformation and activity. It contains three microsatellite tri-nucleotide repeats, two of which encode poly-glutamine (poly-Q) and poly-glycine (poly-G) tracts (Choong and Wilson 1998). Recent studies have shown that variations in this repeat length may affect the stability of AR-NTD and –LBD-ligand binding (N/C interaction) with an inverse relationship that is associated with repeat-length and AR expression or activity (Chamberlain, Driver et al. 1994, Ding, Xu et al. 2004). The poly-Q (CAG) repeats are responsible for a number of neuromuscular diseases, like Kennedy syndrome, where there is progressive neuromuscular atrophy and ataxia (Gelmann 2002). The poly-Q repeats range from 11-31 repeats (Edwards, Hammond et al. 1992) and these repeats have been thought to be associated with a number of diseases such as, greater risk of BPH (≤
19 repeats), defective spermatogenesis ($\geq 28$ repeats) and prostate cancer ($\leq 18$ repeats) (Montgomery, Price et al. 2001, Mononen, Ikonen et al. 2002). It has been shown that there is a greater risk of prostate cancer in African-American men with an average repeat size shorter than those found in Caucasian men (Platz, Rimm et al. 2000). However, a recent clinical study showed no evidence of any such associations with repeat size and prostate cancer risk (Palazzolo, Gliozzi et al. 2008).

A number of co-regulators interact with AR via small amino acid sequence motifs within the NTD (Hur, Pfaff et al. 2004, Dehm, Regan et al. 2007). These motifs are involved in regulating N/C interactions and also compete with LXLL-motif containing co-regulators such as the SRC/p160 family of co-activators (Simental, Sar et al. 1991, He, Kemppainen et al. 1999). SRC/p160 family of co-activators such as transcriptional intermediary factor 2 (TIF2), increase AR transcription by binding with AR-NTD and DBD (He, Kemppainen et al. 1999, Hong, Darimont et al. 1999, Xu and Li 2003). TIF2 is involved in stabilizing N/C interactions and also plays a role in recruiting other co-activators and histone acetyl transferase (HAT) for chromatin remodeling (He, Kemppainen et al. 1999). The NTD consists of an activation function-1 (AF-1), which comprises of two transcriptional activation units (TAU), namely, TAU-1 (amino acids 100-370) and TAU-5 (360-528). Both these units have similar function but different triggers (Chamberlain, Whitacre et al. 1996, Callewaert, Van Tilborgh et al. 2006); for example, full-length AR requires TAU-1 for ligand-dependent transactivation, whereas deletions in the LBD lead to an inactive TAU-1, but the transactivational activity is shifted to TAU-5 in these deletion mutants (Jenster, van der Korput et al. 1995).
The DBD (aa 559-624) binds to the androgen-responsive gene promoter and enhancer regions. The AR-DBD has high degree of sequence similarity with other members of the steroid hormone receptor family such as GR, MR and PR; as revealed by amino acid sequence analysis (Jenster, van der Korput et al. 1995). The DBD consists of two zinc-finger domains, which is formed from three α-helices and a C-terminal extension (Verrijdt, Haelens et al. 2003). The first zinc-finger motif consists of a conserved motif (P-Box), which binds to gene specific nucleotides within the DNA major groove (Glass 1994, Schoenmakers, Alen et al. 1999). The second zinc-finger motif (D-Box), functions as a DBD/DBD binding site for receptor dimerization (Umesono and Evans 1989, Schoenmakers, Alen et al. 1999).

At the junction of the DBD and LBD, is the hinge region, which spans approximately 50 amino acids (aa 625-676) and consists of a ligand-dependent nuclear localization signal (NLS) for AR nuclear-translocation. The NLS comprises of two basic amino acid clusters in the DBD and hinge (amino acids 617-633) (Zhou, Sar et al. 1994). The cytoskeletal protein Filamin-A (FlnA) interacts with the hinge region, DBD and LBD of AR leading to nuclear translocation of AR. FlnA-negative cell lines fail to show this nuclear translocation, thus showing the functional significance of the interaction between FlnA and the above mentioned domains of AR (Ozanne, Brady et al. 2000).

The LBD (aa 677-919), which is present at the carboxyl terminus of AR, facilitates binding of natural ligands like testosterone and dihydro-testosterone (DHT), playing a role in controlling the androgen-signaling axis (Rahman, Miyamoto et al. 2000).
Ligand binding to LBD is shown to increase AR stability via formation of AF2 followed by N/C interaction leading to AR translocation into the nucleus (Lee and Chang 2003). The AR-LBD consists within itself a nuclear export signal in amino acids 742-817, which functions in the absence of androgen. In the presence of androgen, the nuclear export signal is rendered inactive and allows nuclear localization of AR (Heinlein and Chang 2002).

2.2.2  Androgen Receptor in Normal Prostate

The prostate gland is composed of epithelial and stromal components. The epithelial component consists of secretory luminal cells, transit-amplifying cells and basal cells (Litvinov, De Marzo et al. 2003). The initiation of prostate development is dependent on the androgen receptor (AR) and androgen (DHT). Even though the fetal testis produces testosterone, the intracellular reduction of testosterone by the action of the enzyme 5α-reductase is essential for prostate morphogenesis. This enzyme, 5α-reductase, is present in the urogenital sinus before and during prostate development and is involved in converting testosterone and testosterone precursors to dihydroxy-testosterone (DHT) (Siiteri and Wilson 1974). After prostate development, androgens continue to promote survival of secretory epithelial cells, the primary cells, which are transformed in prostate adenocarcinoma (De Marzo, Nelson et al. 1998).

AR plays distinct functional roles in the epithelial and stromal components of the prostate. On androgen binding, the AR in the stromal cells activates the transcription and secretion of growth factors such as insulin-like growth factor-1 (IGF-1), epidermal
growth factor (EGF), and fibroblast growth factor (FGF) (Lu, Luo et al. 1999, Litvinov, De Marzo et al. 2003). These growth factors, termed as andromedins, diffuse through the basement membrane and bind to their cognate receptor in the epithelial cells, and support proliferation of the epithelial cells (Litvinov, De Marzo et al. 2003, Isaacs and Isaacs 2004). However, the binding of androgen to AR in the epithelial compartment suppresses the growth of epithelial cells and promotes differentiation. Thus, AR plays an important physiologic role in maintaining the homeostatic balance of the prostate (Ling, Chan et al. 2001, Whitacre, Chauhan et al. 2002, Berger, Febbo et al. 2004, Simanainen, Allan et al. 2007, Wu, Altwaijri et al. 2007). Interestingly, during prostate cancer development, the AR in the epithelial cells supports proliferation, and the tumor growth becomes independent of stromal cells (Isaacs and Isaacs 2004). This signifies an important switch in the biological function of AR from a growth suppressive role to an oncogenic role in the epithelial tumor cells (Cesari, Rennekampff et al. 2004).

2.2.3 Androgen Receptor Variants

The androgen receptor (AR) is a steroid hormone receptor that has critical functions in development, growth and survival of the normal prostate as well the cancerous prostatic tissue. Loss of function alterations and gain of function alterations of AR have been demonstrated in androgen insensitivity syndrome (AIS) and prostate cancer respectively (Dehm and Tindall 2011). Altered AR splice variants have been shown to play a role in prostate cancer progression and development of resistance to androgen ablation therapy, thereby leading to AIS. Many of these variants of AR function as constitutively active, ligand-independent transcription factors that support androgen-
independent expression of AR target genes and also androgen-independent growth of prostate cancer cells.

2.2.3.1 Naturally occurring AR variants:

Alternative splicing events would have profound effects on the AR signaling axis. AR45, a naturally occurring AR variant, was first identified in the human placental tissue (Ahrens-Fath, Politz et al. 2005). The AR45 cDNA was shown to arise from an, earlier unknown, exon within intron 1 of the AR gene. This alternatively spliced variant of AR was shown to have a molecular weight of 45 kDa containing the entire AR DBD, CTD and a novel seven amino acid sequence (Met-Ile-Leu-Trp-Leu-His-Ser) in place of the wild-type AR NTD (Dehm and Tindall 2011). It is unclear if the protein of this splice variant was expressed, as all the studies were a result of real-time-PCR (RT-PCR); however, LNCaP cell lysates have demonstrated the presence of ~45 kDa protein when immunoblotted with an antibody that recognizes the CTD of wild-type AR. Ectopically expressed AR45 has been shown to bind to androgen, localize to the nucleus and interact with wild-type AR, and inhibit full-length AR activity in a ligand-dependent and DBD-dependent manner. Overexpression of AR45 was shown to inhibit cell proliferation in LNCaP cells, thereby demonstrating that AR45 is a negative regulator of AR signaling. However, it has also been shown that AR45 harbors transcriptional activity when co-activators like β-catenin or TIF-2 are overexpressed (Ahrens-Fath, Politz et al. 2005), which is a phenomenon expressed by AR DBD/CTD splice variants observed in other studies (Alen, Claessens et al. 1999, He, Kemppainen et al. 1999).
2.2.3.2 Loss-of-function AR variants – AR alternate splicing in AIS:

Androgen insensitivity syndrome (AIS) is caused due to AR genetic alterations. These alterations result in AR proteins which have impaired function and prevent normal androgen signaling and proper development of internal and external male phenotypes (Brinkmann 2001). AIS is of two types – complete AIS (CAIS), which is the most severe form of AIS where individuals have a complete female appearance; and partial AIS (PAIS), where the individuals primarily have a male appearance. The severity of AIS has been related to the level of impaired AR activity caused as a result of specific AR gene alterations.

In a patient with CAIS, it was observed that the sequences of the coding exon were unaltered, however, there was a GèT mutation within the splice donor site of intron 4. This mutation led to an internal deletion of 41 amino acids, thereby abolishing any ligand-binding activity and any transcriptional activity on androgen-responsive promoter-reporter gene construct (Ris-Stalpers, Kuiper et al. 1990). There have been similar mutations reported in individuals with CAIS in which exons 2 or exon 7 were skipped, the latter yielding a ~98 kDa AR protein as compared to the wild-type AR which is ~110 kDa (Lim, Ghadessy et al. 1997, Hellwinkel, Bull et al. 1999)

Alternatively spliced AR variants have also been reported in individuals with PAIS. A >6 kb deletion in intron 2 of AR led to an AR variant which had 39 amino acids deleted which encode for the zinc finger motif. This variant showed ligand-binding ability, but showed no transcription activity on an AR-responsive promoter-reporter
construct (Ris-Stalpers, Verleun-Mooijman et al. 1994). Similarly, a TëA mutation, about 11 bp upstream of exon 3, yields two different AR splice variants, one of which has been shown to have ligand-binding activity, but has no DNA-binding activity (Bruggenwirth, Boehmer et al. 1997). There have been other splice variants reported as well, as a result of mutations in AR, some which have shown no ligand binding as well as no transcription activity (Hellwinkel, Holterhus et al. 2001).

2.2.3.3 Gain of function AR variants:

It was recently showed that splicing of AR also gives rise to variants which have gain-of-function activity in contrast to the above described loss-of-function AR variants. These AR variants lack the C-terminal LBD, although the NTD and the DBD are intact. Biochemical characterization revealed that AR with the deleted LBD (ΔLBD), was shown to be constitutively localized in the nucleus, even in the absence of androgen (Tepper, Boucher et al. 2002, Libertini, Tepper et al. 2007). These studies provided evidence that AR–ΔLBD could play an important role in development of resistance to prostate cancer therapy. Examination of AR mRNA expression profiles from different prostate cancer models (CWR22, CWR-R1, 22Rv1 etc.), led to the identification of additional AR mRNA that displayed constitutive ligand-independent transcriptional activity (Guo, Yang et al. 2009, Hu, Dunn et al. 2009, Marcias, Erdmann et al. 2010, Hu, Isaacs et al. 2011).
2.2.4 Androgen Receptor Co-regulators

The AR co-regulators acts a transactivating chaperones. They associate with AR in the absence of ligand in many subcellular locations and influence DNA binding, nuclear translocation, chromatin remodeling, AR stability and also influence AR association with the basal transcription machinery (Lee and Chang 2003). There are a number of co-regulators that have been shown to interact with AR and this number continues to grow (Heinlein and Chang 2002, Wang, Hsu et al. 2005, Burd, Morey et al. 2006, Chmelar, Buchanan et al. 2007)

2.2.4.1 Co-activators:

Increased affinity of AR co-activators for AR is associated with enhanced AR transactivation. Co-activators like SRC-1, TIF2 and GRIP1 are capable of recruiting transcription factors and other co-activators with histone acetyl-transferase (HAT) activity. Co-activators with HAT activity like, cAMP response element binding protein (CREB)-binding protein (CBP/p300) and p300/CBP-associated factor (p/CAF), associate with AR leading to chromatin remodeling thereby facilitating AR to interact with the transcriptional machinery (Shen, Buchanan et al. 2005). AR-associated (ARA) proteins are the predominant co-activators of AR and are named according to their molecular weight (e.g. ARA24, ARA54, ARA55, ARA70, ARA160, ARA267, and the co-repressor ARA67). These co-regulators show no structural or functional similarity. ARA70 plays a role in stabilizing ligand bound AR and also broadens the ligand-binding specificity of AR thereby increasing AR transactivation (Glass and Rosenfeld 2000, Heinlein and Chang 2002).
2.2.4.2 **Co-repressors:**

AR co-repressors inhibit transcription initiation from androgen-responsive genes. Two well-known co-repressors are: nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT). SMRT is capable of interacting with the NTD and LBD of AR in presence and absence of AR ligand, while NCoR does so only in the presence of agonist (Cheng, Brzostek et al. 2002, Heinlein and Chang 2002, Liao, Chen et al. 2003). Both SMRT and NCoR mediate disruption of N/C interaction and compete with co-activators like SRC/p160 thereby causing transcription repression. SMRT and NCoR recruit HDACs, which leads to DNA packaging, thereby preventing access of promoter and enhancer regions to the basal transcription machinery and co-activators, eventually leading to repression of transcription (Liao, Chen et al. 2003).

2.2.5 **Androgen Receptor and Prostate Cancer**

Prostate cancer is one of the most common malignancies and the second leading cause of cancer death in men in the United States. Multiple factors contribute to the high incidence of prostate cancer: rise in aging population and improved screening methods probably constitute the major factor for the high report of prostate cancer cases.

2.2.5.1 **Androgen receptor expression in prostate cancer:**

Primary prostate cancer demonstrates AR expression; AR is detected throughout progression in both, androgen-dependent prostate cancer and also hormone refractory prostate cancers (Sadi, Walsh et al. 1991, Chodak, Kranc et al. 1992, Ruizeveld de
Immunohistochemical studies have demonstrated that AR expression is heterogenous in prostate cancer and the degree of heterogeneity cannot be correlated with response to treatment like androgen-deprivation therapy (Sadi, Walsh et al. 1991, Ruizeveld de Winter, Janssen et al. 1994). The heterogeneity in prostate cancer cells suggests that increased AR expression cannot be associated with prostate cancer initiation and also hormone refractory prostate cancers do not arise from cells that are clonally selected to be AR-negative. Loss of the AR gene or protein is extremely rare (Cunningham, Shan et al. 1996, Nupponen, Kakkola et al. 1998, Alers, Rochat et al. 2000). There are a few postulations with respect to loss of AR: (a) epigenetic silencing of the AR gene by promoter methylation, which is observed in approximately 8% of primary prostate cancers (Sasaki, Tanaka et al. 2002), (b) mutations in the AR gene rendering it inactive or non-functional and (c) poly-ubiquitination of AR leading to proteolytic degradation, which is mediated by Akt-kinase driven phosphorylation of AR (Lin, Wang et al. 2002).

2.2.5.2 Androgen receptor and prostate tumorigenesis:

Different model systems have been developed to understand the role of AR in prostate tumorigenesis. Most of the cell line model systems are derived from patients with advanced metastatic and/or hormone refractory disease. Since these models are unable to mimic the early stage of the disease, they have limitations in understanding the molecular alterations that are essential for transformation of prostate epithelial cells. Some lines have shown the oncogenic role of AR in immortalized and transformed prostate epithelial cells in the prostate microenvironment (Glass and Rosenfeld 2000,
The AR in the normal prostate epithelial cells is involved primarily in differentiation and growth suppression. Molecular alterations that alter AR signaling in epithelial cell proliferation represent an important event in prostate tumorigensis. The oncogenic role of AR has been demonstrated in a transgenic mouse model expressing prostate specific AR transgene (Glass and Rosenfeld 2000). However, it has been recently shown that in the early stage of prostate carcinogenesis, epithelial cells of the prostate lose the growth suppressive function of AR without attaining dependence on AR for growth (D'Antonio, Vander Griend et al. 2010).

2.2.5.3 **Androgen receptor and prostate cancer progression:**

The progression of prostate cancer from androgen dependent into castration recurrent stage is a significant switch in the biology of prostate cancer, in which AR plays a critical role. Although, prostate cancer cells are heterogeneous with respect to the expression of AR (van der Kwast, Schalken et al. 1991, Hobisch, Culig et al. 1996), majority of the population of prostate cancer cells depend on AR for their growth and survival. During androgen ablation therapy, the androgen dependent cells undergo apoptosis and the tumor regresses in size. However, a small population of prostate cancer cells, survive this androgen deprivation and repopulate the tumor, eventually leading to the development of hormone refractory prostate cancer. Majority of recurrent tumors express AR (Jenster, van der Korput et al. 1995, Visakorpi, Hyytinen et al. 1995, Latil, Bieche et al. 2001, Edwards, Krishna et al. 2003, Ford, Gregory et al. 2003), and
frequently show express high levels of the antiapoptic protein such as Bcl-2 (Raffo, Perlman et al. 1995, Shand and Gelmann 2006). Furthermore, recurrent tumors show predominant nuclear localization of AR, even in the absence of hormone, which is consistent with the transcriptional role of AR in the tumor progression (Gregory, Hamil et al. 1998, Scher, Buchanan et al. 2004). Both androgen dependent and castration recurrent prostate cancer cells are dependent on AR signaling for their growth and progression. Several studies have shown the continued reactivation of the AR signaling in hormone refractory tumors (Visakorpi, Hyytinen et al. 1995, de Vere White, Meyers et al. 1997, Berrevoets, Doesburg et al. 1998, Zhang, Johnson et al. 2003).

2.2.6 Mechanism of Regulation of AR Mediated Gene Expression

The androgen receptor (AR) signaling axis plays an important role in the development, function and maintenance of the normal prostate. In the normal prostate, androgen binds to AR followed by AR nuclear translocation, where it binds to androgen response elements (ARE) on target genes and recruits or cross talks with transcription factors (Lonergan and Tindall 2011). There are also other alternative mechanisms of activating AR leading regulation of classical androgen target genes and/or other gene targets that may or may not be classical androgen targets.

2.2.6.1 Classical mechanism of AR action:

Androgenic steroids like testosterone are 19-carbon steroids. Testosterone is primarily produced in the testis in males; a small amount also being generated by the

In the absence of ligand, AR is localized in the cytosol, where it is bound to heat shock proteins (HSP) -90, -70, -56, cytoskeletal proteins and other chaperones (Smith and Toft 2008). Binding of ligand to AR causes a conformational change in AR, due to which helices 3, 4 and 12 within the ligand binding domain (LBD) form the AF-2 site. AF-2 is the principal protein-protein interaction surface that is used by nuclear receptors to recruit LXLL motif-containing co-regulators (Heery, Kalkhoven et al. 1997). Due to the conformational change, the formation of the hydrophobic pocket within the LBD facilitates the interaction between the AR N-terminus and C-terminus (N/C interaction) (He, Minges et al. 2002). This N/C interaction results in dimerization of AR and also leads to the exposure of the nuclear localization signal (NLS). This leads to nuclear translocation of AR, where it binds to specific recognition sequences known as androgen response elements (AREs) in the promoter and enhancer regions of target genes. AR then recruits co-regulators there by completing its transcription complex leading to modulation of gene expression (Dehm and Tindall 2006).

### 2.2.6.2 ARE-dependent actions of AR:

The consensus androgen response element is a palindromic sequence that is separated by a 3 base pair spacer. The classical response element for AR is GGTACAnnnTGTTCT (Claessens, Verrijdt et al. 2001); however sequences that deviate from the consensus are reported in the natural promoter of genes (Bolton, So et al. 2007).
In addition, the AR binds naturally occurring selective AR direct repeat (DR) response elements besides the classical inverted repeat (IR) response elements. AR binds to direct repeat response elements in a head to head orientation rather than head to tail. The selectivity of this response element for AR is because of the presence of additional strong interaction interface in the second zinc finger that is involved in stabilizing AR dimers. On the other hand, glucocorticoid receptor (GR) and progesterone receptor (PR) dimers do not have this additional bond. Hence, the dimers are less stable and the interaction with direct repeat response element is suboptimal. This implies the AR generally forms head to head dimerization regardless of the organization of the DNA response elements (DR or IR). This also allows AR to bind divergent non-canonical DNA motifs (Scher, Buchanan et al. 2004, Shaffer, Jivan et al. 2004, Bolton, So et al. 2007). In addition AR binds ARE half sites as monomer and regulates target genes through interaction with other transcription factors (Massie, Adryan et al. 2007).

There are two important questions with respect to the transcriptional activity of AR, the requirement for binding to DNA (AREs) and the role of ligand for DNA binding. Several studies have shown that binding of AR to AREs is mandatory for androgen dependent gene activation. The binding of AR to the androgen response elements (AREs) requires a conformational modification attained through hormone binding. Furthermore, ligand binding is required for the formation of dimer at AREs (Wong, Zhou et al. 1993, Karvonen, Kallio et al. 1997). There are few studies that showed the binding of AR to AREs in the absence of hormone in a cell free system (Xie, Sui et al. 1992, Luke and Coffey 1994, Huang, Li et al. 2002). However, for optimal ARE mediated transcriptional
activity, binding of ligand like DHT to AR is necessary (Wong, Zhou et al. 1993, Karvonen, Kallio et al. 1997).

With respect to the necessity of DNA binding by AR, it is generally assumed that AR regulates target genes through ARE mediated signaling. Even though the requirement for DNA binding is well established for some selected androgen regulated genes such as KLK3 (PSA), KLK2 (Cleutjens, van Eekelen et al. 1996, Cleutjens, van der Korput et al. 1997)(29, 98) there is no direct evidence that rule out the genomic action of AR without direct DNA binding. Rather, few studies have shown that AR modulates target genes without directly binding to the DNA, rather being tethered to DNA via other transcription factors (Kallio, Poukka et al. 1995, Palvimo, Reinikainen et al. 1996, Schneikert, Peterziel et al. 1996).

2.2.6.2 Non-ARE-dependent actions of AR:

Target gene promoter analysis for AR has been done on a gene-by-gene basis for a finite number of genes (Veldscholte, Ris-Stalpers et al. 1990, Riegman, Vlietstra et al. 1991, Cleutjens, van Eekelen et al. 1996). The ability of AR to regulate target genes from a distal enhancer located more than 100 kb from the transcription start site poses a considerable challenge for assigning binding sites to a specific gene. However, it is a commonly accepted notion that androgen receptor regulates its target gene through androgen response elements (ARE). In contrast, non-classical (tethering) transcriptional mechanisms that do not require direct binding to DNA have also been demonstrated for nuclear receptors. Dimerization mutant GR that lacks the ability to bind DNA (GRE) has
shown AP1 dependent gene repression as efficient as the wild type GR (Dahlman-Wright, Wright et al. 1991, Heck, Kullmann et al. 1994).

A dimerization-defective mutant GR has been shown to not bind to GREs but can is capable of repressing AP-1 regulated genes. GR knockout mice die shortly after birth, however, dimerization mutant GR mice are viable, indicating the mutant GR ability to support survival in GRE independent manner (Kim and Coetzee 2004). In a similar fashion, GR has been shown to physically interact and repress the transcriptional activity of NF-κB (Ray and Prefontaine 1994, Caldenhoven, Liden et al. 1995, Scheinman, Gualberto et al. 1995) and AP1 without directly binding to the DNA (Yang-Yen, Chambard et al. 1990, Dehm and Tindall 2006). These studies have shown that GR regulates genes without binding to GRE via protein-protein interactions.

GR and AR show great structural similarity, and it is plausible to assume direct physical interaction between AR and NF-κB. Androgen has been shown to down regulate the expression of some NF-κB target genes that lack AREs (Bellido, Jilka et al. 1995)(123). The attenuation of IL-6 expression by androgen is an example that might suggest interaction between NF-kB and AR.

AR has also been shown to interact physically with Ets-family of transcription factors to modulate the expression of target genes such as matrix metalloproteinases (MMPs) in prostate cancer cells. AR down regulates the expression of target genes like MMP1, 3 and 7 in a manner that is dependent on DNA bound Ets-related family of
transcription factor (ERM). Furthermore it has been shown that, this repression of MMPs is mediated by DNA binding and dimerization mutant of AR which underlies the recruitment of AR to DNA bound ERM to mediate transcriptional repression (Schneikert, Peterziel et al. 1996). A reciprocal co-activation between AR and ETV1 are also reported in prostate cancer (Shin, Kim et al. 2009).

Recent studies from our lab have demonstrated that potential AR tethering proteins, may redirect AR signaling according to the physiological context (Zhang, Gonit et al. 2010). This may be exemplified by the ability of several well established AR tethering proteins to profoundly influence the pattern of gene activation by androgen/AR, which include HoxB13 (involved in development) (Norris, Chang et al. 2009) and C/EBPalpha (involved in terminal differentiation) (Zhang, Wilkinson et al. 2008, Sivakumaran, Zhang et al. 2010, Zhang, Gonit et al. 2010). HOXB13 activates its target genes in AR dependent manner. ORM1 is one example of HOXB13 regulated gene that lacks ARE but contains binding sites for HOXB13 (Norris, Chang et al. 2009).

Our lab has demonstrated that direct binding of AR to DNA elements is not required for it to activate the required growth genes, in case of hormone independent prostate cancer cell growth (Gonit, Zhang et al. 2011). Our studies led to the discovery that Elk1 which is a genetically redundant protein and is obligatory for androgen/AR-dependent malignant growth (Patki, Chari et al. 2013). We also showed, using co-immunoprecipitation, mammalian two-hybrid assays and chromatin immunoprecipitation, that Elk1 is a true AR tethering protein, both in hormone-dependent PC cells and in
CRPC cells (Patki, Chari et al. 2013). The activation of AR target genes through Elk1 was mechanistically distinct from the mode of activation of immediate early genes by Elk1 that requires Elk1 phosphorylation. We also demonstrated that phosphorylation of Elk1 was not required for the Elk1-AR synergy (Patki, Chari et al. 2013). Further, our lab has shown that AR can bind and transactivate through tethering proteins, including Elk1, and that this phenomenon is hormone-independent and insensitive to conventional antiandrogens (Zhang, Gonit et al. 2010, Patki, Chari et al. 2013). However, hormone-dependent cells (e.g., LNCaP and VCaP cells) are still ligand-dependent for this mode of gene activation by AR as androgen is needed for AR to be localized to the nucleus.

### 2.3 Mechanisms of Development of Castrate Recurrent Prostate Cancer

Genetic modification is one of the main factors responsible not only for tumor progression, but also for development of castration or drug resistance which leads to androgen independent prostate cancer (AIPC) (Ruijter, van de Kaa et al. 1999). However, cells have powerful mechanisms that normally guard the genome from mutations. Like many other cancers, it is possible that prostate tumors initially select for genetic changes which lead to subsequent mutations leading to AIPC (Ruijter, van de Kaa et al. 1999). For example Gultathione S-transferase π, a phase II detoxification enzyme, catalyses intracellular detoxification of electrophilic compounds, including some carcinogens, but is not expressed in 90% of prostate cancers owing to methylation of its promoter in a cancer-specific fashion (Lee, Morton et al. 1994). This promoter methylation of glutathione S-transferase gene is thought to be one of the earliest and most common genomic mutations observed in sporadic prostate cancer.
An earlier study suggested that untreated metastatic tumors contain the bulk of chromosomal alterations, which are required to develop resistance during androgen deprivation. This suggested that mutations might be an early event that is independent of the selective pressure due to androgen blockade or deprivation \( ^{1}\) (Cher, Bova et al. 1996). Many studies have found only a few AR mutations in primary prostate tumors as compared to metastatic prostate tumors – possible with a frequency as high as 50\% \( ^{2}\) (Taplin, Bubley et al. 1995, Tilley, Buchanan et al. 1996, Koivisto, Kononen et al. 1997, Taplin, Bubley et al. 1999, Marcelli, Ittmann et al. 2000). Recent studies have shown that androgen ablation therapy provides selective pressure to target the androgen-signaling pathway \( ^{3}\) (Taplin, Bubley et al. 1995, Culig, Hobisch et al. 1998, Craft, Chhor et al. 1999, Taplin, Bubley et al. 1999). For example, treatment with antiandrogen, Flutamide selects for mutant ARs in which flutamide acts an agonist instead of an antagonist \( ^{4}\) (Taplin, Bubley et al. 1999). The timing of development of AIPC still remains unclear, however, intermittent androgen ablation is considered to be a possible means to delay the development of AIPC \( ^{5}\) (Bruchovsky, Klotz et al. 2000). There are five potential mechanisms that lead to the development of resistance followed by AIPC.

\( ^{2.3.1} \) The Hypersensitive Pathway

One possible mechanism by which prostate cancers bypass the effects of androgen ablation is by increasing their sensitivity to much lower concentrations of androgen. Prostate cancers that use this mechanism are not, strictly speaking, androgen independent, but they have a much lower threshold for androgens.
2.3.1.1 *AR amplification:*

There are several mechanisms that lead to increase in prostate tumor cell proliferation despite low androgen levels. One potential mechanism by which this is accomplished is by increasing the expression of AR itself. Approximately 30% of prostate tumors, after androgen ablation, have amplified AR gene. This results in increased AR expression, however, none of the primary tumors from the same patient samples show to have the amplified AR gene (Visakorpi, Hyytinen et al. 1995, Koivisto, Kononen et al. 1997). These results indicate that amplification of AR was due to clonal selection of cells that could proliferate, despite low levels of circulating androgens. Although tumors with AR amplification have increased levels of AR, the signal for the cells to proliferate presumable requires androgen. This is an example of how tumors that seem to be androgen independent are actually dependent on androgen, but proliferate even at very low levels of androgens (Palmberg, Koivisto et al. 2000).

2.3.1.2 *Increased AR Sensitivity:*

This pathway results in high level of AR expression, increased stability and nuclear localization of AR in recurrent tumor cells. The tumor cells were also found to be hypersensitive to dihydrotestosterone (DHT), i.e. the concentration of DHT required by AR for growth stimulation was found to be four orders of magnitude less than that of the androgen-dependent LNCaP cells. These results have shown that AR is transcriptionally active in some models of recurrent prostate cancer and can increase cell proliferation even in the presence of very low circulating androgens, which are produced within the tumor in castrated men (Gregory, Johnson et al. 2001). It is also possible that amplified
AR may not be only sensitive to lower levels of androgen, rather may be constitutively active, which is described in the subsequent sections (outlaw pathway). Alternatively, the tumors may have amplified or decreased expression of coregulators, which help AR in aiding cell proliferation in the presence of either less active adrenal androgens or at very low concentrations of circulating androgens (Gregory, He et al. 2001).

### 2.3.1.3 Increased Androgen Levels:

A third hypersensitive mechanism that is involved in circumventing androgen ablation is by increasing the local synthesis of androgens, which compensates the overall decline in circulating testosterone. Prostate cells may increase the conversion rate of testosterone to DHT by increasing the activity of the enzyme 5α-reductase. This facilitates AR signaling even in the presence of very low levels of circulating testosterone. A recent study showed that, after androgen ablation therapy, although the levels of serum testosterone decreased by 95%, the concentration of DHT was decreased only by 60% (Labrie, Dupont et al. 1986). It has been shown that different racial groups show a difference in the incidence of polymorphism in the gene 5α-reductase – this polymorphism substitutes valine at codon 89 for leucine (V89L) (Ruijter, van de Kaa et al. 1999). For example, men of African decent show a very high incidence of this polymorphism as compared to that of men of Asian decent, who are at low risk of prostate cancer; men of Central- and South-American decent, both have an intermediate incidence of this polymorphism and intermediate risk of prostate cancer (Makridakis, Ross et al. 1997). Apart from genetic predisposition, it is possible that, by selection
during treatment, tumor cells may acquire mutation in the 5α-reductase gene, however, this has not yet been shown to occur in prostate cancer.

2.3.2 The Promiscuous Pathway

Most castrate resistant prostate cancer (CRPC) cells express the AR protein. Resistance may not only develop because of loss of androgen signaling, but also from acquisition of genetic changes that lead to abnormal activation of the androgen signaling axis (Buchanan, Greenberg et al. 2001). These changes mainly include missense mutations in AR that lead to decreased specificity of ligand binding to AR leading to its activation by various non-androgenic steroids and/or androgen antagonists (Buchanan, Yang et al. 2001).

2.3.2.1 AR mutations:

The AR gene is located on the X chromosome and is not necessary for survival, thus the germ-line loss-of-function mutations in AR resulting in androgen-insensitivity syndrome are frequent (Quigley, De Bellis et al. 1995). Recent studies have shown that the frequency of AR mutations increases significantly after androgen ablation whereas most studies have shown very few AR mutations in the primary tumor (Taplin, Bubley et al. 1995, Tilley, Buchanan et al. 1996, Marcelli, Ittmann et al. 2000). This indicates that mutation(s) in the androgen receptor is likely to be one of the potential mechanisms involved in developing resistance to therapy leading to AIPC.
Although only a few mutations in the AR have been studied in great detail, a mechanism for the development of AIPC has emerged from recent studies (Buchanan, Greenberg et al. 2001). In cells with this kind of AR mutation, the androgen-signaling axis remains active by broadening the range of ligands that bind to AR and activate it. Normally AR is activated by DHT specifically, however, in cells that harbor this kind of mutation, AR is activated by other circulating steroid hormones when the levels of androgens is very low. The first AR mutation of this type was found in LNCaP cells which express high levels of AR and are stimulated by androgens to grow and secrete prostate specific antigen (PSA) – a widely used clinical marker for prostate cancer cells (Veldscholte, Berrevoets et al. 1992). Sequencing of AR from LNCaP cells showed that there was a mutation in the ligand-binding domain, where threonine at codon 877 was substituted by alanine (T877A). Molecular studies showed that hormones such as estrogen, progesterone and anti-androgens like flutamide bind to this mutant AR and act as antagonists (Veldscholte, Berrevoets et al. 1992). During androgen ablation therapy, it is possible that cells that harbor this mutation in AR undergo clonal selection which gives these prostate cancer cells a growth advantage (Taplin, Bubley et al. 1999). Promiscuous AR activators also include adrenal androgens and metabolic products of DHT (Culig, Hobisch et al. 1993, Culig, Hobisch et al. 1998).

This promiscuous receptor mechanism also explains the clinically observed phenomenon of ‘flutamide withdrawal syndrome’, in which patients show worsening with flutamide treatment and recover with flutamide withdrawal (Small and Srinivas 1995). In a series of bone marrow metastases, T877A mutations were found in 5 out of 16
patients who received combined androgen blockade and flutamide treatment. Patients harboring the T877A mutation in the cancer cells were stimulated to grow strongly with flutamide treatment, whereas patients in which the cancer cells did not harbor the T877A mutation were not stimulated to grow in response to flutamide treatment. These findings indicate that AR mutations occur in response to a strong selective pressure from flutamide treatment (Taplin, Bubley et al. 1999).

Crystallographic studies of this mutant AR with T877A mutation revealed the reason for this AR to accommodate other steroid receptors and ligands in the ligand-binding pocket that the wild-type AR cannot (Matias, Donner et al. 2000). Similar to the T877A mutation, the CWR22 tumor cell line harbors a H874Y mutation in the ligand-binding domain of AR that influences the binding of co-activator proteins by affecting the conformation of helix 12 (McDonald, Brive et al. 2000). The MDA PCa 2a and 2b cell lines established from bone metastasis in a patient also harbor promiscuous ARs. These cells, like LNCaP cells, harbor the T877A mutation along with another mutation – L701H (Suzuki, Sato et al. 1993, Zhao, Boyle et al. 1999). The L701H mutation in AR, alone decreases the ability of AR to bind and respond to DHT. However, the T877A mutation has a synergistic effect in this mutant AR having both these mutations – it increases the affinity of AR for glucocorticoids by 300% more than L701H mutation alone (Zhao, Malloy et al. 2000).

Not all AIPC cells, which have promiscuous ARs harbor mutations in the AR. LNCaP cells that are grown in androgen-depleted media over many passages, which are
referred to as LP50 or LNCaP-abl cells – have a fourfold higher expression of AR and a 30-fold increase in basal AR transcription activity compared to the parent LNCaP cell line. In these cells, casodex, which is an AR antagonist, functions as an agonist (Culig, Hoffmann et al. 1999). However, in spite of these changes, the AR in these cells harbored the same T877A mutation as that of the parental LNCaP cells. Clearly, a mechanism other than mutations in the AR is promoting tumor progression.

2.3.2.2 Co-regulator alterations:

Modulation of co-regulatory proteins can be a likely mechanism by which prostate cancer cells develop resistance to therapy followed by progression to AIPC. It has been shown in breast cancers that an imbalance in the expression of co-regulatory proteins leads to development of resistance to hormonal adjuvant therapies like tamoxifen or aromatase inhibitors. For example, steroid receptor co-activator 1 (SRC1) family of nuclear receptor co-activators, AIB1 is amplified in breast and ovarian tumors (Anzick, Kononen et al. 1997). Although SRC1 family members have been shown to be co-activators in a variety of tissues, co-activator ARA70 has been shown to be specific for androgen responsive genes (Yeh and Chang 1996). Decreased expression of nuclear receptor co-repressor (N-CoR), has been found to be directly linked to development of resistance to adjuvant therapy in breast cancer patients (Lavinsky, Jepsen et al. 1998). Although, similar events have not yet been reported in prostate cancer, increase in expression of AR co-activators or loss or decreased expression of AR co-repressors may create a promiscuous AR, which would allow molecules that normally do not activate AR to take on the function of agonists.
2.3.3 The Outlaw Pathway

Steroid hormone receptors that are activated in the absence of ligand are referred to as ‘outlaw receptors’. In breast cancer, an outlaw estrogen receptor (ER) was discovered, which contained mutations that would render the transactivation domain of ER either dominant positive or dominant negative (McGuire, Chamness et al. 1991). Such mutations, if any, are yet to be discovered in AR; however AR could be subverted into becoming an outlaw receptor by other pathways.

2.3.3.1 Growth-factor activated outlaw pathways:

Growth factors like insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF) activate AR and render it an outlaw receptor leading to activation of AR target genes in the absence of its ligand, androgen. It has also been shown that the AR antagonist, casodex, completely blocks AR activation by IGF-1, KGF and EGF, thus showing that the ligand binding domain of AR is essential for this outlaw activation (Culig, Hobisch et al. 1994). However, this mechanism fails to explain the development of AIPC in patients where casodex completely blocks IGF-induced AR activation. It is a possibility that AR mutations in combination with upregulation of growth factors may cause APIC in such patients (Culig, Hobisch et al. 1994).

2.3.3.2 Receptor-tyrosine-kinase activated outlaw pathways:

Studies in breast and ovarian cancers have provided evidence of a connection between nuclear receptor signaling and receptor tyrosine kinases. HER-2/neu, which is
also known as ERBB2, an EGF-receptor (EGFR) family of receptor tyrosine kinases, has been shown to activate ER in the absence of its ligand, estrogen (Borg, Baltedorp et al. 1994). EGFR has been found to be upregulated in 20-30% of breast and ovarian cancer patients (Slamon, Godolphin et al. 1989). HER-2/neu has been shown to cause estrogen independent stimulation leading to activation of ER-mediated signal transduction pathways (Borg, Baltedorp et al. 1994).

AR can also be turned into an outlaw receptor in a similar way - HER-2/neu has been shown to be consistently overexpressed in AIPC cell lines; also, androgen independent cell lines have been generated by overexpressing HER-2/neu in androgen-dependent cell lines. HER-2/neu has been shown to activate AR-dependent genes in the absence of androgen, but not in the absence of AR (Craft, Shostak et al. 1999, Yeh, Lin et al. 1999). However, unlike the effect of IGF-1, HER-2/neu mediated outlaw AR-dependent gene activation could not be blocked by casodex, indicating that this pathway is independent of the ligand binding domain of AR (Craft, Shostak et al. 1999). These findings indicate that HER-2/neu is an important mechanism, which leads to hormone-refractory prostate and breast cancers. Trastuzumab (Herceptin), a monoclonal antibody against HER-2/neu was developed to block this mechanism of activation of ER in breast cancer patients (Slamon, Leyland-Jones et al. 2001). It was used as a first-line therapeutic agent until tamoxifen and second and third-generation aromatase inhibitors. Herceptin has showed anti-proliferative effects in androgen-dependent prostate cancers. In combination with the chemotherapeutic agent, paclitaxel, Herceptin has shown to have additive effects in both androgen-dependent and androgen-independent prostate cancer...
model systems (Agus, Scher et al. 1999). Activation of HER-2/neu leads to the activation of downstream mitogen-activated-protein kinase (MAPK) proteins which in-turn lead to the phosphorylation and activation ER or AR in breast and prostate cancer respectively. MAPK inhibitors like Trametinib, which inhibits MEK were found to decrease HER-2/neu mediated AR activation (Yeh, Lin et al. 1999). Besides all the evidence, this mechanism is to be further investigated to be certain that this pathway is active in patients who develop AIPC.

2.3.3.3 The AKT pathway:

The AKT pathway has been suspected to contribute to tumorigenesis in glioblastoma, breast and prostate cancers owing to its anti-apoptotic activity. AKT phosphorylates and inactivates pro-apoptotic proteins like BAD and procaspase-9 (Zhou, Li et al. 2000). PTEN, which is a tumor suppressor, in normal cells, allows the cells to undergo apoptosis by blocking the AKT pathway. However, cancer cells, in which PTEN has lost its function due to mutations in it, have increased AKT activity leading to cancer cell survival and proliferation. AKT has also been shown to downregulate p27, which is involved in cell cycle inhibition (Medema, Kops et al. 2000).

Her-2/neu, which is an EGFR, is also shown to activate the phosphotidyl inositol 3-kinase (PI3K)/AKT pathway. This is an alternate means for AKT pathway to be activated (Wen, Hu et al. 2000, Zhou, Hu et al. 2000). AKT that has been activated via this pathway phosphorylates AR at serine (Ser) 213 and Ser791, turning it into and androgen-independent outlaw receptor. Her-2/neu-mediated activation of AR was found
to be blocked by expressing a dominant-negative AKT (Wen, Hu et al. 2000). It has been observed that Her-2/neu expression increases with progression to AIPC; thus, therapeutic targeting of Her-2/neu might be necessary in some cases of prostate cancer (Signoretti, Montironi et al. 2000).

2.3.4 The Bypass Pathway

The pathways that are discussed above require AR and its signaling cascade for the development of AIPC. However, it is possible that alternative pathways may be induced, which are capable of bypassing AR completely. Bypassing that androgen-signaling cascade in the absence of androgens leading to cell proliferation and inhibition of apoptosis would be an effective bypass pathway (Feldman and Feldman 2001).

The *BCL2* gene is a potential bypass candidate gene, which is involved in blocking apoptosis. In secretory prostatic epithelial cells, *BCL2* is not expressed, however, *BCL2* expression has been found to be upregulated in prostatic intraepithelial neoplasia (PIN) and also in AIPC. It has also been shown that, in mice xenografts of LNCaP cells, *BCL2* expression is upregulated in tumors that initially did not express it. In these mice, blocking *BCL2* with antisense oligos, delayed the emergence of AIPC. *BCL2* upregulation, was then found to bypass the signal for apoptosis, which is normally generated by androgen ablation (Colombel, Symmans et al. 1993, Gleave, Tolcher et al. 1999). Although, the above studies describe the role of *BCL2* leading to development of AIPC, *BCL2* may not be essential or may not be the only factor involved in the formation
of AIPC (Furuya, Krajewski et al. 1996). Further studies are essential in understanding the mechanism in which these bypass pathways interact with AR signaling.

### 2.3.5 The Lurker Cell Pathway

As described in this above section, there could be several pathways by which a cell can become androgen independent and lead to failure of androgen ablation therapy. A recent study postulated that a sub-population of androgen-independent tumor cells was present even before therapy was initiated (Isaacs 1999). These putative epithelial stem cells, which are present among the basal cells, are believed to be androgen-independent and hence are not affected by androgen ablation (Denmeade, Lin et al. 1996). According to this model, if the epithelial stem cell transformed and became the origin of a prostate cancer, the following events would occur: first, in the presence of androgens, most of the epithelial stem cells would differentiate into androgen-dependent cells that would comprise most of the tumor; second, after androgen ablation, the androgen-dependent cells would be eliminated and the androgen-independent malignant epithelial stem cells, which have been ‘lurking’ in the background, would prevail; and third, these stem cells would continue to proliferate leading to cancer relapse –AIPC (Isaacs 1999).

A recent study (Craft, Chhor et al. 1999), in support of this hypothesis, showed that, androgen independence occurs due to clonal expansion of androgen-independent cells that are present at a frequency of 1 per $10^5$-$10^6$ androgen-dependent cells. This study indicated that prostate cancers consist of a heterogeneous mixture of cells that vary on their dependence on androgen for growth and survival, and that treatment with anti-
androgens provides selective pressure that alters the frequency of these cells leading to androgen-independent cancers (Craft, Chhor et al. 1999).

2.4 The ETS transcription factors

The E-twenty six (ETS) transcription factors are nuclear phosphoproteins that are involved in hematopoiesis, vasculogenesis, neuronal development, proliferation, differentiation and oncogenic transformation (Jakacka, Ito et al. 2001). Several studies have shown that Ets transcription factors are activated as a result of the Ras-MAPK signaling pathway. The transactivation of target genes, protein-protein interaction and stability of Ets transcription factors is modulated by their phosphorylation status (Wasylyk, Hagman et al. 1998, Jakacka, Ito et al. 2001, Safe and Kim 2004).

There are more than 20 ETS domain family members; however, two subfamilies are most extensively studied - (a) The Ets subfamily that includes Ets1, Ets2, and Pointed (P2) that have an amino terminal pointed domain and C-terminal Ets DNA binding domain. This group has a single MAPK phosphorylation site in their pointed domain, and (b) the ternary complex factor (TCF) subfamily which includes Elk-1, SAP-1, SAP-2/Net, which, in contrast have N-terminal Ets DNA binding domain and C-terminal transactivation, protein–protein interaction domain. TCFs have multiple phosphorylation sites on their transactivation domain (Wasylyk, Hagman et al. 1998, Jakacka, Ito et al. 2001, Safe and Kim 2004).
2.4.1 Elk-1 Gene Structure and Domains

Elk-1 is a modular protein that consists of four domains. The N-terminus of Elk-1 that contains the ETS DNA binding domain forms domain A. The A domain also contains a conserved motif that recruits mSin3A-histone deacetylase co-repressor complex and act as a repressor domain (Yang, Vickers et al. 2001). The B domain of Elk-1 contains a highly conserved sequence motif termed as B-box that forms interaction with SRF. This domain is also involved in the interaction of other MADS box family of transcription factors. The C-terminus of Elk-1 forms the C domain that functions as transcriptional activation domain. The activation domain is the target of MAPK phosphorylation and consists of multiple serine/threonine phosphorylation sites. The docking site for MAPK forms the D domain. The D domain facilitates the recruitment of MAP kinase to the correct substrate and confers additional specificity (Sharrocks, Yang et al. 2000). Elk-1 also consists of the R motif that is a repressor domain that dampens the transcriptional activation (Fronsdal, Engedal et al. 1998, Yang, Bumpass et al. 2002).

There are three isoforms of Elk-1; the full length Elk-1 contains 428 amino acid
residues, the short form of Elk1 (sElk-1) lacks the first 54 amino acid residues and it is
generated by utilizing an alternative translation start site. The third isoform, ΔElk-1,
contains 285 amino acid residues and it is an alternative splice variant of Elk-1 that has
lost SRF binding site and part of DNA binding site (Rao and Reddy 1993, Fronsdal,

2.4.2 Elk-1 and Normal Prostate

The Ets transcription factors play a vital role in a wide range of physiological
functions that include cell proliferation, cell differentiation, survival and development
(Macleod, Leprince et al. 1992). Elk-1 has also been shown to support neuronal
proliferation and differentiation and probably involved in memory (Cammarota,
Bevilaqua et al. 2000). However, disruption of Elk-1 in mouse has shown minor
phenotypic abnormalities (Cesari, Rennekampff et al. 2004). This might be probably due
to the functional redundancy of TCF transcription factors. Hence it would be necessary
and interesting to generate double or triple knockout mice to characterize the
physiological role of these molecules.

2.4.3 Elk-1 and Cancer

As mentioned earlier, Elk-1 is involved in cell proliferation, survival and
tumorigenesis. The oncogenic role of Elk-1 occurs through the regulation of immediate
early genes such as c-Fos, whose role in tumorigenesis has been demonstrated in in vitro
cell culture and animal model studies (Jakacka, Ito et al. 2001). In a recent study, it was
shown that the proliferative action of Elk-1 in breast cancer was attenuated by the
recruitment of BRCA1 co-repressor (Maniccia, Lewis et al. 2009). Elk-1, through SUMO dependent repression of Egr-1, promotes cell survival by inhibiting apoptosis in neuroblastoma cells (Demir and Kurnaz 2008). In MLL fusion leukemia, Elk-1 is activated by oncogenic chromosomal rearrangement of mixed lineage leukemia (MLL) (Ng, Ng et al. 2010). Furthermore, the oncogenic role of Elk-1 has been demonstrated in human hepatocellular carcinoma (Ying, Hsieh et al. 2008). It has also been shown that epidermal growth factor promotes breast tumor development and metastasis by regulating plasminogen activator inhibitor 1 (PAI-1) expression via Elk-1 (Wyrzykowska, Stalinska et al. 2010).

2.4.3.1 Elk1 and Prostate Cancer

Growth factor receptors such as the epidermal growth factor receptor (EGFR), insulin like growth factor receptor (IGFR), platelet-derived growth factor receptors are often up-regulated in most prostate cancer tumors (Hellawell, Turner et al. 2002, Uehara, Kim et al. 2003, Renehan, Zwahlen et al. 2004). Activation of these signaling pathways initiates a signaling cascade that promotes anti-apoptotic and pro-mitotic signals. The chronic activation of MAPK pathways in tumors leads to phosphorylation and activation of Elk-1, which in turn activates Elk-1 target genes that are involved in the proliferation and subsequent progression of tumor. A recent study showed that Elk-1 and ATF-2, through p38, activates inter-leukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) that leads to increased proliferation of prostate cancer cells. The growth promoting function of Elk-1 has been correlated with elevated levels of pElk-1 in prostate cancer tissue samples compared to normal control (Ricote, Garcia-Tunon et al. 2006).
Our studies led to the discovery that Elk1, which is a genetically redundant protein and is obligatory for androgen/AR-dependent malignant growth (Patki, Chari et al. 2013). We also showed, using co-immunoprecipitation, mammalian two-hybrid assays and chromatin immunoprecipitation, that Elk1 is a true AR tethering protein, both in hormone-dependent PC cells and in CRPC cells (Patki, Chari et al. 2013). The activation of AR target genes through Elk1 was mechanistically distinct from the mode of activation of immediate early genes by Elk1 that requires Elk1 phosphorylation. Indeed phosphorylation of Elk1 was not required for the Elk1-AR synergy (Patki, Chari et al. 2013). In addition, we demonstrated that AR can bind and transactivate through tethering proteins, including Elk1, and that this phenomenon is hormone-independent and insensitive to conventional antiandrogens (Zhang, Gonit et al. 2010, Patki, Chari et al. 2013). However, hormone-dependent cells (e.g., LNCaP and VCaP cells) are still ligand-dependent for this mode of gene activation by AR as androgen is needed for AR to be localized to the nucleus.

We have also observed that Elk3, which is the closest functional substitute for Elk1, does not interact with AR (Patki, Chari et al. 2013); rather Elk3 levels were significantly higher in normal prostate epithelial cells compared to standard models of early stage PC and CRPC. Clinical prostate tumors also showed elevated Elk1 concomitant with loss of Elk3 (Singh, Febbo et al. 2002), although the tissue samples contained undefined amounts of stromal cells.
We also have shown using mammalian 2-hybrid assays and promoter analyses that the N-terminal A/B domain of AR, which lacks the ligand binding domain, alone can synergize with Elk1 (Patki, Chari et al. 2013), in contrast to other known AR tethering proteins (Zhang, Wilkinson et al. 2008, Zhang, Gonit et al. 2010). This observation is highly significant and parallels recent findings which show that major splice variants of AR that have C-terminal deletions and lack the ligand binding domain strongly support the growth and progression of prostate tumors (Guo, Yang et al. 2009, Hu, Dunn et al. 2009, Haile and Sadar 2011) and are refractory to both conventional and experimental androgen antagonists. The ability of the AR A/B domain to synergize with AR strongly suggests that the AR splice variants may also synergize with Elk1 as a critical growth supporting mechanism as they have an intact N-terminal domain.

Based on previous work published by our lab, we hypothesize that disrupting the interaction of AR with a tethering protein such as Elk1 that is only critical for growth signaling in PC is a potential means of functionally targeting interventions, selectively to prostate tumors, in both early stage PC and CRPC including those resistant to conventional AR targeting drugs. Our interest in Elk1 is also heightened due to the fact that the protein is genetically redundant; therefore targeting the interaction of AR and Elk1 should not pose the problem of side effects that accompanies current AR-targeted therapies, as they would also allow the patient to retain normal hormone status.

2.4.4 Transcriptional Regulation by Elk-1

Elk-1 exerts its transcriptional regulation by forming a complex with a
heterologous transcription factor SRF in a subset of target genes. Studies using the FOS

gene promoter as a model system have shown that SRF is an essential partner of Elk-1

that confers *in vivo* selectivity of the target genes (Aarnisalo, Palvimo et al. 1998,

Fronsdal, Engedal et al. 1998). Alternative ETS transcription factors are also capable of

binding and regulating the promoter of some Elk-1 targets. Redundant promoter

occupancy by different ETS family members has been demonstrated in HeLa and Jurkat

T cells (Hollenhorst, Shah et al. 2007, Norris, Chang et al. 2009). It has been shown that

phosphorylation of Elk-1 facilitates SRF independent DNA binding of Elk-1 (Sharrocks

1995). Using microarray analysis and knockdown approaches direct regulation of genes

by Elk-1 independent of SRF has been demonstrated (Norris, Chang et al. 2009). A

recent study that employed ChIP-chip analysis also showed that the co-occupancy of

Elk-1 and SRF accounts for only 22 % of the Elk-1 target genes in HeLa cells

(Vlahopoulos, Zimmer et al. 2005).

Specificity of gene regulation has also been demonstrated among the ternary

complex factor (TCF) subfamily of ETS transcription factor. Unique amino acid residues

have been found to play a critical role to achieve this gene-specificity. Amino acid

residues D38 and D69 of Elk-1 have shown to confer differential binding specificity

between Elk-1 and Sap1 and mutation of these residues abrogate specificity (Shore,

Whitmarsh et al. 1996). In-silico structural/binding studies have shown that TCF family

members exhibit different DNA binding property. This differential binding property is

mediated by non-conserved amino acid residues, which are located distal to the DNA

binding domain and confer different interaction between the protein recognition helix and
Elk-1 is involved in the activation and repression of genes. The transcriptional effect of Elk-1 is determined by the level co-regulators and in cellular context (Yang, Vickers et al. 2001). The DNA binding and transcriptional activity of Elk-1 is regulated by phosphorylation (Fronsdal, Engedal et al. 1998, Yang, Shore et al. 1999). In the absence of activation signal intermolecular interaction between the ETS DNA binding and transactivation domain inhibits DNA binding. Phosphorylation of the transactivation domain abrogates intermolecular interaction and relives the autoinhibition. In addition, interaction between Id basic helix-loop-helix proteins (bHLH) with the ETS DNA binding domain has been shown to sequester Elk-1 and prevents DNA binding (Yates, Atherton et al. 1999).

Elk-1 is a MAP kinase inducible transcription factor that regulates the transient expression of immediate early genes such as c-Fos, Egr1, Egr2, pip92 (Fronsdal, Engedal et al. 1998, Yang, Vickers et al. 2001, Shaw and Saxton 2003). In addition, Elk-1 can also be activated by MAP-kinase (MAPK) independent pathways that involve novel kinases (Chung, Gomes et al. 1998). Studies have shown that Elk-1 constitutively bound the promoter region of its target genes in the absence of activation stimulus. Upon activation by kinase, phosphorylated Elk-1 forms a complex with serum response factor (SRF) at the serum response element (SRE) of c-Fos promoter and recruits co-activators such as CBP, p300 and Sur-2 (Janknecht and Nordheim 1996, Boyer, Martin et al. 1999). In contrast, the phosphorylation of Elk-1 is not essential for the recruitment co-activators.
MAPK independent interaction between Elk-1 and CBP (Janknecht and Nordheim 1996, Nissen, Gelly et al. 2001) and between Elk-1 and p300 (Li, Yang et al. 2003) have also been demonstrated. However, phosphorylation of both Elk-1 and CBP is required for functional cooperation (Janknecht and Nordheim 1996, Nissen, Gelly et al. 2001). The histone acetyl transferase (HAT) activity of co-activator proteins catalyzes chromatin remodeling for transcriptional activation.

The activation of immediate early genes is tightly regulated by cycles of repression-activation-repression. Recent studies have shown rapid induction of immediate early genes followed by down regulation. Hence, Elk-1 mediated gene activation triggers the recruitment of co-repressor complex to down regulate expression. In a prototypic model it has been shown that MAP kinase induced activation of c-Fos stimulates the recruitment of mSin3A-HDAC repressor complex by Elk-1 to turn off the activation signal to the basal/repressed state. The histone deacetylase (HDAC) activity of the repressor complex facilitates chromatin remodeling and transcriptional repression (Yang, Vickers et al. 2001).

In the absence of activation, Elk-1 has been shown to repress target genes through the recruitment of mSin3A-HDAC repressor complex (Yang, Vickers et al. 2001)(233). This Elk-1 mediated repression is often times dependent on modification of Elk-1 by conjugation of small ubiquitin like modifiers (SUMO). SUMOylation of Elk1 confers additional repression of the target gene promoter (Yang, Jaffray et al. 2003). Modification of the phosphorylation status of Elk-1 by protein phosphatase has also been
shown to inhibit the transcriptional output of MAP kinase signaling (Sugimoto, Stewart et al. 1998, Tian and Karin 1999). The transcriptional output of Elk-1 is profoundly dependent on the balance between phosphorylation and dephosphorylation. Calcenurin (PP2B), an Elk-1 phosphatase that is activated by increased levels of nuclear calcium, down regulates Elk-1 through dephosphorylation (Tian and Karin 1999). On the other hand, factors that enhance the phosphorylation of MAPK activate Elk-1. Of note, Elk-1 plays a central role in the transcriptional activation and repression of target genes in response to a number of extracellular/intracellular stimuli (Fronsdal, Engedal et al. 1998).

2.4.5 Role of Elk-1 isoforms in Transcriptional Regulation

The role of Elk-1 isoforms (sElk-1 and ΔElk-1) in the transcriptional regulation has not been adequately investigated. The short form of Elk1 (sElk-1), which has the first 54 amino acids deleted, is expressed in the neuronal tissues. Because of this deletion, sElk-1 does not form complex with SRF and has low DNA binding properties. The sElk-1 isoform has been shown to antagonize the transcriptional effect of Elk-1 (Vanhoutte, Nissen et al. 2001).

Alternative splice variant of Elk-1, ΔElk-1, has been isolated from cDNA clones and the expression pattern of this isoform has not been studied. ΔElk-1 lacks the SRE interaction motif, the repressor sequence of the A domain and a part of the DNA binding domain. It shows different DNA binding properties and fails to form a complex with SRF. It has also been shown to compete with full length Elk-1 and blocks SRF dependent gene activation (Rao and Reddy 1993).
Chapter 2

Development of RARα Targeted Agents to Improve the Outcome of Hormonal Adjuvant Therapy in Breast Cancer

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Adjuvant therapies that target the estrogen-signaling axis, including tamoxifen and aromatase inhibitors are a mainstay in the treatment of estrogen receptor (ER)-positive breast cancer. The treatments are only partially successful presumably due to progressive genetic or epigenetic changes within cells in the residual tumor that confer drug resistance. For such changes to occur and to be propagated, the residual tumor cells must divide at a basal rate during the adjuvant treatment. We have previously demonstrated that the retinoic acid receptor α1 (RARα1) apoprotein supports a basal level of proliferation in ER-positive breast cancer cells that are either hormone depleted or treated with tamoxifen through ligand-independent mechanisms. As RARα1 is genetically redundant, we hypothesize that including targeted downregulation of RARα1 will improve the outcome of hormonal adjuvant therapy in breast cancer. Tissue array analysis indicated prevalence of RARα1 in clinical breast tumors covering a wide pathologic spectrum. We have synthesized a high affinity RARα-specific antagonist, AGN194301, as a carrier molecule to selectively targeting RARα. AGN194301 by itself did not affect the ability of RARα1 to support basal cell proliferation and did not alter the level of RARα1 protein. Preliminary structure-activity-relationship (SAR) studies showed the functional groups of the compound that could be modified without abrogating its ability to bind to RARα. Further studies and strategies for attaining downregulation of RARα1 using modified versions of this compound will be discussed.
INTRODUCTION

Most breast tumors in both premenopausal and postmenopausal women express estrogen receptor (ER) type alpha. Tamoxifen is a Selective Estrogen Receptor Modulator (SERM) that is widely used for adjuvant therapy in the treatment of ER-positive breast cancer. In case of hormone-sensitive tumors, tamoxifen behaves like a partial antagonist, where it binds to ER with an affinity much higher than that of estrogen and inhibits transcriptional activation by ER (Ali and Coombes 2002). However, 3-5 years of tamoxifen treatment only results in approximately 50% reduction in the incidence of invasive breast cancer in women at high risk, whereas about a third of ER-positive breast tumors are intrinsically resistant to tamoxifen (Dunn and Ford 2001, Cuzick, Powles et al. 2003).

Third generation aromatase inhibitors (AI) are a valuable alternative to tamoxifen adjuvant therapy in postmenopausal women with ER-positive breast cancer (Howell, Cuzick et al. 2005, Mauri, Pavlidis et al. 2006, Group, Mouridsen et al. 2009). Aromatase is essential for the conversion of steroid precursors in peripheral tissues to estrogen. This is the major source of estrogen production in postmenopausal women. Upon treatment with AI, aromatase activity is decreased by proximately 96% and circulating estrogen is virtually absent, inhibiting hormone-dependent tumor growth (Brodie 2002). However, long-term treatment with AI also leads to development of resistance, resulting in disease recurrence and aggressive tumor growth (Howell and Dowsett 2004, Masri, Phung et al. 2008). Clinical trials are currently being carried out to assess if the development of
resistance is delayed by administering AI for two to three years following two to three years of tamoxifen treatment (Winer, Hudis et al. 2005, Dowsett, Cuzick et al. 2010). Selective Estrogen Receptor Downregulators (SERDs, for example, Faslodex) have also been found to be effective inhibitors of ER-positive breast tumor growth. However, their utility is limited as they are used as second or third line therapeutics in postmenopausal women with metastatic cancer due to their broader impact on physiological ER signaling pathways in normal tissues (Osborne, Wakeling et al. 2004). Therefore, it is imperative to continue to identify critical downstream events of ER signaling in breast cancer.

Breast cancer therapy trials have also tested the effect of retinoid compounds either alone or in combination with tamoxifen (Zanardi, Serrano et al. 2006). In in vitro studies and pre-clinical models of breast cancer using MCF-7 cell xenografts, all-trans-retinoic acid (ATRA) alone or in combination with tamoxifen caused induction of cell cycle arrest and apoptosis, leading to tumor regression through activation of multiple signal transduction pathways (Toma, Isnardi et al. 1997, Zhou, Stetler-Stevenson et al. 1997, Yang, Tin et al. 1999). Combination of retinoids and tamoxifen have shown synergistic anti-tumor effects (Danforth 2004, Wang, He et al. 2007); however, ATRA treatment caused increased cytotoxicity in patients with advanced breast cancer during phaseI/II clinical trials (Budd, Adamson et al. 1998). Fenretinide, a synthetic amide of retinoic acid, has been shown to act on both ER-positive and ER-negative breast tumors by inducing apoptosis by both retinoic acid receptor (RAR) -dependent and -independent mechanisms; however, this drug showed a modest che- mopreventive effect only in younger premenopausal women (Decensi, Zanardi et al. 2007).
Hormonal adjuvant therapy of breast cancer is overall tumoristatic with cell death balancing a basal level of cell proliferation (Millar and Lynch 2003). For resistance to develop during long term treatment with either hormone depletion or tamoxifen adjuvant therapy, the latent tumors must maintain a basal level of cell cycling, during which they grow at a very slow rate and it is during this period of slow cell growth, that the cells develop progressive mutations or epigenetic changes eventually leading to development of resistance during treatment (Badia, Oliva et al. 2007). Since this basal level of cell cycling was supported by RARα via its non-classical (ligand-independent) mechanism of action (Salazar, Ratnam et al. 2011), it was the goal of this study to explore targeting RARα by downregulating it at the protein level to enhance the outcome of anti-estrogen therapy. As RARα1 is genetically redundant, specifically targeting it for down-regulation should not result in the cytotoxicity associated with the conventional RAR agonists.

The strategy envisioned in this study for down-regulating RARα is to generate a Protac molecule (Proteolysis Targeting Chimeric molecule) that is specific for RARα. Protac molecules have been successfully used to down regulate other nuclear receptors, including ER and the androgen receptor (AR) (Nawaz, Lonard et al. 1999, Lonard, Nawaz et al. 2000, Cardozo, Michaud et al. 2003, Sakamoto, Kim et al. 2003). Our lead molecule was AGN194301, which was previously synthesized and reported to be a RARα specific antagonist (Teng, Duong et al. 1997). In this study we used a more efficient route to synthesize AGN194301, characterized its binding specificity and identified the positions at which Protac moieties may be attached. We have also
established the prevalence of the target RARα1 in a large number of clinical breast tumors of different classifications.

Estrogen-sensitive breast cancer cell lines (MCF-7, T47 D and ZR-75-1) have proven to be exceptionally reliable predictive models both in vitro and in vivo for clinical drug response and the development of clinical drug resistance in breast cancer (O'Regan, Gajdos et al. 2002, Simstein, Burow et al. 2003, Zhu, Wang et al. 2006, Creighton, Massarweh et al. 2008). We have observed that the expected basal proliferating state of hormone-depleted or tamoxifen treated breast cancer cells may be reproduced in vitro in established cell lines for an indefinite period by avoiding the common practice of intermittently replenishing the culture media, thus avoiding depletion of autocrine growth factors. We, therefore, used in vitro models to investigate the potential impact of hormone-independent actions of ER on the survival or proliferation of hormone-sensitive breast cancer cells and the related mechanisms under conditions that mimic hormonal adjuvant therapy, that is, estrogen-depletion and tamoxifen treatment.
MATERIALS AND METHODS

Chemicals and reagents: Dulbecco’s minimum essential medium (DMEM), glutamine and penicillin/streptomycin/glutamine stock mix were purchased from Life Technologies, Inc. (Carlsbad, CA, USA). Fetal bovine serum (FBS) and charcoal-stripped FBS were from Invitrogen (Carlsbad, CA, USA). Fugene 6 was purchased from Roche Diagnostics (Indianapolis, IN, USA). Affinity purified rabbit and mouse antibodies to human RARα (sc-551) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-47724) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibody was from Vector Laboratories (Burlingame, CA, USA). PI/RNase staining buffer was from BD Pharmigen (San Diego, CA, USA). The protease inhibitor cocktail kit was obtained from Pierce Biotechnology (Thermo Scientific, Rockford, IL, USA). 17β-estradiol (E₂) and all-trans-Retinoic acid (ATRA) were purchased from Sigma Aldrich (Saint Louis, MO USA). ATRA stock solution (5 mmol/L) was made in a mixture of 50% ethanol and 50% DMSO (Fisher Chemical, Fair Lawn, NJ, USA). First strand cDNA from human peripheral blood leukocytes (PBL), thymus and spleen were obtained from Biochain Institute Inc., Hayward, CA, USA).

Synthesis of AGN194301: Modified synthesis route for a known RARα antagonist (AGN194301), 2-fluoro-4-[[[8-bromo-2,2-dimethyl-4-(4-methylphenyl)chroman-6-yl]carbonyl]amino]benzoic acid and a synthesis of its unknown, desfluoro analog, 4-[[8-bromo-2,2-dimethyl-4-(4-methylphenyl)chroman-6-yl]carbonyl]amino]benzoic acid. The
modified route increased yields, lowered cost and incorporated a green alternative step (Jetson, Malik et al. 2013).

**Cell Culture, Transfection and reporter Luciferase assays:** MCF-7 and T47 D (American Type Culture Collection) cells were cultured in DMEM supplemented with FBS (10%), penicillin (100 unit/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM). ZR-75-1 (American Type Culture Collection) cells were cultured in RPMI 1640 supplemented with FBS (10%), penicillin (100 unit/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM). For transfection, MCF7 cells were plated at 20% confluence in low glucose phenol red free medium supplemented with 5% charcoal stripped FBS and glutamine 24 hours to 48 hours prior to transfection transfected with RARE-Tk-Luciferase construct (kind gift from Dr. Lirim Shemshidini), using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). On the day of transfection, the cells were treated with vehicle (DMSO) or ATRA (10 nM) alone or ATRA (10 nM) in combination with AGN194301 (0.1 nM, 1 nM or 10 nM) or AGN194301 derivatives (0.1 nM, 1 nM and 10 nM) for 48 hours, replenishing ATRA or AGN194301 or AGN194301 derivatives after every 24 hours. At the end of 48 hours of treatment, the cells were lysed with Passive Lysis Buffer (Promega Madison, WI) and the luciferase activities were measured using substrates for either Firefly luciferase or Renilla luciferase provided in the Luciferase Assay System (Promega Madison, WI) in a luminometer (Centro LB960; Berthold; Wildbad, Germany). In all cases, uniformity of transfection was confirmed using the pRL-null plasmid expressing Renilla luciferase.
**Cell cycle analysis:** Cells were trypsinized and harvested in phenol red free medium supplemented with charcoal stripped FBS. Cells \((1 \times 10^6)\) were washed and resuspended in 500 µl PBS. The cells were fixed by adding 500 µl 100% ice cold ethanol, drop-wise with agitation and incubated on ice for 20 minutes. The cells were sedimented by brief centrifugation at 200 xg for five minutes and the excess ethanol decanted. After the remaining ethanol was dried off, the cells were resuspended in 500 µl of PI/RNase solution. The cells were incubated in the dark at room temperature for 20 minutes and the cell cycle distribution determined by flow cytometric analysis using a FACSCalibur cell analyzer (BD Biosciences, San Jose, CA, USA). The data were acquired with BD CellQuest Pro software and analyzed using ModFit LT software.

**Western blot:** Cells were harvested by trypsinization, lysed in a high salt- detergent buffer \((400 \text{ nM NaCl}; 10 \text{ nM Tris, pH 8.0}; 1 \text{ mM EDTA}; 1 \text{ mM EGTA}; β-mercaptoethanol; and 0.1% Triton x-100)\) containing a protease inhibitor cocktail kit and incubated on ice for 30 minutes. Cell lysates were heated to 95°C for five minutes. Protein samples \((10 \text{ to } 20 \text{ µg})\) were resolved by electrophoresis on 8% sodium dodecylsulfate-polyacrylamide gels and electrophoretically transferred to PVDF membranes (Millipore Corporation, Bedford, MA, USA). The blots were probed with the appropriate primary antibody and the appropriate horse- radish peroxidase conjugated secondary antibody and the protein bands visualized using enhanced chemiluminescence as described (Hao, d'Alincourt-Salazar et al. 2007). The chemiluminescent signals were quantified using the FluorChem HD2 imaging system (Alpha Innotech/Cell Biosciences, Inc., Santa Clara, CA, USA) and normalized to GAPDH.
Tissue Array and Immunohistochemistry: Breast intraductal cancer tissue microarray (US Biomax Inc., Rockville, MD, Catalog No. BR8011), consisting of 50 cases of intraductal carcinoma, 4 lobular carcinoma in situ and 26 adjacent normal tissue, single core per case, was performed. Immunohistochemical staining was performed using RARα1 and RARα2 antibodies ([http://www.biomax.us/pdfs/ImmunohistochemistryStaining.pdf](http://www.biomax.us/pdfs/ImmunohistochemistryStaining.pdf)). RARα1 and RARα2 staining scores were obtained ranging from 1-4, 1 being the lowest staining and 4 being the highest.
RESULTS

*Irrespective of ER/PR/Her2 status clinical breast tumors are frequently positive for RARα1 and negative for RARα2:*

Tumor samples from 80 different patients were analyzed for RARα1 and RARα2. From each patient, a section of the tumor sample and matched normal breast tissue were collected and immuno-stained for RARα1 and RARα2. In almost all clinical breast tumors observed, irrespective of the stage/grade of the disease or the status of ER/PR/Her2, they were found to be frequently positive for RARα1 and negative for RARα2 (Table 2-1 and Fig 2-1).

*AGN194301 antagonizes the classical mechanism of action of RARα:*

The gene sets that were upregulated or downregulated via the apo-ER/apo-RAR axis were found to be either involved in cell proliferation/mitosis or apoptosis/cell cycle inhibition respectively (Salazar, Ratnam et al. 2011). These genes although they had RARα binding sites, were insensitive to ATRA. Thus RARα maintained this basal level of cell cycling via its non-classical (ligand-independent) mechanism of action. MCF7 breast cancer cells were hormone-depleted for 48 h, followed by transfection with RARE-TK-Luc construct. On the day of transfection, the cells were treated with either vehicle (DMSO) or 10nM ATRA alone, or 10nM ATRA in combination with AGN194301 (10nM, 1nM or 0.1nM). 48h post-treatment, the cells were harvested and luciferase assay was performed. AGN194301 dramatically decreased RARα mediated promoter activity at concentrations of 1nM and 0.1nM (Fig 2-2 B). These results indicate
that AGN194301 antagonizes the classical (ligand-dependent) mechanism of action of RARα.

**AGN194301 is an RARα specific antagonist:**

It was also of interest to assess the binding specificity of AGN194301 with respect to RARα, RARβ and RARγ. MCF7 breast cancer cells were hormone-depleted for 48 h, followed by co-transfection with RARE-TK-Luc construct and RARα1, RARα2, RARβ or RARγ expression vector (pSG5- RARα1, pSG5-RARα2, pSG5-RARβ and pSG5-RARγ). On the day of transfection, the cells were treated with either vehicle (DMSO) or 10nM ATRA alone, or 10nM ATRA in combination with 1 nM AGN194301. 48h post-treatment, the cells were harvested and luciferase assay was performed. AGN194301 specifically decreased the RARα-dependent (RARα1 and RARα2) promoter activity (Fig. 2-2 C). These results show that AGN194301 is an RARα specific antagonist and does not show selectivity for RARβ or RARγ.

**AGN194301 does not alter RARα at the protein level:**

Since AGN194301 was found to be a high affinity antagonist of RARα at concentrations ≤100 nM, it was of interest to see if it downregulates RARα at the protein level. MCF7, ZR-75 and T47D breast cancer cell lines were hormone depleted for 48 h; this was followed by treatment with either vehicle (DMSO) alone or 10 nM ATRA alone or 10 nM AGN194301 alone or 10 nM ATRA in combination with 10 nM AGN194301 for 48 h, after which the cells were harvested and immunoblotted for RARα. In all the
three cell lines, AGN194301 failed to exhibit any effect on RARα i.e. the RARα protein level remained unchanged in all cases (Fig 2-2 D).

**AGN194301 exerts no effect on cell cycle in MCF7 cells:**

The basal level of cell cycling that is maintained during tamoxifen or aromatase inhibitor treatment is via the apo-ER/apo-RARα axis (Salazar, Ratnam et al. 2011). Thus it was of interest to assess if AGN194301 exhibited any effect on cell cycle of breast cancer cells. MCF7 cells were hormone depleted for 48 h, followed by treatment with either vehicle (DMSO) or 1nM estrogen (E₂) or 10 nM AGN194301. 1nM E₂ was used as a control to stimulate cell growth. AGN194301 did not alter the percentage of cells in the S-phase (Fig 2-2 E), thus exerting no effect on cell cycle of MCF7 breast cancer cells.

**SAR studies with AGN194301 revealed that fluorine (F) group could be modified with abrogating its specificity and binding affinity for RARα:**

Previous studies from our lab have shown that RARα is involved in maintaining the basal level of cell cycling via its non-classical mechanism of action (Salazar, Ratnam et al. 2011), and since AGN194301 antagonizes the classical (ligand-dependent) mechanism of action of RARα, it is necessary to modify AGN194301 to inhibit the non-classical mechanism of action of RARα. Structure-activity-relationship (SAR) studies we performed by testing derivatives/variations of AGN194301 (Fig 2-3 A and C). MCF7 breast cancer cells were hormone-depleted for 48h, followed by transfection with RARE-TK-Luc construct. On the day of transfection, the cells were treated with either vehicle
(DMSO) or 10nM ATRA alone, or 10nM ATRA in combination with AGN194301 (10nM, 1nM or 0.1nM), M7 (10nM, 1nM or 0.1nM), CD-880-01 (10nM, 1nM or 0.1nM), M8 (10nM, 1nM or 0.1nM) or M2F (10nM, 1nM or 0.1nM) (Fig. 2-3 B and D). 48 h post-treatment, the cells were harvested and luciferase assay was performed. These studies revealed that the fluorine (F) group attached to the ortho-carbon atom of the carboxylic group of AGN194301, is not necessary for its binding affinity or specificity to RARα and can be modified in order to inhibit the non-classical mechanism of action of RARα by down-regulating it at the protein level.

Docking simulation studies predict that the fluorine (F) atom, attached to the ortho-carbon atom of the carboxylic group of AGN194301, could be substituted:

AutoDock 4.2 (Morris, Huey et al. 2009) was used to perform in-silico docking studies for AGN194301 using the protein databank (PDB) information for RARα complexed with its antagonist BMS195614 (PDB: 1DKF). AGN194301 was shown to bind within the same binding pocket of RARα as BMS195614 (Fig 2-4 A and B). It was also seen that the ortho-carbon atom of the carboxylic group of AGN194301 was exposed to the aqueous environment (Fig 2-4 C, D, E and F).
RESULTS

Table 2.1: Irrespective of ER/PR/Her2 status clinical breast tumors are frequently positive for RARα1 and negative for RARα2

<table>
<thead>
<tr>
<th>Age</th>
<th>Pathology diagnosis</th>
<th>Grade</th>
<th>ER</th>
<th>PR</th>
<th>Ki67</th>
<th>HER2</th>
<th>Type</th>
<th>RARα1</th>
<th>RARα2</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>Normal Breast tissue</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
<td>70%</td>
<td>Normal</td>
<td>3</td>
<td>-ve</td>
</tr>
<tr>
<td>46</td>
<td>Normal Breast tissue</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
<td>30%</td>
<td>Normal</td>
<td>4</td>
<td>-ve</td>
</tr>
<tr>
<td>43</td>
<td>DCIS</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>50%</td>
<td>Malignant</td>
<td>3</td>
<td>-ve</td>
</tr>
<tr>
<td>67</td>
<td>DCIS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>Malignant</td>
<td>2</td>
<td>-ve</td>
</tr>
<tr>
<td>49</td>
<td>IDC</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>Malignant</td>
<td>3</td>
<td>-ve</td>
</tr>
<tr>
<td>58</td>
<td>IDC</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>Malignant</td>
<td>2</td>
<td>-ve</td>
</tr>
</tbody>
</table>

Figure 2-1

A.  
RARα1  RARα2  IgG

C.  
RARα1  RARα2  IgG

B.  
RARα1  RARα2  IgG

D.  
RARα1  RARα2  IgG
Panels A, B, C, D: Tissue array was performed in 80 cores from 80 patients to analyze the expression of RARα1 and RARα2 in normal, benign and malignant breast tissue. The ER, PR and Her2 status was assessed for A. DCIS (ER+/PR-/Her2+), B. IDC (ER+/PR+/Her2+), C. IDC (ER-/PR+/Her2+), D. IDC (ER-/PR-/Her2-) and correlated with RARα1 and RARα2 status; IgG was used as a negative control. DCIS: Ductal carcinoma in situ, IDC: Invasive ductal carcinoma.
Figure 2-2

A. AGN194301

B. Relative Light units (RLU) x 10^2

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative Light Units (RLU) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>0</td>
</tr>
<tr>
<td>10 nM ATRA</td>
<td>3000</td>
</tr>
<tr>
<td>10 nM ATRA + Veh</td>
<td>3500</td>
</tr>
<tr>
<td>10 nM ATRA + 0.1 nM AGN194301</td>
<td>2500</td>
</tr>
<tr>
<td>10 nM ATRA + 1 nM AGN194301</td>
<td>3000</td>
</tr>
<tr>
<td>10 nM ATRA + 10 nM AGN194301</td>
<td>&lt;500</td>
</tr>
</tbody>
</table>
Figure 2-2

Panel A: Chemical structure of AGN194301, an RARα specific antagonist. Panel B: MCF7 cells were grown in media containing charcoal stripped FBS for 48 hours; they were then transfected with RARE-tk-luciferase plasmid, followed by treatment with vehicle (DMSO) or ATRA (10 nM) alone or 0.1 nM or 1 nM of AGN194301 in combination with 10 nM of ATRA for 48 hours. Following treatment, cells were harvested and luciferase assay was performed and relative light units (RLU) were measured using a luminometer. Panel C: MCF7 cells were grown in media containing charcoal stripped FBS for 48 hours; they were then co-transfected with RARE-tk-luciferase plasmid and pSG5-RARα1 or pSG5-RARα2 or pSG5-RARβ or pSG5-RARγ, followed by treatment with 1 nM of AGN194301 in combination with 10 nM of ATRA for 48 hours. 48 h post-treatment, the cells were harvested, luciferase assay was performed and relative light units (RLU) were measured using a luminometer. Panel D:
MCF7, T47D and ZR-75 breast cancer cells were grown in media containing charcoal stripped FBS for 48 hours, followed by treatment with AGN194301 for four days, after which cells were harvested and western blot for RARα was performed. *Panel E*: MCF7 breast cancer cells were grown in media containing charcoal stripped FBS for 48 hours, followed by treatment with AGN194301 for four days, after which cells were harvested and cell cycle analysis was performed.
Figure 2-3

A.

AGN194301

M7

CD-880-01

B.

[Graph showing relative light units (RLU) x 10^4 for different treatments]
C.

AGN194301

M8

M2F

D.

![Graph showing Relative Light Units (RLU) vs. treatments](image)

- Veh
- 10 nM ATRA
- 10 nM ATRA + AGN194301 0.1 nM
- 10 nM ATRA + AGN194301 10 nM
- M8 0.1 nM
- M8 1 nM
- 10 nM ATRA + M8 0.1 nM
- 10 nM ATRA + M8 1 nM
- M2F 0.1 nM
- M2F 1 nM
- 10 nM ATRA + M2F 0.1 nM
- 10 nM ATRA + M2F 1 nM
- 10 nM ATRA + M2F 10 nM
Figure 2-3

Panels A and C: Chemical structure of AGN194301 and its derivatives M7 and CD-880-01. Panel B: MCF7 cells were grown in media containing charcoal stripped FBS for 48 hours; they were then transfected with RARE-tk-luciferase plasmid, followed by treatment with 0.1 nM, 1 nM and 10 nM of AGN194301 or M7 or CD-880-01 in combination with 10 nM of ATRA for four days. 48 h post-treatment, the cells were harvested, luciferase assay was performed and relative light units (RLU) were measured using a luminometer. Panel D: MCF7 cells were grown in media containing charcoal stripped FBS for 48 hours; they were then transfected with RARE-tk-luciferase plasmid, followed by treatment with 0.1 nM, 1 nM and 10 nM of AGN194301 or M8 or M2F in combination with 10 nM of ATRA for four days. Following four days of treatment, cells were harvested and luciferase assay was performed and RLU was measured using a luminometer.
Figure 2-4

A.  

B.  

C.  

D.  

E.  

F.
**Figure 2-4**

*Panel A:* RARα antagonist AGN194301 (magenta stick molecule) modeled into the ligand-binding pocket of RARα (PDB code: 1DKF). The green stick molecule is RARα antagonist BMS195614, which binds in the ligand-binding domain of RARα. *Panel B:* An enlarged view of the boxed region in A. *Panel C:* Predicted conformation of AGN194301 complexed with RARα surface model. *Panel D:* An enlarged view of the boxed region in C showing the ortho carbon atom of the carboxylic group of AGN194301 exposed to the aqueous environment. *Panel E:* Predicted conformation of AGN194301 complexed with RARα mesh model. *Panel F:* An enlarged view of the boxed region in E.
DISCUSSION

ER is known to regulate genes in a ligand-independent manner (Cvoro, Tzagarakis-Foster et al. 2006, Cardamone, Bardella et al. 2009). Hormone-independent actions of ER play an important role in supporting the growth of hormone-refractory breast tumors (Musgrove and Sutherland 2009). Previous findings from our lab indicate that under conditions hormone depletion or tamoxifen treatment, apo-ER maintains a basal level of cell cycling by supporting the expression of apo-RARα. The gene sets that were commonly upregulated or downregulated by the apo-ER/apo-RARα axis were found to be involved in mitosis/cell proliferation/cell cycle progression or inhibition of cell cycle/apoptosis/immune response respectively. Furthermore, these gene sets were found to have RARα binding sites, however, they were ATRA-insensitive (Salazar, Ratnam et al. 2011). This indicates that RARα maintains a basal level of cell growth, during hormone depletion or tamoxifen treatment, via its non-classical (ligand-independent) mechanism of action. Based on these results, we hypothesized that targeted downregulation of RARα, in combination with current available hormonal adjuvants (tamoxifen or aromatase inhibitors), will improve the outcome of hormonal adjuvant therapy in breast cancer. The findings of this study support the development of a potential RARα-specific antagonist, which would downregulate RARα at the protein level and thereby decrease cell proliferation.

Our lab has previously shown, using competitive PCR, that several breast cancer cell lines and clinical breast tumor samples are positive for RARα1 and negative RARα2. It has also been shown that in breast cancer cell lines, RARβ is epigenetically silenced
(Sirchia, Ren et al. 2002, Fackler, McVeigh et al. 2003) and RARγ is expressed at a very low level (Widschwendter, Daxenbichler et al. 1996). However, normal breast tissue expresses significant levels of RARα, β and γ. RARα being genetically redundant, targeting it would specifically affect breast cancer cells leaving the normal cells unaffected, because of its function overlap with RARβ or RARγ. The present studies have expanded the panel of breast tumors examined for the expression of RARα1 and RARα2 using a large tissue array. The results demonstrate that breast tumors do not express RARα2 but do express RARα1 across the different pathological sub-types, including Her2-amplified and triple-negative tumors. This confirms the feasibility of targeting ER-positive tumors RARα down-regulators and further suggests the possibility that they may also be effective in inhibiting the growth of a variety of ER-negative tumors.

AGN194301, a high affinity antagonist of RARα, originally synthesized by Teng, Duong et al. 1997, was synthesized by the CD3 (Center for Drug Design and Development) group at University of Toledo using shorter synthesis steps and increasing the yield of the end product (Jetson, Malik et al. 2013). We tested this compound for its ability to affect RARE-driven promoter activity and also its ability to selectively affect RARα at the level of transcriptional activity and protein stability. Our results demonstrated that (i) AGN194301 inhibits the classical mechanism of action of RARα. (ii) AGN194301 is highly selective for to RARα compared with RARβ and RARγ. (iii) AGN194301 does not affect stability of the RARα protein and (iv) AGN194301 does not affect cell cycle progression in MCF7 breast cancer cells depleted of estrogen. Since
RARα maintains a basal level of cell cycling in breast cancer cells via its non-classical (ligand-independent) mechanism of action, AGN194301 will need to be modified in order to selectively down regulate the RARα protein or to prevent RARα from associating with its target growth genes.

A limited amount of structure-activity-relationship (SAR) studies were undertaken to identify positions in the AGN194301 molecule that would be amenable to further modification without significant loss of binding to and selectivity for RARα. The studies revealed that the fluorine (F) group attached to the ortho-carbon atom of the carboxylic group of AGN194301 is not necessary for binding to RARα. This position could therefore potentially be modified in order to inhibit the non-classical mechanism of action of RARα. Our approach would be to attach a Protac (Proteolysis targeting chimeric) molecule to replace the fluorine atom. Protac molecules have been developed for the estrogen receptor (ER) as well as the androgen receptor (AR). In the case of ER, the protac molecule was linked to its ligand estrogen (E2). On binding of E2 to ER, the protac molecule attracts the ubiquitin ligase complex, thereby targeting ER to the proteasome for degradation. We took a similar approach to design a modified version of AGN194301.

In-silico docking studies revealed that the fluorine (F) attached to the ortho-carbon atom of the carboxylic group of AGN194301 attached to the aromatic ring of the acidic domain, is accessible within the ligand-bound structure of RARα. The modeling predicts that substitution at this position with the protac, will reveal the protac molecule for recognition by the ubiquitin ligase complex, allowing it to direct RARα to the
proteasome, thereby leading to proteolytic degradation of RARα.

In conclusion, we have observed that AGN194301 is an RARα specific antagonist that antagonizes the classical (ligand-dependent) mechanism of action of RARα. Further, since RARα is genetically redundant, selectively down-regulating RARα might significantly enhance hormone ablation therapy in breast cancer since this would further decrease the number cycling cells in the residual tumor. Further modification of AGN194301 is necessary and feasible for attaining selective downregulation of RARα at the protein level.
Chapter 3

Mapping Peptide motifs Required for the Interaction Between Elk1 and AR in Supporting the Growth of Prostate Cancer Cells

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ABSTRACT

We have reported that a critical component of growth signaling by the androgen receptor in cell line models of both hormone-dependent and castration recurrent prostate cancer is mediated by the tethering of AR to a set of its growth supporting target genes by Elk1. Here, we discuss detailed mapping of the peptide sequences within AR and Elk1 that are required for this interaction. Using mammalian two-hybrid assays and promoter analysis, we found that the N-terminal A/B domain of AR is alone sufficient for its interaction with Elk1 and for Elk1-dependent transactivation by AR. We have further used deletion analysis to demonstrate that aa 156-329 within the A/B domain selectively inhibits Elk1-dependent gene activation by androgen as well as androgen-dependent growth of LNCaP cells. Synthetic peptides corresponding to sequence aa 156-206 and aa 181-231 inhibited the binding of the AR A/B domain to Elk1 and also inhibited the androgen-dependent growth of LNCaP cells. Deletional mapping of Elk1 and the use of mammalian two-hybrid assays also identified the sequence aa 287-317 and aa 387-397 as essential for the binding of AR to Elk1. Finally, a phosphorylation mutant of Elk1 retained the ability of Elk1 to associate with AR. The results suggest that major splice variants of AR that lack the C-terminal ligand binding domain and that are known to support hormone-independent growth of prostate cancer cells may utilize Elk1 for growth signaling. They also confirm that phosphorylation of Elk1 is not involved in the Elk1-AR synergy. Finally, the results identify peptides that may be used to selectively disrupt the synergy between Elk1 and AR without interfering with other aspects of AR signaling.
INTRODUCTION

A distinct feature of prostate cancer development and progression is its dependence on stimulation by androgen, which acts by binding to and activating transcriptional signaling by the androgen receptor (AR). Both androgen responsive and advanced prostate tumors are dependent on the androgen receptor (AR) for their growth (Ruizeveld de Winter, Janssen et al. 1994, Linja, Savinainen et al. 2001, Zegarra-Moro, Schmidt et al. 2002, Li, Zhao et al. 2007). This growth could be driven by circulating androgen, post ablation synthesis of intratumoral androgen or by AR acting completely independent of androgen (Chen, Welsbie et al. 2004, Miyamoto, Messing et al. 2004, Guo, Yang et al. 2009, Hu, Dunn et al. 2009, Cai, Chen et al. 2011, Gonit, Zhang et al. 2011). In all cases, the current clinical paradigm for adjuvant therapy is total and ubiquitous attenuation of AR signaling by androgen ablation and the use of AR ligands that antagonize, sequester or deplete AR (Larsson, N et al. 2011). Recurrent prostate cancer (PC) is generally treated by androgen deprivation therapy (ADT) either by surgical castration or by chemical castration using LHRH agonists (Loblaw, Virgo et al. 2007, Dunn and Kazer 2011). Within a few years of such treatments the initial responders generally develop hormone refractory disease, more accurately referred to as castration recurrent prostate cancer (CRPC). In addition, adverse effects may be associated with ADT that are both acute (fatigue, hot flashes, flares) and long-term (hyperlipidemia, insulin resistance, cardiovascular disease, anemia, osteoporosis, sexual dysfunction and cognitive defects) effects (Holzbeierlein, McLaughlin et al. 2004, Dunn and Kazer 2011,
Myklak and Wilson 2011). The recurrent and metastatic disease is then treated by chemotherapy, which is typically non-curative and has its own adverse side effects.

Newer treatments that include Enzalutamide (MDV3100) (Tran, Ouk et al. 2009) or MEK inhibitors (Gioeli, Wunderlich et al. 2011) offer valuable treatment options, but only show moderate improvements (Nilsson, Franzen et al. 2007, de Bono, Oudard et al. 2010, Kantoff, Higano et al. 2010, de Bono, Logothetis et al. 2011, Fizazi, Carducci et al. 2011). The above discussed treatment options have limitations: (i) ineffectiveness against advanced tumors in which functional AR has been restored (ii) the need to deprive the patient of androgen or AR function in all tissues and the consequent multiple long-term side effects as mentioned above.

Mechanisms by which prostate cancer cells reprogram androgen/AR signaling to primarily support tumor growth are poorly understood. In the classical mechanism of action of AR, the ligand (androgen) binds to AR in the cytosol, homodimerizes and enters the nucleus where it binds to well characterized androgen response elements (AREs) associated with target genes (Beato, Herrlich et al. 1995, Pratt and Toft 1997, Glass and Rosenfeld 2000, McKenna and O'Malley 2002, Shaffer, Jivan et al. 2004). Androgen binding also enables phosphorylation of AR that is required for its stabilization and activity (Gioeli, Wunderlich et al., Chen, Xu et al. 2006). When the bound ligand is an agonist, AR then recruits co-activators; in contrast, when bound to antagonists, corepressors are preferentially recruited (Glass and Rosenfeld 2000, McKenna and O'Malley 2002). AR contains sites of coregulator binding that are either ligand-dependent
or -independent. However, in prostate cancer cells that are adapted to grow in the absence of hormone, the AR apoprotein is localized in an active form in the nucleus. In such cells, the AR apoprotein activates a set of genes that is distinct from the genes that require androgen for activation in the same cells (Wang, Li et al. 2009, Gonit, Zhang et al. 2011). This set of genes strikingly overlaps the signature gene overexpression profile of clinical castration recurrent prostate tumors and is enriched for gene clusters primarily supporting mitotic cell division (Wang, Li et al. 2009, Gonit, Zhang et al. 2011). Further, we have demonstrated that in those cells the AR apoprotein can support growth through gene activation that occurs without the direct binding of AR to AREs, and likely through tethered associations of the receptor with its target genes (Gonit, Zhang et al. 2011).

Our goal has been to identify and selectively disrupt a functional arm of AR that is: (i) necessary for tumor growth but not for the physiological functioning of AR in differentiated normal tissues and (ii) preserved as a critical mechanism for supporting growth throughout progression to CRPC. This results of this study demonstrate that targeting this downstream mechanism of growth signaling by AR would not only confer tumor selectivity but also would evade resistance mechanisms to conventional treatments including ADT, androgen synthesis inhibitors and antiandrogens. This strategy will also obviate the need for androgen deprivation.

Recent studies from our lab (Zhang, Gonit et al. 2010) have demonstrated that potential AR tethering proteins, during development, differentiation and malignant transformation of the prostate, could redirect AR signaling according to the physiological
context. This may be exemplified by the ability of several well established AR tethering proteins to profoundly influence the pattern of gene activation by androgen/AR. These proteins include HoxB13 (involved in development) (Norris, Chang et al. 2009) and C/EBPalpha (involved in terminal differentiation) (Zhang, Wilkinson et al. 2008, Sivakumaran, Zhang et al. 2010, Zhang, Gonit et al. 2010). Our lab has demonstrated that in PC cells growing robustly in the complete absence of hormone, direct binding of AR to DNA elements is not required for it to activate the required growth genes (Gonit, Zhang et al. 2011). Our studies led to the discovery that Elk1 which is a genetically redundant protein and is obligatory for androgen/AR-dependent malignant growth (Patki, Chari et al. 2013). We also showed, using co-immunoprecipitation, mammalian two-hybrid assays and chromatin immunoprecipitation, that Elk1 is a true AR tethering protein, both in hormone-dependent PC cells and in CRPC cells (Patki, Chari et al. 2013). The activation of AR target genes through Elk1 was mechanistically distinct from the mode of activation of immediate early genes by Elk1 that requires Elk1 phosphorylation. Indeed phosphorylation of Elk1 was not required for the Elk1-AR synergy (Patki, Chari et al. 2013). In addition, we demonstrated that AR can bind and transactivate through tethering proteins, including Elk1, and that this phenomenon is hormone-independent and insensitive to conventional antiandrogens (Zhang, Gonit et al. 2010, Patki, Chari et al. 2013). However, hormone-dependent cells (e.g., LNCaP and VCaP cells) are still ligand-dependent for this mode of gene activation by AR as androgen is needed for AR to be localized to the nucleus.
We have also observed that Elk3, which is the closest functional substitute for Elk1, does not interact with AR (Patki, Chari et al. 2013); rather Elk3 levels were significantly higher in normal prostate epithelial cells compared to standard models of early stage PC and CRPC. Clinical prostate tumors also showed elevated Elk1 concomitant with loss of Elk3 (Singh, Febbo et al. 2002), although the tissue samples contained undefined amounts of stromal cells.

Finally, we have shown using mammalian 2-hybrid assays and promoter analyses that the N-terminal A/B domain of AR, which lacks the ligand binding domain, alone can synergize with Elk1 (Patki, Chari et al. 2013), in contrast to other known AR tethering proteins (Zhang, Wilkinson et al. 2008, Zhang, Gonit et al. 2010). This observation is highly significant and parallels recent findings which show that major splice variants of AR that have C-terminal deletions and lack the ligand binding domain strongly support the growth and progression of prostate tumors (Guo, Yang et al. 2009, Hu, Dunn et al. 2009, Haile and Sadar 2011) and are refractory to both conventional and experimental androgen antagonists. The ability of the AR A/B domain to synergize with AR strongly suggests that the AR splice variants may also synergize with Elk1 as a critical growth supporting mechanism as they have an intact N-terminal domain.

Based on previous work published by our lab, we hypothesize that disrupting the interaction of AR with a tethering protein such as Elk1 that is only critical for growth signaling in PC is a potential means of functionally targeting interventions, selectively to prostate tumors, in both early stage PC and CRPC including those resistant to
conventional AR targeting drugs. This chapter discusses our published and more recent studies that map the interacting peptide motifs between Elk1 and AR. The corresponding peptides are also shown to disrupt Elk1-dependent gene activation by AR as well as androgen-dependent growth of prostate cancer cells.
MATERIALS AND METHODS

Cell culture and reagents: Early passage LNCaP and HeLa cells were purchased from the American Type Culture Collection (Rockville, MD). 293FT cells were from Invitrogen (Carlsbad, CA). Early passage LNCaP cells were routinely grown at 37°C and in 5% CO\textsubscript{2} in RPMI-1640 supplemented with 10% FBS (Life Technologies, Carlsbad, CA), 1x penicillin/streptomycin/L-glutamine stock mix (Life Technologies, Carlsbad, CA) and 1x sodium pyruvate (Life Technologies, Carlsbad, CA). HeLa cells were grown at 37°C and in 5% CO\textsubscript{2} in DMEM supplemented with 10% FBS, 1x penicillin/streptomycin/L-glutamine stock mix. 293FT cells were grown in DMEM supplemented with 10% FBS, 1x non-essential amino acid (Life Technologies, Carlsbad, CA), 500 µg/ml geneticin and 1x penicillin/streptomycin/L-glutamine stock mix. R1881 was kindly provided by Dr Lirim Shemshedini (University of Toledo). For hormone depletion, the EP-LNCaP, LP50 cells were grown at 37°C and in 5% CO\textsubscript{2} in phenol red free RPMI-1640 supplemented with 10 % charcoal-dextran coated FBS, 1x penicillin/streptomycin/L-glutamine stock mix, 1x sodium pyruvate and 1x HEPES for 48h.

Plasmids, constructs and synthetic peptides: GAL4-TATA-Luc plasmid (pG5luc) and expression plasmid for VP16 and Gal4 were purchased from Promega (CheckMate Mammalian Twohybrid System). The (Elk1)\textsubscript{2}-TATA-Luc plasmid was constructed using an EMSA validated oligonucleotide sequence representing a tandem repeat of the optimal binding site for Elk-1 (5’-GAGCCGGAAGATCGGAGCCGGAAG-3’) that was custom
synthesized. The complementary oligonucleotides were annealed to obtain double
stranded DNA. The synthetic DNA was designed with the addition of 5’ KpnI and 3’
NheI sites and substituted for the Gal4 element in the pG5Luc vector (Promega Madison,
WI) upstream of the TATA box. The pRL plasmid encoding Renilla luciferase was
purchased from Promega (Madison, WI). The PSA-Luc plasmid containing 0.6kb DNA
fragment encompassing the promoter and distal enhancer regions of the PSA gene was a
kind gift from Dr. Lirim Shemshedini. The AR expression plasmid (pSG5 vector) was a
kind gift from Dr. Lirim Shemshedini. The expression plasmids for human full length
Elk1, Elk3 and Elk4 in the pCMV plasmid were purchased from OriGene (Rockville,
MD). Gal4-Elk1 fusion plasmid containing Elk1 activation domain (amino acids 307-
428) was a kind gift from Dr. Kam Yeung (University of Toledo). Gal4 fusion of Elk1 in
which the DNA binding domain of Elk1 (amino acids 1-86) was deleted was constructed
by PCR using the Elk1 expression plasmid as the template and the appropriate primers
and subcloned at BamHI (upstream) and NotI (downstream) sites in a vector expressing
Gal4 fusions (pBind). Similarly Gal4 fusion of different regions of Elk1 was made by
PCR using Elk1 expression plasmid as the template and the appropriate primers and
subcloned at BamHI (upstream) and NotI (downstream). The different deletion constructs
of Elk1 (ΔB, Δ49, Δ50, Δ31, ΔD, Δ32, Δ24, Δ19 and Δ29) were a gift from Dr. Peter
Shaw (University of Nottingham Medical School, Queen's Medical Centre, Nottingham,
UK). VP16 fusion constructs for the various domains of AR were constructed using the
VP16 expression plasmid from Promega. The AR(A/B)-NLS construct was generated by
PCR amplification of the A/B domain (residues 1-555) from the full length AR plasmid
and cloning into the pCDH vector with an in-frame insertion of tandem repeats of a
nuclear localization sequence (NLS) at its carboxyl terminus. pCDH-P1 (AR-A/B domain 1-206 a.a.) was generated by PCR using AR expression plasmid (pSG5-AR) as the template and the appropriate primers and subcloned at EcoRI (upstream) and BamHI (downstream). Similarly all the other pCDH constructs P2 (156-329 a.a.), P3 (306-450 a.a.) and P4 (475-555 a.a.).

The synthetic peptides were custom-made and purchased from GenScript (Piscataway, NJ).

**Transfection and reporter Luciferase assays:** HeLa cells were transfected using Lipofectamine™ 2000 transfection reagent (Life Technologies, Carlsbad, CA). The cells were lysed with Passive Lysis Buffer (Promega Madison, WI) and the luciferase activities were measured using substrates for either Firefly luciferase or Renilla luciferase provided in the Luciferase Assay System (Promega Madison, WI) in a luminometer (Centro LB960; Berthold; Wildbad, Germany). In all cases, uniformity of transfection was confirmed using the pRL-null plasmid expressing Renilla luciferase.

**Lentivirus-mediated transduction:** For lentivirus-mediated gene overexpression, the different sub-parts of the AR-A/B domain (P1, P2, P3 and P4 in pCDH) and control (empty pCDH) were packaged in 293FT cells using lentiviral packaging plasmids as previously described (Gonit, Zhang et al. 2011). The virus containing supernatant was harvested 48 h and 72 h after transfection, filtered and stored at -80°C until the time of infection. 24 h before infection, 5 x 10^5- 6 x 10^5 LNCaP cells were plated in poly-D-lysine coated 6-well plates in phenol red free medium supplemented with 10% heat
inactivated charcoal-stripped FBS and L-glutamine (2mM). The next day cells were infected with either control lentivirus or lentivirus containing the different parts of the AR-A/B domain with polybrene (8µg/mL) for duration of 5 h followed by a similar second lentiviral infection for an additional 5 h. 10 h after the infection, the virus was replaced with fresh phenol red free medium containing 10% charcoal stripped FBS.

**Cell proliferation assay:** Cells were trypsinized and 4000-6000 cells per well were seeded in 96-well plates in phenol red free medium supplemented with 10% charcoal-stripped FBS and grown at 37°C and in 5% CO₂ for different time periods. For LNCaP cells it was necessary to use plates coated with poly-D-lysine. 24 h after seeding in 96-well plates, the cells were treated with vehicle (ethanol) or R1881 (1nM). The culture media was not changed during the entire time course. At the end of each time point cell viability was determined using the MTT assay. Briefly, 10µl of MTT (5mg/mL) was added to each well and incubated for 2 h at 37°C. The formazan crystal sediments were dissolved in 100µl of DMSO and the absorbance at 570nm was measured using the SpectraMax Plus spectrophotometer (Molecular Devices Corp, Sunnyvale, CA). The assay was conducted in sextuplicate wells and all values were normalized to day 0.

**RNA isolation, reverse transcription and quantitative real time-PCR:** Total RNA from cells was isolated using the RNeasy mini kit (Qiagen, Georgetown, MD) per the manufacturer’s protocol. Reverse transcription was performed using 500ng of total RNA and the High-Capacity cDNA Archive kit (Applied Biosystems, Life Technologies Corp, Carlsbad, CA) according to the vendor’s protocol. cDNA was measured by quantitative
real time PCR using the StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies Corp, Carlsbad, CA) and TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Life Technologies Corp, Carlsbad, CA). Primers and TaqMan probes for KLK3 (PSA), DTL, CDC6, CDCA3, CCNB2, CDCA5, MLF1IP and GAPDH were purchased from the Applied Biosystems inventory (Life Technologies Corp, Carlsbad, CA). All samples were measured in triplicate and normalized to the values for GAPDH.

**Mammalian two-hybrid assay:** The Checkmate Mammalian two-hybrid assay (Promega) system was used. HeLa cells were plated in 24 well plates in hormone-free phenol red-free DMEM without antibiotics. When the cells were about 90% confluent, they were co-transfected with pG5Luc, pBind vector expressing Gal4 or Gal4-Elk1 fusion proteins and pACT vector expressing VP16 or VP16 fusion proteins using Lipofectamine™ 2000 transfection reagent. After 48 h of transfection, the cells were lysed with passive lysis buffer and the luciferase activity was determined as described above.
RESULTS

*Elk1 binds to the N-terminal A/B domain of AR*

The interacting domains of AR and Elk1 (Figure 3-1 A) were mapped *in situ* using the mammalian two-hybrid assay. In the Gal4-Elk1 (87-428) fusion construct, the N-terminal DNA binding domain (DBD) of Elk1, which is highly conserved among ETS proteins, was replaced by the DBD of Gal4. VP16 fusion constructs of various domains of AR were used (Figure 3-1 A). The N-terminal A/B domain (residues 1-559) of AR interacted exclusively and strongly with Elk1 (Figure 3-1 B). These results indicate that the Elk1 physically interacts with the N-terminus AR-A/B domain.

*Nuclear targeting of the AR A/B domain recapitulates promoter activation through the Elk1 element by androgen and full length AR:*

The A/B domain of AR comprises about half the length of the AR polypeptide (~555 amino acids) including sites of ligand-independent transactivation and coactivator recruitment. Despite its small size, we attached a carboxyl-terminal nuclear localization signal to the A/B domain construct [AR(A/B)-NLS] to ensure optimal nuclear localization. When expressed in HeLa cells, AR(A/B)-NLS was able to activate (Elk1)$_2$-TATA-Luc in the absence of hormone to the same extent that wtAR did in the presence of androgen (Figure 3-1 C, top panel). The expression level of the A/B domain was comparable to the androgen-stabilized level of wtAR (Figure 3-1 C, top panel inset). In contrast, ARE-driven promoter activation was only observed in the presence of wtAR plus androgen (Figure 3-1 C, bottom panel). The results demonstrate that the A/B domain
is the functional entity required for the action of AR as a coactivator of Elk1 and that the role of ligand binding is for translocation of AR to the nucleus.

**Elk3 suppresses the synergy between AR and Elk1**

As Elk3 is the closest structural and functional analog of Elk1, the effect of ectopically overexpressing Elk3 on androgen-stimulated activation of the (Elk1)₂-TATA-Luc promoter was tested. Ectopic Elk1 increased the androgen-response (Figure 3-2 A, B). In contrast, ectopic Elk3 (Figure 3-2 B) virtually completely suppressed the androgen response (Figure 3-2 A). Therefore, AR cannot synergize with Elk3 to activate target genes, in contrast to Elk1.

It was of further interest to confirm the inability of Elk3 to support activation of endogenous genes that are the targets of Elk1-AR. Accordingly the ability of ectopic Elk3 to rescue gene activation by androgen in the absence of Elk1 was tested. In LNCaP cells, overexpression of Elk3 using lentivirus following knockdown of Elk1 using lentiviral shRNA (Figure 3-2 C) failed to rescue the activation of Elk1-dependent androgen target genes (Figure 3-2 D). This result further confirms that the AR does not cooperate with Elk3.

**Elk4 supports transcriptional activation by AR**

Elk4 is the remaining member of the TCF sub-family to which Elk1 belongs. The effect of ectopically overexpressing Elk4 on androgen-stimulated activation of the (Elk1)₂-TATA-Luc promoter was tested. In contrast to Elk3 overexpression of Elk4
greatly enhanced the promoter activation by androgen (Figure 3-2 E). This result indicates that Elk4 may synergize with AR, similar to Elk1.

**Over expression of an AR A/B domain polypeptide downregulates Elk1-AR mediated androgen dependent gene activation:**

Since the AR A/B domain was found to be the functional entity of AR that interacts with Elk1 and regulates gene expression, it was of interest to further map the AR A/B domain. The parts of AR A/B domain (P1, P2, P3 and P4) (Fig 3-3A) were cloned into pCDH lentiviral vector that were packaged in 293FT cells. LNCaP cells were infected with the packaged virus followed by treatment with vehicle (ethanol) or 1nM R1881. The P2 (aa 156-329) of the AR A/B domain was found to dramatically downregulate the expression of genes like DTL, CDCA3, CDCA5, CCNB2 and MLF1IP, which are AR-Elk1 mediated, androgen dependent genes (Fig 3-3 B-F). However, it failed to have an effect on the expression of PSA, TMPRSS2 and PMEPA1 (Fig 3.3 G, H and I) that are classical androgen target genes. The AR A/B domain P1 (aa 1-206) had a partial effect in decreasing gene expression of the AR-Elk1 mediated androgen dependent genes and did not have any effect on PSA, TMPRSS2 and PMEPA1. P3 (aa 306-450) and P4 (aa 475-555) failed to have any effect on gene expression on any of the above-mentioned genes as compared to that of the empty vector control (Figure 3-3 B-I).
**Over expression of AR A/B domain polypeptide 156-329 decreases androgen dependent growth of LNCaP cells:**

LNCaP cells were infected with the AR-A/B domain polypeptide 156-329(P2) expressing lentivirus and were treated with vehicle or 1nM R1881. 1nM R1881 stimulated cell growth by more than three-fold in cells that were infected with the empty vector control (Figure 3-3 H). However, AR-A/B domain P2 significantly reduced the androgen dependent cell growth of LNCaP cells. This decrease in androgen dependent cell growth was observed to be two-fold or more (Figure 3-3 H). The above results indicate that AR A/B domain P2 not only decreases AR-Elk1 mediated androgen dependent gene activation, but also inhibits androgen dependent cell growth of prostate cancer cells.

**Synthetic peptides VC1 and VC2 decrease androgen-dependent growth of LNCaP cells in a dose-dependent manner:**

Based on results obtained as described above, fifty-mer synthetic peptides (VC1 and VC2, Fig 3-4 A) were custom-designed and ordered from GenScript USA, Inc. (Picataway, NJ). The N-terminus of VC2 had a 25 amino acid overlap with the C-terminus of VC1. Upstream of the fifty-mer for both VC1 and VC2 was the TAT-leader sequence that enables cellular entry of the synthetic peptides. LNCaP cells were hormone depleted for 48 hours and treated with either control (ethanol), VC1 (0.1 μM, 1 μM and 10 μM) or VC2 (0.1 μM, 1 μM and 10 μM) and 10 nM R1881, followed by which cell growth assay was performed for 0-7 days by MTT assay. R1881 stimulated growth in the cells that were treated with ethanol (control); however, the growth of
LNCaP cells was seen to progressively decrease over the seven-day period in a dose-dependent manner (Fig 3-4 B and C).

**Discrete peptide sequences of Elk1 are essential for its interaction with AR:**

Since the interaction domain of AR was narrowed down to 50 amino acids, it was of interest to identify the region(s) of Elk1 responsible for its interaction with AR. Gal4 fusions with Elk1 and parts of Elk1 were made as described in materials and methods (Fig 3-5 A, C and E). HeLa cells were co-transfected with pG5-Luc, pBind (Gal4) or Gal4-Elk1 fusion proteins and pAct or pAct-A/B as described earlier. 48h post transfection, HeLa cells were harvested and luciferase assay was performed. The overlapping sets of Elk1 fragments (Fig 3-5 A) did not show any significant level of interaction with AR (Fig 3-5 B). Next, N-terminus variants of Elk1 (Fig 3-5 C) and C-terminus variants (Fig 3-5 E) of Elk1 were tested using the mammalian two-hybrid assay in Hela cells. It was observed that aa 287-317 from the N-terminus and aa 387-428 from the C-terminus are essential for the interaction of Elk1 with AR. These results indicate that the N-terminus and C-terminus of Elk1 are absolutely essential for its ability to bind to AR. These results indicate that discrete peptide sequences from N-terminus (aa 287-317) and C-terminus (aa 387-397) of the activation domain of Elk1 are essential for its interaction with AR (Fig 3-5 D and F).

**Interaction of Elk1 with AR is not dependent on Elk1 phosphorylation:**

Since Elk1 is activated by phosphorylation through the MAP kinase pathway, it was of interest to explore if interaction of Elk1 with AR was associated with Elk1
phosphorylation. Hela cells plated in regular medium were co-transfected with pCMV5 or Elk1 (87-428) wild-type or Elk1 (87-428) deletion mutant (Ser 383/389 Ala) and (Elk1)_{2}-TATA-Luc. 48h post-transfection, HeLa cells were harvested, lysed and luciferase assay was performed. Elk1 87-428 (wild-type) showed increased promoter activity when co-transfected with pAct-AR-A/B. The mutant Elk1 87-428 (Ser383/389Ala) did not show a decrease in promoter activity as compared to Elk 87-428 (wild-type), which indicates that Elk1 phosphorylation is not necessary for its interaction with AR (Fig 3-5 G).
RESULTS

Figure 3-1

A.  

<table>
<thead>
<tr>
<th>AR</th>
<th>NTD</th>
<th>DBD</th>
<th>H</th>
<th>LBD</th>
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<tbody>
<tr>
<td>1</td>
<td>559</td>
<td>671</td>
<td>919</td>
<td></td>
</tr>
</tbody>
</table>

NTD = N-terminal domain (A/B)  
DBD = DNA binding domain (C)  
H = Hinge region (D)  
LBD = Ligand binding domain (E/F)

B.  

![Bar graph comparing Gal4-TATA-Luc activity with Gal4 and Gal4-Elk1 (87-428) constructs with error bars indicating significance.](https://example.com/bar_graph.png)

- **Gal4**  
- **Gal4-Elk1 (87-428)**

Relative Reporter Activity

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP16</td>
<td>50</td>
</tr>
<tr>
<td>VP16-AB</td>
<td>100</td>
</tr>
<tr>
<td>VP16-CD</td>
<td>150</td>
</tr>
<tr>
<td>VP16-CDE</td>
<td>200</td>
</tr>
<tr>
<td>VP16-DE</td>
<td>250</td>
</tr>
<tr>
<td>VP16-E</td>
<td>300</td>
</tr>
</tbody>
</table>

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*Significance level 0.001.*
Figure 3-1

Panel A: Schematics drawn roughly to scale indicating the structural organization of AR (top) and Elk1 (bottom) including the positions of the major functional domains. Panel B: Mammalian two-hybrid assay was conducted as described under Materials and Methods. HeLa cells plated in hormone-depleted media were co-transfected with pG5Luc, pBind (Gal4 expression plasmid), pBind expressing the Gal4 fusion proteins with Elk1 (307-428) or Elk1 (87-428) together with VP16 fusion proteins of different AR domains (A/B, CD, CDE, DE and E). The hybridization signal was measured by assaying for luciferase activity. Panel C: HeLa cells plated in hormone-depleted media were transfected with...
either (Elk1)$_2$-TATA-Luc (top panel) or ARE-TATA-Luc (bottom panel) and co-transfected with expression plasmids for AR, AR(A/B)-NLS or the vector control. At the same time, the cells were treated with either vehicle or testosterone. Cells were harvested 48 hours later to measure reporter luciferase activities (Panel C) or for western blot analysis using anti-AR antibody (Panel C inset). *$P<0.001$. Panel D:
Figure 3-2

A

(B(Elk1)_2-TATA-Luc

Promoter Activation by androgen (RLU x 10^4)

- Vector
- Ectopic Elk1
- Ectopic Elk3

B

Relative mRNA Level

- Veh
- Test

C

ELK3

Vehicle
Testosterone

D

Fold induction by R1881

- Ctrl shRNA + pCDH vector
- Elk1 shRNA + pCDH vector
- Elk1 shRNA + pCDH-Elk3

E

Relative Light Units (RLU) x 10^4

- Vehicle
- Testosterone

pCMV
Elk3
Elk4
Figure 3-2

Panels A and B, HeLa cells plated in hormone-depleted medium were transfected with (ELK1)$_2$-TATA-Luc. The same amount of either the ELK1 or ELK3 expression plasmid or the pCMV vector control plasmid was co-transfected. In all cases, the AR expression plasmid was co-transfected. The cells were treated with vehicle (Veh) or testosterone (Test) (10 nM) and harvested 48 h after transfection to measure luciferase activity (A) or to measure relative mRNA levels of ELK1 and ELK3 (B). C, LNCaP cells plated in hormone-depleted medium were infected with either ELK1 shRNA or control (Ctrl) shRNA lentivirus along with pCDH empty vector lentivirus or pCDH-ELK3 lentivirus. 72 h after infection, cells were harvested. The expression levels of mRNAs for ELK3 (top panel) and ELK1 (bottom panel) were measured by quantitative real time PCR. D, LNCaP cells plated in hormone-depleted medium were infected with either ELK1 shRNA or control shRNA lentivirus along with pCDH empty vector lentivirus or pCDH-ELK3 lentivirus. 72 h later, the cells were treated with either vehicle or R1881 (1 nM) for 48 h and harvested. The expression levels of mRNAs for the indicated genes were measured by quantitative real time PCR. * and **, $p < 0.001$. Error bars represent S.D. Panel E: Hela cells were hormone depleted for 48h followed by which they were co-transfected with (Elk1)$_2$-TATA-Luc and pCMV or pCMV-Elk3 or pCMV-Elk4 and pSG5-AR. On the day of transfection, the cells were treated with vehicle (ethanol) or 1 nM Testosterone. 48 hours after treatment, the cells were harvested and luciferase assay was performed. Panel E: Hela cells were hormone depleted for 48h, followed by co-transfection with (Elk1)$_2$-TATA-Luc, pSG5-AR and pCMV or pCMV-Elk3 or pCMV-Elk4. On the day of transfection, the cells were treated with 10 nM testosterone. 48h post
treatment, the cells were harvested for luciferase assay and relative light units were measured using a luminometer.
Figure 3-3

A.

- AR
  - 1
  - 920
- AR-A/B
  - 1
  - 555
- P1
  - 1
  - 206
- P2
  - 156
  - 329
- P3
  - 306
  - 450
- P4
  - 475
  - 555

B. CDCA3

C. CDCA5
Figure 3-3

Panel A: Schematics drawn roughly to scale indicating AR A/B domain and parts of the AR A/B domain cloned into the pCDH lentiviral vector. Panels B-G: LNCaP cells plated in hormone depleted media were infected with either empty vector (EV) virus or the different parts of AR A/B domain packaged in pCDH lentiviral vector. 72 hours later, cells were treated with either vehicle (ethanol) or R1881 (1nM) for 48 hours. Total RNA from the cells was used to measure the relative mRNA levels for the endogenous CDCA3, CDCA5, CCNB2, MLF1IP, DTL, PSA, TMPRSS2 and PMEPA1 genes by real-time qRT-PCR. Panel H: Hormone depleted LNCaP cells were infected with control lentivirus (pCDH) or AR A/B domain Part 2 (P2) lentivirus. 72 hours later cells were treated with vehicle or R1881 (1nM) and cell growth was monitored by the MTT assay.
Figure 3-4

A.

Viable Cells Percentage of Control (%)

AR

AR-A/B

VC1

VC2

B.

Viable Cells Percentage of Control (%)

Day 0  Day 3  Day 5  Day 7

Control  VC1 (0.1μM)  VC1 (1μM)  VC1 (10μM)

C.

Viable Cells Percentage of Control (%)

Day 0  Day 3  Day 5  Day 7

Control  VC2 (0.1μM)  VC2 (1μM)  VC2 (10μM)
**Figure 3-4**

*Panel A*: Schematics drawn roughly to scale indicating full-length AR, AR A/B domain and synthetic peptides VC1 and VC2. *Panels B and C*: LNCaP cells were hormone depleted for 48 h, followed by treatment with control (ethanol) or synthetic peptides VC1 (0.1 µM, 1 µM and 10 µM) or VC2 (0.1 µM, 1 µM and 10 µM). 3 h post-treatment with control or peptide, the cells were treated with 10 nM R1881. Cell-growth was monitored by MTT assay from 0-7 days following the day of treatment.
Figure 3-5

A.

B.
G.

Figure 3-5

Panel A, C and E: Schematics drawn to scale indicating Elk1 and parts of Elk1 cloned into pBind. Panels B, D and F: HeLa cells were co-transfected with pG5Luc, pBind (Gal4 expression plasmid), pBind expressing the Gal4 fusion proteins with Elk1 (87-428), Elk1 (307-428), Elk1 (337-428), Elk1 (367-428), Elk1 (397-428), Elk1 (87-367), Elk1 (187-287), Elk1 (237-337), Elk1 (287-367), Elk1 (287-428), Elk1 (297-428), Elk1 (317-428), Elk1 (327-428), Elk1 (297-347), Elk1 (87-377), Elk1 (87-387), Elk1 (87-397), Elk1 (87-407) or Elk1 (87-417) together with pAct (VP16 plasmid) or VP16 fusion protein of AR-A/B domain. The hybridization signal was measured by assaying for luciferase activity 48 hours after transfection. Panel G: Hela cells plated in regular medium were co-transfected with pCMV5 or Elk1 (87-428) wild-type or Elk1 (87-428) deletion mutant (Ser 383/389 Ala) and (Elk1)2-TATA-Luc. 48h post-transfection, HeLa cells were harvested, lysed and luciferase assay was performed.
DISCUSSION

The studies described here represent preliminary efforts to identify peptides that are suitable to develop as agents to selectively disrupt the Elk1-AR interaction in prostate cancer cells. Such peptides will help to validate the Elk1-AR synergy as a druggable target for the treatment of prostate cancer. As the crystal structures of the AR A/B domain and of Elk1 are not known, the best available approach to identifying the structural motifs in these proteins required for their physical association is by deletional and mutational analysis using functional assays. The functional assays used in this study are a binding assay using a mammalian two-hybrid system and also an Elk1-driven promoter activation assay.

The studies established that it is the N-terminal A/B domain of AR that is the functional entity in the synergy between AR and Elk1. This finding is significant because the A/B domain lacks the ligand binding site of AR as well as the ligand-dependent activation function of AR. This result is consistent with our previously reported ligand-independence of the Elk1-AR synergy when AR is localized in the nucleus (Patki, Chari et al. 2013). This in turn provides a basis for the role of the Elk1-AR synergy in prostate tumors that can grow independent of hormone or that are insensitive to conventional anti-androgen drugs. This finding also implies that major AR splice variants, that lack the ligand binding domain, may also utilize Elk1 for the activation of critical growth genes.
Elk1 is a well-known downstream effector of MAPK signaling (Fronsdal, Engedal et al. 1998), although the protein is itself genetically redundant. Phosphorylated forms of Elk1 as well as the other TCF subfamily proteins, Elk3 and Elk4, activate immediate early genes by association with serum response factor. The observation in this study, that mutational inactivation of the major phosphorylation sites of Elk1 did not affect its ability to associate with AR further supports our earlier studies that suggested that Elk1 phosphorylation is not involved in the synergistic gene activation by Elk1 and AR.

Our studies further show that whereas Elk3 does not synergize with AR and potentially could act as a prostate tumor suppressor by suppressing the Elk1-AR synergy, Elk4 behaves similar to Elk1 by supporting transcriptional activation by AR. This is significant because unpublished studies from our lab as well as gene expression profiling databases show that a slightly elevated expression of Elk1 and a marked decrease in Elk3 expression is associated with malignant transformation of prostate epithelial cells. Elk4 expression on the other hand is unaltered in malignant prostate tissues.

Further mapping of the peptide motifs of the AR A/B domain required for binding to Elk1 resulted in the identification of both long peptide fragments and short synthetic peptides of AR that might interfere with the Elk1-AR synergy and also inhibit androgen-dependent growth. These preliminary observations need to be confirmed and explored further by including additional control experiments and in additional experimental systems. By the same token, deletional mapping suggests that it may be possible to
identify short Elk1 peptides to develop in the future as agents to disrupt the Elk1-AR synergy.
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