REGULATION OF ESTROGEN RECEPTOR FUNCTION BY MOLECULAR CHAPERONES

by

Jason Allyn Beliakoff

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Jason Allyn Beliakoff entitled Regulation of Estrogen Receptor Function by Molecular Chaperones

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Luke Whitesell, M.D.	10/6/03 Date
G. Tim Bowden, Ph.D.	10/6/03 Date
Margaret Briehl, Ph.D.	10/6/03 Date
Jesse Martinez, Ph.D.	10/6/03 Date
Garth Powis, D.Phil.	10/6/03 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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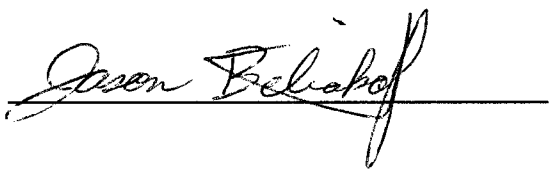
Signed: 

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DEDICATION

Cancer affects people of all ages, both young and old. I would like to dedicate this dissertation to Courtney Page Zillman, a brave child who lost her battle with cancer, but who provided the inspiration for Tee Up for Tots, Inc., an organization that provides fellowship support for the training of young cancer research scientists. It is my hope that the work presented in this dissertation has provided some small advance towards a cure for cancer, so that someday children like Courtney will not have to suffer.

I would also like to dedicate this dissertation to several important people in my life who gave me the strength and courage to pursue my Ph.D. First, to my mother and father, Cheryl Monteith and Bill Beliakoff, whose love, guidance, and values gave me the desire to succeed. Secondly, to Kristy Schwee, who was there to love and support me when I began this journey, and was there when I finished it.

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ABSTRACT

The estrogen receptor (ER) plays a major role in breast cancer progression, and ER+ tumors respond favorably to hormonal manipulation. The selective estrogen receptor modulator (SERM) tamoxifen (Tam) induces remissions in most ER+ patients. However, acquired resistance is often observed. Tam-resistant breast cancer is sensitive to other antiestrogenic compounds, but resistance to these agents has also been described, illustrating a major limitation to antiestrogen therapy. Therefore, we investigated a ligand-independent approach for treating Tam-resistant breast cancer by targeting the molecular chaperone Hsp90.

The ER exists in a multi-protein complex containing Hsp90, which regulates the activity and stability of the receptor. Hsp90 regulates the stability of other proteins relevant to breast cancer, including Akt and Raf-1. The benzoquinone ansamycin antibiotic geldanamycin (GA) and its clinically relevant analog, 17-demethoxy-17-allylaminogeldanamycin (17AAG), bind to Hsp90 and induce the degradation of Hsp90 clients. In these studies, we show that GA depletes ER levels in Tam-resistant cell lines, and the Hsp90 clients Akt and Raf-1. Unexpectedly, Tam inhibited GA-induced degradation of the ER, but not Akt and Raf-1. This effect was consistent *in vivo*, where ER levels were measured in tumor xenografts growing in Tam-supplemented mice. However, Tam-stimulated tumor growth was inhibited by 17AAG, and tumor Akt and Raf-1 levels were downregulated. Immunoprecipitation experiments showed that Tam does not

inhibit GA-induced changes in the ER-chaperone complex, suggesting an alternate mechanism for the inhibition of GA-mediatied ER degradation. Through cell fractionation, immunostaining, and chromatin immunoprecipitation experiments, we have found that the mechanism involves prolonged association of the ER with the DNA in the presence of Tam, which leads to nuclear accumulation of the ER and sequestration from the proteasome. Furthermore, inhibition of GA-induced ER degradation was inhibited by another SERM, Raloxifene, indicating that the effect is not Tam-specific.

Based on its ability to downregulate critical signaling proteins involved in breast cancer, including the ER, 17AAG may provide a useful alternative for patients that have failed hormonal therapy. Because SERMs inhibit the degradation of ER protein induced by GA, they may compromise the efficacy of GA on ER activity, and combined therapy should be approached with caution.

I. INTRODUCTION

Breast Cancer Epidemiology

Incidence and Mortality

Invasive breast cancer is the most commonly diagnosed non-skin cancer in women, and will account for an estimated 211,300 of 658,800 female cancer cases in 2003 (1). Approximately 40,000 deaths from breast cancer are anticipated for 2003, making it the second leading cause of cancer death among women. In women age 35-54, breast cancer is the leading cause of all deaths (2). The frequency of invasive breast cancer in men is exceedingly low, accounting for less than one percent of all cases. In addition to invasive breast cancer, 55,700 new cases of *in situ* breast cancer will be diagnosed in 2003. The vast majority of these cases will be ductal carcinoma *in situ* (DCIS). The incidence of DCIS has increased significantly in recent years, and is likely due to increased screening with mammography. Importantly, patients with breast cancer identified *in situ* have a 97% 5-year survival rate following treatment. Five-year survival decreases to 78% for regionally localized disease, and to 23% for metastatic/disseminated breast cancer (1).

Risk Factors

Gender and age are the two most important risk factors for the development of breast cancer. Over 99% of all breast cancers occur in women (1), and breast tumors in the 55 and over age group are malignant 85% of the time (2). Several other risk factors have been identified, and include: family history, prior diagnosis of breast hyperplasia, prior diagnosis of breast, endometrial, or ovarian cancer, menarche at age 11 or younger, menopause at age 55 or older, never carrying a pregnancy, first delivery after age 30, or uninterrupted menstrual cycles for 30 years or more. The use of oral contraceptives may increase breast cancer risk by as much as 24% (3). However, these studies have also demonstrated that the women at increased risk present with less advanced disease (4). The use of hormone replacement therapy (HRT) for women in menopause may also increase risk, but the data in the literature is conflicting. A pooled meta-analysis of 50 epidemiological studies showed an increased risk of 35% for women on HRT (5). Several other studies, however, have been unable to find an association with HRT and increased risk for breast cancer (6-9).

Although 95% of all breast cancers are sporadic, mutations in the BRCA1 and BRCA2 genes have been identified as predisposing factors that greatly increase the risk of developing breast and ovarian cancers. For carriers of BRCA1 or BRCA2 mutations, the risk of developing breast cancer by age 70 is as high as

85%, and the risk of developing ovarian cancer is 50% (10, 11). This translates to an approximately 20-fold increase in breast cancer risk compared to the general population. For those with a family history of breast cancer, genetic testing for mutations in BRCA1 and BRCA2 by polymerase chain reaction (PCR) is available. Because the likelihood that a carrier will develop breast cancer in their lifetime approaches 100%, drastic surgical procedures such as radical mastectomy or oophorectomy are available, which have been shown to reduce the risk of breast cancer in BRCA1/BRCA2 positive patients by 90% and 70%, respectively (12-14). Understandably, controversy surrounds the idea of removing healthy breast tissue from asymptomatic patients. However, many women choose radical mastectomy rather than the alternative: living with the emotional strain of knowing that they will very likely develop breast cancer.

Diagnosis and Prevention

Mammography is now a common screening technique for breast cancer prevention, and may lower mortality by as much as 40% (15, 16). With this procedure, physicians are able to identify tumors at earlier, non-palpable stages, often while the breast cancer is still *in situ*. At the localized stage, surgical excision yields a 97% 5-year survival rate (1). Since the introduction of mammography as a screening technique, breast cancer incidence has increased, while breast cancer mortality rates have decreased. This suggests that more

cases of localized breast cancer are being identified by mammography, and that these women are being cured by surgical excision of the neoplastic mass.

Although mammography does detect most non-palpable tumors, it has a false negative rate which is 10-15% for women in their forties, and even higher for younger women (15, 17). Therefore, the American Cancer Society recommends that women age 40 and older combine an annual mammogram with an annual clinical breast exam and monthly self-breast exams. Women ages 20-39 are advised to have a clinical breast exam every three years and to perform monthly self breast exams (1). In women where the above prevention techniques have failed, or have not been utilized, the patient will present with symptoms. These include: a breast lump, nipple pain, distortion, thickening, swelling, scaliness, ulceration or tenderness.

Hormonal therapy for the prevention of breast cancer in high-risk women has been shown to significantly decrease the likelihood of developing the disease. Studies with the selective estrogen receptor modulator (SERM) tamoxifen have shown that risk for both invasive and noninvasive breast cancer in patients taking the drug is decreased by as much as 50% (18, 19). Based on these studies, the Food and Drug Administration has approved tamoxifen for breast cancer prevention in high-risk women. Other hormonal therapies are under investigation and may provide better risk reduction than tamoxifen. Raloxifene, another

SERM, was shown to decrease the risk of invasive breast cancer in high-risk, postmenopausal women by 72% (18, 20). In contrast to tamoxifen, raloxifene does not increase the risk of endometrial cancer, and may be a preferable treatment in the future.

Treatment

Breast cancer is 100% fatal if left untreated, and the mean survival of untreated patients is 2.7 years (2). Advances in breast cancer therapies over recent years have prolonged the lives of patients significantly. Relative 5-year survival rates have increased from 75% in the mid-1970s to 86% in the mid-1990s (1). This trend most likely reflects both better detection techniques and better treatments. Depending on the stage of the disease and the patient's preference, treatment may include one or more of the following: lumpectomy, mastectomy, radiation, chemotherapy, or hormonal therapy.

In general, the treatment regimen will be determined by whether the tumor is local or systemic. Several factors are involved in this determination including primary tumor size, lymph node involvement, detection of distant metastases, and histologic grade of the tumor (2).

Localized Disease

DCIS is a localized form of breast cancer, which can develop into invasive breast cancer if left untreated. The prognosis for DCIS is very favorable, as removal of the tumor results in a 10-year survival rate of 95% (2). Although removing the entire DCIS-containing breast is an effective therapy, it is usually not necessary. Instead, breast conservation therapy is preferred. This involves regional removal of the tumor, followed by localized radiation therapy. Adjuvant tamoxifen administration has recently been shown to prevent breast cancer recurrence in DCIS patients, and may also be prescribed (2).

Invasive Breast Cancer

If a primary tumor is 1cm or larger and determined to be invasive by histology, the patient will most likely receive systemic therapy in addition to surgery. Systemic therapy is recommended even if there is no evidence of regional lymph node involvement or distant metastases. This treatment protocol is based on evidence from several clinical trials that show prolonged disease-free survival with a combination of surgery and systemic treatment versus surgery alone (2). The most frequently prescribed systemic medication following surgery is tamoxifen. The efficacy of adjuvant tamoxifen therapy can be predicted based on whether the tumor is estrogen receptor positive. Receptor positive tumors are

much more likely to respond to tamoxifen than receptor negative tumors. Patients will usually discontinue adjuvant tamoxifen therapy after a 5-year period, as longer treatment periods have not been shown to produce any added benefit (21). Clinical studies using adjuvant tamoxifen therapy have shown that 10-year survival is increased by 5-10%, depending on the extent of lymph node involvement (15, 22). Toxicity is relatively low, and major side effects include vaginal dryness and hot flashes. For ER negative tumors, chemotherapy is utilized. Common treatment regimens include combinations of the following agents: Cytoxan, methotrexate, and 5-fluorouracil or Adriamycin and Cytoxan with or without 5-fluoruracil. Taxol as a single agent therapy is becoming more common in the treatment of node-positive disease. Duration of treatment is much shorter than for tamoxifen, and agents are usually administered in 3 to 4 week cycles for 4-6 cycles. Side effects are more severe, and include alopecia, nausea and vomiting, weakness, and fatigue.

In patients with advanced metastatic disease, systemic therapy will preclude surgical removal of the primary tumor. The prognosis for these patients is poor, and 75% will die of their disease within 5 years after diagnosis. The goal of therapy in this case is often management, rather than eradication, of the cancer. Quality of life for patients with advanced metastatic disease has improved with chemotherapy, and remissions for months to years are often achieved. Clearly, new treatments are required to increase the survival rates of patients with

advanced metastatic breast cancer. New therapies with agents such as taxol show promise, and may significantly increase 5-year survival rates (23, 24).

Summary

Breast cancer is the most diagnosed form of cancer in women and the second-leading cause of cancer death. Prognosis for localized disease is excellent, while 5-year survival for women with metastatic disease is only 25%. Major risk factors include age, gender, and family history. Only 5% of all breast cancers are due to heritable mutations, and involve alterations in the BRCA1 and BRCA2 genes. Because these individuals have an 85% chance of developing breast cancer in their lifetime, radical mastectomy or oophorectomy are available preventative measures. For sporadic cases, treatment options include surgery, hormonal therapy, or chemotherapy. These treatments may be used alone or in combination depending on whether the cancer is localized or metastatic. Newer treatments such as autologous bone marrow transplant and taxol show promise and may help to increase 5-year survival rates for patients with metastatic disease. Currently, the best treatment is prevention. Advances in mammography have helped to identify breast cancer at earlier stages when the prognosis is more favorable, and hormonal therapy has been shown to drastically decrease the risk of developing invasive and noninvasive breast cancer in high-risk women.

The Estrogen Receptor (ER)

Estrogen Receptor Structure and Function

The ER is a member of the steroid hormone receptor superfamily of nuclear receptors, which includes receptors for androgens, glucocorticoids, progestins, thyroid hormone, and retinoids. A shared characteristic between all members of the family is the ability to directly activate gene transcription upon ligand binding. The ER is a particularly attractive target for the treatment of breast cancer because protein levels are elevated in many premalignant and malignant lesions, and constitutive ER transcriptional activation is seen in over 50% of diagnosed breast cancers (25, 26). Two known isoforms of the ER exist as separate genes, and are termed ERα and ERβ. The alpha and beta isoforms are similar in structure and activity. However, the significance of ERβ in breast development and breast cancer is unclear. It is expressed at very low levels compared to ERα in mouse mammary tissue, and the mammary gland in ERβ knockout mice develops normally. In contrast, ERα knockout mice have severe developmental abnormalities (27, 28). Expression patterns differ between mice and humans, and ERβ mRNA is detectable in normal and cancerous human breast tissues and cell lines (27, 29-34). Several studies have shown, however, that binding of the antiestrogen tamoxifen is specific for ERα (34, 35). Thus, ERα appears to play

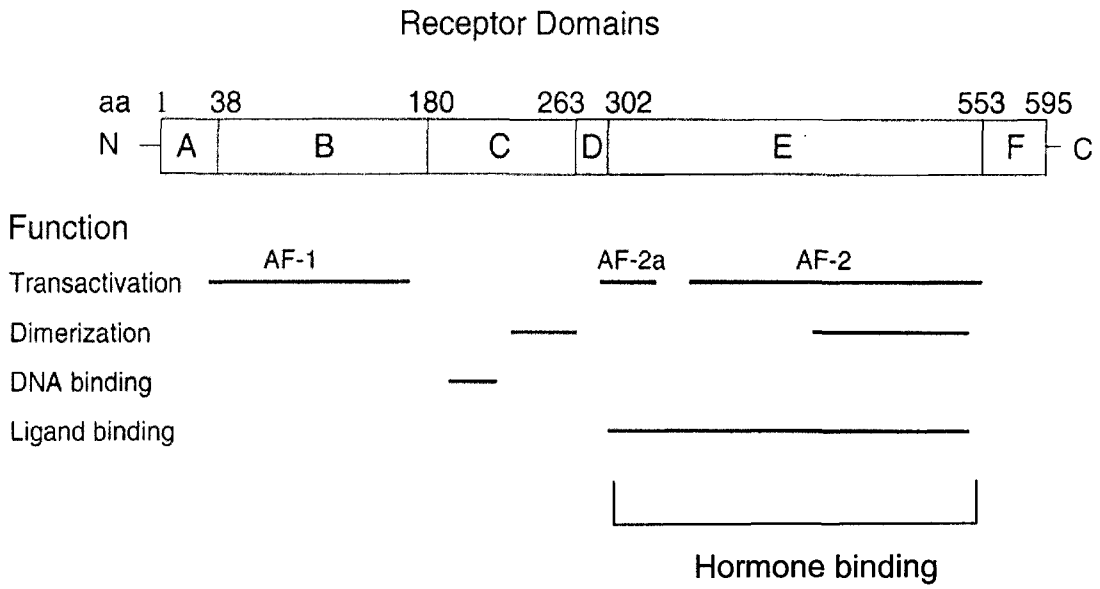

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Figure 1: Estrogen Receptor Structure

The 6 functional domains of the ER: The A/B region contains the ligand-independent AF-1 activation domain. The central "C" domain is involved in DNA binding and ER dimerization. The hinge region, D, is implicated in cofactor binding, and the E/F region contains the ligand-dependent activation function and is the site for hormone binding.

at least a dominant role in the progression of breast cancer and will most likely be the most relevant target for new therapies. The focus of this dissertation is on ERα, which will be referred to simply as ER in the following pages.

The human ER gene is located on chromosome 6 and contains 8 exons that span 140kb of chromosomal DNA (36-38). It is highly conserved across species (36, 39-41), and recent evolutionary data suggests that the ER is the ancestor of all other vertebrate steroid hormone receptors (42). The ER protein is 595 amino acids with an approximate molecular mass of 67 kDa (37, 43). It is comprised of six functional domains, which are designated A-F (figure 1). The amino-terminal A/B domain contains the hormone-independent activation function (AF-1). This region shows the most variation between members of the steroid receptor superfamily (44). The C domain contains two zinc finger motifs, which are responsible for ER binding to estrogen-responsive elements (EREs) in the promoters of estrogen-responsive genes. This region is also responsible for ER dimerization (45). The D domain is a hinge region that has been implicated in binding of coregulators (46). At the carboxy-terminal region, the E and F domains contain the ligand-dependent activation function (AF-2). The ligand-binding domain (LBD) comprises a large part of the E/F region and has been implicated in nuclear localization (47), dimerization (48), and heat shock protein 90 (Hsp90) binding (49), in addition to ligand binding.

Classical Estrogen Receptor Activation

The classical model of ER activation involves direct activation of estrogen-responsive target genes by the ER in a ligand-dependent manner. The unliganded ER exists as a monomer and is localized to the nucleus in a multi-protein complex that includes the chaperones Hsp90, p23, and cyclophillin 40 (50). Ligand binding results in a conformational change in the ER, which causes chaperone dissociation and dimerization of the ER at EREs. The activated receptor associates with the basal transcription machinery (RNA polymerase II), coactivator proteins (A1B1, PBP, p300), and histone deacetylases, resulting in transcriptional activation of ER target genes. Estrogen-responsive genes include: cyclin D1 (51), cathepsin D (52), PS2 (53), Raf-1 (54), IGF-1 (55), the type1 insulin-like growth factor receptor (IGF-1R) (56), the progesterone receptor (57), and egr-1 (54). While all of these genes are responsive to estrogen stimulation, not all have EREs in their promoters. This observation led to the discovery of indirect gene activation through a non-classical mechanism, which involves stimulation of gene activation by association of the ER with other transcription factors or by phosphorylation of the receptor.

Non-Classical Estrogen Receptor Activation

There are two major mechanisms of non-classical ER activation: ligand-

dependent activation and ligand-independent activation. In the model of ligand-dependent non-classical ER activation, the ligand-bound ER interacts with other transcription factors rather than binding directly to an ERE. Transcription factors that are activated by ligand-bound ER include AP-1, Sp1, and NF-κB (58-61). By interacting with these transcription factors, the ER can activate genes without EREs in their promoters. Genes that are upregulated by this mechanism encode proteins that are involved in mitogenesis (cyclin D1, myc) and apoptosis (bcl-2). To further complicate ER signaling, there is evidence to suggest that liganded ER interacts directly with signaling proteins involved in mitogenesis and survival (62). The ER can interact with the IGF-1R and the p85 subunit of phosphatidylinositol 3'-kinase (PI3K) upon estrogen stimulation (63, 64). These interactions may provide a mechanism for estrogen-induced activation of potent mitogenic (MAP kinase) and survival (PI3K/Akt) pathways by bridging the IGF-1R to its downstream target, PI3K.

The second mechanism of non-classical ER activation involves phosphorylation of serine residues within the AF-1 region. Several studies have shown that growth factor stimulation causes increased phosphorylation of the ER (65-68). The AF-1 region appears to be necessary for the activation of the ER by growth factors. In ER mutants where the AF-1 region is deleted but the AF-2 region remains intact, ER activation by EGF and IGF-1 is inhibited. Estrogen-mediated activation, however, is unaffected by loss of the AF-1 domain (69-71).

Conversely, in mutants where the AF-2 domain is deleted and the AF-1 region remains intact, the ER is stimulated by EGF and IGF-1 but not by estrogen. These data strongly suggest that the AF-1 region is necessary for growth factor-mediated activation of the ER. Several kinases have been implicated in ligand-independent ER activation and include MAPK (34), Akt (55), JNK (72), pp90rsk (73), and p38 MAPK (74). Ligand-independent activation of the ER is a likely mechanism for acquired resistance to antiestrogens. Indeed, studies have found that overexpression of signaling proteins involved in breast cancer progression (ErbB2, EGFR, cyclin D1, IGF II) results in ligand-independent ER activation and antiestrogen resistance. Figure 2 illustrates the classical and non-classical mechanisms of ER activation.

Estrogen Receptor Coregulators

Ligand-bound ER is present on the DNA in a multi-protein complex, which includes the basal transcription machinery and coregulatory proteins. The coregulators can be separated into two categories: the coactivators and the corepressors. The type of coregulator bound to the ER varies depending upon cellular context and which ligand is bound to the receptor. For instance, the antiestrogen tamoxifen is inhibitory in the breast, but stimulates endometrial cell growth (75). This selective modulation of ER function is likely due to the differences in cofactor expression patterns between the two cell types. In a

recent study by *Shang et al.*, it was shown that stimulation of an endometrial carcinoma cell line (Ishikawa) with Tam resulted in recruitment of the coactivator steroid receptor coactivator-1 (SRC-1) to an estrogen-responsive promoter (76). In contrast, the same promoter in MCF-7 breast cancer cells displayed no SRC-1 binding. Ishikawa cells expressed significantly higher levels of SRC-1 when compared to MCF-7, suggesting a possible mechanism for Tam resistance in breast cancer. Indeed, several lines of investigation have shown that Tam alters ER conformation in the breast, resulting in the recruitment of corepressors instead of coactivators (77).

There are dozens of coregulatory proteins known to interact with the ER, and undoubtedly more to be discovered. The coactivators function as histone acetyltransferases (HAT), helicases, and ubiquitin ligases (78), and include p300, SRC-1, and E6-associated protein (E6-AP). Although less is known about the functional characteristics of the corepressors, they are linked to histone deacetylase activity (HDAC) (79, 80) and interaction with the basal transcription machinery (81). Corepressors that bind to the ER transcriptional complex include N-CoR (82, 83) and SMRT (79).

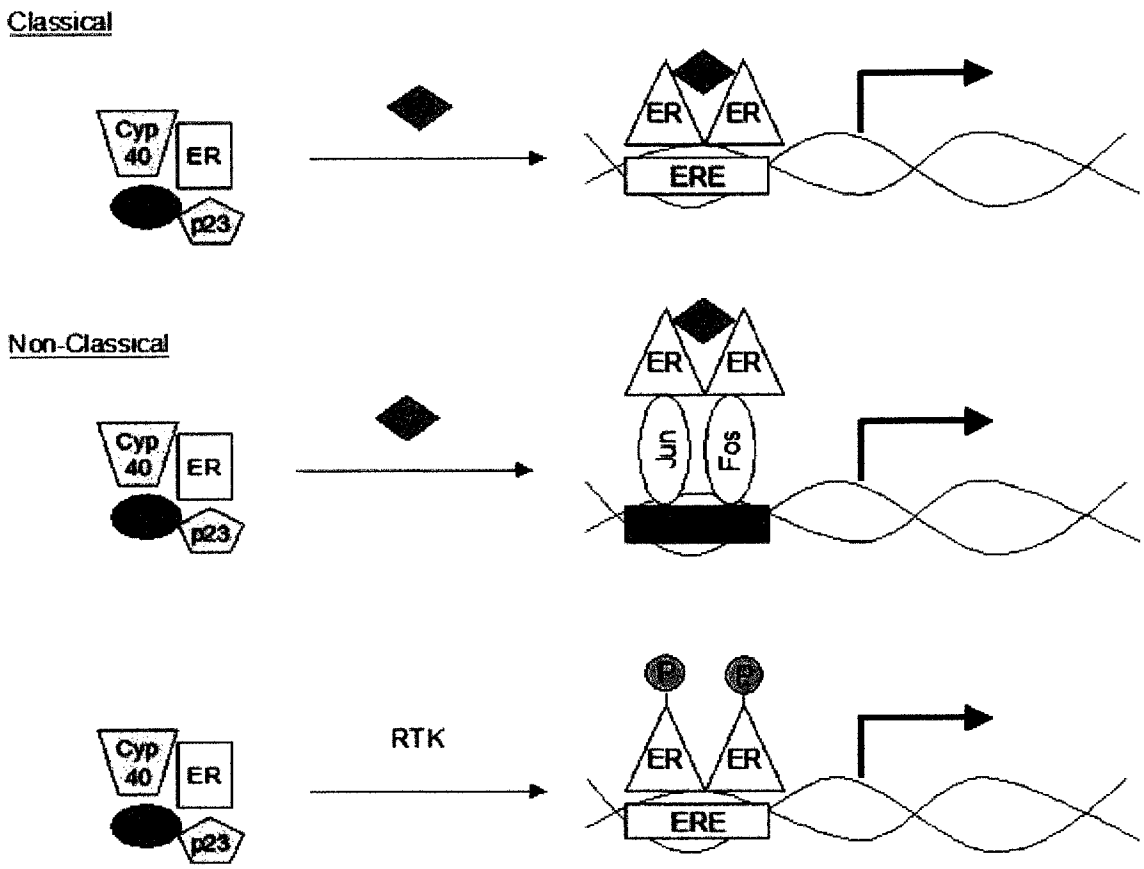

Figure 2: Mechanisms of ER Activation

The ER exists in the nucleus as a monomer and is associated with the molecular chaperones Hsp90, Cyp40, and p23. ER activation occurs through both classical and non-classical mechanisms. *Classical*, E2 binds to the ER and alters its conformaion. The ER dissociates from the chaperone compex and dimerizes at estrogen responsive elements (EREs), where transcription is initiated. *Non-classical*, The ER binds to E2, but associates with other nuclear transcription factors, such as Jun and Fos to initiate transcription at AP-1 sites, or signaling

pathways result in phosphorylation of the ER, which allows the ER to activate transcription through EREs. RTK: Receptor tyrosine kinase

Estrogen Receptor Ubiquitination

Early studies investigating ER stimulation described a rapid decrease in cellular protein with no corresponding decrease in ER mRNA levels. Subsequent reports identified the ER as a target for proteosomal degradation by the ubiquitin-proteasome pathway, and showed that estrogen stimulation increases ER turnover (84, 85). The proteasome inhibitors MG132 and lactacystin abrogated ER degradation, confirming that the major mechanism for destruction of the ER is through the proteasome. It is believed that enhanced degradation of stimulated ER provides a way for cells to rapidly control gene expression in response to hormone. Interestingly, ubiquitination of the ER appears to be essential for it's transcriptional activity. Proteasome inhibition by MG132 or lactacystin inhibits E2-stimulated expression of luciferase reporter constructs with EREs in their promoters, suggesting that ubiquitination is required for ER function (86). Furthermore, the ER coactivator, E6-AP, is a ubiquitin ligase and is localized to the PS2 promoter following estrogen stimulation in chromatin immunoprecipitation (ChIP) experiments (87). Transcriptional activation is a prerequisite for degradation of the ER, as inhibition of RNA polymerase II-mediated transcription by actinomycin D prevents its degradation. Recently, it has been hypothesized that the increased ER levels observed in cells following Tam exposure may, in part, be due to conformational changes in the protein that inhibit the association of ubiquitin ligases, possibly by inhibiting ER transcriptional

initiation (88).

Role of the Estrogen Receptor in Breast Cancer Progression

ER protein levels are elevated in a majority of breast tumors when compared to adjoining normal tissue, indicating that the receptor may play a key role in the development of the disease (77). Additionally, transcriptional upregulation of the ER is observed in over 50% of all newly diagnosed breast cancers (26). The mechanism for increased ER protein and transcriptional activity is not due to mutations, since alterations in the ER gene are rarely observed in breast carcinomas (26, 89). Based on these findings, it has been suggested that upregulation of ER protein and/or transcriptional activity is one of the earliest and most common alterations in breast cancer. Of course, breast cancer is a complex disease and several other genetic changes likely occur during disease progression. Some of the most common are: overexpression of the ErbB2 receptor tyrosine kinase, mutations in the p53 tumor suppressor gene, and amplification of the c-myc transcription factor (26, 90). ER negative tumors undoubtedly have several of these alterations, which provide them with an ER-independent mechanism for malignant growth and insensitivity to antiestrogen therapies.

Current Methods for Inhibition of Estrogen Receptor Function

The fact that the ER is dysregulated in a majority of breast carcinomas makes it an excellent target for anti-cancer therapy. Furthermore, the observation that the ER is often overexpressed and/or overactive in breast cancer provides a potential therapeutic window for selective inhibition of cancer cells. Several pharmacological approaches for inhibiting ER function have been investigated to date, and include antiestrogens, aromatase inhibitors, and leutinizing hormone releasing hormone (LHRH) analogs. Second and third generation compounds have already been developed and the search continues for more effective agents.

Antiestrogens

The antiestrogens are compounds that compete with E2 for the ER ligand-binding pocket. They can be grouped into two categories: the SERMs and the pure antiestrogens. The SERMs are selective modulators of ER function, and act as antagonists in some tissues while acting as agonists in others. In contrast, the pure antagonists inhibit ER activity in all tissues. Several antiestrogenic agents in both categories have been described, but the most well-studied are Tam, Raloxifene (Ral), and ICI 182,780 (fulvestrant) (figure 3).

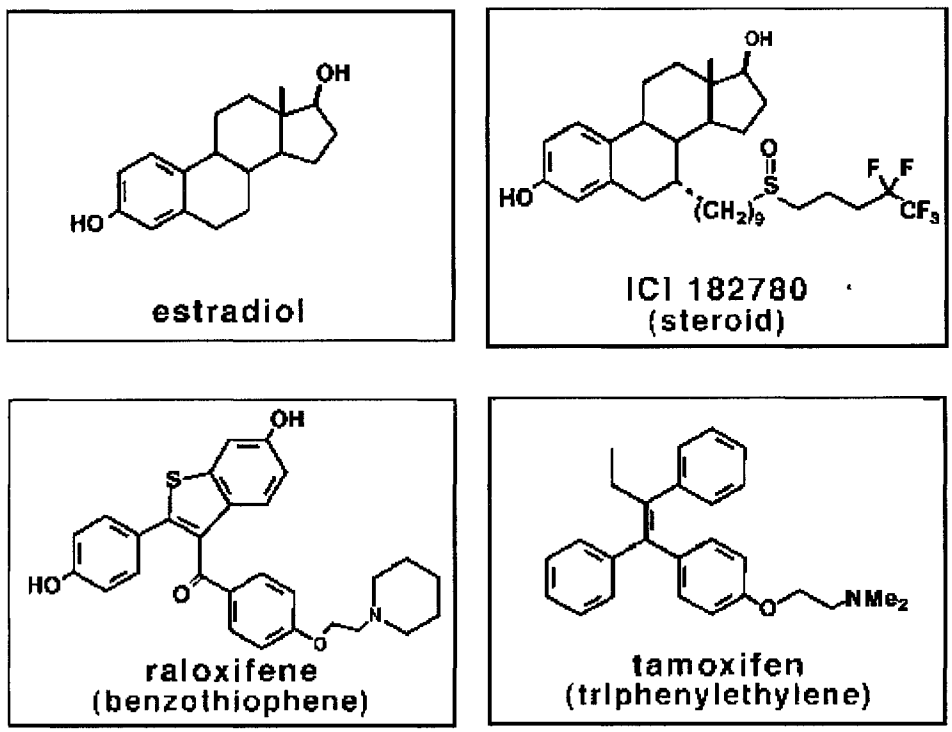

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Figure 3: Structures of ER Ligands

Estradiol is the physiological ligand for the ER. Tam and Ral are SERMs that inhibit ER function in the breast, but have agonistic properties in other tissues. The pure antiestrogen ICI 182,780 (fulvestrant) inhibits ER function in all tissues.

Selective Estrogen Receptor Modulators (SERMs)

Tam and Ral are the most widely studied SERMs, and several related compounds have been developed that are currently being tested in the clinic (91). These agents bind to the ER and stabilize it in a unique conformational state. Each antiestrogen appears to induce a distinct ER conformation, and it has been shown that ER conformation varies depending upon which ligand is bound (92). The SERM-bound ER binds to DNA, but inhibits transcription by associating with corepressor proteins rather than coactivators (93). The partial agonist properties of Tam are likely due to differences in cofactor availability in different tissues, and will be discussed in further detail below.

Tam is a triphenylethylene derivative that has been used clinically in the treatment of invasive breast cancer since 1977 (90). Tam is metabolized to 4-hydroxytamoxifen, which binds to the ER with an affinity similar to that of E2 (94). ER status is a powerful predictor of clinical response to Tam therapy, and Tam induces remissions in nearly all ER+ patients treated. The remissions are temporary, however, and the tumors that reappear are resistant to further Tam treatment (95). Laboratory studies in the late 1970s and early 1980s showed that Tam could prevent mammary carcinogenesis in rats (96, 97), and that long-term treatment was better than short-term treatment (98). Subsequently, it was

shown in clinical trials that 5-year adjuvant Tam therapy reduced breast cancer recurrence rates by as much as 60% and reduced mortality by as much as 36% (99). In addition, the Food and Drug Administration (FDA) recently approved Tam for the chemoprevention of breast cancer in high-risk women. The approval was based on clinical studies showing that women receiving Tam were 50% less likely to develop invasive and non-invasive breast cancers (19). In addition to its antitumor effects, there appear to be secondary effects of Tam exposure that may be of benefit to the patient. Tam increases bone density in ovariectomized rats (100, 101), and women undergoing Tam therapy have a reduced rate of hip and wrist fractures (19). Circulating levels of low-density lipoprotein cholesterol are reduced by about 15% in women taking Tam (102, 103), and studies suggest that the incidence of fatal myocardial infarction is significantly reduced (104, 105). Despite the array of clinical and experimental data detailing the benefits of Tam therapy, a major caveat is increased incidence of endometrial cancer. It has been known for some time that Tam acts as an agonist in cultured endometrial carcinoma cells and in animal models (106, 107), and clinical investigations have determined that post-menopausal women taking adjuvant Tam are 3-4 fold more likely to develop endometrial carcinoma (reviewed in (19)). The agonistic properties of Tam in the endometrium are detrimental to its efficacy as an anticancer agent, and new antiestrogenic compounds that lack this characteristic are of considerable interest.

Over the years, much effort has been focused on understanding the mechanisms of Tam action. It is clear that Tam competes with E2 for ER binding, and inhibits gene activation. However, it has been much more difficult to understand the partial agonist activity displayed by Tam in various tissues. The Tam-bound ER adopts a unique conformation that binds DNA and associates with a different set of cofactor proteins than the E2-bound ER (figure 4). Indeed, a potential mechanism has been outlined where the availability of cofactors in particular tissues determines Tam responsiveness. Supporting evidence for this hypothesis comes from a study by *Shang et al.* showing that the ER associates with the corepressors N-CoR and SMRT at EREs after Tam exposure (93). The same group later determined that in the Tam-stimulated endometrial cell line Ishikawa, levels of the coactivator protein SRC-1 were increased compared to Tam-inhibited MCF-7 breast cancer cells (76). Disruption of SRC-1 expression by interfering RNA abrogated Tam-mediated, but not E2-mediated, expression of ER target genes in Ishikawa cells. These data strongly suggest that cofactor availability plays a role in the partial agonist properties of Tam. A second theory for the partial agonist activity of Tam is differential expression of ERα versus ERβ. It has recently been shown that ER-mediated activation of transcription at AP-1 sites by ERβ is stimulated by Tam (108). Since different tissues express varying ratios of ERα and ERβ, this may explain why Tam acts as a partial agonist in some tissues. A third possibility is that there are antiestrogen responsive elements in the DNA where Tam-bound ER binds and activates gene

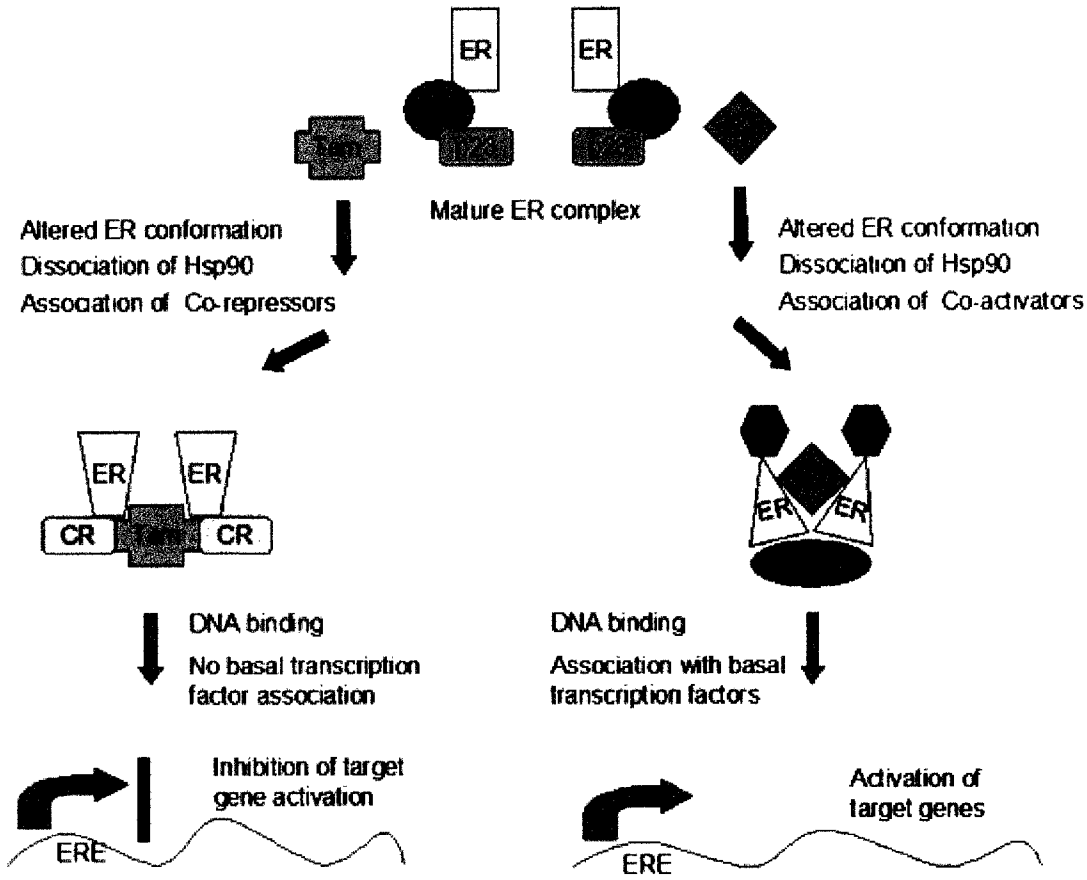


Figure 4: Inhibition of ER Function by Tam

Ligand-dependent ER activation involves the binding of E2 to the ER, which induces a conformational change in the protein that results in dissociation of Hsp90 and other co-chaperones, and allows the association of coactivators (CA) and basal transcription factors (BTF). The Tam-bound ER adopts a distinct conformation compared to E2-bound ER, and recruits corepressors (CR). The Tam-bound ER binds to EREs, but cannot activate transcription.

transcription. In support of this hypothesis, a response element has been identified that is preferentially activated by antiestrogens (109). Thirty years after it's approval by the FDA, the mechanisms of Tam action are still the subject of intense investigation. The problem is complex, and may involve many, if not all, of these proposed mechanisms.

Ral is a benzothiophene derivative that has properties similar to Tam, yet it has less agonist activity in the endometrium (110). In fact, Ral prevents Tam-stimulated endometrial cancer growth in animal models (111). Like Tam, Ral inhibits the growth of cultured breast cancer cells and mammary tumor growth in animal models (112, 113), increases bone density (110), and decreases circulating levels of low-density lipoprotein cholesterol (114). Ral is already approved for the treatment of osteoperosis, and is currently being compared to Tam in clinical breast cancer prevention trials. The results are promising, as a 72% reduction in the risk of invasive breast cancer was seen in post-menopausal women taking Ral (20). Although Ral inhibits the growth of breast cancer cells in culture, it appears to be less effective than Tam in *in vivo* models (reviewed in (115)). This effect may be due to rapid conversion of the drug into less effective metabolites. The clinical efficacy of Ral as an adjuvant therapy is unclear, since studies have used patients with highly advanced disease who have often already undergone, and have become resistant to, Tam therapy (116). It is uncertain whether Tam-resistant tumors are also resistant to Ral therapy, and this issue

must be addressed before the clinical efficacy of adjuvant Ral can be determined.

The mechanism of Ral action is similar to that of Tam, although the partial agonist activities are significantly reduced. However, studies by *Yang et al.* have identified a Ral-responsive element in the promoter of the TGFβ3 gene, indicating that Ral is not entirely antiestrogenic (109). The crystal structure of the Ral-bound ER has been recently solved, and provides some valuable insights into the structural determinants of Ral action (117). Helix 12 appears to be critical for antiestrogen action and its position is significantly altered when Ral is bound. It has been shown that amino acids within helix 12 are critical for coactivator binding (118). Furthermore, mutation of a single amino acid within helix 12 changes the properties of Ral from an antagonist to an agonist (119). When this mutant receptor was transfected into the ER negative cell line MDA-MB-231, expression of the ER-responsive TGFα gene was induced. In contrast, no induction was observed with the wild type receptor.

Pure Antiestrogens

In the late 1980s, Wakeling and colleagues discovered a new class of antiestrogens (120). These compounds were termed "pure antiestrogens" because they lacked the partial agonist activity seen with previous agents. The

drug ICI 182,780 (fulvestrant) is a more potent inhibitor than the original lead compound, ICI 164,384, and has been studied extensively (121, 122). Although the SERMs and the pure antiestrogens are competitive inhibitors of E2 binding, their mechanisms of actions are quite divergent. The precise mechanism(s) of pure antiestrogen action is still unclear, yet several changes in the ER have been observed that likely contribute to inhibition of ER activity. There is experimental evidence to suggest that the ICI compounds inhibit ER dimerization and impair shuttling from the cytoplasm to the nucleus (123-125). However, a number of studies have shown that the ER binds to EREs and is localized to the nucleus in the presence of pure antiestrogens (126-128). Of great interest is the observation that the pure antiestrogens induce degradation of ER protein, a characteristic unique to this class of ER antagonists (129-131). Tam, by contrast, causes stabilization of ER protein (132). ICI 182,780 inhibits the growth of Tam-stimulated MCF-7 tumor xenografts (133), blocks Tam-stimulated uterine growth in rats (121), and can inhibit the growth of Tam-resistant cells (134). Consequently, ICI 182,780 is now in clinical trials for the treatment of Tam-refractory breast cancer. Recent reports have shown that ICI 182,780 is well tolerated and slightly more effective than the aromatase inhibitor anastrozole at inhibiting the progression of Tam-resistant breast cancer (135, 136). ICI 182,780 is not orally bioavailable because of its poor solubility, and intra-muscular injections are required. An orally active compound, EM-800, has been developed and retains a structure similar to ICI 182,780. It inhibits the growth of

cultured breast cancer cells stimulated by a variety of ligands, and prevents the E2-stimulated growth of breast cancer xenografts in athymic mice (137, 138). An orally available pure antiestrogen is yet to enter clinical trials, but may prove very useful in the treatment of Tam-resistant disease. A major concern with the use of any pure antiestrogen in the clinic is reversal of the beneficial side effects seen with SERM therapy. Although the effects of long-term pure antiestrogen therapy are currently unknown, it will be important to determine whether bone density or low-density lipoprotein levels are adversely impacted.

Aromatase Inhibitors and Luteinizing Hormone-Releasing Hormone (LHRH) Analogs

An alternative strategy for inhibiting ER function in breast cancer is to reduce the levels of circulating estrogens. Two approaches are currently available, both aimed at reducing the amount of E2 levels in patients. Aromatase inhibitors block the biosynthesis of E2, and long-term treatment with LHRH analogs reduces the level of circulating estrogens by reducing gonadotropin secretion.

Aromatase is a cytochrome p450 enzyme at the last step in E2 biosynthesis. It catalyzes an aromatization reaction that converts androstenedione and testosterone into estrone and estradiol (139). The position of aromatase in the biosynthetic pathway makes it an excellent target for specific inhibition of E2

production, since disruption of aromatase will not likely affect the synthesis of other steroid hormones. In the 1970s, aminoglutethimide was the first aromatase inhibitor introduced. It exhibited activity in patients with ER+ breast tumors, however it had limited specificity and also inhibited the production of glucocorticoids and mineralocorticoids (140). Today, several third-generation aromatase inhibitors with increased selectivity are under clinical investigation for breast cancer prevention, the treatment of Tam-refractory disease, and as front-line endocrine therapies (141-144). The aromatase inhibitors can be classified into two groups based on their binding characteristics. Type I inhibitors are steroidal compounds that bind to aromatase irreversibly, and include the compound exemestane. Anastrazole and letrozole are nonsteroidal agents that bind aromatase reversibly (145). These agents are well tolerated, and have shown efficacy against Tam-resistant breast cancer in clinical trials (146-150). Significantly, plasma E2 levels are reduced by as much as 98% in patients treated with the aromatase inhibitors (151, 152). Aromatase inhibition is likely effective against Tam-resistant breast cancer because the two agents have divergent mechanisms of action, and cross-resistance is not an issue. Furthermore, the ER is not mutated or lost in a majority of Tam-resistant breast cancers, suggesting that secondary endocrine manipulation may be effective. Indeed, numerous clinical studies have determined that aromatase inhibition is a useful second-line endocrine therapy. Currently letrozole, anastrozole, and exemestane are approved for the treatment of antiestrogen-resistant breast

cancer in the United States (153). Anastozole and letrozole are also approved for first-line endocrine therapy (154). As with the pure antiestrogens, aromatase inhibitors exhibit no partial agonist activity. Therefore, the side effects of long-term E2 deprivation must be carefully considered. Results from an ongoing clinical trial comparing anastrozole to Tam as an adjuvant have shown that fractures of the spine (23 vs 10) and wrist (36 vs 25) are significantly increased in the anastrozole group (155). These data were acquired after approximately half of a 5-year treatment regimen.

LHRH is a hypothalamic hormone that affects gonadotropin secretion and ovarian function. Therefore, analogs of LHRH have been investigated as potential agents for the endocrine therapy of ER+ breast cancer. The LHRH analogs buserelin and goserelin have been studied clinically. Prolonged treatment with LHRH analogs decreases gonadotropin secretion and is equivalent to surgical removal of the ovaries (156). Furthermore, buserelin causes the regression of metastatic breast cancer in premenopausal women, and goserelin reduces E2 levels in >90% of premenopausal women to below postmenaupausal levels (157, 158). Recent clinical studies have focused on combination therapy with Tam and LHRH analogs in premenopausal women, and have shown promise (159).

Mechanisms of Resistance to Estrogen Receptor-Targeted Therapy

Antiestrogen therapy of breast cancer is severely limited by the emergence of resistance. Tam has been available for clinical use for nearly 30 years, and induces remissions in approximately 80% of ER+ patients treated (160). However, nearly all patients relapse and require second-line treatment. Clinical cross-resistance to other Tam-like triphenylethylene derivatives has been observed, and these compounds are not effective as treatments following Tam failure. The aromatase inhibitors, however, are effective against a significant number of tumors that no longer respond to Tam (146, 147, 161). Indeed, these agents are currently being investigated as potential front-line therapies for the treatment of ER+ breast cancer (162). Although Tam-resistant tumors initially respond to the aromatase inhibitors, acquired resistance to these compounds is also observed, resulting in breast cancer progression. The pure antiestrogen ICI 182,780 is also effective as a second-line therapy after Tam failure. Clinical studies have shown that patients with Tam-resistant breast cancer respond to treatment with ICI 182,780, and cell lines that are resistant to Tam retain sensitivity to pure antiestrogens (134, 163, 164). Therefore, no cross-resistance appears to exist between these agents. Resistance to the pure antiestrogens, however, has been observed (165), and cross-resistance to Tam has been described in an ICI 182,780-resistant cell line (166). Clearly, the mechanisms underlying responsiveness to antiestrogen therapy are complex, and many

mechanisms for resistance have been described (91). The majority of the work in this field has focused on Tam, although many of the proposed mechanisms of resistance are likely relevant to the LHRH analogs, aromatase inhibitors, and pure antiestrogens.

Estrogen Receptor Mutation or Deletion

An obvious potential mechanism for resistance to antiestrogen therapy is through mutation or deletion of the ER, as a cancer cell that no longer requires a functional ER for growth and survival would not be affected by treatments targeting the ER. Surprisingly, only a small fraction of Tam-resistant tumors display ER mutations or deletions (167, 168). In a study by *Karnik et al.,* 20 Tam-resistant and 20 Tam-sensitive breast tumor biopsies were screened for ER mutations or deletions. Only two mutations were found (169). Although the identification of ER mutations/deletions in human breast cancers are rare, cell culture studies have shown that T47D and ZR-75-1 breast cancer cell lines switch to an ER- phenotype following prolonged Tam exposure, indicating that deletion does occur (170, 171). The crystal structure of the ER-Ral complex has been solved (117), and has provided critical insights into the structural alterations induced by antiestrogens. The most striking difference between E2-bound and Ral-bound ER is the position of helix 12. It has been proposed that the hydrophobic residues on helix 12 are critical for cofactor binding, and that the

unique position of helix 12 induced by Ral results in the recruitment of an alternate set of cofactors. Indeed, cell culture studies have shown that a point mutation at amino acid Asp-538 within helix 12 is important for modulation of ER function and stability by Tam (172). A second point mutation at aspartate 351 within the ligand-binding region switches Ral activity from an antagonist to an agonist based on expression patterns of TGFα, an ER-responsive gene (119, 173). Indeed, *in vivo* models of antiestrogen-stimulated growth have been useful to determine whether mutant receptors play a role. Interestigly, the mutation in amino acid 351 was originally identified in a model of Tam-stimulated MCF-7 breast cancer cells grown in athymic mice (107, 174). Taken together, these data suggest that mutation or deletion is a mechanism for the development of antiestrogen resistance. However, the rarity of these events in human cancer suggests that there are other mechanisms involved in the majority of cases. Several labs have investigated gene silencing by methylation of the ER promoter as an alternate mechanism for producing a functionally ER-null phenotype (175, 176). However, the data are conflicting and further study is required to determine if methylation of the ER promoter plays a role in acquired resistance to antiestrogens.

Altered Expression of Estrogen Receptor β

The identification of a second ER gene, ERβ, added yet another layer of

complexity to the mechanisms of ER function (177). Although ERα is the predominantly expressed form in breast tissue, ERα and ERβ can form heterodimers (30). This significantly impacts the ER's response to estrogens because ERβ stimulates the expression of ER-responsive genes less efficiently than ERα (178). *In vivo*, ERβ does not seem to play a significant role in the breast since mammary development in ERβ knockout mice is comparable to wild type controls (28). However, there is experimental evidence to suggest that ERβ is expressed in human breast cancer (32, 33, 179). If increased ERα-ERβ heterodimers exist in breast cancer as a result of increased ERβ expression, estrogen responsiveness may be reduced. This could potentially force breast cancer cells to seek alternative pathways for growth and survival, rendering them insensitive to ER modulation by antiestrogens. The current data on ERβ is limited, and future studies will undoubtedly provide more insights into the role of ERβ in breast cancer and its potential role in resistance to antiestrogen therapy.

Altered Antiestrogen Metabolism

Tamoxifen is converted into two main metabolites, 4-hydroxytamoxifen (4-OHT) and N-desmethyltamoxifen. Although 4-OHT is the minor metabolite, it binds to the ER with an affinity comparable to that of E2 (91). In contrast, the major metabolite, N-desmethyltamoxifen, has a weak affinity for the ER. 4-OHT can exist as a *trans* or a *cis* isomer. The *trans* form is stable and acts as a potent

antiestrogen. The *cis* form, however, is not stable and has significantly weaker antiestrogenic activity (180, 181). Furthermore, alternate metabolic pathways can catalyze the conversion of Tam into the estrogenic compounds Met E and bisphenol (182-185). Based on these observations, it has been hypothesized that altered metabolism of Tam into estrogenic or less-potent antiestrogenic compounds can lead to Tam resistance and Tam-stimulated tumor growth. Indeed, reports have described reduced intratumoral levels of Tam in Tam-stimulated tumors, and correlative evidence showed that there were increases in the cis/trans 4-OHT ratio and the estrogenic Met E metabolite (182, 184, 185). However, studies with non-isomerizable forms of 4-OHT that cannot form estrogenic metabolites have shown that they are comparable to Tam at stimulating tumor growth (186, 187). Therefore, altered metabolic conversion of Tam is not a likely mechanism for the emergence of Tam-resistant/Tam-stimulated tumors.

Growth Factor Signaling

It is now clear that ligand-mediated mechanisms of ER activation only partially explain ER activity. The AF-1 region of the ER is ligand-independent and has been implicated in the partial agonist activity of SERMs and in Tam resistance (188). Several growth factor signaling pathways, including the EGFR/Her2 and IGF-1R pathways, induce phosphorylation of the ER N-terminal domain at

multiple sites, resulting in ligand-independent activation (65, 73, 189). The signaling kinases p42/44 mitogen-activated protein kinase (MAPK), Akt, protein kinase A, and the JNK and p38 MAPKs are known to directly phosphorylate the ER (65, 74, 189, 190). Importantly, several studies have indicated that acquired and *de novo* Tam resistance are associated with increased levels of these kinases (191, 192). The hypothesis that growth factor signaling contributes to antiestrogen resistance through phosphorylation of the ER is supported by a recent study showing that overexpression of Her2/neu enhances MAP kinase activity and renders MCF-7 cells Tam-resistant (193). Treatment with a Her2/neu inhibitor resulted in reduced MAP kinase activity and reversion to a Tam-sensitive phenotype. In a separate study, transfection of a dominant-negative Akt into a Tam-resistant, Her2/neu-overexpressing cell line partially restored Tam inhibition of ER function (194). Thus, the MAPK and PI3-kinase pathways seem to play a role in Tam resistance in tumors that overexpress Her2/neu. Interestingly, several investigators have established Tam-resistant cell lines through prolonged Tam exposure and have identified increased MAP kinase activity (195, 196). These data suggest that any growth factor receptor that can induce the MAP kinase pathway may have the ability to render cells resistant to antiestrogens.

The ER is known to increase the expression of a number of growth factor signaling proteins involved in cancer, including the IGF-1R, epidermal growth

factor receptor (EGFR), Insulin receptor substrate-1 (IRS-1), platelet-derived growth factor (PDGF), and transforming growth factor α (TGFα) (56, 197-201). Importantly, these genes are regulated by multiple transcription factors, including the ER. Thus, another possible mechanism for growth factor-mediated antiestrogen resistance is by causing the upregulation of these genes in an ER-independent manner through stimulation of other transcription factors.

Altered Cofactor Availability

There are dozens of coregulatory proteins that can interact with the ER transcriptional complex (77, 78, 202). The coactivators enhance ER-mediated transcription, while the corepressors inhibit ER transcriptional activity. An emerging body of evidence suggests that these coregulatory molecules may play a significant role in antiestrogen resistance, and may explain the tissue-specificity of SERMs. This is because relative levels of coregulators seem to determine whether Tam will act as an agonist or an antagonist (46, 203). Further, coactivators have been shown to be associated with the E2-stimulated ER at EREs, while the Tam-bound ER is associated with corepressors (93). The steroid receptor coregulatory-1 (SRC-1) protein was one of the first steroid hormone receptor coactivators described, and it has been shown that SRC-1 activates E2-bound ER (203). In contrast, Tam-bound ER is not activated by SRC-1. However, a study by *Shang et al.* suggests that high levels of SRC-1

protein may contribute to the agonistic properties of Tam (76). When the Tam-stimulated Ishikawa endometrial carcinoma cell line was compared to the Tam-inhibited MCF-7 breast cancer cell line, it was shown that Ishikawa cells expressed significantly higher levels of SRC-1 protein. Disruption of SRC-1 levels by interfering RNA abrogated Tam-stimulated gene activation in Ishikawa cells, indicating that SRC-1 was at least partially responsible for the observed stimulatory effects of Tam.

The corepressors NCoR and SMRT are the most widely studied, and have been shown to be associated with the Tam-inhibited ER transcriptional complex (93). Overexpression of these proteins can reduce the partial-agonist activity of Tam without affecting ER stimulation by estrogens, and decreased levels of NCoR are associated with hormone resistance in mice (46, 203, 204). In the clinic, reduced corepressor levels are seen in advanced disease, indicating that this may be a mechanism for resistance to endocrine manipulation (205).

As with the ER itself, the ER coregulators are phosphorylated by cell signaling pathways, which modulate their activity. Although the precise mechanisms are unclear, studies suggest that phosphorylation of coactivators leads to increased activity, while phosphorylation of corepressors leads to suppressed function. Phosphorylation of coactivators can lead to increased nuclear subcellular localization (206), enhanced ER association (207), and may stimulate the

recruitment of other cofactors necessary for ER-mediated transcription (208). Phosphorylation of corepressors by growth factor signaling pathways appears to inhibit their ability to associate with the ER (193). A recent study identified nuclear export of phosphorylated corepressors as a potential mechanism for attenuation of corepressor function by growth factor signaling (209).

Summary

The ER is a member of the steroid hormone receptor superfamily of nuclear receptors, and members of this family directly activate transcription of target genes upon ligand binding. The ER contains both ligand-dependent (AF-2) and ligand-independent (AF-1) activation domains. Estrogens and antiestrogens modulate ER activity through the AF-2 domain, while AF-1 activity is controlled by phosphorylation. The ER is critical for normal breast development, and is overexpressed or overactive in a large number of breast cancers, making it an attractive target for therapy. A second ER isoform exists, and has been termed ERβ. ERβ is expressed at low levels in the breast and does not appear to be required for activation of ER target genes in this tissue. However, ERβ is a less potent activator of ER-mediated transcription and may reduce the activity of ERα through heterodimerization. There are two mechanisms for ER activation: classical and non-classical. In the model of classical ER activation, ligand-bound ER binds to EREs and activates transcription. In the non-classical model, ligand-

bound ER associates with other transcription factors, such as AP-1, to activate transcription, or phosphorylation of the ER by growth factor signaling pathways leads to ligand-independent activation of the ER. Ligand-bound ER associates with coregulators at EREs, which modulate ER activity. Coactivators enhance transcription, while corepressors suppress transcription. The coregulators function as histone acetyl transferases, ubiquitin ligases, and DNA helicases. Following activation of gene transcription, the ER is ubiquitinated and presented to the 26S proteasome for degradation. Several approaches for inhibiting ER activity have been described, and all have been investigated in clinical trials. The antiestrogens act as competitive inhibitors for E2 binding, and include the SERMs (Tam and Ral) and the pure antiestrogens (ICI 182,780), and are effective at inhibiting ER function in breast cancer cells and in breast tumors. Inhibition of E2 production using aromatase inhibitors and LHRH analogs has also been successful. A key problem with current ER-targeted therapies is the development of resistance. The complexity of ER signaling has made it difficult to completely understand the mechanisms of resistance to antiestrogen therapy. However, several mechanisms have been proposed. These include ER mutation/deletion, altered expression of ERβ, altered antiestrogen metabolism, changes in growth factor signaling pathways, altered cofactor availability, and enhanced phosphorylation of the ER or it's coregulators. It is likely that antiestrogen resistance is due to a combination of several of these proposed mechanisms, and the mechanism(s) may vary from tumor to tumor. Further

studies should help to clarify which resistance mechanisms are most important.

Molecular Chaperones and the Estrogen Receptor

The Molecular Chaperone Superfamily

The molecular chaperones are ubiquitous proteins that act to maintain proper protein folding within the cell. They are required for several critical cellular processes, including folding of nascent polypeptide chains, prevention of protein aggregation, and protein transport across cell membranes (210). They were first identified as proteins that were highly expressed under conditions of elevated temperature, and were named "heat shock proteins", or Hsps (211). It was subsequently shown, however, that expression of these proteins was induced by a variety of cellular stresses, including exposure to heavy metals, oxidative stress, and inflammation (212). There are six major Hsp families, grouped according to their molecular size: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small hsps (20-25kDa in size) (table 1). Members of these Hsp families are localized to the cytosol, the endoplasmic reticulum, and the mitocondria, where they carry out their functions. Although there is significant overlap in function between members of the molecular chaperone superfamily, Hsp90 is unique in its ability to stabilize a number of oncogenic signaling proteins. The functions of other Hsp family members are beyond the scope of this dissertation, and will only

be discussed as they relate to the estrogen receptor and Hsp90. For the the interested reader, recent reviews by Hartl (210) and Buchner (213) focus on the diverse functions of molecular chaperones.

Heat Shock Protein 90 (Hsp90)

Hsp90 is one of the most abundant proteins in the unstressed cell, comprising about 1-2% of the total soluble cytosolic protein (214). The Hsp90 protein is highly conserved from bacteria to animals, indicating that its function is important for vital cellular processes (212). Unlike many other Hsp family members, which are involved in the general maintenance of protein folding, Hsp90 has a unique set of proteins with which it interacts. These substrate proteins have been termed Hsp90 "client proteins", and depend on Hsp90 interactions for their activity. Approximately 100 Hsp90 clients are currently known (table 2), many of which are involved in signaling. These include signaling kinases (ErbB2, c-Src, Akt) and proteins involved in gene transcription (SHRs, Hif-1α, telomerase). Because many oncogenic signaling proteins are dependent upon Hsp90 for function, the Hsp90 protein has emerged as a potential target for anticancer therapy.

Table 1. Heat Shock Protein Family Members

Hsp 100	Helps to resolubilize heat-inactivated protein aggregates
Hsp90	Stabilizes signaling proteins, role in protein refolding and maintenance, regulator of the heat shock response (through HSF-1 transcription factor)
Hsp70	Interacts with nascent polypeptide chains, regulator of the heat shock response, role in membrane transport, refolds and maintains denatured proteins, associates with tumor-derived antigenic peptides
Hsp60	Refolds denatured proteins and prevents their aggregation, potential cofactor in proteolysis
Hsp40	An Hsp70 cofactor that enhances the rate of Hsp70 ATPase activity and substrate release
Small Hsps	Antiapoptotic activity, suppress heat inactivation and aggregation of protens, stabilization of microfilaments

Structure and Function

There are two isoforms of Hsp90 in humans: Hsp90α and Hsp90β. In most tissues, Hsp90α is induced under cellular stress and Hsp90β is constitutively expressed (215). The proteins are 76% identical, and are the likely result of a gene duplication 500 million years ago (216). Thus, the Hsp90 isoforms are expressed from separate genes. Hsp90α has been mapped to chromosome 14, and the Hsp90β gene is located on chromosome 6 (217-220). It is unclear what functional differences exist between Hsp90α and Hsp90β, and in most cases the two isoforms are not distinguished from one another. However, the proteins are probably not entirely redundant, since a knockout mouse strain for Hsp90β displays embryonic lethality (221). The Hsp90 genes are regulated, at least in part, by a family of cell stress-inducible transcription factors called the heat shock factors (HSFs). Both Hsp90 genes contain heat shock-responsive elements (HSEs) in their promoters (222-224), which are bound by the HSFs to initiate transcription.

The Hsp90 protein contains three functional domains: an ~25 kDa N-terminal domain, a "charged linker" domain, and an ~55kDa C-terminal domain (225). The crystal structure of the N-terminal domain has been solved, and contains a unique ATP-binding pocket similar to that of DNA gyrase and the histidine kinase

Table 2. Hsp90 Client Proteins

Transcription Factors and Polymerases	Siganling Protein Kinases	
Glucocorticoid receptor	v-Src, c-Src	Insulin-like growth
Progesterone receptor	v-fps	factor receptor
Estrogen receptor	v-yes	Pim-1
Androgen receptor	v-fes	ErbB2
Mineralocorticoid receptor	v-frg, c-frg	Epidermal growth
Aryl hydrocarbon (Ah) receptor	Lymphoid cell kinase $p56^{lck}$	factor receptor
v-*erb*A	Hck	Platelet-derived
Retinoid receptor	Wee1	growth factor
Heat-shock factor	Sevenless PTK	receptor
Sim	Heme-regulated eIF-2 kinase	c-Mos
P53 (mutant)	Casein kinase II	Cdc2
Ecdysone receptor	Raf family kinases	Cdk4
Heme activator protein (Hap-1)	MEK	Cdk6
Hypoxia-inducible factor-1α	Kinase suppressor of Ras	Cdk9
Telomerase	Phosphatidylinositol 4-kinase	Polo mitotic
SV40 large T antigen	Focal adhesion kinase	kinase
MTG8 myeloid leukemia protein	Bcr-abl	Tropomyosin
Tumor promoter-specific binding protein	Receptor-interacting protein	related kinase B
Hepatitis B virus reverse transcriptase	Translation initiation factor	(trkB
	kinase Gcn2	Mitogen-activated
	Insulin receptor	protein kinase
	PKR	MOK
	Akt kinase	Male germ cell-
	Mik1, Swe1	associated kinase
	3-Phosphoinositide-	MAK
	dependent kinase-1	MAK-related
		kinase
		dsRNA-
		dependent kinase

Table 2 - *continued.* Hsp90 Client Proteins

Other Hsp90 Clients	
G protein βγ	Proteasome
$G\alpha_o$	Lysosome
Gα12	Centrin/centrosome
Nitric oxide synthase (NOS)	Actin
Endothelial NOS	Tubulin
Neuronal NOS	Apoprotein B
Inducible NOS	β-galactosidase M15 truncation mutant
Protease-activated receptor 1 (PAR-1)	Pancreatic bile salt-dependent lipase
Cystic fibrosis transmembrane conductance regulator (CFTR)	Unassembled immunoglobulin chains
	Fanconi anemia group C protein
Atrial natriuretic peptide receptor	Thyroglobulin
Mammalian aminoacyl-tRNA synthetases	Macrophage scavenger receptor
Reovirus cell attachment protein σ1	*Plasmodium falciparum* erythrocyte membrane protein
HETE binding complex	
Apaf-1	
Cna2 catalytic unit of calcineruin	

MutL (226-228). The N-terminal domain has weak ATPase activity, which is enhanced by binding of client proteins (229-231). The highly charged linker region bridges the N and C-termini. It is specific for eukaryotic Hsp90, and may facilitate binding to some client proteins through alternating lysine and glutamic acid residues (so-called "KEKE motifs") (232). However, this region is dispensible for most Hsp90 functions (233). The C-terminal portion of Hsp90 contains a constitutive dimerization domain, and recent studies with the Hsp90 binding agent novobiocin suggest that it may contain a second ATP binding domain (234, 235). The far end of the C-terminus contains the sequence MEEVD, which is the binding site for the tetratricopeptide repeat (TPR) region that is present on most Hsp90 cochaperones (236). Given the recent information about the structural domains of Hsp90, a model for Hsp90 function has emerged (figure 6). The current model suggests that Hsp90 is constitutively dimerized at the C-terminus, with the ADP-bound N-terminal domains pointing in opposing directions. Upon ATP binding, the N-terminal regions dimerize, forming a molecular clamp around the client protein (237). The Hsp90 cochaperones appear to regulate the clamp by altering the ability of Hsp90 to bind/hydrolyze ATP (231, 238).

Hsp90 Cochaperones

The Hsp90 cochaperones are critical for regulation of the Hsp90 ATPase cycle

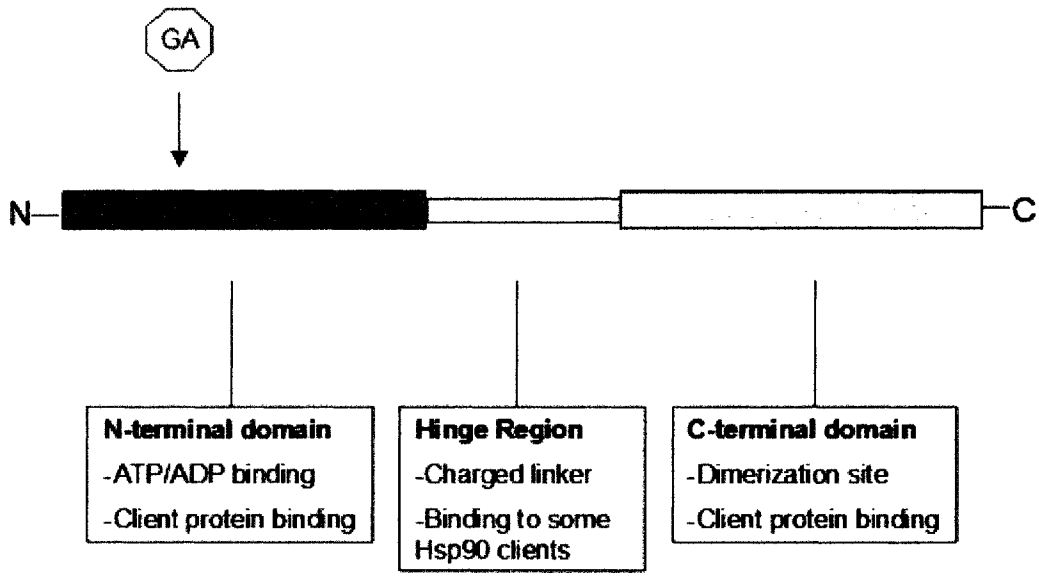


Figure 5: Hsp90 Structure

Hsp90 has two distinct funtional domains, which are separated by a highly charged linker. The N-terminal domain contains the ATP/ADP binding site and is important for binding to client proteins. The Hsp90-binding agent geldanamycin (GA) binds to the N-terminus and locks the protein in its ADP-bound conformation. The C-terminal domain is important for dimerization, and also binds client proteins. A role for the hinge region has been proposed in the binding of some Hsp90 clients, but it is generally dispensible for Hsp90 function.

and for substrate binding specificity. The role of cochaperones in the maturation of client proteins to an activatable complex has been intensively studied with the SHRs, and will be discussed further in the following section. Most Hsp90-cochaperones contain TPR domains, and interact with Hsp90 through TPR acceptor sites in the C-terminus of the molecule. Proteins that interact with Hsp90 in this manner include p60/Hop, protein phosphatase 5 (PP5), the E3 ubiquitin ligase CHIP, and the immunophillins FKPB51, FKBP52, and cyclophillin 40 (cyp40) (239). To date, p23 and p50/Cdc37 are the only Hsp90 cochaperones that do not contain the TPR motif. A newly identified protein, Aha1, accelerates Hsp90 ATPase activity and may bind to the central region of Hsp90 (240, 241). The main function of p60/Hop appears to be as a "protein bridge", which facilitates the physical association of Hsp70 and Hsp90 (242, 243). However, p60/Hop can also modulate Hsp90 activity. Studies have shown that binding of p60/Hop blocks ATP binding and the ATPase activity of Hsp90, locking it in an ADP-bound conformation (231, 238). The immunophillins bind to the mature Hsp90-cochaperone-client protein complex (the complex capable of high-affinity ligand binding), but their function has not yet been determined. Interestingly, they are not required for the formation of mature SHRs (244, 245), although there is some evidence to suggest that they may link Hsp90 to a dyenin motor protein to facilitate SHR translocation to the nucleus (246). CHIP recruitment to Hsp90 complexes leads to ubiquitination and proteasomal

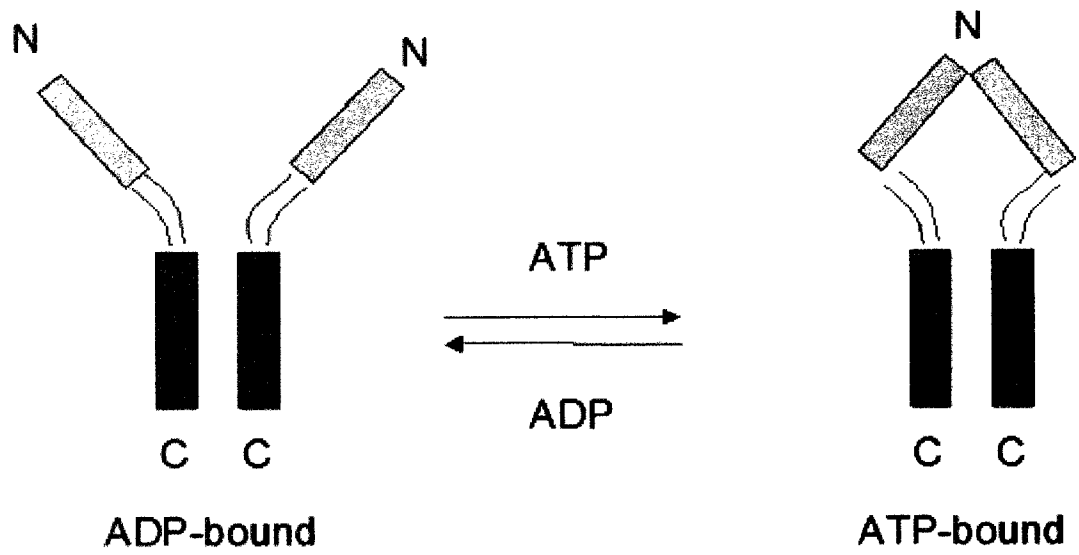


Figure 6: Proposed Mechanism for Hsp90 Function

In its ADP-bound form, Hsp90 is dimerized at the C-termini and the N-termini do not associate. ATP binding facilitates a conformational change in Hsp90, where the N-termini dimerize and form a "molecular clamp" around the client protein.

degradation of the client protein, and PP5 association is important for regulating client protein activity (246-248). Although the function of p23 is still uncertain, it can inhibit the aggregation of denatured proteins, indicating that it has some chaperoning activity independent of Hsp90 (249, 250). p23 binds to the N-terminal domain of Hsp90, but only when Hsp90 is in its ATP-bound conformation (251). It is associated with Hsp90 in mature Hsp90-client protein complexes, and is unable to bind when cells are treated with the ADP mimetics geldanamycin or radicicol (226, 252). Like p23, p50/Cdc37 does not interact with Hsp90 through TPR domains. It has been implicated in specifying the Hsp90-cochaperone complex to kinases such as Raf-1 (253).

The Role of Molecular Chaperones in Estrogen Receptor Function

Nearly twenty years ago, it was discovered that Hsp90 associates with steroid hormone receptors (254-256). Since then, extensive studies have been performed to determine the dynamics of SHR-chaperone interactions. Indeed, studies with the SHRs have determined that the assembly of receptors into mature complexes is preceded by the formation of several distinct SHR-chaperone complexes (246). Although the SHRs differ in structure, ligand binding specificity, and localization, they share similar chaperone requirements for their function (257). Most studies on SHR-chaperone interactions have been performed with the glucocorticoid receptor (GR) and progesterone receptor (PR),

and to a lesser extent, the ER. The Hsp90-chaperone complex that regulates these SHRs appears to be quite similar, and it has been suggested that other Hsp90 clients may require the same multichaperone machinery for activity (258).

Chaperone Requirements for Formation of the Mature Estrogen Receptor Complex

Early studies with the GR and PR utilized the cell-free rabbit reticulocyte lysate system to identify chaperone components required for assembly of the mature complex. It was initially shown that the *in vitro* GR translation product from reticulocyte lysate was indistinguishable from the native receptor in terms of steroid binding and association with DNA (259). It was subsequently determined that purified PR would assemble into a mature complex when added to reticulocyte lysate, indicating that protein translation was not a requirement for SHR maturation (260). Further study identified Hsp70, p60/Hop, p23, and the immunophillins as the components present in reticulocyte lysate that bind to the SHRs (261). Based on the findings in the reticulocyte lysate system, a minimal system for mature SHR complex assembly has now been determined. The chaperone reqirements for this system are: Hsp90, Hsp70, p60/Hop, Hsp40, and p23 (244, 245).

It has now been determined that Hsp90 binds to the ligand binding domain (LBD)

of SHRs. Interestingly, the interaction between Hsp90 and the ER LBD is much less stable than that of the GR or PR (262). It has recently been shown that the Hsp90 N-terminal ATP-binding domain (aa 1-224) is required for its interaction with the ER (263). Hsp90 is critical for maintaining the LBD of SHRs in a conformation that is capable of high-affinity ligand binding (264), and SHRs cannot be activated by ligand in the absence of Hsp90 (265). As with Hsp90, Hsp70 also binds the LBD of SHRs, and deletion of the LBD abrogates the Hsp70 interaction (262, 266). Recent studies have also shown that Hsp70 binds directly to Hsp90 and that these two chaperones cooperate to maintain the LBD in an activatable state (267, 268). Another mechanism for Hsp90-Hsp70 association during SHR maturation is through interactions with the cochaperone p60/Hop. p60/Hop acts as a physical linker by binding to Hsp70 via a TPR domain at the N-terminus, and to Hsp90 through a separate TPR domain at the center of the protein (269, 270). Further, the binding of p60/Hop to Hsp90 inhibits ATP binding and its ATPase activity (231, 238). This suggests that p60/Hop influences Hsp90's function as well as its association with Hsp70. p23 is a 23 kDa protein that associates only with the ATP-bound form of Hsp90 (271), and appears to stabilize mature Hsp90-SHR complexes (272). The Hsp90 binding site for p23 appears to be localized to the N-terminus, but requires an Hsp90 dimer for association (273). Hsp40 is not essential for the formation of a ligand binding-competent SHR, although it greatly enhances ligand binding activity (244, 245, 274). This is likely due to the ability of Hsp40 to bind to Hsp70

and increase its ATPase activity. The chaperones described above associate with SHRs in an ordered cycle to facilitate the formation of a mature receptor-multichaperone complex.

Mechanism of Estrogen Receptor-Hsp90 Multichaperone Complex Assembly

It is now clear that SHRs go through a series of ordered steps on the way to becoming mature complexes. The process has been recently reviewed (246), and can be segmented into three distinct steps: early complex formation, intermediate complex formation, and mature complex formation (figure 7). Initially, the hydrophobic hormone-binding cleft of the ER is closed and inaccessible to ligand. The function of the early chaperone complex is to open the hormone-binding region to facilitate ligand interactions. To form the early complex, Hsp70 and Hsp40 associate with the ER in an ATP-dependent manner (275, 276). The ADP-bound form of Hsp90 binds with high affinity to hydrophobic substrates (271, 277), and it appears that the initial association of Hsp70 and Hsp40 with the LBD of the receptor partially exposes the hydrophobic residues in the LBD. This allows for subsequent recruitment of ADP-bound Hsp90 and p60/Hop to the LBD. The ER complex containing Hsp 90, Hsp70, Hsp40, and p60/Hop is known as the intermediate complex. As the hydrophobic ligand-binding cleft is opened, Hsp90 converts to its ATP-bound conformation

and Hsp70, Hsp40 and p60/Hop dissociate from the complex. p23 associates with and stabilizes the ATP-bound form of Hsp90, and an immunophillin (Cyp40, FKPB52) binds to the free TPR acceptor site following the release of p60/Hop. The ER-chaperone complex is now in its mature conformation, and the ligand-binding domain is accessible. It is important to note that this process is dynamic (264), and the stages in the cycle likely represent equilibrium states of ER-chaperone interactions.

Chaperone Involvement Following Estrogen Receptor Activation

Until recently, there appeared to be no role for molecular chaperones in SHR regulation following ligand binding. The currently accepted model for ER activation involves E2 binding to the ER in the nucleus, which induces conformational changes that cause dissociation of the chaperone machinery (278). The ER then dimerizes and associates with EREs to activate transcription of target genes. However, a recent report by *Freeman et al.* suggests that Hsp90 and p23 may be involved in disassembly of the SHR transcriptional complex following receptor stimulation (279). In this study, it was shown that targeted

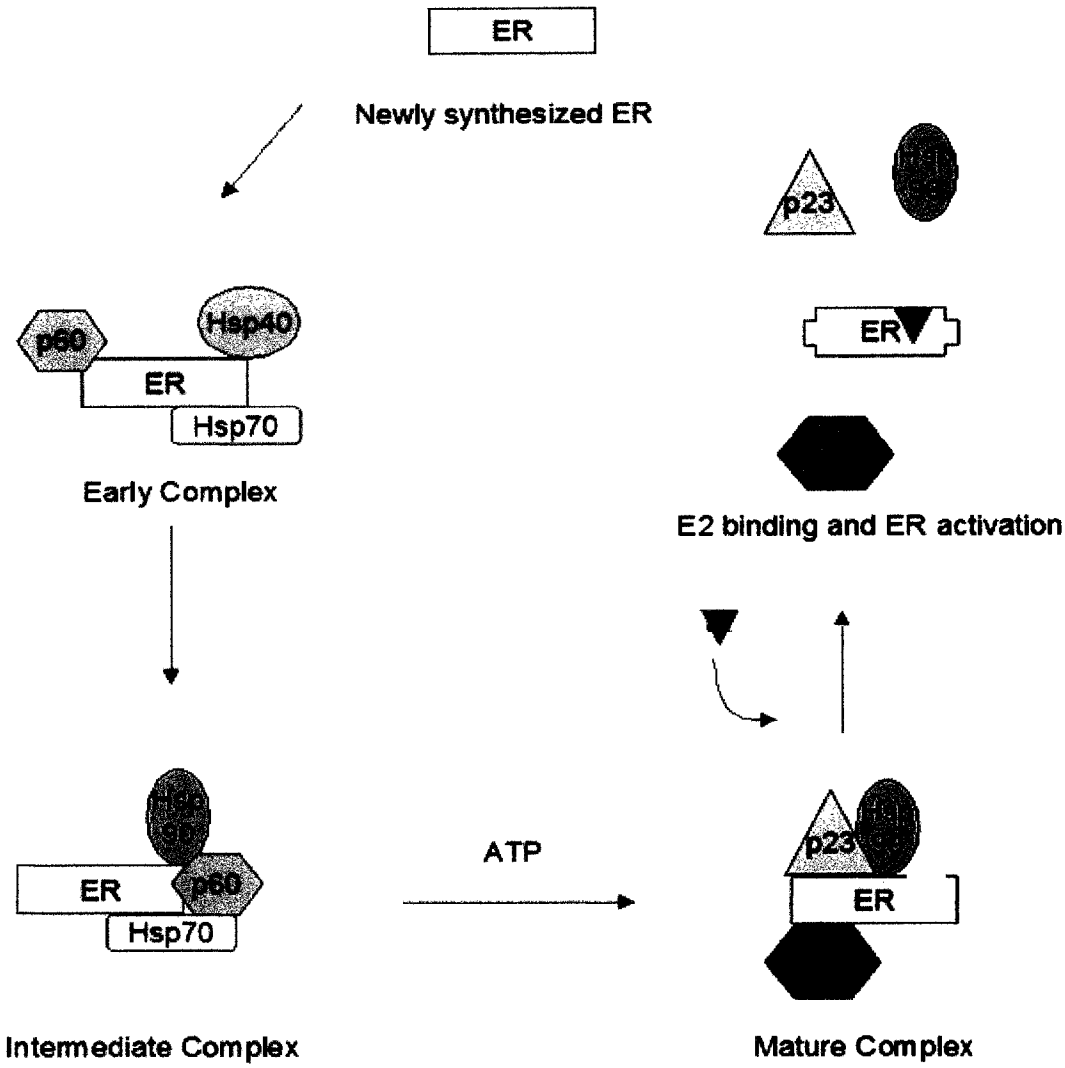


Figure 7: The Role of Molecular Chaperones in ER Maturation

The three steps in ER maturation: The newly-synthesized ER associates with Hsp70, Hsp40, and the co-chaperone p60/Hop. The hydrophobic hormone-binding domain is partially exposed in this complex, and Hsp90 binds to this region and displaces Hsp40 to form the intermediate complex. In an ATP-

dependent step, the ATP-bound form of Hsp90 fully exposes the hormone-binding domain, and the cochaperone p23 stabilizes the ATP-bound Hsp90. Cyclophillin 40 fills the open TPR-acceptor site on Hsp90 to complete the mature complex. Estrogen binding results in a conformational change in the ER, which causes release of the chaperone components and subsequent transcriptional activation of ER target genes.

expression of p23, and to a lesser extent Hsp90, at glucocorticoid responsive elements inhibited GR transcriptional activity in reporter construct assays. Furthermore, using chromatin immunoprecipitation techniques, p23 and Hsp90, but not Hsp70, were found to be localized to a GRE following stimulation of rat hepatoma HTC cells with dexamethasone. These findings indicate that molecular chaperones may indeed play a role in SHR regulation following hormone binding. Specifically, p23 and Hsp90 appear to act as negative regulators of SHR-mediated transcription by facilitating disassembly of SHR transcriptional complexes.

Heat Shock Protein 90 as a Target for Anti-Cancer Therapy

Given the unique ability of Hsp90 to chaperone signaling proteins involved in cell proliferation and survival, Hsp90 has emerged as a potential target for anticancer therapy. Hsp90 is an essential protein that is not mutated in cancer cells, making it a stable target for therapeutic intervention. This provides advantages over current targeted small molecule therapies, such as STI-571 (gleevec), where mutation of the target protein leads to acquired resistance (280). Additionally, Hsp90 is frequently overexpressed at 2-10 fold higher levels in cancer cells versus normal cells (281). One possible reason for this enhanced expression may be related to the inherent genetic instability that cancer cells possess. For example, mutated oncogenic signaling proteins likely require enhanced Hsp90

association to maintain proper folding. The Hsp90 requirements for a normal cell are low, and studies in yeast have demonstrated cell survival with only 5% of normal Hsp90 levels (282, 283). Thus, the increased requirement for Hsp90 in cancer cells may provide a therapeutic advantage against tumors selectively. In a recent study by *Kamal et al.*, further evidence for tumor selectivity of Hsp90 binding agents was described (284). It was shown that in tumor cells, most of the Hsp90 is present in multi-chaperone complexes, while the Hsp90 in normal cells is not. Competitive binding assays determined that 17AAG bound to complexed Hsp90 with 100-fold greater affinity, providing a possible mechanism for its tumor selectivity.

In 1992, it was discovered that the benzoquinone ansamycin antibiotics possessed potent antitumor activity that was unrelated to their proposed mechanism of action as inhibitors of the tyrosine kinase src (285). Two years later, the target for these agents was identified as Hsp90 (286). The compound geldanamycin (GA), in particular, bound to Hsp90 with high affinity and inhibited its association with v-src. It was also shown that disruption of this interaction led to destabilization of v-src, suggesting a possible mechanism for drug-mediated inhibition of v-src activity. The GA binding site on Hsp90 has now been identified as the ATP-binding pocket in the N-terminus (226, 252, 277). GA acts as an ADP mimetic, and binds with much higher affinity than ADP or ATP (287). GA binding locks Hsp90 in its ADP-bound conformation, and prevents maturation to

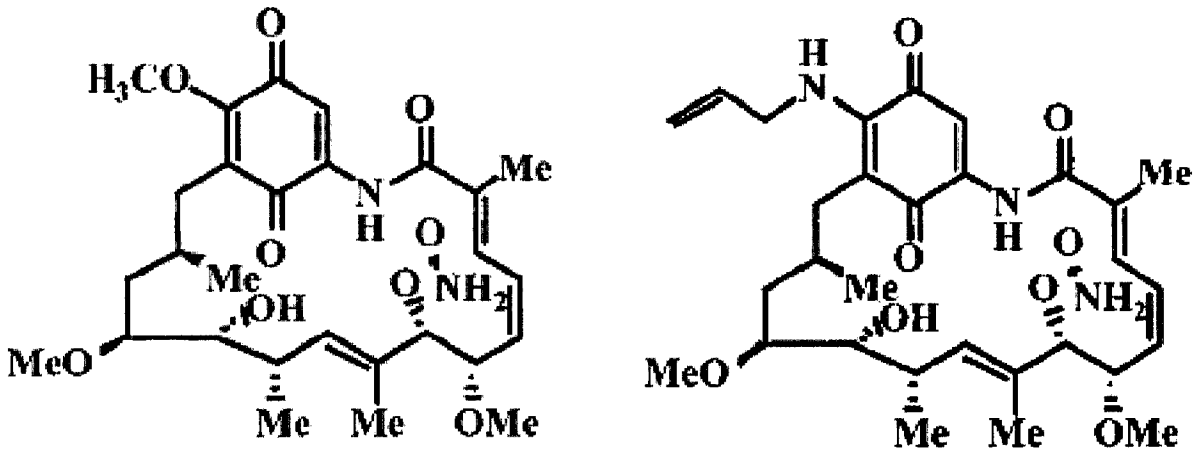
the Hsp90-client protein multichaperone complex containing p23. This results in accumulation of the client in the intermediate complex containing p60/Hop and Hsp70. The client protein is then ubiquitinated and targeted for proteasomal degradation. Recent reports have shown that the E3 ubiquitin ligase CHIP associates with the intermediate complex and promotes the ubiquitination and subsequent degradation of some Hsp90 client proteins (288, 289). Therefore, the general mechanism for disruption of Hsp90 client protein activity is through enhanced degradation. A number of Hsp90 clients that are mutated and/or overexpressed in cancer are sensitive to GA-induced degradation. The list includes mutant p53 (290, 291), ErbB2 (292-294), Raf-1 (295), Akt (296, 297), the Bcr-Abl fusion protein (298, 299), the cyclin-dependent kinases Cdk4 and Cdk6 (300), hypoxia-inducible factor 1α (HIF-1α) (301), and steroid hormone receptors (302-306). Of specific interest to our lab, GA has been shown to downregulate the levels of Hsp90 clients *in vitro* and *in vivo* in breast and prostate cancer cell lines (304, 307). Furthermore, GA inhibits tumor growth in E2-dependent breast cancer and androgen-dependent and independent prostate cancer models, suggesting that it could be a useful agent for the treatment of hormone-dependent and independent tumors in the clinic.

The observation that GA leads to the degradation of many oncogenic client proteins has led to clinical development of the drug. The less hepatotoxic GA derivative, 17-demethoxy-17-allylaminogeldanamycin (17AAG), is now in phase I

clinical trials as a single agent, and biological activity has been achieved at tolerable drug concentrations (308) (for structure comparison, see figure 8). The Hsp90 binding agents radicicol and novobiovin retain similar activities to GA, but are not ansamycin antibiotics. Thus, these compounds, due to their divergent structure, may prove more effective in the clinic by avoiding the hepatocellular toxicity observed with 17AAG. However, this remains to be determined. Other approaches for more specific targeting of GA have involved derivitization of the drug to E2 and the PI3-kinase inhibitor LY294002 (309, 310). Another potential use for Hsp90 binding agents in the clinic is for sensitization to chemotherapy. Overexpression of some Hsp90 client proteins in cancer can lead to resistance to chemotherapy (311), and several labs have reported sensitization following 17AAG treatment. *Blagosklonny et al.* showed that 17AAG sensitizes bcr-abl positive leukemias to doxorubicin therapy (312), and other reports have shown enhanced cytotoxicity with taxol in breast cancer (313, 314) and in non-small-cell lung cancer (315, 316). Thus, the Hsp90 binding agents represent a class of anticancer drugs with a unique mechanism of action, and may be effective in the clinic, either alone or in combination with other agents.

Summary

The molecular chaperones are a family of highly conserved proteins that act to maintain proper protein folding within the cell. Their functions are diverse, and



GA **17AAG**

Figure 8: The Hsp90 Binding Agents GA and 17AAG

Geldanamycin (GA) is a benzoquinone ansamycin antibiotic that binds to the ATP/ADP-binding pocket of Hsp90 with greater affinity than ATP or ADP. GA locks Hsp90 in it's ADP-bound form, and does not allow formation of the mature ER-chaperone complex. This results in accumulation of the intermediate complex and enhanced degradation of the ER. 17-demethoxy-17-allylaminogeldanamycin (17AAG) displays less toxicity and greater activity *in vivo*, and is currently in phase I clinical trials.

include the stabilization of nascent polypeptide chains, prevention of protein aggregation, and transport through cell membranes. They were first discovered as proteins that displayed increased expression under elevated temperature, and were termed "heat shock proteins". There are six distinct subclasses of molecular chaperones: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small hsps (20-25kDa). There are two isoforms of Hsp90 in humans: the constitutive Hsp90β and the inducible Hsp90α. A unique function of Hsp90 is its ability to stabilize a specific set of client proteins, many of which are involved in signal transduction. Hsp90 cooperates with a subset of chaperones and cochaperones to maintain client proteins in a conformation that facilitates activation. The Hsp90 protein contains an N-terminal ATP-binding domain, a highly charged central linker, and a C-terminal domain containing a dimerization and TPR acceptor site. Regulation of Hsp90 clients has been extensively studied with the SHRs, and seems to involve at least three ordered steps that are likely applicable to other Hsp90 clients. In the first step, Hsp40 and Hsp70 bind to the LBD in an ATP-dependent manner. This "early complex" partially opens the hydrophobic hormone-binding region. ADP-bound Hsp90 has a high affinity for hydrophobic residues and associates with the partially exposed LBD. p60/Hop has high affinity for ADP-bound Hsp90, and associates with the complex. The two TPR domains on p60/Hop bind to acceptor sites on Hsp90 and Hsp70, linking the two chaperones. This is called the "intermediate complex". Finally, Hsp90 binds ATP, and the chaperone members of the intermediate complex dissociate. The

free TPR acceptor site is bound by an immunophillin, and the ATP-bound conformation of Hsp90 is stabilized by p23. The LBD is now exposed and the SHR is in a mature conformation that is capable of high affinity ligand binding. Upon activation, the chaperone complex dissociates, and receptor-mediated gene transcription occurs. Interestingly, p23 and Hsp90 overexpression inhibits dexamethasone-induced gene transcription at GREs, indicating that these chaperones may be important in disassembly of SHR multiprotein complexes from the DNA.

Hsp90 is overexpressed 2-10 fold in many malignancies. Furthermore, no Hsp90 mutants in cancer have been identified, making it a stable target for therapeutic intervention. The Hsp90 binding agent GA binds to Hsp90 with high affinity and modulates its function. GA is an ADP mimetic, and binds to the ATP-binding pocket of Hsp90 with greater affinity than ATP or ADP. Thus, GA binding locks Hsp90 in its ADP-bound form and inhibits the association of p23. This blocks the normal maturation cycle for client proteins, and results in the accumulation of clients in the intermediate complex with p60/Hop and Hsp70. Proteins in this complex are then targeted for ubiquitination and subsequent proteasomal degradation. Indeed, it has been shown that the E3 ubiquitin ligase CHIP associates with the intermediate complex and targets proteins to the proteasome. Signaling proteins that are downregulated by GA and play a role in malignant transformation include: ErbB2, Akt, Hif-1α, Raf-1, Cdk4 and Cdk6, bcr-abl, and

the steroid hormone receptors. The GA derivative, 17AAG, displays less hepatocellular toxicity and is currently in clinical trials as a single agent. Interestingly, recent reports using mouse models of human breast and prostate cancer have indicated that 17AAG may be effective against both hormone-sensitive and hormone-resistant tumors. Another potential use for Hsp90 inhibitors in cancer therapy is for sensitizing tumors to chemotherapeutic agents. Indeed, several investigators have shown that 17AAG sensitizes tumor cells to doxorubicin and taxol. The Hsp90 binding agents radicicol and novobiocin also cause destabilization of Hsp90 clients, and they may display less hepatocellular toxicity than 17AAG. Thus, the Hsp90 binding agents represent a class of anticancer drugs with a unique mechanism of action, and may be effective in the clinic, either alone or in combination with other agents.

Statement of the Problem

The ER is a well-validated target for breast cancer therapy. It is essential for the growth of normal mammary epithelium, and its levels/activity are elevated in a majority of breast tumors. Therefore, the identification and characterization of pharmacological modulators of ER function has been the subject of intensive study over the last several decades. Current methods for ER inhibition involve the use of agents that directly compete for E2 binding to the ER (SERMs, pure antiestrogens), or compounds that reduce the levels of circulating E2 (aromatase

inhibitors, LHRH analogs). Tam is the best-characterized ER modulator, and has been used in the clinic for nearly 30 years. It is initially effective in nearly all ER+ patients treated. However, recurrence of Tam-resistant breast cancer is common. The observation that Tam-resistant cells are sensitive to pure antiestrogens is promising. However, resistance to these compounds has also been observed, indicating that treatment with both agents may only delay the resistant phenotype. The aromatase inhibitors and LHRH analogs are less specific, and decrease circulating E2 levels. Again, Tam-resistant tumors are sensitive to these agents, but they eventually progress. The mechanisms of resistance to ER-targeted therapy are complex and poorly understood. However, mutation/deletion of ER is not common in resistant tumors, indicating that the ER is still a valid target for anticancer therapy. Furthermore, breast cancers with acquired resistance to one agent are sensitive to other ER modulators, suggesting that a functional ER is important in the progression of resistant tumors.

The ER is an Hsp90 client protein, and requires interaction with Hsp90 for its stability. Treatment with GA/17AAG enhances ER degradation through the proteasome *in vitro* and inhibits the growth of ER+ breast cancer xenografts *in vivo*. Furthermore, both androgen-dependent and independent prostate cancers are sensitive to 17AAG *in vitro* and *in vivo*. Therefore, we sought to determine whether GA/17AAG is an effective agent for the treatment of Tam-resistant

breast cancer by inhibiting the function of several key proteins involved in the progression of the disease, including the ER, by inducing their degradation. Importantly, GA/17AAG induces degradation of ER protein in a ligand-independent manner, and acquired resistance to Hsp90 binding agents has not been described. Thus, Hsp90 represents a stable therapeutic target for ER modulation in Tam-resistant breast cancer. In addition to ER modulation, GA/17AAG induces the degradation of a number of signaling proteins implicated in the progression of breast cancer, including ErbB2, Raf-1, and Akt. Thus, the combined decrease in the levels of these Hsp90 clients may potentially lead to increased antitumor activity. In the following studies, we sought to determine whether the Hsp90 binding agents GA and 17AAG could prove useful as anticancer agents against tumors with acquired Tam-resistance and could induce the degradation of the ER and other oncogenic signaling proteins in those tumors (chapter III), and to understand more about the mechanisms by which these drugs stimulate ER degradation (chapter IV).

II. EXPERIMENTAL PROCEDURES

Cells and Reagents

MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD). TAMR-1, TAMR-7, and TAMR-8 cells were generated in the laboratory of A. E. Lykkesfeldt and have been previously described (134, 317). Cells were cultured at 37°C under 6% CO_2 in air using phenol red-free DME/F12 medium (Invitrogen, Inc.) containing 1% fetal bovine serum (Irvine Scientific, Santa Ana, CA) and 6ng/ml insulin. For chromatin immunoprecipitation (ChIP) assays, MCF-7 cells were maintained in 2% or 10% fetal bovine serum, and cells were grown for at least 3 days in 2% or 10% charcoal-stripped serum prior to experimentation. Cells were re-fed twice weekly and passaged 1:5 once weekly. To maintain high-level resistance in TAMR cell lines, medium was supplemented with Tam (1μM). All cell lines routinely tested negative for *Mycoplasma* by ELISA. The C3 luciferase reporter plasmid (C3-luc) was provided by D.P. McDonnell, and has been described previously (118). All biochemical reagents were from Sigma unless otherwise specified. GA (NSC 122750) and 17AAG (NSC 330507) were provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute. Stock solutions were formulated in DMSO and maintained at -70°C in the dark prior to use within one month. For immunodetection of the ER, a cocktail of monoclonal antibodies

1D5 and 6F11 was used at 1:1000 for immunoblotting and 1:100 for immunofluorescent cell staining (Neomarkers, Fremont, CA). For ER immunoprecipitation, clone AER310 was used (Neomarkers, 3μg/reaction). Anti-Akt (Cat# 9272) and Phospho-Akt (Ser473, Cat# 9271) were used at 1:1000 for immunoblotting (Cell Signaling; Beverly, MA). Additional immunoblotting antibodies included: Anti-raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA; clone E-10; 1:250), anti-Hsp 72 (Stressgen, Victoria, B.C.; clone C92F3A-5; 1:5000), anti-p60/Hop (clone F5, D. Smith, Mayo Clinic, Scottsdale, AZ) and anti-Hsp 90 (Stressgen; clone AC88; 1:500). Anti-p23 ascites was provided by D. O. Toft and purchased from Affinity Bioreagents (clone JJ3; 1:1000). Antibodies (5μg total antibody/immunoprecipitation) used in ChIP experiments were: anti-ER Ab-1, Ab-3, and Ab-9 (Neomarkers); anti-p23 clone JJ3 (D.O.Toft and Affinity Bioreagents); anti-Hsp90 AC88 (Stressgen) and F8 (Santa Cruz)

Immunoblotting

Chapter 3

To analyze protein levels, breast cancer cells were harvested by scraping into non-ionic detergent buffer supplemented with protease and phosphatase inhibitors as previously described (318) Tumor lysates were prepared by grinding snap-frozen tissue into a powder over a dry ice/ethanol bath. Lysis

buffer was then added directly to the powder followed by extraction on ice for 15 minutes. All lysates were clarified by centrifugation at 14,000 X *g* for 30 min at 4°C. Supernatants were collected, and equal amounts of protein as determined by bicinchoninic acid assay (BCA; Pierce, Rockford, IL) were resolved by SDS-PAGE using 7.5% or 12% gels. Proteins were electro-blotted to nitrocellulose membranes, which were subsequently stained with Ponceau S to confirm that equal amounts of protein were loaded and transferred. Membranes were then blocked with 3% milk and immunoblotted for the protein of interest. Species-appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoLabs, PA, 1:100,000) and chemiluminescent substrate (Pierce) followed by exposure to Kodak XAR-5 film were used for detection. Multiple exposure times were evaluated for each blot to ensure that the band intensities were within the dynamic response range of the film.

Chapter 4

Cells were grown for at least 24 hours in DME/F12 medium supplemented with 1% charcoal-stripped serum. Treatments were prepared in fresh, pre-warmed medium and cells were exposed for the indicated times. Cell lysates were prepared by scraping cells into TNES buffer (50mM Tris, 2mM EDTA, 100mM NaCl, 1% NP-40) or TNEK buffer (50mM Tris, 2mM EDTA, 100mM KCl, 1% NP-40) containing protease inhibitors. For salt extraction experiments, cells were

lysed with TNEK buffer supplemented with 400mM KCl. Lysates were cleared by centrifugation and, where applicable, the pellet fractions were lysed in the appropriate lysis buffer containing 2% SDS at 100°C for 15 min. Needles (25 gauge) were used to shear genomic DNA in the pellet fractions prior to gel loading. Equal amounts of protein were determined by bicinchoninic acid assay (BCA; Pierce, Rockford, IL) and resolved by SDS-PAGE using 7.5% gels. Proteins were electro-blotted onto nitrocellulose membranes, stained with Ponceau S to determine equal gel loading, blocked with 3% milk, and immunoblotted for the relevant protein. Species-specific horseradish peroxidase secondary antibodies (Jackson Immunolabs, PA) and chemiluminescent substrate (Pierce) were used for detection on Kodak XAR-5 film.

Reporter Construct Assays

MCF-7 and TAMR-1 cells were inoculated into 24 well plates at 1-1.5x10^5 cells/well. The following day, wells were transfected with 0.9ug C3-luc inducible reporter and 0.1ug pRLnull constitutive Renilla reporter (Promega) using Lipofectamine Plus reagent (Invitrogen). After 3 hours, transfection medium was replaced with pre-warmed serum free media +/- 2μM 17AAG. After an additional 3-5 hours, wells were supplemented with 17β estradiol (E2, 5μM) or E2 (5μM) plus 17AAG (2μM). After 24 hours, the firefly and renilla luciferase activities of cell lysates were quantitated using a dual luciferase assay system (Promega)

and a Femtomaster FB 12 single tube luminometer (Zylux Corporation) per manufacturer's recommendations.

Immunostaining

Immunofluorescent staining was performed on monolayer cultures and on frozen tumor sections mounted on glass slides. Monolayer cultures were established using $1x10^5$ cells/well in 8-well chamber slides and allowed to adhere overnight (Nalge Nunc International). After incubation with test compounds overnight in serum-free medium, cells were fixed in 4% freshly prepared paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 for 30 min. Slides were then blocked for 30 min in phosphate-buffered saline (PBS) containing 10% goat serum and 0.3% BSA. Anti-ER primary antibody was applied in PBS containing 1% goat serum, 0.3% BSA and Rnase A (20 μg/ml) and incubation carried out overnight at 4°C. After extensive washing, slides were incubated for 60 min with Alexafluor-conjugated goat anti-mouse $F(ab')_2$ fragment secondary antibody (Molecular Probes; Eugene, OR). Cells were then incubated with 4',6 diamidino-2-phenylindole (DAPI, 1μg/ml) in PBS for 15 min followed by extensive washing and mounting with Cytoseal 60 under coverslips (VWR Scientific). The staining procedure for snap-frozen tumor sections was identical to that used for chamber slides, except that 1% Triton X-100 was used to permeabilize the tissue. Images in chapter IV were acquired by confocal microscopy (MRC 5000, BioRad) using a

60x objective with identical gain, black level, and iris settings.

Estrogen Receptor Quantitation by Immunofluorescent Staining and Laser-Scanning Cytometry

Laser-scanning cytometry (LSC) is a microscope-based cytofluorometric technique that combines the advantages of flow cytometry with image analysis (319, 320). To measure relative ER levels, immunostained chamber slides and frozen tumor sections were analyzed on a Model LSC-1 instrument using sequential violet and argon laser excitation and the appropriate filter sets (CompuCyte Corp; Cambridge, MA). Individual cells were identified by contouring on their DAPI-stained nuclei. The relative ER level for each cell identified was defined by maximum pixel intensity and frequency histograms were acquired for each experimental sample in a manner analogous to standard flow cytometry. Appropriate size gates were used to omit multi-cell events. At least 3,000 events per sample were analyzed per chamber slide and at least 5,000 events per sample were acquired to evaluate tumor sections. To complement the quantitative data generated by LSC, the same slides examined by LSC were also evaluated qualitatively by confocal microscopy (MRC 5000, BioRad). Images in chapter III were acquired using a 20X objective using identical gain, black level and iris settings.

Immunoprecipitation

MCF-7 cells were plated in 10-cm dishes, grown to ~70% confluence and then hormone-deprived by incubation in medium containing 1% charcoal-stripped serum for 48h. To examine drug effects on the composition of ER-chaperone protein complexes, cells were incubated for 2hr with various test compounds and then scraped into cold, molybdate-containing lysis buffer as previously described (321). After brief sonication on ice, lysates were cleared by centrifugation at 14,000 X *g* for 30 min at 4°C, and supernatants were incubated with ER-specific primary antibody, for 3-4 hours at 4°C. Protein G-Sepharose beads (GammaBind Plus, Pharmacia;15μl resin/precipitation) were added, and incubation continued for an additional hour with gentle agitation in the cold. Beads were spun down, washed 4 times, and bound proteins eluted into 1X Laemmli sample loading buffer by heating at 95°C for 5 min. Samples were then resolved on 7.5% or 12% SDS-PAGE gels and immunoblotted as described above.

In vivo Studies

To examine the anti-tumor activity of 17AAG against hormone-refractory breast tumors, TAMR-1 cells ($5x10^6$) suspended in 200 μl of a 1:1 mixture of saline and Matrigel (Discovery Labware, Inc.; Bedford, MA) were injected into the mammary

fat pad of SCID mice (Arizona Cancer Center breeding colony, C.B.-17/IcrACCscid). The following day, hormone supplementation was begun as daily subcutaneous injections of Tam (500μg/dose) dissolved in peanut oil. Injections were continued 5 times per week for the duration of the experiment. Eleven days post cell inoculation, mice with palpable tumors were randomized to receive i.p. injections of 17AAG (100mg/kg) or an equal volume of DMSO vehicle 3X weekly for two weeks. To assess drug effects on tumor progression, serial caliper measurements were performed for 77 days (8 mice/group) and tumor volume calculated using the equation: length x width^2/2. To assay for 17AAG-mediated target modulation, TAMR-1 tumor xenografts were established and mice treated as described above. Eighteen hours after the second 17AAG injection, mice were sacrificed, and tumors were removed. Half of each tumor was snap-frozen in OCT embedding medium (Tissue-Tek, Torrance, CA), and cryotome sections were prepared for immunofluorescence staining. The other half was snap-frozen for preparation of lysates and immunoblot analysis. For quality control purposes, tumor lysates were routinely fractionated by 7.5% SDS-PAGE, followed by coomassie staining, to evaluate the overall integrity of the sample. Only those samples found to be free from extensive proteolysis or heavy contamination by serum albumin were evaluated by immunoblotting for specific protein levels. All *in vivo* experiments were performed under protocols approved by the University of Arizona Institutional Animal Care and Use Committee.

Chromatin Immunoprecipitation

This procedure was developed based on previously published techniques (76, 279), with modifications. MCF-7 cells were grown in 5% charcoal-stripped serum for at least 3 days prior to experimentation. After treatments, ER-DNA complexes were crosslinked by adding 11X crosslinking buffer (50mM Hepes-KOH, pH 8, 1mM EDTA, 0.5mM EGTA, 100mM NaCl, 11% paraformaldehyde) directly to the culture medium to attain a final concentration of 1X. Cells were incubated at room temperature for 10 min with agitation. Cells were then washed twice with cold PBS, scraped into cold PBS, and centrifuged at ~800g for 5 min. Cells were lysed in 300μl ChIP lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1, aprotinin, leupeptin, and PMSF) and chromatin was sheared by sonication using a Fisher model F60 sonic dismembrator at maximum power. Lysates were sonicated on ice using 10 x 15 sec bursts and cooled on ice for 1 min between bursts. Immunoprecipitation, reversal of formaldehyde crosslinks, and DNA purification was performed as described by *Shang et al.* (322). For PCR, 1μl of input DNA and 20μl of ChIP DNA was amplified using the following PCR cycle protocol: 94°C 4 min; 94°C 45 sec, 50°C 45 sec, 72°C 20 sec x 23 cycles; 72°C 2 min. PCR products were resolved on 1.2% agarose gels and visualized by staining with ethidium bromide. Images were acquired using the Gel Doc imaging system (Bio Rad).

III. HSP90 INHIBITION AS AN ALTERNATIVE THERAPY FOR THE TREATMENT OF ANTIESTROGEN-RESISTANT BREAST CANCER

Introduction

The estrogen receptor has become an important target in the management of hormone-responsive breast cancer and the anti-estrogen tamoxifen is now a standard component of front-line therapy for ER+ breast cancers, inducing remissions in over half of patients treated (323). Unfortunately, most tumors eventually become Tam resistant. The mechanisms that underlie this resistance are poorly understood, but appear to include alterations in ER expression, structure, or the association of cofactors involved in ER transactivation (324, 325). Furthermore, Tam is known to exhibit partial agonist activity in breast cancer cell lines, which may compromise its antitumor activity (326). The antiestrogens ICI 164,384 and ICI 182,780 exhibit pure antagonist properties, and are able to inhibit the growth of Tam resistant cell lines (134, 327). However, resistance to these pure antiestrogens has also been observed (165). Thus, while it is possible to delay breast tumor progression with currently available hormone antagonists, the frequent development of resistance remains a major limitation to their overall effectiveness as anticancer agents.

To address the problem of resistance, we have been examining the feasibility of

targeting heat shock proteins to disrupt ER function in a manner that does not rely on the interaction of antagonist with the hormone binding subunit of the receptor (304). Steroid hormone receptors exist in multi-protein complexes containing heat shock protein 90 and other essential molecular chaperone proteins (reviewed in (328)). Iterative, low affinity interactions with these complexes are required to maintain the receptor in a mature conformation that is capable of binding ligand with high affinity (329, 330). Upon ligand binding, these interactions are altered, allowing the receptor to bind DNA tightly, recruit co-activators and/or repressors and regulate target gene expression (331). Over the past decade, we and others have shown that the antitumor antibiotics geldanamycin and radicicol act as selective Hsp90 inhibitors with the ability to alter the stability and activity of steroid hormone receptors (303) as well as numerous cancer-associated growth factor receptors, kinases, and transcription factors (reviewed in (311, 332)). Due to this novel mechanism of action, considerable enthusiasm exists for the development of Hsp90 inhibitors as anticancer agents. NCI-sponsored Phase I trials of the GA derivative 17-allylaminogeldanamycin are nearing completion (333-336).

In the case of steroid receptors, drug-induced alterations in Hsp90 function clearly lead to rapid loss of hormone binding and enhanced proteasome-mediated degradation of the progesterone (321), androgen (307, 337), and glucocorticoid receptors (306). Consistent with previous reports that the ER does

not require ongoing Hsp90 interaction to maintain its high affinity hormone binding conformation (338, 339), we found that Hsp90 inhibitors do not immediately disrupt estrogen binding in whole cells. However, they do deplete cellular ER levels and impair receptor function both *in vitro* and in estrogen-supplemented mice bearing hormone-responsive breast tumor xenografts (304, 326, 340). In the current study, we now extend these observations to hormone-refractory breast cancer and assess the feasibility of disrupting hormone signaling in this setting using a chaperone-targeted, ligand-independent approach. We found that GA and its clinically relevant derivative 17-allylamino-17-demethoxygeldanamycin can destabilize the ER and inhibit its ability to activate gene expression in both Tam-sensitive and Tam-resistant breast cancer cells. Furthermore, 17AAG treatment of tumor-bearing mice inhibited the growth of Tam-resistant tumors. As expected, growth inhibition was associated with modulation of several well-recognized Hsp90 client proteins. Surprisingly, however, we found that Tam, unlike estrogen, inhibited ER destabilization by GA and 17AAG both *in vitro* and *in vivo*. The precise mechanism underlying this Tam-related effect is under investigation currently, but it does not appear to involve inhibition of GA-induced alterations in the composition of ER-associated chaperone complexes. Taken together, our results indicate that 17AAG can disrupt ER function in hormone-refractory breast cancer but that combined therapy with Tam and Hsp90 inhibitors may prove problematic.

Results

Geldanamycin Depletes the Estrogen Receptor and Other Hsp90 Client Proteins in Hormone-Refractory Cells

To evaluate the effects of GA on Tam-sensitive and Tam-resistant breast cancer cells, we used MCF-7 and three Tam-resistant subclones of MCF-7 (TAMR-1, TAMR-7, and TAMR-8), which have been described previously (317). All TAMR cell lines express readily detectable amounts of ER protein, although at somewhat reduced levels compared to the parental MCF-7 cells. Studies with the TAMR-1 cell line have shown that it is E2-responsive and contains no ERα mutations (341). Cells were exposed to GA, Tam, E2, or combinations thereof overnight in low serum-containing media. Western blot analysis revealed that GA depleted ER (figure 9*A*), Akt (figure 9*B*), and Raf-1 (figure 9*C*) levels in all cell lines tested. Membranes were stained with Ponceau-S prior to western blotting, and equal protein loading was confirmed (figure 10). A clear induction of the Hsp90-regulated gene Hsp72 is observed on the Ponceau S-stained membranes in lysates exposed to GA, as has been previously described (304). As expected from previous reports, Tam exposure resulted in a slight increase in ER levels (figure 9*A*, lane 2) (132, 342), while there was a substantial decrease in ER protein upon E2 stimulation (figure 9*A*, lane 3) (343, 344). Unexpectedly,

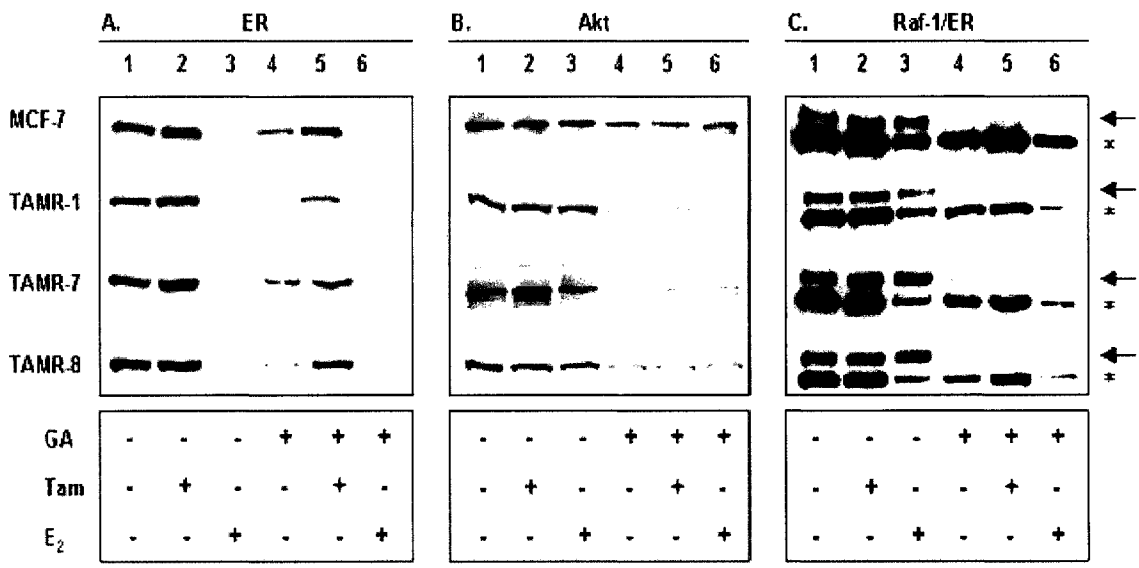


Figure 9: Modulation of Hsp90 Client Proteins by GA in Tam-Sensitive and Tam-Resistant Cell Lines

Modulation of ER, Akt, and Raf-1 levels by GA was compared in Tam-sensitive MCF-7 breast cancer cells and three Tam-resistant variants of MCF-7. Cells were treated overnight with EtOH vehicle (lane 1), Tam (1μM, lane 2), E2 (1μM, lane 3), GA (1μM, lane 4), Tam + GA (1μM each, lane 5), or E2 + GA (1μM each, lane 6). Lysates were prepared and analyzed by immunoblotting for ER (panel A), Akt (panel B), or Raf-1 (panel C). In panel C, the same blot presented in panel A was re-probed for Raf-1. The Raf-1 band is indicated by an arrow and the ER band is indicated by an asterisk.

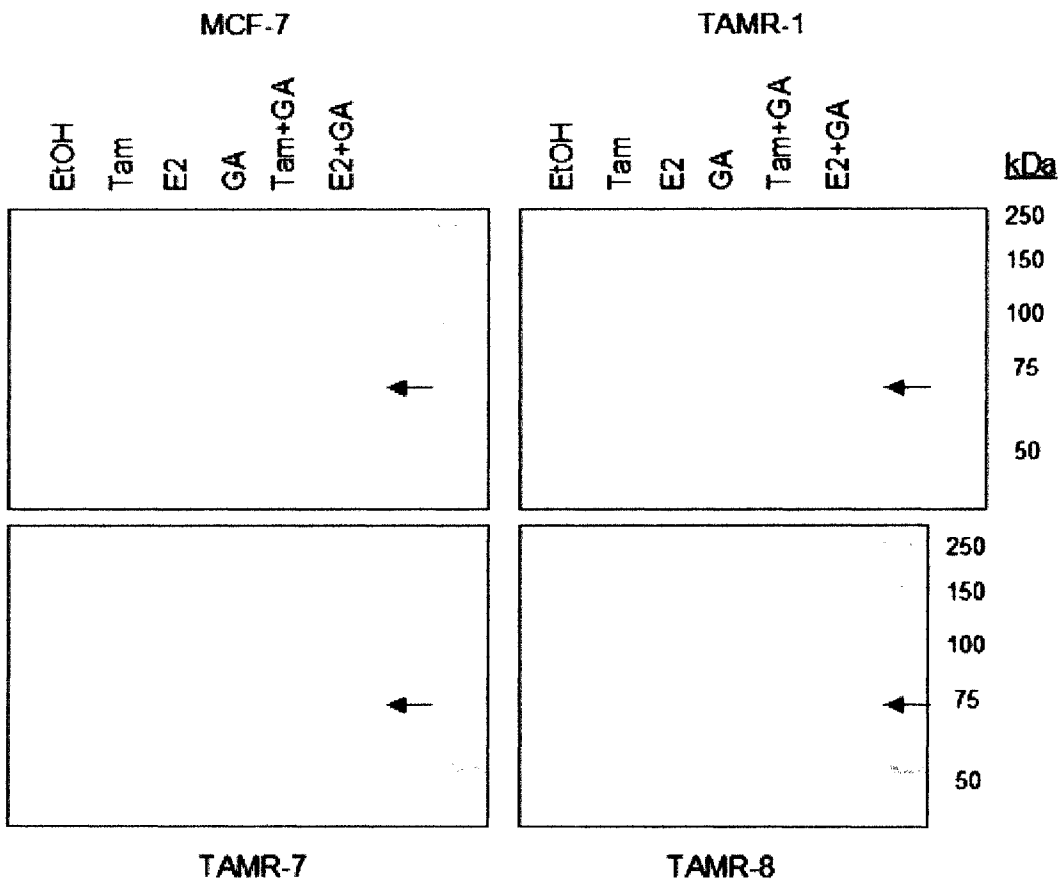


Figure 10: Ponceau S Stains of Blots Shown in Figure 9 Show Equal Protein Loading and Induction of Hsp72 Following GA Exposure

Nitrocellulose membranes used in Figure 9 were stained with Ponceau S for 5 min, washed with water, and scanned prior to immunoblotting. The GA-induced expression of the Hsp90-regulated protein Hsp72 is indicated by an arrow.

Tam appeared to limit the ability of GA to deplete ER levels in MCF-7 and all TAMR cell lines (figure 9*A*, lane 5). This effect was not seen with Akt or Raf-1 (figures 9*B* and 9*C*, lane 5), suggesting that Tam selectively inhibits the enhanced ER degradation induced by GA. A similar effect was not seen with the GA + E_2 combination (figures 9*A-C*, lane 6). Since all TAMR cell lines responded similarly to GA treatment, we chose the TAMR-1 cell line for further study.

Modulation of Estrogen Receptor Levels by 17AAG is Detectable by Immunoblotting, Confocal Microscopy, and Laser Scanning Cytometry (LSC)

It is problematic to monitor ER protein levels in tumor tissue by western blotting due to interference by co-migrating serum albumins present in tissue lysates. Therefore, we developed a method involving immunofluorescent staining and LSC as an alternative approach. To validate the method and prepare for *in vivo* studies, MCF-7 cells were grown as confluent monolayers *in vitro* and treated with ethanol vehicle (EtOH), Tam, 17AAG, or Tam + 17AAG. 17AAG is a clinically relevant GA derivative that retains activity in whole animals while GA does not. After overnight exposure to drugs, lysates were prepared and immunobloted for ER, Akt, or phospho-Akt (ser 473). As shown in figure 11, there was a marked reduction in ER, Akt, and phospho-Akt levels after exposure to 17AAG (lane 3), while treatment with Tam had little effect. Consistent with the GA data in figure 9, Tam inhibited depletion of ER levels by 17AAG, but had no

effect on downregulation of Akt or phospho-Akt. To compare the changes in ER protein levels documented by immunoblotting to those detectable by LSC, we next grew cells to confluence in chamber slides and exposed duplicate wells to the identical drug treatment conditions used to generate immunoblotting lysates. Cell monolayers were then fixed and stained for ER using an indirect immunofluorescence technique. DAPI counterstain was included in the procedure to identify cell nuclei during cytometry. We confirmed specific nuclear staining for ER under these conditions by confocal microscopy, and qualitative differences between treatment groups were readily apparent (figure 12). Robust nuclear ER staining was observed with EtOH vehicle while the signal was markedly reduced after 17AAG treatment. ER staining following Tam + 17AAG treatment was intermediate, consistent with the data in figure 11. The no primary antibody control displayed very little background staining. To quantitate these observations, the same chamber slides were scanned by LSC using a sequential violet and argon laser excitation protocol that keys on DAPI-stained nuclei to identify events and then acquires relative ER immunofluoresence signal for that same event. As demonstrated in figure 13, frequency distribution histograms of relative fluorescence intensity (as a measure of ER protein levels) were generated and compared between the EtOH, 17AAG and Tam+17AAG treatment groups. Consistent with immunoblotting and confocal microscopy data, ER levels were markedly decreased after 17AAG treatment, and this effect was substantially inhibited when Tam and 17AAG were combined.

Estrogen-Stimulated Gene Expression is Inhibited by 17AAG in MCF-7 and TAMR-1 Cells

To determine if depletion of ER protein by 17AAG had a functional effect on ER transcriptional activity, we used cells transiently transfected with a reporter plasmid encoding an estrogen response element (ERE) upstream of the firefly luciferase gene. Previous work had already demonstrated that a reporter plasmid containing the vitellogenin ERE upstream of the CAT gene could be activated upon E2 treatment of TAMR-1 cells (134). For the experiment presented in figure 14, we transiently transfected MCF-7 and TAMR-1 with a reporter construct encoding a different ERE, namely promoter elements of the human C3 gene (118). Co-transfection with the plasmid pRLnull that constitutively expresses *Renilla* luciferase was used to normalize for transfection efficiency. Following transfection, cells were treated with media +/- 17AAG for 3 hrs and then stimulated overnight with E2 or E2 + 17AAG. Analysis of the lysates revealed a 3 to 4-fold induction of luciferase activity by E2 in both cell types. Not only was E2 stimulation of luciferase activity inhibited by 17AAG, reporter activity was actually reduced below basal levels in both cell types. This reduction was not due to non-specific cytotoxicity because 17AAG treatment had little effect on the absolute level of reporter activity generated by the co-transfected constitutive *Renilla* reporter plasmid (not shown). Of note, TAMR-1

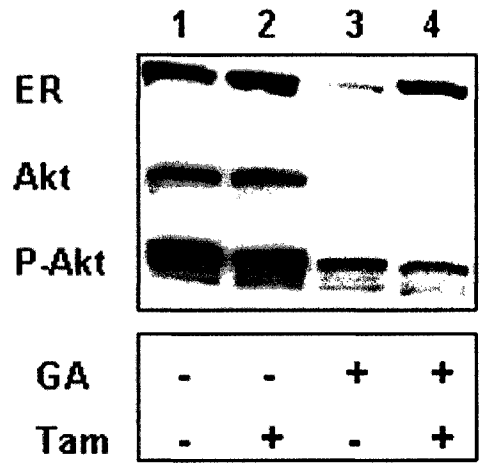


Figure 11: Modulation of Hsp90 Client Protein Levels by 17AAG is Detectable by Immunoblotting

MCF-7 cells were exposed to EtOH control (lane 1), Tam (1μM, lane 2), GA (1μM, lane 3), or Tam + GA (1μM each, lane 4) overnight. Lysates were prepared, and relative levels of ER, Akt, or phospho-Akt were determined by immunoblotting.

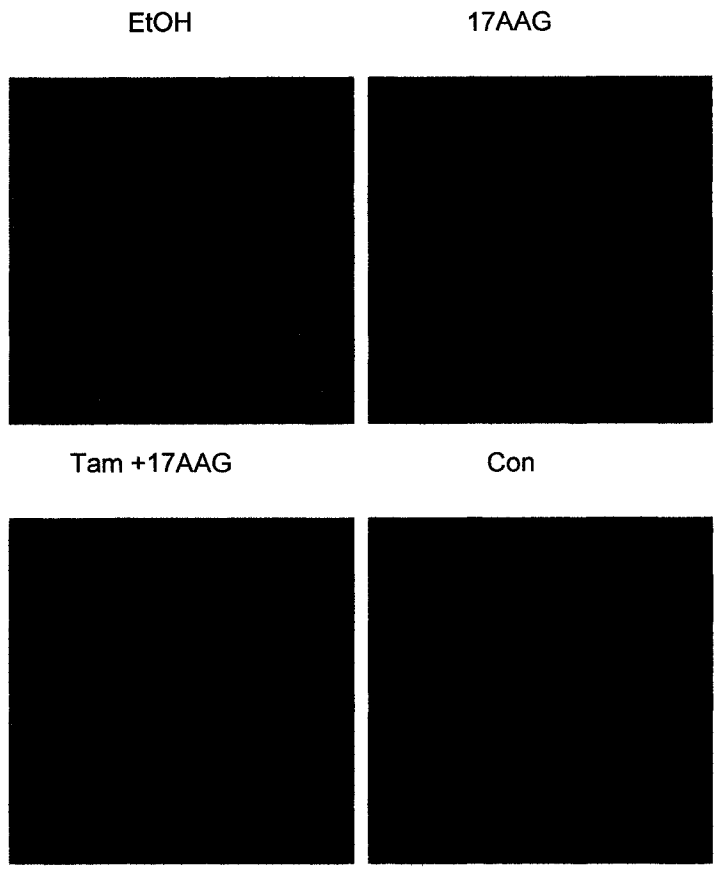


Figure 12: Modulation of ER Protein Levels by 17AAG is Detectable by Immunofluorescent Staining

MCF-7 cells growing in chamber slides were treated with EtOH control, 17AAG (1μM), or Tam + 17AAG (1μM each) overnight. Cells were then fixed, immunostaining for the ER was performed, and images were acquired using confocal microscopy. Identical gain, iris, and magnification were used for each treatment group. Con represents a negative control where an EtOH-treated well was stained using only fluorescent secondary antibody.

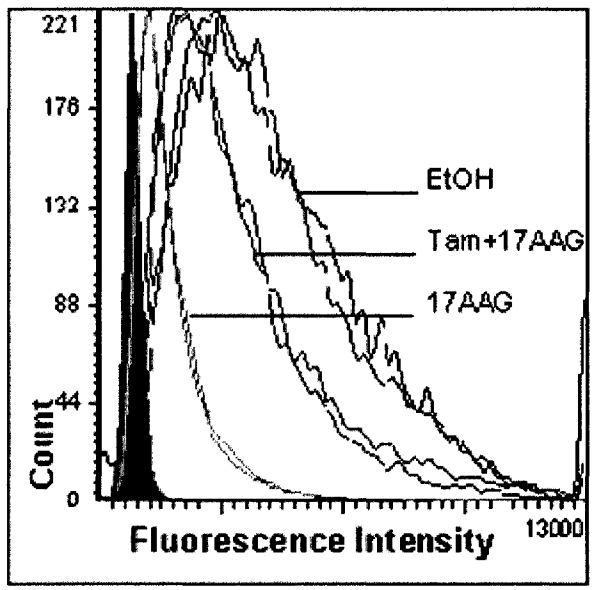


Figure 13: Modulation of ER Protein Levels by 17AAG is Detectable Using Laser-Scanning Cytometry (LSC)

The immunostained samples shown in Figure 12 were analyzed by LSC. Duplicate wells were scanned and fluorescence distribution histograms were created and are represented as open traces. Filled trace: No primary antibody control.

cells displayed lower basal ERE reporter activity than parental MCF-7 cells. This may be due to the lower level or altered activity of the ER in this cell line.

Tamoxifen does not Interfere with Geldanamycin-Induced Alterations in Chaperone Complexes Physically Associated with the Estrogen Receptor

Mature steroid hormone receptors exist in multi-protein complexes containing Hsp90, the co-chaperone p23 and one of several large immunophilins (328). GA is known to alter the composition of these complexes, most notably by inhibiting the participation of p23. It is GA-induced alterations in chaperone associations with receptor protein that are thought to enhance receptor ubiquitination and subsequent proteasome-mediated degradation (306). Since ER antagonists such as Tam are known to induce distinct conformational changes in the receptor protein (92), we wondered whether Tam might alter the composition of the chaperone complexes that associate with the ER and thus alter its stability and sensitivity to GA. To examine this possibility, we treated MCF-7 cells for 2 hours with E2, Tam, GA or Tam + GA and then lysed the cells and immunoprecipitated the ER. In figure 15, precipitates were analyzed by immunoblot to assess levels of the ER and its associated Hsp90 and p23. Lanes 4-8 contain ER immunoprecipitates derived from cells exposed to the indicated drugs. Unlike our findings in figure 9, where overnight exposure to E2 and GA resulted in marked depletion of ER, it was possible to precipitate approximately equal

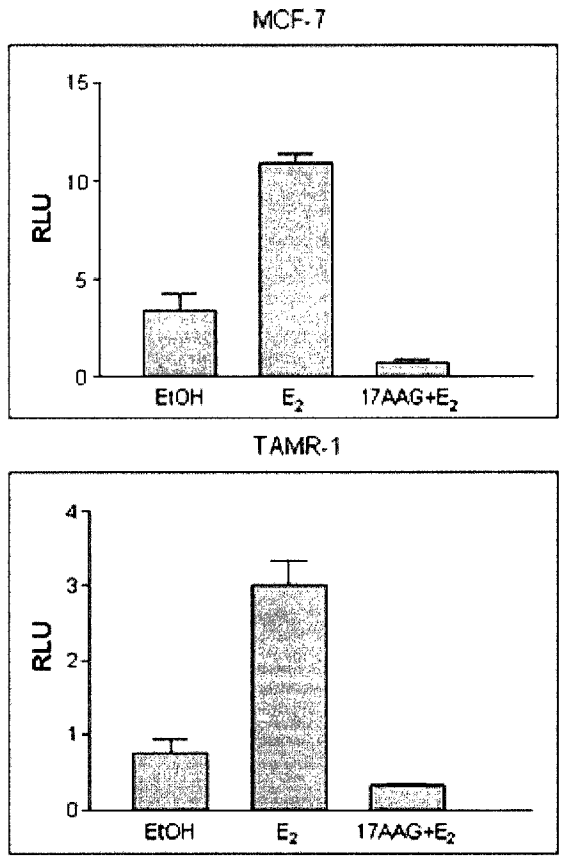


Figure 14: Estrogen-Induced Transcriptional Activation is Inhibited by 17AAG in Tam-Sensitive and Tam-Resistant Breast Cancer Cells

MCF-7 (upper panel) and TAMR-1 (lower panel) cells were transfected with the E2-responsive C3-luciferase reporter plasmid and the constitutive renilla reporter plasmid. Following transfection, cells were treated with media with (17AAG + E_2) or without (EtOH, E_2) 2μM 17AAG for 3h. Cells were re-fed with media containing EtOH vehicle control, 5μM E2, or 5 μM E2 +2 μM 17AAG. Lysates were prepared and luminescence was quantified. RLU refers to relative light units of the inducible firefly luciferase over the constitutive renilla luciferase. Data presented are the means of quadruplicate determinations. Bars: SE ($p<0.0001$).

amounts of ER in all treatment groups because drug exposure was limited to 2 hrs. Hsp90 co-precipitation remained relatively constant regardless of treatment. Likewise, ER-associated p23 decreased only slightly with E2 (lane 5) and Tam (lane 6) treatment. However, p23 association was completely lost with GA treatment in the presence or absence of Tam (lanes 7 and 8), indicating that Tam did not directly alter Hsp90/p23 interactions with the ER and did not interfere with at least one of GA's expected effects on Hsp90 function. As controls, lane 1 contains an aliquot of total cell lysate before immunoprecipitation (IP), and lane 2 contains an aliquot of the same lysate after immunoprecipitation was performed. As expected, total Hsp 90 and p23 levels did not decrease after IP because only a small fraction of the total cellular pools of these proteins are associated with the ER. The ER levels in lysate before and after IP are not visible on the exposure presented because the ER is a much less abundant protein than the chaperones seen in lanes 1 and 2. Longer exposures did reveal depletion of the ER in the sample run in lane 2 (not shown). Lane 3, in which no primary antibody was added to the immunoprecipitation, is a control to identify proteins that bind protein G beads non-specifically. A prominent non-specific band (NS) was detected that migrates just below the p23 band. This same experimental design was repeated two more times with similar results.

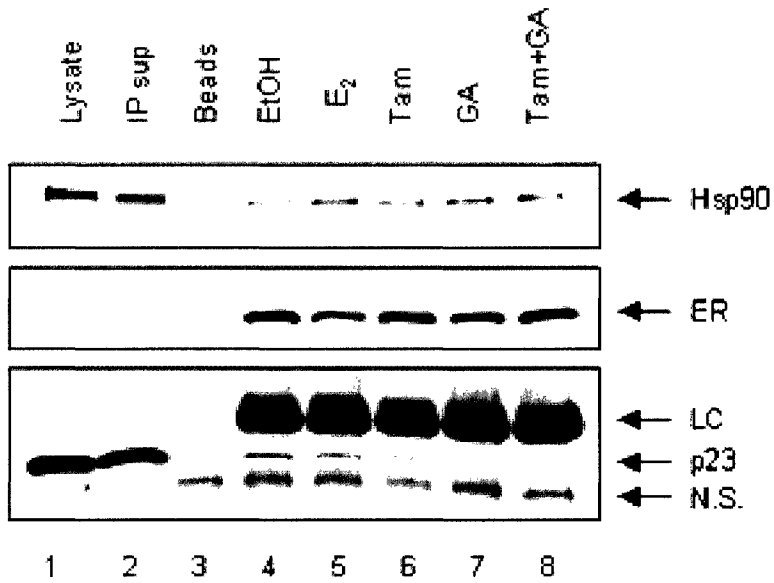


Figure 15: GA Induces p23 Dissociation from the ER Complex in the Presence or Absence of Tam

MCF-7 cells growing in 10 cm dishes were exposed to media containing EtOH vehicle control, E2 (5μM), Tam (5μM), GA (5μM), or Tam + GA (5μM each) for 2 h. Cells were lysed and the ER was immunoprecipitated. ER-associated p23, Hsp 90, and the level of ER protein itself, were detected by immunoblotting as indicated. An aliquot of total cell lysate before (lane 1) and after (lane 2) IP was loaded to indicate the migration position of the proteins of interest. In lane 3, the IP was performed without primary antibody to assess non-specific binding. Arrows indicate the position of Hsp 90, ER, antibody light chain (LC), p23, and a non-specific band that precipitates with the protein G sepharose beads (NS).

17AAG does not Deplete Estrogen Receptor Levels in Breast Tumors Growing in Tamoxifen-Supplemented Mice but does Modulate Other Hsp90 Clients

To examine the activity of 17AAG against Tam-resistant breast cancer *in vivo*, we developed a xenograft model that employed the same TAMR-1 cells used for *in vitro* studies. Consistent tumor engraftment and reproducible growth kinetics were achieved by injecting cells suspended in Matrigel into the mammary fat pad of female SCID mice. Like parental MCF-7 cells (134), we found that TAMR-1 cells displayed an absolute requirement for hormone supplementation to support their tumorigenicity (figure 19). Unlike MCF-7 cells, however, Tam supplementation (500 μg/mouse/day) was much more effective than standard E2 supplementation (not shown). To examine alterations in Hsp90 client protein levels in this model, mice bearing well-established TAMR-1 tumors were treated twice with 17AAG (4 mice) or DMSO vehicle control (4 mice), and sacrificed 18 h after the final treatment. Cryostat sections and protein lysates were prepared from each tumor. Sections were stained for ER. Specific staining was verified by confocal microscopy, and levels were quantitated using LSC. Confocal microscopy showed that tissue integrity was preserved sufficiently to achieve specific ER staining in four DMSO-treated but only three 17AAG-treated tumors (figure 16). Visually, there appeared to be no decrease in ER levels with 17AAG treatment. LSC histograms were then generated for each of these DMSO (figure 17, upper panel) or 17AAG-treated tumors (lower panel). Included in each panel

is a tumor where no primary antibody was added to control for non-specific binding of the fluorescent secondary antibody to the tumor tissue (filled trace). Each trace represents a single tumor, and the average of the median channel fluorescence (MCF) determinations for each tumor is indicated within the relevant panel. We observed the highest variability in ER staining within the DMSO treated group, with one tumor exhibiting a noticeably higher level of fluorescence intensity than the other three. The histograms for the 17AAG-treated tumors tracked more closely, with two tumors nearly superimposable and a third displaying only slightly less fluorescence intensity. Comparison of the average MCF for each group showed no statistically significant difference (4197 for DMSO-treated and 4800 for 17AAG-treated; two-tailed t test, p= .38). The staining and scanning of additional sections from each of these tumors was repeated once with similar results.

Because TAMR-1 tumors required Tam supplementation for *in vivo* growth, it seemed likely that Tam antagonized ER depletion by 17AAG *in vivo*, just as we had observed with GA and 17AAG in cell culture (figures 9 and 11). To evaluate this hypothesis, we examined whether other 17AAG target proteins had been modulated in TAMR-1 tumors by drug treatment as expected. Lysates from three DMSO-treated and three 17AAG-treated tumors were immunoblotted to

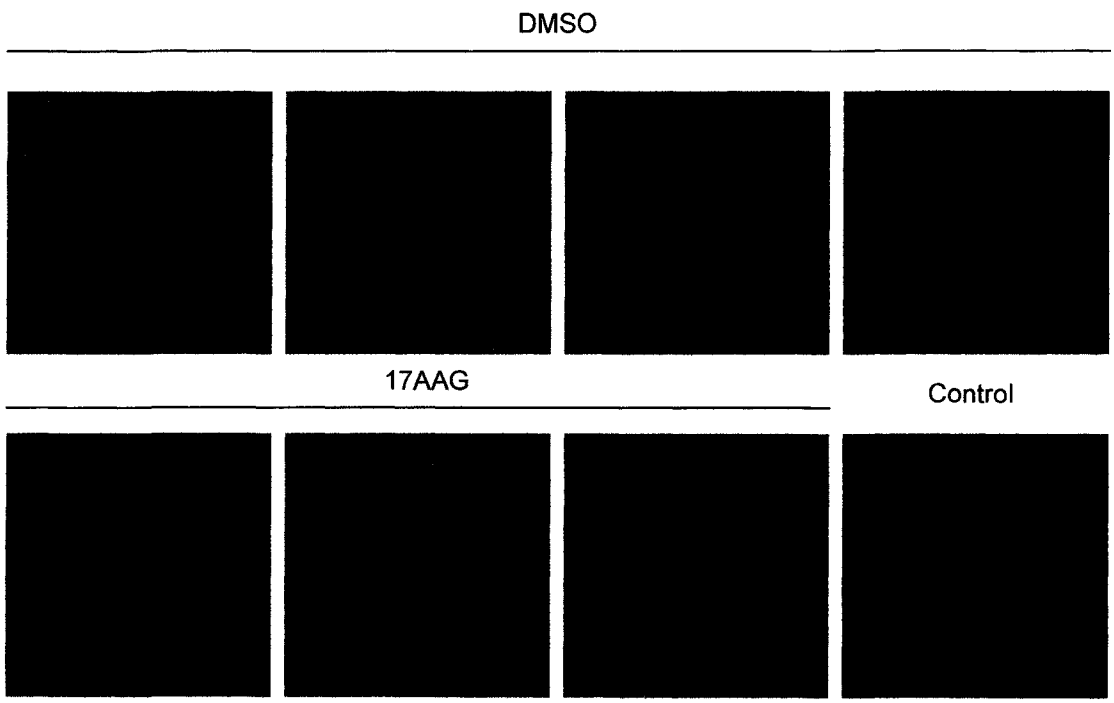


Figure 16: 17AAG does not Decrease ER Levels in TAMR-1 Tumor Xenografts Growing in Tam-Supplemented Mice

Tumors were resected 18h after drug (17AAG) or control vehicle (DMSO) treatment and snap frozen. Cryostat sections from 4 DMSO-treated and 3 17AAG-treated tumors were stained for ER using an immunofluorescent technique. Confocal images (20x) were obtained using identical magnification, gain, and iris settings. Control represents a tumor section from a DMSO-treated mouse that was stained using no primary antibody.

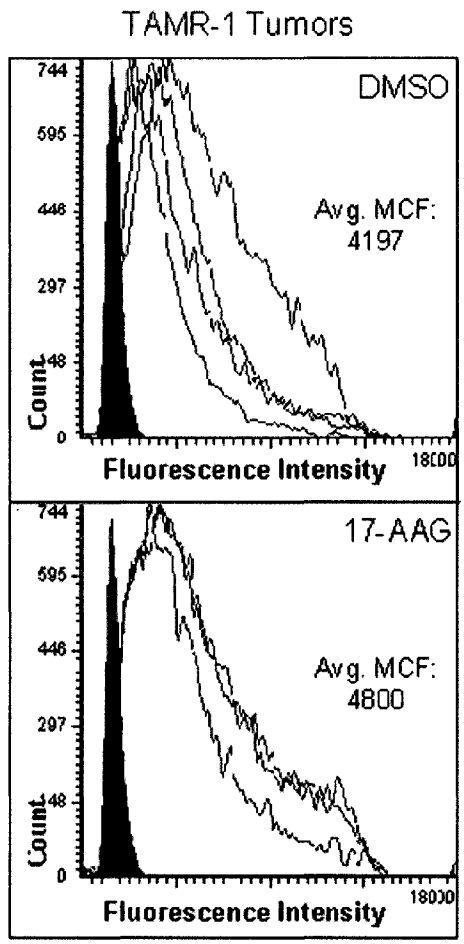


Figure 17: Quantitation of ER Levels in TAMR-1 Tumor Xenografts by LSC

The immunostained sections in Figure 16 were analyzed by LSC. Open traces represent individual tumors. Filled trace: No primary antibody control. MCF: Average median channel fluorescence of the traces depicted.

determine relative levels of Akt, Raf-1, and Hsp 72. An equal amount of TAMR-1 cell lysate was included as a positive control for each of the immunoblots. 17AAG treatment clearly downregulated Raf-1 levels in two of three tumors tested compared to DMSO control, while Akt levels were decreased in all three tumors, but to a varying extent (figure18). Interestingly, the tumor in which the Raf-1 level was not modulated by 17AAG still showed marked depletion of Akt. An increase in Hsp 72 was observed with 17AAG treatment in all three tumors tested, consistent with our previous findings in a different xenograft model (304).

17AAG Inhibits the Growth of Tamoxifen-Resistant Tumor Xenografts

Given the demonstrated ability to modulate several Hsp90 client proteins, but not ER, in Tam-resistant tumors, we next evaluated the effects of 17AAG on tumor growth. Tam-supplemented SCID mice bearing established TAMR-1 tumors were treated with 17AAG (100 mg/kg) or DMSO vehicle control on Monday/Wednesday/Friday for 2 weeks starting on day 11 post-tumor cell inoculation. Tumor growth was monitored by serial caliper measurements for 77 days. In animals treated with 17AAG, there was a significant reduction (two-way ANOVA, $p<0.0001$) in Tam-stimulated tumor growth compared to DMSO control (figure 19). This experiment was repeated using a twice weekly dosing schedule that extended for three weeks and significant reduction in tumor growth was again observed ($p<0.001$).

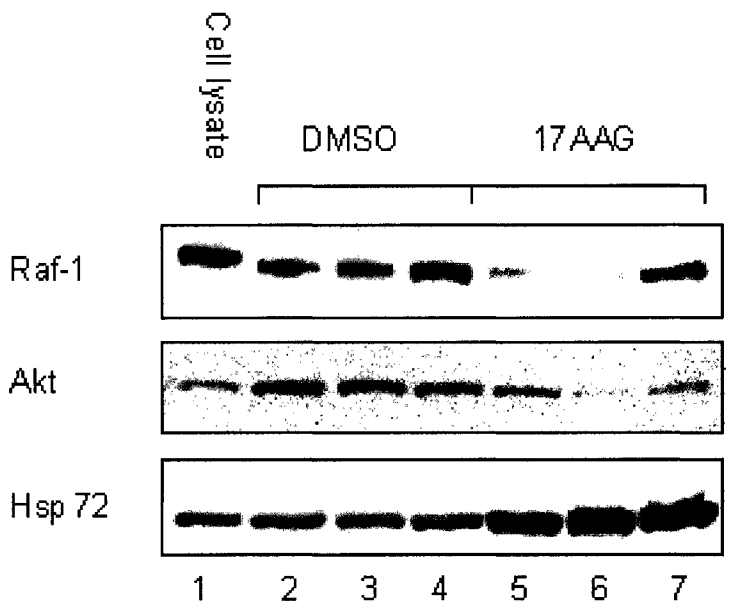


Figure 18: 17AAG Modulates Hsp90 Client Protein Levels in TAMR-1 Tumor Xenografts Growing in Tam-Supplemented Mice

Tumors were resected 18 h after drug (17AAG) or control vehicle (DMSO) treatment, snap frozen and pulverized over a dry ice-ethanol bath. Lysates were prepared and equal amounts of protein were analyzed by immunoblotting. Blots were probed for the 17AAG-sensitive proteins Raf-1, Akt, and Hsp 72 as indicated. Lysate from TAMR-1 cells grown *in vitro* was analyzed in lane 1 as a control to confirm the migration positions of the proteins of interest. Lanes 2-4: Lysate from three different DMSO-treated tumors. Lanes 5-7: Lysate from three different 17AAG-treated tumors.

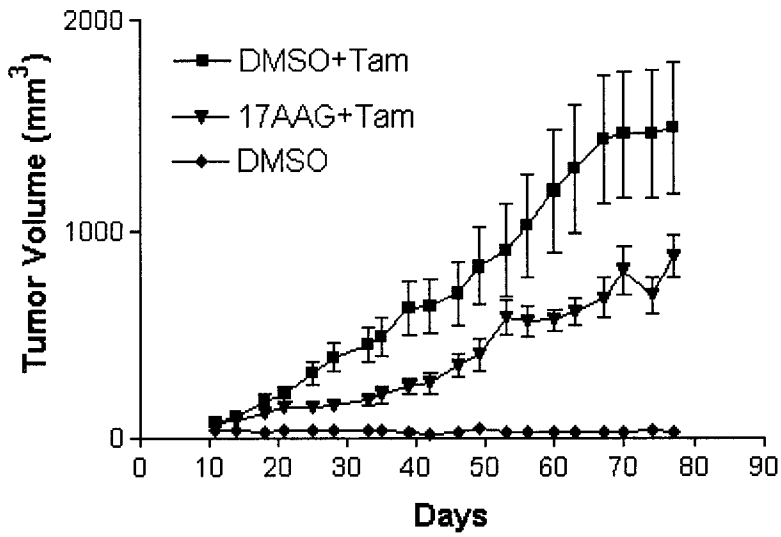


Figure 19: 17AAG Inhibits the Growth of TAMR-1 Tumor Xenografts

SCID mice bearing established TAMR-1 tumor xenografts were treated with DMSO + Tam, or 17AAG + Tam as indicated. As a control, a cohort of mice were injected with tumor cells, but given no Tam supplementation ("cells only" group). Mean tumor volumes for each treatment group as determined by serial caliper measurements over a 77 day interval are depicted. Bars: SE.

Discussion

The anti-estrogen Tam has been used as front-line hormonal therapy for ER+ breast cancer for decades (323). Acquired resistance, however, limits its long-term efficacy. Although the mechanisms of Tam resistance are not completely understood, they include alterations in cofactor association, mutations in the ER itself, and variety of compensatory changes in other growth factor signaling pathways (323, 345). Loss of ER expression, however, is not commonly observed in Tam-resistant tumors (325). A recent study has shown that 77% of patients who developed contralateral breast cancer despite Tam treatment had ER+ tumors (346). These findings suggest that the ER may continue to play a key role in tumor progression despite Tam therapy. In recent years, efforts have been made to develop alternative ER antagonists for treatment of Tam-resistant breast cancer (326). The pure anti-estrogens ICI 164,384 and ICI 182,780 have been shown to inhibit the growth of Tam-resistant breast cancer both *in vitro* (134) and in patients (163), but acquired resistance to these agents has been described (165). Thus combinations of anti-estrogens may delay tumor progression more effectively than single agent therapy, but the growth of resistant tumors remains likely. An alternative hormonal approach to antagonist therapy for breast cancer is to reduce levels of circulating E2. Aromatase inhibitors reduce E2 biosynthesis while LHRH analogs such as SB-75 (Cetrorelix) pharmacologically mimic ovarian ablation. These hormone deprivation strategies

have shown some activity against Tam-resistant breast cancer (347). Recent evidence indicates, however, that during the development of resistance to E2 deprivation (and Tam), cross-talk between critical signal transduction pathways occurs via the ER protein and this essential cross-talk facilitates tumor survival and proliferation (348). In light of these observations, therapies that do not depend on receptor-ligand interactions or that deplete cellular levels of the ER could prove particularly useful in dealing with the problem of resistance.

We have previously described a novel strategy for the disruption of ER signaling in breast cancer cells, making use of drugs that bind to the chaperone Hsp90 (304). Hsp90 is a critical component of the multi-protein chaperone complexes that have been shown to be required for stabilization of hormone receptors including the ER (Reviewed in (329)). GA binds to the ATP-binding pocket of Hsp90, altering its function and the chaperone complexes associated with steroid hormone receptors. As a result of these alterations, the receptor undergoes ubiquitination and is targeted for proteasomal degradation (349). We now show that ER levels can be decreased in both Tam-sensitive and Tam-resistant breast cancer cells as a result of exposure to Hsp90 inhibitors *in vitro*. Unlike the ER itself, however, Hsp90 provides a stable target for pharmacological intervention. While overexpression of certain chaperone proteins has been documented in a variety of cancers, mutation of Hsp90 has never been reported. Furthermore, Hsp90 is known to be involved in the regulation of at least 40 different client

proteins, many of these involved in mitogenic and survival signaling pathways critical to hormone-refractory breast cancer. Depletion of several of these client proteins may simultaneously contribute to the anti-tumor activity of Hsp90 inhibitors (350) and make the emergence of resistance less likely. The fact that Hsp90 inhibitors affect multiple signaling pathways also makes them attractive for the treatment of breast cancer given the evidence that several signaling pathways other than the ER pathway are involved in the development of Tam resistance and breast cancer progression (327, 348). Because Akt and Raf-1 have been shown to be important for the survival and proliferation of breast cancer cells (351, 352), and because these proteins are also known to associate with Hsp90 (296, 353), we evaluated the effects of GA/17AAG on Akt and Raf-1. We found that the levels of these proteins as well as that of the ER were decreased following exposure to GA in Tam-sensitive and Tam-resistant cells. We also found that levels of Akt and Raf-1 were decreased in TAMR-1 tumors from mice treated with 17AAG compared with levels in tumors from control animals. Although no changes in ER protein levels in these tumors were observed (see below), a significant inhibition of tumor growth in 17AAG-treated animals was nonetheless observed, suggesting that modulation of other targets independent of the ER contribute to the anti-tumor activity of 17AAG.

In order to evaluate effects of Hsp90 binding agents on ER levels in tumor tissues, we used laser-scanning cytometry (LSC) to overcome a methodological

problem. Although the ER is readily detectable in cultured cells using immunoblotting, serum albumins from tumor tissue migrate very closely with the 67 kDa ER during SDS-PAGE electrophoresis. It is therefore difficult to detect ER in tumor homogenates using this technique. LSC permits highly sensitive, reproducible quantitation of ER levels with minimal manipulation of the experimental sample since tumor homogenization and electrophoresis are not required for analysis. We validated the technique by comparing our ability to detect 17AAG-mediated ER modulation in MCF-7 cells using immunoblot and LSC (figures 11-13). LSC findings corresponded well with immunoblot results in these experiments. To confirm the ER specific staining of tissue culture cells detected by LSC, every slide evaluated by LSC was also examined using confocal microscopy. LSC and confocal results were concordant in each case. We then used LSC to analyze tumors from *in vivo* experiments. As shown in figures 16 and 17, we were able to quantitate relative ER levels in TAMR-1 xenograft sections following exposure to 17AAG or DMSO. As might be expected, there was variation in ER specific staining among tumor samples within the same treatment group, likely due to a number of factors including degree of vascularization, interaction between tumor cells and supporting stromal tissue, and systemic levels of growth factors in different animals. Nonetheless, we were able to clearly demonstrate that 17AAG had little effect on ER protein levels in tumors from Tam-supplemented mice. This result was concordant with our *in vitro* finding that Tam antagonizes the ability of 17AAG to destabilize the

ER.

It is not yet clear why Tam inhibits modulation of ER levels by Hsp90 binding drugs, but there is evidence to suggest that Tam itself may decrease the turnover of the ER protein. We have shown that the half-life of unliganded ER in wild type MCF-7 cells is 2 to 4 hours (304). In contrast, other investigators have demonstrated that only 10% of the ER is turned over in 6 hours if it is covalently labeled by a tamoxifen aziridine derivative (132). Consistent with this finding, we and others have also shown that unlike E2, Tam treatment does not cause a decrease in the level of ER in breast cancer cells (figure 9; (354)). Tam clearly induces a unique change in the conformation of the ER protein (92, 355), and it is possible that this change in turn alters the interaction of ER with Hsp90 and/or other chaperone proteins. If this were the case, the Tam-bound ER could become less sensitive to Hsp90-binding drugs. To begin investigating this possibility, we performed co-precipitation experiments designed to detect changes in the binding of critical chaperone proteins to the ER in the presence of GA and Tam. We did not find any Tam-induced changes in the association of Hsp90 or p23 with the ER protein. As expected, we did find that GA alone caused the dissociation of p23 from the ER-chaperone complex. If Tam induced a conformational change that effectively negated GA effects on the receptor-chaperone complex, a failure to dissociate p23 would have been observed. Instead, as seen in Figure 15, the presence of both Tam and GA still led to

dissociation of p23 from the ER-associated protein complex. At this point, alterations in the ER-chaperone complex do not explain Tam-induced stabilization of the ER in the presence of GA. Interestingly, a recent report has shown that helix 12 of the ER protein is critical for it's stability (172). The position of this helix within the receptor protein's structure appears to vary in a ligand-specific manner leading to differential association of coregulator proteins and the distinct transcriptional responses induced by various Selective Estrogen Receptor Modulators (SERMs). Consistent with this model, these investigators found that a point mutation at Asp-538 that alters the conformation of this domain results in enhanced ER turnover in the presence of Tam rather than stabilization as seen with its wild type counterpart. Likewise, another group has shown that accumulation of wild type ER in the presence of Tam results from reduced ubiquitination of the protein, apparently as a result helix 12 alterations (52). Further work examining changes in ER ubiquitination and turnover in the presence of Tam and GA is in progress and should provide insights into the mechanism of the ER stabilization observed in our experiments.

Our findings regarding effects of the combination of Tam and GA have several important therapeutic implications. While ER levels in breast tumors did not decrease following Hsp90 inhibitor exposure in the presence of Tam, 17AAG treatment nonetheless resulted in decreased growth of these Tam-resistant tumors. This finding, together with the observation that 17AAG modulates the

levels of other proteins known to be important in breast cancer, including Raf-1 and Akt (figure 18), as well as IGF-1R (L. Whitesell, unpublished observations) and erbB2 (356), suggests that 17AAG may be effective in the treatment of recurrent breast cancer. Based on our *in vitro* results (figures 9-14) it is likely that in the absence of Tam, ER levels would be depleted by 17AAG and its anti-tumor activity could be even more pronounced. Preliminary work in our lab indicates that raloxifene, a SERM in current clinical use, also antagonizes GA-induced ER depletion, indicating that the effect is not Tam-specific. The pure antiestrogen ICI 182,780 (fulvestrant) stimulates degradation of the ER, and is effective in about 20% of patients with Tam-resistant breast cancer in clinical trials (135, 136). Experiments in our lab are ongoing to examine the effects of combining GA and fulvestrant on ER stability and to see whether fulvestrant can enhance the anti-tumor activity of 17AAG in mice bearing TAMR-1 tumors.

Data from on-going Phase I trials have already indicated that 17AAG is well-tolerated in adults with refractory solid tumors at doses that modulate Hsp90 client protein levels in the lymphocytes of patients receiving the drug (333, 334). Our findings now provide strong pre-clinical support for the pursuit of Phase II trials of 17AAG in patients with recurrent, hormone-refractory breast cancer. Furthermore, they suggest that discontinuation of hormonal therapy, especially Tam, prior to study entry may be important.

IV. THE DEGRADATIVE EFFECT OF HSP90 BINDING AGENTS ON THE ESTROGEN RECEPTOR IS INHIBITED BY ANTIESTROGENS: POTENTIAL MECHANISMS

Introduction

Over 200,000 cases of breast cancer are diagnosed each year, making it the number one diagnosed malignancy in women (1). Although recent advances in mammographic imaging has led to an increase in early detection where surgical excision is nearly curative, a significant fraction of these women progress and require systemic therapy. The estrogen receptor (ER) plays a key role in breast cancer progression, and has been a major target in breast cancer treatment for decades. The selective estrogen receptor modulator (SERM) tamoxifen (Tam) competes with estrogen for ER binding and inhibits ER activity (91). Indeed, Tam induces regressions in nearly all women with ER+ breast cancer. However, resistance is frequently observed. The mechanisms that underlie Tam resistance are unclear, but likely involve alterations in ER-cofactor associations at estrogen responsive elements (EREs) on the DNA (77, 204). Indeed, altered cofactor association in some breast cancers may eventually lead to Tam-stimulated growth. Tam acts as an agonist in the endometrium, and recently *Shang et al.* described differential expression and ERE association of the coactivator SRC-1 between MCF-7 breast cancer cells and the Ishikawa endometrial carcinoma cell

line (76). The pure antiestrogens ICI 182,780 and ICI 164,384 are effective against Tam-resistant breast cancer, although resistance to these agents is also observed (165). Clearly, the development of resistance limits the overall efficacy of antiestrogens as anticancer agents, and alternative approaches for the treatment of antiestrogen-resistant breast cancer are needed.

Recently, our lab has shown that degradation of ER protein can be induced by exposure to heat shock protein 90 (Hsp90) binding agents in Tam-resistant breast cancer, providing an alternative to ligand-targeted therapy (304). Importantly, the ER is still present in a majority of Tam-resistant breast cancers in a non-mutated form, and Tam resistant breast cancer is responsive to further hormonal therapy, indicating that it is still required for disease progression (161). Therefore, the ER likely remains a relevant target for the treatment of antiestrogen-resistant breast cancer. The ER and other steroid hormone receptors require interactions with Hsp90 and other essential molecular chaperones to remain in a mature conformation capable of high-affinity ligand binding (261). The ER-chaperone interactions are altered following ligand binding, allowing the ER to localize at EREs and to associate with coactivators or corepressors, which positively or negatively regulate gene expression (246). It is now clear that the anti-tumor antibiotics geldanamycin (GA) and radicicol bind Hsp90 with high affinity and alter basal chaperone interactions with a variety of client proteins. These include steroid hormone receptors and a variety of growth

factor receptors, signaling kinases, and transcription factors involved in cancer progression (292, 296, 301, 304). The clinically relevant analog to GA, 17-allylamino-17-demethoxygeldanamycin (17AAG), is nearing completion in NCI-sponsored Phase I clinical trials.

Under basal conditions, ER half-life is approximately 4-6h (304). ER turnover is accelerated following E2 stimulation, resulting in decreased half-life of the protein (84). This effect is due to increased ER ubiquitination and subsequent proteosomal degradation through the 26S proteasome. Indeed, the E3 ubiquitin ligase E6-AP has been identified as an ER cofactor (85). Furthermore, ubiquitination appears to play an integral role in ER transcriptional activity, as disruption of the normal proteolytic cycle with proteasome inhibitors abrogates ER transactivational ability (86). Tam induces a conformational change in the ER that is distinct from the E2-bound form, and causes protein stabilization (92, 132). In a study by *Wijayaratne et al.*, it was shown that ER protein levels following 4h E2 stimulation were reduced to 20% of normalized control. ER levels following 4h Tam treatment, however, were 120% of control (357). Tam was also shown to be the least effective at inducing ER ubiquitination, suggesting a mechanism for stabilization. Helix 12 appeared to be critical for the stability induced by Tam, as a mutation in this region resulted in ER degradation following Tam exposure. Interestingly, we have recently shown that Tam significantly inhibits GA-induced degradation of the ER both *in vitro* and *in vivo* (358). Although a mechanism was

not determined, it suggested that Tam may inhibit the degradative effects of GA by altering the normal ER proteolytic cycle.

Recent studies have shown that the ER cycles on and off of EREs (87, 93). Inhibition of the proteasome disrupts this process, suggesting that ubiquitination may be required for ER removal from the DNA. Furthermore, inhibition of RNA polymerase II phosphorylation by 5,6 Dichlorobenzimidazole-1β-D-ribofuranoside (DRB) caused a continuous association of the ER with the DNA, indicating that transcriptional initiation is required for ER cycling and subsequent degradation. Also of interest, *Freeman et al.* have shown that Hsp90 and the co-chaperone p23 are recruited to glucocorticoid responsive elements following exposure to dexamethasone, and are involved in removal of the glucocorticoid receptor from the DNA (279). Because Tam does not induce ER-stimulated transcription in MCF-7 cells, we hypothesized that the ER may become immobilized on the DNA, resulting in sequestration of the ER in the nuclear compartment. This event would likely inhibit ubiquitination and subsequent proteosomal degradation of the ER, and could explain why Tam inhibits ER degradation induced by GA. In this study, we show that Tam and the related SERM raloxifene (Ral) both inhibit ER degradation induced by GA. Tam exposure led to the accumulation of the ER in the nucleus but the protein was extractable when protein-protein and/or protein-DNA interactions were disrupted by high salt. Furthermore, immunofluorescent localization of the ER showed that both Tam and Ral inhibit ER nuclear export

and the accumulation of cytoplasmic aggregates normally induced by the proteasome inhibitor MG132. Inhibition of ER transcriptional initiation with DRB caused nuclear accumulation similar to that observed with Tam and Ral, suggesting that SERMs may cause the ER to become immobilized on the DNA. Indeed, chromatin immunoprecipitation (ChIP) experiments determined that Tam exposure resulted in prolonged ER-DNA association compared to E2. Furthermore, consistent with the findings of *Freeman et al.* with the glucocorticoid receptor, we report that p23 and Hsp90 are associated with an ERE following E2 stimulation. Taken together, these data indicate that Tam inhibits GA-induced degradation of the ER by prolonging its association with EREs and sequestering it away from the proteasome by causing nuclear retention of the ER.

Results

Antiestrogens Inhibit Geldanamycin-Mediated Degradation of Estrogen Receptor Protein

Previous studies in our lab have shown that Tam inhibits ER degradation induced by GA, and its clinically relevant analog 17AAG, both *in vitro* and *in vivo* (358). To examine whether this effect is Tam-specific, we compared the degradative effects of GA on the ER in the presence of another SERM, Raloxifene (Ral). The Food and Drug Administration has approved Ral for the treatment of

osteoperosis, and clinical trials are underway to determine its efficacy as an agent for breast cancer therapy (20). MCF-7 cells were exposed to EtOH vehicle, GA (1μM), or GA in combination with Tam or Ral (1μM each) overnight and ER protein levels from cell lysates were determined. As shown in figure 20, GA exposure resulted in depletion of ER to below detectable levels. In contrast, both Tam and Ral significantly inhibited GA-induced degradation of ER protein. Even in the presence of Tam and Ral, GA caused ER levels to decrease by approximately 50%, suggesting that antiestrogens do not completely abolish the ability of GA to induce ER degradation. These data suggest that the ability to block GA-mediated degradation of the ER is not exclusive to Tam, but is rather a general characteristic of SERMs.

Tamoxifen does not Inhibit Geldanamycin-Induced Formation of the Intermediate Estrogen Receptor-Chaperone Complex

Steroid hormone receptors are dependent on chaperone interactions for their activity (246). The form of the ER that is capable of high affinity ligand binding exists in a mature complex with Hsp90, p23, and cyclophillin 40. GA inhibits the formation of the mature complex, instead causing the ER to form a premature, or intermediate, complex containing Hsp70 and p60/Hop. ER in this intermediate complex is then marked for degradation through the ubiquitin-proteasome pathway. Importantly, Tam induces conformational changes in the ER that are

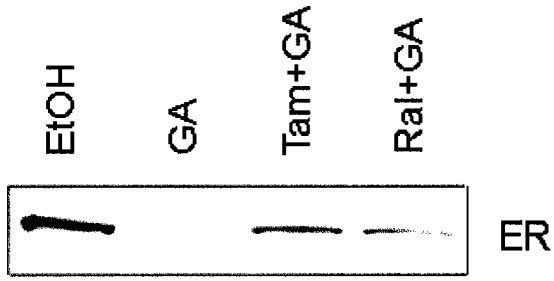


Figure 20: GA-Induced Degradation of ER Protein is Inhibited by Tam and Ral MCF-7 cells were treated overnight with EtOH vehicle, GA (1μM), Tam + GA (1μM each), or Ral + GA (1μM each). Lysates were prepared and ER protein levels were analyzed by immunoblotting.

distinct from E2 (92). Thus, we hypothesized that the conformation assumed by Tam-bound ER may inhibit the formation of the intermediate complex following treatment with GA. To determine whether Tam inhibits the degradative effects of GA by altering ER-chaperone association, immunoprecipitation experiments were performed using lysates from cells exposed to EtOH vehicle, E2 (1μM), Tam (1μM), GA (1μM) or Tam + GA (1μM each) for 2h. An ER-specific antibody was used for the immunoprecipitation, followed by immunoblotting for the ER itself, p23, or p60/Hop. We limited the duration of treatment to two hours to avoid the downregulation of ER levels that has been described with prolonged E2 or GA exposure (304). Consequently, approximately equal amounts of ER protein were recovered from all treatment groups (figure 21). Only background levels of p60 are evident in cells treated with EtOH, E2 or Tam (lanes 4-6). However, a p60-ER association is observed in cells treated with either GA alone, or a combination of Tam + GA (lanes 7 and 8). Converse to p60, p23 is associated with the ER following EtOH, E2, and Tam exposure (lanes 4-6), but not with Tam or Tam + GA (lanes 4-8). Because association of p60/Hop and dissociation of p23 was detected with both GA and Tam + GA treatments, it suggests that Tam-induced conformational changes do not alter the expected effects of GA on the mature ER-chaperone complex. As controls, lane 1 contains cell lysate before immunoprecipitation to identify the migration positions of the proteins of interest. Lane 2 contains the same lysate after immunoprecipitation with ER antibody. Interestingly, there is only a slight depletion of ER protein from the lysate after

immunoprecipitation, which is more evident with lighter exposures. It is unclear why the ER was poorly depleted from the lysate (compare lanes 1 and 2). However, a significant amount of ER protein was immunoprecipitated (lanes 4-8). As expected, p23 and p60/Hop levels did not decrease following immunoprecipitation because only a small fraction of the total cellular pool of these proteins is associated with the ER. To control for non-specific binding of proteins to the protein G beads used in the precipitation, a "beads only" condition was used where no primary antibody was included. A prominent non-specific (NS) band migrates just below the p23 band on immunoblots. This experiment was repeated twice with similar results.

Nuclear Accumulation of Estrogen Receptor Protein Following Tamoxifen Exposure

Tam is known to increase ER half-life, and elevated ER protein levels are detected in cells treated with Tam (132). Furthermore, export of the ER from the nucleus is required for degradation by the 26S proteasome (87). Therefore, we investigated whether Tam-induced nuclear accumulation of the ER could be a possible mechanism for inhibition of GA-mediated ER degradation. Localization of ER protein was analyzed by differential centrifugation of cell lysates followed by immunoblotting. MCF-7 cells were treated with EtOH vehicle or 1μM Tam overnight. To determine if the nuclear-localized ER is present in a multi-protein

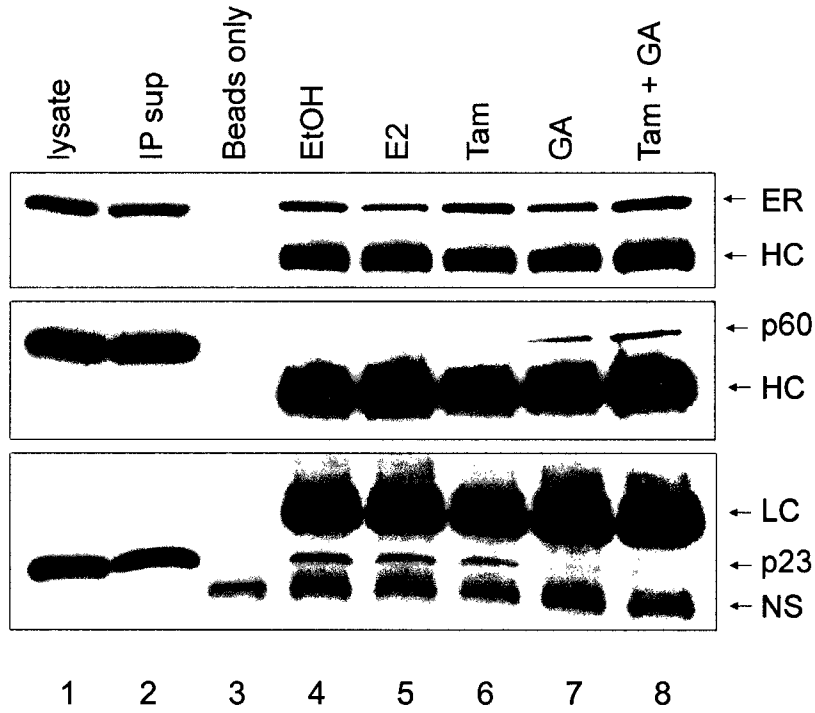


Figure 21: Tam does not Inhibit GA-Induced Formation of the Intermediate ER-Chaperone Complex

MCF-7 cells were treated for 2h with EtOH vehicle, E2 (5μM), Tam (5μM), GA (5μM), or Tam + GA (5μM each). Cells were lysed and the ER was immunoprecipitated. ER-associated p23, p60/Hop, and the level of the ER protein itself, were detected by immunoblotting. An aliquot of total cell lysate before (lane 1) and after (lane 2) IP was loaded to indicate the migration position of the proteins of interest. In lane 3, the IP was performed without primary antibody to assess non-specific binding. Arrows indicate the position of the ER, p60, antibody heavy chain (HC), antibody light chain (LC), p23, and a non-specific band that precipitates with the protein G sepharose beads (NS).

complex, indicating that it is bound to EREs with other cofactors, we lysed cells in the presence or absence of high salt. NP-40 soluble and insoluble fractions were separated by centrifugation, and the insoluble fractions were dissolved in 2% SDS-containing buffer. Proteins were then resolved by SDS-PAGE and immunoblotted for the ER. As shown in figure 22, the ER is detected in the insoluble fraction under basal conditions (lane 5). However, this level increases dramatically following exposure to Tam (lane 7). Interestingly, the ER is completely extractable under high salt conditions (lanes 6 and 8), indicating that the ER may be present in a multi-protein complex bound to DNA within the nucleus. Because NP-40 lysis permeabilizes nuclei, it was not unexpected that a significant amount of ER was recovered in the soluble fraction. This likely represents ER that is not associated or loosely associated with DNA and/or protein complexes within the nucleus. In the soluble fraction, Tam caused the expected increase in ER levels. However, there was not a corresponding increase in soluble ER protein following extraction with high salt (compare lanes 1 and 2, 3 and 4). This may be because the majority of ER protein was isolated in the NP-40 soluble fraction, and small contributions from the ER released from the DNA by high salt lysis were not detected.

Tamoxifen does not Induce Accumulation of Estrogen Receptor Protein Aggregates

Proteasome inhibition results in the accumulation of insoluble protein aggregates that are localized to NP-40 isoluble fractions (291). These aggregates, however, cannot be extracted to the soluble fraction using high salt buffer. To rule out aggregation of the ER as a mechanism for Tam-induced accumulation in the NP-40 insoluble fraction, we compared the salt extractability of the ER from the pellet fraction of lysates treated with Tam or the proteasome inhibitor MG132. Cells were treated with Tam (1μM) or MG132 (10μM) overnight, and low or high salt lysates were prepared and separated into NP-40 soluble and insoluble fractions as described in experimental procedures. As expected, nearly all of the cellular ER was distributed in the insoluble fraction following MG132 exposure, which was not extractable with high salt (Figure 23). In contrast, the Tam-bound ER in the pellet fraction was salt extractable, indicating that Tam does not simply cause aggregation of ER protein.

Pharmacological inhibition of the proteasome disrupts the normal ER ubiquitin-mediated degradation process and abrogates the degradative effects of GA (87, 291). Based on the data presented in figures 22 and 23, the Tam-mediated effects on ER stability appear to be related to nuclear accumulation, rather than proteasome inhibition or aggregate formation. Because the ubiquitinated ER

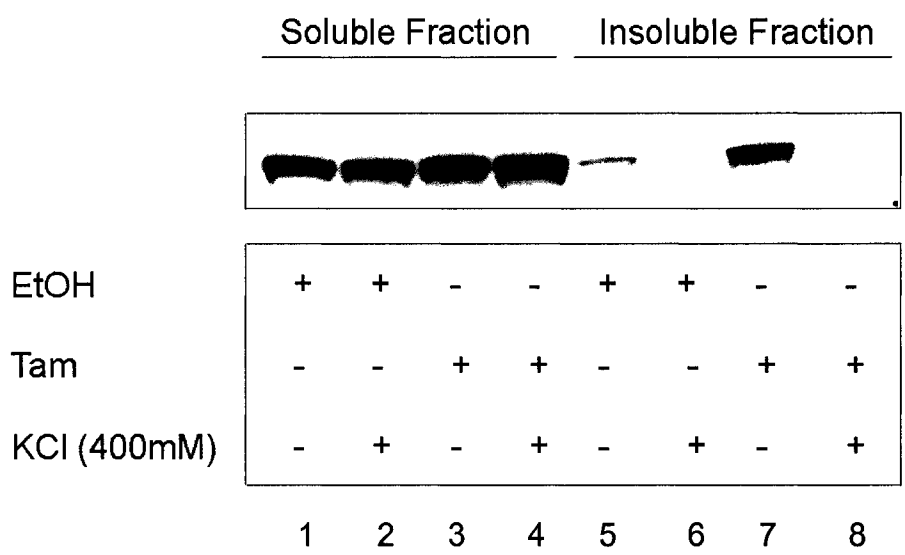


Figure 22: Nuclear Accumulation of ER Protein Following Tam Exposure

MCF-7 cells were treated with EtOH vehicle control or Tam (1μM) overnight. Cell lysates were prepared using NP-40 lysis buffer containing 40mM or 400mM KCl, and soluble and insoluble fractions were isolated by centrifugation. Insoluble fractions were solubilized in buffer containing 2% SDS. Lysates were resolved by SDS-PAGE and immunoblotted for the ER.

must be exported from the nucleus for subsequent degradation by the proteasome, we hypothesized that antiestrogens may reduce the efficacy of ER export by causing nuclear accumulation of the receptor, thereby inhibiting the accumulation of cytosolic aggregates induced by MG132 exposure. To investigate this possibility, we visualized ER cellular localization with various ER ligands in the presence or absence of MG132 using an imunofluorescent staining technique. MCF-7 cells were exposed to EtOH vehicle, E2 (1μM), Tam (1μM), or Ral (1μM) overnight in the presence or absence of MG132 (10μM). As shown in figure 24, MG132 treatment caused the ER to accumulate in cytosolic aggregates. The intensity of staining appeared weaker than controls, and may be due to reduced immunoreactivity of the staining antibodies for heavily ubiquitinated/aggregated ER protein. Consistent with previous reports, E2 treatment resulted in decreased ER levels in the absence of MG132 (84). No decrease was observed with Tam alone, and Ral appeared to cause a modest decrease. Interestingly, all ligands inhibited the accumulation of cytosolic aggregates induced by MG132. However, much more ER was retained in the nucleus following Tam, and to a lesser extent Ral, exposure.

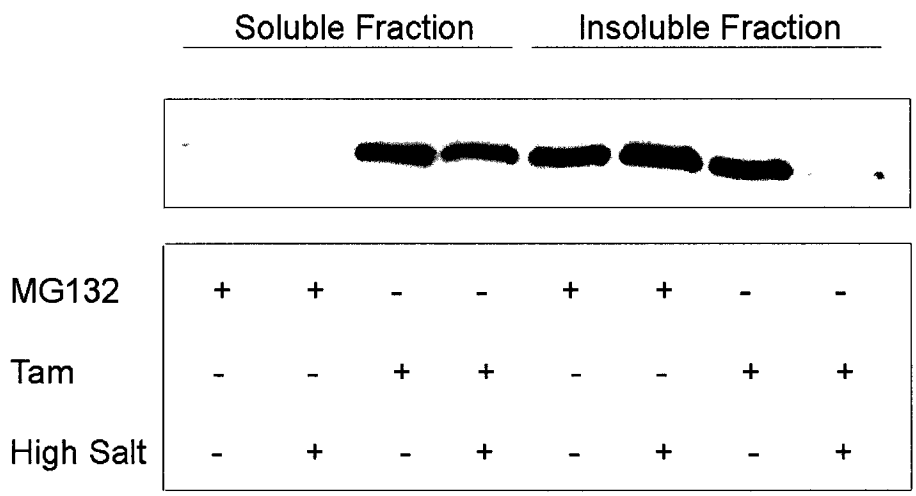


Figure 23: Tam does not Induce Aggregation of ER Protein

MCF-7 cells were treated with MG132 (10μM) or Tam (1μM) overnight. Cell lysates were prepared using NP-40 lysis buffer containing 40mM or 400mM (High Salt) KCl, and soluble and insoluble fractions were isolated by centrifugation. Insoluble fractions were solubilized in buffer containing 2% SDS. Lysates were resolved by SDS-PAGE and immunoblotted for the ER.

Inhibition of Transcriptional Initiation Results in Nuclear Estrogen Receptor Accumulation and Abrogation of Geldanamycin-Induced Estrogen Receptor Degradation

In hormone-responsive breast cancer cells, the Tam-bound ER associates with DNA but does not activate gene transcription (77). Recent reports have shown that inhibition of RNA polymerase II C-terminal phosphorylation with DRB abrogates ER-stimulated transcriptional initiation, resulting in prolonged association with EREs (93). To investigate whether this may be a mechanism for the observed accumulation of Tam-bound ER in the nucleus, we treated MCF-7 cells for 6h with EtOH, Tam (1μM), Ral (1μM), or DRB (10μM) in the presence or absence of 1μM GA. NP-40 soluble and insoluble lysate fractions were prepared and ER levels were analyzed by SDS-PAGE and immunoblotting. As shown in figure 25, the expected accumulation of ER protein in the insoluble fraction was observed with Tam and Ral compared to EtOH control. Interestingly, DRB also caused accumulation of insoluble ER. Furthermore, GA had no effect on ER levels in the presence of the three compounds, while soluble ER levels in EtOH control cells decreased by approximately 50%. These results suggest that inhibition of transcriptional initiation can mimic the effects observed with SERMs. Because DRB does not bind to the ER directly, the observed inhibition of GA-induced ER degradation by DRB in the soluble fraction is likely due to nuclear ER that was retained in the nucleus during treatment, but leaked into the cytosolic

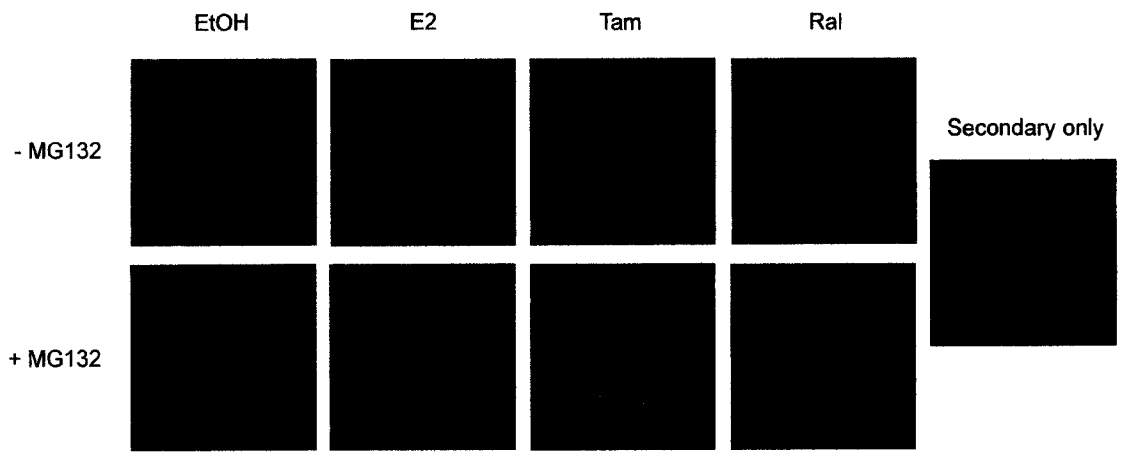


Figure 24: Nuclear Accumulation of ER Protein by ER Ligands in the Presence of MG132

Cells growing in chamber slides were treated with EtOH vehicle control, E2 (1μM), Tam (1μM), or Ral (1μM) in the absence or presence of 10μM MG132 overnight. Cells were fixed and stained for the ER using an immunofluorescent technique. Secondary only represents a negative control where no primary antibody was used.

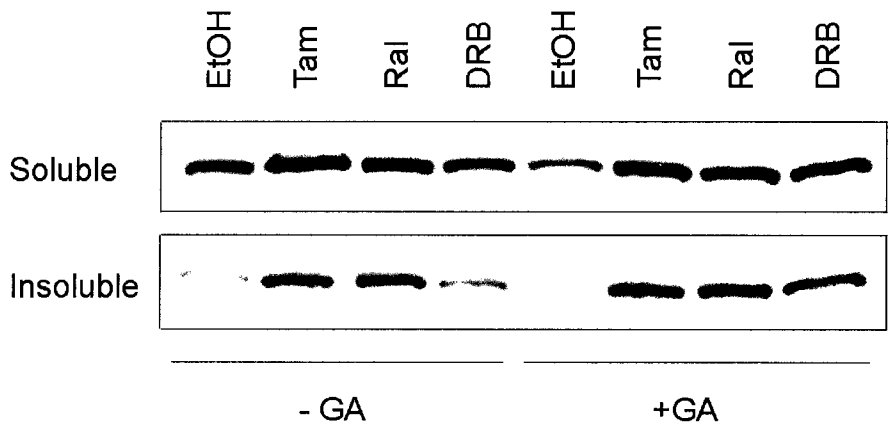


Figure 25: Inhibition of ER Transcriptional Initiation Abrogates GA-Induced ER Degradation and Causes Nuclear ER Accumulation

MCF-7 cells were treated with EtOH vehicle control, Tam (1μM), Ral (1μM), or DRB (50μM) for 6h in the absence or presence of 1μM GA. NP-40 soluble and insoluble fractions were prepared and ER levels were analyzed by immunoblotting.

fraction during the cell lysis procedure. This is also likely the case with Tam and Ral, since we determined that the ER is primarily localized to the nucleus after overnight treatment in the presence of these compounds (figure 24).

Prolonged Association of the Estrogen Receptor with an Estrogen-Responsive Element Following Tamoxifen Exposure

To directly assess whether Tam stabilizes ER association with the DNA, we analyzed ER binding to the ERE in the PS2 promoter using ChIP analysis. The ChIP procedure allows for evaluation of ER promoter occupancy through formaldehyde crosslinking of ER protein to DNA, followed by ER immunoprecipitation and amplification of bound EREs by PCR. MCF-7 cells were grown in media containing 5% charcoal-stripped serum for at least 3 days prior to experimentation. Cells were then exposed to E2 (1μM) or Tam (1μM) for 30, 60, and 90 min, and ChIP was performed using ER-specific antibodies as described in experimental procedures. Under basal conditions, ER binding to the PS2 promoter was not detected (figure 26*A*, Time 0). Following 30 min E2 stimulation, however, a strong induction of ER binding was observed. The association was significantly less after 60 min, and was completely absent after 90 min of E2 exposure. Interestingly, the dynamics of ER binding were significantly altered following Tam exposure. ER-ERE association was delayed with Tam, and maximal binding was not achieved until the 60 min time point

(figure 26*B*). Unlike E2, where a rapid decline in ER binding was observed at later time points, the association was sustained with Tam, and DNA-bound ER was readily detected 90 min after Tam stimulation. Of note, Tam appeared to induce somewhat less ER binding to the PS2 promoter overall when compared to E2 (compare E2 30' and Tam 60'). Importantly, DNA amplified from chromatin extracts before ChIP (Inputs) showed that equal amounts of DNA were present in each sample before immunoprecipitation.

Chaperone Association at an Estrogen-Responsive Element Following Estrogen Stimulation

Studies with the GR have shown that Hsp90 and p23 bind to GREs following GR stimulation (279). Therefore, we used ChIP analysis to determine whether these chaperones also bind to the ER following E2 stimulation. MCF-7 cells growing in 2% charcoal-stripped serum for 3 days were treated with E2 (1μM) for the indicated times. The binding of the ER, p23, and Hsp90 to an ERE was determined by ChIP analysis, and the results are shown in figure 27. Little basal association was observed before E2 treatment (Time 0). However, E2 stimulation resulted in rapid ER binding (within 30 min) and subsequent dissociation. Hsp90 and p23 also bound to the ERE within 30 min, and decreased binding was observed over the timecourse. To control for non-specific binding of DNA to the antibody, ChIP was also performed with a non-

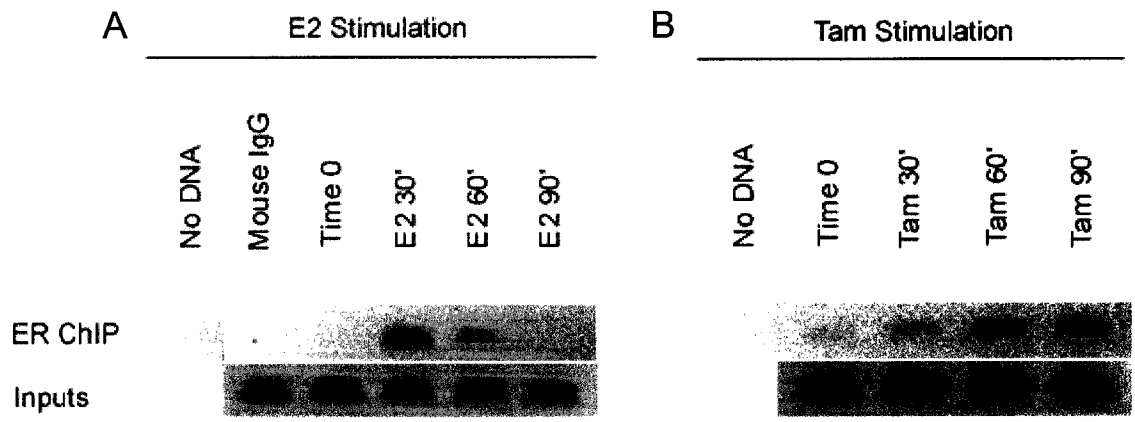


Figure 26: Delayed and Prolonged Association of the ER with an ERE Following Tam Treatment

MCF-7 cells were grown in 5% charcoal-stripped serum for at least 3 days. Cells were then treated with 1μM E2 (panel A) or 1μM Tam (panel B) for the indicated times. Cells were crosslinked with 1% paraformaldehyde for 10min, lysates were prepared, chromatin was sheared, and the ER was immunoprecipitated. Following immunoprecipitation, crosslinks were reversed by heating the samples, and occupancy of the ER at the PS2 promoter was analyzed by PCR. Time 0 represents basal levels of ER binding and Mouse IgG indicates a sample that was immunoprecipitated with a non-specific mouse IgG. No DNA is a PCR reaction where no template DNA was included. Inputs: PCR on lysates before immunoprecipitation indicates that equal amounts of DNA were precipitated in all samples.

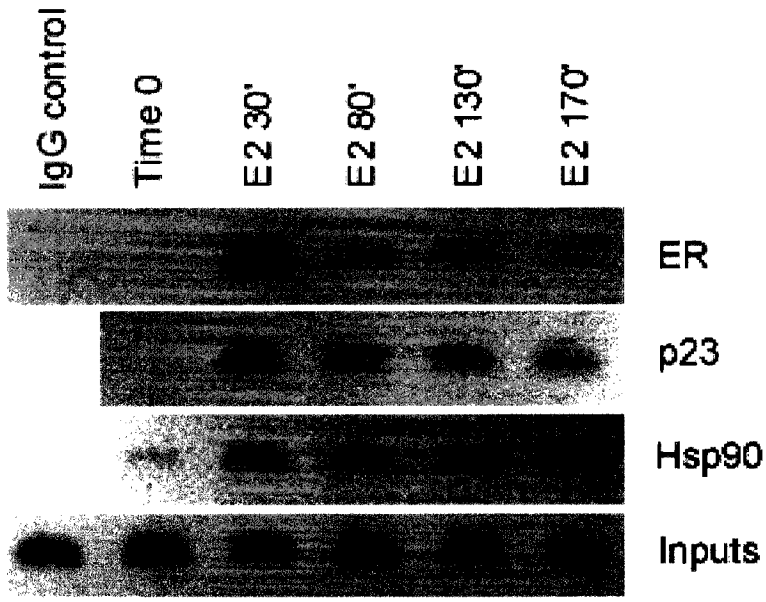


Figure 27: Occupancy of Hsp90 and p23 at an ERE Following E2 Stimulation MCF-7 cells were grown in 2% charcoal-stripped serum for at least 3 days. Cells were then treated with 1μM E2 for the indicated times. Cells were crosslinked with 1% paraformaldehyde for 10min, lysates were prepared, chromatin was sheared, and the ER was immunoprecipitated. Following immunoprecipitation, crosslinks were reversed by heating the samples, and occupancy of the ER at the PS2 promoter was analyzed by PCR. Time 0 represents basal levels of ER binding and Mouse IgG indicates a sample that was immunoprecipitated with a non-specific mouse IgG. Inputs: Equal amounts of lysates before immunoprecipitation were saved and PCR was performed to confirm that comparable amounts of DNA were present in each sample.

specific mouse IgG (IgG control). DNA amplified from chromatin extracts before ChIP (Inputs) showed that equal amounts of DNA were present in each sample before immunoprecipitation.

Discussion

The SERMs represent a class of agents that effectively inhibit ER activity and induce remissions in the majority of patients with ER+ tumors. Indeed, Tam has been used as a front-line breast cancer treatment for over 20 years. However, acquired Tam resistance and Tam-stimulated growth have been observed in animal models and in the clinic. Thus, the therapeutic efficacy of Tam, and almost certainly other SERMS, is severely limited by acquired resistance. Interestingly, the ER is not mutated or deleted in a majority of Tam-resistant breast cancer, and Tam-resistant cells respond to further treatment with aromatase inhibitors and the pure antiestrogen ICI 182,780 (134, 148). These observations suggest that the ER still plays an integral role in the progression of Tam-resistant tumors. While further endocrine manipulation with other agents following Tam failure is promising, resistance to these agents has also been observed (165). The mechanisms of Tam resistance are unclear, but likely involve altered cofactor expression/association with the ER and enhanced N-terminal ER phosphorylation through increased stimulation of growth factor signaling pathways (345). Regardless of the mechanism, it is clear that a

functional ER is required for the growth of Tam-resistant tumors, and that further hormonal manipulation using other ligand-dependent therapies may only delay tumor progression. Therefore, alternative approaches for inhibiting ER function in a ligand-independent manner may be of great benefit in treating breast cancers that no longer respond to endocrine therapy. Our lab has been investigating the efficacy of such an approach by inducing ER degradation with the heat-shock protein 90 (Hsp90) binding agent, geldanamycin (GA).

Steroid hormone receptors (SHRs) require the molecular chaperone Hsp90 and other cochaperones to maintain a mature conformation that is capable of ligand binding and subsequent activation (246). This interaction is ATP-dependent, and GA inhibits formation of the mature complex by blocking ATP binding to the ATP/ADP binding pocket of Hsp90. In the presence of GA, the SHR accumulates in an intermediate complex consisting of a different set of chaperones. The receptor in this complex is then ubiquitinated and degraded by the 26S proteasome. In addition to the SHRs, GA induces the degradation of several other signaling proteins involved in breast cancer progression, including erbB2 (359), Akt (307), and Raf-1 (295). We have previously shown that ER, Akt, and Raf-1 protein levels are decreased in Tam-resistant cells following GA exposure (358). However, Tam selectively inhibits ER degradation by GA both in cell culture and *in vivo*. Given the clear role of the ER in the progression of Tam-resistant tumors, it seems likely that the *in vivo* antagonism of Tam on GA's

degradative effects may result in decreased antitumor activity of the drug. Inhibition of GA-mediated ER degradation by Tam is seen in both MCF-7 cells and in Tam-resistant variants of MCF-7. Therefore, we chose the MCF-7 cell line to investigate potential mechanisms for Tam-mediated inhibition of GA-induced ER degradation.

Ral and Tam are both SERMs, and display similar inhibitory effects in the breast. However, Ral has little agonistic activity in the endometrium (360). Since both compounds inhibit ER transcriptional activity, we wondered whether Ral could also inhibit ER degradation by GA. In figure 20, we show that Ral does indeed partially inhibit the degradative effects of GA on the ER. This result suggests that the inhibition is not a specific effect of Tam, but is instead a general characteristic of the SERMs. Antiestrogens can be grouped into at least four classes based on the ER structural changes that they induce, all of which are distinct from the E2-bound conformation (92). E2 binding to the ER results in chaperone dissociation, and inhibition of ATP binding to Hsp90 by GA inhibits the mature ER-chaperone complex. These observations indicate that the ER-chaperone complex is extremely sensitive to changes in ER and/or Hsp90 conformation. Therefore, we tested the possibility that the conformation adopted by the Tam-bound ER inhibits the ability of GA to induce accumulation of the intermediate ER-chaperone complex. In figure 21, we determined that the intermediate complex-associated cochaperone p60 was bound to the ER following GA treatment. Tam

did not inhibit GA-induced association of p60 with the ER, indicating that GA-induced accumulation of the intermediate complex is not inhibited by Tam.

The ER is localized to the nucleus under basal conditions, and must be exported to the 26S proteasome in the cytoplasm for degradation (87). Furthermore, the degradative effects of GA require nuclear export of ER protein for subsequent degradation by the proteasome. The ER is rapidly ubiquitinated and degraded following E2 stimulation. However, Tam increases ER half-life (132). Because the Tam-bound ER associates with the DNA, we investigated the possibility that Tam causes nuclear retention of the ER, thus inhibiting the degradative effects of GA. Using differential centrifugation, we crudely separated nuclei from cytosolic material and analyzed the presence of the ER following various treatments (figures 22 and 23). We determined that Tam does indeed increase nuclear retention of ER protein, and this retention was inhibited when the cells were lysed in buffer containing high salt. Studies have shown that high salt dissociates multi-protein complexes associated with SHRs (361), and the ligand-bound ER associates with a variety of proteins on the DNA to form a functional transcriptional complex (93). Thus, the observation that Tam induces nuclear ER accumulation that is salt-extractable suggests that the Tam-bound ER may be in a multi-protein complex on the DNA. Clearly, Tam does not simply cause aggregation of ER protein because when ER aggregates were induced with the proteasome inhibitor MG132 the protein in the NP-40 insoluble fraction was not

salt extractable (figure 23). Interestingly, a significant amount of ER protein is isolated in NP-40 soluble fractions (figures 20, 22, 23, 25). This observation seemingly refutes previous reports that the majority of ER protein is localized to the nucleus (362). However, it is likely that the NP-40 lysis procedure allows a significant portion of ER protein to leak out of the nucleus and into the soluble fraction. When we used an approach that does not require cell lysis with NP-40 (immunofluorescent staining), ER protein is clearly localized predominantly to the nucleus (figure 24), consistent with previous reports (362). Interestingly, in this experiment we observed inhibition of ER cytoplasmic aggregate formation in the presence of Tam and the proteasome inhibitor MG132. This effect appeared to be due to inhibited nuclear export of ER protein, since nuclear ER staining was observed with MG132 + ER ligands, but not with MG132 alone. The levels of nuclear ER in the presence of MG132 + ligands varied depending on which ligand was used, and Tam was the most effective at causing retention of ER protein in the nucleus (figure 24, bottom panel). Interestingly, it has been shown recently that MG132 inhibits E2-induced transactivation of an ERE reporter and ER ubiquitination (86), suggesting that proteasome inhibitors may alter the normal degradative cycle of the ER, inhibiting ER export to the proteasome. Thus, DNA-associated ER may become immobilized on the DNA in the presence of MG132. This may explain why we saw inhibition of MG132-induced cytoplasmic aggregate formation with all ligands, including E2.

To determine whether inhibition of ER transcriptional initiation inhibits the degradative effects of GA on the ER, we utilized the transcriptional initiation inhibitor DRB. This compound inhibits RNA pol II phosphorylation and causes prolonged association of the ER with EREs. In figure 25, we show that DRB Tam, and Ral have similar effects on GA-induced ER degradation. Specifically, nuclear ER accumulation and inhibition of GA-induced ER degradation was observed with all three compounds. This suggested that the SERMs may inhibit GA's effects on the ER by inducing a "stalled" ER transcriptional complex on the DNA. To directly test this possibility, we determined the dynamics of ER binding to an ERE using ChIP analysis (figure 26). E2 stimulation resulted in rapid association and dissociation of the ER with the ERE over a 90' timecourse. In contrast, Tam caused a delayed and prolonged ER association. Therefore, the mechanism for SERM-mediated inhibition of GA-induced ER degradation appears to be through prolonged association of the ER with the DNA. This results in inhibited nuclear export and nuclear retention of ER protein.
The ER is effectively sequestered in the nucleus and GA-induced degradation through the proteasome is impaired.

A recent report has shown that Hsp90 and p23 associate with glucocorticoid responsive elements following GR stimulation, and may be important in disassembly of the GR transcriptional complex (279). Indeed, we have shown that Hsp90 and p23 also associate with an ERE following E2 stimulation (figure

26), and it is likely that these chaperones may play a similar role in disassembly of the ER transcriptional complex. The consequences of Tam or GA treatment on Hsp90 and/or p23 associations with the ERE are currently unknown, but this work is ongoing in our laboratory.

In summary, we have shown that SERMs inhibit GA-induced degradation of ER protein. The mechanism for this inhibition appears to be due to prolonged association of the Tam-bound ER with the DNA, resulting in diminished nuclear export and subsequent degradation of ER protein. Significantly, inhibition of GA and 17AAG-induced ER degradation by Tam has also been observed *in vitro* and *in vivo* (358). Given the importance of the ER in the progression of Tam-resistant tumors, it appears likely that the ER stabilization by Tam in the presence of 17AAG/GA may reduce the antitumor activity of 17AAG by inhibiting the ability of the drug to reduce the levels of a key protein (the ER) involved in the progression of Tam-resistant breast cancer. Studies with 17AAG have shown that it displays antitumor activity in both androgen-sensitive and androgen-independent prostate tumors (307). Thus, in cases where the AR is required for antiandrogen-resistant tumors, antiandrogens may also have detrimental effects on the antitumor activity of 17AAG. A second class of antiestrogens that display unique functional characteristics are the pure antiestrogens. The compound ICI 182,780 (faslodex) is currently in clinical development, and induces degradation, rather than stabilization, of ER protein (122). Thus, it seems likely that ICI 182,780 may

enhance, rather than antagonize, the effects of 17AAG on the ER. Although currently unknown, combination therapy with faslodex and 17AAG may potentially result in increased antitumor activity than treatment with either agent alone. It is clear, however, that SERMs antagonize GA-induced ER degradation by sequestering it in the nucleus and away from the proteasome. Therefore, it is possible that SERMs may inhibit the clinical efficacy of 17AAG, and combination therapy should be viewed with caution.

V. CONCLUDING STATEMENTS

Breast cancer is a major health problem in the United States, affecting the lives of over 200,000 American women each year. Major advances in screening and prevention techniques have been made in the last two decades, and more breast cancers are being identified *in situ*, where surgical removal of the tumor is often curative. However, a significant fraction of women present with more advanced disease, and systemic therapy is required. While several conventional chemotherapeutic agents have shown efficacy in the clinic, hormonal therapy provides a more specific, less toxic approach for the treatment of patients with metastatic disease.

Many breast cancers are uniquely sensitive to hormonal manipulation, and the ER has been identified as a key target for breast cancer therapy. Indeed, ER protein levels and/or function are increased in many breast cancers, and ER+ tumors respond well to hormonal manipulation. Over the past several decades, a number of ER antagonists have been described that inhibit ER function. These compounds are collectively called antiestrogens, and compete with E2 for the ligand-binding pocket of the ER. Tam is the most well characterized antiestrogen, and has been used clinically for nearly thirty years. Although most patients with ER+ tumors initially respond to Tam therapy, acquired resistance is almost always observed. Resistance to other antiestrogenic compounds has

also been described, illustrating a major limitation to current ER-targeted therapies. Importantly, ER function is still required for the progression of Tam-resistant disease. Therefore, inhibition of ER function in a ligand-independent manner may prove quite useful in the treatment of patients that have failed therapy with Tam or other antiestrogens.

In this dissertation, the efficacy of the Hsp90 binding agent GA as an alternative therapy for Tam-resistant breast cancer has been investigated. Although Hsp90 is not a protein that is directly implicated in cancer progression, it is required for the stability of many proteins that do play a role in cancer. Therefore, Hsp90 represents a unique target for cancer therapy because modulation of its function by Hsp90-binding drugs can result in degradation of important signaling proteins involved in cancer progression, including the ER, the erbB2 receptor, Akt, and Raf-1. Antitumor activity of the clinically relevant GA analog, 17AAG, has been observed in antiandrogen-sensitive and resistant prostate cancer xenografts. Furthermore, our lab has shown that 17AAG inhibits the growth of Tam-sensitive breast cancer xenografts. Taken together these reports and the data presented in this dissertation indicate that 17AAG may also be effective in the treatment of Tam-resistant breast cancer.

GA binds to the ATP/ADP-binding pocket of Hsp90, effectively altering the Hsp90-client protein complex. This results in enhanced ubiquitination and

proteasomal degradation of Hsp90 client proteins. Since the antitumor activity of 17AAG is likely due to decreased levels of oncogenic Hsp90 clients, we determined whether GA/17AAG induced the degradation of the ER, Akt, and Raf-1 in Tam-sensitive and Tam-resistant cancer cell lines. Indeed, the levels of all three Hsp90 clients were decreased following GA exposure in all cell lines tested, indicating that Tam-resistance does not result in altered sensitivity to GA-induced degradation of Hsp90 client proteins. Furthermore, we determined that 17AAG-induced degradation of ER protein had a functional consequence on ER transcriptional activity in both Tam-sensitive and Tam-resistant cell lines. Specifically, E2-stimulated expression of a luciferase reporter was inhibited by 17AAG.

To measure ER protein levels *in vivo*, we had to overcome a methodological problem. ER levels in tumor lysates are not easily measured by immunoblotting because contaminating serum albumins run at approximately the same molecular weight and disrupt antibody binding. Therefore, we used an alternative approach based on immunofluorescent staining and laser-scanning cytometry (LSC). In MCF-7 cells treated +/- 17AAG and immunostained for the ER, we showed quantitative differences in ER levels using LSC. These differences were consistent with confocal images and immunoblot data, indicating that ER immunostaining followed by LSC is a useful alternative for measuring ER levels *in vivo*.

An interesting observation from these studies was that Tam antagonized the degradative effects of GA both *in vitro* and *in vivo*. Tam is known to induce a distinct conformational change in the ER, and we hypothesized that this altered conformation may be less sensitive to GA-induced alterations in the multichaperone-ER complex. This was not the case, however, as we showed that GA is able to alter ER-chaperone interactions in the presence or absence of Tam using an immunoprecipitation technique. Inhibition of GA-induced ER degradation was also observed with Ral *in vitro*, indicating that this may be a general characteristic of SERMs.

MCF-7 tumor xenografts require E2 supplementation for growth. In our studies, we determined that Tam was much more effective than E2 at stimulating the growth of our Tam-resistant cells *in vivo*. This was not entirely surprising, since Tam-stimulated tumor growth has been documented in *in vivo* models and in the clinic. Inhibition of Tam-stimulated tumor growth by 17AAG was observed, which correlated with modulation of the Hsp90 clients Raf-1, Akt, and Hsp72 in the tumors. ER levels were not reduced, indicating that Tam antagonizes the degradative effects of GA on the ER both *in vitro* and *in vivo*.

The Tam-bound ER associates with the DNA and associates with corepressor proteins to inhibit transcription. While E2 stimulation results in rapid turnover of

ER protein, Tam stabilizes ER half-life. Since Tam does not inhibit the ability of GA to induce alterations in the ER-multichaperone complex, we hypothesized that the Tam-bound ER accumulates in the nucleus through prolonged association with the DNA and inhibits nuclear export and subsequent presentation to the proteasome. Using differential centrifugation of cell lysates, we show that the ER accumulates in the nucleus following Tam exposure. Furthermore, the nuclear accumulation is disrupted when cells are lysed in high salt buffer, indicating that the Tam-bound ER is likely associated with the DNA in a multi-protein complex. Unlike the proteasome inhibitor MG132, Tam does not induce the accumulation of ER aggregates, and immunolocalization of the ER showed that Tam inhibits the accumulation of MG132-induced ER cytosolic aggregates by retaining the ER in the nucleus.

Inhibition of transcriptional initiation results in a "stalled" ER transcriptional complex, which causes prolonged association of the ER with the DNA. If prolonged association of the ER with the DNA is indeed the mechanism for inhibition of GA-induced ER degradation by Tam, then inhibition of transcriptional initiation should produce a similar effect. In these studies we show that the transcriptional initiation inhibitor, DRB, like Tam, induces nuclear accumulation and inhibits GA-mediated degradation of ER protein. To directly assess whether Tam and DRB inhibit ER degradation by the same mechanism (ie. prolonged association with the DNA), we investigated the DNA binding dynamics of the

Tam-bound ER by ChIP. Upon E2 stimulation, the ER rapidly associated and dissociated with an ERE. In contrast, there was a delayed and prolonged association of the Tam-bound ER. Taken together, these results suggest that Tam causes the nuclear accumulation of the ER by inducing a prolonged association with EREs. As a consequence, the ER accumulates in the nucleus, and is not exported to the cytoplasm for presentation to the proteasome.
A recent study has shown that Hsp90 and p23 associate with GREs following GR stimulation. It has been hypothesized that these proteins play a role in disassembly of the GR transcriptional complex. In these studies, we also show association of Hsp90 and p23 with EREs following E2 stimulation. Presumably, these chaperones play a similar role in disassembly of the ER transcriptional complex. The consequence of GA on this interaction is not yet known, but is currently under investigation in our laboratory.

Based on our findings in chapter IV, a model for Tam inhibition of GA-induced ER degradation is presented in figure 28. Under basal conditions, the ER is predominantly localized to the nucleus with Hsp90 and co-chaperones. Stimulation with E2 results in rapid DNA binding and transcriptional activation. This ER-DNA association is transient, however, and the ER is rapidly dissociated from EREs and presented to the proteasome for degradation. In contrast, Tam binding causes the ER to associate with the DNA in a "stalled" transcriptional complex that cannot be readily dissociated. Thus, the ER is sequestered in the

nucleus and away from the proteasome. In these studies, the inhibition of GA-induced degradation was only partially inhibited by Tam/Ral, suggesting that a fraction of the cellular ER pool is still degraded by GA treatment. One possible explanation for this observation is that the cells were treated simultaneously with both agents. Indeed, the inhibitory effects may be enhanced if cells are first pre-treated with Tam or Ral.

Taken together, these data indicate that 17AAG may be an effective treatment for Tam-resistant breast cancer. However, combined therapy with SERMs and 17AAG could be a problem because Tam and Ral inhibit GA/17AAG-induced ER degradation. Given the critical role for the ER in the progression of Tam-resistant breast cancer, it seems possible that the antitumor effects of 17AAG may be enhanced in the absence of Tam. Clinically, combined therapy of Tam + 17AAG would not occur in patients with Tam-resistant tumors. Thus, maximal inhibition of the ER by 17AAG could presumably be achieved. Interesingly, the pure antiestrogen ICI 182,780 (fulvestrant) induces degradation of ER protein and is effective against Tam-resistant breast cancer. Although combination therapy with fulvestrant and 17AAG has not been investigated, it is intriguing to postulate that these two agents may act synergistically in Tam-resistant tumors. The mechanism for Tam-mediated inhibition of GA-induced ER degradation appears to be through prolonged association with the DNA, resulting in sequestration away from the proteasome. This inhibitory effect is not specific for Tam, but

seems to be a general characteristic of the SERMs. Furthermore, it seems likely that this effect may also be observed with competitive inhibitors for other SHRs (ie. antiandrogens), provided that the antagonist does not cause degradation of the receptor protein. The data presented in this dissertation provide pre-clinical support for the use of 17AAG in the treatment of hormone-refractory breast cancer. Given its efficacy against Tam-resistant breast cancer in the clinic, the antitumor effects of the pure antiestrogen fulvestrant in combination with 17AAG may be particularly interesting.

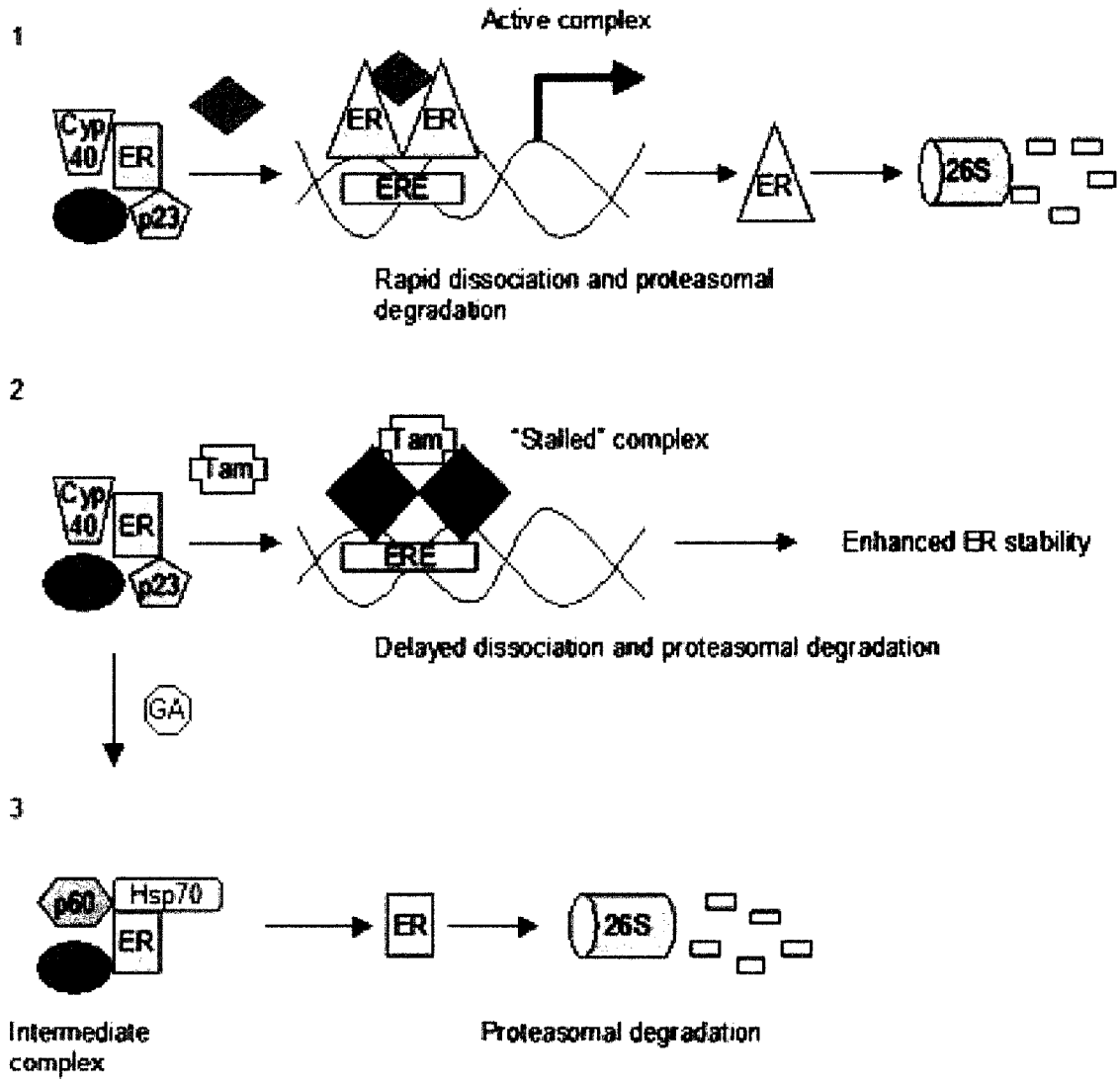


Figure 28: A Model for Inhibition of GA-Induced ER Degradation by Tam

The ER is primarily localized to the nucleus in a complex with Hsp90, p23, and Cyp40. 1: E2 binds to the ER and alters its conformation, resulting in

dissociation of the multichaperone complex. The E2-bound ER rapidly forms an active transcriptional complex at EREs on the DNA. This complex is rapidly dissociated, and the receptor is exported from the nucleus and degraded by the 26S proteasome. 2: Tam-bound ER adopts a unique conformation that is distinct from the E2-bound form, which also results in dissociation of the multichaperone complex and binding to EREs. Tam inhibits transcriptional activation, and causes the ER complex to be stalled on the DNA. This results in nuclear retention of ER protein, and sequestration from GA-induced degradation. 3: The inhibition of degradation by Tam is only partial, and suggests that there is a pool of ER protein that is degraded by GA through accumulation of the intermediate ER-chaperone complex.

APPENDIX: HUMAN/ANIMAL SUBJECTS APPROVAL

Institutional Animal Care
and Use Committee

THE UNIVERSITY OF
ARIZONA.
TUCSON ARIZONA

P.O. Box 210101
Tucson, AZ 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

#01-073 - "Experimental Mouse Shared Service"

PRINCIPAL INVESTIGATOR/DEPARTMENT:

Joyce A. Schroeder, PhD - MCB/AZCC

GRANTING AGENCY:

Various

SUBMISSION DATE: **May 22, 2001**

APPROVAL DATE: **August 14, 2001** APPROVAL VALID THROUGH*: **August 13, 2004**

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **May 5, 2003**

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell

Richard C. Powell, PhD, MS
Vice President for Research

DATE: May 5, 2003

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

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