**BRCA1-Mimetic Compound NSC35446.Hcl Inhibits *IKKB* Expression
by Reducing Estrogen Receptor- α Occupancy in the *IKKB* Promoter
and Inhibits NF- κ B Activity in Antiestrogen-Resistant Human Breast
Cancer Cells**

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degree of
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In Tumor Biology

By

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ABSTRACT

This dissertation examines the mechanism of action of the BRCA1-mimetic compound NSC35446.HCl and identifies a potential mechanism by which it is able to overcome antiestrogen resistance in human breast carcinoma cell lines. Previous work has identified small molecule compounds that fit into a BRCA1-binding cavity within estrogen receptor-alpha ($ER\alpha$), mimic the ability of BRCA1 to inhibit $ER\alpha$ activity ("BRCA1-mimetics"), and function differently from other antiestrogens such as Tamoxifen and Fulvestrant. These BRCA1-mimetics inhibit $ER\alpha$ activity in both antiestrogen-sensitive and antiestrogen-resistant cells. One such compound, the hydrochloride salt of NSC35446 ("NSC35446.HCl"), also inhibited growth of antiestrogen-resistant LCC9 tumor xenografts. In this dissertation, I show that NSC35446.HCl inhibits proliferation of antiestrogen resistant LCC9, T47DCO, MCF-7/RR, and LY2 cells but not of $ER\alpha$ -negative breast cancer cell lines (MDA-MB-231, MDA-MB-468, and HCC1806) and causes apoptosis in LCC9 and T47DCO cells but not in $ER\alpha$ -negative breast cancer cell lines. I identified *IKKB*, an upstream activator of NF- κ B, as a BRCA1-mimetic-regulated gene, based on an RNA-seq analysis. I showed that NSC35446.HCl inhibited *IKKB* mRNA and protein expression in LCC9 cells. NSC35446.HCl also inhibited NF- κ B

transcriptional activity and expression of NF- κ B target genes in antiestrogen resistant cell lines. *In silico* analysis of the *IKKB* (*IKBKB*) promoter identified nine estrogen response element (ERE) half-sites and one ERE-like full-site (which differs from the canonical ERE full-site by one nucleotide). Chromatin immunoprecipitation (ChIP) assays revealed that ER α was recruited to the ERE-like full-site and five of the nine half-sites and that ER α recruitment was strongly inhibited by compound NSC35446.HCl in LCC9 and T47DCO cells. These studies identified functional EREs within the *IKKB* promoter and identified the *IKKB* gene as a target of NSC35446.HCl. They further suggest that NF- κ B, which has previously been linked to antiestrogen resistance, may be a target for NSC35446.HCl in reversing antiestrogen resistance.

DEDICATION

This dissertation is dedicated to all those who helped me out at my time in Georgetown.

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION	1
1.1 Descriptive Epidemiology of Breast Cancer	1
1.1.1 Incidence and Mortality	1
1.1.2 Breast Cancer Classification.....	4
1.2 Nuclear Receptors	6
1.2.1 Structure	7
1.2.2 Nuclear Receptor Activation	8
1.2.3 Mechanism of Action	10
1.3 Estrogen Receptors	11
1.3.1 Estrogen Receptor Structure.....	13
1.3.2 Estrogen Receptor Mechanism of Action.....	15
1.3.3 The Roles of Estrogen and Estrogen Receptor α in Breast Cancer.....	16
1.4 BRCA1 Function in Breast Cancer.....	18
1.5 Endocrine Resistance in Breast Cancer	19
1.6 BRCA1-mimetics.....	22
CHAPTER 2: BRCA1: ESTROGEN RECEPTOR α INTERACTION	24
2.1 BRCA1: Estrogen Receptor α Interaction.....	24
2.1.1 BRCA1 Inhibition of Estrogen Receptor α Activity by Direct Interaction	24
2.1.2 Further Characterization of Structure/Function Relationship for the BRCA1: Estrogen Receptor α Interaction.....	27
2.1.3 Role Tamoxifen in Mammary Cancer Development in BRCA1 Mutation Breast Cancer Mouse Model	32
2.1.4 Estrogen Signaling and BRCA1 Loss	36
CHAPTER 3: MATERIALS AND METHODS	39
3.1 Cell Lines and Culture	39
3.2 Reagents.....	39
3.3 Cell Proliferation Assays	40
3.4 Cell-Cycle Analysis	40
3.5 Mammosphere Assays	40
3.6 RNA-seq	41
3.7 qRT-PCR	41
3.8 Western Blotting.....	42
3.9 Assays of NF- κ B Transcriptional Activity	43
3.10 TUNEL Assays	44
3.11 Annotating Putative ERE Half-Sites and ERE-like Full-Sites in the <i>IKKB/IKBKB</i> Promoter.....	44
3.12 Chromatin Immunoprecipitation (ChIP) Assays	45
3.13 Statistical Analysis.....	46
CHAPTER 4: RESULTS	47
4.1 NSC 35446.HCl Alters Cell Growth in Antiestrogen Resistant Cell Lines	47
4.1.1 NSC 35446.HCl Cell Proliferation Assays	47
4.1.2 NSC35446.HCl Cell Cycle Assays	48
4.1.3 NSC 35446.HCl Mammosphere Assays	49
4.2 RNA-seq Analysis of LCC9 Cells Treated with BRCA1-mimetic	50

4.2.1 Compound A7 RNA-seq	50
4.2.2 qRT-PCR Validation of RNA-seq	51
4.2.3 Pathway Studio Analysis of RNA-seq Data	52
4.3 NSC35446.HCl Alters Expression of IKKB and Other IKK Proteins in LCC9 cells	53
4.3.1 Protein Expression Following NSC35446.HCl Treatment at 24, 48, 72 hr	53
4.3.2 mRNA Expression Following NSC35446.HCl Treatment at 6, 12, 24, and 48 hr	58
4.4 NSC35446.HCl affects NF- κ B Transcriptional Activity and NF- κ B-Responsive Gene Expression in Antiestrogen-Resistant Cells	58
4.4.1 NF- κ B Luciferase Assays	58
4.4.2 Effect of Compound NSC35446.HCl on Protein Levels of NF- κ B-Regulated Genes	59
4.5 Effect of Compound NSC35446.HCl on Apoptosis in LCC9 Cells	63
4.5.1 Caspase-7 Cleavage After Treatment with NSC35446.HCl	63
4.5.2 Effect of Compound NSC35446.HCl without and with Caspase Inhibitors on Proliferation of LCC9 Cells	65
4.5.3 Caspase 7 Expression After Treatment with Compound NSC35446.HCl Without and With Caspase Inhibitors	65
4.5.4 Effect of Compound NSC35446.HCl on Apoptosis in Human Breast Cancer Cell Lines	68
4.6 The <i>IKKB</i> Promoter Contains ERE Half-Sites and an ERE-like Full-Site	69
4.6.2 Effect of Compound NSC35446.HCl Alters the Recruitment of Estrogen Receptor α to the <i>IKKB</i> promoter	72
4.6.3 ChIP Control Experiments	75
4.7 Effect of Compound NSC35446.HCl on ER α Protein Expression in LCC9 cells	76
4.8 Additional Experiments to Satisfy the Comments of the Reviewers of Our Manuscript	78
4.8.1 Additional Proliferation Assays for Antiestrogen-Resistant Cells.	78
4.9 Heat map of Genes that Influence NF- κ B Signaling from RNA-seq Experiment of LCC9 Cells treated with vehicle (control) vs compound A7 (NSC31303) (5 μ M for 24-hr).	79
4.10 Dose-Response for Effect of Compound NSC35446.HCl on IKKB, IKKG, and IKKA Protein Levels in LCC9 Cells.	80
4.11 Dose-Response for Effect of Compound NSC35446.HCl on NF- κ B-Regulated Protein Expression in LCC9 Cells	82
4.12 Additional Time Course Experiments Showing the Effects of Different Doses of Compound NSC35446.HCl on <i>IKKB</i> mRNA Expression in LCC9 Cells and of a Single Dose of Compound on Two Other MCF-7-Derived Estrogen Receptor α -Positive Antiestrogen-Resistant Cell Lines, MCF-7/RR and LY2.	83
4.13 Dose-Response and Time Course for the Effect of Compound NSC35446.HCl on Estrogen Receptor α Recruitment to the <i>IKKB</i> promoter in LCC9 Cells.	84
4.14 Dose-Response for the Effect of Compound NSC35446.HCl on Development of Apoptosis in LCC9 Cells.	87
CHAPTER 5: DISCUSSION	89
REFERENCES	96

LIST OF FIGURES

Figure 1. Age of incidence and morality curves for breast cancer in the US	1
Figure 2. Breast cancer incidence and mortality rates by race and ethnicity	3
Figure 3. Nuclear receptor ligand as a switch	9
Figure 4. Structure/function/sequence relationships within the nuclear hormone receptor superfamily	11
Figure 5. Sequence arrangement of two gene products (ESR1 and ESR2) of Estrogen Receptor.	12
Figure 6. ER α and ER β bound to estradiol	13
Figure 7. Secondary structural predictions for the NTDs of both ER receptors	15
Figure 8. Classical pathway of estrogen action	16
Figure 9. Summary of resistance mechanisms in breast cancer.....	21
Figure 10. Mutant BRCA1 domains fail to inhibit ER α activity	24
Figure 11. Interaction of BRCA1 and ER α in vitro	26
Figure 12. Interaction of BRCA1	28
Figure 13. N-terminal BRCA1 N-terminal, Proteins to inhibit ER activity	28
Figure 14. BRCA1 1-100 point mutations	29
Figure 15. BRCA1 aa 1-100 mutant peptide relative binding to ER α	30
Figure 16. BRCA1 1-100 mutations inhibition of ER activity.	30
Figure 17. Model structure of BRCA1:ER complex	31
Figure 18. Detection of apoptotic cells through apoptag and detection of nuclear ER and ER	33
Figure 19. Detection of tumors in placebo and tamoxifen treated BRCA1Co/C0 MMTV-Cre/p53+/- female mice	34
Figure 20. Effect of reduced BRCA1 expression in MCF-7 cells on Tamoxifen agonist activity	35
Figure 21. Mammary glands ducts as a result of BRCA1 ablation	36
Figure 22. Alveolar differentiation as a result of BRCA1 ablation	37
Figure 23 Result of exogenous estrogen exposure in BRCA1 ablated mice	38
Figure 24. Chemical structure of compound NSC35446.HCl	47
Figure 25. Effect of compound NSC35446.HCl on the growth of various human breast cancer cell lines	48
Figure 26. Effect of compound NSC35446.HCl on cell cycle distribution of LCC9 cells	49
Figure 27. Effect of NSC35446.HCl on mammosphere formation in human breast cancer cells	50
Figure 28. Chemical structure of compound A7 (NSC31303)	51
Figure 29. Validation of selected RNA-seq data by qRT-PCR	52
Figure 30. Sample tree of biologically relevant signaling pathways based on RNA-seq of compound A7 (NSC31303)-treated cells	53
Figure 31. Effect of compound NSC35446.HCl on the expression of IKKB protein and that of two other IKK isoforms	57

Figure 32. Time course for effect of compound NSC35446.HCl on IKKB mRNA expression .	58
Figure 33. Effect of compound NSC35446.HCl on NF- κ B transcriptional activity.	59
Figure 34. Effect of compound NSC35446.HCl on NF- κ B-responsive gene expression in LCC9 and T47DCO cells.	63
Figure 35. Effect of compound NSC35446.HCl on cleavage of caspase-7 in LCC9 cells	64
Figure 36. Effect of compound NSC35446.HCl without and with caspase inhibitors on proliferation of LCC9 cells .	65
Figure 37. Effect of compound NSC35446.HCl alone or in combination with caspase inhibitors on caspase-7 cleavage in LCC9 and other cell lines	67
Figure 38. TUNEL apoptosis assays of four human breast cancer cell lines treated without or with compound NSC35446.HCl for 48-hr	69
Figure 39. Effect of compound NSC35446.HCl on the recruitment of ER α to the <i>IKKB</i> promoter in LCC9 cells	74
Figure 40. Additional ChIP assays	75
Figure 41. Control ChIP assays	76
Figure 42. Effect of compound NSC35446.HCl on ER α protein expression in LCC9 cells.	77
Figure 43. Additional proliferation assays for antiestrogen-resistant cells	79
Figure 44. Heat map of genes that influence NF- κ B signaling from RNA-seq experiment of LCC9 cells treated with vehicle (control) vs compound A7 (NSC31303) (5 μ M for 24-hr)	80
Figure 45. Dose-response for effect of compound NSC35446.HCl on IKKB, IKKG, and IKKA protein levels in LCC9 cells	81
Figure 46. Dose-response for effect of compound NSC35446.HCl on NF- κ B-regulated protein expression in LCC9 cells	83
Figure 47. Additional time course experiments showing the effects of different doses of compound NSC35446.HCl on IKKB mRNA expression in LCC9 cells and of a single dose of compound on two other MCF-7-derived ER α -positive antiestrogen-resistant cell line	84
Figure 48. Dose-response and time course for the effect of compound NSC35446.HCl on ER α recruitment to the <i>IKKB</i> promoter in LCC9 cells	86
Figure 49. Dose-response for the effect of compound NSC35446.HCl on development of apoptosis in LCC9 cells	88

LIST OF TABLES

Table 1. Summary of breast cancer subtypes.....	4
Table 2. Primers utilized for qRT-PCR studies.	42
Table 3. RNA-seq data for eight selected genes regulated by compound A7 (NSC 31303) in LCC9 cells.	52
Table 4. ChIP assay PCR primers for ERE half-sites and an ERE-like full-site in the <i>IKKB</i> promoter.....	70

CHAPTER 1: INTRODUCTION

1.1 Descriptive Epidemiology of Breast Cancer

1.1.1 Incidence and Mortality

Breast cancer is the leading cancer among women in the U.S. It affects around 233,000 women a year, with incidence rates increasing with age after 30, and is responsible for 40,000 deaths per year in the female population [Figure 1]. However, lifetime (birth to death) risk of death due to breast cancer for the general female population is 3.4%. As of 2012, 2.9 million women will survive breast cancer (1).

Figure 1. Age of incidence and mortality curves for breast cancer in the US.

Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) Research Data (1973-2014), National Cancer Institute, DCCPS, Surveillance Research Program, released April 2017, based on the November 2016 submission.

Mortality and incidence rates in breast cancer can vary extensively due to race and ethnicity. Ethnicities with lower incidence rate of breast cancer include Asian, Hispanic, and American Indian women. Ethnicities with higher incident rates of breast cancer include non-Hispanic white and African American women (2)

In the U.S., the incidence and mortality trends in breast cancer are influenced by many factors, which cause them to vary over time. For example, increases in incidence rates in the 1980s can be explained by a significant increase in breast cancer screening, such as the use of mammograms (1). Today, worldwide reporting of breast cancer incidence and mortality can have a great deal of variability. Some of this variability can be due to factors such as differences in data collection and reporting, risk factor exposure, and screening methods. Most experts posit that breast cancer rates worldwide differ in part, due to lifestyle factors and environmental differences. Environmental differences could include exposure to different kinds of carcinogens such as pesticides, paint removers, plastic additives and other synthetic chemicals. Social inequalities, nationally and worldwide, can be tied to environmental factors. For example, low-income individuals may have greater exposure to toxins and less access to quality healthcare. Lifestyle factors that can contribute to breast cancer risk include alcohol consumption, obesity, lack of physical activity, and hormone therapy after menopause. (3).

Figure 2. Breast cancer incidence and mortality rates by race and ethnicity. Surveillance, Epidemiology, and End Results. (SEER) Program (www.seer.cancer.gov) Research Data (1973-2014), National Cancer Institute, DCCPS, Surveillance Research Program, released April 2017, based on the November 2016 submission

Breast cancer is screened for by mammography, breast ultrasound, and breast MRI (1) and diagnosed upon fine needle aspiration and core/surgical biopsy (1). Upon diagnosis, the tumor is staged according to invasiveness, molecular genotypes, and hormone status for proper prognosis and personalized treatment (1). Breast cancer is classified according to tumor nuclear grade, stage of cancer, protein and gene status, and histological grade. Tumor grade is determined by histopathology, which focuses on the appearance of the cancer cell nuclei and how it differs from the normal breast epithelial cell nuclei. The stage of cancer refers to the size, status of nodal involvement, status of metastatic involvement, and presence or absence of evidence of invasion. Molecular status refers to

any receptors found on the plasma membrane or in the nucleus or cytoplasm that can affect prognosis and therapeutic options (2).

1.1.2 Breast Cancer Classification

Based on molecular profiling, breast cancer can be classified according to four major subtypes, which are important in determining clinical outcome, and treatment options (1).

The major subtypes of human breast cancer are: luminal A, luminal B, HER2/Neu-positive, and triple-negative (or basal-like) (1).

Table 1. Summary of Breast cancer subtypes

Subtype	Markers	Prevalence (%)	Clinical characteristics
Luminal A-like	ER+ and/or PR +/HER2-/low grade	42-59	Less aggressive, slow growing, endocrine sensitive. Low recurrence rates
Luminal B-like	< ER+ and/or PR > +/HER2 or ER+ and/or PR +/HER2-/ high grade	10-20	Aggressive, poor-prognosis, less estrogen sensitive. High recurrence rates
HER2+ type	ER-/ PR-/HER2+	10-20	Aggressive, poor short-term prognosis, more common in younger women. High recurrence rates
Triple negative/Basal-like	ER-/ PR-/HER2-	10-20	Fast growing, aggressive, often has a higher grade, and tends to metastasize, High recurrence rates. More common in African Americans, premenopausal women, and those with the BRCA1 mutations

Tamimi RM. Pathology and Epidemiology of Cancer. Epidemiology of Breast Cancer. 2016; 2: 51-17

The luminal A subtype is the most common classification of breast cancer and includes markers ER-positive, PR (progesterone receptor)-positive or negative, and HER2-

negative. This suggests that the cancer cells, like the normal cells of the breast, contains receptors for the hormones estrogen and progesterone and may receive signals from these hormones that could promote their growth; but these cells do not over-express the HER2 receptor. Estrogen receptor-alpha (ER α) and PR are the primary drivers of tumors belonging to this subtype. It is often associated with a good prognosis; and the recurrence rate is low as antiestrogen therapy (tamoxifen, aromatase inhibitors, fulvestrant) is available for use in women with this cancer subtype. In premenopausal women, the selective estrogen receptor modulator (SERM) tamoxifen may be used in combination with cytotoxic chemotherapy as an adjuvant therapy (1). Postmenopausal women, who are ER-positive, may also be treated with aromatase inhibitors as initial treatment or following tamoxifen administration (4).

Luminal B breast cancers express the ER-marker similar to luminal A (ER-positive, PR-positive or negative, but may also have abnormally high levels of HER2 expression. They also has a higher expression of the proliferative marker Ki67, and they are usually treated with a combination of chemotherapy with antiestrogen therapy. Luminal B cancers usually have a worse prognosis than Luminal A (chi square = 4.76, P=.029) (5).

HER2-positive breast cancers are generally negative for ER and PR expression, but over-express HER2/Neu. Chemotherapy combined with anti-HER2 therapy is the standard form of treatment. These cancers have had worse short-term prognosis and tend to be biologically more aggressive. However with the introduction of trastuzumab (Herceptin), a monoclonal antibody that binds to the HER2 receptor and blocks its activity, the ten year overall survival rate has improved significantly (6). The overall survival rate is 37%

better with adjuvant therapy combining Herceptin plus chemotherapy than with chemotherapy alone.

Triple-negative breast cancer (TNBC) does not express any of the three markers. Prognosis is the worst among the four subtypes (5 year survival rates show that 77% of women with TNBC survived at least 5 years compares to 93% with other breast cancer subtypes) (5). At present, systemic chemotherapy and locoregional therapy (surgery and or radiation therapy) are the mainstays of treatment. Since the epidermal growth factor receptor (EGFR) is frequently over-expressed in TNBC, it is now being investigated as a potential therapeutic target for TNBC. Thus, the EGFR tyrosine kinase inhibitor erlotinib is being assessed for use in TNBC in clinical trials (7). Pembrolizumab is an antibody that targets the immunotherapeutic target PD-1 (programmed cell death 1, CD279). This drug was initially used for metastatic melanoma but now is being investigated for TNBC. The anti-tumor immune response is evaded in cancer through the PD-1 adaptive immune resistance pathway. A direct link to the PD-1 pathway in TNBC was found in the “Cancer Genome Atlas”; and several studies support the investigation of immunotherapy in TNBC (8).

At present, segmental mastectomy with axillary nodal sampling, also known as breast-conserving surgery (BCS), is used in conjunction with radiation therapy (RT) to greatly minimize recurrence to the locoregional area (9).

1.2 Nuclear Receptors

Normally, cells control their growth and their function through tight programs of transcriptional regulation. Each subtype of breast cancer exhibits deregulation of various genes, such as transcription factors, that result in tumor heterogeneity. Binding of

transcription factors to their respective DNA response sites is not only important to control the rate of transcription of gene products but also to regulate other transcription factors in order to achieve multiple modes of control and regulation. Members of one family of transcription factors, the nuclear receptor superfamily, are activated by binding to specific ligands and directly regulate gene expression by binding to specific regions of DNA, known as enhancers or response elements (10).

1.2.1 Structure

Nuclear receptors (NRs) are characterized by several structural domains:

1. A DNA binding domain (DBD) that recognizes and interacts with DNA in a sequence-specific manner. This response element sequence can vary depending on the type of receptor and its conformation. The DBD recognizes and binds to DNA utilizing zinc finger motifs, which comprise 25-28 amino acids and are stabilized by zinc ions along with zinc coordinating amino acids, such as histidines and/or cysteines.
2. A ligand binding domain (LBD) containing 200-300 amino acid residues within the carboxyl-terminus of the protein. The LBD contains the transcriptional activation function-2 (AF-2) domain, which is formed by α helices 3, 3', 4 and α helix 12. AF-2 is essential for ligand interactions and complexes with the LxxLL (where x = any amino acid) motifs of transcriptional coregulators. The LBD can form one of the surfaces of nuclear receptors when they homo- or heterodimerize. Amino acids in helices 3, 7, and 10 act as a scaffold to provide the primary shape of the LBD although

they might not necessarily provide residues of interaction with other ligands or co-factors. The monomeric structure of ER contains three anti-parallel α helix structures that funnel into 2 anti-parallel β -sheets. This is the Ligand Binding Pocket and is dependent on α helices H1, H3, H4, H9, H10, and H11 for formation of the pocket. The LBD is the main focus of drug discovery as it is the site of both ligand binding and co-activator interaction.

3. The N-terminal region, which is highly variable and contains another transcriptional activation domain called activation function-1 (AF-1) and which can activate transcription independently of AF-2 and the ligand-LBD interaction.
4. The hinge region is a variable portion of the nuclear receptor, which generally contains binding residues for the DNA minor groove and link the DBD and LBD (11).

1.2.2 Nuclear Receptor Activation

Nuclear receptor (NR) ligands are hydrophobic molecules that are derivatives of cholesterol, fatty acids, retinoids, vitamins, lipophilic hormones, antibiotics, xenobiotics, and synthetic drugs. Ligand binding to the NR causes a conformational change in the receptor. The lipophilic nature of the ligand allows it to diffuse through the cell to the cytoplasm and bind to the highly alpha-helical and hydrophobic ligand-binding pocket of the LBD, inducing an allosteric change in the receptor that causes critical residues to appear on the surface of the receptor for interactions with cofactors and DNA. The size of the ligand-binding pocket can range from 220 Å to 1600 Å that contain 12 alpha-helices

that center around a hydrophobic pocket. Amino acids in helices 3, 7, and 10 provide the primary shape of the ligand-binding pocket. The size and shape of the pockets are highly inducible or able to respond to a wide variety of stimuli and are, therefore, accessible to a variety of ligands and molecules besides endogenous ligands, including synthetic ligands. The nuclear receptor ligand can act as a transition switch between co-activator and co-repressor binding [Fig3a] (12)(13).

Utilizing LxxLL motifs, co-regulators can bind to nuclear receptors and cause helix 12 to dramatically shift in position as a result of ligand binding to ligand-binding pocket. Agonist ligands position helix 12 for co-activator binding, whereas, antagonists will position helix 12 in a different position for co-repressor binding [Fig 3b].

AR Huang P, et al. 2010.
Annu. Rev. Physiol. 72:247–72

Figure 3. Nuclear receptor ligand as a switch.

1.2.3 Mechanism of Action

NR's can be classified into four categories. These categories or classes are based on their dimerization and DNA binding properties. Class I (steroid hormone receptors) are ligand-induced, homodimerize, and bind to DNA half sites that are inverted repeats. Class II NR's heterodimerize with the retinoid X receptor (RXR), bind to DNA sites that contain direct repeats and/or symmetrical repeats, and includes all the other known ligand receptors. Class III NR's homodimerize and bind to direct repeats.

Class IV NR's are monomers and bind to extended core sites (14). Class III and Class IV receptors belong to the orphan nuclear receptor family. These receptors share significant sequence homology with other nuclear receptors but have no known ligands identified with them, so far.

Figure 4 represents structure/function as well as sequence relationships among the nuclear hormone receptor superfamily, which encompass all known nuclear hormone receptors. Superfamilies are further divided into family and subfamilies based on sequence homology. The N-terminal variable region containing AF-1 is denoted "A/B", while the DBD is denoted as domain "C", the hinge region is denoted as "D", and the LBD is denoted as "E". In addition, there is an "F" domain at the C-terminus of NRs that may bind co-regulators.

Figure 4. Structure/function/sequence relationships within the nuclear hormone receptor superfamily.

Cell Volume 83, 835-839 December 15, 1995 Copyright© Cell Press

1.3 Estrogen Receptors

Estrogen receptors (ER) are members of subfamily 3 of Class I nuclear receptors and contain two isoforms: ER α and ER β . These two isoforms contain conserved domain structures. Similarities include ligand binding modulation by ligand binding domain (LBD), receptor dimerization, and transactivation of target genes upon ligand binding to AF-2 domain. The DBDs of ER α and ER β are 96% conserved, with the DNA recognizing zinc subdomains being 100% identical. However the LBD of both isoforms only share 60% homology, a difference that allows for subtype-specific ligands (15). Early published data showed that transcriptional activation in ER α is dependent upon ligand-independent AF-1, while, transcriptional activation in ER β is more dependent on the ligand-dependent AF-2. ER α and ER β have antagonistic functions in breast cancer,

where ER α is associated with cell proliferation and ER β is associated with anti-proliferative effects (16).

Most ligands that bind to both ER α and ER β tend to display a higher selectivity toward ER α . However, there have been a number of ER β selective ligands reported, as well, despite the similarity between the two ligand-binding pockets. An example of ER β selective ligands are diphenylmethane and its analogues such as bisphenol A. ER α and/or ER β are expressed in a wide variety of tissues, including the uterus, breast, brain, liver, salivary gland, colon, and prostate. Estrogen receptors homodimerize and bind to a consensus inverted-repeat palindrome in order to facilitate transcription, whereby they are bound to either co-activators or co-repressors at a consensus estrogen response element (ERE) (GGTCAnnnTGACC) (where n=any nucleotide). Alternatively, estrogen receptors can function as co-activators for other transcription factors, such as activator protein-1 (AP-1) or specificity protein-1 (Sp-1). Estrogen receptors can be further regulated via post translational modifications such as phosphorylation, acetylation, SUMOylation, and ubiquitination (17).

Figure 5. Sequence arrangement of two gene products (ESR1 and ESR2) of Estrogen Receptor.

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1.3.1 Estrogen Receptor Structure

The C-terminal E/F region of both estrogen receptors, characterized by Montona et al., contains 42 amino acids in the F region alone. The E/F region was shown to be important receptor in dimerization and in transcription in ligand bound receptor. The LBD is in the E region of the estrogen receptor and contains 12 helices, the ligand- binding pocket, and dimerization function. The structure of ER α /ER β LBD is similar to other nuclear receptors and shares similarity in function (18).

Figure 6. ER α and ER β bound to estradiol. Copyright © 2011 Raj Kumar et al.
<http://dx.doi.org/10.4061/2011/812540>

The DBD recognizes DNA sequences proximal or distal to a particular transcription start site, usually recognizing the sequence “GTCCAnnnTGACC”, which is recognized by both ER α and ER β .

Most EREs are not perfect matches to the above consensus ERE sequence. Thus, EREs play a key regulatory role in determining the level of binding of ERs to the ERE and determining co-activator binding as well. Two distinct mechanisms control ERE

regulation: ligand-mediated direct binding of ER to the ERE or ER mediating transcription through binding of multiprotein complexes, in which the ER does not directly bind to DNA (*eg.*, AP-1).

The N-terminal domain (NTD), which contains the ligand independent activation function AF-1 that is responsible for recruitment of co-regulator proteins (ex p68/p72), is an intrinsically disordered structure that may exist in this state to help it facilitate proper binding with transcriptional machinery or other co-modulators at suitable concentrations of protein. The amino acid structure of the NTD in ER α is predicted to be 67% as random coil configuration, while in ER β it is 80% as random coil configuration (Fig 6). Therefore, it seems that both receptors remain unstructured until selective binding has occurred. The variability found in the NTDs of ER α and ER β , as seen in the random coil configurations, may explain the different binding affinity each receptor has for the TATA box-binding protein (TBP). ER α binds the TBP through its NTD, while ER β is unable to bind TBP through its NTD, which implies differential binding of target proteins through the NTD (19).

Figure 7. Secondary structural predictions for the NTDs of both ER receptors.

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Through surface plasma resonance, the interaction between the NTD and a potential co-activator was found to be a two-step process. The first step involves a fast association of the NTD and co-activator through electrostatic interactions, while the second step involves a folding event, which is slower and stronger, allowing folding to take place due to specific amino acid interactions (20).

1.3.2 Estrogen Receptor Mechanism of Action

Upon translation, ER (ER α and ER β) binds to the 70 kDa heat-shock protein (HSP70), HSP70 interacting protein (HIP), and the 40 kDa heat shock protein (HSP40) (Fig 7). This complex has a partially exposed hydrophobic binding region, which is immediately bound by 90 kDa heat shock protein (HSP90) and HSP70/HSP90-organizing protein (HOP), which displaces HSP40. This intermediate complex now has a fully exposed hormone binding domain, whence ATP binds to HSP90 followed by stabilization of the

ATP/HSP90 interaction by co-chaperone p23. An open tetratricopeptide region (TPR) on HSP90 is now bound by cyclophilin 40 (CYP40). At this state the complex is considered mature. Estrogen (for example 17β -estradiol) diffuses through the plasma membrane and binds to the ER monomer (which is normally located in the cytoplasm). ER ($ER\alpha$ and/or $ER\beta$) then undergoes a conformational change, releases its chaperone components, homodimerizes, and translocates to the nucleus. ER dimer then binds to the DBD, and co-activators are recruited to the ER/ERE complex (21).

Figure 8. Classical pathway of estrogen action.

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1.3.3 The Roles of Estrogen and Estrogen Receptor α in Breast Cancer

The implication of estrogen in breast cancer development has its basis in data from both clinical and animal studies that look at the exposure of breast epithelium to estrogen (21). For example, the development of transgenic mouse models that lack either estrogen synthesis or $ER\alpha$ and randomized clinical trial and epidemiological studies that examine

the risk of breast cancer after treatment with estrogens have been crucial in determining the role of estrogen in breast cancer (21).

In the luminal breast epithelium, estrogen binding to ER α causes proliferation of mammary cells, increases the number of ER α -positive cells within the breast tissue, and increases DNA synthesis and cell division at an accelerated rate (22). This increases the potential for replication errors, which, in turn, increases the risk of missense and nonsense mutations that can disrupt normal cellular processes, including apoptosis or cell death. Additionally, genotoxic side products created by estrogen metabolism can directly damage epithelial cells and cause mutations in the DNA. Some of these metabolites and adducts include estrogen quinone metabolites that directly interact covalently with DNA. Chronic estrogen exposure leads to endogenous DNA adducts in mice, DNA strand breaks, and 8-hydroxylation of purine bases caused by free radical generation due to redox reactions of estrogen metabolites. (22)

Currently, SERMs (*eg.*, Tamoxifen) and aromatase inhibitors are used to treat breast cancer by inhibiting the activity of ER α or the production of estrogen, with some success (22). The role of ER β in breast cancer is still controversial, but recent data suggest that it may be a positive prognostic indicator (23). A meta-analysis of studies involving early stage breast cancer patients found that ER β is positively associated with disease-free survival (DFS). These studies also show that ER β might be a better prognostic factor in ER α -positive as compared with ER α -negative patients (23). ER α -dependent actions in breast cancer were also found to involve amplification of transcriptional co-regulators, such as steroid receptor co-activator (SRC)/p160 family proteins, cAMP responsive

element-binding protein (CREB), and the SWI/SNF chromatin remodeling complex (23). Therefore, there is an array of research devoted to understanding the role of co-regulators in ER α and ER β dependent transcription and cell growth. But more importantly is the context of the steady state level of estrogen receptor, especially ER α , and how its levels are regulated in the context of breast cancer (20).

1.4 BRCA1 Function in Breast Cancer

The major focus in research on BRCA1 has been on its role in the DNA damage response, especially its role in DNA double strand break (DSB) repair by homologous recombination repair (HRR). The importance of BRCA1 in HRR is evidenced by the co-localization of BRCA1 and RAD51 in the nuclei foci and the sensitivity to ionizing radiation of BRCA1 deficient cells. However, the physical association of BRCA1 and ER α and the ability of BRCA1 to negatively regulate ER α activity is also an important function of BRCA1 since estrogen exposure and activation of ER α signaling contribute significantly to breast cancer development. Thus, over-expression of BRCA1 inhibits the estradiol (E2)-stimulated ER α activity in cells transfected with an E2-responsive luciferase reporter (24,25,26). Under-expression of BRCA1 confers increased E2-stimulated ER α activity and E2-independent activation of ER α activity (24,25,26). The ability of BRCA1 to suppress ER α activity is due to a physical interaction between the N-terminus of BRCA1 and the C-terminal AF-2 domain of ER α (24,25,26). Cancer-associated BRCA1 mutations reduce or completely abolish the ability of BRCA1 to inhibit ER α activity. The ability of BRCA1 to inhibit the expression of the ER α co-activator p300 was also found to significantly contribute to the mechanism of BRCA1-

mediated suppression of ER α activity; and the BRCA1 inhibition of ER α could be effectively rescued by the over-expression of p300 (24,25). The ability of BRCA1 to inhibit ER α activity was also found to be dependent upon its ability to regulate the relative level of ubiquitination vs acetylation of ER α (24,25,26).

The BRCA1 inhibition of ER α activity was also documented *in vivo* in mouse models of *Brcal* deficiency, where a mammary-specific deletion of *Brcal* exon 11 caused increased E2 sensitivity and promoted mammary cancer development (24-28).

1.5 Endocrine Resistance in Breast Cancer

The roots of endocrine therapy were observed in 1896 when George Beatson showed regression of metastatic breast cancer by performing an oophorectomy, thereby showing, for the first time, the link between hormones and cancer (29). By 1973, the SERM Tamoxifen was synthesized, and became the standard of care in ER α -positive breast cancer. At present, endocrine therapies, such as Tamoxifen, may be given in the context of adjuvant or combination therapies in addition to chemotherapy or radiation. In general, tamoxifen is given in oral form after chemotherapy or radiation. The main function of endocrine therapy is to interfere with critical endocrine signaling pathways involved in breast cancer by inhibiting the activity of ER α or the synthesis of estrogen. Most of the time, this type of therapy involves the exogenous administration of an antagonist to a specific hormone. Generally, these therapies reduce growth rate (cytostatic), usually by cell cycle arrest in G1/S phase. (29)

A few examples of endocrine therapies being used at present are:

1. Tamoxifen is a SERM that competitively binds to ER α . This causes a conformational change within dimerized ER α , which involves the shift of helix 12 to the AF-2 site. This conformational change prevents co-activator binding, subsequently inhibiting of estrogen signaling (29). However, Tamoxifen therapy still renders partial agonist activity because the AF-1 domain in ER α remains active.
2. Fulvestrant, a selective estrogen receptor down-regulator (SERD) that competitively binds to ER α in the monomer form, inhibiting dimerization and immobilizing ER α in the cytoplasm (29). In this unstable form, ER α is subsequently ubiquitinated and degraded. Fulvestrant does not have any agonist properties, thereby making it a pure antagonist.
3. Aromatase inhibitors inhibit estrogen synthesis by irreversibly binding to and inactivating aromatase (type 1) or reversibly binding to aromatase (type 2) (29). Type 1 drugs include the steroid inhibitor exemestane, and type 2 drugs include the non-steroidal inhibitors letrozole and anastrozole.

Although endocrine therapy has a high success rate in patients with ER α -positive breast cancer, resistance still occurs in a significant number of patients. This can be observed as an increase in the primary tumor mass and/or the development of nodules at distant metastatic sites (30). Studies showing Breast cancer recurrence in ER α -positive breast cancer vary from 30-50% over a period of 15 years (22).

A multitude of resistance mechanisms have been elucidated with the aim of improving the outcome of endocrine treatment (Fig 9). Endocrine-resistant cells maintain a high expression of proliferative genes (*eg.*, cyclin D1) and anti-apoptotic proteins (*eg.*, Bcl-2 and Bcl-X_L). The underlying molecular mechanisms of endocrine resistance are highly complex and heterogeneous. At present, a personalized medicine approach has been taken to stratify subgroups that are considered high risk in order to better predict acquired resistance and provide a tailor-made treatment according to patient need. This could include providing novel and combinatorial therapies to improve patient outcome.

Figure 9. Summary of resistance mechanisms in breast cancer.

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1.6 BRCA1-mimetics

As noted above, BRCA1 inhibition of ER α is dependent upon a direct interaction between the N-terminus of BRCA1 and the C-terminus of ER α (25,26,27). Based upon high-resolution mapping studies, the Rosen lab created a three-dimensional model of the BRCA1: ER α interaction (with ER α ligated to E2) (28). Using this model, a BRCA1-binding surface in ER α was found to contain two pockets suitable to accommodate small molecules (31). A pharmacophore database (from the National Cancer Institute/Developmental Therapeutics Program) was screened to identify small molecules that could fit into one of these pockets. Six compounds that inhibit ER α activity in both anti-estrogen-sensitive MCF-7 cells and anti-estrogen-resistant LCC9 cells were identified (31). These compounds (called “BRCA1-mimetics”) bind to ER α through a pocket distinct from the ligand-binding pocket and, thus, exhibit a different mode of interaction with ER α than Tamoxifen and Fulvestrant, both of which bind to the ligand-binding pocket. The prototype compound, A7 (NSC 31303), exhibited an IC₅₀ of 2-3 μ M for inhibition of ER α activity.

A second BRCA1: ER α interaction model, this time with ER α ligated to Tamoxifen, was then created because prior data suggests that overexpression of BRCA1 re-sensitizes Tamoxifen resistant LCC9 cells back to Tamoxifen. Based on a computer model of the BRCA1-binding pocket in ER α , the same pharmacophore database was screened and six different BRCA1-mimetic compounds were identified (32). These compounds inhibited ER α in MCF-7 cells (IC₅₀ $\leq$ 1.0 μ M); and four of the six exhibited similar activity in antiestrogen resistant LCC9 cells. NSC35446 represents a prototype such compound.

Unlike A7, these compounds partially restored sensitivity of LCC9 cells to Tamoxifen. A protonated formulation of NSC35446 (the hydrochloride salt, designated “NSC35446.HCl”) inhibited the growth of LCC9 tumor xenografts at non-toxic concentrations. Both sets of BRCA1-mimetic compounds appear to function by disrupting the interaction between ER α and an ERE.

Here, we have further elucidated the function of BRCA1-mimetics. We showed that compound NSC35446.HCl inhibits mRNA and protein expression of *IKKB*, an upstream activator of NF- κ B, and inhibits NF- κ B signaling in anti-estrogen-resistant cells. We further showed that NSC35446.HCl targets the *IKKB* promoter and disrupts the accumulation of ER α at ERE sites.

CHAPTER 2: BRCA1: ESTROGEN RECEPTOR α INTERACTION

2.1 BRCA1: Estrogen Receptor α Interaction

2.1.1 BRCA1 Inhibition of Estrogen Receptor α Activity by Direct Interaction

Evidence of direct interaction between BRCA1 and ER α is built upon the finding that overexpression of the gene encoding wild-type (wt) BRCA1 inhibits ER α signaling in multiple breast and prostate cancer cell lines (33). Various mutant/truncated forms of BRCA1 were shown to be ineffective in suppressing ER α transcriptional signaling, including four tumor/cancer associated mutations which includes: 5382insC, C5365G, T300G, and 185delAG; a consensus Rb binding motif mutation **LXCXE** (RXXRH); and three deletion mutations (Δ EcoRI, Δ KpnI, Δ BamHI) (Fig. 10) (33).

Figure 10. Mutant BRCA1 domains fail to inhibit ER α activity.

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Further evidence suggests that the amino-terminal region of BRCA1, which encompasses aa 1-300), binds to the carboxyl terminal region of ER α . Fig. 11 shows results of GST capture assays using *in vitro* translated [IVT] BRCA1 with a series of GST-ER α fusion

proteins. Fig. 11A shows the ER α constructs, while Fig. 11B shows the validation of these constructs through Western blotting. Fig. 11C shows that three carboxyl-terminal ER α peptides (ER 282-420, ER 282 -595, and ER 393 -595) bound to IVT wtBRCA1, while three amino-terminal ER α peptides (ER 1-185, ER 1-282, ER 282-337) failed to bind to the IVT wtBRCA1. Confirmation that the carboxyl-terminal region (AF-2 domain) is the binding site for BRCA1 on ER α is shown by utilizing the mutant BRCA1 domains in Fig. 10, which all showed binding to the carboxyl GST-ER 282-420 (Fig. 11E), with BRCA1 Δ *EcoRI* (aa 1-302) showing the most binding (33). Further experimental evidence shows that the GST-ER 282-420 fusion protein binds to full-length BRCA1 but fails to bind to amino-terminally truncated IVT BRCA1 peptides (BRCA1 772-1863, BRCA1 1314-1863) (Fig. 11G). Some carboxyl-terminally truncated BRCA1 mutants were also shown to exhibit dominant negative inhibitory activity, as evidenced by BRCA1 Δ *EcoRI* rescuing wtBRCA1-mediated inhibition of ER α in a dose dependent fashion in DU-145 cells transfected with wtBRCA1 protein (Fig. 11H) (33).

Furthermore, the inability of BRCA1 Δ *EcoRI* gene to inhibit ER α activity (Fig. 10) suggests that binding of a BRCA1 fragment to the AF-2/ligand binding (LBD) carboxyl-region of ER α , although necessary, is not by itself sufficient for the inhibition of ER α activity (33). The above model suggests that the amino-terminus of BRCA1 (aa 1-302) is involved in interaction with ER α but a more carboxyl-terminus domain of BRCA1 is required for ER α inhibition. Consistent with this model is evidence that suggests that BRCA1 may function as an inhibitory adapter that recruits repressor proteins to ER α that is bound to its estrogen response element (ERE) (34) and observations that several BRCA1 fragments that are missing the carboxyl-terminal region (BRCA1 aa 1-302, aa 1-772, and aa 1-1313) rescued wtBRCA1-mediated suppression of ER α activity.

2.1.2 Further Characterization of Structure/Function Relationship for the BRCA1: Estrogen Receptor α Interaction

Further characterization of the structural determinants of the BRCA1: ER α interaction revealed two separate points of contact for ER α on the amino-terminus of BRCA1: BRCA1 aa 1-100 and BRCA1 aa 101-200. Specifically, BRCA1 aa. 67-100 and 101-134 are required for ER α interaction, whereas the BRCA1 RING domain (aa 20-64) is not essential for ER α binding but is required for transcriptional repression of ER α activity (35). Essentially, the majority of BRCA1 binding to ER α could be contained within aa 1-200, with aa 1-100 being the primary interaction site as seen in co immunoprecipitation Western blots in Fig. 12a,b. Either one of these truncated BRCA1 peptides can repress ER α activity separately or when combined, as seen in ER α luciferase reporter assays, but the level of repression is significantly less than that of full-length BRCA1 (Fig. 13a,b),

suggesting that regions outside of aa 1-200 of BRCA1 are needed in order for maximal repression of ER α (35).

Figure 12. Interaction of BRCA1.
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Figure 13. N-terminal BRCA1 N-terminal, Proteins to inhibit ER activity.
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Further studies of the BRCA1: ER α interaction identified a more specific region of ER α involved in the binding to BRCA1. Within the aa 282-420 region of ER α lies a strong region of interaction within ER α aa 338-379. This primary binding site on ER α can interact with both BRCA1 aa 1-100 and BRCA1 aa 101-200, and further examination of the BRCA1 sequences that binds to this primary binding region of ER α revealed a

leucine and isoleucine-rich region (aa 67-100, 86-95) which occurs within a previously identified function HIV Rev-type nuclear export sequence (NES) (36). The HIV Rev-type NES-like sequence of BRCA1 (**LxxIxxFxL**) is somewhat similar to a consensus extended helix co-repressor motif (**Lxx(I/H)Ixxx(I/L)**) (35). When utilizing different L/I → A substitutions on the above motif within BRCA1 aa 1-100 peptides (Fig. 14), it was observed that peptides that incorporated either one (**AxxIxxFxL**) or two (**LxxAAxxFxL**) of the substitutions had little or no effect on repression (Fig. 16) and no effect on *in vivo/in vitro* binding of BRCA1 to ERα (Fig. 15). However, a BRCA1 aa 1-100 peptide that harbored three mutations (**AxxAAxxFxL**) strongly inhibited the ability of the peptide to bind (Fig. 15) and to repress ERα activity (Fig. 16) (35).

Figure 14. BRCA1 1-100 point mutations.
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Figure 15. BRCA1 aa 1-100 mutant peptide relative binding to ER α .

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Figure 16. BRCA1 1-100 mutations inhibition of ER activity.

Fig. 17 shows a model structure for the BRCA1: ER α complex (Global Range Molecular Matching), which is based on the published structures of the BRCA1 amino-terminus (aa 1-103) and the ER α AF-2/LBD. This model shows BRCA1: ER α interactions involving paired antiparallel α -helices, an interaction with aa 420-595 of ER α to the RING domain of BRCA1, and a prominent interaction involving helix three of BRCA1 (aa 80-96) and

the α -helix of ER α (aa 338-379) (35). The amino-terminus of BRCA1 is shown to interact with BARD1 (BRCA1-associated RING domain 1), which is involved in ubiquitin ligase activity that contributes to tumor suppression (37). Several key residues were also identified as important for the BRCA1: ER α complex for their role in hydrophobic interactions critical for the stability of the complex. Some of these residues are: L-82, L-86, I-89, A-92, and L-95. Note that this model predicts a relatively broad interaction surface for BRCA1 on ER α , but our subsequent studies showed that this surface contains several small pockets wide and deep enough to accommodate small molecules.

Figure 17. Model structure of BRCA1:ER complex.
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2.1.3 Role Tamoxifen in Mammary Cancer Development in BRCA1 Mutation Breast Cancer Mouse Model

The direct interaction of BRCA1 with ER α causes inhibition of ER α transcriptional activity and points to a possible role for estrogen in the early stages of BRCA1 mutation-related cancer development. SERMS (Selective Estrogen Modulators) such as Tamoxifen target ER α and can cause regression of significant size tumors. Most BRCA1 mutation-related breast cancers are ER α negative but estrogen and ER α still may play a role during the early stages of ER α -negative cancer development and hence may still be targeted by Tamoxifen in this context. However, it has been shown that tamoxifen is not protective in a MMTV-Cre Brca1 conditional exon 11 deleted mouse model (38). In fact, tamoxifen behaved more as a growth agonist than an agonist in this model as evidenced by increased mammary epithelial cell proliferation (Fig. 18 a,b), ductal growth and promotion of mammary adenocarcinoma development (Fig. 19). Recurring exposure to tamoxifen resulted in downregulation of nuclear localized ER β , but not ER α (Fig 18 e,f) (38). Whereas, recurring exposure to estrogenic resulted in downregulation of nuclear localized ER α (38). Note that this model uses a haplo-insufficient p53 gene (p53^{+/-}), which promotes tumor formation.

Figure 18. Detection of apoptotic cells through apotag and detection of nuclear ER and ER.

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Figure 19. Detection of tumors in placebo and tamoxifen treated BRCA1Co/C0 MMTV-Cre/p53+/- female mice.

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Consistent with the mouse model observation, decreased levels of BRCA1 expression resulted in increased agonist activity of tamoxifen in the MCF-7 cell line (Fig. 20) (38).

Figure 20. Effect of reduced BRCA1 expression in MCF-7 cells on Tamoxifen agonist activity.

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A possible mechanism that explains this increase in Tamoxifen agonist activity in this model is that a conformational change in ER α induced by Tamoxifen alters the ability of ER α to interact with corepressors relative to coactivators, thereby altering ER α signaling. Changes in coactivator/corepressor expression levels associated with BRCA1 may explain the agonist activity of Tamoxifen in this model. Consistent with this idea is that loss of BRCA1 has been observed to increase ER α transcriptional activity by enhancing p300 expression levels (39).

2.1.4 Estrogen Signaling and BRCA1 Loss

Using the above conditional *Brcal* knockout model, loss of *Brcal* exon 11 (the largest exon in *Brcal*, encoding 60% of the protein) in mouse mammary epithelial cells resulted in an increased response to estrogen stimulation that was consistent with enhanced development of pre-neoplasia and invasive mammary cancer (Figs. 20,21) as well as weakened differentiation responses after exogenous estrogen and progesterone exposure (Fig. 22). The data are consistent with the fact that either mutation of *Brcal* does not terminate mammary gland development but, alternatively, it produces a mammary gland that is overly sensitive to hormonal stimulation and is predisposed towards cancer development (40).

Figure 21. Mammary glands ducts as a result of BRCA1 ablation.
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Figure 22. Alveolar differentiation as a result of BRCA1 ablation.
(a,c,e,g- *Brca1* ablated: b,d,f,h – wt) *Oncogene* (2008) 27, 794-802 © 2008 Nature Publishing Group

Figure 23 Result of exogenous estrogen exposure in BRCA1 ablated mice.
(a,c,e,g-Placebo: b,d,f,h- Treated) Oncogene (2008) 27, 794-802 © 2008 Nature Publishing Group

Cell proliferation induced by estrogen is normally a paracrine mediated response, involving pathways such as epidermal growth factor (EGF) and insulin-like growth factor (IGF) signaling. Brca1 can inhibit the above signaling pathways that are involved in proliferation and increase activity of pathways, such as TGF- β , that are involved in inhibition of proliferation. Therefore, the loss of Brca1 and p53 insufficiency may be combining with decreased regulatory factors such as TGF- β signaling and increased proliferation pathways such as IGF signaling to contribute to an abnormal response to estrogen (40)

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell Lines and Culture

Human breast cancer cells (LCC9, T47DCO, MCF-7, MDA-MB-231, MDA-MB-468, and HCC1806) were obtained from the Lombardi Comprehensive Cancer Center (LCCC) Tissue Culture Shared Resource (TCSR). MCF-7, MD-MBA 231, and HCC1806 were grown in DMEM plus 5% fetal calf serum (FCS), L-glutamine (5 mM), non-essential amino acids (5 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Lonza, Walkersville, MD). LCC9 and T47DCO were grown in phenol-red-free IMEM (Corning Cellgro, Manassas, VA) plus 5% charcoal-stripped serum (CSS) (from the TCSR). MDA-MB-468 cells were grown in RPMI 1640 (Corning Cellgro) plus 5% FCS. All cell lines were tested for mycoplasma using the MycoAlert™ Mycoplasma Detection Kit (Lonza) (cell lines were tested for mycoplasma yearly) and were mycoplasma-free. All cell lines were authenticated at LCCC by DNA fingerprinting using the PowerPlex^R 16 System (Promega, Madison, WI).

3.2 Reagents

Trypan blue was purchased from Thermo Scientific (Waltham, MA). Compound A7 (NSC31303) was obtained from the National Cancer Institute/Developmental Therapeutics Program and was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) (51). Compound NSC35446.HCl was synthesized as described earlier (50) and dissolved in DMSO (vehicle). Small molecule cell-permeable caspase inhibitors were purchased in solution in DMSO from Millipore (Billerica, MA): InSolution™ Caspase Inhibitor I (pan caspase inhibitor) (Z-VAD-FMK) and InSolution™ Caspase-8 Inhibitor II (selective caspase-8 inhibitor) (Z-IETD-FMK). Fulvestrant (Sigma-Aldrich) was a gift from Dr. Robert Clarke (Department of Oncology, Georgetown University). 17β-Estradiol (E2) was purchased from Sigma-Aldrich and dissolved in ethanol (Sigma-Aldrich).

3.3 Cell Proliferation Assays

Growth media for proliferation assays were phenol red-free IMEM containing 1% CSS for LCC9 and T47DCO, DMEM containing 1% FCS for MDA-MB-231 and HCC1806, and RPMI 1640 plus 1% FCS for MDA-MB-468. Subconfluent proliferating cells were seeded into 6-well dishes at 1×10^5 cells/well on Day 0 and allowed to attach and recover for 24-hr. The cells were treated with vehicle or NSC35446.HCl (10 μ M for T47DCO and 5 μ M for all others) starting on Day 1, with daily re-feeding with fresh medium containing vehicle or compound, respectively. Trypan blue dye-excluding cells were counted in triplicate wells by hemocytometer on Days 1, 3 and 5. For experiments using caspase inhibitors, the cells were pre-incubated for 12-hr with the indicated inhibitor(s) (10 μ M). Each independent experiment was performed three times.

3.4 Cell-Cycle Analysis

Subconfluent proliferating cells were seeded into 100-mm dishes at 6×10^5 cells/dish, allowed to attach for 24-hr, and treated with vehicle or NSC35446.HCl (5 μ M) for 48-hr. The cells were then harvested using trypsin, fixed using 70% ethanol, and stained with propidium iodide (BD Biosciences, San Jose, CA). Samples were analyzed on a BD LSRFortessaTM Cell Analyzer (LCCC Flow Cytometry Shared Resource). DNA histograms were analyzed using ModFit software (Verity Software House, Topsham, ME).

3.5 Mammosphere Assays

LCC9 or MDA-MB-231 cells were treated with vehicle or NSC35446.HCl (5 μ M) for five days, after which the cells were re-plated in six-well non-adherent dishes (Corning, NY) at 5.0×10^3 cells/well. Mammospheres/well or non-mammosphere aggregates/well were counted after seven days with an inverted phase contrast microscope (Olympus IX-71), using a grid. Three independent experiments were performed.

3.6 RNA-seq

Subconfluent proliferating LCC9 cells were treated with vehicle (DMSO) or A7 (5 μ M) for 24-hr and harvested for RNA extraction. Two independent experiments were performed. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA) and submitted to Otogenetics Corporation (Norcross, GA) for RNA-seq analysis. After verifying the integrity and purity of total RNA, cDNA was generated using a SMARTer^R PCR cDNA Synthesis Kit (Clontech Laboratories, *Inc.*, Mountain View, CA). Reference measurements were composed of 21,366,072 total reads/sample for vehicle-treated control with 89.5% of RNA-seq reads mapped to the human genome and 21,619,802 total reads/sample for A7-treated cells with 87.4% of RNA-seq reads mapped to the human genome. Cufflinks suite was used to map reads to Human genome 19 and was done James Li of Georgetown Lombardi Cancer center. BAM files that showed FPKM values for specific genes up/downregulated as a result of treatments was submitted to NCBI GEO public database. The FPKM cutoff was $P < .05$. The results were analyzed using Ingenuity Pathway Analysis software (Qiagen Bioinformatics, Redwood City, CA).

3.7 qRT-PCR

After the indicated cell treatment, RNA was extracted using the miRNeasy Mini Kit (Qiagen) and converted to cDNA using Taqman^R Reverse Transcription Reagents (Applied Biosystems-Roche, Foster City, CA), using 3 μ g of total cellular RNA. Five μ L (5%) of the cDNA was used for each PCR reaction. All PCRs were carried out in a 25- μ L reaction volume, containing 5 μ L of cDNA, 12.5 μ L of Power SYBR^R Green PCR Master Mix [containing SYBR Green I dye, AmpliTaq Gold DNA Polymerase, deoxynucleotide triphosphates, passive reference, optimized buffer components (Applied Biosystems)], 2.5 μ L of nuclease-free water (Ambion-Life Thermo Fisher, Waltham, MA), and 5 μ L (2

μg) of primers (IDT Technologies, Coralville, IA) (see Table 1). β-Actin was used as a control for loading. The thermal cycling conditions were as follows: denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min, for a total of 45 cycles.

Table 2. Primers utilized for qRT-PCR studies.

Gene name	Forward	Reverse	Start position (bp)	Amplicon size (bp)
<i>ACP5</i>	5'-GGGTGCAGACTTCATCCTGT-3'	5'-GAAGTGCAGGCGGTAGAAAAG-3'	+488	241
<i>CUTA</i>	5'-AAAGGGAAGATCGAGGAAGA-3'	5'-GTACGGAAGTTCCCCTGTT-3'	+486	153
<i>PHC2</i>	5'-GAGCAGAGCCAGACAGACAT-3'	5'-GAGGAAGCCAGTGCTATCAA-3'	+2678	209
<i>MAP3K7</i>	5'-CAGCCAAGACGTAGATCCATC-3'	5'-GGTCCTGAGGTAGTAATCATTCTG-3'	+200	110
<i>PIK3R3</i>	5'-GTCACACACAAGAACAACATAGC-3'	5'-TCATCAACAAGTGGGTAGTCAG-3'	+1385	80
<i>ZNF684</i>	5'-CAAGGTTAGAGCAAGGACAAGA-3'	5'-TCATCAACAAGTGGGTAGTCAG-3'	+520	90
<i>GRB2</i>	5'-GAAGCCATCGCCAAATATGAC-3'	5'-TTAAGCTCTGCCTTGACCAG-3'	+315	122
<i>IKKB</i>	5'-CAGCAAGGAGAACAGAGGT-3'	5'-ACTGTGTACTTCTGCTGCTC-3'	+2678	209
<i>pS2</i>	5'-TGACACCAGGAAAAACCACAA-3'	5'-CCATGGAGAACAAGGTGATCT-3'	+48	132
<i>β-Actin</i>	5'-CCTTGCACATGCCGGAG-3'	5'-ACAGAGCCTCGCCTTTG-3'	+30	110

3.8 Western Blotting

Subconfluent proliferating cells were seeded into 100-mm dishes (@6 x 10⁵ cells/dish), incubated for 24-hr, and treated with vehicle or NSC35446.HCl for the indicated time before lysis in RIPA buffer. Equal aliquots of whole-cell protein (30 μg) were electrophoresed on 4–20% SDS-polyacrylamide gradient gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), and blotted overnight using primary antibodies directed against: IKKA (3G12, mouse monoclonal, 1:1000 dilution,

Cell Signaling, Danvers, MA); IKKB (D30C6, rabbit monoclonal, 1:1000, Cell Signaling); IKKG (DA10-12, mouse monoclonal, 1:2000, Cell Signaling); pS2/TFF1 (rabbit polyclonal, 1:2000, Cell Signaling); IRF-1 (D5E4, rabbit monoclonal, 1:2000, Cell Signaling); Caspase-7 (C7, mouse monoclonal, 1:1000, Cell Signaling); Caspase-8 (1C12, mouse monoclonal, 1:1000, Cell Signaling); cIAP1 (D569, rabbit monoclonal, 1:1000, Cell Signaling); TRAF-2 (54H6, rabbit monoclonal, 1:1000, Cell Signaling); Bcl-XL (D569, rabbit monoclonal, 1:1000, Cell Signaling); Bcl-2 (50E3, rabbit monoclonal, 1:1000, Cell Signaling); ER α (6F-11, mouse monoclonal, 1:500, Vector Laboratories, Burlingame, CA); and GAPDH (D16H11, rabbit monoclonal, 1:2000, Cell Signaling). The membranes were blotted for 1 hr with the appropriate secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). The blotted proteins were visualized with the Hyglo chemiluminescent HRP detection system (Denville Scientific, Holliston, MA) using the AI-600 imager (Amersham Biosciences, Marlborough, MA), with colored molecular size markers (Bio-Rad Laboratories, Hercules, CA). For each condition tested, three or four independent experiments were performed. Densitometry was performed using ImageJ software (NIH) (<https://imagej.nih.gov/ij/>). All primary antibodies were validated by Cell Signaling by under-expression (*eg.*, siRNA) and/or over-expression.

3.9 Assays of NF- κ B Transcriptional Activity

Subconfluent proliferating cells in 6-well dishes were transfected overnight with 1 μ g of NF- κ B reporter pGL3-NF- κ B-luc or 1 μ g of control plasmid pGL3-luc (gifts from Dr. Robert Clarke, Department of Oncology, Georgetown University) and 1 μ g of Renilla luciferase plasmid (a gift from Dr. Rebecca Riggins, Department of Oncology, Georgetown University) in serum-free IMEM containing Lipofectamine^R LTX (Life Technologies, Washington, DC). The cells were washed, incubated in IMEM containing 5% CSS, and treated with vehicle or NSC35446.HCl (5 μ M) for 48-hr. Lysates were

analyzed using the Dual-Luciferase^R Reporter Assay System (Promega), with a MicroLumat^{Plus} LB 96V Microplate Luminometer (Eg&G, Gaithersburg, MD). Luciferase activity was normalized to the *Renilla* signal. Each assay condition was tested in quadruplicate, and each independent experiment was performed three times.

3.10 TUNEL Assays

Cells were seeded into 100-mm dishes (6×10^5 cells/dish), allowed to attach for 24-hr, and treated with vehicle or NSC35446.HCl at the indicated concentration for 48-hr. TUNEL assays were performed using an APO-BRDUTM Kit (BD Biosciences). After treatment, the cells were counted and fixed with paraformaldehyde/ethanol to cross-link the DNA. The volume equivalent of 5×10^5 cells was used for fixation. The fixed cells were treated with terminal deoxytransferase (TdT) enzyme and Br-dUTP, stained with an FITC-labeled anti-BrdU monoclonal antibody, and incubated for 60 min at 37°C. The cells were washed, re-suspended, and analyzed to detect FITC-labeled fragmented apoptotic DNA by flow cytometry on a BD LSRFortessaTM Cell Analyzer at the LCCC Flow Cytometry Shared Resource. FITC-labeled DNA histograms were analyzed using ModFit software.

3.11 Annotating Putative ERE Half-Sites and ERE-like Full-Sites in the *IKKB/IKBKB* Promoter

R packages (R project free software environment for statistical computing and graphics (<https://cran.r-project.org/>) for genomic coordinate and sequence manipulation were used to extract sequence corresponding to the promoter region of the *IKKB* gene up to 10,000 bp upstream of the transcription start site (TSS). Genomic sequence for this region was extracted using the *BSgenome* and *BSgenome.HsapiensUCSC.hg19* R packages (Bioconductor). Pattern matching was then performed on the extracted sequence using the *Biostrings* package (Bioconductor), looking for "TGACC" or "GGTCA" with no mismatches (half-sites) or for "GGTCANNNTGACC" (where N=any nucleotide) with up

to two mismatches, permitting indels (insertion or deletion of nucleotides). The discovered coordinates were annotated for ChIP verification. See Table 4 in the Results section for a listing of the locations of nine ERE half-sites and one ERE-like full-site within the *IKKB* promoter. One such half-site ERE (TGACC half-site 1) was adjacent to a specificity protein 1 (Sp1) site.

3.12 Chromatin Immunoprecipitation (ChIP) Assays

Subconfluent proliferating LCC9 cells were seeded into 100-mm dishes (@6 x 10⁵ cells/dish). After a 24-hr incubation to allow for attachment, cells were treated with vehicle or NSC35446.HCl (5 μM) for 48-hr. ChIP assays were performed using a combination of two commercial kits: the truChIP™ Chromatin Shearing Reagent Kit (Covaris, Woburn, MA) and the Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit (Millipore). Cross-linking of the DNA-protein complexes was carried out by addition of 11.1% formaldehyde and incubation for 5 min @ 25°C. Cells were lysed by addition of Lysis Buffer A (Covaris) followed by Shearing Buffer (1 mM EDTA, 10 mM Tris-HCl/pH 7.6, 0.1% SDS). The lysates were sonicated using the M220 Focused-ultrasonicator™ (Covaris) @ 6°C; and the concentration of sheared DNA was measured by NanoDrop. Four μg of antibody [ERα antibody (clone AER304, mouse monoclonal, Millipore) or mouse non-immune IgG (Millipore) as a control] was utilized for IPs. The ERα IP antibody was validated by Millipore (Validated by chromatin immunoprecipitation followed by PCR using primers for TFF1). The antibody-sheared DNA was incubated with protein A/G magnetic beads (Millipore) for pre-linking @ 4°C for 2 hr. Five percent of undiluted sheared chromatin was set aside as input. A volume of sheared chromatin corresponding to 7 x 10⁵ cells was incubated with the beads at 4°C overnight. Beads were washed with low stringency IP wash buffer (Millipore). The DNA was eluted from the beads by overnight treatment with Proteinase K and RNase H at

57°C. It was purified using the QIAquick PCR Purification Kit (Qiagen). Purified DNA was subjected to qPCR amplification of sequences surrounding the previously identified EREs (see Table 2 in the Results section). Primers and were obtained from IDT Technologies. The thermal cycling conditions were as follows: denaturation at 95°C for 15 sec annealing/extension at 60°C for 1 min, for a total of 45 cycles.

In some experiments, antiestrogen-resistant T47DCO cells were treated $\pm$ NSC35446.HCl (10 μ M) for 48-hr, and ChIP assays were performed to quantify ER α at the known full-site ERE of the *pS2/TFF1* promoter (21) or the ERE-like full-site of the *IKKB* promoter. In other experiments, antiestrogen-sensitive/estrogen-sensitive MCF-7 cells were treated $\pm$ E2 (10 nM) and $\pm$ Fulvestrant (1 μ M) for 120 min and assayed to detect ER α at the known EREs of the *pS2* or *GREB1* (22) promoters (see Table 4 in the Results section). In other experiments, MCF-7 cells were treated with vehicle or NSC35446.HCl (5 μ M) for 48-hr and then exposed to E2 (10 nM) for 120 min and assayed to quantify ER α at the EREs of the *pS2* or *GREB1* promoters.

3.13 Statistical Analysis

All values are reported as means $\pm$ SEMs of three or four independent experiments.

Statistics were calculated using the two-tailed Student's t-test, with $p < 0.05$ being considered significant. Statistical significance was analyzed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA).

CHAPTER 4: RESULTS

4.1 NSC 35446.HCl Alters Cell Growth in Antiestrogen Resistant Cell Lines

4.1.1 NSC 35446.HCl Cell Proliferation Assays

NSC 35446.HCl (see Fig.23) significantly inhibited cell proliferation in anti-estrogen-resistant LCC9 and T47DCO cells but not in ER α -negative MDA-MB-231 and HCC 1806 cells (Fig.24). Similarly, the compound did not inhibit proliferation in ER α -negative MDA-MB-468 cells (data not shown). Note that a higher concentration of NSC 35446.HCl was used for T47DCO cells (10 μ M) than for LCC9 cells (5 μ M) because T47DCO cells were slightly less sensitive to the compound than LCC9.

Figure 24. Chemical structure of compound NSC35446.HCl.

Figure 25. Effect of compound NSC35446.HCl on the growth of various human breast cancer cell lines. Growth curves were performed for two anti-estrogen-resistant cell lines (LCC9 and T47DCO) and two ER α -negative cell lines (HCC 1806 and MD-MBA-231). Cells were treated with vehicle (DMSO) or compound NSC35446.HCl (5 μ M for LCC9, 10 μ M for T47DCO, and 5 μ M for HCC1806 and MDA-MB-231) starting on Day 1. Viable cells were counted using trypan blue dye exclusion. Curves are means $\pm$ SEMs of three independent experiments

4.1.2 NSC35446.HCl Cell Cycle Assays

Although the compound did not significantly alter the cell-cycle distribution of LCC9 cells, there was a significant sub-G1 population in the compound-treated cells (40%), consistent with induction of apoptosis by the compound (Fig. 25).

Figure 26. Effect of compound NSC35446.HCl on cell cycle distribution of LCC9 cells. LCC9 cells were treated with vehicle or NSC35446.HCl (5 μM) for 48 hr and analyzed to determine the cell-cycle distribution by flow cytometry. Typical flow cytometry histograms for vehicle- and compound-treated cells are shown. The bar graph shows represents the means ± SEMs of three independent experiments.

4.1.3 NSC 35446.HCl Mammosphere Assays

Consistent with its ability to inhibit cell proliferation, NSC35446.HCl significantly inhibited mammosphere formation by LCC9, whereas formation of mammospheres and non-mammosphere aggregates by MDA-MB-231 was unaffected (Fig. 26). Thus, the compound may target the mammosphere-forming capacity, which correlates with the number of cancer stem cells, in the LCC9 cell line.

Figure 27. Effect of NSC35446.HCl on mammosphere formation in human breast cancer cells. LCC9 and MDA-MB-231 cells were initially treated with vehicle or compound NSC 35446.HCl (5 μ M) for five days, after which they were re-plated in six-well non-adherent dishes at 5.0×10^3 cells/well. Mammospheres or non-mammosphere aggregates were counted after seven days by microscopy. Values are means $\pm$ SEMs of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns, not significant”.

4.2 RNA-seq Analysis of LCC9 Cells Treated with BRCA1-mimetic

4.2.1 Compound A7 RNA-seq

We initially evaluated the effects of compound A7 (NSC31303) (see Fig. 27) on LCC9 gene expression using RNA-seq. Cells were treated for 24 hr with vehicle or A7. We identified a large set of genes differentially expressed in A7-treated cells as compared with vehicle-treated cells (data not shown).

Figure 28. Chemical structure of compound A7 (NSC31303).

4.2.2 qRT-PCR Validation of RNA-seq

A selected set of eight genes (these genes were selected because they consistently showed up in canonical pathways relevant to cancer) that were up- or down-regulated by A7 (see Table 1) were validated by qRT-PCR (Fig. 28). The validation study revealed that all eight genes were altered in the same direction as in the RNA-seq analysis. We next tested the ability of NSC35446.HCl to alter expression of the same gene set. This validation revealed that seven of the eight genes (all except *MAP3K7*) were regulated in the same direction by NSC35446.HCl as by A7 (Fig. 28). As a positive control, both A7 and NSC 35446.HCl significantly down-regulated expression of *pS2*, a known ER α -responsive gene (Figs. 2B and 2C).

Table 3. RNA-seq data for eight selected genes regulated by compound A7 (NSC 31303) in LCC9 cells.

Gene name	Fold change ¹	p value
<i>ACP5</i>	+6.1	0.017
<i>CUTA</i>	+13.3	0.007
<i>PHC2</i>	+9.8	0.022
<i>MAP3K7</i>	+2.5	0.022
<i>PIK3R3</i>	-4.1	0.0096
<i>ZNF684</i>	-1.9 x 10 ⁹	0.00012
<i>GRB2</i>	+2.8	0.00024
<i>IKKB</i>	-2.0	0.032

¹A7-treated cells relative to control (vehicle)-treated cells

Figure 29. Validation of selected RNA-seq data by qRT-PCR. LCC9 cells were treated with vehicle or A7 (5 μ M) for 24 hr, after which the RNA was isolated and the samples were analyzed by RNA-seq. Validation of selected RNA-seq data was carried out with qRT-PCR using A7 (B) or compound NSC 35446.HCl

4.2.3 Pathway Studio Analysis of RNA-seq Data

Pathway Studio Analysis of RNA-seq data revealed differential expression around the NF- κ B signaling pathway (Fig.29), consistent with down-regulation of *IKKB* expression shown in both validations. We, therefore, selected *IKKB* and the NF- κ B signaling

pathway for further study. These studies were performed using NSC 35446.HCl because this compound was more potent than A7 and worked in animal experiments (51).

Figure 30. Sample tree of biologically relevant signaling pathways based on RNA-seq of compound A7 (NSC31303)-treated cells. The data were analyzed using Ingenuity Pathway Analysis software.

4.3 NSC35446.HCl Alters Expression of IKKB and Other IKK Proteins in LCC9 cells

4.3.1 Protein Expression Following NSC35446.HCl Treatment at 24, 48, 72 hr

We next tested the effect of NSC35446.HCl on the expression of IKKB and two other IKK complex proteins. Western blotting with densitometry revealed that NSC35446.HCl caused a non-significant effect at 24 hr (Fig. 30A, D) and a significant decrease of IKKB,

IKKA, IKKG protein levels in LCC9 cells at 48 and 72 hr (Figs. 30B and 30C, 30E and 30F). As a positive control, NSC35446.HCl caused significant decreases in pS2 protein levels at 48 and 72 hr (Figs. 3B and 3C; 3G).

A

B

C

D

E

F

G

Figure 31. Effect of compound NSC35446.HCl on the expression of IKKB protein and that of two other IKK isoforms. (A-C) LCC9 cells were treated with vehicle or compound NSC35446-HCl (5 μ M) every 24 hr and harvested at 24, 48, and 72 hr, respectively. Protein expression of each IKK isoform and pS2 (positive control) were analyzed by Western blotting. The results of three independent experiments are shown on each blot (D-G). Densitometry quantification of pixel images was performed using Image J software and the data were normalized to the GAPDH values. Bar graphs show means $\pm$ SEMs of three independent experiments, expressed in terms of arbitrary units (AU). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns, non significant.”

4.3.2 mRNA Expression Following NSC35446.HCl Treatment at 6, 12, 24, and 48 hr

The effect of the compound on *IKKB* mRNA levels was quantified by qRT-PCR at various time points (Fig. 31). At the 6-hr time point, there was no significant reduction in *IKKB* transcript expression, but transcript levels were significantly decreased at 12, 24, and 48 hr. Thus, a reduction in *IKKB* mRNA precedes that of the protein.

Figure 32. Time course for effect of compound NSC35446.HCl on *IKKB* mRNA expression. *IKKB* mRNA expression was measured after a 6, 12, 24, or 48hr incubation with vehicle or compound NSC35446.HCl (5 μ M) by qRT-PCR, using β -actin as the control gene. Values are means $\pm$ SEMs of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns , not significant.”

4.4 NSC35446.HCl affects NF- κ B Transcriptional Activity and NF- κ B-Responsive Gene Expression in Antiestrogen-Resistant Cells

4.4.1 NF- κ B Luciferase Assays

Because NF- κ B stimulates the expression of pro-proliferative and anti-apoptotic genes (23), we investigated whether NSC35446.HCl alters NF- κ B activity, using the NF- κ B-responsive reporter pGL3-NF- κ B-luc and control plasmid pGL3-luc. Vehicle-treated LCC9 cells showed easily measurable NF- κ B activity, which was significantly decreased by a 48-hr exposure to NSC35446.HCl. However, NSC35446.HCl treatment did not reduce NF- κ B activity in ER α -negative MDA-MB-231 cells (Fig. 32).

Figure 33. Effect of compound NSC35446.HCl on NF-κB transcriptional activity.

Cells were transfected overnight with plasmid pGL3-NF-κB-luc or control plasmid pGL3-luc and with Renilla luciferase plasmid and were treated with vehicle or compound NSC35446.HCl (5 μM) for 48 hr. The cells were harvested and luciferase activity was detected and normalized to the *Renilla* signal. Values shown are means ± SEMs of three independent experiments.

4.4.2 Effect of Compound NSC35446.HCl on Protein Levels of NF-κB-Regulated Genes

We examined the effects of the compound on protein levels of several NF-κB-regulated genes. Western blotting with densitometry showed a significant decrease in the levels of anti-apoptotic proteins cIAP1, TRAF2, Bcl-2, and Bcl-X_L in LCC9 and T47DCO cells (Fig. 33 B,C; Figs. D, E, F). NSC35446.HCl also caused a significant increase of IRF-1 protein in LCC9 and T47DCO cells (Fig. 33C; Figs D, E, F). IRF-1, an NF-κB-regulated gene (24), is a tumor suppressor that induces apoptosis (25,26). Thus, our compound inhibits NF-κB activity, consistent with its ability to reduce protein expression of IKKB, IKKA, and IKKG.

B

C

D

E

T47DCO

F

Figure 34. Effect of compound NSC35446.HCl on NF-κB-responsive gene expression in LCC9 and T47DCO cells. A,B,C,E. Western blotting of NF-κB-responsive genes was performed after a 48-hr treatment with vehicle or compound NSC35446.HCl (5 μM for LCC9 and 10 μM for T47DCO cells). Data are shown for three or four independent experiments. D,F. Densitometric quantification of the above Western blots. Values are normalized to GAPDH and expressed in arbitrary units (AU). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns, not significant”

4.5 Effect of Compound NSC35446.HCl on Apoptosis in LCC9 Cells

4.5.1 Caspase-7 Cleavage After Treatment with NSC35446.HCl

We next tested whether the compound could induce apoptosis in LCC9 cells. These cells lack caspase-3, so we tested the ability of the compound to cause cleavage of caspase-7. Although NSC35446.HCl did not cause a reduction in uncleaved caspase-7, it caused a significant increase in cleaved caspase-7 (Fig. 34A; Supplementary Fig 34B).

A

B

Figure 35. Effect of compound NSC35446.HCl on cleavage of caspase-7 in LCC9 cells. (A) Cells were treated with vehicle or 5 μ M of compound NSC35446.HCl for 48 hr. Whole cell lysates were analyzed by Western blotting for uncleaved and cleaved caspase-7. Four independent experiments are shown. (B) Quantification of each Western blot was performed using Image J software and the data were normalized to the GAPDH values. Values plotted are means $\pm$ SEMs of three independent experiments and are expressed in arbitrary units (AU). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns, not significant”

4.5.2 Effect of Compound NSC35446.HCl without and with Caspase Inhibitors on Proliferation of LCC9 Cells

Treatment with a pan caspase inhibitor, a caspase-8 inhibitor, or both in combination with compound NSC35446.HCl blocked the inhibition of cell proliferation due to NSC35446.HCl, suggesting that some of this inhibition is due to apoptosis (Fig. 35).

Figure 36. Effect of compound NSC35446.HCl without and with caspase inhibitors on proliferation of LCC9 cells. LCC9 cells were treated $\pm$ the indicated caspase inhibitor(s) (10 μ M) and $\pm$ compound NSC35446.HCl (5 μ M), and growth curves were performed. Curves are means $\pm$ SEMs of three independent experiments.

4.5.3 Caspase 7 Expression After Treatment with Compound NSC35446.HCl Without and With Caspase Inhibitors

Another Western blot study revealed that a combination of caspase inhibitors with compound blocked the increase in cleaved caspase-7 due to the compound (Fig. 36D; Fig. 36E). Western blotting was also performed to detect caspase-8, but no caspase-8 protein was detected (data not shown). Unlike LCC9, NSC35446.HCl did not cause down-regulation of IKKB protein or caspase-7 cleavage in two ER α -negative cell lines (MDA-MB-468 and HCC 1806) (Fig. 36F).

A

LCC9

B

MDA-
MB-468

Vehicle (DMSO)	+	+	+	-	-	-	Vehicle (DMSO)	+	+	+	-	-	-
35446.HCl (5 μ M)	-	-	-	+	+	+	35446.HCl (5 μ M)	-	-	-	+	+	+
IKKB 87 kDa							IKKB 87 kDa						
Uncleaved CASP7 35 kDa							Uncleaved CASP7 35 kDa						
Cleaved CASP7 20 kDa							Cleaved CASP7 20 kDa						
GAPDH 37kDa							GAPDH 37kDa						

Figure 37. Effect of compound NSC35446.HCl alone or in combination with caspase inhibitors on caspase-7 cleavage in LCC9 and other cell lines. (A) Antiestrogen-resistant LCC9 cells were treated with compound NSC35446.HCl (5 μ M) for 48-hr without or with caspase inhibitors and subjected to Western blotting for caspase-7 cleavage. The Western blot shown is representative of three independent experiments. Panel B shows densitometric quantification of the Western blots in panel A. (C) ER α -negative MDA-MB-468 and HCC1806 cells were treated with compound NSC35446.HCl (5 μ M) for 48-hr and subjected to Western blotting to detect IKKB and caspase-7 cleavage. Western blots were quantified by densitometry and normalized to GAPDH (arbitrary units, AU). The data shown reflect three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, ““ns, not significant”” significant.

4.5.4 Effect of Compound NSC35446.HCl on Apoptosis in Human Breast Cancer Cell Lines

Finally, we directly tested the effect of a 48-hr treatment with vehicle or compound on apoptosis using a flow cytometric TUNEL assay to detect FITC-labeled apoptotic fragmented DNA. This experiment revealed less than 10% apoptosis in vehicle-treated cells but a large apoptotic fraction (*ca.* 50%) in compound-treated cells (Fig. 37G). Finally, NSC35446.HCl also caused apoptosis in T47DCO cells but not in ER α -negative MDA-MB-231 or HCC 1806 cells (Fig. 37H, 37I). These data are consistent with the proliferation and cell-cycle data in Figs. 24 and 25.

A LCC9

B T47DCO

C

Figure 38. TUNEL apoptosis assays of four human breast cancer cell lines treated without or with compound NSC35446.HCl for 48-hr. (A-C) Cells were treated daily with vehicle or compound NSC35446.HCl (10 μ M for T47DCO and 5 μ M for all other cell lines) for 48-hr, after which a TUNEL assay was performed using the APO-BRDUTM Kit (BD Biosciences) and flow cytometry to detect FITC-labeled apoptotic fragmented DNA. The histograms shown are representative of three independent experiments. The ordinate represents cell count; and the abscissa represents the FITC label. Values plotted are means $\pm$ SEMs of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. “ns, not significant”.

4.6 The *IKKB* Promoter Contains ERE Half-Sites and an ERE-like Full-Site

We sought to determine if inhibition of *IKKB* expression by NSC35446.HCl correlates with decreased ER α occupancy on the *IKKB* promoter. Thus, BRCA1-mimetics were shown to reduce expression of the known E2/ER α -responsive gene *pS2* by reducing ER α

occupancy at the known ERE in the *pS2* promoter (19,20). Since *IKKB* is not known to be an E2- or ER α -regulated gene, we first performed an *in silico* analysis to identify putative EREs in the *IKKB* promoter upstream to -10,000 bp from the TSS. Based on this analysis (see Methods section), we identified one ERE-like full-site (differing from the canonical ERE by one nucleotide) and nine ERE half-sites (five TGACC sites and four GGTCA sites) (Table 2). These sites were all located well upstream in the *IKKB* promoter, between bp -9539 and -4607. Our *in silico* analysis also revealed that one such half-site (TGACC half-site 1), which showed the highest accumulation of ER α of any site, is adjacent to an Sp1 site.

Table 4. ChIP assay PCR primers for ERE half-sites and an ERE-like full-site in the *IKKB* promoter.

ERE (half/full)	Forward	Reverse	Amplicon size (bp)	Location from transcription start site (bp)
TGACC (half-site 1)	5'- GCTGATGACCTCTCTTTGAACT A-3'	5'- CAGGGACACCCAGGAATATAAC-3'	78	-9539 to -9535
TGACC (half-site 2)	5'-CAGCCCACACTTCTCTTTCT- 3'	5'-TGTGCAGGCAACCAACT-3'	82	-9440 to -9436
TGACC (half-site 3)	5'- CGTATGTAACTAACCTGCACA ATG-3'	5'-CTCCTGGATCTGTGTGATGTC-3'	81	-8994 to -8990
TGACC (half-site 4)	5'- TTAGCCAGGATGGTCTCGAT-3'	5'-GCCTGTAATCCCAGCACTTT-3'	102	-8634 to -8630
TGACC (half-site 5)	5'- CCACCACTATGCCTGGATAATT T-3'	5'-TCACCTGAGGTCAGGAGTTC-3'	93	- 7270 to -7266

Table 4 (Cont.)

ERE (half/full)	Forward	Reverse	Amplicon size (bp)	Location from transcription start site (bp)
GGTCA (half-site 1)	5'- TAGAGACAGGGCTTCACCAT-3'	5'-CACCTGTAATCCCAGCACTTT-3'	105	-6540 to -6536
GGTCA (half-site 2)	5'-CATGTTGGTCAGGCTGGT-3'	5'-ATGGCTCACGCCTGTAATC-3'	72	-6234 to -6230
GGTCA (half-site 3)	5'- GCTTCAGCCTCTGGGATAGA-3'	5'-CTGAGAAACAGCAACCGAAGA A-3'	80	-6000 to -5996
GGTCA (half-site 4)	5'-AGCCCAGGAAGGACTAGAG- 3'	5'-GACGTCTAAGAAACCAACAGGT A-3'	80	-5731 to -5727
GGTCATAA AGACC (full-site)	5'- CCCAGATGGTTAAGGCATTCTA -3'	5'-GTGCTGGTGGGAGTATAACAG- 3'	182	-4620 to -607
GGTCATAA AGACC (full-site)	5'- CCCAGATGGTTAAGGCATTCTA -3'	5'-TTAGAGCCGGCTTCTTTAC-3'	80	-4620 to -4607
Non-ERE site in <i>IKKB</i> promoter	5'-CTGCCGACAGGTGAGTC-3'	5'-AACTCCAACCCGCACAA-3'	174	-15 kbp
<i>pS2</i> ERE (full-site) GGTCACG GTGGCC	5'-TGGGCTTCATGAGCTCCTTC- 3'	5'- TTCATAGTTGAGAGATGGCCCGG- 3'	202	-0.4 kbp
<i>GREB1</i> ERE (full-site) GGTCATTC TGACC	5'- GTGGCAACTGGGTCATTCTGA- 3'	5'- CGACCCACAGAAATGAAAAGG=3'	190	-1.6 kbp

4.6.2 Effect of Compound NSC35446.HCl Alters the Recruitment of Estrogen Receptor α to the *IKKB* promoter

We performed ChIP assays to quantify the accumulation of ER α protein in a segment of DNA surrounding each of these ERE sites. We compared the amount of amplified product (as a percentage of input) found in vehicle-treated vs compound-treated cells. For the ERE-like full-site, we utilized two different PCR amplicons, and for each half-site, we utilized a single PCR amplicon. Non-immune IgG IP of vehicle-treated cells was used as a negative control. NSC35446.HCl blocked the recruitment of ER α to the full ERE-like site and to five of the nine half-sites in LCC9 cells (Figs. 38A and 38B). Four of the nine half-sites showed no significant amplified product (Fig.38C). Similarly, a non-ERE site 15 kbp upstream of the TSS showed no significant ER α recruitment (Fig.38D). We also showed that NSC35446.HCl blocked the ER α signal from the known ERE in the *pS2* promoter and from the ERE-like full-site in the *IKKB* promoter in T47DCO cells (Fig. 39).

A

B

C

D

Figure 39. Effect of compound NSC35446.HCl on the recruitment of ERα to the *IKKB* promoter in LCC9 cells. (A) Cells were treated with vehicle or 5 μM of compound NSC35446.HCl for 48-hr. ChIP assays were performed using two sets of primers for the ERE-like full-site, using normal IgG IP (negative control for vehicle-treated cells) or anti-ERα antibody IP. (B) ChIP assays are shown for the five ERE half-sites that exhibited significant recruitment of ERα in vehicle-treated cells. (C) ChIP assays are shown for the four ERE half sites in the *IKKB* promoter that showed negligible recruitment of ERα. (D) ChIP assay for an irrelevant amplicon (non-ERE) located 15-kbp upstream of the transcription start site (TSS) of the *IKKB* gene. Values plotted are means ± SEMs of three independent experiments and are normalized to percent of Input. *p < 0.05, **p < 0.01, ***p < 0.001, “ns, not significant” significant.

A

B

Figure 40. Additional ChIP assays. T47DCO cells were treated with vehicle or compound NSC35446.HCl (10 μ M) for 48 hr and ChIP assays were performed to evaluate recruitment of ER α to the known *pS2* full ERE site (A) or the full ERE-like site of the *IKKB* promoter (large amplicon) (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.6.3 ChIP Control Experiments

As another control, we tested the ability of Fulvestrant (ICI 182,780), which causes ER α degradation (27,28), to inhibit the E2-stimulated recruitment of ER α to the known EREs in the *pS2* and *GREB1* promoters in anti-estrogen sensitive/E2-responsive MCF-7 cells (Fig.40A) 120-min exposure to E2 caused accumulation of ER α at these two EREs, while co-treatment with Fulvestrant (1 μ M) inhibited the E2-stimulated accumulation of ER α . Furthermore, NSC35446.HCl blocked the subsequent E2-stimulated recruitment of ER α to the EREs of *pS2* and *GREB1* in MCF-7 cells (Fig. 40B).

A

B

Figure 41. Control ChIP assays. (A) Estrogen-sensitive ERα-positive MCF-7 cells were treated with 17β-estradiol (E2) (10 nM) and/or Fulvestrant (1 μM) for 120 min and then ChIP assays were performed to detect recruitment of ERα to the known full site EREs within the *pS2* promoter (*left* panel) or the *GREB1* promoter (*right* panel). (B) MCF-7 cells were treated with vehicle or compound NSC35446.HCl (5 μM) for 48 hr and then exposed to E2 (10 nM) for 120 min, and ChIP assays were performed to detect recruitment of ERα to the known full-site EREs within the *pS2* promoter (*left* panel) or the *GREB1* promoter (*right* panel). Values plotted are means ± SEMs of three independent experiments. Values are normalized to the percent of input. *p < 0.05, **p < 0.01, ***p < 0.001.

4.7 Effect of Compound NSC35446.HCl on ERα Protein Expression in LCC9 cells

We performed Western blotting to rule out the possibility that loss of ERα occupancy at ERE sites in the *IKKB* promoter due to NSC35446.HCl is caused by a decrease in ERα protein levels. This experiment revealed no change in ERα protein due to a 48 hr

treatment with compound (Fig. 41). In contrast, the compound significantly reduced the pS2 protein levels.

Figure 42. Effect of compound NSC35446.HCl on ER α protein expression in LCC9 cells. Cells were treated with vehicle or compound NSC35446.HCl (5 μ M) for 48-hr and then Western blotted to detect ER α or GAPDH. Western blots show three independent experiments. Densitometry was performed, and the values were normalized to GAPDH and expressed in arbitrary units (AU). Bar graphs show means $\pm$ SEMs of three independent experiments, * p < 0.05, ** p < 0.01, *** p < 0.001, “ns, not significant” significant.

4.8 Additional Experiments to Satisfy the Comments of the Reviewers of Our Manuscript

4.8.1 Additional Proliferation Assays for Antiestrogen-Resistant Cells

Growth curves utilizing different concentrations of NSC35446.HCl showed significant inhibition of cell proliferation in anti-estrogen-resistant LCC9 cells for concentrations of 0.3, 1, and 5 μM but no significant change in growth for 0.01 μM concentration (Fig. 42A). Similarly, as with the LCC9 cells, the compound inhibited proliferation in MCF-7-derived ER α - positive anti-estrogen-resistant MCF-7/RR and LY2 cells (Fig. 42B). Note that a single concentration of NSC35446.HCl (5 μM) was used for MCF-7/RR and LY2 cells. Consistent with the above cell proliferation data, NSC 35446.HCl significantly inhibited mammosphere formation in LCC9 cells for concentrations 0.3, 1, and 5 μM ; whereas there was no significant effect measured at 0.01 μM .

Figure 43. Additional proliferation assays for antiestrogen-resistant cells. (A) Growth curves were performed for LCC9 cells as described for Fig. 1B, using different concentrations of compound NSC35446.HCl, as indicated. (B) Growth curves were performed for ER α -positive MCF-7-derived antiestrogen-resistant cell lines MCF-7/RR and LY2, using a concentration of 5 μ M of compound. (C) Mammosphere assays were performed in LCC9 cells as described for Fig. 1D, using different concentrations of compound, as indicated. The data shown in panels (A), (B), and (C) reflect three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns, not significant” significant.

4.9 Heat map of Genes that Influence NF- κ B Signaling from RNA-seq Experiment of LCC9 Cells treated with vehicle (control) vs compound A7 (NSC31303) (5 μ M for 24-hr)

Pathway Studio Analysis of RNA-seq data revealed differential expression of genes that directly influence NF- κ B activity/signaling pathway (Fig.43), along with down-regulation of *IKKB* expression. Here we show the ability of BRCA1-mimetic compound to suppress ER α activity may also affect other genes besides *IKKB* that influence NF- κ B activity or that influence *IKKB* expression/activity, thereby giving a better understanding of the interactions of BRCA1-mimetic and NF- κ B as seen in the RNA-seq data .

Figure 44. Heat map of genes that influence NF- κ B signaling from RNA-seq experiment of LCC9 cells treated with vehicle (control) vs compound A7 (NSC31303) (5 μ M for 24-hr).

4.10 Dose-Response for Effect of Compound NSC35446.HCl on IKKB, IKKG, and IKKA Protein Levels in LCC9 Cells

We next tested the effects of different concentrations of NSC35446.HCl on the expression of IKKB and the two other IKK complex proteins (IKKA, IKKG) at the 48-hr time point. Western blotting with densitometry revealed that NSC35446.HCl caused a non-significant effect at the 0.01 μ M concentration for all three isoforms of IKK, as well as non-significant effect at 0.3 μ M on the expression of IKKA protein (Fig. 44). A significant decrease of IKKB, IKKA, IKKG protein levels in LCC9 cells at concentrations of 0.3, 1, and 5 μ M was observed at the 48-hr time point (Fig. 31).

Figure 45. Dose-response for effect of compound NSC35446.HCl on IKKB, IKKG, and IKKA protein levels in LCC9 cells. Cells were treated with different concentrations of compound, as indicated, for 48-hr. The Western blot shown on the left is representative of three independent experiments. Quantification of pixel images was performed using Image J software and the data were normalized to the GAPDH values.

Bar graphs show means $\pm$ SEMs of three independent experiments, expressed in terms of arbitrary units (AU). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns, non significant.”

4.11 Dose-Response for Effect of Compound NSC35446.HCl on NF- κ B-Regulated Protein Expression in LCC9 Cells

We examined the effects of different concentrations of the compound on the protein levels of NF- κ B-regulated genes, CIAP1, TRAF2, and BCL2. Western blotting with densitometry showed significant decreases in the levels of anti-apoptotic proteins CIAP1, TRAF2, BCL2, in LCC9 for concentrations 0.3, 1, and 5 μ M (Fig. 45). NSC35446.HCl also caused no significant increase of these anti-apoptotic proteins in LCC9 cells at the 0.01 μ M concentration (Fig. 45). Thus, our compound inhibits NF- κ B-regulated protein expression in a dose-dependent manner, consistent with its ability to reduce protein expression of IKKB, IKKA, and IKKG in a dose-dependent manner.

Figure 46. Dose-response for effect of compound NSC35446.HCl on NF- κ B-regulated protein expression in LCC9 cells. Cells were treated with different concentrations of compound, as indicated, for 48-hr. The Western blot shown on the left is representative of three independent experiments. Quantification of pixel images was performed using Image J software and the data were normalized to the GAPDH values. Bar graphs show means $\pm$ SEMs of three independent experiments, expressed in terms of arbitrary units (AU). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns non significant.

4.12 Additional Time Course Experiments Showing the Effects of Different Doses of Compound NSC35446.HCl on *IKKB* mRNA Expression in LCC9 cells and of a Single Dose of Compound on Two Other MCF-7-Derived Estrogen Receptor α -Positive Antiestrogen-Resistant Cell Lines, MCF-7/RR and LY2

The effect of different concentrations of the compound on *IKKB* mRNA levels was quantified by qRT-PCR at various time points in LCC9 cells (Fig. 46A). At the 6-hr time point, there was no significant reduction in *IKKB* transcript levels for any of the concentrations of compound NSC35446.HCl. Transcript levels were significantly decreased at 48-hr for concentration of 0.3 μ M and at 12, 24, and 48hr for concentrations of 1 μ M and 5 μ M of compound. Thus, a dose-dependent reduction in *IKKB* mRNA is induced by the compound. Additionally, the effect of a single 5 μ M concentration of the NSC.35446HCl compound on *IKKB* mRNA levels was quantified by qRT-PCR at

various time points in antiestrogen-resistant ER α + cell lines MCF7/RR and LY2 (Fig. 46B). The 6-hr time point yielded no significant reduction in *IKKB* transcript for either cell line, but both cell lines showed significant decreases in *IKKB* transcript levels at the 12, 24, and 48hr time points.

Figure 47. Additional time course experiments showing the effects of different doses of compound NSC35446.HCl on *IKKB* mRNA expression in LCC9 cells and of a single dose of compound on two other MCF-7-derived ER α -positive antiestrogen-resistant cell line. (A) *IKKB* mRNA expression in LCC9 cells was measured after a 6, 12, 24, or 48hr incubation with different concentrations of compound, as indicated, by qRT-PCR, using β -actin as the control gene. (B) *IKKB* mRNA expression in MCF-7/RR and LY2 cells was measured after a 6-, 12-, 24-, or 48-hr incubation with 5 μ M of compound. All values plotted are means $\pm$ SEMs of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, “ns, not significant” significant.

4.13 Dose-Response and Time Course for the Effect of Compound NSC35446.HCl on Estrogen Receptor α Recruitment to the *IKKB* promoter in LCC9 Cells

We performed ChIP assays as in Fig. 38 after treatment with compound NSC35446.HCl at concentrations of 0.01, 0.3, 1, and 5 μ M for the ERE-like full-site, utilizing the large

PCR amplicon. Non-immune IgG IP of vehicle-treated cells was used as a negative control. NSC35446.HCl treatment at concentrations of 0.3, 1, and 5 μ M for 48-hr blocked the recruitment of ER α to the full ERE-like site in a dose-dependent manner (Fig. 47A). We also performed ChIP assays after treatment with a 5 μ M concentration of NSC35446.HCl at shorter time points of 12-hr and 24-hr (Fig. 47B and C). For the ERE-like full-site (Fig. 47B), we utilized the large PCR amplicon. Non-immune IgG IP of vehicle-treated cells was used as a negative control. NSC35446.HCl treatment at a concentration of 5 μ M significantly inhibited the recruitment of ER α to the full ERE-like site for both time points and for the TGACC 1 ERE half-site at the 24-hr time point. Similar results were observed for other ERE sites that were previously shown to be affected by compound at 48-hr. Thus, a reduction in ER α occupancy at ERE sites in the IKKB promoter could be detected at time points earlier than 48-hr.

Figure 48. Dose-response and time course for the effect of compound NSC35446.HCl on ER α recruitment to the *IKKB* promoter in LCC9 cells. (A) Cells were treated with different concentrations of compound, as indicated, for 48-hr; and ChIP assays were performed to determine ER α occupancy at the full ERE large amplicon in

the *IKKB* promoter. Values are means $\pm$ SEMs of three independent experiments. (B) Cells were treated with 5 μ M of compound for 12-hr or 24-hr (as indicated); and ChIP assays were performed to determine ER α occupancy at the full ERE large amplicon in the *IKKB* promoter. Note: data for the 48-hr time point are shown in panel (A). Values are means $\pm$ SEMs of three independent experiments. (C) Cells were treated with 5 μ M of compound for 12-hr or 24-hr (as indicated); and ChIP assays were performed to determine ER α occupancy at the TGACC ERE half-site 1 in the *IKKB* promoter.*p < 0.05, **p < 0.01, ***p < 0.001, “ns, not significant” significant.

4.14 Dose-Response for the Effect of Compound NSC35446.HCl on Development of Apoptosis in LCC9 Cells

We directly tested the effect of a 48-hr treatment with different concentrations of the compound NSC35446.HCl on apoptosis in LCC9 cells using a flow cytometric TUNEL assay to detect FITC-labeled apoptotic fragmented DNA. This experiment revealed less than 10% apoptosis in vehicle-treated cells and a dose-dependent increase in apoptotic fraction starting with around 25% at 0.3 μ M, 38% in 1 μ M, and ending in 50% apoptosis at 5 μ M in compound-treated cells (Fig. 48).

Figure 49. Dose-response for the effect of compound NSC35446.HCl on development of apoptosis in LCC9 cells. Cells were treated daily with vehicle or NSC35446.HCl (concentration as indicated) for 48-hr, after which a TUNEL assay was performed using the APO-BRDU™ Kit (BD Biosciences) and flow cytometry to detect FITC-labeled apoptotic fragmented DNA. The values plotted are means $\pm$ SEMs of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns, not significant” significant.

CHAPTER 5: DISCUSSION

Antiestrogen-resistant LCC9 cells, which were derived from antiestrogen-sensitive MCF-7 cells, are resistant to both Fulvestrant and Tamoxifen (63,64) but are sensitive to both A7 and NSC35466 (the free base form of 35466.HCl) (31,32); and NSC35466.HCl inhibits both ER α activity (with an IC₅₀ of 0.3 μ M) (data not shown) and cell proliferation (this study) in LCC9 cells. In addition, NSC35466 (free base) partially restored the sensitivity of LCC9 cells to Tamoxifen (32). We will assume that NSC35466.HCl has the same qualitative activities as NSC35446, since the former is merely a more water soluble and potent form of the latter.

The ability of compound NSC35466.HCl to inhibit NF- κ B signaling may, in part, explain its ability to inhibit cell proliferation and cell survival in antiestrogen resistant LCC9 cells but not in ER α -negative cell lines. NSC35466.HCl also inhibited proliferation and induced apoptosis in T47DCO cells and blocked their expression of NF- κ B-regulated genes. T47DCO cells are antiestrogen resistant, and express a mutant form of ER α (73). In contrast, NSC35466.HCl does not inhibit cell proliferation or NF- κ B signaling in ER α -negative MDA-MB-231, HCC1806, or MDA-MB-468 cells and does not cause down-regulation of IKKB protein expression in ER α -negative MDA-MB-468 or HCC1806 cells. Consistent with the idea that NF- κ B regulates LCC9 resistance to antiestrogens, it has been shown that curcumin, a proposed cancer chemoprevention agent, inhibits NF- κ B activity and restores LCC9 sensitivity to Tamoxifen (32); and COUP-TFII expression, which contributes to endocrine sensitivity, was reduced in LCC9 cells, which had five-fold greater NF- κ B activity than parental MCF-7 cells (65). Finally, and most directly, Parthenolide (a selective inhibitor of NF- κ B transcriptional activity) or

over-expression of a dominant-negative mutant I κ B each restored sensitivity of LCC9 cells to Fulvestrant (71,72). This effect could be reversed by a caspase-8-specific inhibitor. The latter finding is consistent with our finding that a caspase-8-specific inhibitor blocked the ability of compound NSC 35466.HCl to inhibit LCC9 cell proliferation and caspase-7 cleavage.

Interferon γ (IFNG), an inflammatory cytokine that induces the expression and function of interferon regulatory factor-1 (IRF-1), induces IRF-1 expression in LCC9 cells, restores sensitivity to Fulvestrant, inhibits NF- κ B activity, inhibits expression of anti-apoptotic genes (*eg.*, *Bcl-2*), and stimulates expression of pro-apoptotic genes (78). IRF-1 is down-regulated in LCC9, as compared with parental MCF-7 cells; while NF- κ B activity is up-regulated in LCC9 cells (79). These findings are consistent with the idea that IRF-1 induces antiestrogen sensitivity in LCC9 cells (79) and are consistent with our finding that compound NSC35446.HCl up-regulates IRF-1 protein expression in LCC9 and T47DCO cells.

Our *in silico* analysis revealed one imperfect full-site ERE (with a one nucleotide mismatch relative to the consensus ERE sequence) and nine perfect half-sites, including one Sp1/ERE site (TGACC half-site 1), in the *IKKB* promoter. ChIP assays revealed that the imperfect full-site and five of the nine half-sites (including TGACC half-site 1) showed easily measurable ER α accumulation that was significantly reduced by compound NSC35446.HCl in LCC9 and T47DCO cells. We are unaware of any prior reports of ERE sites within the *IKKB* promoter or E2/ER α -sensitivity of the *IKKB* gene. However, several prior reports suggest that ERE half-sites in other genes can strongly bind ER α and are functional (80-88). This includes Sp1/ERE half-sites, which can bind a ternary Sp1-ER α -DNA complex (73,66,78,80). Our study shows that the greatest

quantitative accumulation of ER α in vehicle-treated LCC9 cells was at the Sp1/ERE half-site. ER α is also known to bind to imperfect full-site EREs (89,90). Even the known full-site ERE of the *pS2* promoter shows a one nucleotide mismatch relative to the canonical ERE sequence (65). The quantitative magnitude of ER α accumulation at the imperfect ERE full-site and the five ERE half-sites of the *IKKB* promoter was similar in vehicle-treated LCC9 cells as was E2-induced ER α accumulation at the known *pS2* and *GREB1* EREs in E2-sensitive MCF-7 cells, suggesting that these *IKKB* sites are functional.

We showed that compound NSC35446.HCl down-regulates the protein expression of three different IKK isoforms, IKKB (IKBKB, IKK β), IKKA (IKK α) and IKKG (IKK γ)/NEMO, in antiestrogen-resistant LCC9 cells but did not down-regulate protein expression of IKKB in ER α -negative MDA-MB-468 or HCC1806 cells. [We did not test whether the compound affects protein expression of IKKA or IKKG/NEMO in ER α -negative cell lines.] Thus, other proteins within the NF- κ B signaling pathway may be targeted by our compound in LCC9 cells. Interestingly, IKKG/NEMO has been shown to be a component of a signaling network that impinges on the key nodes of NF- κ B, IRF-1, and X-box binding protein-1 (XBP1) and regulates the antiestrogen sensitivity of LCC9 cells (91). Our findings suggest that components of this same network may also be involved in regulation of the response to compound NSC35446.HCl.

Building on IKKB's potential role in resistance, Stender et al, shows that resistance to endocrine treatment could be caused by accumulation of inflammatory cytokines (96). This inflammatory environment causes an increase in the pro-inflammatory cytokines tumor necrosis factor alpha (TNF α) and interleukin 1-beta (IL1 β), which stimulate NF- κ B activity. These pro-inflammatory cytokines activate unliganded ER α by phosphorylating the S305 site through IKKB. Stender et al. showed that the IKKB-

mediated phosphorylation of ER α causes a conformational change by bringing helix 12 to the CoRNR groove thereby blocking co-repressor protein interactions and enabling constitutive AF-1 activity (96). IKK β phosphorylation of ER α also causes a salt bridge formation in the LBD/F domain interface, which is associated with SRC3 recruitment to proliferative genes (*eg.*, MYC). Our study brings to light that ER α directly influences *IKKB* transcription. Thus, NSC35446 HCl could potentially also overcome antiestrogen resistance by directly inhibiting IKKB production. As shown by Stender et al, IKKB can directly phosphorylate ER α and thereby activate ER α and promote its resistance to Tamoxifen (96).

To fully elucidate this role of NF- κ B in the context of endocrine resistance and, furthermore, to have a complete understanding of signaling pathways affected as a result of NSC35446.HCl treatment, ChIP-seq combined with RNA-seq could be employed in future studies. Data integration can be very useful in trying to gain new insights in complex biological networks and, in this particular instance, could give insights into how ER α occupancy in ERE sites can predict gene expression and infer gene regulatory networks in endocrine resistant cells (95). For example, ChIP-seq may allow us to identify other genes in the NF- κ B signaling pathway that contain functional EREs and for which treatment with NSC35446.HCl reduces ER α occupancy at the gene promoters. ChIP-seq may also allow us to identify genes for which our compound increases ER α occupancy at promoter sites. (97).

Expanding on this, we have shown that NSC35446.HCl inhibits ER α occupancy at ERE sites within the *IKKB* promoter, but the next question is, functionally, how does BRCA1 mimetic block ER α recruitment to DNA. What are the allosteric changes that are taking place in ER α with BRCA1-mimetic binding that do not occurring with Tamoxifen

binding in antiestrogen-resistant but ER α breast cancer cells. Previous studies utilizing electrophoretic mobility shift assays suggest that both sets of BRCA1-mimetic compounds (see Introduction) block the interaction of ER α with duplex consensus ERE oligonucleotides (31,32). This finding is consistent with the ability of compound NSC35446.HCl to reduce ER α occupancy at various ERE sites on the *IKKB* promoter. We hypothesize that the binding of NSC35446.HCl and other BRCA1-mimetic compounds to ER α induces a profound conformational change that causes dissociation of ER α from DNA. One method to identify such conformational perturbations for certain signaling outcomes is the use of X-ray crystallography.

In this case, crystallography can be done with NSC35446.HCl bound to ER α with and without E2, Tamoxifen, or Fulvestrant bound to ER α . These experiments may be feasible because E2, Tamoxifen, and Fulvestrant bind to the ligand-binding pocket; while NSC35446.HCl binds to a site outside of the ligand-binding pocket, thus allowing the possibility of simultaneous binding. Important structural and atomistic information can be obtained with crystallography such as the position of ER α helix 12 and the positions of critical binding residues in ligand-receptor binding. Yet another approach is the use of mass spectrometry, which can also ascertain global conformational change of the ER α with NSC35446.HCl binding. One advantage of combining certain MS techniques (*eg.*, Ion mobility MS, MS, HDX-MS) is to separate multiple conformation states to see how each conformation state affects ligand and/or DNA binding, as well conformational changes as a result of co-regulator binding (97). Although important, both of the above methods are costly and are time consuming.

A preliminary method to suggest structure/activity relationships is to use BIACORE's surface plasmon resonance (SPR) technology, to examine the reaction kinetics for small

molecule receptor interactions. ER α would be immobilized on a chip and different binding partners could be passed over it, such as NSC35446.HCl, Tamoxifen, and DNA (oligonucleotides containing the ERE sequence). Association rates would be observed for each binding parameter and the combination of these binding parameters (*eg.*, binding kinetics of ER α to compound NSC35446.HCl and DNA). This information could be used to predict the biological effect of the above ligands.

Another avenue of study could be the assessment of BRCA1 mimetics in aromatase inhibitor resistance. Aromatase inhibitors are only given in the context of estrogen dependent breast cancers in post-menopausal women (Aromatase inhibitors cannot be given in the above model of tamoxifen resistance because the resistance is estrogen independent). Resistance may still develop in this context whereby the ER α is activated independent of estrogen and the subsequent breast cancer becomes resistance to aromatase inhibitor therapy. BRCA1 mimetics may prove to overcome resistance in this setting as well. Hypothetically, BRCA1 mimetics can function as anti-estrogens, as long as the small segment of the BRCA1 binding pocket in ER α , where the mimetics bind remains intact and free of mutations in critical binding residues. Nonetheless, it is quite possible that resistance may develop if BRCA1 mimetics are administered as a single anti-estrogen therapy. One way to understand potential BRCA1 mimetic resistance mechanisms is to develop a BRCA1 resistant cell line from both an estrogen dependent as well as an estrogen independent/Tam resistant cell line. However, in an effort to minimize the evolution of resistance, BRCA1 mimetics would be given in combination with Tamoxifen.

About 70% of all breast cancers are ER α -positive at presentation. These ER α -positive cancers are candidates for first-line antiestrogen therapy, such as Tamoxifen (98).

However, about 50% of patients who present with advanced breast cancer who receive Tamoxifen fail to respond, despite ER α -positivity. Moreover, all patients with metastatic disease eventually develop Tamoxifen resistance (56); and about 40% of patients with early stage ER α -positive disease who receive Tamoxifen as an adjuvant therapy relapse and die of disease (98). In most cases of acquired antiestrogen resistance, breast cancers retain the expression of ER α . Thus, patients with *de novo* or acquired antiestrogen resistance are candidates for novel therapeutic approaches to target ER α . NSC35446.HCl, a specific ER α inhibitor that works by a distinct mechanism from Tamoxifen or Fulvestrant (31) and can target ER α signaling and antiestrogen-resistance mechanisms in antiestrogen-resistant cell lines such as LCC9 and T47DCO.

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