THE NUCLEAR FACTOR KAPPA B (NFκB) PLAYS A CRITICAL ROLE IN THE DEVELOPMENT OF ANTIESTROGEN RESISTANCE

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THE NUCLEAR FACTOR KAPPA B (NFκB) PLAYS A CRITICAL ROLE IN THE DEVELOPMENT OF ANTIESTROGEN RESISTANCE

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ABSTRACT

Resistance to endocrine therapies remains a major problem in the management of estrogen receptor (ER) positive breast cancer. Precise mechanisms that contribute to acquired resistance remain undefined. While over-expression of nuclear transcription factor-κB (NFκB) has been implicated in drug resistance, its role in affecting responsiveness to antiestrogens (TAM and ICI 182,780; Faslodex) is unknown. We show here that inhibition of NFκB either by over-expression of a mutant IκB”super-repressor” (IκBSR) or a small molecule inhibitor (parthenolide) provides a means to overcome resistance to both SERMs (4-hydroxytamoxifen; 4HT) and SERDs (ICI 182,780). Parthenolide not only reverses resistance to antiestrogens in resistant cells (MCF7/LCC9; MCF7/RR) but also increases the responsiveness of sensitive cells (MCF-7; MCF7/LCC1) to both SERMs and SERDs. These effects are independent of changes in the level of autophagy, measured by cleavage of LC3 or inhibition of p62/SQSTM1 expression, or in cell cycle distribution.

We then proceed to show that treatment with 4HT in the presence of an inhibited NFκB restores TAM-induced cell death in resistant cells by decreasing the expression of BCL2 (anti-apoptotic protein), increasing mitochondrial membrane permeability (MMP), and inducing apoptosis. These activities of NFκB involve the regulation of CASP8 action upstream of
mitochondria. The pancaspase inhibitor (PI) and a specific CASP8 inhibitor (C8I), both reverse the effects of IκBSR and parthenolide on BCL2 expression, MMP, and responsiveness to 4HT. In addition to establishing a role for NFκB in the development of antiestrogen resistant phenotype, we provide evidence that NFκB activation in resistant cells is necessary but not sufficient to drive their antiestrogen resistant phenotype.

Taken together, these data show that parthenolide acts primarily through its inhibition of NFκB, providing important and relevant new insights into how NFκB signaling affects antiestrogen (primarily tamoxifen; TAM) responsiveness in breast cancer cells. These data also strongly suggest that a combination of parthenolide and SERM/SERD may offer a novel therapeutic approach to the management of some ER+ breast cancers. In this era of personalized medicine, measuring NFκB and markers of its functional signaling may help us to tailor specific therapies to individual breast cancer patients while improving prognosis and clinical outcomes for women.
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<tbody>
<tr>
<td>4HT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>AEBS</td>
<td>Antiestrogen binding sites</td>
</tr>
<tr>
<td>AF-1</td>
<td>Hormone-independent transactivation domain</td>
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<tr>
<td>AF-2</td>
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<td>Charcoal stripped-improved minimal essential medium</td>
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<tr>
<td>CDK1</td>
<td>Cyclin-dependent kinase 1</td>
</tr>
</tbody>
</table>
CML  Chronic myelogenous leukemia
COX-2  Cyclooxygenase 2
CYP2D6  Cytochrome P450, family 2, subfamily D, polypeptide 6
cyPG  Cyclopentenone prostaglandin
DBD  DNA binding domain
DD  Death domain
DDE  1, 1-dichloro-2,2’-bis-p-chlorophenylethylene
DDT  Dichloro-diphenyl-trichloroethane
DIM  3,3’-diindolylmethane
DISC  Death inducing signaling complex
E2  Estrogen; 17 beta estradiol
EBV  Epstein-Barr virus
ELISA  Enzyme-linked immunosorbent assay
ERα  Estrogen receptor-alpha
ERβ  Estrogen receptor-beta
ERE  Estrogen response elements
EV  Empty vector
FACS  Fluorescence activated cell sorting
FADD  Fas-associated death domain
FasL  Fas-ligand
FBS  Fetal bovine serum
FITC  Fluorescein isothiocyanate
FLIP  c-Flice-like inhibitory proteins
GADD  Growth arrest and DNA-damage-inducible
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
GC  Glucocorticoids
HBV  Hepatitis B virus
HCV  Hepatitis C virus
HNSCC  Squamous cell carcinomas of the head and neck
HRT  Hormone replacement therapy
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>HTLV-1</td>
<td>The human T-cell leukemia virus type I</td>
</tr>
<tr>
<td>I3C</td>
<td>Indole-3-carbinol</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of apoptosis</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>Faslodex; Fulvestrant; ICI</td>
</tr>
<tr>
<td>IxB</td>
<td>Inhibitors of NF kappa B</td>
</tr>
<tr>
<td>IxBSR</td>
<td>IkappaB super-repressor; dominant-negative NFkappaB inhibitor</td>
</tr>
<tr>
<td>IKK</td>
<td>IkappaB kinase (IKK) complex</td>
</tr>
<tr>
<td>IKKγ/NEMO</td>
<td>NFkappaB essential modulator</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRF1</td>
<td>Interferon regulatory factor-1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LC3</td>
<td>Light Chain 3</td>
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<tr>
<td>LHRH</td>
<td>Luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane permeability</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloprotease</td>
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<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
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<tr>
<td>NFκB</td>
<td>Nuclear transcription factor-kappa B</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>Par</td>
<td>Parthenolide</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP-ribose polymerase</td>
</tr>
<tr>
<td>PI</td>
<td>Pancaspase Inhibitor; zVADfmk</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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xx
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RI</td>
<td>Relative Index</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAGE</td>
<td>Serial analysis of gene expression</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>SERD</td>
<td>Selective estrogen receptor degrader</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>si-Ctrl</td>
<td>Nonsilencing control</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SV</td>
<td>Sindbis virus</td>
</tr>
<tr>
<td>TAD</td>
<td>C-terminal transactivation domain</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>tBID</td>
<td>Truncated BID; Truncated BCL2-interacting domain protein</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered saline and Tween-20</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>Tumor necrosis receptor-associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-receptor associated factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VAM-1</td>
<td>Vascular adhesion molecule-1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>XBP1</td>
<td>Human X-box binding protein</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
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Chapter 1: Introduction

1.1. Trends in Breast Cancer Incidence:

Today, breast cancer is the second most common cause of cancer related death in women after lung and bronchus cancer. Over 40,000 women will die of breast cancer this year in the United States and over 190,000 women will be diagnosed with breast cancer in this country this year (1). Approximately 70% of all these newly diagnosed breast cancers will be estrogen receptor-α positive (ER+; HUGO Gene Symbol; ESR1). However, over the past twenty years, death rates associated with breast cancer in women have decreased by 37% (1). This significant progress made in reducing mortality rates is potentially attributable to timely detection of the disease and its treatment. Despite the significant progress made in reducing mortality rates, metastatic breast cancer still remains an incurable disease.

1.2. Risk factors for the development of Breast Cancer:

It remains difficult to explain why some women develop breast cancer and others do not, as there are multiple factors that appear to control the etiology of breast cancer in women. Age, family history, reproductive factors, and a previous history of benign breast disease are all recognized factors of risk. Breast cancer incidence in general increases with age and studies have shown that the incidence almost doubles every 10 years (2). Breast cancer risk also varies by geographical variation. There exists a difference in incidence for breast cancer between Eastern and Western countries. Although this difference between countries is diminishing, American women are still at an approximately five-fold increased risk of developing breast cancer compared to Asian women. Studies have shown that upon migration and adoption of a western
lifestyle, these populations lose their protection within one or two generations, suggesting that environmental factors are perhaps more important than the genetic factors (3,4).

The associations between age at menarche and menopause are known to be important risk factors for breast cancer. Women who start menstruating before the age of 14 increases their risk of developing breast cancer by two-fold compared to women who begin to menstruate after the age of 14 (5). Similarly, in women who experience menopause before the age of 45, the risk of developing breast cancer is reduced by half compared to women who experience menopause after the age of 55 years (6). Another reproductive factor that correlates with the risk for breast cancer is the age at first pregnancy. The risk in women is known to increase by about two-fold if they deliver their first child after the age of 30 compared to women delivering before the age of 20 (7,8).

One of the most widely recognized risk factor for breast cancer is family history. About 5-10 percent of breast cancers are thought to be due to a specific inherited mutation (9,10). Two breast cancer genes associated with a genetic predisposition to cancer: the BRCA1 and BRCA2 genes that are located on long arms of chromosomes 17 and 13 respectively (11,12). Women with mutations in these two genes have been identified to be at high risk of breast cancer development. The risk of developing breast cancer in women with mutated BRCA1 is about 50 percent before the age of 50 that increases to about 80 percent by the age of 65 (13). Easton et al., have also demonstrated that carriers of mutated BRCA1 gene are not only at high risk of developing breast cancer, but also have a 10 percent incidence of developing ovarian cancer by the age 60. More
recently, it has been reported that 5-15% of all ovarian cancer cases carry mutations in BRCA1 and BRCA2 (14). Thus, it is important to identify individuals who are carriers of the abnormal gene (13,15) to help reduce their risk of ovarian cancer.

Our lifestyle and the environment are some of the other factors that also correlate with breast cancer risk. A high fat diet has been associated with increased incidence of breast cancer most likely because a high fat diet is shown to increase the plasma estrogen levels (16,17). There is strong evidence that high levels of estrogen are a major risk factor for breast cancer, while progesterone may have a protective effect (18). The study shows that premenopausal women with low progesterone concentrations have a five-fold higher risk of developing breast cancer than those with normal concentrations. Additionally, the study supports the idea that if serum progesterone levels are found to be low in women who are either at high risk or those who have breast cancer; these women can be supplemented with progesterone giving them a protective effect to the disease. However, overwhelming evidence in the postmenopausal women demonstrates that the use of hormone replacement therapy (HRT): a combination of synthetic estrogen and progesterone (progestin) given essentially to “replace” body’s depleted hormone levels increases the incidence of breast cancer (19) and furthermore, the risk of breast cancer is greater in users of combined estrogen-progestin than in users of estrogen alone (19).

Additional lifestyle factors that may be associated with the increasing risk of breast cancer are obesity and alcohol intake. Although some studies have shown a positive correlation between alcohol consumption and increased risk of breast cancer, the studies are highly
inconsistent. Several epidemiological studies indicate that obesity is a risk factor for the development of postmenopausal breast cancer (20),(21). It is known that for postmenopausal women, increases in estrogen serum levels are associated with increasing levels of BMI (Body Mass Index) contributing to an increased risk of breast cancer (22,23). However, breast cancer risk is reduced in women with a higher physical activity resulting in low serum levels of estrogens (24, 23). Importantly, an inverse relationship between obesity and breast cancer risk has been reported among premenopausal women. Obesity in premenopausal women has been associated with a reduced incidence of breast cancer (25,26).

Within the last few years, among the various environmental factors, exposure to pesticides and especially organochlorine pesticides such as dichloro-diphenyl-trichloroethane (DDT), phenylethylene 1, 1-dichloro-2,2’-bis-p-chlorophenylethylene (DDE) and others have gained considerable attention. This group of chemicals is now implicated in affecting breast cancer risk. These organochlorine pesticides can act as agonists on ERα and/or antagonists on ERβ (27) and thus disrupt the endocrine process. In addition to tumor promotion properties, these pesticides are also known to produce free radicals that may either directly or indirectly be mutagenic (28). Thus, some organochlorine pesticides may cause both tumor initiation followed by tumor promotion.

1.3. Estrogen and the Estrogen-Receptor Pathway:

Estrogens have long been implicated in promoting breast cancer. Various studies in rodents have shown that estrogen in various tissues, including kidneys, liver, uterus and mammary glands can be carcinogenic (29-33). The increased levels of estrogens in serum are
consistently associated with risk of developing breast cancer in many studies (34). Evidences suggest that prolonged use of hormone replacement therapies (HRT) (35,36) that combine estrogens and progestins have an increased risk of breast cancer. The mechanisms through which estrogens contribute to carcinogenesis (initiation, promotion and progression) are very complex. The role of estrogen as tumor promoter is firmly established. However, their role as an initiator in breast cancer is largely controversial. Lacassagne et. al., in 1932 were the first to demonstrate that estrogens alone can produce mammary tumors in rodents suggesting that estrogens can affect carcinogenesis by acting as tumor promoters. A role of estrogen as an initiator in breast cancer is also suggested, (37) most likely by interacting with certain environmental toxicants, producing DNA adducts and enhancing tumorigenicity in human breast cancer cells (38).

Estrogen (primarily 17β-estradiol) exerts its effect through two structurally related, nuclear receptor transcription factors, ERα (estrogen receptor alpha) and ERβ (estrogen receptor beta) (39). ERs form homo and heterodimers. Both ERα and ERβ contain a central conserved DNA binding domain (DBD) flanked by transactivation domains (AF-1 and AF-2). The N-terminal AF-1 domain is hormone-independent, whereas the carboxy-terminal AF-2 domain is hormone-dependent. Binding of estrogen induces distinct conformational changes in the ligand binding domain (39) that allow ER to bind to DNA sequences termed “estrogen response elements” (ERE’s) in target promoters (40), subsequently turning on the transcription of estrogen–dependent genes that are involved in cell proliferation and growth [Figure 1]. In the process, ERs recruit other proteins to the transcriptional complex (these proteins may act as co-activators or co-repressors of transcription) that attract multiprotein complexes including histone acetyltransferases to the target promoters that modulate gene transcription [Figure1] (41). In
addition to direct regulation of various genes like those encoding for cell survival and cell proliferation proteins, ER can also activate transcription by interacting with the transcriptional machinery at the binding sites for other transcription factors such as AP-1, SP-1 or NFκB without directly binding to DNA (42-44).

Apart from acting through the classical genomic mechanisms as discussed above, ERs can also act through less well-characterized non-genomic, non transcriptional mechanisms that involve a membrane bound form of ERα and/or ERβ (45). Estrogen causes activation of various protein kinases, such as mitogen-activated protein kinases (MAP kinases; MAPK), and increases the levels of second messengers like cyclic AMP. This non-genomic pathway is also referred to as a second-messenger pathway (46,47).

Depending on the cell type and the specific stimulus, ER has also been shown to inhibit NFκB activity at several steps in the canonical NFκB pathway (refer to Figure 5 for the pathway)(48). ER can inhibit NFκB by mechanisms that either act in the cytoplasm (mainly include the upstream NFκB signaling steps) or by those that act in the nucleus (these mainly include inhibition of DNA binding activity). ER can affect IκB processing, which in turn can inhibit nuclear translocation of NFκB. For example, E2 (estrogen) treatment can increase IκBα gene expression and inhibit its degradation, which subsequently inhibits NFκB activation in HeLa cells (49). Furthermore, estrogen treatment in MCF-7 human breast cancer cells can block the processing of NFκB precursor protein, p105 and inhibit NFκB’s nuclear translocation and activation (50). Some in vitro studies also suggest that estrogen induced activation of ER can
inhibit the expression of IL-6 by blocking the binding of NFκB to the IL-6 promoter (51,52). Additionally, some studies show that estrogen can also inhibit NFκB’s transcriptional activity with no effect on either IκBα levels or on NFκB DNA binding activity, but possibly by affecting the ability of NFκB to interact with the coactivators such as Bcl-3, p300 and CBP (53,54).

1.4. Endocrine Therapies for Breast Cancer:

Many breast tumors depend on estrogen for their growth. Endocrine manipulation aims to specifically eliminate the cell survival stimulus that is required for breast cancers to proliferate. Endocrine manipulation, such as ovariectomy, chemical ovarian ablation, antiestrogen, or aromatase inhibitor therapy are all effective and established approaches for the treatment of ER+ breast cancers. It is now well established that estrogen stimulates the growth of some breast cancer cells and it is estimated that one third of all breast cancer patients will respond to some type of endocrine therapy. Currently, there are three different endocrine therapies with different agents in each category that are very successful in reducing the growth stimulatory signals of estrogen: 1). Agents that prevent estrogen from binding to the estrogen receptor (ER) such as the use of Tamoxifen (TAM), a non steroidal antiestrogen in both pre and post menopausal women (55). 2). Agents like ICI 182, 780 also known as Faslodex (Fulvestrant; a steroidal antiestrogen) that stimulate ER degradation and completely destroy the estrogen receptor (ER) (56). 3). Finally, agents that decrease the levels of estrogen in the blood and tumors such as third generation aromatase inhibitors such as Letrozole or Exemestane [Figure 1] (57). Temporary suppression of the ovarian function (i.e., suppression of estrogen synthesis by the ovaries) by luteinizing hormone releasing hormone (LHRH) agonists such as goserelin and leuprorelin in adjuvant
setting for premenopausal women has also been reported to be beneficial (58).

Although ovariectomy/ovarian ablation in premenopausal women, first reported by Dr. George Beatson in 1896, was one of the oldest forms of endocrine manipulation known to produce benefits in a majority of patients with early stage breast cancer (59), antiestrogen treatment is the most widely used form of first line endocrine manipulation for ER+ breast tumors. (55).

1.5. **Antiestrogens and their mechanism of action:**

1.5.1. **The Triphenylethlenes (e.g. Tamoxifen; TAM):**

Currently, the antiestrogen Tamoxifen (TAM) is among the most widely used hormonal treatments for treating patients with early stage breast cancer, both in the metastatic as well as in the adjuvant setting. Two types of selective estrogen receptor modulators (SERMs) that differ in their chemical structure are used in clinical settings; the triphenylethlenes (e.g. Tamoxifen; TAM) and the benzothiophenes (e.g. Raloxifene). Tamoxifen originally known as “Nolvadex” was first synthesized in 1963 as a fertility agent, but subsequently its activity against breast cancer became evident and it was approved for marketing by United States FDA for the treatment of breast cancer in 1977 (60).

Over the last 30 years, triphenylethylene tamoxifen or TAM (a nonsteroidal SERM) has
played an important role in both adjuvant setting as well as in treating metastatic breast cancer. In the metastatic setting, tamoxifen has been shown to delay progression and cause tumor shrinkage (61). In the adjuvant setting, TAM has been shown to increase both disease free and overall survival, and also decrease the recurrence in ER+ breast tumors (62). Randomized trials have shown that 5 years of adjuvant therapy with TAM for early stage ER+ breast cancer can proportionally reduce mortality by 28%. In addition to the established antitumor activities, TAM therapy can also provide protection against bone loss in postmenopausal women (63).

Tamoxifen is extensively metabolized by the human cytochrome P450 enzyme system into several metabolites. Of these, 4-hydroxytamoxifen (4HT) obtained by the hydroxylation of the parent compound TAM, is the most potent metabolite of TAM. 4HT binds to ERα with a higher affinity compared to 17β-estradiol (estrogen). More recently, it has been reported that endoxifen (4-hydroxy-N-desmethyl-tamoxifen) which is catalyzed by cytochrome P450 2D6 (CYP2D6), is another active metabolite of TAM that is as potent as 4HT with respect to relative affinities for ER (64). Moreover, it has been reported that patients with decreased CYP2D6 metabolism or activity produce lower levels of endoxifen and show a poor therapeutic benefit from tamoxifen (65,66). Thus, the altered metabolism of CYP2D6 has been proposed to be one of the several mechanisms responsible for tamoxifen resistance (67).

The primary mechanism of action of TAM is to compete with available estrogens (estradiol) for binding to the ER and subsequently block or inhibit estrogenic effects in the breast. When TAM binds to the ER, ER dimerizes and binds to DNA but the conformational change in the ligand binding domain (LBD) or the AF-2 domain of ER is different from that seen with
estrogen (68). This different conformational change alters the relative orientation of the AF-2 domain of the ER and changes the TAM-bound ER balance of co-activators and co-repressors in such a way that gene transcription through the AF-2 domain is blocked, but gene transcription mediated through the AF-1 domain of ER can still occur (69) leading to a much less efficient transcription of ER target genes involved in cell proliferation and growth [Figure 1]. The fact that AF-1 domain of ER remains active in the TAM-ER complex somewhat explains the partial agonist/antagonist activity of TAM.

Although the clinical usefulness of TAM is well established, it is still associated with side-effects. TAM acts as a partial agonist in the breast in rats and humans, (70) but it is not a pure antagonist of estrogen action. Based on the cell type, TAM is known to have mixed agonist and antagonist properties (partial agonist), and this has led to the term selective estrogen receptor modulator or the SERM. TAM is known to have agonist effects in the endometrium in humans, and its clinical use can increase the risk of endometrial cancer (71). However, the majority of cases of endometrial cancer after TAM treatment are low-grade stage I tumors (72), with only a few high grade sarcomas (73).

Other side-effects of TAM, such as the increased risk of thromboembolic disorders, are known to originate when TAM is administered along with chemotherapy (74). Despite the positive effects of TAM, the therapeutic index of TAM is affected due to its side-effects that are mainly attributed to partial agonist effects of TAM.
Several lines of evidence suggest that this partial agonism of TAM may also be responsible for disease progression after an initial response to TAM therapy in patients. For this reason other newer hormonal agents like ICI 182,780 and third generation aromatase inhibitors are gaining increased attention as subsequent endocrine therapies in TAM-resistant patients. But, it is important to note that TAM is effective in both premenopausal and postmenopausal women. So elucidating the molecular mechanisms of endocrine resistance and resistance to TAM in particular are of great interest.

1.5.2. The Benzothiophenes (e.g. Raloxifene):

Other antiestrogens, like the benzothiophene Raloxifene (SERM) have also shown utility. Raloxifene appears to have a better toxicological profile compared to TAM (55). Raloxifene is approved for the treatment and prevention of osteoporosis in postmenopausal women. Interestingly, Raloxifene was tested in clinical settings on postmenopausal osteoporotic women, where a significant decrease in the rate of newly diagnosed breast cancers was observed. In addition, there were no stimulatory effects seen on the endometrium. Since, Raloxifene still exhibited the antagonistic effects in the breast similar to TAM (a triphenylethylenes) (71), it may have more advantages when comparing the toxicity profile with TAM. Recent clinical studies have shown that Raloxifene not only has fewer side-effects compared to TAM, but is also as effective as TAM in preventing breast cancer (75).

1.5.3. The SERDs (e.g. Faslodex; Fulvestrant; ICI 182, 780):

The search for a specific or a “pure antiestrogen” without any agonist or estrogenic
effects that also has a very high affinity for the ER led to the development of ICI 182,780 (Faslodex; Fulvestrant). ICI 182,780 is a “pure” antagonist of ERα. It enhances the ubiquitin-mediated degradation of ERα (76), inhibits receptor dimerization (77) and inhibits estrogen-dependent gene transcription [Figure 1]. Since ICI 182,780 acts as a selective estrogen receptor degrader, it is also known as a SERD. ICI 182,780 is effective as a second line agent in TAM-resistant patients (78), where it is at least as affective as the aromatase inhibitor anastrazole (79). Many clinical trials have shown that ICI 182,780 can be used as a first line of endocrine treatment and is a good alternative to TAM and aromatase inhibitors (80). ICI 182,780 was first approved by the FDA in 2002 for the treatment of ER+ metastatic breast cancer in postmenopausal women where other antiestrogen therapy fails. Since ICI 182,780 is a pure antagonist of the ER and does not possess a partial agonist activity, it is not associated with the increased risk of endometrial cancer as seen in adjuvant TAM therapy (81).

1.6. Aromatase Inhibitors:

Recently, the class of agents that block the synthesis of estrogen, known as the Aromatase Inhibitors, are gaining attention. Aromatase inhibitors work by inhibiting the enzyme aromatase, a cytochrome P-450 enzyme (encoded by CYP19) that is responsible for the conversion of androgens (e.g. testosterone, androstenedione) into estrogens (e.g. estradiol, estrone) [Figure 1] (57). Aminogluthethimide, the first aromatase inhibitor used in clinical practices was introduced in the late 1970’s. While the drug showed comparable efficacies to TAM, its use was limited due to its nonselective nature (in addition to estrogen, it suppressed cortisol and aldosterone production) that led to many side effects (82). The second and third generation aromatase inhibitors were then developed with much greater specificity. The third
Third-generation aromatase inhibitors are mainly divided into two categories: Class I and the Class II Inhibitors. The Class I inhibitors are steroidal and bind irreversibly to the aromatase enzyme (e.g. exemestane). Class II inhibitors (e.g. anastrozole, letrozole) are nonsteroidal compounds that bind to the aromatase enzyme in a reversible manner (83). Both Class I and Class II agents are potent enough to decrease the circulating levels of estrogen below the level of detection (84-86).

The use of aromatase inhibitors is particularly effective in postmenopausal women, where peripheral aromatase is mainly responsible for synthesizing most of body’s estrogen. However, in premenopausal women with active ovaries, this approach is inappropriate because the suppression of estrogen production in the ovaries is associated with significant toxicity. Inhibition of estrogen production in premenopausal women is associated with increased gonadotropins, which can subsequently stimulate the growth of the follicles (87). Importantly, for these premenopausal women, the antiestrogenic drug tamoxifen still remains the drug of choice. The clinical experience with TAM now exceeds 15 million patient years (55). Despite the positive clinical activities seen in these other endocrine therapies, the antiestrogen therapy with TAM in particular is still the preferred first line endocrine therapy in premenopausal ER+ breast cancer patients.
1. Aromatase Inhibitors

Androgens (e.g. testosterone, androstenedione) → Estrogens (e.g. estradiol, estrone)

2. Tamoxifen (TAM)

Estradiol + ER → EREs → ER target genes

3. Faslodex (ICI)

ER degradation → Impaired ER dimerization

Adapted from Dowsett et al., Cancer, 2003.

Figure 1: Endocrine Therapies for Breast Cancer.
Figure 1. Endocrine Therapies for Breast cancer.

The enzyme aromatase converts androgens to estrogens. Estrogen (Estradiol) then diffuses into the cell and binds the estrogen receptor (ER). Two transactivation domains AF-1 and AF-2 in the ER are involved in the regulation of gene transcription. Binding of estrogen leads to receptor dimerization, a conformational change in the AF-2 domain of the ER, and binding to the estrogen response elements (EREs) in the promoters of the target genes that are involved in cell proliferation.

1) Aromatase Inhibitors inhibit the function of the enzyme aromatase and thereby reduce estrogen synthesis. Estrogen depletion reduces estrogen-dependent transcription. 2) Tamoxifen (TAM), a nonsteroidal antiestrogen on the other hand competes with available estradiol for binding to the ER. Binding of TAM to the ER induces a conformational change that is different from that seen with estradiol leading to a less efficient transcription of ER target genes. 3) ICI 182,780 (Faslodex; ICI), a steroidal antiestrogen binds to the ER with similar affinity as estradiol. ICI stimulates ER degradation, prevents receptor dimerization, and inhibits estrogen-dependent transcription.
1.7. Cytostatic and Cytotoxic Effects of Antiestrogens:

Antiestrogens are capable of inducing both cell cycle arrest and apoptosis in endocrine-sensitive cells (55,88).

1.7.1. Antiestrogen-mediated Cell Cycle Arrest:

The eukaryotic cell cycle is typically divided into four non overlapping phases. DNA synthesis and mitosis occur in the S and the M phase respectively, these phases are separated by G1 and G2 gap phases. In the G1 phase of the cell cycle, cell prepares itself for DNA replication. This is followed by S phase during which DNA synthesis occurs. Before the cell moves on to the M or the mitotic phase, the cell continues through the G2 phase of the cell cycle and prepares itself for cell division. Cells that are not actively cycling are in the G0 phase of the cell cycle (89). Defects in DNA repair, or DNA damage, or the loss of cell-cycle checkpoints, can often predispose cells to cancer (90).

The non steroidal antiestrogen TAM exerts an antiproliferative effect by inducing an early G0/G1 cell cycle arrest and inhibiting cell proliferation (91,92). Sutherland et al. also showed that concentrations of TAM that inhibited growth or resulted in slow growth were not lethal to MCF-7 cells in culture. Both the steroidal ICI 182,780 and the non-steroidal antiestrogens (TAM) have shown reduction in antigen Ki67 (a proliferation biomarker) in human breast cancers (93,94). The primary mechanism through which antiestrogens exert this cytostatic effect appears to be down-regulation of cyclin D1 (G1 cyclin) gene expression in ER+ breast cancer cells (95). Other studies have shown that antiestrogens can suppress DNA polymerase
activity (96).

1.7.2. Antiestrogen-mediated Apoptosis:

For long it was thought that antiestrogens are merely cytostatic (reduce proliferation). However, clinically antiestrogens have been shown to reduce breast tumor size and increase overall survival (62,97). Also, TAM has been shown to be useful in chemopreventive settings to inhibit the development of ER+ tumors (98). These significant effects seen in patients are not possible if antiestrogens only have a cytostatic effect, as eventually some cells may develop a tendency to escape this cell cycle arrest and start proliferating again. It is now clear that in addition to the cytostatic nature of these antiestrogens, they also exert a cytotoxic effect with induction of apoptosis or programmed cell death as a key mechanism of these drugs (55). Both the steroidal ICI 182,780 and the non-steroidal antiestrogens (TAM) have been shown to induce apoptosis in ER+ human breast cancer (99). When Raloxifene (benzothiophene prototype of SERM) was tested in primary breast cancer patients, it showed significant antiproliferative effects in ER+ breast cancer but no significant effects on apoptosis in ER+ breast cancers was detected (100).

The mechanisms of induction of apoptosis by antiestrogens are complex. One mechanism, by which antiestrogens can regulate the apoptotic pathway in breast cancer cells, may be by regulating the expression of BCL2 family of proteins. Various studies have shown that steroid sex hormones such as, estrogen (E2) can regulate apoptosis by regulating the BCL2 (anti-apoptotic protein) and BAX (pro-apoptotic protein) protein levels. Increased level of estrogen is often correlated with increased level of BCL2 (both mRNA and protein levels) that can thus provide protection against apoptosis (101). Importantly, Wang et al., showed that while increased
levels of E2 positively correlated with increased levels of BCL2 mRNA, the BAX mRNA levels were unaffected, suggesting that it is the ratio of BCL2:BAX or BAX:BCL2 that plays an important role in deciding the cell fate.

Thus, the use of antiestrogens in human breast cancer cells that mainly work by inhibiting the function of ER and thereby reducing the circulating levels of E2 (estrogen), can have some important implications. Apart from suppressing cell proliferation, these antiestrogens can inhibit the E2-mediated up-regulation of BCL2: BAX ratio and enhance apoptosis. For example, both TAM and ICI 182,780 have been shown to be effective in inducing apoptosis in vitro in MCF-7 breast cancer cells by down-regulating BCL2 protein expression, although ICI 182,780 was found to be more effective in inducing apoptosis (102,103). ICI 182,780 has also been shown to induce apoptosis in rat mammary glands by down-regulating anti-apoptotic proteins BCL2 and BCLX and up-regulating the pro-apoptotic protein BAX (104). Furthermore, it has been shown that while estrogen (E2) can increase the levels of BCL2 protein, and render the MCF7 cells resistant to chemotherapeutic drug Adriamycin, the antiestrogen ICI 182,780 can antagonize the effects of estrogen and thereby reduce or reverse the resistance to the chemotherapeutic drugs such as Adriamycin (105).

Although, removal of tumor cells by apoptotic cell death is the main mechanism of action of antiestrogens, more recently, it has been shown that antiestrogens can also trigger an autophagic cell death pathway in cancer cells such as MCF-7 breast cancer cells (106). Interestingly, the cancer cells have the ability to switch between apoptotic cell death and autophagic cell death based on the cell death stimulus (107). More importantly, it has been shown that antiestrogens such as tamoxifen can induce both apoptosis and autophagy simultaneously in
MCF-7 breast cancer cells through the induction of sterol accumulation (108).

1.8. Cell Death Pathways in Cancer Development:

Apoptosis, necrosis and autophagy are among the three major pathways of cell death and a cross-talk among these different pathways of cell death is known to exist. Apoptosis is a tightly regulated form of cell death characterized by certain morphological changes that take place during the process, such as membrane blebbing, cell rounding, nuclear condensation, DNA fragmentation and finally the formation of apoptotic bodies (109). The balance of pro-apoptotic and anti-apoptotic signals, regulated by the BCL2 family members (BCL2; B cell lymphoma) present within the mitochondria, plays a major role in regulating apoptosis and the characteristic morphological changes discussed above. It is now well established that resistance to apoptotic cell death can lead to tumorigenesis and also lead to resistance to anti-cancer therapies (90,110,111). Thus, much research is focused on overcoming this resistance and forcing tumor cells to undergo apoptosis. There is also increasing interest in apoptotic-resistant cells, where anticancer drugs can cause other types of non-apoptotic cell death, such as senescence, mitotic catastrophe, autophagy and necrosis (112,113). The mechanisms underlying the apoptotic and the non-apoptotic cell death are discussed below.

1.8.1. Apoptotic Pathways:

Traditionally, two major pathways are involved in apoptosis, the transmembrane or the “extrinsic” pathway and the mitochondrial or the “intrinsic” pathway (114) [Figure 2]. Both apoptotic pathways depend on the activation of caspases, which are cysteine proteases that cleave at specific peptide residues to degrade the cell or cause apoptosis. Accordingly, inhibitors
of caspase enzymes can prevent cell death (115). The caspases are first synthesized as proenzymes also called as “initiator caspases” that are activated by adaptor proteins. Once activated, the initiator caspases in turn activate the “effector caspases” that act on the target proteins and are responsible for the bulk of cellular proteolysis.

1.8.1.1. Extrinsic or the Death-Receptor Apoptotic Pathway:

The extracellular ligands such as tumor necrosis factor (TNFα), TNF-related apoptosis-inducing ligand (TRAIL), or Fas-ligand (FasL) bind to their receptors. The intracellular death domain (DD) of the ligand-bound receptors is recognized by adaptor proteins such as FADD and TRADD. The adaptor proteins then recruit initiator caspases 8 and 10 and form a death inducing signaling complex (DISC) (116,117). The initiator caspases 8 and 10 then undergo an autocatalytic reaction and get activated at the DISC. Activated caspase-8 (CASP8) is then able to propagate apoptosis via direct cleavage and activation of downstream caspases such as caspase-3, 6 and 7 (CASP3, 6 and 7) [Figure 2] (118).

Several proteins such as c-Flice-like inhibitory proteins (FLIP) (119) are involved in regulating this pathway. Different family members of the IAPs (Inhibitors of apoptosis) such as XIAP, IAP1 and IAP2, and Survivin are also known to control the activation of caspases. Importantly, NFκB may play a crucial role in up-regulation of XIAP, IAP1 and IAP2 (120). Binding of IAPs to caspases can be inhibited by several proteins such as SMAC/DIABLO (121,122) and the serine protease Omi (123) released from the mitochondria. It is believed that these proteins are released into the cytosol during changes in mitochondrial membrane permeability (MMP), as is cytochrome c, to transduce the apoptotic signal.
1.8.1.2. Mitochondrial or the Intrinsic Apoptotic Pathway:

The intrinsic or the mitochondrial pathway on the other hand depends on the release of pro-apoptotic and anti-apoptotic BCL2 family of proteins from the mitochondria. The balance between the pro-apoptotic and anti-apoptotic BCL2 family members usually determines the cell fate. Intracellular signals, such as DNA damage or oxidative stress can induce the intrinsic pathway and favor the release of pro-apoptotic members of the BCL2 family from the mitochondria. The outer mitochondrial membrane is thus permeabilized, leading to the release of cytochrome c that binds to apoptosis activating factor-1 (APAF1), which in turn binds to pro-caspase 9 to form an apoptosome. Once CASP9 (caspase 9) gets activated, it can activate CASP3 or 7 ultimately leading to apoptosis [Figure 2].

The pro-apoptotic and the anti-apoptotic members of the BCL2 family are known to tightly regulate the intrinsic or the mitochondrial pathway. All the BCL2 family members have at least one of the four BCL2 homology (BH) domains, BH1-BH4. While the anti-apoptotic members (such as BCL2, BCL-XL, BCL-w) have all the four BH domains BH1-BH4, the pro-apoptotic members (such as BAX, BAK) lack the BH4 domain and contain BH1-BH3 domains only or the pro-apoptotic members (such as BID, BIK) have only BH3 domain (124) [Table 1].

There is cross-talk between two apoptotic pathways (intrinsic and the extrinsic pathway) at the level of mitochondria, since activated CASP8 can initiate both the activation of a caspase cascade as well as cleavage of the pro-apoptotic BID (the BH3 only protein). Once the pro-apoptotic BID is cleaved, the truncated fragment (tBID) translocates to the mitochondria to activate BAX and BAK and bring about a conformational change in BAX (125). BID’s induced conformational change forces BAX to oligomerize (126) and insert itself into the outer
mitochondrial membrane to form pores (126,127). These pores lead to permeabilization of the mitochondrial membrane and ultimately lead to cytochrome c release (along with other proapoptotic factors) from within the mitochondria, either directly or indirectly. Once cytochrome c is released, apoptosomes are formed that activates CASP9 (128), which in turn activates CASP3 or 7 ultimately leading to apoptosis and cell death.

<table>
<thead>
<tr>
<th>BCL2 Family Members</th>
<th>BH1</th>
<th>BH2</th>
<th>BH3</th>
<th>BH4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-apoptotic proteins (BCL2, BCL-XL, BCL-w)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Pro-apoptotic proteins (BAX, BAK, BOK)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✗</td>
</tr>
<tr>
<td>Pro-apoptotic proteins (BID, BIM, BAD, BIK); BH3-only proteins</td>
<td>✗</td>
<td>✗</td>
<td>✓</td>
<td>✗</td>
</tr>
</tbody>
</table>

**Table 1:** BCL2 homology (BH) domains (BH1-4) present in some representative members of BCL2–related proteins.
Figure 2: Two Major Pathways of Cellular Apoptosis: Extrinsic and the Intrinsic Pathway. (Please refer to text 1.8.1 for details).

Adapted from MacFarlane M, Williams AC, EMBO reports, 2004.
1.8.1.3 Defects in Apoptosis and Tumorigenesis:

Several mechanisms for resistance to apoptosis have been described. The tumor suppressor protein p53 can activate apoptosis (129) and provide protection against cancer(130) by causing cells to undergo a cell cycle arrest and repair the damage or if the damage is beyond repair, cells that are proliferating aberrantly undergo apoptosis (129). But if p53 is mutated or somehow absent in the cells with such lesions, the cells can proliferate inappropriately, inactivate apoptosis and can become malignant and subsequently develop cancer. p53 deficient mice are highly prone to developing tumors (131,132).

A functional defect of a pro-apoptotic protein (e.g., BAX, BAK) (133,134) or over-expression of anti-apoptotic protein (e.g., BCL2) (135) can cause resistance to apoptosis and lead to drug resistance. Furthermore, alterations in cell-survival pathways can also suppress apoptosis. For example, the PI3K-AKT survival signaling pathways activated by various intra and extracellular stimuli can both activate anti-apoptotic molecules and inhibit the activity of pro-apoptotic proteins (136). The dysregulation of PI3K-AKT signaling pathway is linked to breast cancer and many other cancers (137). Another survival factor that can be activated by various cytokines is nuclear transcription factor-κB or NFκB, which can prevent apoptosis induced by death receptors (138,139).

1.8.2. Non-Apoptotic Pathways:

1.8.2.1. Autophagy:

The three types of autophagy known include macroautophagy, Chaperone-mediated autophagy and microautophagy. Macroautophagy also referred to here as autophagy is a process where cell degrades the long lived proteins and damaged organelles such as ribosomes,
mitochondria by lysosomal proteases. It is well conserved in various species from yeast to mammalian cells (140). The morphology of autophagic cells is very distinct from apoptotic cells. In autophagy, the cell is characterized by the formation of autophagosomes, which are double-membrane-bound vesicles that contain cytoplasmic organelles that fuse with lysosomes (the degradative organelle in a eukaryotic cell) and degrade the contents of the autophagosome (141).

Autophagy is activated under limited-nutrient conditions/starvation to enhance cell survival, but excessive autophagy can result in cell death. Hence, based on the nutritional status of the cells, autophagy can exhibit a role in either cell survival or cell death (142). Under limited-nutrient conditions, autophagy can provide the essential building blocks, the amino acids, back to the cell and enhance cell survival (143). Furthermore, autophagy can provide a protective effect against infection by self-ingestion or degradation of the damaged organelles (144).

In contrast, autophagy can also induce cell death. Although cells that are deficient in both BAX and BAK have been shown to be resistant to apoptosis, it was shown that in these cells autophagy can compensate for apoptosis as a mode of cell death (145).

Autophagy is regulated by a set of autophagy–associated proteins (ATG proteins) that are involved in the autophagosome formation. The mammalian autophagy protein ATG6/BECN1 (also known as Beclin 1) is a tumor suppressor protein (146,147) and has been identified as a binding partner for the anti-apoptotic protein BCL2 (148). BECN1 plays a crucial role in activation and complex formation with class III phosphatidylinositol 3-kinase, which is required for autophagosome formation (149). It had been thought that the apoptotic protein BCL2 primarily acts by inhibiting apoptosis. Increasing evidence now suggests that BCL2 also has an
important role in regulation of autophagy. BCL2 proteins can block autophagy by binding to BECN1 and subsequently interfering with the formation of BECN1/hVps34 complex (150,151).

For detection of autophagy, the presence of LC3 protein (Light Chain 3), located in the membrane of autophagosome (152), as well as conversion of LC3 to LC3-II, have been used as indicators of autophagy. Cellular depletion of p62/SQSTM1 adaptor protein (153) is also used as a marker of autophagy. The p62/SQSTM1 protein binds tightly to LC3 as well as to ubiquitinated substrates and helps the latter to aggregate to the autophagy machinery (154). However, the use of p62/SQSTM1 protein alone to monitor autophagy is often discouraged. Instead, it is recommended to use p62/SQSTM1 protein in addition to LC3 to monitor autophagy (153). Other techniques like electron microscopy are also used for distinguishing autophagic vacuoles from other structures.

1.8.2.1.1. **Defects in Autophagy and Tumorigenesis:**

Defects in the autophagic pathway may be connected to cancer. Decreased expression of BECN1 as well as mutation of BECN1 can contribute to breast cancer and other malignancies and that transfection of BECN1 into the transformed cell can have a protective effect on tumorigenesis (155). Monoallelic deletion of BECN1 which is seen in 40-75% of human breast, ovarian and prostate cancer can promote tumorigenesis (146) and lead to death in mice during early embryogenesis (147). In addition, constitutive activation and mutations in PI3K-AKT survival pathway that are common in many cancers may suppress autophagy (156,157). Since PTEN can negatively regulate the PI3K-AKT survival pathway and positively regulate autophagy, loss of PTEN function may also result in autophagy suppression (158).
1.8.2.2. Mitotic Catastrophe:

Mitotic Catastrophe is a form of cell death where cells die due to aberrant mitosis (159). Activation of CDK1 controls the entry of cells into the M (mitotic) phase from the G2 phase. When the cell detects DNA damage, the G2 checkpoint blocks mitosis. However, deficiencies in cell cycle checkpoints usually allow the cell to enter M phase without the DNA damage being repaired, and this aberrant mitosis can then cause the cell to undergo death by mitotic catastrophe (160). The typical characteristic of cells undergoing mitotic catastrophe is formation of multinucleate cells that contain uncondensed chromosomes. Resistance to mitotic catastrophe can lead to aneuploid cells that are tumorigenic (161).

1.8.2.3. Necrosis:

Necrosis is an unregulated form of cell death. However, recent studies demonstrate that in addition to an uncontrollable and un-regulated form of necrosis (also referred to as accidental necrosis), there exists a regulated form of necrosis or the programmed necrosis (162). For example, a form of necrosis can be induced by either Fas or TNFα ligands via their respective death receptors (163). In addition to death receptors, DNA damage can also trigger necrosis (164). Further evidence of programmed necrosis comes from the studies that show that mode of cell death can switch between apoptosis and programmed necrosis and in certain cell types apoptotic and necrotic cell death can occur simultaneously referred to as “Necrapoptosis” (165).

Morphologically, necrosis is characterized by organelle degradation, swelling of the cell and the loss of membrane integrity which results in the release of intracellular components in the microenvironment initiating an inflammatory response (109). In such a scenario, necrosis may have a protective effect against tumor development. However, chronic inflammation or sustained
inflammatory response is known to promote tumor development (166). Since necrosis can lead to inflammation and chronic inflammation is associated with increased risk of cancer development, it is very likely that necrosis may play a role in tumor development.

1.8.2.4: Senescence:

Senescence is a process where the normal cells are prevented from dividing indefinitely. After a finite number of cell divisions, normal cells acquire one or more critically short telomere and enter a state of irreversible growth arrest (167). Senescent cells are typically large and are characterized by a flattened cytoplasm, induction of senescence-associated β galactosidase activity (168), and a distinct heterochromatic structure (senescence-associated heterochromatic foci) (169). One of the main functions of senescence response is to protect cells against tumor development. The involvement of tumor suppressor proteins such as p53, cyclin dependent kinase inhibitors (170) and other proteins such as Retinoblastoma (RB) (171) is implicated in senescence. Cells that are not capable of entering senescence are known to develop cancer at an early age (172,173).

1.9. Antiestrogen Resistance:

Antiestrogen therapy has been used to treat women with breast cancer for more than 30 years, and it remains among the most effective and least toxic of the systemic therapies currently available for the treatment of estrogen receptor-alpha (ERα) - positive breast cancers (55,88). Antiestrogens, both SERMs (e.g. TAM) and SERDs (e.g. Faslodex; ICI 182,780) are very effective in treating ER+ breast tumors as they mainly act by inhibiting the function of the ER as seen in Figure 1. However, the triphenylethylene Tamoxifen (TAM), also known as the selective
estrogen receptor modulator (SERM), is still the most widely used antiestrogen in clinical settings. Unfortunately, resistance to these antiestrogen therapies is a major limitation to the effectiveness of current antiestrogen/hormonal treatments.

Whether an individual patient will receive benefit from endocrine therapy is based on the presence of the estrogen receptor (ER) in that individual patient. In theory, all ER+ and PR+ (progesterone receptor-positive) breast tumors should respond to endocrine therapy. While the majority of ER+/PR+ breast tumors do respond to endocrine therapy, many ER+/PR+ tumors do not respond to antiestrogens. Other tumors that initially respond to antiestrogens, eventually acquire resistance to these treatments (88).

There are two major classes of antiestrogen resistance known: acquired resistance and de novo or intrinsic resistance. Approximately 70% of newly diagnosed breast cancers are ER-positive (174) of which ~30% do not respond to TAM and exhibit de novo or intrinsic resistance (55,87). The lack of ER (estrogen receptor) and PR (progesterone receptor) expression is one of the major forms of de novo antiestrogen resistance known. However, most patients that initially respond are at risk for relapse and the development of an acquired antiestrogen-resistant breast cancer. The steroidal antiestrogen ICI 182,780, a pure antagonist of the ER is often the second-line agent in patients with TAM-resistant disease (78).

Despite over 15 million patient years of experience with TAM, the precise mechanisms that contribute to progression to acquired antiestrogen resistance remain uncertain. Resistance mechanisms may include heterogeneity of ER expression within tumors, ER mutation, mitogenic
growth factor production, and loss of ER expression, culminating in the deregulation of cell survival and cell cycle progression functions (55,88,175). However, the genes or proteins responsible for making cells resistant to antiestrogens are not known. ER-regulated functions appear to be important; as most tumors that become antiestrogen resistant still express ER (176-178) and inhibition of ER in antiestrogen resistant cells is growth inhibitory (179).

Several other mechanisms have also been proposed to explain TAM resistance. One theory suggests that during the long term exposure of TAM, breast cancer cells are exposed to low levels of estrogen. During the process, tumors can adapt themselves to this low estrogen environment and develop an increased sensitivity to estrogen and the agonist/estrogenic effects of TAM (180). Another proposed mechanism involved in TAM resistance is the presence of excessive antiestrogen binding sites (AEBS) that can alter the bioavailability of antiestrogens to the tumor (181).

The antiestrogen resistant phenotype is complex and involves many changes at the cellular and molecular levels. It is highly unlikely that a single gene/signaling pathway drives antiestrogen resistance in ER+ breast tumors. Antiestrogens can affect proliferation end points and apoptosis in antiestrogen-sensitive cells (55, 87), hence it seems likely that breast cancer cells that acquire resistance to antiestrogens have altered the expression and/or function of some key components of the gene network that controls cell proliferation and cell fate (113). We hypothesize that in antiestrogen resistant breast cancer cells, the function of some of the key components of this gene network has changed in such a way that the antiapoptotic signals dominate in the tumor cells, and that these tumor cells no longer respond to the proapoptotic signals.
To test this hypothesis, we developed and used a series of estrogen-independent and antiestrogen-resistant models (as discussed below) to identify the key genes that are a part of such a network and contribute to antiestrogen resistance. Differences in the transcriptomes of estrogen independent (aromatase inhibitor resistant phenotype) but antiestrogen-sensitive (MCF7/LCC1) (182) and estrogen-independent, TAM (selective estrogen receptor modulator; SERM) and ICI 182,780 (selective estrogen receptor degrader; SERD) cross-resistant (MCF7/LCC9) (183) cells were explored using two approaches/technologies i.e., serial analysis of gene expression (SAGE) and gene expression microarray analyses. Several genes were found to be altered when the transcriptomes of these MCF7/LCC1 and MCF7/LCC9 cells were compared (184). Among the key genes identified in this network was the nuclear transcription factor-κB (NFκB) (184). The study also identified XBP1 as being up-regulated (XBP1 is known to be induced by NFκB) and showed the down-regulation of interferon regulatory factor-1 (IRF1), a protein partner of NFκB involved in regulation of iNOS expression (185).

1.10. Cellular Models of Antiestrogen Resistance:

We previously generated a series of unique cell models for ER+ antiestrogen resistance (MCF7/LCC1, MCF7/LCC2, and MCF7/LCC9) through in vitro and in vivo selection in our laboratory. MCF7/MIII cells [Figure 3] were first derived from the estrogen-dependent MCF7 human breast cancer cell line, following selection for growth in ovariectomized nude mice. MCF7/LCC1 cells [Figure 3] were then selected in vivo from MCF7/MIII cells in ovariectomized athymic nude mice, and reestablished in vitro in media supplemented with 5% calf serum stripped of estrogens (5%CCS). These MCF7/LCC1 cells retain ER expression, are E2 (estrogen)-independent for growth, and are inhibited by antiestrogens (186,187).
Subsequently, MCF7/LCC1 cells were selected against increasing concentrations of either the nonsteroidal antiestrogen 4HT (4-hydroxytamoxifen) or the steroidal antiestrogen ICI 182,780 (Faslodex; Fulvestrant) in vitro. Cells selected against 4HT produced stable, TAM resistant cells (MCF7/LCC2; [Figure 3] that are E2-independent for growth but are not crossresistant to ICI 182,780 (182). On the other hand, ICI 182,780-resistant cells (MCF7/LCC9; [Figure 3] were generated by in vitro stepwise selection of the antiestrogen responsive MCF7/LCC1 cells against ICI 182,780. The MCF7/LCC9 cells are ER+, E2-independent for growth, ICI 182,780 resistant, and TAM cross-resistant. TAM cross-resistance emerges at early passages during selection and before stable ICI 182,780 resistance occurs (183). LCC cells, therefore, are a novel and powerful series of genetically related variants to identify new antiestrogen resistance mechanisms.

We also used MCF7/RR cells [Figure 3], a MCF7 variant generated by selection against tamoxifen that showed cross-resistance to both 4HT and all-trans retinoic acid (atRA), hence the name RR (188). Interestingly enough, these MCF7/RR cells are phenotypically similar to the MCF7/LCC2 cells. Just like the MCF7/LCC2 cells, the MCF7/RR cells are TAM resistant that are not cross-resistant to ICI 182,780. While these MCF7/RR cells do not require estrogen for growth, they may still respond to estrogens. Selection against the steroidal antiestrogen ICI 182,780 generated a cell line that is cross-resistant to 4HT. However, the selection against 4HT generated a cell line that is resistant to 4HT only and is not cross-resistant to the steroidal antiestrogen ICI 182,780.

Because antiestrogen resistant and sensitive cells provide controls for each other, a direct comparison between them was done in order to be most efficient in identifying genes that confer
Cellular Models of Endocrine Resistance

Figure 3: Cellular Models for ER+ Antiestrogen Resistance.
1.11. The NFκB Family of Transcription factors:

It is well established that the nuclear factor-κB (NFκB) plays an important role in regulating the transcription of genes that drive inflammation and immune responses (189). However, NFκB has been recently connected with several aspects of oncogenesis, including control of apoptosis, cell cycle progression, differentiation, and cell migration (190). Moreover, NFκB is now shown to be involved in development and progression of cancer (191,192).

1.11.1. NFκB Structure and the Pathway:

The NFκB complex is found in almost all cells (189,193,194). In mammals, the NFκB family consists of five known members that belong to the Rel family: RelA (p65/RELA), RelB, cRel (Rel), p50/p105 (NFκB1) and p52/p100 (NFκB2) that either form homodimeric or heterodimeric complexes (189,195). However, in most cell types, NFκB is present as a p50/p65 heterodimer. All NFκB proteins share a conserved 300 amino acid N-terminal Rel homology domain (RHD) that is important for DNA binding, dimerization, inhibitor association (with IκB proteins) and nuclear localization [Figure 4] (196). Three members of the NFκB family including Rel A (p65; RELA), RelB, and cRel (Rel) also have a C-terminal transactivation domain (TAD) whereas the other two members of the NFκB family [p50/p105 (NFκB1) and p52/p100 (NFκB2)] that are synthesized as large precursors (p105 and p100 respectively) lack this TAD domain. Instead they have ankyrin repeat motifs (ANK) [Figure 4] for interaction with RHD to inhibit their DNA binding activity (196).

In the canonical/classical pathway, under the non-stimulated conditions, the NFκB proteins reside in the cytoplasm where they are sequestered by one or more members of the IκB
protein family (IκBα, IκBβ, IκBε, Bcl-3 and the precursor p100 or p105 Rel proteins) also known as inhibitors of NFκB. The IKK complex composed of three subunits: IKKα, IKKβ (the catalytic subunits) and the regulatory subunit IKKγ or NEMO (NFκB essential modulator) (197) phosphorylates the IκB molecules and regulates the transcriptional activity of NFκB proteins. The ankyrin repeat motifs (ANK) of the IκB proteins interact with the RHD of NFκB proteins (198,199) that either inhibit nuclear transport or block NFκB’s nuclear translocation signal (200).

The Bcl-3 member of the IκB protein family usually functions as a transcriptional co-activator for p52 and p50 heterodimers (189). Activation of NFκB is thought to occur in response to a broad range of stimuli such as cytokines, lipopolysaccharide, various stress signals, bacterial or viral products, growth factors, chemotherapeutic agents, free radicals and various other stimuli. Activation leads to phosphorylation of IκB by the IκB kinase (IKK) complex at two specific serine residues (serines 32 and 36), which tags IκB for ubiquitination and subsequent proteolysis by the 26S proteasome [Figure 5] (201). The active NFκB dimers are then released and are free to translocate to the nucleus to activate transcription of the various target genes that encode proteins involved in immune and inflammatory responses (such as TNFα, IL-8) and in cell proliferation (e.g. cyclin D1), apoptosis (e.g. BCL2), differentiation [Figure 5] (202,203).

Several studies have shown that IKKγ or NEMO is required for the IKK activation of NFκB through the canonical pathway and IKKγ/NEMO deficient cells can trigger cell death (204). However, it appears that IKKγ/NEMO is not a requirement for activation of NFκB through the non-canonical pathway (205). Instead, in the non-canonical pathway, IKKα activity appears to be necessary for proteasomal processing of p100. IKKα homodimers that are activated by specific stimuli such as BAFF (B cell activating factor of TNF family) and lymphotoxin-β phosphorylate
p100 at the C-terminus. This phosphorylation initiates IKKα proteasomal degradation and produces a transcriptionally active form of p52-RelB complex that translocates to the nucleus (196,206).

Several aspects of normal mammary gland development appear reliant upon NFκB activity (207), perhaps partly reflecting its regulation by both estrogens and growth factors (208,209). It has been shown that if NFκB activity is blocked in the mammary gland, mice can undergo severe lactation deficiency (210). In addition, while NFκB activity appears to be required for normal function of the immune system as well as for normal organ development, its dysregulation is observed in a variety of cancers including breast cancer. Elevated NFκB activity may be a consequence of various prolonged chronic inflammatory disorders (190) and organ aging (211).
Figure 4: Structure of different members of the NFκB family.

The five known members of the NFκB/Rel family are shown: Rel A (p65/RELA), RelB, cRel (Rel), p50/p105 (NFκB1) and p52/p100 (NFκB2). These all share a conserved 300 amino acid N-terminal Rel homology domain (RHD) that is responsible for DNA binding, dimerization, association with IκB proteins (inhibitors of NFκB) and nuclear localization. One class of NFκB family comprising Rel A (p65/RELA), RelB, and cRel (Rel) have a C-terminal transactivation domain (TAD), whereas the second class of NFκB family comprising of p50/p105 (NFκB1) and p52/p100 (NFκB2) lack this TAD domain. The latter class of NFκB family is synthesized as large precursors (p105 and p100 respectively) and contains ankyrin repeat motifs (ANK) for association with the RHD. They are converted into active p50 and p52 forms respectively upon ubiquitin-mediated processing and removal of C-terminus of large precursors (p105 and p100 respectively).

Adapted from Gilmore TD et al., Trends in Endocrinology and Metabolism, 2005.
Figure 5: The Canonical NFκB Activation Pathway.

NFκB is maintained in an inactive state in the cytosol bound to members of the IκB family that either inhibit nuclear transport or block NFκB’s nuclear translocation signal. Following several stimuli, activation of NFκB occurs by phosphorylation of IκB by the IKK kinase complex, which tags IκB for ubiquitination and degradation. NFκB then translocates to the nucleus and activates expression of various genes including cytokines (e.g. TNFα), chemokines (e.g. IL-8), cell adhesion molecules (e.g. vascular adhesion molecule-1; VAM-1), cell proliferating proteins (e.g. cyclin D1, c-myc) and inhibitors of apoptosis (e.g. BCL2).
1.12. Dual Role of NFκB: Anti-apoptotic and Pro-apoptotic mechanisms.

1.12.1. Anti-apoptotic role of NFκB:

NFκB regulates the expression of several genes involved in cell proliferation and cell survival; hence NFκB activation is associated with suppression of apoptosis. Activation of NFκB is known to regulate apoptotic cell death in different cell lines (212). Binding of TNFα (tumor necrosis factor) to the TNF receptor (TNFR) can activate the NFκB pathway, which activates expression of IAP’s (family of anti-apoptotic proteins), TRAF1 and 2 (TNF-receptor associated factors) that block the activity of caspase-8 (CASP8) (213,214), thereby suppressing apoptosis. However, in the absence of NFκB activity, TNF-induced activation of JNK pathway leading to apoptosis is favored (213). Lack of NFκB, especially the p65/REL A subunit, can cause massive liver apoptosis leading to embryonic lethality in mice (215).

While NFκB is known to control the expression of many antiapoptotic genes such as Bcl-x and Bfl-1/A1 (216), NFκB can also regulate the expression of pro-apoptotic proteins of the BCL2 family such as BAX. Decreased NFκB activity is associated with increased BAX expression (217). Importantly, activation of NFκB can also antagonize with the pro-apoptotic function of p53 and suppress p53-mediated apoptosis (218).

Since activation of NFκB inhibits apoptosis, inappropriately high levels of NFκB have been linked to the development of cancers.
1.12.2. Pro-apoptotic role of NFκB:

While there is strong evidence that NFκB blocks apoptosis and promotes cell proliferation and growth, there is equally compelling evidence that, based on the cell type and under certain stimuli, NFκB can be pro-apoptotic and thus prevent tumor development (219). For example, certain antioxidants such as N-acetylcysteine (NAC; that also inhibit NFκB) have been shown to inhibit virus (SV; Sindbis virus)-induced apoptosis in prostate cancer cell lines (220) correlating NFκB activity with apoptosis. Moreover, studies have implicated activation of NFκB as an absolute requirement for stress-induced FasL expression and apoptosis in T cells (221). NFκB is also involved in apoptosis that takes place during avian development and in bone marrow cells (222).

NFκB target genes include both anti-apoptotic genes such as BCL2 (223) and pro-apoptotic genes including tumor suppressor gene p53 (224). While a direct antagonism between p53 and NFκB has been reported, emphasizing the anti-apoptotic role of NFκB (218), several groups have shown that transcriptional activation of p53 requires activation of NFκB for p53-mediated apoptosis (224,225). Due to the conflicting results, further investigation is clearly needed to clarify the relationship between p53 and NFκB.

1.13. NFκB and Inflammatory Diseases:

Activation of NFκB can induce transcription of various pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8), chemokines, adhesion molecules such as E selectin, ICAM-1 (intercellular adhesion molecule); VCAM-1(vascular cell adhesion molecule), MMPs (matrix metalloprotease-2 and 9) that are involved in the degradation of extracellular matrix and
metastasis (226), COX-2 (cyclooxygenase 2) and inducible nitric oxide (iNOS). The constitutive activation of NFκB is observed at the major sites of inflammation in many diseases such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and asthma (227).

Since NFκB has been identified to play an important role in the pathogenesis of inflammation (227), NFκB-targeted therapies were tried against various inflammatory diseases. Importantly, it was found that many anti-inflammatory drugs that are already in use to treat human inflammatory diseases also have effects on NFκB activity (228) such as corticosteroids, aspirin, and other non-steroidal anti-inflammatory drugs (NSAIDs). While, these drugs are not primarily recognized as NFκB inhibitors, they have been shown to inhibit NFκB activity (229).

On the other hand, inhibition of NFκB activity by molecular approaches has also been effective in controlling inflammatory diseases in various animal models. For example, inhibition of NFκB activity inhibited the inflammatory response as well as further tissue destruction in rheumatoid synovium (230). Moreover, administration of NFκB decoy oligonucleotides that block NFκB, were also found to be effective against rheumatoid arthritis (231). Furthermore, inhibition of NFκB by locally administering antisense p65 was shown to block colitis in mice (232).
1.14. **NFκB and its importance in cancer:**

Since activation of NFκB regulates the expression of several genes involved in cell proliferation and cell survival/apoptosis, it has been implicated in cancer development as well as in resistance to several antineoplastic drugs used in cancer therapies (190,233-235).

Numerous studies have shown that NFκB is activated and over-expressed in a variety of cancers reviewed in Ref (191). The role of NFκB in cancer development was first evident when c-Rel was identified to be a counterpart of the viral oncogene v-Rel that causes aggressive tumors in chickens (236,237). The gene amplification or chromosome translocation of genes such as c-Rel, NFκB2 (p100/p52), and Bcl-3 are seen in many cancers (236,238-240). Studies have shown that mutations in IκBα (241) can render NFκB constitutively active in various hematological malignancies, such as Hodgkin’s lymphoma (242) and inhibition of NFκB activity can block cell growth and enhance apoptotic cell death in Hodgkin’s tumor cells (243).

NFκB can be activated during the cell transformation process by several viral oncoproteins, such as Tax from the human T-cell leukemia virus type I (HTLV-1) (244,245), and Epstein-Barr virus-encoded (EBV-encoded) proteins (246).

Several oncoproteins, such as Ras can also activate NFκB indicating that NFκB is a downstream target of activated Ras in Ras-signal transduction pathway. Importantly, studies with p65 -/- fibroblasts revealed that inhibition of NFκB can block NFκB dependent gene transcription in response to oncogenic Ras (247), thereby strengthening the involvement of NFκB in cancer. NFκB has also been shown to regulate the expression of certain proto-oncogenes such as *c-myc*
Furthermore, the fusion oncoprotein BCR-ABL, which is strongly implicated in myelogenous leukemia (CML) can also activate NFκB and drive tumorigenesis (249).

Studies have demonstrated that TNFα secreted in multiple myeloma (MM) cells can activate NFκB and promote growth and survival of MM cells, while inhibition of NFκB may promote apoptosis in MM cells (250).

NFκB is found to be constitutively active in human pancreatic adenocarcinoma (251) and inhibition of NFκB was able to decrease the expression of NFκB regulated genes such as COX-2 (cyclooxygenase-2) known be implicated in human pancreatic cancer (252). Recently, NFκB (NFκB1 specifically) is found to be activated in some gastric cancers (253).

Persistent activation of NFκB is also seen in squamous cell carcinomas of the head and neck (HNSCC) (254,255) leading to the expression of cytokine IL-8 involved in inflammation and angiogenesis (256). Importantly, NFκB can control expression of the anti-apoptotic protein BCL2 in HNSCC (257).

Many inflammatory diseases that show an increased activation of NFκB are now associated with an increased incidence of cancer. Various studies that establish a link between inflammation and cancer (258,259) suggest that prolonged/chronic inflammation caused by various infectious agents can increase the risk of cancer development and such chronic inflammations account for >15% of all the malignancies (258). Various chronic infections and chronic inflammatory diseases are found to be major risk factors for various cancers (260,261). For example: chronic infections with HBV (Hepatitis B virus) or HCC (Hepatitis C virus) can
predispose to liver cancer; chronic infections with *H. pylori* (*Helicobacter Pylori*) that causes gastritis can lead to gastric cancers; and chronic inflammation of airways due to tobacco smoke or airborne particles can predispose to lung cancer (262).

Activation of NFκB is also frequently seen in breast and prostate cancer cells (208,263). In breast cancer cells, the primary form of NFκB appears to be the p50/p65 heterodimer, which is composed of two homologous protein subunits produced by different genes. The gene for the p50 subunit (NFκB1) is located on chromosome 4, and the p65 (RELA) gene is on chromosome 11. Although less common, some human breast cancers may also show activation of p52 (NFκB2) (264). While normal function of the immune system requires NFκB (265), and more importantly normal mammary gland development requires NFκB (207), its deregulation is observed in variety of cancer including breast cancers. Elevated NFκB activity is detected during early stages of neoplastic transformation in the rat mammary gland (266) as well as in the mouse mammary gland (267). NFκB is widely expressed in human and rat mammary tumors (264,268).

NFκB expression is increased in breast cancer cells that exhibit an estrogen-independent phenotype (53,208). An estrogen-independent, protein-protein interaction between ER (estrogen receptor) and NFκB, but not IκB, has been demonstrated between *in vitro* translated proteins. NFκB and ligand-activated ER mutually repress their transactivation activities of the IL-6 promoter in MCF-7 cells (51). The ability of ER to block NFκB activation of IL-6 expression is consistent with an effective sequestration of NFκB by ER (52), and may explain the ability of estrogens to block cytokine-mediated bone resorption (269).
1.15. **NFκB Inhibition:**

Chemotherapeutic agents have been highly successful in treating patients with different types of cancer; however acquired resistance to chemotherapy remains a major limitation to effective cancer treatment. Since clinical drug resistance remains a serious problem, much research today focuses on understanding the molecular mechanisms underlying drug resistance in cancer cells. While most cancer cells become susceptible to apoptotic cell death following treatment with chemotherapeutic drugs, blockage of apoptosis is thought to be one of the mechanisms that result in failure to chemotherapy. Since NFκB is known to have an anti-apoptotic role in cancer cells, studies have shown that activation of NFκB can confer resistance to different chemotherapies or radiation in cancer cells (270).

Therefore, with several studies as discussed above, it has become clear that deregulation of NFκB pathway can contribute to the development of many human cancers, and also lead to resistance of cancer cells to the chemotherapeutic agents/ anticancer drugs. Therefore, inhibition of NFκB activity is a potential therapeutic strategy in the prevention and treatment of cancer and also in overcoming resistance to chemotherapeutic agents in cancer.

NFκB inhibitors may be used as a mono therapy in certain types of cancer, such as Hodgkin’s lymphoma where proliferation of tumors cells completely depend on NFκB activation (242). However, in most cases, cancers are driven by a network of gene signaling that involves up regulation of several anti-apoptotic factors and down regulation of many tumor suppressor genes (113,175). Therefore, NFκB inhibition alone may not be entirely successful in reducing the tumor growth in a majority of cancers. Instead, a combination therapy combining NFκB inhibitor and
chemotherapy may be a much more effective and logical therapeutic approach to not only improve overall survival for cancer patients but also to improve the clinical efficacy of many anticancer drugs used in chemotherapy.

1.15.1. Different approaches to inhibit NFκB:

The NFκB pathway can be inhibited at several steps. Inhibition of IKK activation, IκB phosphorylation and degradation, and inhibition of NFκB nuclear translocation [Figure 4] can all ultimately lead to inhibition of NFκB activity. Inhibitors of NFκB pathway include both natural and synthetic compounds: such as proteasome inhibitors, anti-oxidants molecules, peptides, small molecules, dominant negative or constitutively active polypeptides. These inhibitors of NFκB may gain importance as anticancer agents in near future (229).

1.15.1.1. Inhibition of NFκB by non-steroidal anti-inflammatory drugs (NSAIDS):

NFκB activity can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, sodium salicylate by competitive inhibition of the ATP-binding site of IKKβ (271). Sulfasalazine can also inhibit NFκB activation by suppressing IκB phosphorylation and its subsequent degradation (272). Mesalmine, a related aminosalicylate derivative with anti-inflammatory properties can inhibit IL-1 mediated NFκB transcription without affecting IκB phosphorylation and degradation (273). Sulindac and its metabolites, fall under non-steroidal anti-inflammatory agents that inhibit IKK activity in the NFκB pathway (274).
1.15.1.2. Inhibition of NFκB by small molecules such as parthenolide:

Recently, the sesquiterpene lactone Parthenolide, isolated from Mexican Indian medicinal plants and European herb feverfew (Tanacetum parthenium) has gained considerable attention because of its pro-apoptotic activity. Parthenolide, is a small molecule that is well recognized as a potent inhibitor of NFκB (275,276). Since parthenolide is known to have anti-inflammatory properties, it is in clinical use for the treatment of migraines (277). More recently, it has attracted considerable attention for its anti-tumor activity in vitro and in vivo. It is well tolerated in phase I clinical trials with no significant toxicity in patients with cancer (278). Several studies have shown that parthenolide either alone or in combination with other cytotoxic drugs can induce apoptosis (279-281). More recently, parthenolide has been shown to have anti-cancer and anti-angiogenic properties, and has the potential to improve the efficacy of both chemotherapy as well as hormonal therapy in vivo (282). It has been shown that the combined treatment with parthenolide and the NSAID sulindac can synergistically target NFκB pathway in pancreatic cancer cells and inhibit cell growth (283). The proapoptotic nature of parthenolide along with several other advantages of being relatively specific, non-toxic in vivo, and already in clinical trials makes parthenolide an excellent candidate for cancer treatments.

Several mechanisms for parthenolide’s biological effect on NFκB have been proposed. For example, parthenolide can inhibit NFκB by targeting the IκB kinase complex (284) and/or by directly modifying the p65/RELA protein (275,276). In breast cancer cells, parthenolide can inhibit NFκB DNA binding activity and increase sensitivity to paclitaxel (285). Interestingly, it has also been shown that parthenolide can induce apoptosis by other mechanisms in human cancer cells, such as induction of low levels of glutathione S-transferase (detoxification enzyme) resulting in caspase activation and over-expression of GADD153 (anticancer agent inducible
gene) (279, 286). More recently, parthenolide has been shown to induce TNF-related apoptosis by
inducing JNK, independent of NFκB inhibition, and reverse resistance to breast cancer cells
(281). Furthermore, it has been shown that parthenolide can also exert its anticancer activity by
inhibiting the enzyme tubulin carboxypeptidase (TCP), independent of parthenolide’s inhibitory
effect on NFκB (287). The enzyme TCP is responsible for removing the COOH-terminal tyrosine
residue of tubulin (the main constituent of microtubules involved in cell division) and its
inhibition by parthenolide can prevent the abnormal accumulation of detyrosinated tubulin in
tumor cells and restore the normal tyrosinated tubulin levels which in turn could inhibit tumor
progression (287).

1.15.1.3. Inhibition of NFκB by dominant negative NFκB inhibitor:

Phosphorylation of IkB and its subsequent degradation is an important step in the NFκB
pathway required for NFκB to translocate to the nucleus and induce its specific target genes.
Mutant IkBα protein (also known as IkB super-repressor; IkBSR), with mutations in IkBα at
serine residues 32 and 36, cannot be phosphorylated by IKK and targeted for degradation by the
proteasome. Thus, IkBSR acts as a dominant negative NFκB inhibitor and NFκB is sequestered
in the cytoplasm and prevented from inducing its target genes. The expression of IkBSR can
enhance TNFα-induced apoptosis in Jurkat cells (139). Furthermore, the adenoviral delivery of
IkBSR to mice resistant to chemotherapeutic drug camptothecin can resensitize these cells to the
drug (camptothecin) by enhancing apoptosis (235).

1.15.1.4. Other methods to Inhibit NFκB:

Glucocorticoids (GCs), such as dexamethasone and prednisone, having anti-inflammatory
properties are also widely used to inhibit NFκB pathway though several proposed mechanisms
These agents exert their effects by interacting with the glucocorticoid receptor (GR) and down-regulating the genes involved in inflammation such as COX-2 and TNF.

Cyclopentenone prostaglandins (cyPGs), the intracellular regulators of inflammatory and immune response, can also regulate NFκB pathway by acting as irreversible inhibitors of IKKβ (290,291).

Several naturally occurring phenolic compounds (with anti-oxidant properties) known as Flavonoids including isoflavones, I3C (Indole-3-carbinol), (DIM (3,3’-diindolylmethane), curcumin, lycopene, and resveratrol (red wine polyphenol) have also been shown to inhibit NFκB activation and subsequent transcription of anti-apoptotic and pro-inflammatory genes by NFκB (292).

The 26S proteasome targets the phosphorylated and ubiquitinylated IκBα for degradation and thus regulates the NFκB pathway [Figure 5]. Inhibitors that block function of the proteasome, such as PS-341 and MG-132, are of interest because they can reduce the degradation of IκBα and can prevent NFκB dependent activation of target genes (189,198). Both PS-341 and MG-132 have been found to sensitize cancer cells to apoptosis induced by cancer therapeutic drugs (293,294).

Finally, some synthetic cell-penetrating peptides constructed with NFκB nuclear localization sequence (NLS), such as peptides based on NFκB p50, can inhibit nuclear translocation of NFκB following lipopolysaccharide and TNF-α stimulation (295). However, since the peptide carrying the NLS of NFκB p50 can also inhibit the nuclear import of AP-1,
NFAT, and STAT1 whose NLS’s differ from that of NFκB p50, (296), suggests that the effect of NLS for NFκB p50 is not specific. Thus, a potent peptide specific to NFκB is highly desirable.

In summary, the studies discussed above suggest that NFκB is an important target for cancer prevention, treatment, and also for reversing resistance to chemotherapy. Although normal levels of NFκB activity is required for normal functioning of the cell, the constitutive activation of NFκB is likely involved in enhanced cell growth and blockage of apoptosis. Thus, combination therapeutic approaches that use NFκB inhibitors to inhibit NFκB pathway along with conventional chemotherapy may become promising for enhancing the cytotoxic effects of conventional chemotherapeutic/anticancer drugs in the treatment of cancer.

1.16. Implication of NFκB in Antiestrogen Resistance:

We have shown that NFκB can confer both antiestrogen resistance and estrogen-independence (53,184,297). On comparing the differences in the transcriptomes of antiestrogen-sensitive MCF7/LCC1 and antiestrogen-resistant MCF7/LCC9 human breast cancer cells by SAGE and gene expression microarrays, the mentor’s laboratory previously found that NFκB p65/RELA mRNA expression and transcriptional activation are both significantly increased in the cross-resistant MCF7/LCC9 cells (184). Also, these antiestrogen-resistant MCF7/LCC9 cells are found to be more sensitive to growth inhibitory effects of parthenolide, which is a small molecule inhibitor of NFκB (184) suggesting that resistant MCF7/LCC9 cells exhibit a greater reliance upon NFκB signaling for growth. Furthermore, a loss of endocrine regulation of NFκB was also observed in resistant MCF7/LCC9 cells. These data strongly implicate signaling that involves NFκB in altered hormone responsiveness.
Therefore, the aim of the studies presented here was to identify key functions that mechanistically drive antiestrogen responsiveness in human breast cancer cells, with a specific focus on the transcription factor NFkappa B (NFκB) and its downstream signaling.

1.17. Hypothesis and Aims:

The research presented in this dissertation was based on the following hypothesis and on our previously published studies that suggest that NFκB signaling may be active in some breast cancers.

We hypothesize that up-regulation of NFκB’s transcriptional activation contributes to an antiestrogen resistant phenotype; whereas a reduced activation increases sensitivity to these drugs and may reverse the antiestrogen resistant phenotype.

To test this hypothesis the following two aims were performed:

**Aim 1:** We will study the functional ability of NFκB over-expression to confer antiestrogen resistance.

- We will use both molecular (e.g., mutant IκB) and pharmacological (e.g., parthenolide; small molecule inhibitor of NFκB) approaches and see if down-regulation of NFκB will reverse antiestrogen resistance in resistant cells (e.g., MCF7/LCC9, MCF7/RR, MCF7/LCC2).

- We will also study if inhibition of endogenous NFκB will increase sensitivity in cells that are already sensitive to antiestrogens, e.g., MCF7/LCC1, MCF-7.

**Aim 2:** We will then study the functional ability of NFκB inhibition to sensitize breast cancer cells to antiestrogens (TAM and ICI 182,780).
We will overexpress NFκB in antiestrogen responsive cells (e.g., MCF-7) and see if overexpression of NFκB will confer antiestrogen resistance.

Experiments that specifically address these aims are presented here within and provide an assessment of NFκB’s relevance in breast cancer and antiestrogen resistance.
Chapter 2: Materials and Methods

2.1. Cell culture and reagents:

MCF-7 (ER+, estrogen dependent for growth, antiestrogen sensitive), MCF7/p65 (estrogen dependent for growth, antiestrogen sensitive), MCF7/p65/ICI, and MCF7/p65/TAM cells were routinely grown in improved minimal essential media (IMEM; Biofluids, Rockville, MD, USA) with phenol red and supplemented with 5% fetal bovine serum (FBS; FBS-IMEM). MCF-7 cells were originally obtained from Dr. Marvin Rich (Barbara Ann Karmanos Cancer Institute, Detroit, MI) and MCF7/p65 cells stably transfected with p65/RELA component of NFκB were obtained as a part of the collaboration with Dr. Nakshatri (Indiana University, Indianapolis). MCF-7 derived MCF7/LCC1 (ER+, estrogen independent for growth, antiestrogen sensitive); MCF7/LCC2 cells (ER+, estrogen independent for growth, TAM resistant and ICI 182,780 sensitive) (182); MCF7/LCC9 cells (ER+, estrogen independent for growth, antiestrogen cross-resistant) (298,299) and MCF7/RR cells (ER+, estrogen independent for growth, TAM resistant and ICI 182,780 sensitive, MCF-7 variant generated directly from MCF-7 cells by selection against tamoxifen (300) were routinely grown in phenol red-free improved minimal essential media (IMEM; Biofluids, Rockville, MD, USA) supplemented with 5% charcoal-stripped calf serum (CCS; CCS-IMEM). MDA-MB-231 cells (ER-, antiestrogen cross-resistant) were obtained from Lombardi Comprehensive Cancer Center’s Tissue Culture Shared Resource and were routinely grown in IMEM with phenol red and supplemented with 5% fetal bovine serum. These cells represent a standard model of de novo resistance. All cells were shown to be free of Mycoplasma spp. contamination and maintained in a humidified incubator at 37°C in an atmosphere containing 95% air: 5% CO₂.

4-hydroxytamoxifen (4HT) and parthenolide (Par) were purchased from Sigma-Aldrich.
(St. Louis, MO, USA), and ICI 182,780 (Fulvestrant; Faslodex; ICI) was obtained from Tocris Bioscience (Ellisville, MO, USA). The concentration of 4HT, ICI 182,780 and parthenolide used were 1 M, 100nM and 500nM respectively, unless otherwise indicated. The Insolution caspase inhibitor I (cell-permeable, irreversible, pancaspase inhibitor, catalog# 627609) and the CASP8/caspase-8 Inhibitor II (C8I; catalog# 218759, potent, cell-permeable, irreversible inhibitor of CASP8; the Z-IETD-FMK sequence binds to CASP8 and blocks it’s binding to the substrate) were purchased from Calbiochem (San Diego, CA, USA); a 20 M concentration of each was used.

2.2. Stable transfection with IκBSR:

MCF7/LCC9 cells were seeded at a density of 8 x 10^5 cells/dish in 10-cm² dishes and grown for 24 hrs before transfection. Cells were stably transfected with 4 µg of either an empty pCMV4 plasmid or S32/36A mutant pCMV4-FLAG IκBα, a mutant IκB that acts as a dominant negative NFκB inhibitor also referred to as IκBSR (kindly provided by Dr. Marty Mayo, University of Virginia, Charlottesville, VA, USA) and 1 µg of the puromycin-resistance cassette (pBABE plasmid), using Fugene 6 as recommended by the manufacturer (Roche Diagnostics, Indianapolis, IN, USA). Stably transfected cells were selected for growth in the presence of 1 g/ml puromycin. Puromycin-resistant colonies were selected and expanded from 10 cm² dishes to 6-well dishes and then to T-75 cm² plastic tissue culture flasks.

For each colony selected, cells were lysed as described below and screened for FLAG and IκBα protein expression by western blot analysis. Cells transfected with the empty control vector were designated LCC9/EV and those transfected with the S32/36A mutant pCMV4-FLAG IκBα were designated LCC9/IκBSR. The MCF7/LCC9/IκBSR and MCF7/LCC9/EV cells were
routinely grown in phenol red-free improved minimal essential media (IMEM; Biofluids, Rockville, MD, USA) supplemented with 5% charcoal-stripped calf serum (CCS; CCS-IMEM).

2.3. **Cell lysis, immunoblotting and coimmunoprecipitation:**

To determine the effects of 4HT, ICI 182,780, parthenolide (Par), pancaspase inhibitor (PI), and CASP8 inhibitor (C8I) on protein expression, MCF7/LCC1 and MCF7/LCC9 cells were seeded into 6-well dishes at 3 x 10⁵ cells/well and cultured in normal growth media for 24 hrs. Cells were then treated with vehicle, 1 M 4HT (IC₅₀ for the parental MCF7/LCC1 cells), 100nM ICI 182,780; IC₅₀ for the parental MCF7/LCC1 cells) or 500nM parthenolide (Par; IC₅₀ for the cross-resistant MCF7/LCC9 cells) singly or in combination with or without the 20 M caspase inhibitor (pancaspase inhibitor; PI or C8I as indicated) in CCS-IMEM for 72 hrs. For the determination of basal NFκB (p6/RELA, p50, and p52), IKKγ/NEMO, IκBα and BCL2 protein expression, cells were grown in T-25 cm² tissue culture flasks. Cells were washed once with saline solution (1X PBS) then lysed on ice in modified radioimmunoprecipitation assay buffer; RIPA [150mM NaCl, 50mM Tris (pH 7.5), 1% Igepal CA-630, and 0.5% deoxycholate] supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and 1mM sodium orthovanadate phosphatase inhibitor (Sigma). Lysates were clarified by centrifugation at 4°C and total protein was quantified using the bicinchoninic acid assay (BCA) purchased from Pierce Biotechnology (Rockford, IL, USA).

Whole cell lysate (20-40 g) was resolved by PAGE using NuPAGE 10% precast acrylamide gels (Invitrogen, Carlsbad, CA), then transferred onto nitrocellulose membranes. Nitrocellulose membranes were washed briefly in Tris Buffered saline and Tween-20 (TBST) and blocked in a solution of TBST containing 5% nonfat dry milk for 15 min with constant agitation.
After blocking, the nitrocellulose membrane was incubated with the following primary antibodies overnight at 4°C in the block: mouse monoclonal BCL2 primary antibody AAM-072 (1:500; Stressgen Biotechnologies, Vancouver, BC, Canada), mouse monoclonal BAD primary antibody 610391 (1:500; BD Transduction Laboratories), rabbit polyclonal BAX primary antibody 06-499 (1:500, Upstate Biotechnology, Charlottesville, VA), mouse monoclonal BCL10 primary antibody AAM-073(1:500; Stressgen Biotechnologies, Vancouver, BC, Canada), rabbit polyclonal p65/RELA NFκB primary antibody 06-418 (1:1000, Upstate Biotechnology, Charlottesville, VA, USA), rabbit p50 NFκB primary antibody 06-886 (1:500, Upstate Biotechnology, Charlottesville, VA), rabbit monoclonal IκBα primary antibody sc-371(1:200, Santa Cruz Biotechnology Inc.), rabbit polyclonal IKKγ/NEMO primary antibody sc-8330(1:200, Santa Cruz Biotechnology Inc.), rabbit polyclonal LC3B primary antibody #2775 (1:1000, Cell Signaling, Beverly, MA), rabbit polyclonal p62/SQSTM1 primary antibody ab64134(1:1000, Abcam, Cambridge, MA), rabbit polyclonal PARP primary antibody #9542(1:500, Cell Signaling, Beverly, MA), rabbit monoclonal XIAP primary antibody #2045(1:500, Cell Signaling, Beverly, MA), rabbit polyclonal TNFα primary antibody #3707(1:200, Cell Signaling, Beverly, MA), mouse monoclonal TNFR1 primary antibody sc-8436(1:200, Santa Cruz Biotechnology Inc.), mouse monoclonal p300 primary antibody 554215 (1:1000; BD Biosciences, San Jose, CA, USA), rabbit polyclonal JNK primary antibody 06-748 (1:1000, Upstate Biotechnology), rabbit active-JNK pAb primary antibody v-7931(1:500, Promega, Madison, WI, USA), mouse monoclonal CASP8 primary antibody #9746(1:500, Cell Signaling, Beverly, MA), rabbit polyclonal CASP9 primary antibody #9502(1:500, Cell Signaling, Beverly, MA). Membranes were washed in TBST (3x for 15 min) and were incubated for 1hr with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ, USA) at a 1:5000 dilution at room temperature with constant agitation before enhanced
chemiluminescence (Amersham Biosciences) and exposure to film. For rabbit polyclonal BAX primary antibody 06-499 (1:500; Upstate Biotechnology) 10% horse serum in TBST was used as a block. The membrane was blocked for 1hr and incubated with the primary antibody overnight at 4°C in the block. The secondary antibody was also diluted in the block, incubated for 1hr at room temperature. Finally, membranes were reprobed as above with β-actin monoclonal antibody (1:5000; Sigma, St. Louis, MO) to confirm equal loading of the gels.

Quantification was done by densitometry. Data (mean ± SE) are presented as the ratio of protein: β-actin ratio where indicated. To screen LCC9/EV and S32/36A mutant pCMV4-FLAG IκBα (LCC9/IκBSR) clones for FLAG and IκBα protein expression mouse monoclonal FLAG-M5 primary antibody F4042 (1:500, Sigma) and rabbit polyclonal IκBα primary antibody sc-371 (1:200, Santa Cruz Biotechnology) were used, respectively.

For coimmunoprecipitations, 400 g of treated (as indicated) cell lysate was incubated with 2.5 l of p65/RELA NFκB antibody overnight at 4°C with rotation. The following day, 50 l of Protein A-Sepharose beads (Amersham Biosciences) were added and the tubes were returned for additional rotation for 1hr at 4°C to recover the immune complexes. Samples were centrifuged and supernatant was aspirated. The beads were then washed once with lysis buffer and twice with Tris-saline [TN; 50mM Tris (pH 7.5) and 150mM NaCl], resuspended in 2x Laemmlli sample buffer and boiled for 5 min. The immune complexes and 20 g of corresponding cell lysate were then resolved by PAGE as described above.
2.4. Transient transfections and luciferase reporter assays:

Cells were seeded at a density of approximately $8 \times 10^4$ cells/well into 12-well dishes and allowed to grow for 24 hrs before transfection. Cells were cotransfected with 0.4 µg of Luc reporter plasmids [pNFκB-Luc, or pAP-1-Luc (Stratagene, La Jolla, CA) or ERE-tk-Luc (Promega) or pBCL2-Luc {P1 or P1+P2} as indicated] and 0.1 µg of pRL-SV40 plasmid (Promega, Madison, WI) containing the Renilla luciferase gene under the control of a constitutive SV40 promoter per well using the Fugene 6 transfection reagent (Roche, Indianapolis, IN, USA). To determine if IκBSR inhibits NFκB dependent transcription transiently in MCF7/LCC1 and MCF7/LCC9 cells, these cells were also cotransfected with a third plasmid that was either a standard control comprising an empty pCMV4 plasmid or S32/36A mutant pCMV4-FLAG IκBα (IκBSR). Three hours post-transfection, cells were treated with either 500nM parthenolide (Par) and/or 1 M 4HT/100nM ICI 182,780 in CCS-IMEM, or media were changed to IMEM containing no drug as indicated for 24 hrs. Subsequently, cells were lysed and activation of the pNFκB-Luciferase construct was measured using the Dual Luciferase Assay Kit (Promega) according to the manufacturer’s instructions. Luminescence was quantified using a Lumat LB 9501 luminometer (EG&G Berthold, Bundoora, Victoria, Australia). Three independent experiments were done each at least in quadruplicate, and the luciferase values were normalized to Renilla Luminescence. Data are presented as the mean ± SE.

2.5. Cell proliferation:

To study effects of NFκB inhibition on response to antiestrogens 4HT/ICI 182,780 or to caspase inhibition (using 20 µM of either PI or C8I), the TAM resistant (MCF7/LCC9, MCF7/RR, MDA-MB-231) and the LCC9/IκBSR cells were seeded at a density of 1 to $1.8 \times 10^4$ cells/well in
24-well plates, and 24 hrs later were treated with the indicated concentrations of drug in appropriate media for 7 days, with redosing on days 3 and 5. On the day of counting, cells were washed twice with warm 1x PBS, trypsinized, resuspended in PBS, and counted using a Beckman Coulter Counter (Beckman Coulter Corp., Fullerton, CA, USA). To study the effects of NFκB inhibition on response to 4HT/ICI 182,780 in antiestrogen sensitive (MCF-7, MCF7/LCC1) cells and response to ICI 182,780 in TAM resistant but ICI 182,780 sensitive (MCF7/RR) cells, these cells were seeded at a density of 2 x 10⁴ cells/well in 24-well plates. Twenty-four hours post plating, cells were treated with increasing concentrations of 4HT (0-1µM) or ICI 182,780 (0-1 M) in the presence or absence of 500nM parthenolide as shown in appropriate media for 5 days, with redosing once on day 3. On day 5, cells were trypsinized, resuspended in PBS, and counted as described above. Data were normalized to vehicle-treated (control) cells and three independent experiments were done each at least in quadruplicate. Data are presented as the mean ± SE.

To study estrogen independence, MCF7/p65 cells were estrogen deprived by washing the monolayers with CCS-IMEM and maintaining the cultures for 72 hrs in T-75 cm² plastic tissue culture flasks. Cells were then trypsinized and seeded at a density of 1 to 1.8 x10⁴ cells/well into 24-well plastic tissue culture plates in CCS-IMEM ± E2 (10nM; day 0). On days 1, 4, and 7, the cells were trypsinized, resuspended in PBS, and counted using a Beckman coulter counter (Beckman Coulter Corp., Fullerton, CA).

2.6. Mitochondrial membrane permeability:

Cells were seeded at a density of 5 x 10⁵ cells/well in 6-well dishes and cultured in normal growth media. Twenty-four hours later, cells were treated with ethanol vehicle, 4HT (1 M), parthenolide (Par; 500nM) singly or in combination with or without the caspase inhibitor (20 M
pancaspase inhibitor, 20 M CASP8/Caspase-8 Inhibitor: C8I) for 18-20 hrs. Cells were gently washed once with warm 1x PBS and trypsinized to remove adherent cells from the culture dish. Cells were then centrifuged at 350 x g for 5 min. 1 l of MitoSensor Reagent (Clontech Laboratories, Inc, Mountain View, CA, USA) was added to 1ml Incubation Buffer/1ml 1x warm PBS (final concentration: 5 g/ml). Cells were vortexed and then centrifuged for 5 min at 14,000 rpm. The cell pellet was gently resuspended in 1 ml diluted MitoSensor Reagent per sample and incubated at 37°C in a 5% CO₂ incubator for 15-20 min. Cells were again centrifuged at 350 x g for 5 min to obtain a cell pellet, resuspended in 500 l of 1x PBS, and fluorescence measured by the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource. Apoptotic cells show primarily green fluorescence (due to collapse of the electrochemical gradient across the mitochondrial membrane) and are easily differentiated from healthy cells that exhibit red and green fluorescence. Data are presented as the mean ± SE for at least three independent experiments.

2.7. Cell cycle and apoptosis analyses:

Cells were seeded at a density of 5 x 10⁵ cells/dish in 10 cm² dishes and cultured in growth media (CCS-IMEM) for 24 hrs. The following day, cells were treated with ethanol vehicle, 4HT (1 M) and/or parthenolide (500nM) in CCS-IMEM for an additional 72 hrs. For cell cycle analysis, cells were harvested, fixed in ethanol and analyzed for alterations in cell cycle via fluorescence activated cell sorting (FACS) at the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource according to the method of Vindelov et al. (301). Data are presented as the mean ± SE for three or more independent experiments. For detecting apoptosis, Staining for Annexin V was performed according to the manufacturer’s instructions as described in TACS™ Annexin V Kit # 4830-250-K (In Situ Cell Detection Kit, Trevigen, Gaithersburg,
After staining with FITC annexin V and PI (propidium iodide) in the binding buffer provided, apoptotic cells show green fluorescence that was measured by the Flow Cytometry Shared Resource (Lombardi Comprehensive Cancer Center). Data are presented as the mean ± SE for three independent experiments.

2.8. RNA isolation and real time qPCR:

MCF7/LCC9 cells were plated in T-25cm² tissue culture flasks and treated with ethanol vehicle, 4HT (1 M), parthenolide (Par; 500nM), singly or in combination for 72 hrs before RNA isolation. Total RNA was isolated from proliferating subconfluent flasks using the Triazol reagent (Life Technologies, Gaithersburg, MD). Briefly, cells were rinsed with 1X PBS and lysed using the Triazol reagent. RNA was isolated by chloroform extraction and precipitated using isopropanol. Total RNA was quantified by comparing the optical density ratios (OD_{260}/OD_{280}) obtained spectrophotometrically using a Beckman DU640 Spectrophotometer (Beckman, Fullerton, CA). 1 µg of RNA was then treated with DNase I (Invitrogen) prior to reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen) and oligo d(T)₁₆ primers (Applied Biosystems, Foster City, CA). qPCR reactions for each cDNA sample and a standard curve were performed using TaqMan Universal PCR Master Mix and the following TaqMan Gene Expression Assay primers (Applied Biosystems): BCL2, Hs00608023_m1; and the housekeeping gene RPLP0 (Hs99999902_m1). 10 µl reactions were run in triplicate in 384-well plates on an ABI Prism 7900HT Sequence Detection System, using the absolute quantification protocol specified by the manufacturer. Expression data for each gene was estimated relative to the housekeeping control, and these data were used to calculate the ratio of expression relative to that in the MCF7/LCC9 cells treated with ethanol vehicle. Data are presented as the mean ± SE.
2.9. **BrdUrd ELISAs:**

Cells were seeded at a density of $1 \times 10^4$ cells/well in 96-well plastic tissue culture dishes. 24 hrs post plating, cells were treated with increasing concentrations of 4HT (0-1 M) or ICI 182,780 (0-1 M) as shown and cultured in appropriate media for 5 days, with redosing once on day 3. On day 4, BrdUrd (final concentration 10 M) was added for an additional 18 hrs (total incubation in drug, 5 days) before performing the Cell Proliferation ELISA, BrdUrd (colorimetric) assay as directed by the manufacturer (Roche). At least three independent assays were performed with five replicate wells per treatment group. Data were normalized to vehicle-treated (control) cells and are presented as the mean ± SE.

2.10. **Statistical analyses:**

One-way ANOVA was used to compare the effects of treatment on cell proliferation, apoptosis, mitochondrial membrane permeability assay, and in immunoblot assays with more than two treatment groups. Student’s $t$ test was used to compare two group-design experiments. The nature of the interactions between 4HT and parthenolide and between ICI 182,780 and parthenolide were defined by determining the R index (RI) (302). RI values were obtained by calculating the expected cell survival ($S_{exp}$; the product of survival obtained with drug A alone and the survival obtained with drug B alone) and dividing this $S_{exp}$ by the observed cell survival in the presence of both drugs ($S_{obs}$). $S_{exp}/S_{obs} >1.0$ indicates a synergistic interaction, $<1.0$ indicates an antagonistic interaction, and $= 1$ is indicative of an additive interaction between the two drugs used.
Chapter 3: Inhibition of NFκB by parthenolide increases sensitivity to antiestrogens and reverses the antiestrogen resistant phenotype

3.1. Functional ability of NFκB over-expression to confer antiestrogen resistance (Aim 1):

To address AIM 1, we studied antiestrogen responsiveness to two antiestrogens (ICI 182,780 and 4HT) in different cellular models of antiestrogen resistance as described in detail in chapter 1 of this dissertation [Figure 3].

- We used pharmacological small molecule inhibitor of NFκB (parthenolide) to test if down-regulation of NFκB will reverse antiestrogen resistance in resistant cells (e.g., MCF7/LCC9, MCF7/RR, MCF7/LCC2, and MDA-MB-231 cells that are ER-ve).
- Since inhibition of endogenous NFκB may also increase sensitivity in cells that are already sensitive to antiestrogens, we used parthenolide to see if inhibition of endogenous NFκB will increase sensitivity in antiestrogen sensitive cells (e.g., MCF7/LCC1, MCF-7).

A direct comparison between antiestrogen resistant and sensitive cells was done because they provide controls for each other. Furthermore, we identified key functions that mechanistically drive antiestrogen responsiveness in human breast cancer cells, with a specific focus on transcription factor NFkappa B (NFκB) and its downstream signaling.
3.2. Re-testing the relative responsiveness to antiestrogens (ICI 182,780 and 4HT) in the Cellular Models of Antiestrogen Resistance:

We re-confirmed the phenotype of different cell models for ER+ antiestrogen resistance used in the study. As expected, our proliferation experiments [Figures 6 and 7] show that MCF7/LCC9 cells are resistant to ICI 182,780 and are cross-resistant to 4HT. However, the MCF7/RR and MCF7/LCC2 cells are resistant to 4HT but retain sensitivity to ICI 182,780.

Figure 6: Sensitivity to ICI 182,780 in different cell line models.
We also confirmed that MCF-7 and MCF7/LCC1 cells are responsive to antiestrogens, 4HT and ICI 182,780 [Figures 6 and 7].

Figure 7: Sensitivity to 4-hydroxy tamoxifen (4HT) in different cell line models.

Cells were seeded in quadruplicate and treated with either 0 to 100nM ICI 182,780 [Figure 6] or with 0 to 1000nM (1 µM) 4HT [Figure 7] in CCS-IMEM for 5 days before counting, with redosing on day 3. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment.
3.3. **Published studies from our laboratory:**

3.3.1. **NFκB activity is overexpressed in resistant MCF7/LCC9 cells:**

Our published studies show that NFκB is likely to play an important role in breast cancer and specifically in antiestrogen resistance. Differences in the transcriptomes of MCF7/LCC1 cells (estrogen-independent, TAM and ICI 182,780 responsive but aromatase inhibitor resistant phenotype) and MCF7/LCC9 (estrogen-independent, TAM and ICI 182,780 non-responsive cross-resistant) human breast cancer cells explored by SAGE and gene expression microarrays showed NFκB p65 (HUGO gene symbol, RELA) mRNA expression and transcriptional activation to be significantly increased in the cross-resistant MCF7/LCC9 cells (184). The study also identified XBP1 as being unregulated (XBP1 can be induced by NFκB) and showed the down-regulation of interferon regulatory factor-1 (IRF1), a protein partner of NFκB involved in regulation of iNOS (185). Our previous studies also showed that the basal activity of NFκB is 10-fold higher in MCF7/LCC9 cells relative to MCF7/LCC1 cells. Additionally, the study identified a 2-fold up-regulation of NFκB p65/RELA mRNA in resistant MCF7/LCC9 cells by expression microarray analysis (184). These data strongly implicate signaling that involves NFκB in altered hormone responsiveness.

More recently, we reported that the expression of the NFκB regulator IKKγ/NEMO is up-regulated in antiestrogen resistant MCF7/LCC9 cells, likely explaining their increased expression of NFκB mRNA (297).
3.3.2. Loss of endocrine regulation of NFκB in resistant cells:

Since NFκB is a survival factor, its 10-fold up-regulation could confer antiestrogen resistance. However, this would not be likely if an antiestrogen still inhibited this NFκB activity in resistant cells. While ICI 182,780 inhibits NFκB activity (promoter-reporter assay) in the MCF7/LCC1 cells (TAM/ICI 182,780 responsive), this regulation is lost in the TAM and ICI 182,780 cross-resistant MCF7/LCC9 cells (184). These data suggest that some breast cancer cells may survive antiestrogen treatment by deregulating some ER functions. The ER in these cells is still functional because studies done by a collaborator (Dr. S. Conrad; Michigan State University) have shown that ER in the LCC cells can still regulate an ER-driven promoter in a promoter-reporter assay.

3.3.3. Antiestrogen resistant cells are more dependent on NFκB signaling for growth:

Increased activation of NFκB, and loss of its antiestrogenic regulation in MCF7/LCC9 cells, suggests that these cells might be partly dependent upon NFκB signaling for growth. We have also reported that MCF7/LCC9 cells are more sensitive than MCF7/LCC1 cells to growth inhibition by parthenolide, a potent inhibitor of NFκB (184). Parthenolide produces a dose dependent growth inhibition of the antiestrogen resistant MCF7/LCC9 cells. Our data in [Figure 8] re-confirms these observations. These data indicate that cells that acquire ICI 182,780 resistance exhibit a greater reliance upon NFκB signaling for growth.
Figure 8: Resistant MCF7/LCC9 cells are more sensitive to growth inhibition by parthenolide.

Cells were seeded in quadruplicate and treated with 0 to 1000nM (1 M) parthenolide in CCS-IMEM for 5 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment. The experiment was independently done at least thrice. *, P=0.003 versus MCF7/LCC1 cells with 500nM parthenolide by Student’s t test.

$t$-test: * $p=0.003$ relative to MCF7/LCC1 cells treated with 500nM.
3.3.4. **Parthenolide restores ICI 182,780 sensitivity to MCF7/LCC9 cells and increases apoptosis:**

To determine whether inhibition of NFκB activity could restore ICI 182,780 sensitivity, MCF7/LCC9 cells were treated with increasing concentrations of parthenolide in the presence or absence of 100nM/L ICI 182,780 (this dose approximates the IC₅₀ in MCF7/LCC1 cells but is ineffective in MCF7/LCC9 cells). While the NFκB inhibitor parthenolide inhibits the proliferation of MCF7/LCC9 cells in the absence of ICI 182,780 (297), it is important to note that the combined treatment with ICI 182,780 and parthenolide synergistically (302) inhibit MCF7/LCC9 cell proliferation, where 50% growth inhibition occurs at a concentration of 100nM/L parthenolide. [Figure 9; *p= 0.034 for ICI 182,780 plus parthenolide compared with parthenolide alone; RI = 1.82]. We have also reported that combined treatment with ICI 182,780 and parthenolide synergistically (302) promotes apoptosis (Table 2; RI= 2.28) and it is important to note that the percentage of apoptotic cells observed in the presence of both the drugs (19%) is comparable to that seen when MCF7/LCC1 cells are treated with the same dose of ICI 182,780 (19.9%; Table 2). These data supports our hypothesis that the up-regulated NFκB activity in the resistant MCF7/LCC9 cells is a major contributor to their antiestrogen resistant phenotype.

3.3.5. **NFκB p65/RELA is expressed in breast cancer:**

Our study of NFκB expression and other genes in breast cancer specimens from 58 patients, shows a positive association of NFκB expression with XBP1 (p=0.018) and a negative association (p=0.034) with nuclear IRF1 (IRF1n; expected to be active IRF1), two genes indicated in our transcriptome studies and involved in NFκB signaling. Coexpression of NFκB p65/RELA and XBP1 are preliminary data but suggest that NFκB may be active in some breast cancers.
*p=0.034 vs 100nM parthenolide without ICI 182,780; RI=1.82.  #p=0.05 vs 600nM parthenolide without ICI 182,780; RI= 1.48.

* Riggins et al., MCT, 2005.

Figure 9: Parthenolide and ICI 182,780 synergistically restore sensitivity to ICI 182,780 in resistant MCF7/LCC9 cells.

<table>
<thead>
<tr>
<th>Cell line/drug</th>
<th>% Apoptosis ± SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCC1 vehicle</td>
<td>4.22 ± 0.98</td>
<td>—</td>
</tr>
<tr>
<td>LCC1 ICI</td>
<td>19.96 ± 4.43</td>
<td>0.03*</td>
</tr>
<tr>
<td>LCC9 vehicle</td>
<td>3.20 ± 1.96</td>
<td>—</td>
</tr>
<tr>
<td>LCC9 ICI</td>
<td>4.41 ± 0.90</td>
<td>0.61*</td>
</tr>
<tr>
<td>LCC9 parthenolide</td>
<td>9.95 ± 1.21</td>
<td>0.04*</td>
</tr>
<tr>
<td>LCC9 ICI + parthenolide</td>
<td>18.34 ± 1.45</td>
<td>0.003*, 0.001 †, 0.01 ‡</td>
</tr>
</tbody>
</table>

NOTE: RI = 2.28 for combination of ICI and parthenolide.

* Versus vehicle.
† Versus ICI.
‡ Versus parthenolide.

* Riggins et al., MCT, 2005.

Table 2. Parthenolide and ICI 182,780 synergistically increase apoptosis in resistant MCF7/LCC9 cells.
3.4. Relative sensitivity to parthenolide in different cell line models:

We expect inhibition of NFκB to reverse, at least partly, antiestrogen resistance in MCF7/LCC9, MCF7/LCC2 and MCF7/RR cells. To address this aim we first used a small molecule inhibitor (parthenolide) of NFκB to determine which of the above resistant models are most sensitive to NFκB. Parthenolide, a small molecule inhibitor of NFκB (276) is a sesquiterpene lactone isolated from the European herb feverfew (Tanacetum parthenium) that has attracted considerable attention for its anti-tumor activity in vitro and in vivo. Parthenolide is well tolerated with no significant toxicity in patients with cancer (278) and several studies have shown that parthenolide, either alone or in combination with cytotoxic drugs, can induce apoptosis (279,281). We already know from Figure 8 and also from our previously published studies (184) that MCF7/LCC9 cells are sensitive to growth inhibition by parthenolide. We now show that the resistant MCF7/LCC2 and MCF7/RR cells are also sensitive to growth inhibition by parthenolide [Figure 10]. It is important to note that the resistant cells (MCF7/LCC9, MCF7/LCC2 and MCF7/RR) are more sensitive to growth inhibition by parthenolide than the sensitive (MCF7/LCC1 and MCF-7) breast cancer cells that also serve as their respective controls [Figures 8 and 10]. Thus, the antiestrogen resistant cells (MCF7/LCC9, MCF7/LCC2 and MCF7/RR cells) that also have up-regulated p65/RELA may exhibit a greater reliance upon NFκB signaling for growth.

The MCF7/LCC9 and MCF7/RR cells appear to be the most sensitive models, as the effects of NFκB inhibition are more pronounced in these cells compared to the MCF/LCC2 cells, where the difference in relative growth response of MCF7/LCC1 and MCF7/LCC2 cells to parthenolide is not significant until 1 M dose of parthenolide [Figure 10]. Since resistant MCF7/RR cells are phenotypically similar to the resistant MCF/LCC2 cells, the experiments that
specifically focus on the given four models of antiestrogen resistance are presented here within. It should be most efficient to do antiestrogen resistant and sensitive cells together as the sensitive and resistant cells provide controls for each other.

We compared:

MCF7/LCC1 cells (ICI 182,780 –s; 4HT-s) to MCF7/LCC9 cells (ICI 182,780 –r; 4HT-r)
MCF-7 cells (ICI 182,780 –s; 4HT-s) to MCF7/RR cells (ICI 182,780 –s; 4HT-r)

3.5. NFκB family members are differentially expressed in resistant and sensitive breast cancer cells:

We have previously shown that p65/RELA mRNA is increased by ~2-fold in antiestrogen cross-resistant MCF7/LCC9 cells (selected against ICI 182,780: ER-positive, E2-independent for growth, ICI 182,780 resistant, TAM cross-resistant) and that this translates into a 10-fold higher basal promoter-reporter activity of NFκB when compared to the MCF7/LCC1 cells (ER-positive, E2-independent for growth, TAM and ICI 182,780 sensitive) (184). To confirm altered expression of p65/RELA at the protein level and to examine the expression of other members of the NFκB family (i.e., p50 (NFκB1), p105 (p50 precursor), p52 (NFκB2) and p100 (p52 precursor), which are the only NFκB components known to be expressed in breast cancer cells, we measured the expression of the above mentioned NFκB family members at the protein level in antiestrogen resistant (MCF7/LCC9; MCF7/RR) and antiestrogen sensitive (MCF7/LCC1; MCF-7) cells. Similar to mRNA levels, MCF7/LCC9 cells exhibit an increase in p65/RELA protein expression by ~2-fold compared with their MCF7/LCC1 cell controls (297). Expression of the p50 and p52 NFκB subunits is not different between the two cell lines [Figure 11 also re-confirms these observations].
To determine if this also occurs in TAM resistant models, we measured expression of p65/RELA at the protein level in MCF7/RR cells, an antiestrogen resistant variant of MCF-7 generated following in vitro selection against tamoxifen (TAM resistant but retains sensitivity to ICI 182,780) (188). As seen in MCF7/LCC9 cells (297), MCF7/RR cells also exhibit an increase in p65/RELA protein expression by 8-fold compared with their MCF-7 cell controls [Figure 12; p<0.05]. In contrast, expression of the p50 and p52 subunits of the NFκB heterodimer is not different between the resistant and sensitive cell lines [Figure 12]. Similar results were also seen with resistant MCF7/LCC2 cells, where only the protein expression of the p65/RELA subunit of NFκB was increased by ~8-fold, and no change was seen in the expression of the p50 and p52 subunits of the NFκB heterodimer when compared with antiestrogen sensitive MCF7/LCC1 cells (data not shown).

3.6. Basal NFκB activity is increased in Resistant MCF7/RR cells:

To measure transcriptional activation of NFκB, we used a dual-luciferase promoter-reporter assay where MCF-7 and MCF7/RR cells were cotransfected with a NFκB-luciferase reporter vector and a phRL-SV40-Renilla control vector. Figure 13 shows that basal activity of the NFκB promoter is increased by ~3-fold (p<0.001) in antiestrogen resistant MCF7/RR cells compared to the antiestrogen sensitive MCF-7 control cells. These data further implicate increased NFκB signaling in reduced TAM responsiveness.
Figure 10: Parthenolide produces a dose dependent inhibition of growth in resistant (MCF7/LCC2 and MCF7/RR) and sensitive (MCF7/LCC1 and MCF-7) cells.

Cells were seeded in quadruplicate and treated with 0 to 1000nM (1 M) parthenolide (Par) in CCS-IMEM for 5 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment. The experiment was independently done at least thrice. A) MCF7/LCC1 and MCF7/LCC2 cells. P<0.001 for all treatment groups by one-way ANOVA. B) MCF-7 and MCF7/RR cells. P<0.001 for all treatment groups by one-way ANOVA, and *, P=0.008 versus MCF-7 cells with 500nM Par by Student’s t test.
Figure 11: Basal p65/RELA protein expression is increased in resistant MCF7/LCC9 cells.

Quantitation and representative immunoblots for p65/RELA, p50 (NFκB1), p105 (p50 precursor), p52 (NFκB2) and p100 (p52 precursor) in MCF7/LCC1 and MCF7/LCC9 cells. 20μg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted. β-actin, loading control.
Figure 12: Basal p65/RELA protein expression is increased in resistant MCF7/RR cells.

Quantitation and representative immunoblots for p65/RELA, p50 (NFκB1), p105 (p50 precursor), p52 (NFκB2) and p100 (p52 precursor) in MCF-7 and MCF7/RR cells. 20µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted. β-actin, loading control. Bars represent the mean ± SE of the relative p65:actin ratio (normalized to MCF-7 control cells) for three independent experiments. P values were calculated by Student’s t test.
Figure 13: Basal transcriptional activity of NFκB is increased in resistant MCF7/RR cells.

Basal transcriptional activity of NFκB in MCF-7 and MCF7/RR cells. Data represent mean ± SE of four determinations and are presented as fold induction relative to MCF-7 cells. Student’s t test, P<0.001 for MCF7/RR versus MCF-7 cells.
3.7. **Parthenolide restores sensitivity to 4HT in resistant MCF7/LCC9 and MCF7/RR cells and also increases the responsiveness to 4HT in sensitive MCF7/LCC1 and MCF-7 cells:**

Parthenolide, a small molecule NFκB inhibitor, produces a dose-dependent inhibition of antiestrogen resistant MCF7/LCC9 cell growth (184) but has only a minor effect on the growth of the antiestrogen sensitive MCF7/LCC1 cells, suggesting that these cells in which p65/RELA is up-regulated, exhibit a greater reliance upon NFκB signaling for growth (184,297). We have recently reported that inhibition of NFκB by parthenolide can synergistically restore sensitivity to ICI 182,780 in MCF7/LCC9 cells [Figure 9] (297).

Resistance to TAM and ICI 182,780 can occur independently (78,187) and a role for NFκB in affecting TAM responsiveness has not been determined. We have now determined that NFκB can also reverse resistance to 4HT in resistant cells (MCF7/LCC9, MCF7/RR) and increase sensitivity to 4HT in highly responsive cells (MCF7/LCC1, MCF-7). These four models were each treated with increasing concentrations of antiestrogen 4HT (0-1 M) in the presence or absence of three different concentrations of parthenolide (50nM, 100nM, 500nM). While 1 M 4HT alone is ineffective in both the resistant MCF7/LCC9 and MCF7/RR cells [Figure 14; one-way ANOVA: LCC9, p<0.001; MCF7/RR, p=0.004], a combination of 4HT and exposure to the IC_{50} of parthenolide (~500nM) significantly inhibits growth and restores sensitivity to 4HT in both resistant variants (MCF7/LCC9, MCF7/RR). Ineffective concentrations of parthenolide as a single agent for cell proliferation (50nM, 100nM) do not restore sensitivity to 4HT in these cells (data not shown). This result is to be expected, as antiestrogen cross-resistant MCF7/LCC9 cells are dependent upon NFκB (297). Thus, concentrations of parthenolide that inhibit NFκB activity should also inhibit basal proliferation in other antiestrogen resistant cell models. The interaction
between parthenolide and 4HT is clearly synergistic in both resistant cells (MCF7/LCC9, MCF7/RR), generating a relative index (RI) of 3.3 for MCF7/LCC9 and RI=1.8 for MCF7/RR cells (values >1.0 indicate synergy; and values closer to 2.0 are clinically relevant) (302).

Since blocking NFκB might increase sensitivity to 4HT in sensitive cells (MCF7/LCC1; MCF-7), we also measured the effects of parthenolide on the response of these two antiestrogen sensitive models to antiestrogen 4HT. While inhibition of endogenous NFκB by parthenolide increases sensitivity to 4HT in the antiestrogen sensitive cells (MCF7/LCC1, MCF-7), this interaction is additive rather than synergistic [Figure 15; A and B].

3.8. Parthenolide increases sensitivity to ICI 182,780 in resistant MCF7/RR as well as in the sensitive MCF7/LCC1 and MCF-7 cells:

Since the TAM resistant MCF7/RR cells still retain sensitivity to ICI 182,780, we also subjected these MCF7/RR cells to increasing concentrations of ICI 182,780 (ICI) in the presence or absence of 500nM dose of parthenolide (~IC₅₀). While MCF7/RR cells are sensitive to ICI 182,780 [Figure 16], the combined treatment with parthenolide and ICI 182,780 further robustly and synergistically increase their sensitivity to ICI 182,780 which is evident from their Relative Index values that are closer to 2 for each dose of ICI 182,780 as shown in Figure 16.

To see if down-regulation of NFκB will increase sensitivity in cells that are already sensitive to ICI 182,780, (e.g. MCF7/LCC1, MCF-7) these cells were also treated with increasing concentrations of ICI 182,780 in presence or absence of three different doses of parthenolide (50nM, 100nM, 500nM). While these sensitive MCF-7 and MCF7/LCC1 cells are growth inhibited by both parthenolide [Figure 17; A and C] and ICI 182,780 [Figure 17; B and D;
vehicle treated] alone, a combination of ICI 182,780 and an IC$_{50}$ dose of parthenolide (~500nM) further sensitizes MCF-7 and MCF7/LCC1 cells to ICI 182,780 [Figure 17; B and D]. Lower and ineffective doses of parthenolide (50nM, 100nM) were not able to restore sensitivity to ICI 182,780 in these cells [Figure 17B]. While inhibition of endogenous NFκB by parthenolide increases sensitivity to ICI 182,780 in the antiestrogen sensitive cells (MCF7/LCC1, MCF-7), this interaction is additive at best [Figure 17D] for MCF-7 cells generating a relative index (RI) of 0.82 (values>1 imply synergy) (302). However, the nature of interaction between parthenolide and ICI 182,780 was synergistic in MCF7/LCC1 sensitive cells as indicated by the RI values [Figure 17B].

Parthenolide also does not restore sensitivity to either 4HT or ICI 182,780 in MDA-MB-231 cells (an ER negative model of de novo endocrine cross-resistance; [Figure 19]. Unlike MCF-7 and MCF-7 TAM-resistant or sensitive variants, the MDA-MB-231 cells have lost expression of estrogen receptor (ER). Although parthenolide produces a dose dependent growth inhibition of these cells [Figure 18 A], parthenolide does not induce sensitivity to either 4HT [Figure 19A] or ICI 182,780 [Figure 19B] in these ER- cells, implying that the reversal of resistance in ER+ cells requires expression of functional ER protein. Since the primary action of antiestrogens is to block ER function, it is therefore concluded that the mechanism of antiproliferative synergy between antiestrogens and parthenolide works in ER+ cells where antiestrogens can antagonize with the function of ER and thereby reduce proliferation.

Thus, up-regulation of NFκB’s transcriptional activity contributes to an ER+, TAM resistant phenotype. Furthermore, reducing NFκB activation by parthenolide both reverses the antiestrogen resistant phenotype and can also increase (but to a lesser degree) TAM sensitivity in
cells that are already sensitive to 4HT. Since some ER+ breast cancers are heterogeneous and may contain both TAM sensitive and resistant cells, the ability to reverse resistance and increase responsiveness in sensitive cells implies a potentially significant advantage to including parthenolide in TAM regimens.
Figure 14: Parthenolide inhibits proliferation and synergistically restores 4HT sensitivity in resistant MCF7/LCC9 and MFC7/RR cells.

Parthenolide inhibits the proliferation of antiestrogen-resistant cells and restores sensitivity to 4HT. Cells were seeded in quadruplicate and treated with ethanol vehicle, 4HT (1 M), parthenolide (Par; 500nM) in presence or absence of 4HT (1 M) in CCS-IMEM for 7 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment. The experiment was independently done at least thrice. For MCF7/LCC9 cells: P<0.001 for ANOVA, and *, P=0.029 versus Par without 4HT; RI= 3.3. For MCF7/RR cells: P=0.004 for ANOVA, and ~, P=0.015 versus Par without 4HT; RI= 1.8.
A. Parthenolide increases sensitivity to 4HT in sensitive MCF7/LCC1 (A) and MCF-7 (B) cells.

Figure 15: Parthenolide increases sensitivity to 4HT in sensitive MCF7/LCC1 (A) and MCF-7 (B) cells.

Cells were seeded in quadruplicate and treated with 0 to 1000nM (1 M) 4HT in the presence or absence of 500nM parthenolide in CCS-IMEM for 5 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment. The experiment was independently done at least thrice.

A) MCF7/LCC1 cells. P≤0.001 for all treatment groups by one-way ANOVA, and *, P≤0.008 versus 4HT without parthenolide by Student’s t test; RI values for each dose of 4HT are indicated.
Figure 15. Parthenolide increases sensitivity to 4HT in sensitive MCF7/LCC1 (A) and MCF-7 (B) cells.

Cells were seeded in quadruplicate and treated with 0 to 1000nM (1 M) 4HT in the presence or absence of 500nM parthenolide in CCS-IMEM for 5 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment. The experiment was independently done at least thrice.

B) MCF-7 cells. P≤0.001 for all treatment groups by one-way ANOVA, and *, P≤0.001 versus 4HT without parthenolide by Student’s t test; RI values for each dose of 4HT are indicated.
Figure 16: Parthenolide synergistically enhances sensitivity to ICI 182,780 in resistant MCF7/RR cells.

Cells were seeded in quadruplicate and treated with 0 to 1000nM ICI 182,780 in the presence or absence of 500nM parthenolide in CCS-IMEM for 5 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment. The experiment was independently done at least thrice. P≤0.001 for all treatment groups by one-way ANOVA, and *, P≤0.029 versus ICI 182,780 without parthenolide by Student’s t test; RI values for each dose of ICI 182,780 are indicated.
Figure 17: Parthenolide increases sensitivity to ICI 182,780 in sensitive MCF7/LCC1 and MCF-7 cells.
Figure 17. Parthenolide increases sensitivity to ICI 182,780 in sensitive MCF7/LCC1 and MCF-7 cells.

(A) and (C) Parthenolide produces a dose dependent inhibition of the antiestrogen sensitive (MCF7/LCC1 and MCF-7) cells.

MCF7/LCC1 (A) and MCF-7 (C) cells were seeded in quadruplicate and treated with 0 to 500nM parthenolide in CCS-IMEM for 5 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment.

(B) and (D) Parthenolide increases sensitivity to antiestrogen ICI 182,780 in Antiestrogen-sensitive MCF7/LCC1 and MCF-7 cells.

MCF7/LCC1 (B) and MCF-7 (D) cells were seeded in quadruplicate and treated with increasing concentrations of ICI 182,780 as indicated in the presence or absence of three different concentrations of parthenolide (50nM, 100nM, 500nM) in CCS-IMEM for 5 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment. Relative Index (RI) values are indicated. The experiment was independently done at least thrice. B) MCF7/LCC1 cells. P<0.001 for all treatment groups by one-way ANOVA. D) MCF-7 cells. P<0.001 for all treatment groups by one-way ANOVA.
Figure 18: MDA-MB-231 cells are resistant to 4HT and ICI 182,780, but are growth inhibited by parthenolide.

Cells were seeded in quadruplicate and treated with A) 0 to 1000nM (1 µM) parthenolide B) 0 to 1000nM (1 µM) 4HT and C) 0 to 100nM ICI 182,780 or ethanol vehicle in CCS-IMEM for 5 days before counting. Data represent mean ± SE of relative proliferation (relative to their respective ethanol treated control) of four determinations from a single representative experiment. The experiment was independently done at least thrice.
Figure 19: Parthenolide does not restore sensitivity to either 4HT (A) or ICI 182,780 (B) in ER-negative MDA-MB-231 cells.

Cells were seeded in quadruplicate and treated with 1 M 4HT, 100nM ICI 182,780, 500nM parthenolide (Par) singly or in combination as indicated above or with ethanol vehicle in CCS-IMEM for 7 days before counting. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of four determinations from a single representative experiment. A) P≤0.001 for ANOVA, and P=0.082 for Par versus Par + 4HT; B) P≤0.001 for ANOVA, and P=0.351 for Par versus Par + ICI 182,780.
3.9. Parthenolide inhibits NFκB-dependent transcription in all breast cancer cells:

Parthenolide restores 4HT sensitivity in resistant MCF7/LCC9 and MCF7/RR cells and increases sensitivity to antiestrogens both 4HT and ICI 182,780 in antiestrogen sensitive (MCF7/LCC1; MCF-7) cells. Thus, we determined whether this exposure (i.e., exposure to IC₅₀ dose of parthenolide; ~500nM) also inhibits NFκB’s transcriptional activity. To measure transcriptional activation of NFκB, we used a dual-luciferase promoter-reporter assay where the sensitive (MCF7/LCC1; MCF-7) and resistant (MCF7/LCC9; MCF7/RR) cells were co-transfected with a NFκB-luciferase reporter vector and a phRL-SV40-Renilla control vector prior to treatment with 50, 100 or 500nM parthenolide for 24 hrs before lysis and luminescent detection. As shown in Figure 20, 500nM parthenolide (corresponds to an IC₅₀ dose of parthenolide in antiestrogen-resistant cells) inhibits NFκB-dependent transcription in both resistant (MCF7/LCC9; MCF7/RR) and sensitive (MCF7/LCC1; MCF-7) cells. However, inhibition of NFκB activity is significantly greater in resistant cells (MCF7/LCC9; MCF7/RR; ~50% inhibition) compared with sensitive cells (MCF7/LCC1; MCF-7; ~30% inhibition). These observations are fully consistent with the effects of parthenolide on responsiveness to TAM as measured by changes in cell proliferation (Figures 14-17) and with the effects of parthenolide on ICI 182,780 sensitivity (297).

However, lower doses of parthenolide (50nM, 100nM) do not inhibit NFκB activity in any cell line (Figure 21; A and B). This result is to be expected, as these concentrations of parthenolide (50nM, 100nM) are also inactive as a single agent for cell proliferation and do not restore sensitivity to 4HT in the resistant cells.
Figure 20: Parthenolide inhibits NFκB-dependent transcription in all breast cancer cell lines.

Cells were cotransfected with pNFκB-Luc and pRL-SV40 Renilla constructs prior to treatment with 500nM parthenolide (Par) for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations from a single representative experiment. The experiment was independently done at least thrice. Student’s t test; *, P=0.011 for MCF7/LCC9 versus MCF7/LCC1; ^, P=0.016 for MCF7/RR versus MCF-7.
A. Lower doses of parthenolide do not inhibit NFκB-dependent transcription in any breast cancer cell line. Cells were cotransfected with pNFκB-Luc and pRL-SV40 Renilla constructs prior to treatment with either 50 or 100nM parthenolide for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations from a single representative experiment. The experiment was independently done at least thrice. A) MCF7/LCC1 and MCF7/LCC9 cells. B) MCF-7 and MCF7/RR cells.

Figure 21: Lower doses of parthenolide do not inhibit NFκB-dependent transcription in any breast cancer cell line.
3.10. Restoration of sensitivity to antiestrogens (4HT; ICI 182,780) by parthenolide may not involve additional inhibition of NFκB-dependent transcription:

Parthenolide inhibits NFκB-dependent transcription in TAM resistant cells [Figure 20]. Thus, we asked whether the combination of parthenolide and 4HT is more effective than parthenolide alone in inhibiting NFκB-dependent transcription. We measured NFκB-activity in the resistant cells (MCF7/LCC9; MCF7/RR). While parthenolide alone significantly inhibits NFκB-dependent transcription in both the resistant cells, parthenolide in combination with 4HT [Figure 22] or with ICI 182,780 [Figure 23] has no additional effect on NFκB-luciferase activity in the resistant MCF7/LCC9; MCF7/RR cells.

3.11. Association of p65/RELA with p50 or with the coactivator p300 does not change regardless of the treatment:

Because MCF7/LCC9 cells exhibit the strongest interaction between parthenolide and antiestrogens (both 4HT and ICI 182,780), we used these cells to understand the underlying mechanism of synergy between parthenolide and antiestrogens. We asked whether the combination treatment of parthenolide and antiestrogens (4HT and ICI 182,780) alter the association of p65/RELA with p50 or with the coactivator p300 in MCF7/LCC9 cells. The physical association of p65/RELA with either NFκB p50 or with the coactivator p300 was measured using co-immunoprecipitation. We find no change in the association of p65/RELA with either p50 or with the coactivator p300 following any of the treatments [Figure 25], indicating that parthenolide may be acting through other alternative mechanisms to synergize with antiestrogens.
Figure 22: Parthenolide inhibits NFκB-dependent transcription, but has no additional effect on NFκB-luciferase activity in combination with 4HT.

MCF7/LCC9 and MCF7/RR cells were cotransfected in quadruplicate with p NFκB-Luc and pRL-SV40 Renilla constructs prior to treatment with 1 M 4HT, 500nM parthenolide (Par) singly or in combination with 4HT (1 M), or ethanol vehicle in CCS-IMEM for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations from a single representative experiment. The experiment was independently done at least thrice. $P \leq 0.001$ by one-way ANOVA for MCF7/LCC9 cells and $P \leq 0.001$ by one-way ANOVA for MCF7/RR cells.
Figure 23: Parthenolide inhibits NFκB-dependent transcription, but has no additional effect on NFκB-luciferase activity in combination with ICI 182,780.

MCF7/LCC9 and MCF7/RR cells were cotransfected in quadruplicate with pNFκB-Luc and pRL-SV40 Renilla constructs prior to treatment with ICI 182,780 (MCF7/LCC9:100nM and MCF7/RR: 1nM), 500nM parthenolide (Par) singly or in combination with ICI 182,780, or ethanol vehicle in CCS-IMEM for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the ratio of luciferase: Renilla activity for four determinations from a single representative experiment. A) MCF7/LCC9 cells: P≤0.001 by one-way ANOVA. Student’s t test; P=0.093 for Par versus Par+ICI. B) MCF7/RR cells: P≤0.001 by one-way ANOVA.
Figure 24: Combined treatment with parthenolide and antiestrogens (both 4HT/or ICI 182,780) does not inhibit AP-1-dependent transcription in resistant MCF7/LCC9 cells.

MCF7/LCC1 and MCF7/LCC9 cells were cotransfected in quadruplicate with pAP1-Luc and pRL-SV40 Renilla constructs prior to treatment with 1 M 4HT, 100nM ICI 182,780, 500nM parthenolide (Par) singly or in combination or with ethanol vehicle as indicated in CCS-IMEM for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations. A) Par+4HT combination. B) Par+ICI combination.
Figure 25: Physical association of p65/RELA with NFκBp50 or with coactivator p300 measured using co-immunoprecipitation.

NFκB p65/RELA was immunoprecipitated from 400 µg of MCF7/LCC9 lysates treated with 1 M 4HT, 100nM ICI 182,780, 500nM parthenolide (Par) singly or in combination, or ethanol vehicle in CCS-IMEM for 72 hrs with polyclonal anti-p65 antibodies; immune complexes were isolated and resolved by SDS-PAGE, and immunoblotted for p65/RELA, p50 and p300. Immunoblots of 20 µg of corresponding cell lysate are shown alongside.
3.12. **Synergistic interaction between parthenolide and antiestrogens (4HT or ICI 182,780) in MCF7/LCC9 cells may not involve activation of the JNK pathway:**

Since parthenolide can reverse the resistance of breast cancer cells to tumor necrosis factor (TNF)-related apoptosis by inducing c-Jun NH2-terminal kinase (JNK) independent of NFκB inhibition (281), we determined whether parthenolide-induced JNK activity may play a role in its restoration of antiestrogen sensitivity. Antiestrogen-sensitive MCF7/LCC1 and resistant MCF7/LCC9 cells were treated with ethanol vehicle, parthenolide, 4HT or ICI 182,780 in the presence or absence of parthenolide for 72 hrs. Whole cell lysates were then collected and immunoblotted for active-JNK. While the protein levels of JNK and p-JNK are both higher in resistant MCF7/LCC9 cells compared to sensitive MCF7/LCC1 cells, protein levels of JNK and p-JNK do not change in MCF7/LCC9 cells following treatment [Figure 26; A and B]. These data suggest that the synergistic interaction between parthenolide and antiestrogen 4HT or ICI 182,780 may not involve activation of the JNK pathway.

3.13. **Combined treatment with parthenolide and 4HT does not affect the cell cycle profile in resistant MCF7/LCC9 and MCF7/RR cells:**

Because 4HT can affect proliferation by affecting both cell cycle changes and apoptosis, we measured these endpoints *in vitro*. MCF7/LCC9 and MCF7/RR cells were both treated with ethanol vehicle or 4HT in the presence or absence of parthenolide for 72 hrs and the relative proportions of cells in different phases (G0/G1; S; G2/M) were analyzed by Flow Cytometry. We have previously reported that parthenolide alone or in combination with ICI 182,780 has no effect on the cell cycle profile of MCF7/LCC9 cells (297). We now show that parthenolide alone or in
combination with antiestrogen 4HT does not alter the cell cycle profile of MCF7/LCC9 cells [Figure 27] suggesting that the synergistic interaction between parthenolide and 4HT is not due to changes in cell cycling.

3.14. Combined treatment with parthenolide and 4HT induces apoptosis in resistant MCF7/LCC9 cells:

Our previous studies reported that ICI 182,780 and parthenolide synergistically enhance apoptosis in MCF7/LCC9 cells also shown in Table 1 (297). Since 4HT can also induce apoptosis (175), we subsequently studied the effects of ethanol vehicle or 4HT in the presence or absence of parthenolide on apoptosis as measured by FITC-conjugated Annexin V and propidium iodide staining. As expected, 4HT alone does not induce apoptosis in resistant MCF7/LCC9 cells [Figure 28; A] whereas 4HT in the presence of parthenolide statistically significantly induces apoptosis by almost ~1.7 fold [Figure 28; A; One-way ANOVA: p=0.042; p<0.05 relative to 4HT; p<0.05 relative to control]. In contrast, while 4HT alone significantly induces apoptosis by almost ~3 fold in sensitive MCF7/LCC1 cells [Figure 28; B], parthenolide in combination with 4HT has no additional induction of apoptosis in these sensitive MCF7/LCC1 cells [Figure 28; B; One-way ANOVA: p=0.022; p=0.591 for 4HT versus Par+4HT]. This result is to be expected as MCF7/LCC1 cells are sensitive to 4HT and that 4HT can induce apoptosis. These results are also consistent with the effects of parthenolide on responsiveness to 4HT, where parthenolide further sensitizes these antiestrogen sensitive MCF7/LCC1 cells to 4HT but the nature of interaction between the two drugs is additive [Figure 15A].

Samaddar et al. (303) have recently implicated autophagy in responsiveness to TAM. For detection of autophagy, the presence of LC3 protein (Light Chain 3; LC3-I), located in the
membrane of autophagosome (152), as well as conversion of LC3 to LC3-II (i.e., the increase in LC3-II, a decrease in LC3-I, and/or a change in the overall ratio between LC3-II and LC3-I) have been used as indicators of autophagy. During autophagy, LC3-I converts to LC3-II that subsequently associates with autophagic vesicles. The p62/SQSTM1 protein binds tightly to LC3 as well as to ubiquitinated substrates and helps the latter to aggregate to the autophagy machinery (154). Cellular depletion of p62/SQSTM1 (p62; scaffold protein/adaptor protein) (153) can also be used as a marker of autophagy. However, the use of p62/SQSTM1 protein alone to monitor autophagy is often discouraged. Instead, it is recommended to use p62/SQSTM1 protein in addition to LC3 to monitor autophagy (153).

We measured the effects of parthenolide ± 4HT or ethanol vehicle on LC3 (Light chain 3) and p62/SQSTM1 expression (304) in antiestrogen-sensitive MCF7/LCC1 and resistant MCF7/LCC9 cells. While the protein levels of LC3-I and LC3-II are both higher in resistant MCF7/LCC9 cells compared to sensitive MCF7/LCC1 cells, protein levels of LC3-I and LC3-II do not change in either MCF7/LCC9 or MCF7/LCC1 cells following treatment [Figure 29]. Moreover, we found no evidence of depleted levels of p62 in MCF7/LCC9 cells when treated with parthenolide in the presence or absence of 4HT [Figure 29] suggesting that the ability of NFκB inhibition to restore 4HT-induced cell death most likely involves an apoptotic, rather than autophagic, cell death pathway.
Figure 26: Protein levels of JNK and p-JNK do not change in MCF7/LCC9 cells irrespective of the treatment.

MCF7/LCC1 and MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, 100nM ICI 182,780, Par+4HT, Par+ICI or ethanol vehicle as indicated in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for active-JNK and then total JNK. β-actin, loading control. Representative immunoblot is shown.
A.

![MCF7/LCC9 cells graph](image)

**Figure 27:** Combined treatment with parthenolide and 4HT does not alter the cell cycle profile of resistant cells.

**MCF7/LCC9 (A)** and **MCF7/RR (B) cells** were each treated with 500nM parthenolide (Par), 1 µM 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs before cell cycle analysis. Data represent mean percentage of total cells ± SE for three independent experiments.
A.

Figure 28: Combined treatment with parthenolide and 4HT induces apoptosis in resistant MCF7/LCC9 (A) but not in sensitive MCF7/LCC1 (B) cells.

MCF7/LCC9 cells were each treated with parthenolide (Par; 500nM), 4HT (1µM); Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs before determination of apoptosis by Annexin V assay. Data represent mean ± SE for relative apoptosis normalized to ethanol-treated cells for at least three independent experiments A) MCF7/LCC9 cells: $P=0.042$ for all treatment groups by one-way ANOVA, and $P<0.05$ for 4HT versus +4HT.
Figure 28 (B). 4HT induces apoptosis in sensitive MCF7/LCC1 cells, but has no additional effect on apoptosis in combination with parthenolide.

MCF7/LCC1 cells were each treated with parthenolide (Par; 500nM), 4HT (1 µM); Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs before determination of apoptosis by Annexin V assay. Data represent mean ± SE for relative apoptosis normalized to ethanol-treated cells for at least three independent experiments. **B) MCF7/LCC1 cells:** $P=0.022$ for all treatment groups by one-way ANOVA, and $P=0.591$ for 4HT versus Par+4HT.
Figure 29: Protein levels of LC3-I and LC3-II do not change in MCF7/LCC9 cells irrespective of the treatment.

MCF7/LCC1 and MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT, or ethanol vehicle as indicated in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for LC3 and p62/SQSTM1 (referred to as p62) adaptor protein. β-actin, loading control. Representative immunoblot is shown.
3.15. Defining the underlying mechanism of synergy between parthenolide and antiestrogens (primarily Tamoxifen; 4HT):

We now know that both 4HT and ICI 182,780 synergize with parthenolide and increase apoptosis in resistant MCF7/LCC9 cells. We now elucidate mechanistically how NFκB affects cell signaling to modify antiestrogen (primarily Tamoxifen; 4HT) responsiveness; and how affecting NFκB action leads to cell death in resistant MCF7/LCC9 cells.

3.15.1. Combined treatment with parthenolide and 4HT increases mitochondrial membrane permeability (MMP) in resistant MCF7/LCC9 cells:

The induction of apoptosis is often accompanied by the mitochondrial permeability transition, where the electrochemical gradient across the mitochondrial membrane collapses due to the formation of pores driven by the activation of pro-apoptotic members of the BCL2 family. Once the mitochondrial membrane is permeable, cytochrome c is released into the cytoplasm. To determine if the combination of parthenolide and 4HT in resistant MCF7/LCC9 cells induces changes in the mitochondrial membrane integrity, we measured the effects of parthenolide on mitochondrial membrane permeability (MMP) after ~20 hours of drug treatment. Consistent with the effects on apoptosis shown in Figure 28A, Figure 30 shows a statistically significant increase in MMP in the presence of both drugs [Figure 30; One-way ANOVA: p=0.008; p<0.05 relative to 4HT; p<0.05 relative to control]. No significant increase in MMP is seen in response to either parthenolide or 4HT alone. Thus, combined treatment with parthenolide and 4HT synergistically reduces cell growth and restores 4HT-induced cell death in resistant cells, at least in part, by activating the intrinsic apoptotic pathway leading to permeabilization of the outer mitochondrial membrane.
Figure 30: Combined treatment with parthenolide and 4HT enhances MMP in MCF7/LCC9 cells.

MCF7/LCC9 cells were each treated with parthenolide (Par; 500nM), 4HT (1 µM); Par+4HT or ethanol vehicle in CCS-IMEM for 18-20 hrs before measuring mitochondrial membrane permeability; MMP. Data represent mean ± SE of relative MMP normalized to ethanol-treated cells for at least three independent experiments (P=0.008 for all treatment groups by one-way ANOVA, and P<0.05 for 4HT versus Par+4HT).
3.15.2. Parthenolide in combination with 4HT inhibits expression of PARP in resistant MCF7/LCC9 cells:

There is increasing evidence that the family of cysteine proteases, also known as caspases, play a very important role in the pathway that mediates the highly ordered process leading to cell death or apoptosis (305). At the onset of apoptosis, these caspases have been identified to target number of specific proteins including PARP (poly ADP-ribose polymerase), for proteolytic cleavage (306). PARP, an 116kDa nuclear protein, is required for cells to maintain their viability. In human PARP, the cleavage occurs between Asp214 and Gly215, which separates the PARP amino-terminal DNA binding domain (24 kDa) from the carboxy-terminal catalytic domain (89kDa) (306). In the last few years, cleavage of PARP has been used extensively as a marker of cells undergoing apoptosis (307).

![Diagram of PARP in Apoptotic Pathways](image)

**Figure 31: Role of PARP in Apoptotic Pathways.**
To confirm how PARP protein expression is affected by antiestrogens 4HT/ICI 182,780 and parthenolide, MCF7/LCC1 and MCF7/LCC9 cells were treated with ethanol vehicle, parthenolide, and 4HT/or ICI 182,780 in the presence or absence of parthenolide for 72 hrs. Whole cell lysates were collected and subjected to Western blot analysis. While we do no see any cleaved PARP with 4HT alone in either MCF7/LCC1 or MCF7/LCC9 cells, cleaved PARP was detected with 100nM paclitaxel (positive control). 4HT alone does not affect PARP expression in MCF7/LCC9 cells [Figure 32 A and B], but parthenolide in combination with 4HT strongly inhibits PARP protein expression in these resistant MCF7/LCC9 cells [Figure 32 A and B; p<0.05 for 4HT versus Par+4HT).

In contrast, ICI 182,780 alone inhibits PARP expression in sensitive MCF7/LCC1 cells, but no significant decrease in PARP expression is seen in presence of parthenolide and ICI 182,780 in MCF7/LCC9 cells [Figure 32 C and D; P=0.048 for all treatment groups by one-way ANOVA, and P=0.052 for ICI versus Par+ICI).

Since PARP can be cleaved by many caspases in vitro directly or indirectly as shown in Figure 31, we measured the effects of parthenolide ± 4HT or ethanol vehicle on caspase expression (caspase 8 and 9; upstream of PARP) as shown in [Figure 33]. While parthenolide in combination with 4HT decreases Caspase 8 and Caspase 9 expression in resistant MCF7/LCC9 cells, no cleaved fragments of either Caspase 8 (p43/p41) or Caspase 9 (p35/p37) were seen. The cleaved products of both Caspase 8 and Caspase 9 were clearly seen in our positive control cells (MCF7/LCC9 cells treated with 100nM Paclitaxel [Figure 33].
Figure 32: PARP protein expression is down-regulated by the combined treatment with parthenolide and 4HT in MCF7/LCC9 cells.

MCF7/LCC1 and MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, 100nM ICI 182,780, singly or in combination or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for PARP. β-actin, loading control. A) Representative immunoblot for Par+ 4HT combination. B) Data from at least three independent
experiments are presented as the mean ± SE of the relative PARP: β-actin ratio in MCF7/LCC9 cells (P=0.016 for all treatment groups by one-way ANOVA, and P<0.05 for 4HT versus Par+4HT. C) Representative immunoblot for Par+ICI combination. D) Data from at least three independent experiments are presented as the mean ± SE of the relative PARP: β-actin ratio in MCF7/LCC9 cells (P=0.048 for all treatment groups by one-way ANOVA, P=0.052 for ICI versus Par+ICI).

Figure 33: Caspase 8 and 9 protein expression in MCF7/LCC1 and MCF7/LCC9 cells treated with combination of parthenolide and 4HT.

MCF7/LCC1 and MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for A) Caspase 8. β-actin, loading control B) Caspase 9. β-actin, loading control. Antibodies to both Caspase 8 and 9 detect endogenous levels of full length protein as well as the cleaved fragments.
3.15.3. **Combined treatment with parthenolide and antiestrogens (4HT/ICI 182,780)** has no effect on the expression of p65/RELA or p50 or on the stability of IκBα or IKKγ/NEMO expression:

Parthenolide, a specific potent inhibitor of NFκB pathway can enhance stabilization of inhibitor IκBα and lead to cytosolic sequestration of p65/RELA in the cytoplasm in an inactive state (276). Since the protein kinase IKK (*i.e.*, IκB complex kinase) that phosphorylates IκBα, and is composed of at least two catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ/NEMO (308) can also be targeted by parthenolide (309), we measured the effects of parthenolide ± 4HT/ICI 182,780 or ethanol vehicle on IκBα and IKKγ/NEMO expression [Figure 34; A to D]. While the protein levels of IKKγ/NEMO are higher in resistant MCF/LCC9 cells compared to sensitive MCF7/LCC1 cells as shown in Figure 34; A and B and also shown in our previously published studies (297), protein levels of IKKγ/NEMO do not change in MCF7/LCC9 cells irrespective of the treatment [Figure 34; A and B]. The protein levels of IκBα were also unchanged regardless of treatment in MCF7/LCC9 cells [Figure 34; C and D].

We measured the effects of parthenolide ± 4HT/ICI 182,780 or ethanol vehicle on p65/RELA and p50 expression [Figure 35; A to D]. In agreement with our previously published studies, although the protein levels of p65/RELA are higher in resistant MCF/LCC9 cells compared to sensitive MCF7/LCC1 cells (297), protein levels of the p65/RELA or the p50 subunit of the NFκB heterodimer do not change in MCF7/LCC9 cells irrespective of the treatment [Figure 35; A to D].
3.15.4. Effect of the combined treatment with parthenolide and antiestrogens (4HT/ICI 182,780) on the expression of TNFα, TNFR1 and BCL10:

The proinflammatory cytokine TNFα (tumor necrosis factor) can trigger signaling pathways through two distinct cell surface receptors, TNFR1 and TNFR2 (TNFR1 is known to initiate majority of the biological activities) that converge on the activation of the transcription factor NFκB (213). Since, TNFα is known to activate NFκB through a kinase module that involves the canonical IKKα,β,γ complex (310), we determined the effects of antiestrogen (4HT/ICI 182,780) ± parthenolide or ethanol vehicle on TNFα and TNFR1 protein expression [Figure 36 and 37]. While TNFα protein expression is strongly inhibited by ICI 182,780, 4HT has no affect on TNFα expression in the sensitive MCF7/LCC1 cells [Figure 36; A and B]. Importantly, the protein levels of TNFα were unchanged in resistant MCF7/LCC9 cells regardless of the treatment [Figure 36; A and B].

In contrast, parthenolide in combination with antiestrogen 4HT strongly inhibits TNFR1 protein expression in resistant MCF7/LCC9 cells [Figure 37; A and B; P=0.039 for all treatment groups by one-way ANOVA, and P<0.05 for 4HT versus Par+4HT], but the protein levels of TNFR1 were unchanged in resistant MCF7/LCC9 cells when treated with a combination of parthenolide and ICI 182,780 [Figure 37; C]. These data suggest that molecular signaling that affects 4HT responsiveness is perhaps different from ICI 182,780 responsiveness in these breast cancer cells.

TNFα is also known to up-regulate the expression of BCL10 (B-cell lymphoma 10) (311) and that over-expression of BCL10 is sufficient to activate NFκB (312). Since Cheng et al., have also recently shown that an NFκB binding site resides in the BCL10 5'-UTR, we wanted to
determine if NFκB controls the protein expression of BCL10. MCF7/LCC1 and MCF7/LCC9 cells were treated with ethanol vehicle, parthenolide, and 4HT/ICI 182,780 in the presence or absence of parthenolide for 72 hrs. Whole cell lysates were collected and subjected to Western blot analysis to see if BCL10 protein expression is affected by antiestrogens (4HT and ICI 182,780) and parthenolide. While BCL10 protein expression is strongly inhibited by ICI 182,780, 4HT has no affect on BCL10 expression in the sensitive MCF7/LCC1 cells [Figure 36; C and D]. Importantly, the protein levels of BCL10 were unchanged in resistant MCF7/LCC9 cells regardless of the treatment [Figure 36; C and D]. These data suggest that parthenolide may be acting through other alternative mechanisms to restore the apoptotic response in MCF7/LCC9 cells.
Figure 34: Combined treatment with parthenolide and antiestrogens (4HT/ICI 182,780) has no effect on the stability of IkBα or IKKγ/NEMO expression.

MCF7/LCC1 and MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, 100nM ICI 182,780, Par+4HT, Par+ICI or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for IkBα or IKKγ/NEMO (the regulatory molecules upstream of NFκB). β-actin, loading control.
Figure 35: Combined treatment with parthenolide and antiestrogens (4HT/ICI 182,780) has no effect on the expression of p65/RELA or p50.

MCF7/LCC1 and MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, 100nM ICI 182,780, Par+4HT, Par+ICI or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for p65/RELA or p50. β-actin, loading control.
Figure 36: Combined treatment with parthenolide and antiestrogens (4HT/ICI 182,780) has no effect on the expression of TNFα or BCL10 in resistant MCF7/LCC9 cells.

MCF7/LCC1 and MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, 100nM ICI 182,780, Par+4HT, Par+ICI or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for TNFα or BCL10. β-actin, loading control.
Figure 37: TNFR1 protein expression is down-regulated by the combined treatment with parthenolide and 4HT in MCF7/LCC9 cells.

MCF7/LCC1 and MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, 100nM ICI 182,780, Par+4HT, Par+ICI or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40μg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for TNFR1. β-actin, loading control. A) Representative immunoblot for Par+ 4HT combination. B) Data from at least three independent experiments are presented as the mean ± SE of the relative TNFR1: β-actin ratio in MCF7/LCC9 cells (P=0.039 for all treatment groups by one-way ANOVA, and P<0.05 for 4HT versus Par+4HT. C) Representative immunoblot for Par+ ICI combination.
3.15.5. Parthenolide in combination with 4HT inhibits expression of BCL2 in resistant cells:

The changes in MMP seen in Figure 30 implicate the BCL2 family of proteins as key players in signaling downstream from NFκB in the context of antiestrogen responsiveness. The BCL2 family of proteins can be divided into the anti-apoptotic (such as BCL2, BCL-XL, BCL-W) and pro-apoptotic proteins (including BAX, BAD and BID). The balance between pro-apoptotic and the anti-apoptotic members of the BCL2 family can determine cell fate. To confirm how BCL2 protein expression is affected by 4HT and parthenolide, MCF7/LCC1 and MCF7/LCC9 cells were treated with ethanol vehicle, parthenolide, and 4HT in the presence or absence of parthenolide for 72 hrs. Whole cell lysates were collected and subjected to Western blot analysis. While 4HT strongly inhibits BCL2 protein expression in the sensitive MCF7/LCC1 cells [Figures 39A and 39B; P<0.05 relative to control], it does not affect BCL2 expression in MCF7/LCC9 cells [Figures 38A and 38B]. In contrast, BCL2 protein expression is significantly inhibited in resistant MCF7/LCC9 cells by parthenolide [Figures 38A and 38B; ~50% inhibition; p=0.001 relative to control], and parthenolide in combination with antiestrogen 4HT further inhibits BCL2 protein expression in resistant MCF7/LCC9 cells [Figures 38A and 38B; ~75% inhibition; p=0.003 relative to parthenolide). Similar results were seen in the sensitive MCF7/LCC1 cells [Figure 39B; p<0.05 relative to 4HT] that are consistent with the effects of parthenolide on responsiveness to 4HT [Figure 15A] measured by changes in cell proliferation, but RI values were additive.

To test whether BCL2 protein expression is affected by ICI 182,780 and parthenolide, MCF7/LCC1 and MCF7/LCC9 cells were treated with ethanol vehicle, parthenolide, and ICI in the presence or absence of parthenolide for 72 hrs and immunoblotted for BCL2 as shown in
Figure 38. While ICI almost completely inhibits BCL2 protein expression in the sensitive MCF7/LCC1 cells, it does not affect BCL2 expression in MCF7/LCC9 cells [Figure 40 A]. Similar to data shown in Figure 38, BCL2 protein expression is significantly inhibited in resistant MCF7/LCC9 cells by parthenolide [Figure 40A and 40B; ~50% inhibition], and parthenolide in combination with antiestrogen ICI 182,780 further inhibits BCL2 protein expression in resistant MCF7/LCC9 cells [Figures 40A and 40B; ~80% inhibition; p=0.008 relative to parthenolide].

To evaluate if this decrease in BCL2 protein expression is a transcriptional or posttranscriptional event, BCL2 mRNA levels were measured by RT-PCR in the resistant MCF7/LCC9 cells following treatment. Similar to the protein levels, BCL2 mRNA was inhibited by ≥70% when cells were treated with the combination of parthenolide and 4HT [Figure 41]. Thus, the changes in BCL2 protein expression closely follow changes in BCL2 mRNA transcription.

While parthenolide alone or in combination with 4HT has no statistically significant effect on expression of the pro-apoptotic proteins BAX [Figure 42 A] and BAD [Figure 44A and 45], there is a marked 5-fold increase in the overall BAX:BCL2 ratio when MCF7/LCC9 cells are treated with a combination of parthenolide and 4HT [Figure 42B] primarily because of the decrease in BCL2 expression. These data strongly suggest that parthenolide may synergize with 4HT by acting to decrease expression of BCL2 such that a marked increase in the overall BAX:BCL2 ratio occurs (105,313) shifting the balance towards cell death and restoring the 4HT-induced apoptotic response in resistant cells.
Figure 38: BCL2 protein expression is down-regulated by the combined treatment with parthenolide and 4HT in MCF7/LCC9 cells.

MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BCL2. β-actin, loading control. A) Representative immunoblot for MCF7/LCC9 cells. B) Data from three independent experiments are presented as the mean ± SE of the relative BCL2: β-actin ratio in MCF7/LCC9 cells (P≤0.001 for all treatment groups by one-way ANOVA, and P=0.001 for Par versus ethanol vehicle; P=0.003 for Par versus Par+4HT).
Figure 39: BCL2 protein expression is down-regulated by the combined treatment with parthenolide and 4HT in MCF7/LCC1 cells.

MCF7/LCC1 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BCL2. β-actin, loading control. A) Representative immunoblot for MCF7/LCC1 cells. B) Data from three independent experiments are presented as the mean ± SE of the relative BCL2: β-actin ratio in MCF7/LCC1 cells (P=0.005 for all treatment groups by one-way ANOVA, and P<0.05 for 4HT versus ethanol vehicle; P<0.05 for 4HT versus Par+4HT).
Figure 40: BCL2 protein expression is down-regulated by the combined treatment with parthenolide and ICI 182,780 in MCF7/LCC9 cells.

MCF7/LCC9 cells were each treated with 500nM parthenolide (Par), 100nM ICI 182,780, Par+ICI or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BCL2. β-actin, loading control. A) Representative immunoblot for MCF7/LCC9 cells. B) Data from three independent experiments are presented as the mean ± SE of the relative BCL2: β-actin ratio in MCF7/LCC1 cells (\(P=0.005\) for all treatment groups by one-way ANOVA, and \(P<0.05\) for 4HT versus ethanol vehicle; \(P<0.05\) for 4HT versus Par+4HT).
Figure 41: BCL2 mRNA is down-regulated by the combined treatment with parthenolide and 4HT in MCF7/LCC9 cells.

Total RNA was isolated from MCF7/LCC9 cells treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs, reverse-transcribed, and subjected to RT-PCR to detect BCL2, and the housekeeping gene RPLP0. Data are presented as the mean ± SE of the relative BCL2: RPLP0 ratio analyzed in triplicate. ($P$≤0.001 for all treatment groups by one-way ANOVA, and $P$<0.05 for Par+4HT versus 4HT; $P$<0.05 for Par+4HT versus ethanol vehicle).
MCF7/LCC9 and MCF7/LCC1 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. A) Representative immunoblot for BAX. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BAX. β-actin, loading control. B) Data from three independent experiments are used to present relative BAX:BCL2 ratio in MCF7/LCC9 cells.

Figure 42: Combined treatment with parthenolide and 4HT increases BAX:BCL2 ratio in MCF7/LCC9 cells.
3.15.6. Inhibition of NFκB by parthenolide does not correlate with reduced XIAP expression in resistant MCF7/LCC9 cells:

XIAP (X-chromosome-linked inhibitor of apoptosis protein), one of the members of the IAP (Inhibitors of apoptosis) family, is regulated by NFκB and is known to block caspase activity. XIAP is also known to play an important role in cancer progression (314). Recent studies show that XIAP is a strong candidate among cancer therapeutic targets (315,316) and suppression of NFκB expression can result in down-regulation of XIAP expression (317). So, we measured the effects of parthenolide ± 4HT or ethanol vehicle on XIAP expression [Figure 44B]. Since protein levels of XIAP were unchanged in MCF7/LCC9 cells regardless of the treatment, XIAP is unlikely to be regulated by NFκB in our cell system.

3.15.7. Restoration of sensitivity to antiestrogens by parthenolide may not involve inhibition of BCL2 promoter activity in resistant MCF7/LCC9 cells:

The function of the BCL2 protein family has been largely documented. NFκB has been reported to regulate the expression of several genes that control apoptosis; one of them being BCL2 family of proteins. Inhibition of the NFκB transcription factor can increase BAX expression in cancer cell lines (217) and p52/p100 can induce BCL2 expression (318). Nuclear localization of NFκB is associated with BCL2 and BAX expression and activation of NFκB/BCL2 pathway may be associated with a poor response to neoadjuvant doxorubicin based chemotherapy (319). However, NFκB has not been shown to directly regulate transcription of BAX and BCL2 mRNAs. Since the BCL2 promoter has multiple putative NFκB binding sites (318) and NFκB inhibition on responsiveness to either 4HT or ICI 182,780 leads to a decrease in BCL2 protein expression [Figures 38, 39 and 40], we asked whether NFκB regulates BCL2 promoter activity in our cell system. The human BCL2 upstream region has two promoters: P1
and P2 (318). P1 promoter, with multiple initiation sites, is 1400bp upstream of the ORF; P2 promoter, with one initiation site is located from -750 to -8, just upstream of the ORF but it does not contain any perfect κB binding site. Two κB sites are located at -2306 and -1896 respectively [Figure 43]. P1+P2 promoter has five other κB sites (1-5); all of these sites have one mismatched nucleotide [Figure 43]. We cotransfected MCF7/LCC1 and MCF7/LCC9 cells with pBCL2-Luc (P1, or P1+P2) and pRL-SV40 Renilla constructs prior to treatment with 4HT, ICI 182,780, and/or parthenolide for 24 hrs before performing promoter-reporter assay. While antiestrogens (both 4HT and ICI 182,780) alone significantly inhibit P1 promoter activity in sensitive MCF7/LCC1 cells [Figures 46 and 47], parthenolide alone or in combination with either 4HT or ICI 182,780 does not inhibit BCL2-luciferase activity (P1 or P1+P2) in the resistant MCF7/LCC9 cells [Figures 46 and 47].

![BCL2 promoter (P1, P2 and P1+P2).](image)

*Viatour et al., Leukemia, 2003*
Figure 44: Combined treatment with parthenolide and 4HT has no effect on the expression of XIAP or on the pro-apoptotic protein BAD in MCF7/LCC9 cells.

MCF7/LCC9 and MCF7/LCC1 cells were each treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BAD or XIAP. β-actin, loading control. A) Representative immunoblot for BAD. B) Representative immunoblot for XIAP.
Figure 45: Combined treatment with parthenolide and ICI 182,780 has no effect on the expression of pro-apoptotic protein BAD in MCF7/LCC9 cells.

MCF7/LCC9 and MCF7/LCC1 cells were each treated with 500nM parthenolide (Par), 100nM ICI 182,780, Par+ICI or ethanol vehicle in CCS-IMEM for 72 hrs before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BAD. β-actin, loading control. Representative immunoblot for BAD is shown.
Figure 46: Restoration of sensitivity to antiestrogens (4HT or ICI 182,780) by parthenolide may not involve inhibition of BCL2 (P1) promoter activity in MCF7/LCC9 cells.

MCF7/LCC1 and MCF7/LCC9 cells were cotransfected in quadruplicate with pBCL2(P1)-Luc and pRL-SV40 Renilla constructs prior to treatment with 1 M 4HT, 100nM ICI 182,780, 500nM parthenolide (Par) singly or in combination (or ethanol vehicle) in CCS-IMEM for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations. A) Par+4HT combination. B) Par+ICI combination.
Figure 47: Restoration of sensitivity to antiestrogens (4HT or ICI 182,780) by parthenolide may not involve inhibition of BCL2 (P1+P2) promoter activity in MCF7/LCC9 cells.

MCF7/LCC1 and MCF7/LCC9 cells were cotransfected in quadruplicate with pBCL2 (P1+P2) -Luc and pRL-SV40 Renilla constructs prior to treatment with 1 M 4HT, 100nM ICI 182,780, 500nM parthenolide (Par) singly or in combination (or ethanol vehicle) in CCS-IMEM for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations. A) Par+4HT combination. B) Par+ICI combination.
3.15.8. Caspase inhibitors reverse the effects of NFκB and 4HT on cell proliferation and mitochondrial membrane permeability:

Caspases are central components of the intrinsic apoptotic pathway that ultimately lead to an apoptotic cell death. Since we have shown that NFκB inhibition affects apoptosis by disrupting BCL2 mediated signaling and changing the MMP in TAM resistant cells, we sought to determine whether restoration of 4HT-induced cell death by parthenolide is caspase-dependent. We first used a pancaspase inhibitor that inhibits all caspases upstream and downstream of the mitochondria. We then measured the effects of caspase inhibition on two major endpoints: cell proliferation and MMP.

To measure the effects on cell proliferation, MCF7/LCC9 cells were treated with 4HT (1 M), parthenolide (500nM), parthenolide±4HT or ethanol vehicle for 7 days in the presence or absence of a pancaspase inhibitor. In the absence of the pancaspase inhibitor, combined treatment with 4HT and parthenolide synergistically inhibits growth and restores sensitivity to 4HT in MCF7/LCC9 cells. However, this effect of NFκB inhibition is fully reversed by the pancaspase inhibitor [Figure 48; One-way ANOVA: \( P \leq 0.001; P = 0.029 \) relative to Par; \( P = 0.029 \) relative to Par+4HT]. We then measured the effects of the pancaspase inhibitor on MMP. MCF7/LCC9 cells were treated with 1 M 4HT, 500nM parthenolide, parthenolide±4HT (1 M) or ethanol vehicle in CCS-IMEM for 18-20 hr before measuring the changes in MMP in the presence or absence of the pancaspase inhibitor. While combined treatment of resistant MCF7/LCC9 cells with 4HT and parthenolide significantly increases MMP in the absence of the pancaspase inhibitor (1.6-fold; \( P < 0.05 \) relative to 4HT), the effects on MMP are completely reversed by the pancaspase inhibitor [Figure 50; \( P = 0.012 \) relative to LCC9/Par+4HT]. These data show that a caspase-dependent cell death mechanism is active when cells are treated with 4HT and the NFκB inhibitor parthenolide.
Importantly, these data strongly implicate caspases upstream of the mitochondria because the effects of treatment on MMP were fully reversed by the pancaspase inhibitor.

To further clarify which upstream caspases are involved in restoring 4HT induced cell death in MCF7/LCC9 cells, we first measured the effects of a specific CASP8 inhibitor (C8I) on cell proliferation. We used C8I, an irreversible inhibitor of CASP8; the Z-IETD-FMK sequence binds only to CASP8 and blocks substrate binding. Antiestrogen resistant MCF7/LCC9 cells were treated with 4HT (1 M), parthenolide (500nM), parthenolide±4HT or ethanol vehicle for 7 days in the presence or absence of CASP8 inhibitor (C8I). In the absence of CASP8 inhibitor (C8I), combined treatment with 4HT and parthenolide synergistically inhibits growth and restores sensitivity to 4HT in MCF7/LCC9 cells. This effect of NFκB inhibition is at least partially reversed by the CASP8 inhibitor [Figure 49; One-way ANOVA: P≤0.001; P=0.01 relative to LCC9/Par; P≤0.001 relative to LCC9/Par+4HT]. We then measured the effects of the CASP8 inhibitor (C8I) on mitochondrial membrane permeability. MCF7/LCC9 cells were treated with 1 M 4HT, 500nM parthenolide, parthenolide±4HT (1 M) or ethanol vehicle in CCS-IMEM for 18-20 hrs before measuring changes in MMP in the presence or absence of C8I. The CASP8 inhibitor data [Figure 50; P=0.006 relative to LCC9/Par+4HT] mimics the effects seen with the pancaspase inhibitor, implying that CASP8, acting upstream of the mitochondria, is involved in restoring 4HT induced caspase-dependent cell death in TAM resistant breast cancer cells. Since we do not fully reverse resistance, we cannot exclude a minor role for CASP2 and/or CASP10 in 4HT induced apoptosis as modified by NFκB. Studies to address the role of other caspases in affecting antiestrogen responsiveness in breast cancer cells need to be done.
Figure 48: Pancaspase inhibitor fully reverses the effects of NFκB inhibition on cell proliferation in MCF7/LCC9 cells.

Cells were seeded in quadruplicate and treated with 1 M 4HT, 500nM Par±4HT (1 M) or ethanol vehicle in CCS-IMEM in the presence or absence of a pancaspase inhibitor for 7 days before counting. Data represent mean ± SE of relative proliferation of four determinations from a single representative experiment (the experiment was done at least thrice). P≤0.001 for all treatment groups by one-way ANOVA, and *, P=0.029 for Par versus Par with pancaspase inhibitor; ^, P=0.029 for Par+4HT versus Par+4HT with pancaspase inhibitor.
**Figure 49:** Specific inhibitor to CASP8 partially reverses the effects of NFκB inhibition on cell proliferation in MCF7/LCC9 cells.

Cells were seeded in quadruplicate and treated with 1 M 4HT, 500nM Par±4HT (1 M) or ethanol vehicle in CCS-IMEM in the presence or absence of a CASP8 inhibitor for 7 days before counting. Data represent mean ± SE of relative proliferation of four determinations from a single representative experiment (the experiment was done at least thrice). \( P \leq 0.001 \) for all treatment groups by one-way ANOVA, and *, \( P=0.01 \) for Par versus Par with caspase-8 inhibitor; ^, \( P \leq 0.001 \) for Par+4HT versus Par+4HT with caspase-8 inhibitor.
Figure 50: Pancaspase inhibitor and the specific inhibitor to CASP8, both reverse the effects of NFκB inhibition on MMP in MCF7/LCC9 cells.

Cells were treated with 1 M 4HT, 500nM Par±4HT (1 M) or ethanol vehicle in CCS-IMEM in the presence or absence of either a pancaspase inhibitor or a CASP8 inhibitor for 18-20 hrs before measuring MMP. Data represent mean ± SE of relative MMP normalized to ethanol-treated cells for at least three independent experiments (*, P=0.012 versus Par+4HT with pancaspase inhibitor, and ^, P=0.006 versus Par+4HT with CASP8 inhibitor).
3.15.8. Caspase inhibitors block the ability of parthenolide and 4HT to inhibit BCL2 expression:

Finally, to determine if the down-regulation of BCL2 seen when MCF7/LCC9 cells are treated with 4HT and parthenolide [Figures 38A and 38B] is also caspase dependent, we treated MCF7/LCC9 cells with ethanol vehicle or 4HT in the presence or absence of parthenolide, but in the presence of pancaspase inhibitor for 72 hrs. Whole cell lysates were collected and subjected to SDS-PAGE and immunoblotted for BCL2. While in the absence of pancaspase inhibitor parthenolide alone and in combination with 4HT strongly inhibits BCL2 protein expression in resistant MCF7/LCC9 cells [Figures 38A and 38B; 50% inhibition with parthenolide alone; 75% inhibition achieved with combined treatment of parthenolide and 4HT], addition of the pancaspase inhibitor to parthenolide alone or in combination with 4HT no longer inhibits the expression of anti-apoptotic protein BCL2 [Figure 51: A and B; one-way ANOVA: \( P=0.818 \)]. Furthermore, the significant down regulation of BCL2 protein expression observed with 4HT in the sensitive MCF7/LCC1 cells [Figure 39: A and C] is also no longer seen in the sensitive MCF7/LCC1 cells in the presence of the pancaspase inhibitor [Figure 52: A and B; one-way ANOVA: \( P=0.748 \)]. Importantly, the CASP8 inhibitor (C8I) mimics the effects seen with the pancaspase inhibitor [Figures 53 and 54; one-way ANOVA: \( P=0.377 \) for LCC9; \( P=0.143 \) for LCC1].

Thus, NFκB inhibition restores 4HT- induced cell death and sensitivity to 4HT in resistant cells by increasing MMP, decreasing the expression of specific anti-apoptotic proteins (BCL2) and altering the BAX:BCL2 ratio, contributing directly to a caspase-dependent intrinsic apoptotic cell death pathway.
Figure 51: Combined treatment with parthenolide and 4HT no longer inhibits BCL2 protein expression in the presence of a PI in MCF7/LCC9 cells.

MCF7/LCC9 cells were treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs in the presence of PI (pancaspase inhibitor) before cell lysis. 20-40 µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BCL2. β-actin, loading control. A) Representative immunoblot for MCF7/LCC9 cells. B) Data from three independent experiments are presented as the mean ± SE of the relative BCL2: β-actin ratio in MCF7/LCC9 cells (P=0.818 for all treatment groups by one-way ANOVA).
Figure 52: Combined treatment with parthenolide and 4HT no longer inhibits BCL2 protein expression in the presence of PI in MCF7/LCC1 cells.

MCF7/LCC1 cells were treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs in the presence of PI (pancaspase inhibitor) before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BCL2. β-actin, loading control. A) Representative immunoblot for MCF7/LCC1 cells. B) Data from three independent experiments are presented as the mean ± SE of the relative BCL2: β-actin ratio in MCF7/LCC1 cells (P=0.748 for all treatment groups by one-way ANOVA).
Figure 53: Combined treatment with parthenolide and 4HT no longer inhibits BCL2 protein expression in the presence of a specific inhibitor to CASP8 (C8I) in MCF7/LCC9 cells.

MCF7/LCC9 cells were treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs in the presence of C8I before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BCL2. β-actin, loading control. A) Representative immunoblot for MCF7/LCC9 cells. B) Data from three independent experiments are presented as the mean ± SE of the relative BCL2: β-actin ratio in MCF7/LCC9 cells (P=0.377 for all treatment groups by one-way ANOVA).
Figure 54: Combined treatment with parthenolide and 4HT no longer inhibits BCL2 protein expression in the presence of a specific inhibitor to CASP8 (C8I) in MCF7/LCC1 cells.

MCF7/LCC1 cells were treated with 500nM parthenolide (Par), 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs in the presence of C8I before cell lysis. 20-40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for BCL2. β-actin, loading control. A) Representative immunoblot for MCF7/LCC1 cells. B) Data from three independent experiments are presented as the mean ± SE of the relative BCL2: β-actin ratio in MCF7/LCC1 cells (P=0.143 for all treatment groups by one-way ANOVA).
3.15.9. Caspase inhibitors block the ability of parthenolide and 4HT to inhibit PARP and TNFR1 expression in resistant MCF7/LCC9 cells:

Interestingly, when we treated MCF7/LCC9 cells as shown in [Figures 32A and 37A] with parthenolide or 4HT either singly or in combination, but now in the presence of a pancaspase inhibitor (PI), the down-regulation of PARP [Figure 32: A and B] and TNFR1 [Figure 37: A and B] seen in the presence of Par+4HT in MCF7/LCC9 cells is reversed with the addition of a pancaspase inhibitor (PI) [Figure 55; B and D].

While in the absence of pancaspase inhibitor parthenolide in combination with 4HT strongly inhibits PARP protein expression in resistant MCF7/LCC9 cells [Figures 32A, 32B, and 55A; 50% inhibition achieved with combined treatment of parthenolide and 4HT], addition of the pancaspase inhibitor no longer inhibits the expression of PARP [Figure 55B]. Furthermore, the significant down regulation of TNFR1 protein expression observed with PAR+4HT in the resistant MCF7/LCC9 cells [Figure 37A, 37B and 55C] is also no longer seen in the resistant MCF7/LCC9 cells in the presence of the pancaspase inhibitor [Figure 55D]. How NFκB regulates PARP and TNFR1 expression remains to be determined.

To ensure that the concentration of pancaspase inhibitor used (20 M) is sufficient to inhibit apoptosis in our resistant MCF/LCC9 cells, we included a positive control. MCF7/LCC9 cells were treated with 100nM paclitaxel in the presence or absence of 20 M pancaspase inhibitor (PI) and immunoblotted for PARP (the PARP antibody used detects both endogenous levels of full length PARP (116kDa) as well as the large fragment of PARP (89kDa) resulting from caspase cleavage). While the cleaved PARP was detected with 100nM paclitaxel alone, this
cleaved fragment is no longer seen in the presence of PI confirming the inhibition of caspase cleavage/apoptosis in presence of PI [Figure 50E].

A. [Image of LCC9 - I]  
B. [Image of LCC9 + I]  

C. [Image of LCC9 - I]  
D. [Image of LCC9 + I]  

E. [Image of LCC9 with Par, Par+4HT and Par+4HT]  

Figure 55: Combined treatment with parthenolide and 4HT no longer inhibits PARP and TNFR1 protein expression in the presence of a pancaspase inhibitor (I) in MCF7/LCC9 cells.

Cells were treated with 500nM Par, 1 M 4HT, Par+4HT or ethanol vehicle in CCS-IMEM for 72 hrs in the presence of Pancaspase inhibitor (PI; I; 20 M) before cell lysis. Lysates (20-40µg) were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted. β-actin, loading control. Representative immunoblots for PARP (A and B); ± PI and TNFR1 (C and D); ± PI are shown. E) Positive control: LCC9 cells treated with 100nM Paclitaxel ± 20 M PI.
3.16. Conclusions:

The most significant, novel findings of the study:

Resistance to non steroidal antiestrogen Tamoxifen (SERM; Selective Estrogen Receptor Modulator) is a highly relevant clinical phenotype, as this is the most widely used antiestrogen and it remains the primary endocrine therapy for many breast cancer patients with ER+ disease. We have shown here that inhibition of NFκB by a small molecule antagonist (parthenolide) provides a means to overcome resistance to both SERMs and SERDs (ICI 182,780). Parthenolide not only reverses resistance to Tamoxifen in resistant cells but also increases the responsiveness of sensitive cells to this SERM.

We then proceed to show that NFκB acts by inducing signaling through BCL2 to control mitochondrial membrane permeability and that this signaling involves alterations in the activation of CASP8 acting upstream of both the mitochondria and BCL2. For example, inhibiting CASP8 activity affects the ability of Tamoxifen (acting through NFκB) to inhibit BCL2, induce mitochondrial membrane permeability, and to affect cell survival. However, a role for executioner caspases downstream of mitochondria remains to be determined. Furthermore, we show that when antiestrogen resistance is reversed by inhibition of NFκB, the cells die primarily through apoptosis rather than by an autophagic cell death mechanism.

These data provide important and relevant new insights into signaling in endocrine resistance in breast cancer, showing the central role of NFκB signaling through the mitochondria, as regulated by CASP8 and BCL2, to affect cell survival. The data with parthenolide, which we show acts primarily through its inhibition of NFκB, strongly supports the design of novel clinical trials to prevent, delay, and/or reverse resistance to antiestrogens.
Chapter 4: Dominant-negative NFκB inhibitor (mutant IκB; pCMV4-FLAG-tagged IκB super-repressor; IκBSR) mimics the effects seen with parthenolide

4.1. Selection of a Molecular Approach for NFκB Inhibition:

4.1.1. Stable transfection of a Dominant-negative NFκB inhibitor into breast cancer cells:

Data from our parthenolide studies indicate that NFκB inhibition by parthenolide affects antiestrogen sensitivity by modulating CASP8 activity, with consequent effects on BCL2 expression, mitochondrial function, and apoptosis. Since parthenolide may have “off-target” effects, we took a molecular approach to obtain independent confirmation of the role of parthenolide in NFκB inhibition. We sought several different strategies to knock out the activity and/or the expression of NFκB.

Problems in Accomplishing Task 4.1.1:

We took a molecular approach and introduced a mutant IκB (which cannot be phosphorylated and targeted for degradation and which acts as a dominant negative NFκB inhibitor; also known as the IκBSuper-Repressor; IκBSR) into our most sensitive cells from the parthenolide studies (MCF7/LCC9 and MCF7/RR cells). We first used the ecdysone-inducible expression system to stably and inducibly express IκBSR in MCF7/LCC9 and MCF7/RR cells as this will allow us to control the levels of IκB expression. We transfected this mutant IκB construct, which was a single vector containing both halves of the expression system and was FLAG tagged [Figure 56] into MCF7/LCC9 and MCF7/RR cells. After selection against puromycin, several clones and three pooled populations were selected for each cell line. We then
tried inducing the gene expression using Ponastrone A. While characterizing these cells and controls for expression, we saw no FLAG at all in the clones as well as the pooled population. This approach was thus abandoned as the attempts made to stably and inducibly express IκBSR in MCF7/LCC9 and MCF7/RR cells were unsuccessful.

Ponasterone-inducible Flag-IκBSR vector

![Diagram of Ponasterone-inducible Flag-IκBSR vector](image)

**Figure 56: Ponasterone-inducible FLAG- IκBSR vector.**

We then stably transfected the MCF7/LCC9 and MCF7/RR cells with a constitutively active S32/36A mutant non-FLAG tagged pcDNA3-IκBSR (LCC9/ IκBSR; MCF7/RR/IκBSR) and generated pooled populations for both the cells lines. Control cell populations were generated by stable transfection with the expression vector lacking the pcDNA3-IκBSR insert (LCC9/EV; MCF7/RR/EV). Over-expression of the mutant IκBα (IκBSR) protein was measured using antibodies to IκBα in these cells [Figure 57]. While both pooled populations (Pool 1 and Pool 2) of MCF7/RR/IκBSR cells showed significant over-expression of IκBα, LCC9/IκBSR pooled populations (Pool 1, Pool 2, Pool 3) did not show a significant over-expression of IκBα [Figure 57].
**Figure 57: IκBSR expression in MCF7/LCC9 and MCF7/RR cells.**

Characterization of MCF7/LCC9 and MCF7/RR cells stably expressing IκBSR and their empty vector controls (EV). 40μg of whole cell lysates from IκBSR stable transfectants (pools) and empty vector controls were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with rabbit polyclonal IκBα antibody. β-actin, loading control. MCF7/RR/IκBSR pooled population (Pool 1 and Pool 2) over-express IκBα/IκBSR as shown in a representative immunoblot.
4.1.2. Stable transfection of a pCMV4-FLAG-tagged dominant-negative NFκB inhibitor; (IκB super-repressor; IκBSR) into MCF7/LCC9 cells:

Since MCF7/LCC9 cells exhibited the strongest interaction between parthenolide and antiestrogens (4HT, ICI 182,780) \textit{in vitro} as seen with our parthenolide studies, we wanted to obtain independent confirmation of the role of parthenolide in NFκB inhibition in these cells. MCF7/LCC9/pcDNA3-IκBSR pooled populations (Pool 1, Pool 2, Pool 3) did not show a significant over-expression of IκBα as shown in Figure 57, and so we then chose a FLAG-tagged constitutively active mutant IκB (pCMV4-FLAG-tagged IκB super-repressor; IκBSR) to stably introduce into MCF7/LCC9 cells as shown below. We selected the clonal as well as pooled populations.

MCF7/LCC9 cells were again stably transfected with either an empty pCMV4 plasmid or S32/36A mutant pCMV4-FLAG IκBα, also referred to as IκBSR (kindly provided by Dr. Marty Mayo, University of Virginia, Charlottesville, VA, USA). This IκBSR cannot be phosphorylated and targeted for degradation and so acts as a dominant-negative NFκB inhibitor (285). Stably transfected cells were selected for growth in the presence of 1 µg/ml puromycin. After selection against puromycin, twelve single clones and three pooled populations were selected from the IκBSR-transfected MCF7/LCC9 population and expanded thereafter. Cells transfected with the empty control vector were designated LCC9/EV and those transfected with the S32/36A mutant pCMV4-FLAG IκBα were designated LCC9/IκBSR.

Expression of FLAG and over-expression of mutant IκBα (IκBSR) protein was confirmed by western blot analysis, for each colony and the pooled population selected using antibodies to FLAG and IκBα, respectively [Figure 58].
MCF7/LCC9 cells

\[ \text{pCMV4-FLAG-I} \kappa \text{BSR} \quad + \quad \text{puromycin resistance cassette} \]

Selected by adding puromycin, grown for about 2 weeks

Only the puromycin resistant colonies grow

- Isolated 12 clones and expanded the colonies
- 3 Pooled populations

Screened the pooled and clonal populations for the I\(\kappa\)BSR gene expression
Figures 58 and 59 show the characterization of these LCC9/IκBSR cells and their empty vector controls (EV). While the three different LCC9/IκBSR pooled populations (Pool 1, Pool 2, Pool 3) expressed FLAG, the over-expression of IκBSR was modest [Figure 58C]. We utilized LCC9/IκBSR/Pool 1 and LCC9/EV/Pool 1 for growth curves to confirm the effects seen with parthenolide.

The LCC9/IκBSR clonal populations, when screened, showed a greater level of over-expression of mutant IκBα [Figure 58B; Clone 5 and Clone 7] and also expressed FLAG [Figure 58A; Clone 5 and Clone 7]. We utilized LCC9/IκBSR/Clone 5 and LCC9/EV/Clone 11 cells for growth curves to confirm the effects seen with parthenolide. LCC9/IκBSR/Clone 5 cells express FLAG and over-express IκBα/ IκBSR (~2 fold), whereas LCC9/EV/Clone 11 control cells do not express FLAG [Figure 59].

4.1.3. Additional approaches for inhibiting NFκB:

siRNA knockdown of p65/RELA NFκB in MCF7/LCC9 cells:

In addition to the dominant negative, an antisense approach to inhibiting NFκB expression was also considered. While the stable transfections overcome some of the problems associated with transient transfections, for example variation in transfection efficiencies, controlling the levels of the desired protein expression, these too can be problematic. Apart from the labor intensive screening of clones required to achieve desirable expression levels, the cells can also downregulate or expel plasmids over time. For the purpose of this project, MCF7/LCC9 cells were also transiently transfected with siRNA oligonucleotides directed against p65/RELA NFκB (si-p65) or a nonsilencing control (si-Ctrl) and the expression was measured after 24-96 hrs. A two fold decrease in p65/RELA expression is attained by siRNA at 24 hrs, however almost complete p65/RELA knockdown can be achieved at 96hrs as shown in [Figure 60].
Characterization of MCF7/LCC9 cells stably expressing IkBSR and their empty vector controls (EV). 40µg of whole cell lysates from IkBSR stable transfectants (pools and clones) and empty vector controls were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with mouse monoclonal FLAG-M5 and rabbit polyclonal IkBα antibodies respectively. β-actin, loading control. LCC9/IkBα Clone 5 and Clone 7 cells over-express IkBα/IkBαR and express FLAG as shown in a representative immunoblot.

**Figure 58: Mutant IkBα (IkBSR) and FLAG expression in resistant MCF7/LCC9 cells.**

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**Figure 59: LCC9/IκBSR/Clone 5 cells express FLAG and over-express IκBSR in resistant MCF7/LCC9 cells.**

Characterization of MCF7/LCC9 cells stably expressing IκBSR and their empty vector controls (EV). 40μg of whole cell lysates from IκBSR stable transfectants (Clone 5) and EV controls were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with mouse monoclonal FLAG-M5 and rabbit polyclonal IκBα antibodies respectively. β-actin, loading control. Representative immunoblots for mutant IκBα/IκBSR and FLAG are shown. LCC9/IκBSR Clone 5 and LCC9/EV/Clone 11 cells were studied to confirm the effects of NFκB inhibition.
Figure 60: siRNA to p65/RelA NFκB inhibits BCL2 expression in MCF7/LCC9 cells.

MCF7/LCC9 cells were seeded in 12-well dishes and were transfected with control (si-ctrl) or p65- specific (si-p65/RELA) oligonucleotides for either 24 or 96 hrs before lysis. Lysates were then separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for p65/RELA and BCL2 as shown. GAPDH, loading control. Representative immunoblots for p65/RELA and BCL2 are shown. BCL2 protein expression is inhibited in resistant MCF7/LCC9 cells by p65/RELA NFκB knockdown at 96 hrs.
4.2. pCMV4-FLAG-tagged IκBSR mimics the effects seen with parthenolide:

Figure 59 shows that LCC9/IκBSR Clone 5 cells express FLAG and over-express IκBα/IκBSR (~2 fold), whereas LCC9/EV Clone 11 control cells do not express FLAG. To determine whether IκBSR over-expression mimics the effects of NFκB inhibition, the ability of IκBSR to restore antiestrogen-induced inhibition of cell proliferation was first measured in these clonal population [Figure 61]. Control (LCC9/EV/Clone 11) and IκBSR (LCC9/IκBSR/Clone 5) transfected clones were seeded in 24-well plates one day before treatment with ethanol vehicle, 4HT or ICI. As expected, control cells (LCC9/EV Clone 11) remain resistant to 4HT [Figure 61A] and ICI 182,780 [Figure 61B]. In marked contrast, IκBSR expression restores TAM sensitivity [Figure 61A; p<0.001] and ICI 182,780 sensitivity [Figure 61B; p=0.029] in the resistant MCF7/LCC9 cells confirming the effects seen with parthenolide. These data show that the effects of parthenolide are primarily driven by its inhibition of NFκB.

To see if IκBSR over-expression in pooled clones (LCC9/IκBSR/Pool 1) will also reverse resistance to antiestrogens, control (LCC9/EV/Pool 1) and IκBSR (LCC9/IκBSR/Pool 1) transfected pooled clones were also seeded in 24-well plates one day before treatment with ethanol vehicle, 4HT or ICI. Consistent with our studies in clonal population as stated above, control cells of the pooled population are not responsive to either antiestrogen [Figure 62]. However, LCC9/IκBSR transfected pools are significantly growth inhibited by TAM [Figure 62; p=0.002], but over multiple experiments performed we see that the inhibition of cell proliferation achieved by TAM is greater in clonal population (LCC9/IκBSR/Clone 5 cells) compared with pooled population. (LCC9/IκBSR/Pool 1 cells). In marked contrast, there is no significant inhibition of cell proliferation seen when LCC9/IκBSR/Pool 1 cells were treated with ICI
182,780 [Figure 62]. These observations to some extent were expected, as the LCC9/IκBSR pooled populations did not show a high level of over-expression of IκBSR during the screening process [Figure 58C]. Regardless, these data further imply that inhibition of endogenous NFκB by both molecular (IκBSR) and pharmacological (parthenolide) approaches effectively reverse the antiestrogen resistant phenotype.

Since the expression of FLAG, IκBSR over-expression and the effects of IκBSR over-expression on responsiveness of MCF7/LCC9 cells to inhibition by antiestrogens TAM and ICI 182,780 were more pronounced in clonal population (LCC9/IκBSR Clone 5) compared with the pooled population [Figures 58, 61 and 62], the LCC9/IκBSR/clone 5 cells were utilized for all subsequent experiments to confirm the effects seen with parthenolide.

4.3. **IκBSR expression inhibits NFκB dependent transcription:**

We also measured NFκB driven luciferase activity in cells transiently transfected with IκBSR. MCF7/LCC1 and MCF7/LCC9 cells were co-transfected with a NFκB-luciferase reporter vector and a phRL-SV40-*Renilla* control vector with or without IκBSR for 24 hrs before lysis and luminescent detection using a dual-luciferase promoter-reporter assay. The dominant negative NFκB inhibitor (IκBSR) almost completely inhibits NFκB-dependent transcription in both the resistant MCF7/LCC9 and the sensitive MCF7/LCC1 cells [Figure 63; *P*=0.001 relative to LCC1/EV; *P*=0.001 relative to LCC9/EV].
Figure 61: IκBSR expression in clone 5 cells sensitize the resistant MCF7/LCC9 cells to antiestrogens both 4HT and ICI 182,780.

IκBSR expression restore 4HT (A) and ICI 182,780 (B)-induced inhibition of cell proliferation in MCF7/LCC9 cells. LCC9/IκBSR/clone 5 and LCC9/EV control cells were seeded in quadruplicate and treated with either ethanol vehicle, 4HT (1 M), or ICI 182,780 (100nM) as indicated for 7 days before counting. Data represent mean ± SE of relative proliferation of four determinations. Student’s t test; P<0.001 for IκBSR versus EV controls in presence of 4HT and P=0.029 for IκBSR versus EV controls in presence of ICI.
Figure 62: IκBSR expression in pooled population sensitizes the resistant MCF7/LCC9 cells to antiestrogen 4HT only.

IκBSR expression in LCC9/IκBSR pooled population restores 4HT-induced inhibition of cell proliferation in MCF7/LCC9 cells. LCC9/IκBSR and LCC9/EV pooled clone cells were seeded in quadruplicate and treated with either ethanol vehicle, 4HT (1 M), or ICI 182,780 (100nM) as indicated for 7 days before counting. Data represent mean ± SE of relative proliferation of four determinations. $P=0.003$ for all treatment groups by one-way ANOVA, and $P=0.002$ for IκBSR stable transfectants (Pooled population) versus EV controls in presence of 4HT.
Figure 63: IκBSR expression inhibits NFκB dependent transcription in MCF7/LCC1 and MCF7/LCC9 cells.

IκBSR significantly inhibits NFκB-dependent transcription in both MCF7/LCC9 and MCF7/LCC1 cells. Cells were transiently transfected in quadruplicate with pNFκB-Luc and pRL-SV40 Renilla constructs with or without IκBSR for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations. Student’s t test; *, P=0.001 for MCF7/LCC1/IκBSR; †, P=0.001 for MCF7/LCC9/IκBSR compared to their respective empty vector controls.
4.4. IκBSR inhibits BCL2 expression in MCF7/LCC9 cells stably expressing IκBSR:

The data in Chapter 3 demonstrate that parthenolide affects antiestrogen sensitivity by signaling to the mitochondria through BCL2. To show that parthenolide acts primarily through its inhibition of NFκB, we used the dominant-negative NFκB inhibitor (IκBSR) and measured its effect on BCL2 protein expression. Whole cell lysates were collected from LCC9/EV and LCC9/IκBSR cells, subjected to SDS-PAGE, and immunoblotted to measure the protein expression of BCL2. Similar to the effects of parthenolide seen in MCF7/LCC9 cells in [Chapter 3; Figure 38; A and B], BCL2 protein expression is significantly inhibited in LCC9/IκBSR cells [Figure 64A; ~65% inhibition; *P*=0.029]. It is important to note that similar decreases in BCL2 protein expression are observed in MCF7/LCC9 cells when p65/RELA NFκB expression is knockdown by siRNA [Figure 60; 96hrs]. These data suggest that NFκB plays a key functional role in the regulation of anti-apoptotic protein BCL2 in the TAM resistant phenotype.

To test whether BCL2 protein expression is affected by treatment with the antiestrogens 4HT and ICI 182,780 in IκBSR over-expressing cells, cell lysates from LCC9/IκBSR and control (LCC9/EV) cells treated with either ethanol vehicle, 4HT (1 M) or ICI 182,780 (100nM) for 72 hrs were collected and subjected to SDS-PAGE, and immunoblotted for BCL2. The basal BCL2 protein expression is significantly inhibited in LCC9/IκBSR cells compared to controls [Figure 64A; ~65% inhibition; *P*=0.029 and Figure 64B]. These data confirm the effects seen with parthenolide and in turn suggest that the effects of parthenolide as seen in Figures 38 and 40 (Chapter 3) are primarily driven by its inhibition of NFκB.
While the basal BCL2 protein expression is significantly inhibited in LCC9/ικBSR cells compared to controls [Figure 64A; ~65% inhibition; \( P=0.029 \) and Figure 64B], 4HT and ICI 182,780 have no additional effect on BCL2 protein expression [Figure 64B]. Approximately 50% inhibition of BCL2 protein expression is achieved by parthenolide alone in resistant MCF7/LCC9 cells [Figures 38 and 40]. Importantly, the level of inhibition of BCL2 protein expression in the presence of parthenolide and 4HT/ICI 182,780 [Figures 38 and 40] was essentially similar to that achieved by ικBSR alone in resistant MCF/LCC9 cells [Figure 64A; ~65% inhibition; \( P=0.029 \) and Figure 64B]. The strong inhibition of BCL2 protein expression in resistant MCF7/LCC9 cells by ικBSR alone is sufficient to restore sensitivity to antiestrogens 4HT/ICI 182,780 and any additional effect on BCL2 protein expression by either 4HT or ICI 182,780 may not be necessary to further affect antiestrogen sensitivity in resistant MCF7/LCC9 cells.

4.5. ικBSR increases the overall BAX:BCL2 ratio in MCF7/LCC9 cells stably expressing ικBSR:

There is a marked 5-fold increase in the overall BAX:BCL2 ratio when MCF7/LCC9 cells are treated with a combination of parthenolide and 4HT [Chapter 3; Figure 42B]. Thus, we determined whether BAX protein expression is altered by ικBSR over-expression and whether 4HT treatment affects BAX expression in these LCC9/ικBSR cells that are stably over-expressing ικBSR. For this, LCC9/ικBSR and control (LCC9/EV) cells were treated with either ethanol vehicle, or 4HT (1 µM) for 72 hrs before lysis. Whole cell lysates were then collected from these treated cells, subjected to SDS-PAGE, and immunoblotted to measure the protein expression of BAX.

Similar to our parthenolide studies, while there is again no significant effect on BAX
protein expression in LCC9/IκBSR cells with or without treatment with antiestrogen 4HT compared to their respective controls [Figure 65A], it is important to note that the overall BAX:BCL2 ratio is increased by 2-fold [Figure 65B] primarily because of the decrease in BCL2 expression seen in Figure 64. These data strongly suggest that NFκB inhibition decreases the expression of BCL2 such that a marked increase in the overall BAX:BCL2 ratio occurs (105,313) shifting the balance towards cell death and this perhaps explains the restoration of sensitivity to 4HT in resistant cells.

Since parthenolide in combination with antiestrogen 4HT decreases TNFR1 and PARP protein expression in resistant MCF7/LCC9 cells [Figures 32 and 37; Chapter 3], we also tested if expression of these proteins is affected by antiestrogens 4HT and ICI 182,780 in IκBSR over-expressing cells. The cell lysates from LCC9/IκBSR and control (LCC9/EV) cells treated with either ethanol vehicle, 4HT (1 M) or ICI 182,780 (100nM) for 72 hrs were subjected to Western blot analysis. While LCC9/IκBSR cells express less PARP compared to controls [Figure 66A], 4HT and ICI 182,780 have no additional effect on PARP protein expression [Figure 66A]. In addition, we found no significant effect on TNFR1 expression in LCC9/IκBSR cells with or without treatment with antiestrogen 4HT compared to their respective controls [Figure 66B].
A.

Figure 64: Stable expression of IκBSR inhibits BCL2 protein expression in MCF7/LCC9 cells.

LCC9/IκBSR cells (cells stably expressing IκBSR) and empty vector (EV) controls cells were cultured in the presence of ethanol vehicle, 4HT (1 µM) or ICI 182,780 (100nM) for 72 hrs prior to lysis and SDS-PAGE analysis for BCL2. β-actin, loading control. A). Data from three independent experiments are presented as the mean ± SE of the relative BCL2: β-actin ratio (normalized to EV controls) (Student’s t test; P=0.029). B) Representative immunoblot is shown.
A.

Figure 65: IκBSR has no effect on BAX protein expression, but increases the BAX:BCL2 ratio in MCF7/LCC9 cells.

A) Representative immunoblot of the regulation of BAX expression by IκBSR and TAM in MCF7/LCC9 cells. LCC9/IκBSR cells (cells stably expressing IκBSR) and empty vector (EV) controls cells were cultured in the presence of ethanol vehicle, or 4HT (1 µM) for 72 hrs prior to lysis and SDS-PAGE analysis for BAX and β-actin. B) Data from three independent experiments is normalized to their respective EV controls and used to present the relative BAX:BCL2 ratio.
Figure 66: Regulation of PARP and TNFR1 expression by IκBSR and antiestrogens in MCF7/LCC9 cells.

LCC9/IκBSR cells (cells stably expressing IκBSR) and empty vector (EV) controls cells were cultured in the presence of ethanol vehicle, 4HT (1 µM) or ICI 182,780 (100nM) for 72 hrs prior to lysis and SDS-PAGE analysis for PARP and TNFR1. β-actin, loading control. A). Representative immunoblot of the regulation of PARP expression by IκBSR and antiestrogens in MCF7/LCC9 cells. B) Representative immunoblot of the regulation of TNFR1 expression by IκBSR and antiestrogen 4HT in MCF7/LCC9 cells.
4.6. **IkBSR in presence of antiestrogens (4HT or ICI 182,780) does not inhibit ERE-dependent transcriptional activity in MCF7/LCC9 cells:**

A primary function of antiestrogens is to block the estrogen receptor (ER). While TAM competes with available estrogens for binding to ER, ICI 182,780 stimulates ER degradation, prevents receptor dimerization and therefore, inhibits estrogen-dependent transcription (320). Our previous studies have shown that the basal ERE-activity can be increased by estradiol treatment and almost completely abolished by ICI 182,780 treatment in the antiestrogen sensitive MCF7/LCC1 cells (297) [Figure 67]. In contrast, although the basal ERE-activity is higher in MCF7/LCC9 cells compared to the control MCF7/LCC1 cells, this is also increased by treatment with estradiol but ICI 182,780 treatment does not inhibit the ERE-dependent transcription in these MCF7/LCC9 cells (297) [Figure 67]. This result is to be expected as MCF7/LCC9 cells are resistant to the antiestrogen ICI 182,780. However, it was important to note that NFκB inhibition by parthenolide either alone or in combination with ICI 182,780 did not have any statistically significant effect on the ERE-dependent transcription in these MCF7/LCC9 cells [Figure 67].

Since parthenolide can also have some “off–target” effects, one obvious question to ask was whether expression of the mutant IkBα (IkBSR) can restore antiestrogen-mediated inhibition of ER-dependent transcriptional activity in resistant MCF7/LCC9 cells. MCF7/LCC1 and MCF7/LCC9 cells were transiently transfected with ERE-Luc and pRL-SV40 Renilla constructs with or without pCMV4-FLAG-IkBBSR prior to treatment with either ethanol vehicle, 4HT (1 M) or ICI 182,780 (100nM) for 24 hrs before lysis and luminescent detection using dual-luciferase promoter-reporter assays. Both MCF7/LCC1 and MCF7/LCC9 cells show an increase in the basal ERE-luciferase activity in the presence of IkBSR [Figures 68 and 69]. These data are consistent with our earlier observations that basal ERE-activity can be increased by NFκB inhibition using
parthenolide in both MCF/LCC1 and MCF7/LCC9 cells (297) [Figure 67]. Again as expected, 
ERE-luciferase activity is almost completely abolished by antiestrogens both 4HT [Figure 68] 
and ICI 182,780 [Figure 69] in MCF7/LCC1 cells, but these antiestrogens (4HT or ICI 182,780) 
do not inhibit ERE-activity in MCF7/LCC9 cells as these cells are resistant to both the 
antiestrogens [Figures 68 and 69]. Interestingly, dominant negative inhibitor of NFκB; the 
IκBSR in presence of either 4HT or ICI 182,780 has no statistically significant effect on the ERE-
dependent transcription in these MCF7/LCC9 cells [Figures 68 and 69], suggesting that NFκB 
inhibition may not involve the direct regulation of ER-dependent transcriptional events. It is 
possible that NFκB inhibition may be affecting antiestrogen (4HT/ICI 182,780) sensitivity 
through effects that are either downstream of the ER activity and/or through the effects that 
involvnonclassic ER pathways.
Figure 67: Parthenolide in combination with ICI 182,780 does not inhibit ER-dependent transcriptional activity in resistant MCF7/LCC9 cells.

Cells were transiently cotransfected in quadruplicate with ERE-Luc and pCMV-Renilla constructs prior to treatment with 10nM Estradiol (E₂), 100nM ICI 182,780, and 600nM parthenolide (parth) singly or in combination for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four independent experiments. P<0.001 for all treatment groups by one-way ANOVA.
Figure 68: Combined treatment with IκBSR and antiestrogen 4HT does not inhibit ERE-dependent transcription in resistant MCF7/LCC9 cells.

Cells were transiently transfected in quadruplicate with ERE-Luc and pRL-SV40 Renilla constructs with or without pCMV4-FLAG-IκBSR prior to treatment with either ethanol vehicle, or with 4HT (1 M) for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations. P=<0.001 for all treatment groups by one-way ANOVA.
Figure 69: Combined treatment with IkBSR and ICI 182,780 does not inhibit ERE-dependent transcription in resistant MCF7/LCC9 cells.

Cells were transiently transfected in quadruplicate with ERE-Luc and pRL-SV40 Renilla constructs with or without pCMV4-FLAG-IkBSR prior to treatment with either ethanol vehicle, or with ICI 182,780 (100nM) for 24 hrs before lysis and luminescent detection. Data represent mean ± SE of the relative luciferase: Renilla activity (presented as relative light units) for four determinations. P=<0.001 for all treatment groups by one-way ANOVA.
4.7. Caspase inhibitors reduce sensitivity to the antiproliferative effects of 4HT in MCF7/LCC9 cells stably expressing IκBSR:

We next wanted to establish whether the dominant-negative NFκB inhibitor (IκBSR) mimics the effects seen in [Chapter 3; Figures 48 and 49], where we show that a caspase-dependent cell death mechanism is active when cells are treated with 4HT and the NFκB inhibitor parthenolide, and that caspases upstream of the mitochondria are strongly implicated because the effects of treatment on MMP were fully reversed by the pancaspase inhibitor.

To measure the effects on cell proliferation, the LCC9/IκBSR cells (stably expressing IκBSR) were treated with ethanol vehicle or 4HT for 7 days in the absence or presence of either pancaspase inhibitor or specific CASP8 inhibitor (C8I; an irreversible inhibitor of CASP8; the Z-IETD-FMK sequence binds only to CASP8 and blocks substrate binding). In the absence of both the pancaspase and C8I caspase inhibitors, 4HT effectively inhibits LCC9/IκBSR cell growth by approximately 40% [Figure 70]. However, addition of the pancaspase inhibitor reverses the effects of NFκB inhibition [Figure 70; \( P=0.014 \)]. To further confirm the role of CASP8, a caspase upstream of the mitochondria, in restoring 4HT induced cell death in MCF7/LCC9 cells [Chapter 3; Figure 49], we measured the effects of a specific CASP8 inhibitor (C8I) on cell proliferation in response to 4HT in the LCC9/IκBSR cells. As shown in Figure 70, the CASP8 inhibitor data [Figure 70; \( P=0.036 \)] mimics the effects seen with the pancaspase inhibitor.
Figure 70: Pancaspase and CASP8 inhibitors reduce sensitivity to the antiproliferative effects of 4HT in LCC9/IκBSR cells.

LCC9/IκBSR/clone 5 cells were seeded in quadruplicate and treated with either ethanol vehicle, or with 4HT (1 M) for 7 days in the absence or presence of either a pancaspase inhibitor or a CASP8 inhibitor before counting. Data represent mean ± SE of relative proliferation of four determinations. Student’s $t$ test, $P=0.014$ for 4HT versus 4HT with pancaspase inhibitor, and $P=0.036$ for 4HT versus 4HT with CASP8 inhibitor.
4.8. Caspase inhibitors prevent mitochondrial membrane permeabilization in response to 4HT in MCF7/LCC9/\(\text{I}_{\kappa}\)BSR cells:

To determine how the two caspase inhibitors, pancaspase and C8I, might affect permeability of the mitochondrial membrane in LCC9/\(\text{I}_{\kappa}\)BSR cells, MMP was measured following treatment with ethanol vehicle or 4HT for about 20 hrs in the absence or presence of either pancaspase inhibitor or C8I. Figure 71 shows a significant increase in MMP (≥1.5-fold) in response to antiestrogen 4HT in LCC9/\(\text{I}_{\kappa}\)BSR cells in the absence of caspase inhibitors. In marked contrast, addition of either PI or C8I fully reverses the increase in MMP [Figure 71; \(P=0.024\) for PI; \(P=0.037\) for C8I], implying that CASP8, acting upstream of the mitochondria, is involved in restoring 4HT induced caspase-dependent cell death in TAM resistant breast cancer cells.

These data confirm that NF\(\kappa\)B inhibition by both pharmacological (parthenolide) and molecular (\(\text{I}_{\kappa}\)BSR) approaches effectively restore 4HT induced cell death and sensitivity to 4HT in resistant cells, likely acting through a CASP8-dependent intrinsic apoptotic pathway.
Figure 71: Pancaspase and CASP8 inhibitors prevent MMP in response to 4HT in LCC9/ΙκBSR cells.

LCC9/ΙκBSR(Clone 5 cells) were treated with either ethanol vehicle, or with 4HT (1 M) for 18-20 hrs in the absence or presence of either a pancaspase inhibitor or a CASP8 inhibitor before measuring MMP. Data represent mean ± SE of relative MMP normalized to ethanol-treated cells for at least three independent experiments. Student’s t test; *, P=0.024 for 4HT versus 4HT with pancaspase inhibitor, and ^, P=0.037 for 4HT versus 4HT with CASP8 inhibitor.
4.9. Conclusions:

It is clear from the studies presented here with a dominant negative NFκB inhibitor (the IκBSR), that parthenolide acts primarily through its inhibition of NFκB. The data strongly suggests that NFκB inhibition by both pharmacological (parthenolide) and molecular (IκBSR) approaches have the ability to effectively restore 4HT induced cell death and sensitivity to 4HT in resistant cells, likely acting through a CASP8-dependent intrinsic apoptotic pathway. Inhibition of the expression of the key-anti-apoptotic protein BCL2, alters the ratio of BCL2:BAX expression in favor of an increased destabilization of the mitochondrial membrane and leads to an increase in mitochondrial membrane permeability. These events are caspase dependent and involve the regulation of caspase-8 (CASP8) activity upstream of mitochondria. A role for executioner caspases downstream of mitochondria also is likely. Together, our data strongly suggest that NFκB plays a critical role in the development of the antiestrogen resistant phenotype, and that NFκB inhibition provides a means to overcome resistance to both SERMs and SERDs. These findings provide strong support for designing clinical trials to combine parthenolide and antiestrogens in ER- positive breast cancer patients.
Chapter 5: NFκB activation in resistant cells is necessary but not sufficient to drive their antiestrogen resistant phenotype

5.1. Functional ability of NFκB inhibition to sensitize breast cancer cells to antiestrogens (4HT and ICI 182,780):

The studies presented here in this Chapter further help in understanding the functional role of increased NFκB activity in antiestrogen responsiveness. It is not known if the NFκB activation in resistant cells is necessary and/or sufficient for their antiestrogen resistant phenotype. We studied the role of NFκB by inhibiting its activity in resistant cells using both molecular (IkBαSR) and pharmacological (parthenolide) approaches (Aim 1; Chapters 3 and 4) and by over-expressing its activity in antiestrogen sensitive cells (Aim 2; Chapter 5). These studies provide a direct assessment of NFκB’s relevance in breast cancer and antiestrogen resistance.

5.2. BCL2, IKKγ/NEMO, and IkBα are up-regulated in MCF7/p65 cells:

To address Aim 2 as highlighted under Hypothesis and Aims in Chapter 1 of this dissertation, the antiestrogen responsive MCF-7 cells were stably transfected with p65/RELA component of NFκB as a part of a collaboration with Dr. Nakshatri and were named MCF7/p65 cells (the cells over-expressing NFκB).

Over-expression of the p65/RELA component of NFκB was confirmed by western blot analysis, using antibodies to p65/RELA [Figure 72A]. However, the expression of the p50 (NFκB1) and p52 (NFκB2) subunits was not different between MCF-7 and MCF/65 cells [Figure 73], confirming that the MCF7/p65 cells over-express only the p65/RELA subunit of NFκB.
Our studies in Chapters 3 and 4 show that the anti-apoptotic protein BCL2 is also differentially expressed in resistant MCF7/LCC9 cells that show an up-regulated NFκB as compared to the sensitive MCF/LCC1 cells. Furthermore, we have shown that NFκB acts by inducing signaling through BCL2. For example, inhibiting NFκB activity increased the ability of 4HT (4-hydroxytamoxifen; acting through NFκB) to inhibit BCL2 and increased apoptotic cell death in MCF7/LCC9 cells. Therefore, to determine whether MCF7/p65 cells exhibit changes in BCL2 protein expression, whole cell lysates were prepared from MCF-7 and MCF7/p65 cells and subjected to SDS-PAGE and immunoblot analysis. Similar to our results in Chapter 3, a significant increase in BCL2 protein expression is apparent in MCF7/p65 cells [Figure 72A] further establishing that over-expression of NFκB has consequent effects on BCL2.

The inhibitors of NFκB, termed IxBs, play a major role in modulating the transcriptional activity of NFκB (discussed in detail in Chapter 1), whose activity in turn is negatively regulated by the IKK complex (IκB kinase complex; composed of IKKa, IKKβ, and the regulatory IKKγ/NEMO. To determine whether MCF7/p65 cells show an altered expression of these regulatory molecules at the protein level, lysates from MCF-7 and MCF7/p65 cells were immunoblotted for IKKγ/NEMO and IxBα. An increase in the protein expression of both IKKγ/NEMO and IxBα was observed in MCF7/p65 cells [Figure 72B]. Although the up-regulated IKKγ/NEMO seen in cells over-expressing NFκB agrees with our previously published studies (297), the precise role of these regulatory molecules in breast cancer in the context of antiestrogen resistance requires further investigation.
Figure 72: Basal expression of p65/RELA, BCL2, NEMO and IκBα is up-regulated in MCF7/p65 cells.

A) and B). Representative immunoblots for p65/RELA, BCL2, NEMO and IκBα in MCF7/p65 and MCF-7 cells. 20-40μg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted. β-actin, loading control.
Figure 73: Basal protein expression of p50 (NFκB1) and p52 (NFκB2) subunits of NFκB is not different between MCF-7 and MCF7/p65 cells.

Representative immunoblots for p50 (NFκB1) and p52 (NFκB2) subunits of NFκB in MCF7/p65 and MCF-7 cells. 20–40µg of whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted. β-actin, loading control.
5.3. **Over-expression of NFκB p65/RELA alone does not confer resistance to antiestrogens (4HT and ICI 182,780) in MCF-7 cells:**

Next we used these MCF7/p65 transfectants to assess the ability of NFκB over-expression to affect the responsiveness to the two antiestrogens (4HT and ICI 182,780). Since the studies presented in Chapters 3 and 4 of this dissertation show that a reduced activation of NFκB, either by a molecular approach (IkBSR) or by a small molecule antagonist (parthenolide), increases sensitivity and reverses the antiestrogen resistant phenotype, we expected the over-expression of NFκB in antiestrogen responsive cells (MCF-7; MCF7/LCC1) to confer antiestrogen resistance. We further expected this result, since our studies in Chapter 3 implicate only p65/RELA subunit of NFκB to be up-regulated, the other NFκB components such as p50 (NFκB1) and p52 (NFκB2) were expressed but were not found to be up-regulated in resistant cells (MCF7/LCC9; MCF7/RR). Furthermore, studies have shown that introducing either p65/RELA or p50 (NFκB1) may be sufficient because either gene alone can be active (321).

To measure the responsiveness of MCF7/p65 transfectants to antiestrogens (ICI 182,780 and 4HT) relative to parental MCF-7 cells, both MCF-7 and MCF7/p65 cells were treated with increasing concentrations of either 4HT (0-1 M) or ICI 182,780 (0-1 M). While the antiestrogens inhibited the growth of MCF-7 cells as expected, we found that MCF7/p65 cells are also growth inhibited by both 4HT and ICI 182,780 [**Figures 74 and 75**]. These data imply that over-expression of p65/RELA subunit of NFκB alone is not sufficient to confer resistance to antiestrogens (either 4HT or ICI 182,780) when compared to our data from MCF7/LCC1 and MCF7/LCC9 cells (Chapter 3). Furthermore, these data further suggest that the p65/RELA NFκB is necessary but not sufficient for their antiestrogen resistant phenotype.
Figure 74: Antiestrogen 4HT produces a dose dependent inhibition of growth in MCF7/p65 cells.

Cells were seeded in quintuplicate in 96-well tissue culture dishes 1 day before treatment with the indicated concentrations of 4HT (0-1 M) for a total of 5 days. BrdUrd was added for the last 18 hrs of culture. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of five determinations from a single representative experiment. The experiment was independently done at least thrice. P=0.001 for all treatment groups by one-way ANOVA for MCF-7 cells and P≤0.001 for all treatment groups by one-way ANOVA for MCF7/p65 cells.
Figure 75: Antiestrogen ICI 182,780 produces a dose dependent inhibition of growth in MCF7/p65 cells.

Cells were seeded in quintuplicate in 96-well tissue culture dishes 1 day before treatment with the indicated concentrations of ICI 182,780 (0-1 M) for a total of 5 days. BrdUrd was added for the last 18 hrs of culture. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of five determinations from a single representative experiment. The experiment was independently done at least thrice. P≤0.001 for all treatment groups by one-way ANOVA for MCF-7 cells and P≤0.001 for all treatment groups by one-way ANOVA for MCF7/p65 cells.
5.4. **MCF7/p65 cells are dependent on E2 (estrogen) for cell growth:**

To determine whether p65/RELA over-expression modifies the dependence of MCF-7 breast cancer cells on E2 (Estrogen) for proliferation, the MCF7/p65 transfectants were cultured for 72 hrs in media deprived of estrogens (CCS-IMEM) before being seeded in quadruplicate and grown in either CCS-IMEM (E2 deprived) or in the presence of E2 (10nM) as shown [Figure 76] for a total of 7 days. Proliferation was measured by counting the cells on days 1, 4 and 7. In the absence of E2, the cell number at day 7 is similar to that on day 1 for these MCF7/p65 transfectants, indicating their E2 dependence for proliferation. However, MCF7/p65 cells proliferate much more rapidly in the presence of E2 [Figure 76], which is evident by a 14-fold difference in cell count as seen at day 7 compared to day 1 [Figure 76]. These data suggest that over-expression of p65/RELA alone in MCF-7 cells does not confer an E2-independent phenotype in MCF-7 breast cancer cells. However, it may be likely that double transfections [with p65/RELA and p50 (NFκB1)] together may confer an E2-independent phenotype in these transfectants, which remains to be determined.

The data in Figures 74-76 imply that over-expression of p65/RELA subunit of NFκB alone is not sufficient to confer both estrogen independence and resistance to antiestrogens (either 4HT or ICI 182,780). These data clearly implicate other factors in addition to NFκB to be involved in both acquired antiestrogen resistance and E2-independence. For example, in addition to up-regulated NFκB p65/RELA, our published data implicates loss of a putative tumor suppressor interferon regulatory factor-1 (IRF; a protein partner of NFκB involved in the regulation of iNOS) and over-expression of spliced XBP1 (human X-box binding protein-1) in acquired antiestrogen resistance (184). XBP1 is shown to confer E2-independence in addition to
antiestrogen resistance in breast cancer cells (322). Both IRF1 and XBP1 are known to be involved in NFκB signaling. NFκB expression shows a negative association with IRF1 and a positive association with XBP1 (184). NFκB can form functional heterodimers with IRF1 and can directly regulate gene transcription (185,323).

XBP1 is also predicted to be induced by NFκB (324). Importantly, our published studies implicate XBP1 activity to be sufficient to confer both E2-independence and antiestrogen resistance in breast cancer cells (322). Since antiestrogen resistant MCF7/LCC9 cells clearly show an up-regulated NFκB p65/RELA; XBP1 and a down-regulated IRF1 activity (184), it may be likely that concurrent effects of over-expression of NFκB, XBP1, and loss of IRF1 may be required to confer E2-independence and antiestrogen resistance in breast cancer cells. A further delineation of the factors conferring E2-independence and antiestrogen resistance in breast cancer cells remains critical to a better understanding of acquired E2-independence and antiestrogen resistance in breast cancer cells.
Figure 76: MCF7/p65 cells are dependent on estrogen for growth.

Cells were deprived of E2 (estrogen) for 72 hrs before being seeded in quadruplicate and grown in either CCS-IMEM (E2 deprived) or in the presence of E2 (10nM) as shown for a total of 7 days. Data represent mean ± SE for relative cell proliferation for three independent experiments. A single representative experiment is shown.
5.4. Generation of TAM-resistant (MCF7/p65/TAM) and ICI 182,780-resistant (MCF7/p65/ICI) variants:

Because MCF7/p65 cells were responsive to antiestrogens both 4HT and ICI 182,780, we sought to develop TAM-resistant and ICI 182,780-resistant models using the MCF7/p65 cell line. For this, first MCF7/p65 cells were selected against increasing concentrations of ICI 182,780 in vitro, and the cell population proliferating in 100nM ICI 182,780 was designated MCF7/p65/ICI [Figure 77]. Then, to measure the responsiveness of MCF7/p65/ICI cells to antiestrogens (ICI 182,780 and 4HT), cells were treated with increasing concentrations of either ICI 182,780 (0-1 µM) or 4HT (0-1 M). While the cells selected against ICI 182,780 produced stable, ICI resistant cells (MCF7/p65/ICI) [Figure 78], we found that MCF7/p65/ICI cells also exhibited cross-resistance to the antiestrogen 4HT [Figure 79].

Importantly, MCF7/p65 cells were also selected against increasing concentrations of 4HT in vitro, and the cell population proliferating in 1µM 4HT was designated MCF7/p65/TAM [Figure 77]. When the responsiveness of MCF7/p65/TAM cells to antiestrogens (ICI 182,780 and 4HT) was measured by treating MCF7/p65/TAM cells with increasing concentrations of either ICI 182,780 (0-1 M) or 4HT (0-1 M), we found that as expected, the MCF7/p65/TAM cells are no longer responsive to the antiproliferative effects of 4HT [Figure 80]. However MCF7/p65/TAM cells are still responsive to the antiproliferative effects of antiestrogen ICI 182,780 and do not exhibit cross-resistance to ICI 182,780 [Figure 81].

Since the MCF7/p65/ICI cells are phenotypically similar to MCF7/LCC9 cells and the MCF7/p65/TAM cells are phenotypically similar to MCF7/LCC2 cells (refer to text 1.10 of chapter 1 for details), it appears that cells when selected against increasing concentrations of ICI
182,780, tend to develop a 4HT cross-resistant phenotype, however when cells are selected against increasing concentrations of 4HT they still tend to be responsive to ICI 182,780.

We tried growing the MCF7/p65 cells in the media deprived of E2 (CCS-IMEM) for weeks, however this approach was unsuccessful [Figure 77].

Figure 77: MCF7/p65 cells made resistant to 4HT (MCF7/p65/TAM) and to ICI 182,780 (MCF7/p65/ICI).

MCF7/p65 cells were selected against increasing concentrations of ICI 182,780 (ICI) or 4HT. Cell population proliferating in 100nM ICI 182,780 was designated MCF/p65/ICI and the one proliferating in 1µM 4HT was designated MCF/p65/TAM as shown above. MCF7/p65 cells could not be made estrogen independent for growth.
Figure 78: MCF7/p65/ICI cells are resistant to ICI 182,780.

Cells were seeded in quintuplicate in 96-well tissue culture dishes 1 day before treatment with the indicated concentrations of ICI 182,780 (0-1 M) for a total of 5 days. BrdUrd was added for the last 18 hrs of culture. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of five determinations from a single representative experiment. The experiment was independently done at least thrice. P≤0.001 for all treatment groups by one-way ANOVA for MCF7/p65 cells and P≤0.001 for all treatment groups by one-way ANOVA for MCF7/p65/ICI cells.
Figure 79: MCF7/p65/ICI cells are cross-resistant to 4HT.

Cells were seeded in quintuplicate in 96-well tissue culture dishes 1 day before treatment with the indicated concentrations of 4HT (0-1 µM) for a total of 5 days. BrdUrd was added for the last 18 hrs of culture. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of five determinations from a single representative experiment. The experiment was independently done at least thrice. P≤0.001 for all treatment groups by one-way ANOVA for MCF7/p65 cells and P=0.014 for all treatment groups by one-way ANOVA for MCF7/p65/ICI cells.
Figure 80: MCF7/p65/TAM cells are resistant to 4HT.

Cells were seeded in quintuplicate in 96-well tissue culture dishes 1 day before treatment with the indicated concentrations of 4HT (0-1 M) for a total of 5 days. BrdUrd was added for the last 18 hrs of culture. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of five determinations from a single representative experiment. The experiment was independently done at least thrice. P\(\leq0.001\) for all treatment groups by one-way ANOVA for MCF7/p65 cells and P\(\leq0.001\) for all treatment groups by one-way ANOVA for MCF7/p65/TAM cells.
Figure 81: MCF7/p65/TAM cells retain sensitivity to ICI 182,780.

Cells were seeded in quintuplicate in 96-well tissue culture dishes 1 day before treatment with the indicated concentrations of ICI 182,780 (0-1 M) for a total of 5 days. BrdUrd was added for the last 18 hrs of culture. Data represent mean ± SE of relative proliferation (relative to ethanol treated control) of five determinations from a single representative experiment. The experiment was independently done at least thrice. P≤0.001 for all treatment groups by one-way ANOVA for MCF7/p65 cells and P≤0.001 for all treatment groups by one-way ANOVA for MCF7/p65/TAM cells.
5.5. **Conclusions:**

It is clear from the studies presented here with MCF7/p65 transfectants (the cells over-expressing NFκB p65/RELA only), that expression of an exogenous NFκB p65/RELA alone in antiestrogen sensitive MCF-7 breast cancer cells does not confer resistance to antiestrogens (4HT and ICI 182,780) in these cells. Thus, while over-expression of the p65/RELA subunit of NFκB is necessary, it is not sufficient for the development of an antiestrogen resistant phenotype. Moreover, the data presented here show that MCF7/p65 cells remain dependent on E2 (estrogen) for growth. Whether a double transfectant [with p65/RELA and p50 (NFκB1) in the same cell line] produces antiestrogen resistance in sensitive MCF-7 or MCF7/LCC1 cells remains to be determined. However, the novel cell lines made in the process of understanding the functional role of increased NFκB activity in antiestrogen responsiveness: the MCF7/p65/ICI (ICI resistant; TAM cross-resistant) and MCF7/p65/TAM cells (TAM resistant; ICI sensitive) can serve as unique cell models, whose further investigation can enhance our understanding of the functional role of increased NFκB activity in antiestrogen responsiveness.
Chapter 6: Discussion

6.1. Novel functions of NFκB in affecting phenotype of great clinical significance:

Estrogens have long been implicated in promoting breast cancer. Estrogen mediates its action primarily by binding to the estrogen receptor (ER) alpha and beta. The concentration of the most biologically active form of estrogen (17β estradiol) found in breast tumors is sufficient to occupy all the ERs in breast tumors and promote carcinogenesis (55). Endocrine manipulations, whether in the form of ovariectomy or ovarian ablation, antiestrogen or aromatase inhibitor therapy, are all effective and established approaches for the treatment of ER+ breast cancers. For the last several decades, antiestrogen therapy been used widely in the treatment of hormone-receptor positive (ER+) breast cancer. Antiestrogen therapy, whether in the form of SERD or SERM, primarily prevents the activation of ER by the endogenous estrogen and thus inhibits cell growth.

Currently, Tamoxifen (TAM), a prototype for selective estrogen receptor modulator (SERM) remains the drug of choice for premenopausal women and aromatase inhibitors are viable alternatives to antiestrogens for first line endocrine treatment in postmenopausal women with ER+ breast cancers. Approximately, 70% of all newly diagnosed breast cancers are ER+. TAM produces a significant survival benefit and is effective in approximately 50% of all ER+ tumors (62,97). However, almost 50% of ER+ breast cancers do not respond to TAM, and many of those that initially respond eventually become resistant. ICI 182,780 is effective as a second-line agent in women with TAM-resistant disease (78). Nonetheless, acquired endocrine resistance is a major limitation to the effectiveness of current antiestrogen/hormonal treatments.
The precise mechanism contributing to acquired antiestrogen resistance in ER+ breast cancers is not completely understood. Several putative mechanisms of antiestrogen resistance have been extensively studied (325,326) that may include heterogeneity of ER expression within tumors, ER mutation, mitogenic growth factor production, and loss of ER expression, (55,88,175). Although the lack of ER (estrogen receptor) and PR (progesterone receptor) expression is one of the major forms of de novo antiestrogen resistance known, it is important to note that most tumors that become antiestrogen resistant still express ER (176,178,327). Thus, ER expression is not a strong predictive marker in acquired antiestrogen resistance. More accurate predictive markers are clearly needed to identify patients who are likely to benefit from antiestrogen therapy, and to develop rationale approaches to personalize the combination therapies to reverse the resistant phenotype.

Since, the molecular signaling that drives resistance, and that when inhibited reinstates sensitivity, remains largely unknown, to develop new therapies that prevent and/or reverse resistance we must first understand how resistance arises and then identify molecular targets that, when modulated, restore sensitivity in resistant cells and/or increase sensitivity in sensitive cells.

The work presented in this dissertation has elucidated a functional role for nuclear transcription factor NFκB in antiestrogen (primarily TAM) resistance; how NFκB affects cell signaling to modify antiestrogen responsiveness; and how affecting NFκB action leads to cell death in both antiestrogen sensitive and resistant human breast cancer cells.

It is highly unlikely that a single gene/ signaling pathway confers antiestrogen resistance, rather we believe that antiestrogen resistant phenotype develops through changes in several
mechanisms that are part of a broad gene expression network which controls cell proliferation and apoptosis. We have implicated NFκB as a member of such a gene network that is closely associated with endocrine responsiveness in breast cancer (88,113,184). Importantly, we reported NFκB to be detectable in a high proportion of ER+ breast tumors (328). Our lab has previously reported that NFκB p65/RELA mRNA (2-fold) and the basal activity (~10-fold) are both higher in antiestrogen resistant MCF7/LCC9 (ICI 182,780 resistant; TAM cross-resistant) cells when compared to the antiestrogen sensitive MCF7/LCC1 (ICI 182,780 and TAM sensitive) cells (184). Increased activation of NFκB, and loss of its antiestrogenic regulation in resistant MCF7/LCC9 cells (184) suggests that resistant cells might be partly dependent on NFκB signaling for growth. Further implication of NFκB in antiestrogen resistance came from the observation that parthenolide, a small molecule inhibitor of NFκB (284) produces a dose dependent inhibition of antiestrogen resistant MCF7/LCC9 cells (p<0.01 at both 300 nM and 600 nM parthenolide). In marked contrast, parthenolide only had a minor affect on growth of antiestrogen sensitive MCF7/LCC1 cells suggesting that the antiestrogen resistant MCF7/LCC9 cells exhibit a greater reliance upon NFκB signaling for growth.

Furthermore, we have recently reported that the protein expression of NFκB p65/RELA is up-regulated in antiestrogen resistant MCF7/LCC9 cells which agrees with the up-regulated mRNA levels previously reported (184). Since we also reported the NFκB regulator IKKγ/NEMO to be up-regulated in antiestrogen resistant MCF7/LCC9 cells, it is likely that up-regulated IKKγ/NEMO enhances the kinase activity of the IKK complex and this may likely explain the increased expression of NFκB mRNA in MCF7/LCC9 cells (297). Since our studies implicated IKKγ/NEMO in increased basal activation of NFκB, we further examined whether
hormonal regulation of IKKγ/NEMO is altered in MCF7/LCC9 cells. The studies contained here in this dissertation clarified that protein levels of IKKγ/NEMO were unchanged in MCF7/LCC9 cells regardless of the treatment suggesting that up-regulated IKKγ/NEMO may not explain the synergistic interaction between antiestrogens (4HT or ICI 182,780) and parthenolide. We next asked if parthenolide can re-sensitize MCF7/LCC9 cells to ICI 182,780-mediated apoptosis. Pharmacological inhibition of NFκB by parthenolide restores sensitivity to the SERD ICI 182,780 (Faslodex; Fulvestrant) by synergistically enhancing apoptosis (297).

However, resistance to TAM and ICI 182,780 often occur independently (78,182) and a role for NFκB in affecting TAM responsiveness has not been previously studied. Thus, the studies contained here provide evidence for a potential role of NFκB in TAM resistance, and establish its mechanism of action. In addition, the current study also provides evidence that interfering with NFκB activity might provide a means to improve responses to both TAM and ICI 182,780 therapies. However, the mechanisms by which parthenolide reverses resistance to TAM and ICI 182,780 are potentially different.

In this study we first confirmed the observations from our previous studies (184,297) directly, using another cell culture model of acquired TAM resistance, the MCF7/RR cells (ICI 182,780 sensitive; TAM resistant) (188,329). Similar to resistant MCF7/LCC9 cells, these TAM resistant cells (MCF7/RR) do not exhibit any change in the expression of either the p50 or p52 subunits of NFκB but NFκB p65/RELA protein expression (8-fold) and the basal activity of the NFκB promoter (3-fold) are both significantly increased. Since increased NFκB activation is also associated with acquired estrogen independence (53,208), these data, when considered with the
published studies, strongly implicate NFκB as a central component in cellular signaling in an antiestrogen resistance phenotype.

To determine the functional relevance of these observations, we now show that NFκB inhibition is not restricted to ICI 182,780 (Faslodex; SERD) alone but can provide a means to overcome resistance to TAM (SERM) as well. Both molecular (mutant IκB; IκBSR) and pharmacological (parthenolide) approaches to inhibit NFκB are highly effective in downregulating NFκB’s activity and synergistically restoring sensitivity to 4HT as well as ICI 182,780. This reversal of resistance is seen in both MCF7/RR and MCF7/LCC9 cells. While parthenolide alone strongly inhibits the proliferation of resistant MCF7/LCC9 and MCF7/RR cells, the interaction between parthenolide and 4HT is strongly synergistic (RI=3.3 for MCF7/LCC9 cells; RI=1.8 for MCF7/RR cells). Importantly, While MCF7/RR cells are sensitive to ICI 182,780, the combined treatment with parthenolide and ICI 182,780 further robustly and synergistically increase their sensitivity to ICI 182,780, as is evident from their RI values (RI=2.4 for 1 M ICI 182,780). In addition, NFκB inhibition by parthenolide further sensitizes antiestrogen sensitive MCF-7 and MCF7/LCC1 cells to 4HT and ICI 182,780; the interaction between these two drugs is additive in antiestrogen sensitive cells. Parthenolide does not induce sensitivity to 4HT or ICI 182,780 in ER- cells (MDA-MB-231 cells), implying that the reversal of resistance in ER+ cells requires expression of functional ER protein and provides evidence for the use of this combination therapeutic approach only in patients that are ER+ for the disease.

This restoration of sensitivity to 4HT and ICI 182,780 by parthenolide directly supports our hypothesis that up-regulation of NFκB’s transcriptional activity contributes to the antiestrogen resistant phenotype. A reduced activation reverses the antiestrogen resistant
phenotype and increases sensitivity to both antiestrogens (4HT and ICI 182,780) in antiestrogen sensitive cells. Importantly, the combination treatment with parthenolide and antiestrogens (whether 4HT or ICI 182,780) to inhibit endogenous NFκB in resistant cells (MCF7/LCC9; MCF7/RR) may be very useful in clinical settings owing to their strong synergy/RI values. This approach may not be of lesser clinical relevance in cells that are already sensitive to antiestrogens (MCF-7; MCF7/LCC1) due to their additive interaction between parthenolide and 4HT/ICI 182,780.

The ability of reduced NFκB activity to affect proliferation endpoints in antiestrogen resistant cells could reflect the effects of 4HT on either cell cycle distribution and/or apoptosis (175,330). Combined treatment with parthenolide and 4HT does not affect the cell cycle profile of the resistant MCF7/LCC9 and MCF7/RR cells. We use 500nM concentration of parthenolide to achieve 50% growth inhibition of MCF7/LCC9 cells, a concentration that is several fold lower than that used in several published studies to inhibit NFκB (285,331,332). Thus, the role of parthenolide in regulating cell cycle progression at concentrations much higher than 500nM cannot be completely excluded. In our cell system, an effect of parthenolide and 4HT on cell survival adequately explains the effects seen in the cell proliferation assays. We show that NFκB inhibition by parthenolide significantly induces apoptosis in MCF7/LCC9 cells.

Samaddar et al. (303) have recently implicated autophagy in responsiveness to TAM. We measured LC3 (Light chain 3) cleavage (153) and p62/A170/SQSTM1 (p62; scaffold protein/adaptor protein) expression (304) as markers of autophagy. During autophagy, LC3-I is cleaved to LC3-II that subsequently associates with autophagic vesicles. We found no evidence of either LC3-II conversion or depleted levels of p62 in MCF7/LCC9 cells when treated with
parthenolide in the presence or absence of 4HT, suggesting that the ability of NFκB inhibition to restore 4HT-induced cell death most likely involves an apoptotic, rather than autophagic, cell death pathway.

The mitochondrial membrane permeability transition is a critical step in the induction of intrinsic apoptosis. Small pores form in the outer membrane of the mitochondria, a process driven in part either by dimerized BAX, or activated BID, BAK, or BAD proteins (pro-apoptotic members of the BCL2 family). These proteins cause the mitochondrial membrane potential across the membrane to collapse, leading in turn to the release of cytochrome c into the cytoplasm and an increase in effector caspase activities (333-335). Consistent with the observed effects on apoptosis, NFκB inhibition by both IκBSR and parthenolide markedly increase 4HT induced permeability of the mitochondrial membrane in MCF7/LCC9 cells. Expression of the anti-apoptotic protein BCL2 is also up-regulated in MCF7/LCC9 cells compared with their sensitive MCF7/LCC1 controls {{Crawford et al., manuscript submitted}}. 4HT treatment alone strongly inhibits BCL2 protein expression in MCF7/LCC1 cells by 60%, but not in the resistant MCF7/LCC9 cells. Parthenolide alone inhibits BCL2 protein expression in MCF7/LCC9 cells by 50%, and a greater inhibition (75 %) is achieved with the combined treatment of parthenolide and 4HT. These observations imply at least a partial resensitization to 4HT should occur in cells treated with parthenolide. Similar responses are seen in the antiestrogen sensitive MCF7/LCC1 cells, also consistent with the effects of parthenolide on responsiveness to 4HT as measured by changes in cell proliferation and with the effects of parthenolide on ICI 182,780 sensitivity (297). The fact that BCL2 mRNA level was also inhibited by ≥70% when cells were treated with the combination of parthenolide and 4HT implies that the decrease in BCL2 protein expression seen is a transcriptional event.
An increase in BCL2 expression and a decrease in BAX expression can predict the response to chemotherapy in breast cancer cells (336) where the pro-apoptotic effect of cytotoxic drugs is linked to either a low BCL2:BAX or a high BAX:BCL2 ratio (105,313). While parthenolide alone or in combination with 4HT does not affect the expression of the pro-apoptotic BAX protein, its inhibition of BCL2 leads to a 5-fold increase in the overall BAX:BCL2 ratio driving the cell towards apoptosis. These effects of NFκB inhibition on BCL2 and BAX expression were confirmed by IκBSR, suggesting that the effects of parthenolide are primarily driven by its inhibition of NFκB.

The ability of antiestrogens to induce apoptosis through changing NFκB and BCL2 activity is likely mediated through changes in caspase activation. However, the precise role of caspases in this regard is unknown. Activation of effector caspases following an increase in MMP and cytochrome c release is expected, but a role for the activity of caspases upstream of mitochondria cannot be excluded. To establish the role of caspases, we first used a broad spectrum pancaspase inhibitor that inhibits caspases both upstream and downstream of the mitochondria. The effects of NFκB inhibition (by both parthenolide and IκBSR) on cell proliferation and mitochondrial membrane permeability are statistically significantly reversed by the pancaspase inhibitor. Since the effects on mitochondrial membrane permeability are also reversed, caspases upstream of the mitochondria, such as caspases 2, 8 or 10, are strongly implicated in regulating the effects of NFκB signaling through BCL2 on mitochondrial function and cellular responsiveness to antiestrogens.

We now show that CASP8 is functionally involved in NFκB signaling in the context of affecting endocrine responsiveness. Using a specific inhibitor to CASP8 that works by blocking
the binding of CASP8 to its substrate, we obtain similar results as seen with the use of a pancaspase inhibitor. In addition, the effects of parthenolide alone or in combination with 4HT on BCL2 expression in both MCF7/LCC9 and MCF7/LCC1 cells are reversed with either the pancaspase inhibitor or the specific CASP8 inhibitor. Notably, the decrease in BCL2 expression with 4HT treatment observed in MCF7/LCC1 cells is no longer seen in the presence of either the pancaspase inhibitor or the specific CASP8 inhibitor. While we cannot exclude a minor role for CASP2 and/or CASP10, these are unlikely to be dominant given the significant effects evident with inhibition of CASP8 alone. Thus, NFκB signaling to events upstream of its effects on mitochondrial permeability appears to be transduced primarily by CASP8.

Precisely how this signaling flows is unclear. One possible explanation is that CASP8 also cleaves BID (337) leading to a conformational change in BAX (126) [Figure 82]. Insertion of activated BAX into the outer mitochondrial membrane would increase mitochondrial membrane permeability, facilitate the release of cytochrome c, and ultimately induce apoptosis (338) [Figure 82]. Whether the hormonal regulation of BID is altered in MCF7/LCC9 cells remains to be determined, but the studies presented here show the altered hormonal regulation of several other key proteins both upstream and downstream of CASP8 (such as PARP and TNFR1) that may be involved in the pathway by which NFκB inhibition induces apoptosis in MCF7/LCC9 cells. For example, the cleavage of PARP has been used extensively as a marker of cells undergoing apoptosis (307). While 4HT alone does not affect PARP expression in MCF7/LCC9 cells, a combined treatment of parthenolide and 4HT strongly inhibits PARP protein expression in these resistant MCF7/LCC9 cells. Importantly, while TAM and ICI 182,780 have been shown to induce TNFR1 in MCF-7 breast cancer cells (102), we see an opposite effect in the studies presented here. We show that parthenolide in combination with antiestrogen 4HT
strongly inhibits TNFR1 protein expression in resistant MCF7/LCC9 cells. More importantly, this down-regulation of PARP and TNFR1 seen in the presence of parthenolide and 4HT in MCF7/LCC9 cells is reversed with the addition of a pancaspase inhibitor (PI). Surprisingly, no significant decrease in either PARP or TNFR1 expression is seen in presence of parthenolide and ICI 182,780 in MCF7/LCC9 cells.

The studies presented here provide strong evidence that the mechanism by which NFκB inhibition (by parthenolide) re-sensitizes MCF7/LCC9 cells to TAM involves signaling to the mitochondria through BCL2. When this signaling is disrupted, e.g., by inhibiting NFκB or CASP8, resistance to TAM is reversed in resistant cells and drug sensitivity to TAM is increased in sensitive cells. We believe that inhibition of NFκB activity (i.e., in the presence of parthenolide+4HT) promotes TNFα mediated apoptosis through increased MMP, cytochrome c release, and activation of CASP8 and downstream caspases [Figure 82]. For example, lower levels of TNFR1 inhibit NFκB activation, but may recruit certain key enzymes such as CASP8 (213), where the latter becomes activated and initiates signaling events. Activated CASP8 may cleave BID (337,338), whose C-terminal fragment (tBID) translocates to the mitochondria to bring about a conformational change in BAX and activate BAX (126) resulting in cytochrome c release [Figure 82]. Once cytochrome c is released, an apoptosome is formed that activates CASP9 (128) which in turn can activate CASP3 or 7 [Figure 82]. Caspases 3 or 7 are known to target number of specific proteins including PARP (poly ADP-ribose polymerase; 116kDa nuclear protein) for proteolytic cleavage (306), ultimately leading to apoptosis [Figure 82].

In addition, the current study also provides evidence that the mechanisms by which parthenolide reverses resistance to TAM and ICI 182,780 are potentially different, since the
observations on inhibiting NFκB in presence of 4HT and ICI were potentially different. The pathway by which parthenolide re-sensitizes MCF7/LCC9 cells to ICI 182,780-mediated apoptosis remains to be determined.

Our data strongly suggest that parthenolide may be a useful therapeutic strategy, acting through its effects on NFκB. However, parthenolide is also known to activate c-Jun NH2-termin al kinase (JNK) and reverse resistance to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) (281). Protein levels of JNK and phospho-JNK are higher in MCF7/LCC9 cells but these do not change when these resistant cells are growth inhibited by treating with either parthenolide alone or the synergistic combination of parthenolide and 4HT. Thus, JNK dependent pathways are unlikely to account for the synergistic interaction between parthenolide and 4HT in MCF7/LCC9 cells. Of the several mechanisms known for parthenolide’s biological effect on NFκB (discussed in detail in Chapter 1), one is by preventing degradation of IκB (276). However, we found no evidence of change in protein levels of IκBα when the MCF7/LCC9 cells were treated with a combined treatment of parthenolide and antiestrogens (4HT or ICI 182,780).

NFκB is one of the many targets of phosphatidylinositol 3-kinase (PI3K) and Akt signaling pathways. Studies have shown that activated Akt can induce activation of NFκB (339); promote resistance to TAM as well as other cytotoxic drugs (340) in breast cancer cells. However, we found no evidence of up-regulated or activated phospho-Akt in our resistant MCF7/LCC9 cells, suggesting that pathways other than PI3K and Akt are likely involved in NFκB up-regulation and development of antiestrogen resistant phenotype. Additionally, loss of p53 or its reduced expression can activate NFκB (341,342) but the levels of p53 mRNA and protein expression are comparable in MCF7/LCC1 and MCF7/LCC9 cells. Thus, loss of p53 is
unlikely to account for the increased NFκB activity.

The studies presented here in Chapters 3 and 4 show that a reduced activation of NFκB, either by a molecular approach (IκBSR) or by a small molecule antagonist (parthenolide), increases sensitivity and reverses the antiestrogen resistant phenotype, hence we expected an increased NFκB activity to produce antiestrogen resistance. To address this, NFκB p65/RELA was overexpressed alone in antiestrogen sensitive MCF-7 cells (as a part of collaboration with Dr. Nakshatri) and were named MCF7/p65 cells. However, when we measured the responsiveness of MCF7/p65 transfectants to antiestrogens (ICI 182,780 and 4HT) relative to parental MCF-7 cells, we found that exogenous expression of NFκB p65/RELA alone does not confer resistance to antiestrogens (either 4HT or ICI 182,780). Since inhibition of NFκB can reverse resistance, expression of p65/RELA subunit of NFκB is necessary but is not sufficient for the fully antiestrogen resistant phenotype. Interesting, we found that over-expression of p65/RELA in MCF-7 cells does not make them E2 (estrogen) independent for growth. Although introducing either p65/RELA or p50 subunit of NFκB should be sufficient as either gene alone can be active (321), but whether p50 alone or a combination of p50+p65 confers antiestrogen resistance remains to be determined.
Figure 82: A working model showing how the signaling possibly flows and leads to apoptosis when NFκB is inhibited in resistant MCF7/LCC9 cells.

Following several stimuli, activation of NFκB occurs by phosphorylation of IκB. NFκB then translocates to the nucleus and activates expression of target genes including inhibitors of apoptosis (e.g. BCL2). Inhibition of NFκB activity (i.e., in the presence of parthenolide+4HT) disrupts this signaling and promotes TNFα mediated apoptosis through decreased BCL2 expression, increased MMP, cytochrome c release, and activation of CASP8 and downstream caspases. TNFR1 may recruit certain key enzymes such as CASP8. Activated CASP8 may cleave BID, whose C-terminal fragment (tBID) translocates to the mitochondria to bring about a conformational change in BAX and activate BAX. This results in cytochrome c release, apoptosome formation and activation of downstream caspases that target number of specific
proteins including PARP for proteolytic cleavage, ultimately leading to apoptosis.

6.2. Future Directions:

The studies presented in this body of work report novel functions of the nuclear transcription factor NFkB and its role in affecting a phenotype of clinical significance. These data provide important and relevant new insights into signaling in endocrine resistance in breast cancer, showing the central role of NFkB signaling through the mitochondria, as regulated by CASP8 and BCL2, to affect cell survival. The data with parthenolide, which we show acts primarily through its inhibition of NFkB, strongly supports the design of novel clinical trials to prevent, delay, and/or reverse resistance to antiestrogens. However based upon our current findings further studies are clearly needed to address the questions that remain as discussed below:

6.2.1. A role for BID in the context of TAM responsiveness in MCF7/LCC9 cells?

Based on our data, it is clear that certain defects in the apoptotic pathway such as up-regulated anti-apoptotic protein BCL2, as regulated by NFkB, can contribute to the development of TAM resistant phenotype. However, how these defects contribute to resistance is not completely understood. We have shown in the studies presented here that NFkB affects TAM sensitivity by modulating CASP8 activity, with consequent effects on BCL2 expression and MMP, and apoptosis.

Although CASP8 is part of the extrinsic cell death pathway where it directly cleaves and activates downstream caspases such as CASP3 or 7 and initiates a direct signaling pathway
independent of the mitochondria (343), several lines of evidence suggest that CASP8 can also be a part of the intrinsic/mitochondrial apoptotic pathway (337,338,343). In light of our data and published studies, we propose a possible mechanism explaining parthenolide induced re-sensitization of MCF7/LCC9 cells to TAM-mediated apoptosis. We propose that CASP8 cleaves BID (337) leading to a conformational change in BAX (126). Activated BAX then oligomerizes and inserts tightly into the outer mitochondrial membrane making the mitochondrial membrane permeable. This facilitates the release of cytochrome c, and ultimately induces apoptosis.

Thus, it would be of interest to elucidate mechanistically the functional role of BID in context of TAM responsiveness in breast cancer cells and moreover establish a link between CASP8 and BID in the mitochondrial apoptotic pathway in the context of TAM responsiveness in breast cancer cells (primarily MCF7/LCC9).

6.2.2. The key functions that mechanistically drive ICI 182,780 responsiveness in breast cancer cells?

Although the data provided here supports a role for NFκB in mediating responsiveness to both antiestrogens such as 4HT and ICI 182,780, the results mainly elucidate mechanistically relevant molecular signaling that affects primarily TAM responsiveness in breast cancer cells, with a specific focus on events regulated by NFκB.

The pathway by which parthenolide re-sensitizes MCF7/LCC9 cells to ICI 182,780-mediated apoptosis remains to be determined. While certain observations in the current study imply that the mechanism by which parthenolide reverses resistance to TAM and ICI 182,780 may be potentially different, they are preliminary. Observations in the study suggest that NFκB acts by inducing signaling through BCL2; a study to identify key functions that mechanistically drive
ICI 182,780 responsiveness in breast cancer cells is needed. It is not known if a decrease in BCL2 expression in presence of parthenolide and ICI 182,780 would translate to increases in mitochondrial membrane permeability. Moreover, whether CASP8 is functionally involved in NFκB signaling in the context of affecting ICI 182,780 responsiveness to affect cell survival remains to be determined. Furthermore, it will be interesting to know the effects of inhibition of CASP8 activity on BCL2 (both protein and mRNA) and mitochondrial membrane permeability. Finally, it will be important to obtain an independent confirmation of the role of parthenolide in NFκB using a mutant IκB (which cannot be phosphorylated and targeted for degradation and which acts as a dominant negative NFκB inhibitor; also known as the IκBSuper-Repressor; IκBSR). Similar experiments as done with the combination treatment of parthenolide and 4HT with or without caspase inhibitors should answer these questions.

Certain endpoints measured here, such as increase in the mitochondrial membrane permeability and decrease in BCL2 protein expression, appear to be common to the intrinsic apoptotic pathway as well as to the autophagic cell death pathway. It would be of interest to determine if the primary mechanism of cell death in MCF7/LCC9 cells is through apoptosis as our earlier data suggest (297), or is it a combination of both apoptotic and autophagic cell death. Such a study could help clarify if the resistance to TAM and ICI 182,780 occur independently and if the molecular mechanisms contributing to cell death are different in cells treated with Par+4HT than those treated with Par+ICI.

**6.2.3. Does NFκB affect antiestrogen (both 4HT and ICI 182,780) sensitivity in vivo?**

We show that inhibition of NFκB, either by a molecular approach (IκBSR) or by a small
molecule antagonist (parthenolide), not only reverses resistance to both 4HT and ICI 182,780 in resistant cells, but also increases the responsiveness of sensitive cells to these SERMs and SERDs. Furthermore, we show that inhibition of NFκB attenuates both TAM and ICI 182,780-mediated apoptosis \textit{in vitro}, however the ability of NFκB inhibition (primarily by parthenolide) to do so \textit{in vivo} would firmly establish NFκB as a mediator of responsiveness to both SERMs and SERDs. Thus, animal studies could use parthenolide because this drug is already in clinical trials. Based upon the data presented here, the resistant MCF7/LCC9 cells show the greatest interaction between parthenolide and antiestrogens (both TAM and ICI 182,780) \textit{in vitro}. Hence, it will be appropriate to first study the MCF7/LCC9 cells in nude mice.

Furthermore, our lab has identified NFκB to be present in a high proportion of ER+ breast tumors (328), but measuring NFκB expression in breast cancer patient samples treated with antiestrogens would greatly enhance our ability to determine whether NFκB plays a role in modifying antiestrogen responsiveness in breast cancer.

\textbf{6.2.4. Designing Novel Clinical trials?}

In the era of personalized medicine, measuring NFκB and markers of its functional signaling, and developing additional small molecule antagonists such as parthenolide, are critically important. Such measurements may help us to tailor specific therapies to individual breast cancer patients and improve clinical outcomes for women. The studies presented here identify NFκB as one of the molecular targets with possible predictive value that, when modulated, restores sensitivity in resistant cells and/or increase sensitivity in sensitive cells. Parthenolide has been shown to be well tolerated in phase I clinical trials with no significant toxicity in patients with cancer (278). Furthermore, large-scale randomized trials have shown that
all ER+ breast cancer patients receive significant survival benefit from 5 years of adjuvant TAM therapy given immediately after surgery (62), thus making resistance to TAM a highly relevant clinical phenotype.

It would be worthwhile to design novel clinical trials with parthenolide (which we show acts primarily through its inhibition of NFκB) in combination with a SERM or SERD in ER+ breast cancers. Such combination therapy is predicted to be better than an antiestrogen alone in sensitive cells. Furthermore, since TAM is still the most widely used antiestrogen, and it remains the primary endocrine therapy for many breast cancer patients with ER+ disease, such combination therapy could prove useful in reversing resistance to antiestrogens in patients with TAM-resistant disease where residual tumor cells are still ER+.

6.2.5. Can Expression of an Exogenous NFκB in Antiestrogen Sensitive Cells alter Responsiveness to Antiestrogens (4HT and ICI 182,780)?

The studies presented here in Chapters 3 and 4 show that reduced activation of NFκB, either by a molecular approach (IκBSR) or by a small molecule antagonist (parthenolide), increases sensitivity and reverses the antiestrogen resistant phenotype. Hence, we expected an increased NFκB activity to produce antiestrogen resistance.

We examined MCF-7 cells overexpressing NFκB p65/RELA only (MCF7/p65) from one of our collaborators (Dr.Nakshatri) and tested their responsiveness to antiestrogens (4HT and ICI 182,780). The data in Chapter 5 shows that these MCF7/p65 cells are highly dependent on E2 (estrogen) for growth and that exogenous expression of NFκB p65/RELA alone does not confer resistance to antiestrogens (either 4HT or ICI 182,780). We concluded from this part of the study
that the p65/RELA subunit of NFκB is necessary but is not sufficient for the antiestrogen resistant phenotype.

Importantly, introducing either the p65/RELA or p50 subunit of NFκB should be sufficient as either gene alone can be active (321), but it would still be interesting to study that if p65/RELA alone is not sufficient to affect antiestrogen responsiveness, whether transfection of p50 subunit of NFκB into MCF-7 cells could confer resistance to antiestrogens (either 4HT or ICI 182,780). Finally, if neither p65/RELA nor p50 alone is sufficient, it would be of interest to introduce p50 into our p65/RELA transfected MCF-7 cells (MCF7/p65) and measure their sensitivity to antiestrogens. The same approach can be used in our sensitive MCF7/LCC1 cells, to determine if their response to antiestrogens is any different from MCF-7 cells following introduction of p50, or p65/RELA or p50 + p65/RELA. If the data supports the experiments, it would be worthwhile to do such studies in vivo in nude mice.

In the process of studying if over-expression of p65/RELA could confer resistance to antiestrogens, we made some novel cell lines as discussed in detail in Chapter 5 of this dissertation: MCF7/p65/ICI (ICI resistant; TAM cross-resistant) and MCF7/p65/TAM (TAM resistant; ICI sensitive). It also would be important to conduct additional experiments, such as western blotting to compare the expression of some key proteins that may be implicated in antiestrogen resistance such as p65/RELA, p50, BCL2, NEMO, IκBα, XBP1 (XBP1 is known to be induced by NFκB), ERα, IRF1 (a protein partner of NFκB involved in regulation of iNOS) in MCF-7, MCF7/p65, MCF7/p65/ICI and MCF7/p65/TAM cells. In addition, gene expression microarray analysis of the control MCF7/p65 compared to MCF7/p65/ICI and MCF7/p65/TAM cells might provide useful information on genes that are differentially expressed among the above
mentioned cell lines.

Since we show that BCL2 protein expression is up-regulated in MCF7/p65 cells, it would be of interest to know if promoter activity in these cells correlates with the protein expression. Further, it would be interesting to see if MCF7/p65/ICI and MCF7/p65/TAM cells require estrogen for growth or are these cells estrogen independent for their growth.

6.3. Conclusions:

Our studies clearly show that NFκB has the potential to serve as a putative predictive biomarker for breast cancer recurrence following adjuvant hormonal therapy. We have shown here that antiestrogen sensitive and resistant cells differentially use NFκB to affect cell fate. In resistant cells, NFκB expression and function are increased and act by signaling through CASP8 and BCL2 to affect prevent mitochondrial membrane destabilization and the consequent induction of an apoptotic and/or autophagic cell death. These data further provide a compelling rationale to initiate clinical trials of parthenolide (which we show acts primarily through its inhibition of NFκB) in combination with a SERM or SERD in ER+ breast cancers. Such combination therapy is predicted to be better than an antiestrogen alone in sensitive cells. Furthermore, in heavily pretreated patients where residual tumor cells are ER+ but have become estrogen-independent and cross-resistant to TAM and ICI, this combination could prove useful in producing further clinical responses, and thus restore the activity of TAM. The importance of this is evident since TAM not only remains the most widely used antiestrogen but also remains the primary endocrine therapy for premenopausal breast cancer patients with ER+ disease.
Chapter 7: References


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