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Investigating The Apoptotic Response Of Triple Negative Breast Cancer Cells To Styrene, A Potential Poly(Adp-Ribose) Polymerase (Parp) Inhibitor

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Investigating the Apoptotic Response of Triple Negative Breast Cancer Cells to Styrene, a Potential Poly(ADP-ribose) Polymerase (PARP) Inhibitor

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Biographical Sketch

Shaina Richardson was born August 19, 1987 in Richmond, Virginia. She is the only child to the parents of Pamela and George Lee. In high school she participated in many activities such as competition cheerleading and gymnastics, basketball, and dance. In 2005, she entered North Carolina Agricultural and Technical State University as a freshman in biology. She finished her undergrad degree in December 2009. In August of 2011 she re-entered North Carolina A&T to complete her Master's of Science in Biology. Her future endeavors upon graduating include becoming a successful physician's assistant by continuing her education at an institution in North Carolina.

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Key to Symbols or Abbreviations

Activatin factor-1 ..... Apaf-1

Adenosine Diphosphate ..... ADP

ADP Ribosylation Factor ..... ARF

Amino-terminus ..... N-terminus

Angiotensinogen ..... AGT

Apoptosis antigen 1/ FAS APO1

Apoptotic protease activating factor 1 ..... APAF1

Ataxia telangectasia mutated ..... ATM

Auto-modification domain ..... AMD

Base excision repair ..... BER

B-cell lymphoma 2 ..... Bcl2

Breast Cancer Susceptibility Gene ..... BRCA

Carbon Dioxide ..... CO_2

CarboxyL-terminus ..... C-terminus

Celsius ..... ℃

Cyclin-dependent kinase ..... Cdk

Death-inducing signaling complex ..... DISC

Death receptor 5/KILLER DR5

Deoxyribonucleic acid ..... DNA

Dimethylsulfoxide ..... DMSO

Enzyme-linked Immunosorbent Assay ..... ELISA

Estrogen Receptor ..... ER

Fas-Associated protein with Death Domain .....FADD

Fas Ligand .....FasL

Fas Receptor .....FasR

Fetal bovine serum .....FBS

Grams .....g

Grams/mole .....g/mol

Homeobox A5 .....HOXA5

Homologous recombination .....HR

HT Fluorescent PARP Inhibition Assay .....HFHPIA

Human Epidermal Growth Factor Receptor 2 .....HER2/neu

Inhibitor of Cyclin-dependent Kinase 4 .....INK4

Junia K. Selby .....JKS

Kilobases .....kb

Kilodalton .....kDa

Methylthiazol Tetrazolium .....MTT

Microliter .....µl

Micromolar .....µM

Milliliters .....ml

Minutes .....min

Murine double minute 2.....MDM2

Myelocytomatosis .....Myc

Nanometers .....nm

National Cancer Institute .....NCI

National Institute of Health .....NIH

Nicotinamide adenine dinucleotide .....NAD^+

Nuclear export signal sequence .....NES

Nuclear factor-kappa B .....NF-κB

Nuclear localization signal .....NLS

Protein 53-regulated Apoptosis-Inducing Protein 1 .....p53AIP1

Phosphate Buffer Saline .....PBS

Phosphate Buffer Saline and 1% Tween .....PBST

Platelet derived growth factor .....PDGF

Platelet derived growth factor receptor .....PDGFR

Platelet derived growth factor receptor-alpha .....PDGF-α

Platelet derived growth factor receptor-beta .....PDGF-β

Poly (ADP-ribose) Polymerase .....PARP

Progesterone Receptor .....PR

Protein 14 alternate reading frame .....$p14^{ARF}$

Protein 21 .....p21

Protein 53 .....p53

Receptor-Interacting Protein .....RIP

Reticular activating system .....Ras

Retinoblastoma protein .....Rb

Revolutions per minute .....RPM

Roswell Park Memorial Institute -1640 .....RPMI-1640

Tumor necrosis factor .....TNF

Tumor necrosis factor alpha .....TNF-α

Tumor necrosis factor receptor 1 .....TNFR1

Tumor necrosis factor receptor type 1-associated death domain .....TRADD

Triple Negative Breast Cancer .....TNBC

Ultraviolet .....UV

Abstract

Triple Negative Breast Cancer (TNBC) is a rare basal-like subtype of breast cancer characterized by the absence of the estrogen (ER), progesterone (PR), and human epidermal receptor 2 (HER2) receptors which are normally targeted in other breast cancer subtypes, thereby making TNBC difficult to combat. In this study, four different styrene compounds (1.001, 1.006, 1.007, and 1.009) were used to treat TNBC cell lines HCC1806 and HCC70. Each of the compounds were composed of a 3-nitro group on the A ring and varying aryl groups on the B ring of the styrene. The breast cancer cells were exposed over a 24-hour period to a 10, 100 and 1000 μM styrene concentration. Styrene compounds have a similar structural composition to Resveratrol and some known Poly (adenosine diphosphate (ADP)-ribose) Polymerase (PARP) inhibitors. Previous studies have demonstrated that PARP-1 has a high affinity for breast cancer susceptibility gene-deficient (BRCA) breast carcinomas such as TNBC. Similarities in structure suggest that these compounds may have the same apoptotic effect found in PARP inhibitors used in treating cancer. A cell viability assay revealed that all of the styrene compounds were effective and showed apoptosis at varying concentrations in-vitro. Apoptosis was also verified with phase-contrast microscopy and Methylthiazol Tetrazolium (MTT) Assay. PARP inhibition by styrenyl compounds was verified with the HT Fluorescent Homogenous PARP Inhibition Assay. Preliminary data show that these compounds have the ability to induce apoptosis in triple negative cells and lead us to believe these compounds represent a novel potential chemotherapeutic treatment for TNBC.

CHAPTER 1

Introduction

Cancer is a disease characterized by excessive, uncontrolled growth of abnormal cells that invade and destroy other adjacent tissues. Cancer can also spread to other regions of the body, through metastasis, on the same seek and destroy mission. Cancer can develop in any tissue of any organ at any age and in any race/ethnicity. Various factors influence the onset of this disease, which can include gender, geography, genetics and cultural distinctions. Such factors play an important role in the effect that cancer has on the general population and its prevalence.

In the United States, cancer is the second most common cause of deaths, accounting for nearly 1 of every 4 deaths (American Cancer Society, 2012). Breast cancer is the 2nd leading cause of cancer death of women in the United States, according to the National Cancer Institute (NCI) (National Cancer Institute, Breast). Triple Negative Breast Cancer (TNBC) has a mortality rate that increases in at least one-fourth of those diagnosed and is a rare type of breast cancer that is more prevalent in younger women of color (African American and Hispanic). TNBC is characterized by the absence of ER, PR, and HER2 receptors. Triple negative breast cancer lacks effective therapies due to lack of specific biomarkers. Well-known treatments for breast cancer are unable to combat TNBC and often reappear in a more aggressive form. Therefore, the need for novel chemotherapeutic options, other than chemotherapy, in TNBC still remains. Styrene is a potential chemotherapeutic option for TNBC due to its similar PARP inhibitor characteristics. As a PARP inhibitor, styrene has the ability to attack the cells at the molecular DNA level. Previous literature has identified PARP inhibitors such as Olaparib that produced significant results in treating triple negative breast cancer (Fong, 2009). Styrenyl compounds also have a

similar structure to resveratrol and similarities in structure leads to the impression that styrene will provide the same results in TNBC.

The purpose of this study is to identify a potential novel therapeutic for triple negative breast cancer. With styrenes as our prominent chemical compounds, the objective is to determine the styrene compounds with the effective working concentration that produces a measurable apoptotic effect in triple negative breast cancer. The hypothesis is that these styrenyl compounds will produce a measurable apoptotic effect at a low minimum working concentration. As an increasingly widespread type of cancer that has been growing in diagnostics and mortality, the need for therapeutic options is still needed to diminish the odds and slow down these death rates of TNBC patients. Thus the significance of this study is to reduce the gap in therapeutic treatment options for TNBC.

CHAPTER 2

Literature Review

2.1 Cancer

Cancer, defined as the disease caused by an uncontrolled division of abnormal cells in a part of the body, is primarily caused by environmental factors (90-95%) while the remaining causes are due to genetics (5-10%). Some common environmental factors include tobacco, diet, obesity, infections, radiation, stress, lack of physical activity, and pollutants. Yet, with all these different risk factors, cancer is not caused by only one component. Cancer tends to develop because of numerous factors, also known as the 'multi-hit theory.'

In the multi-hit theory an accumulation of mutations causes the formation of cancer. These mutations can include random, inherited (pre-disposed), viral infections, or environmental factors (chemical/physical). Although cancer may begin with just one mutation, one is not enough. A correlation between age and the incidence of cancer further justify the multi-step process involved with cancer development. As age increases, chromosomal abnormalities occur and genes are further mutated. Oncogenes that normally promote cell proliferation are actively mutated, while tumor suppressor genes that normally inhibit cell proliferation are inactively mutated. Due to the different 'hits' or mutations that can occur to a cell, the theory of cancer development is further justified.

An estimation of 1.6 million new cancer cases are expected to be diagnosed in 2013 and out of these, 580,350 Americans are expected to die (Siegal, 2013). Since the risk of being diagnosed increases with age, most cases occur in adults who are middle age or older. About 77% of all cancers are diagnoses in persons 55 years of age and older. Yet with continued research and early diagnoses, the 5-year survival rate for all cancer diagnoses has increased from

49% in 1975-1977, to 67% in 2001-2007 and is continuously rising (American Cancer Society, 2012). The National Cancer Institute estimates that nearly 12 million Americans were alive in January 2008 with a history of cancer. According to the National Institute of Health (NIH) the overall estimate cost of cancer in 2007 was $226.8 billion, including direct medical cost ($103.8 billion) and indirect mortality cost ($123.0 billion). With great investment being spent into cancer many types take precedence over others, which in turn require the bulk of the investment as well as the attention.

Each organ in the body is made up of different types of tissue. Most cancers arise in one of three main types of tissues: blood forming, connective or epithelial. Sarcomas represent less than 2% of cancers and originate within connective tissue. Two types combined account for 8% of cancers develop in blood cells (leukemia) and the lymphatic system (lymphomas). 90% of all human carcinomas occur in the epithelial tissue throughout the body (Sperry, 2009).

Carcinoma is a term often used interchangeably with cancer. Typically carcinomas are identified based on its type and organ of origination. Some of the most frequently diagnosed cancers in the United States include: melanomas, bladder, prostate, colon and rectal, lung and breast. Melanoma is a form of skin cancer that begins in the melanocytes and has an estimated 76,250 new cases in 2013 (National Cancer Institute, Melanoma). Bladder cancer forms in the tissues of the bladder and begins in the cells that normally make up the inner lining of the bladder, such as, squamous cells and adenocytes. These cells can develop squamous cell carcinoma and adenocarcinoma in the inner lining of the bladder as a result of chronic irritation and inflammation. The estimated new cases in the US were 73,510 in 2013 (National Cancer Institute, Bladder). Prostate cancer forms in the tissue of the male reproductive gland found below the bladder and in front of the rectum. This cancer usually occurs in older men and was

estimated to have 241,740 new cases in 2013 (National Cancer Institute, Prostate). Colon and rectal carcinoma forms in the respective organs. Most colon cancers are adenocarcinomas, a cancer that begins in the cells that make and release mucus and other fluids. The estimated amount of new cases in 2013 was 103,170 (colon) and 40,290 (rectal) (National Cancer Institute, Colon). Lung cancer forms usually in the cells lining air passages. Two of the main types are small cell and non-small cell lung cancer, which are diagnosed based upon its appearance under the microscope. The estimated amount of new cases in 2013 was 226,160. This type of cancer is the leading cause of cancer deaths, estimated to the amount of 160,340 (National Cancer Institute, Lung). Breast cancer forms usually in the ducts and lobules of the breast. This cancer occurs in both men and women and 226,870 (females) and 2,190 (males) new cases were estimated in 2013 (National Cancer Institute, Breast). Breast cancer is the second leading cause of death in the United States and unlike the preventability of lung cancer as the leading cause of cancer deaths; breast cancer results from other non-preventable causes.

2.2 Breast Cancer

Although breast cancer can develop in both men and women, women are the most frequently diagnosed. Understanding the structure of the breast helps one appreciate the development of breast cancer in these women. Each breast has 15-20 sections known as lobes that are arranged like the petals of daisy (Figure 1). Each lobe has many smaller lobules, which end in dozens of tiny bulbs that can produce milk. The ducts, or thin tubes, link all the lobes, lobules and bulbs. These ducts lead to the nipple in the center of a dark area of skin called the areola. Fat occupies the space found in between the lobules and ducts and no muscles are found in the breast, but rather under the breast to cover the ribs. Each breast also contains blood and lymph vessels. The lymph vessels lead to the lymph nodes, the organs found under the arm,

above the collarbone, in the chest and many other parts of the body (The Ohio State). Once an individual is able to comprehend the anatomy of the breast, they can tackle diagnosing and treating the many forms of breast cancer found in many women.

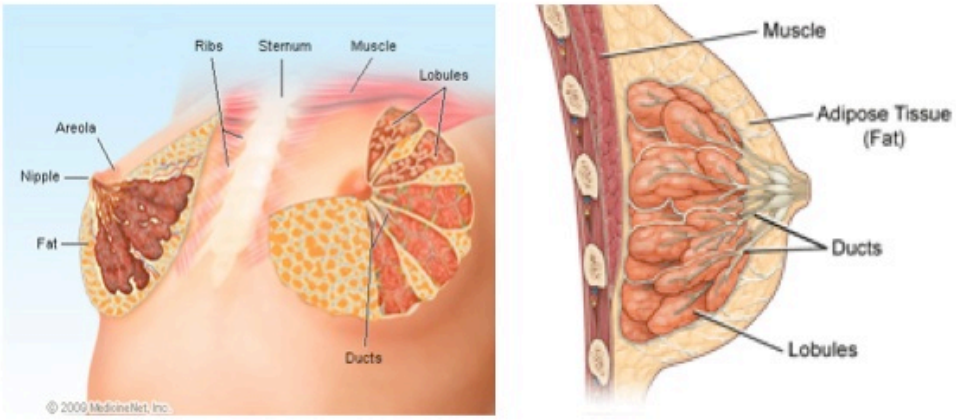

Figure 1. Anatomy of the Breast

Along with the amount of invasive breast cancers, 63,300 new cases of in situ breast cancers were estimated to occur among women in 2012 (American Cancer Society, 2012). However since 2004, in situ breast cancer incidence rates have been stable in white women and increasing in African American women by 2.0% per year (American Cancer Society, 2012). The death rates have also steadily decreased in women since 1990; from 2004 to 2008, women younger than 50 have had rates decrease by 3.1% per year and women 50 and older, 2.1% per year. The decrease in breast cancer mortality represents the progress in earlier detection, improved treatment therapies, and the possibility of decreased incidence. In 2012, an estimated 39,920 breast cancer deaths were expected (American Cancer Society, 2012). These estimations include all invasive and in situ breast cancer subtypes that effect women of all races and ages.

There are several different types of breast cancer that originate in different areas of the breast. Breast cancer is categorized into non-invasive or invasive. In non-invasive breast cancer

abnormal cells grow inside the milk ducts but do not spread to nearby tissue (Susan G. Komen Foundation, 2013a). Abnormal cells from inside the ducts or lobules breaking out and spreading into nearby breast tissue characterize invasive breast cancer. This allows the cancerous cells to spread to lymph nodes and in advanced stages propagate to other organs throughout the body. Molecularly, breast cancer is further classified into four major subtypes: luminal A, luminal B, HER2 type and basal-like.

Luminal A tumors account for most breast cancers and develop in the inner luminal cells lining the mammary ducts of breast. These tumors characteristics tend to be positive in estrogen (ER+) and/or progesterone (PR+) receptors, human epidermal growth factor receptor 2 (HER2/neu-) and have a low or moderate tumor grade. Of the four subtypes, luminal A tumors tend to have the best prognosis, with higher survival rates and low recurrence (Susan G. Komen Foundation, 2013b).

Luminal B tumors also develop in the inner luminal cells lining the mammary ducts of breast. However, these tumors are highly positive for Ki67 and/or HER2/neu-positive. Ki67 is a protein strictly associated with cell proliferation that is present during all active phases of the cell cycle (Scholzen, 2000). High levels of this protein, Ki67, increases the number of cancer cells actively dividing, resulting in recurrence of this tumor. The other characteristics found in luminal A tumors remain the same in luminal B, ER+ and/or PR+ and HER2/neu-. Younger women are often diagnosed with luminal B tumors and have a poorer prognosis due to a poorer tumor grade, larger tumor size, positive lymph nodes and p53 gene mutations. Although those diagnosed with luminal B breast cancer have a poorer prognosis, they usually have a fairly high survival rate (Susan G. Komen Foundation, 2013b).

HER2 type tumors are named for their positive HER2/new status. Characteristics include negative ER and PR, positive lymph nodes and a poor tumor grade. Along with these factors, 75% of HER2 tumors contain a p53 mutation and occur in about 10-15% of breast cancers. HER2 tumors are diagnosed in women younger than those with luminal A and B tumors. These women have a fairly poor prognosis and are prone to early and frequent recurrence and metastases (Susan G. Komen Foundation, 2013b).

Basal-like tumors develop in the outer (basal) cells lining the mammary ducts of breast. These tumors tend to express HER1 and/or cytokeratin 5/6 proteins and most contain p53 mutations (Susan G. Komen Foundation, 2013b). Basal-like tumors tend to have similar characteristics to triple negative tumors. However, not all triple negative tumors are basal-like and not all basal-like tumors are triple negative. But whether basal-like or triple negative, 15-20% young African American women are diagnosed with this breast cancer. In comparison to the estrogen receptor-positive subtypes (luminal A and B), triple negative/basal-like tumors are often aggressive and have a poorer prognosis.

2.3 Triple Negative Breast Cancer

Triple Negative Breast Cancer is characterized by the absence of ER, PR, and HER2 receptors. Anyone can develop TNBC, but research has shown that it occurs more often in younger, African American women, Hispanic/Latina women and women with breast cancer susceptibility gene 1 (BRCA1) mutations. BRCA1 is a gene that normally functions as a tumor suppressor in humans by helping to ensure the stability of deoxyribonucleic acid (DNA) in a cell and prevent uncontrolled cell growth. Mutations made to this gene make one more susceptible to the development of cancer. TNBC is often a very aggressive tumor in comparison to other breast

cancer subtypes. This cancer is often more likely to metastasize to other parts of the body quickly. It also seems to recur more often than other subtypes of breast cancer.

Because of the lack of hormone receptors present in TNBC, treatment options are limited. TNBC is insensitive to most of the known effective therapies available for breast cancer treatment including HER2-directed therapy such as trastuzumab and endocrine therapies such as tamoxifen or aromatase inhibitors due to the lack of these receptors present (National Cancer Institute, Breast). Like most cancers, TNBC can be successfully treated if caught early, however the need for other targeted therapies for this type of breast cancer is still present.

Creating therapeutics for TNBC will instead target the affected pathways rather than the non-present receptors. Research found some of the most significant regulators of TNBC to be angiotensinogen (AGT), nuclear factor-kappa B (NF-κB), platelet derived growth factor receptor (PDGFR), and protein 53 (p53) pathways (Ossovskaya, 2011). AGT has been reported to significantly increase angiogenic proteins in receptor-negative cells. These proteins function in the process of angiogenesis, the formation of new blood vessels. The relationship between AGT and breast cancer has not yet been previously well characterized. However changes in NF-κB, PDGFR and p53 have been previously reported. NF-κB is a transcription factor involved in many functions, including stress-induced, immune, and inflammatory responses, development of certain hemopoietic cells, keratinocytes, and lymphoid organ structures, neoplastic progression, tumorigenesis, and an important regulator in cell fate (Baldwin, 1996). In cancer, NF-κB activation has been linked to cell proliferation, survival, invasion, and angiogenesis, resulting in a desirable target for therapy (Brown, 2008). PDGFRs' coincide with platelet derived growth factors (PDGF) that play an important role in development, cell proliferation, cell migration and angiogenesis. PDGFs act via two receptor tyrosine kinases with common domain structures,

platelet derived growth factor receptor-alpha (PDGFR-α) and platelet derived growth factor receptor-beta (PDGFR-β), each having a distinct function in inducing several signaling pathways (Andrae, 2008). While p53 is a tumor suppressor gene that has been found to be mutated in more than 50% of human cancers and the most mutated gene in TNBC (Azvolinsky, 2012; Bai, 2006). It has also proven to be a key regulator in the induction and progression of apoptosis.

2.4 p53, a tumor supressor

p53 is a nuclear phosphoprotein with a molecular weight 53 kDa (kilodaltons). The gene is 20 kilobases (kb) long and contains 11 exons with the first and second exons being separated by a 10 kb intron (Lamb, 1986). This gene can be found on the small arm of chromosome 17, and contains 393 amino acids, consisting of three functional domains: the N-terminus, central core, and C-terminus (Bai, 2006). The amino-terminus (N-terminus) contains an amino-terminal transactivation domain (residues 1-42) and a proline-rich region (residue 61-94). The central core (residues 102-292) is made up of DNA-binding domain required for sequence-specific DNA binding. This domain is frequently mutated in human cancer cells and tissues and highly conserved. The carboxyl-terminus (C-terminus) contains the tetramerization domain (residues 324-355), regulatory domain (residues 363-393), a nuclear localization signal sequence (NLS), and 3 nuclear export signal sequence (NES). This region is thought to perform regulatory functions as well as posttranslational modifications to residues, such as phosphorylation and acetylation. The C-terminus also functions as a negative regulator by regulating the ability of the core DNA binding domain to lock the DNA binding domain in a dormant conformation. If this interaction is interrupted by a posttranslational modification, the DNA binding domain becomes active, inducing enhanced transcriptional activity (Bai, 2006).

p53 is involved in multiple central cellular processes, including transcription, DNA repair, genomic stability, senescence, cell cycle control, and apoptosis. As a transcription factor, p53 domains are responsible for recognizing and binding to specific DNA elements and interacting with components found in the transcriptional machinery and activation. By binding to these regions, p53 can either activate some genes, or repress transcription of others (Chumakov, 2007). These processes occur in response to the induction of regulatory genes that are activated or repressed by p53. The activity of p53 must also be tightly regulated and controlled to prompt the desired results on cell growth and other processes. Regulation of p53 is seen more at the protein level with stability, control of subcellular localization, posttranslational modifications, and conformational changes that allow activation of DNA binding (Woods, 2001). Many transcription factors are also responsible for regulating p53 expression such as NF-κB, homeobox A5 (HOXA5) and murine double minute 2 (MDM2).

MDM2 has been deemed the master regulator for the stability of p53. This gene interacts with the N-terminal region and functions as an ubiquitin ligase for p53 (Woods, 2001). MDM2 is said to participate with the export of p53 from the nucleus to the cytoplasm, where degradation of p53 occurs. Many tumors have shown increased expression of MDM2, thus inactivating the apoptotic and cell cycle arrest functions of p53, as seen with deletion and mutations of p53 (Iwakuma, 2003).

With mutations seen in p53, the normal function of arresting the growth of cells that contain damaged DNA is compromised. However the presence of wild-type p53 is a strong indicator of apoptosis. The p53 signaling pathway remains in ‘standby’ mode under normal cellular conditions, only becoming activated in response to cellular stress and distinct regulatory kinases found upstream. Some of these include an ataxia telangectasia mutated (ATM)/human

homologue of Rad53 checkpoint kinase 2(Chk2)-dependent pathway activated by DNA double-strand breaks, $p14^{ARF}$, an alternative product of the INK4 gene, activated by expression of oncogenes, and a third pathway whose activity is increased by cytotoxic anti-tumor agents and ultraviolet (UV) light, but is independent of the previous pathways mentioned (Gasco, 2002).

In apoptosis, p53 can initiate, if DNA damage is proved to be irreparable. p53 affects both the extrinsic and intrinsic/mitochondrial pathways by either repressing or activating several different genes involved in the apoptosis process. The extrinsic pathway can be regulated via p53 activating transcription of apoptotic protease activating factor 1 (APAF1) gene and increasing the sensitivity to extrinsic proapoptotic ligands by stimulating transcription of FAS (also known as apoptosis antigen 1 (APO1)) and KILLER (also known as death receptor 5 (DR5)) genes (Chumakov, 2007). While p53s role in the intrinsic pathway involves repressing transcription of B-cell lymphoma 2 (Bcl2), an anti-apoptotic protein, and activating transcription of proapoptotic proteins Bcl2-associated X protein (Bax), Noxa, p53-regulated Apoptosis-Inducing Protein 1 (p53AIP1), and Puma (Chumakov, 2007). Deviations of the many pathways that regulate p53 activity, such as the activation of oncogenes Reticular activating system (Ras) and Myelocytomatosis (Myc), or the loss of tumor suppressor genes retinoblastoma protein (Rb) and ADP Ribosylation Factors (ARF) lead to the activation of p53 and thus the elimination of normal cells.

2.5 Poly (ADP-ribose) Polymerase & PARP Inhibitors

Poly (ADP-ribose) Polymerases (PARPs) are a family of enzymes involved in DNA repair via the base excision repair (BER) pathway. In the late 1980s, PARP was isolated. PARPs use nicotinamide adenine dinucleotide (NAD^+) as a substrate to transfer ADP-ribose onto glutamic acid residues of proteins (Smith, 2001). Of this family, the most well-known and

studied enzyme is PARP-1. PARP-1 plays a key role in repairing single-stranded DNA breaks through BER. It is a 116 kDa protein that contains three main functional domains: a DNA-binding domain, automodification domain (N-terminal), and catalytic domain (C-terminal) (Hong, 2004) (Figure 2).

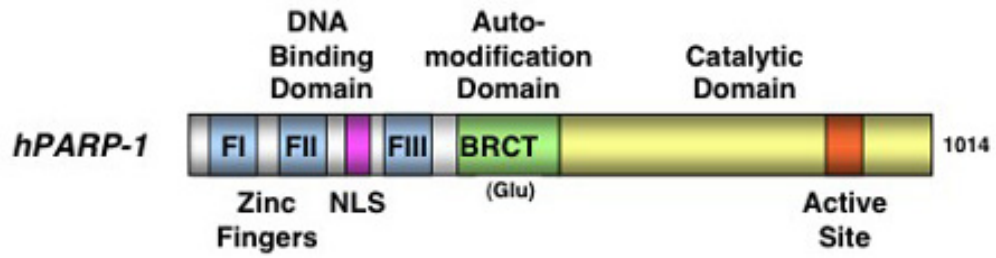

Figure 2. PARP-1 Structure

The DNA-binding domain utilizes three zinc-finger motifs that recognized single or double-stranded breaks in DNA (D'Amours, 1999). This domain also contains a nuclear localization signal (NLS). The auto-modification domain (AMD) serves as the target of covalent auto-poly(ADP-ribosyl)ation. Whereas the catalytic domain (C-terminal) catalyzes poly(ADP-ribosyl)ation reactions using NAD^+ molecules as a donor of ADP-ribose groups (Kim, 2005). All together these domains allow PARP-1 to interact with DNA and chromatin, poly(ADP-ribosyl)ate a variety of nuclear target proteins and regulates nuclear functions (Kraus, PARP-1). PARP-1 can function in regulating gene expression, amplification, cellular differentiation, malignant transformation, cellular division, DNA replication, mitochondrial function, and cell death. Under physiological conditions, inactive PARP-1 resides in the nucleoplasm. Breaks in DNA strand trigger the synthesis of poly(ADP-ribose) at sites if breakage, and PARP-1 binds tightly to nicked DNA (Smith, 2001).

PARP inhibitors mimic the nicotinamide moiety of NAD and bind to the enzyme's catalytic domain, inhibiting automodification and subsequent release of the enzyme from the DNA damage site (Kummar, et al., 2012). PARP inhibitors also prevent other repair proteins

from gaining access to sites of DNA breaks. PARP inhibition enhances the therapeutic index of cytotoxic chemotherapy only if the DNA damage is significantly increased in tumor compared to normal tissues (Kummar, et al., 2012). Therefore, the opportunity for PARP inhibitors to have the greatest killing affect and selectivity is vastly improved in tumors that already have DNA repair defects. Synthetic lethality is the term describing simultaneous dysfunction of two DNA damage repair pathways that decreases the ability of tumor cells to withstand the DNA damage produced during normal cellular replication (Iglehart, 2009). Two genes are said to be in a synthetic lethal relationship if a mutation in either gene alone is not lethal but mutations in both cause the death of the cell (Iglehart, 2009). For tumors harboring somatic or germline defects in non-BER DNA damage repair pathways, synthetic lethality can be accomplished with PARP inhibitors simultaneously blocking BER and non-BER pathways. The schematic seen in figure 3 describes the mechanisms cell death from synthetic lethality in normal cells, BRCA mutated cells, PARP inhibited cells, and cells with both defects (Iglehart & Silver, 2009).

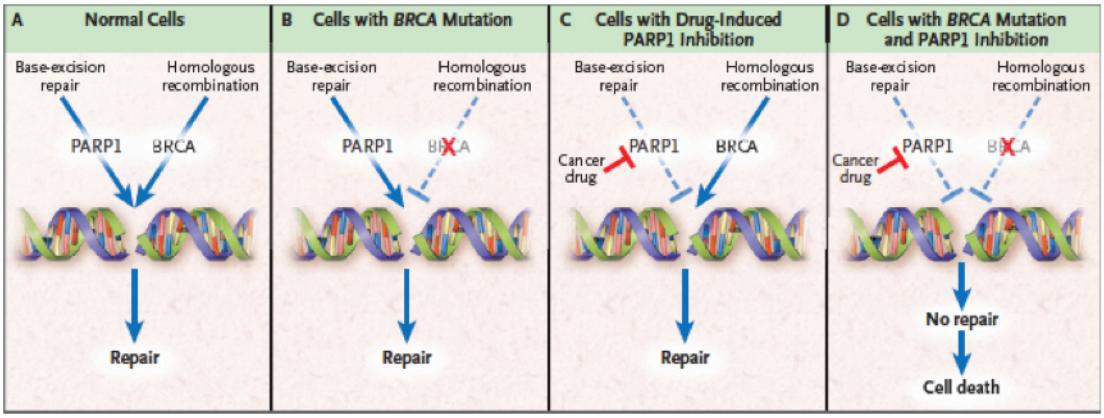

Figure 3. Synthetic Lethality Scheme

As shown in cases A-C, DNA repair is achieved due to the compensation of functioning of repair pathways, BER and/or homologous repair (HR). However in case D, the absence or BRCA along with the inhibition of PARP results in cell death due to the inability of both repair pathways (Figure 3).

PARP inhibitors have the ability to selectivity create cell death in tumors that have mutations to specific genes such as, BRCA1 or BRCA2. These patients are perfect candidates for treatment in being highly sensitive to this treatment through synthetic lethality resulting in apoptosis.

2.6 Apoptosis

Apoptosis is defined as programmed cell death characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. The term was first used in a paper written by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death (Elmore, 2007). The normal function of apoptosis is to serve as a homeostatic mechanism during development and aging, and results in maintaining cell populations in tissues. It also serves as a defense mechanism to immune reactions or cell damage caused by disease or other agents. Some stimuli and conditions that can trigger apoptosis can include irradiation, chemotherapeutics, and/ or hormones. Although a variation of things can initiate apoptosis, not all cells will die or even be affected by the same stimulus.

The apoptotic mechanism has two main pathways, extrinsic and intrinsic, that are highly complex and sophisticated. The extrinsic pathway is also known as the death receptor pathway and involves mediated interactions with transmembrane receptors. These receptors include members of the tumor necrosis factor (TNF) receptor gene superfamily. Members of this family share a similar cystein-rich extracellular domain and have a cytoplasmic domain of ~80 amino acids called the “death domain” (Ashkenazi, 1998). This domain plays a critical role in transmitting the death signals from the cell surface to the intracellular signaling pathways. The extrinsic pathway involves the ligands and corresponding death receptors Fas ligand (FasL)/Fas receptor (FasR) and Tumor necrosis factor alpha (TNF-α)/Tumor necrosis factor receptor

(TNFR1). In these models, clustering of receptors and binding with the homologous trimeric ligand occurs. Upon the ligand binding, cytoplasmic adapter proteins are recruited to bind with the receptors. The binding of FasL to FasR results in the binding of the adapter protein Fas-associated protein with Death Domain (FADD) and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein Tumor necrosis factor receptor type 1-associated death domain (TRADD) with recruitment of FADD and Receptor-interacting Protein (RIP). FADD then connects with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the autocatalytic activation of procaspase-8 (Kischkel, et al., 1995). Once caspase-8 is activated, the execution pathway of apoptosis is triggered.

The intrinsic pathway is also known as mitochondrial pathway and involves a diverse assortment of non-receptor mediated stimuli that produces intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events (Elmore, 2007). These stimuli produce intracellular signals that may act in either a negative or positive manner. Negative signals result from the absence of certain growth factors, hormones and cytokines that lead to failure of suppression of death receptors and trigger apoptosis. While stimuli that act in a positive manner include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, free radicals and DNA damage. This pathway involves a cascade of proteins that induce apoptosis. A healthy cell is characterized by mitochondria's outer membrane containing Bcl-2 on its surface. The Bcl-2 protein functions in inhibiting apoptosis within a health cell. However in a damaged cell, the protein Bax, migrated to the surface, inhibits Bcl-2 and creates holes within the mitochondrial membrane causing cytochrome c to leak out. The cytochrome c then binds to apoptotic protease activatin factor-1 (Apaf-1) and forms a complex known as

apoptosomes. Apoptosomes in turn bind to and activate caspase-9, which functions in cleaving proteins. As Caspase-9 cleaves proteins it activates other caspases in an expanding cascade that results in digestion, degradation, and phagocytosis of the cell.

2.7 Styrene

These styrenes, also known as 3-nitro-2-styrlbenzoic, are acid derivatives that are precursors to indole and share the same structural motif as a well-known chemopreventive, resveratrol. The structure for indole is a bicyclic aromatic with a nitrogen pyrole group (Figure 4).

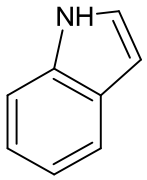

Figure 4. Indole Structure

Resveratrol (3.5.4'-trihydroxy-trans-stibene) (Figure 5) is a stilbenoid and natural polyphenol present in the skin of grapes and red wine (Horvath, 2004).

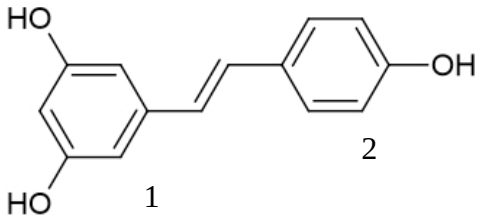

Figure 5. Resveratrol Structure

Resveratrol can also be found in food products and beverages such as peanut butter, dark chocolate, and grape juice. Phenols are of particular interest in the development of chemopreventives. Resveratrol is an important structure to use as a building block for new chemopreventives due to its proven effectiveness in biological function. Resveratrol possesses

antioxidant activity and may decrease reactive oxygen species production in blood platelets and oxidant-induced apoptosis (Stivala, et al., 2001). It can also inhibit platelet aggregation induced by thrombin, collagen, and adenosine diphosphate and reduce inflammation by inhibiting prostaglandin production, cyclooxygenase-2 activity and nuclear factor-B activity (King-Bioactivity). In addition to antioxidant and anti-inflammatory properties, resveratrol can inhibit D-type cyclins and cyclin-dependent kinase (Cdk) 4 expression (Lee, 2008; Liang, 2007). This can also lead to inducing tumor suppressors, p53, and Cdk inhibitor, protein 21 (p21).

These stilbene-based molecules are widely found in nature and have a versatile function in many biological activities. The styrene derivatives used, have a trans-stilbene structure with biologically active amide and nitro groups in addition to electron withdrawing or donating substituents on the second ring. The substituents on ring 2 are biologically active and naturally occurring in the body. A push-pull mechanism will result from placement of these groups to stabilize the styrene structure. Due to the structural similarities, with substituents at the ortho-, meta- and para- positions (as seen in Figure 6), it is likely; the 3-nitro-2-stryrlbenzoic acid derivatives will be effective chemopreventives.

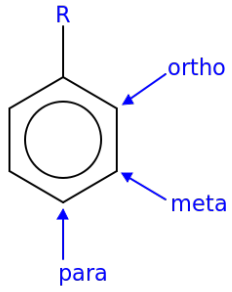

Figure 6. Relative Positions on Benzene Ring

Theses derivatives will maximize the biological activity without increasing cytotoxicity in the body.

CHAPTER 3

Material & Methods

3.1 Cell Lines

Human breast cancer cell lines HCC1806 and HCC70 were used throughout this project and purchased from the American Type Culture Collections (Manassas, VA). Both cell lines are classified as triple negative breast cancer. HCC1806 was derived from a 60-year-old African American female suffering from primary squamous cell carcinoma. HCC1806 lacks the expression of ER, PR, and HER2 receptors. HCC70 is basal A subtype breast cancer cell line derived from the mammary gland of a 49-year-old African American female suffering from primary ductal carcinoma. This cell line lacks the expression of ER, PR, and HER2 as well. Both cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 (Thermo Scientific; Rockford, IL) that contains 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S) (Bio Express: Kaysville, UT).

3.2 Styrene Compounds

Styrene compounds used as treatments were chemically synthesized. Each compound consisted of the common Methyl-2-methyl-3 nitrobenzoate reactant plus an additional type of benzaldehyde reactant to produce a *(E)*-3-nitro-2-styrylbenzoic acid product (labeled JKS 1.001, -1.006, -1.007, -1.009) as seen in the general reaction (Figure 7).

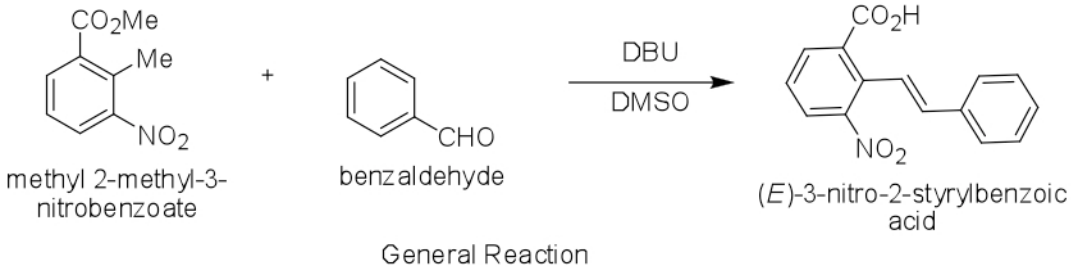

Figure 7. Styrene General Reaction

Compound JKS 1.001 was synthesized by reacting methyl-2-methy-3-nitobenzoate and 2,3-demethoxybenaldehyde in the presence of base to yield 2-[trans-2-(2,3-dimethylphenyl)-vinyl]-3-nitrobenzoic after sufficient workup. Compound JKS 1.006 was synthesized by reacting methyl-2-methy-3-nitobenzoate and 4-chlorobenzaldehyde in the presence of base to yield 2-[trans-2-(4-chlorophenly)-vinyl]-3-nitrobenzoic after sufficient workup. Compound JKS 1.007 was synthesized by reacting Methyl-2-methy-3-nitobenzoate and 2-methoxybenzaldehyde in the presence of base to yield 2-[trans-2-(methoxyphenyl)-vinyl]-3-nitrobenzoic after sufficient workup. Compound JKS 1.009 was synthesized by reacting methyl-2-methy-3-nitobenzoate and 2-(Trifluoromethyl) benzaldehyde in the presence of base to yield 2-[trans-2-(2-9trifluormethylphenyl)-vinyl]-3-nitrobenzoic after sufficient workup (Figure 8).

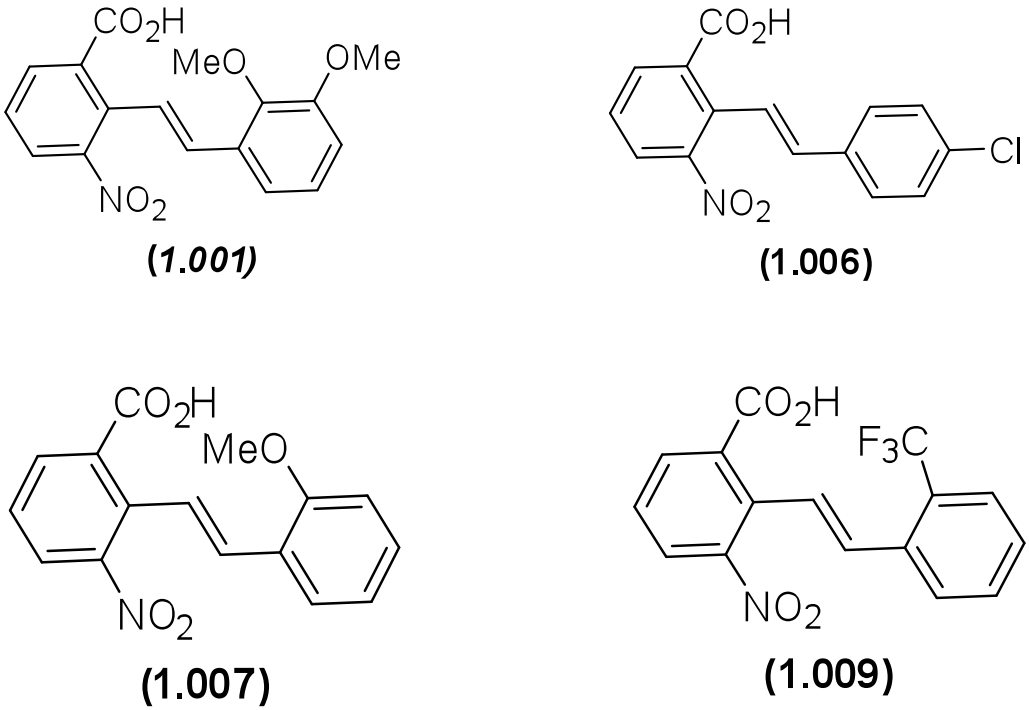

Figure 8. Styrene Compounds

3.3 Chemotherapeutic Drug Treatments

Each compound was diluted in dimethyl sulfoxide (DMSO) to attain a stock concentration of 1000 μM. The following amount of grams was dissolved in 2 ml of DMSO to bring the molarity to approximately 1000 μM (Table 1).

Table 1

Styrene Preparations

Treatment/Chemical	Molecular Weight (g/mol)	Amount Used (g)
JKS 1.001	329.19	0.0006
JKS 1.006	303.70	0.0006
JKS 1.007	299.28	0.0007
JKS 1.009	337.25	0.0008

The working concentration was further diluted into a 100 and 10 μM for further titration treatments. Adherent cells were exposed to chemical treatments. Following exposure, cells were photographed using at Phase-Contrast Microscope at 10x and 20x magnification to assess physical apoptosis. Treatments were pipetted on 10 cm plates for each cell line and incubated up to 24 h at 37 (℃) and 4% carbon dioxide (CO_2). Floating or dead cells were collected and adherent cells were washed with 3 ml of 1X Phosphate Buffer Saline (PBS). All washes were collected and held for further analysis. Approximately 2 ml of trypsin (Hyclone Laboratory; Logan, UT) was added to the plates and incubated until cells detached from plate. To stop trypsin action, 2 ml of media was added to each plate. The chemically treated media, 1X PBS, trypsin, and fresh media were all collected in a designated 15 ml conical per treatment and mixed via pipette.

3.4 Cell Viability

Following treatment, cells were analyzed using the VI-Cell XR Viability Analyzer to quantify apoptotic cells. 500 µl of each treatment was placed into labeled sample cups, in triplicates (total of 15 sample cups). Nine sample cups were placed in the VI-Cell XR Cell Viability Analyzer at a time and analyzed following the user's manual protocol. (CMCresson, User Instructions) The remaining solution within each conical were aliquot out amongst 6 eppendorf tubes per treatment (totaling 36 tubes including both untreated control and vehicle control per concentration). Eppendorf tubes were centrifuges at 5,000 rpms for 5 minutes to produce pellets. Supernatant was removed from each eppendorf again with the aspirator vacuum. Eppendorfs were stored at -80ºC.

3.5 MTT Assay

Cells were grown on a 96-well plate for 24 hours. MTT solution was added to each well and incubated at 37ºC in 4% CO_2 for approximately 4 hours. Solution C was then added to each well and incubated for up to an hour. Absorbance was measured at wavelengths of 570 and 630 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader.

3.6 HT Fluorescent PARP Inhibition Assay (HFHPIA)

Styrene compounds were analyzed with Trevigen's HT F Homogeneous PARP Inhibition assay kit to determine the PARP inhibition potential. The Trevigen protocol was followed with some changes to accommodate using DMSO as a solvent for the styrene compounds. A black 96-well microplate along with all reagents was provided. The protocol was followed except a change in concentration levels due to the lack of availability of the styrene compounds. Concentrations were reduced to 5, 15 and 25x.

CHAPTER 4

Results

4.1 To identify the apoptotic effect of these styrene compounds and the most effective working concentration.

Following 24 hours, both cell lines (HCC1806 and HCC70) treated at various concentrations (10, 100, and 1000 μM) were visualized at 20x magnification under a Phase-Contrast Microscope (Figures 9-14). Exposure of breast cancer cell lines to styrene compounds demonstrates apoptosis within a 24 hour time period. Qualitative analysis determined an approximation of the amount of apoptosis produced by each styrene compound based upon apoptotic characteristics seen in the images such as undefined shape and clustering of the cells.

At a concentration of 10 μM, cell line HCC70 qualitative analysis exhibited compound 1.006 yielding the greatest amount of apoptosis due to morphological integrity of the cells within a 24 hour time period (Figure 9). Cell line HCC1806 exhibited compound 1.007 yielding the greatest amount of apoptosis within 24 hours (Figure 10).

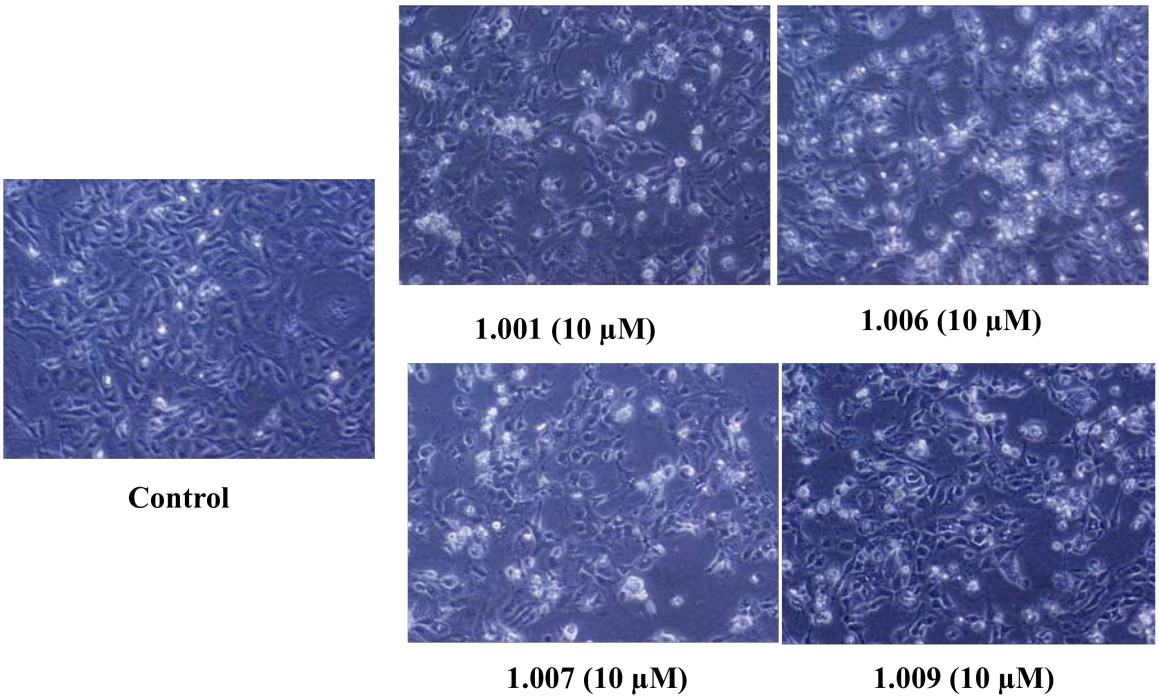

Figure 9. Qualitative Analysis of HCC70 at 10 µM**.**

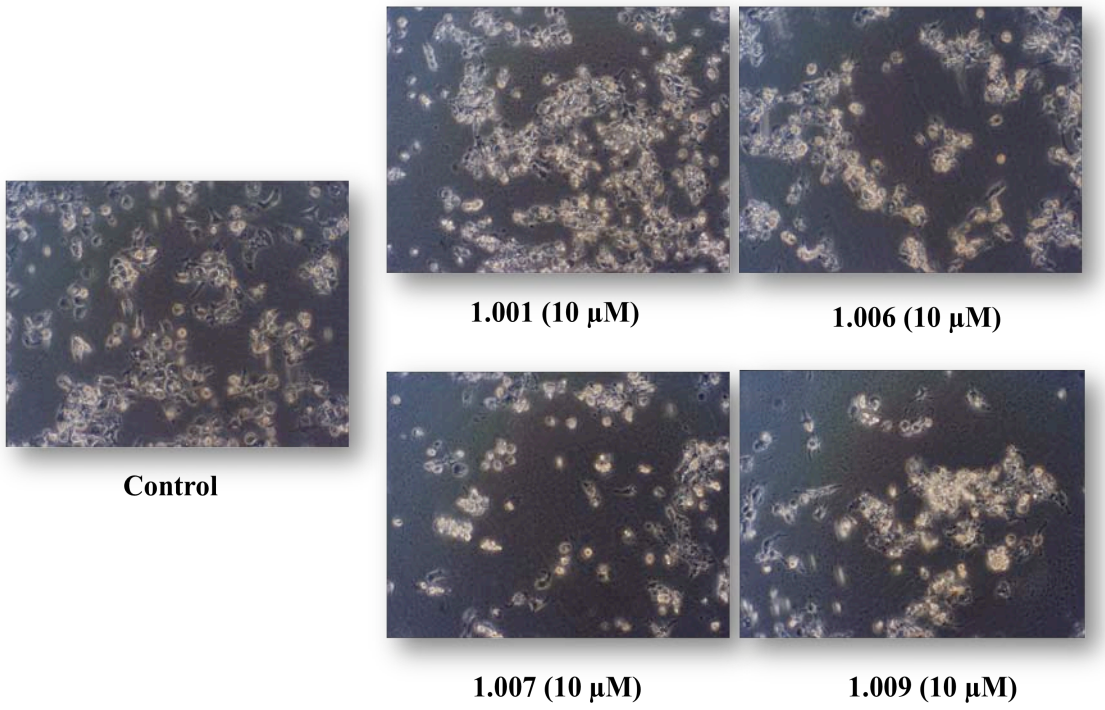

Figure 10. Qualitative Analysis of HCC1806 at 10 µM.

At a concentration of 100 µM, cell line HCC70, qualitative analysis exhibited compound 1.009 yielding the greatest amount of apoptosis. In cell line, HCC1806, 1.007 yielded the greatest amount of apoptosis (Figures 11 & 12).

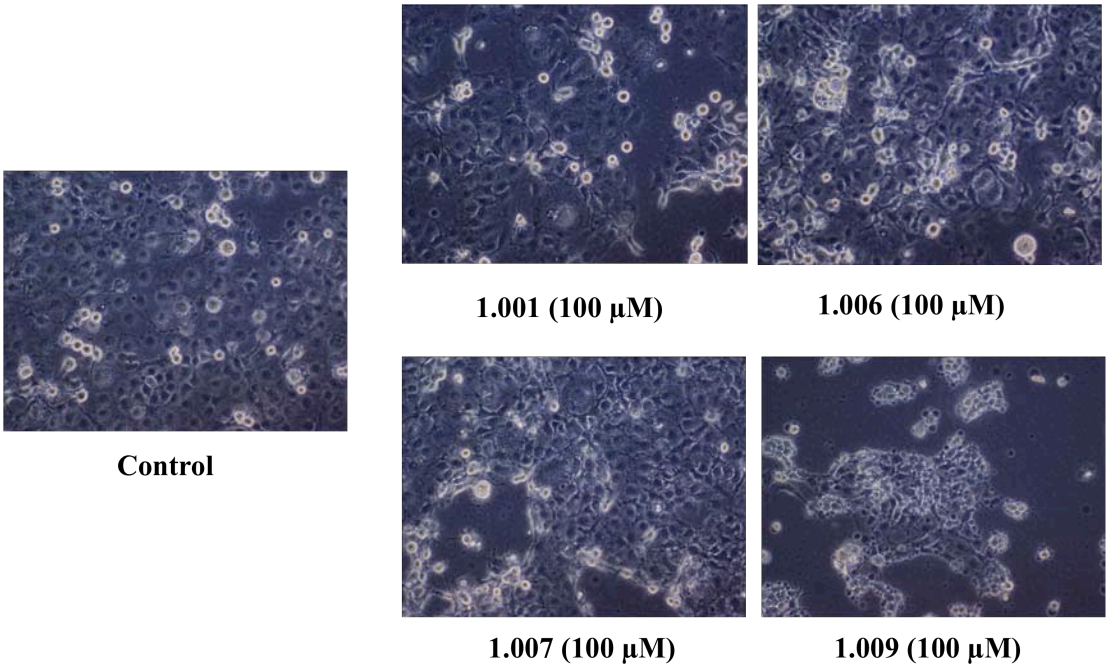

Figure 11. Qualitative Analysis of HCC70 at 100 µM

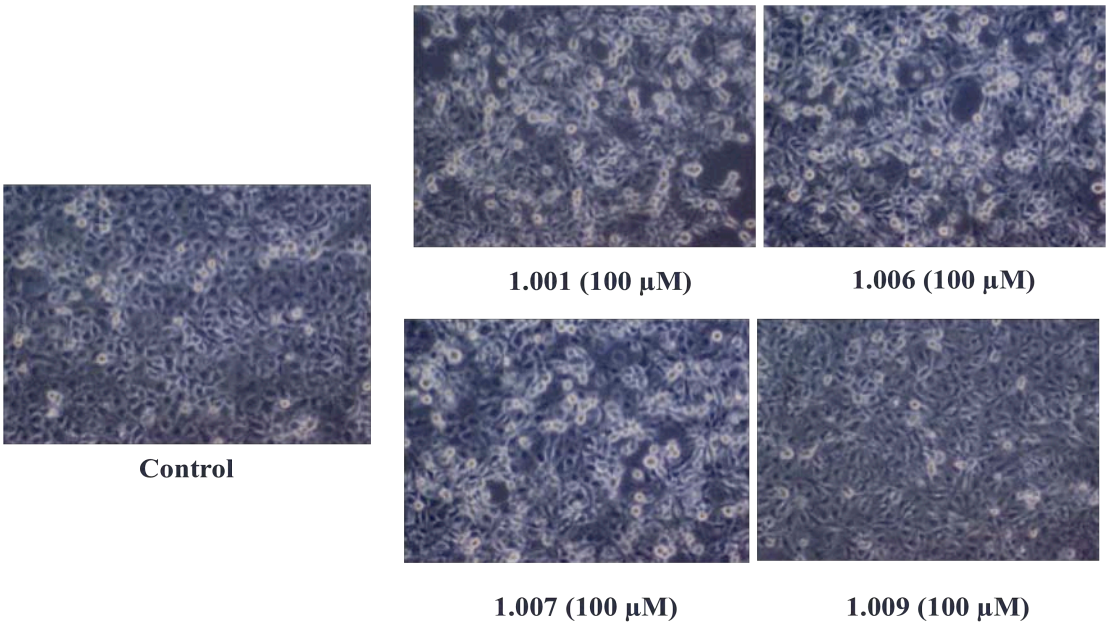

Figure 12. Qualitative Analysis of HCC1806 at 100 µM

At a concentration of 1000 µM, cell line, HCC70, exhibited compound 1.009 yielding the greatest amount of apoptosis. While cell line HCC1806, exhibited compound 1.007 yielding the greatest amount (Figures 13 & 14).

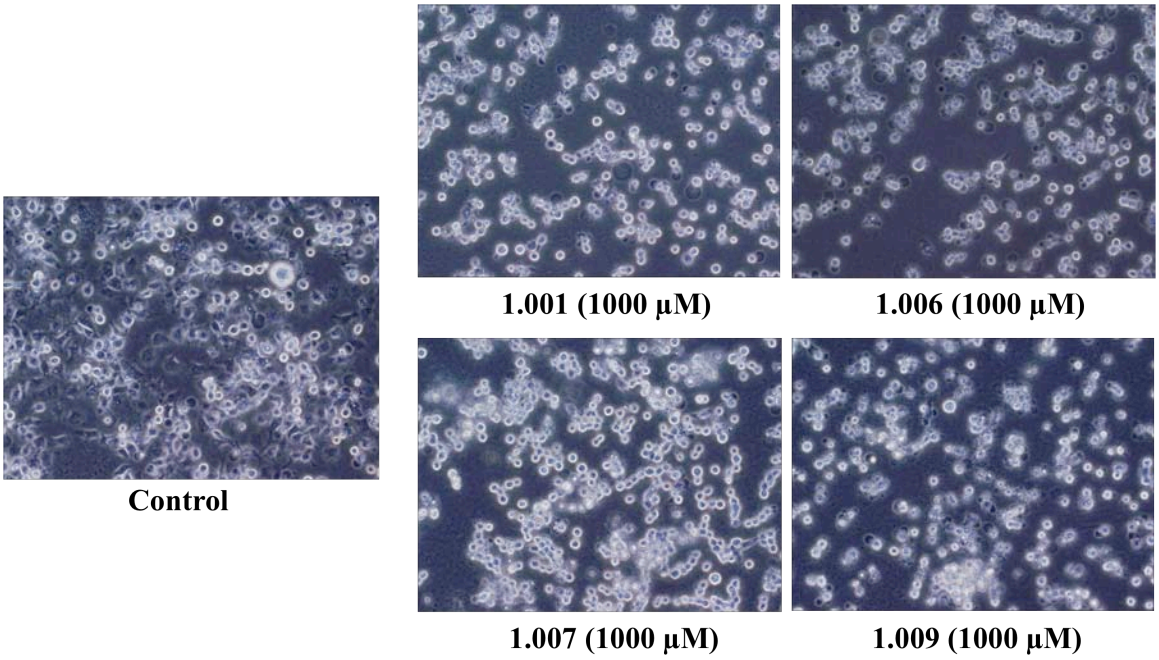

Figure 13. Qualitative Analysis of HCC70 at 1000 µM

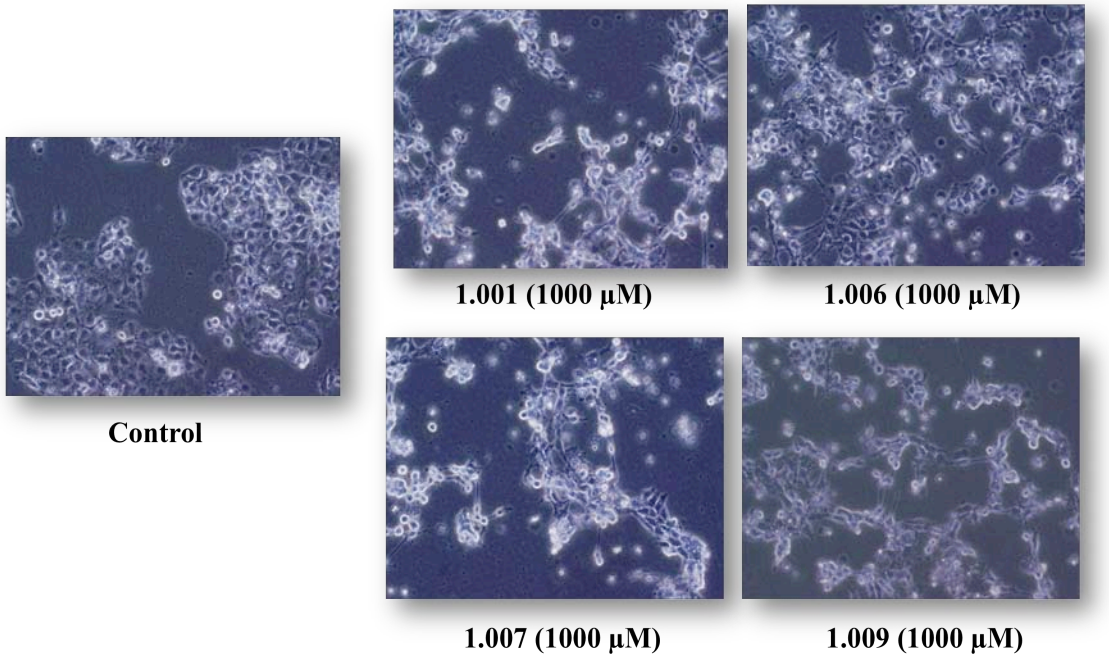

Figure 14. Qualitative Analysis of HCC1806 at 1000 µM

Following qualitative analysis, quantitative analysis was performed and analyzed with the VI-Cell XR Viability Analyzer (Figures 15-20). Quantitative analysis confirmed cell viability of each cell line following 24-hour exposure to each styrene at various concentrations. Viability percent describes the amount of cells living, calculated as the percent viability value subtracted from 100 resulting in the Apoptosis Percentage (%).

Quantitative analysis at 10 µM confirms compound 1.006 to exhibited the greatest amount of apoptosis in HCC70 cell line. However, in the HCC1806 cell line, compound 1.001 exhibited the greatest amount of death (Figures 15 &16).

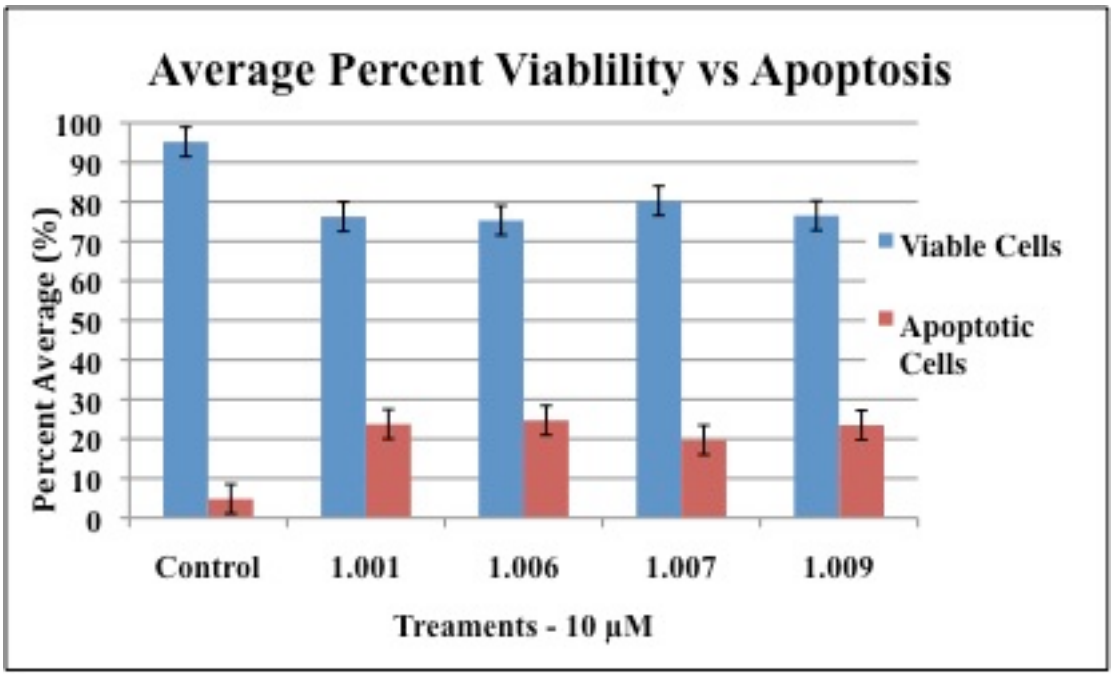

Figure 15. Quantitative Analysis of HCC70 at 10 µM

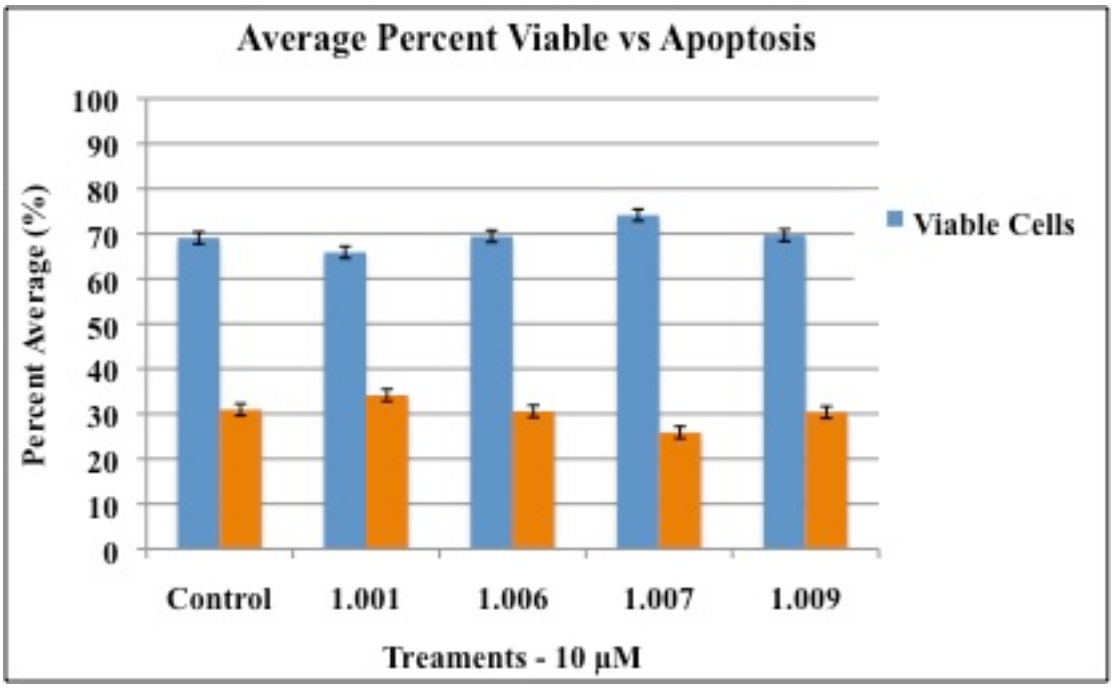

Figure 16. Quantitative Analysis of HCC1806 at 10 µM

Quantitative analysis show in both cell lines that compound 1.009 yielded the maximum apoptotic effect at 100 µM Figures 17 & 18.

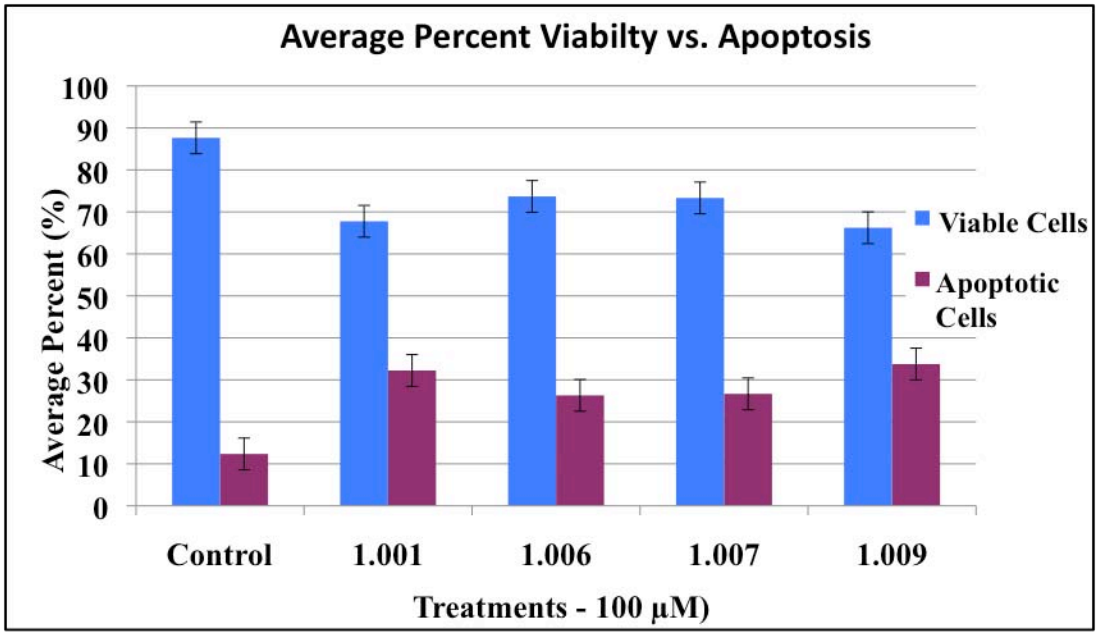

Figure 17. Quantitative Analysis o HCC70 at 100 µM

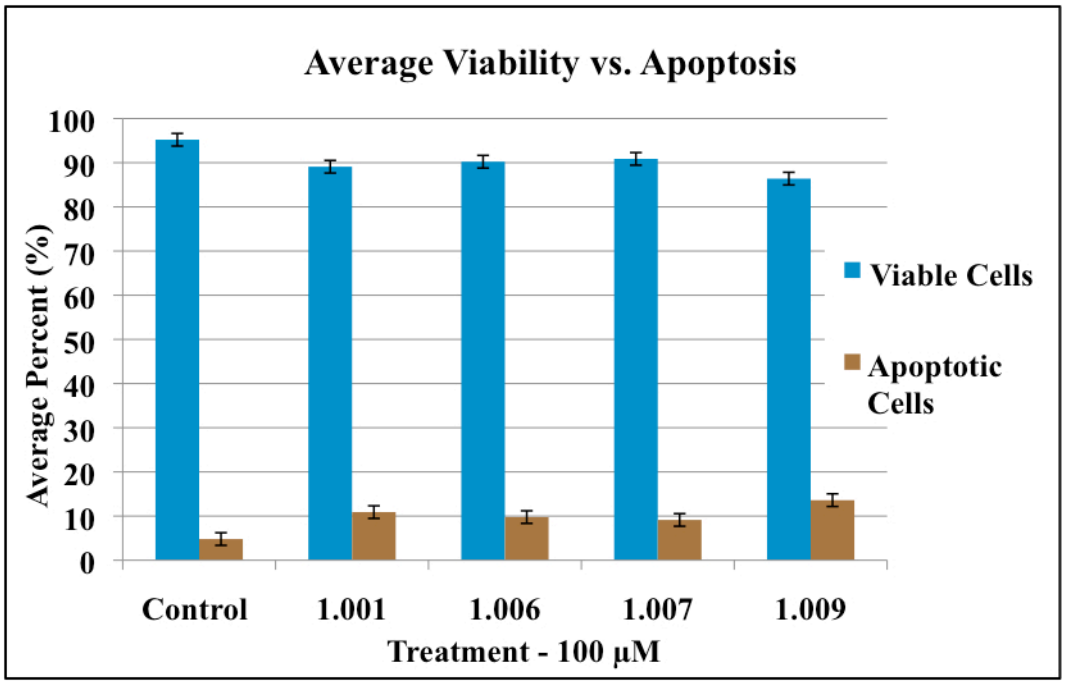

Figure 18. Quantitative Analysis of HCC1806 at 100 µM

Quantitative analysis shows that both compounds yielded maximum apoptotic effect at 1000 µM in Figures 19 & 20. The 70-cell line had a significant amount of apoptosis in compound 1.009 and the 1806-cell line in compound 1.007.

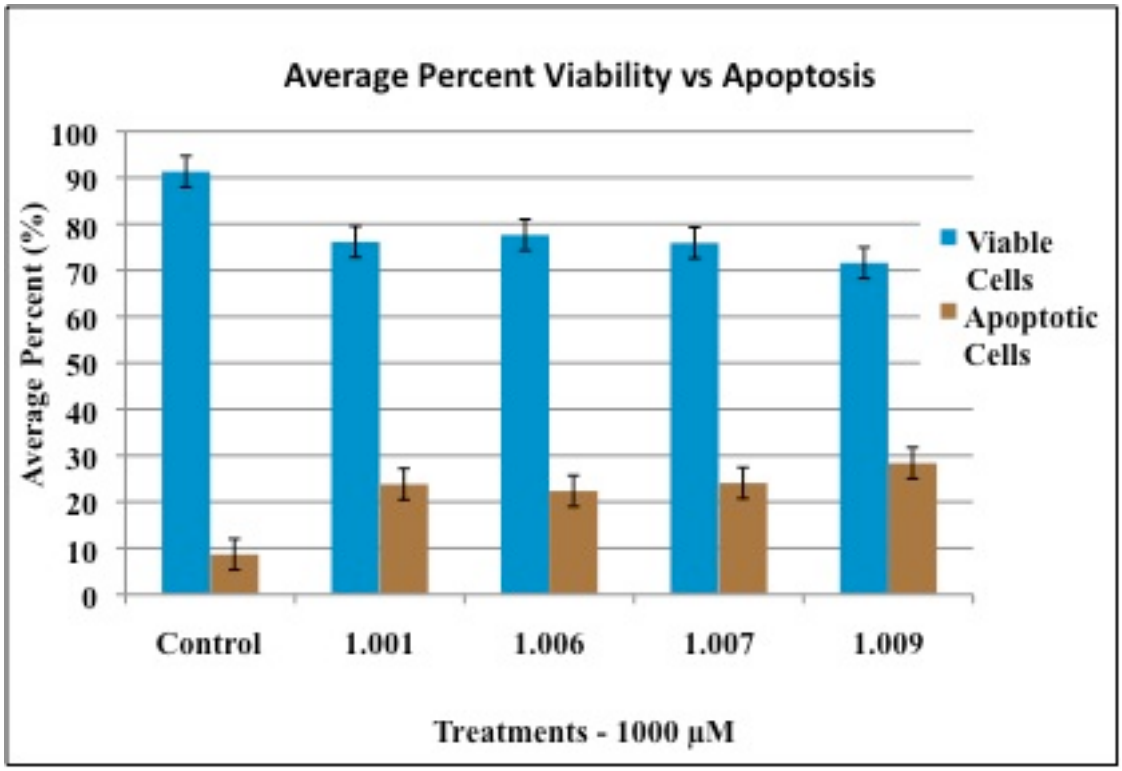

Figure 19. Quantitative Analysis of HCC70 at 1000 µM

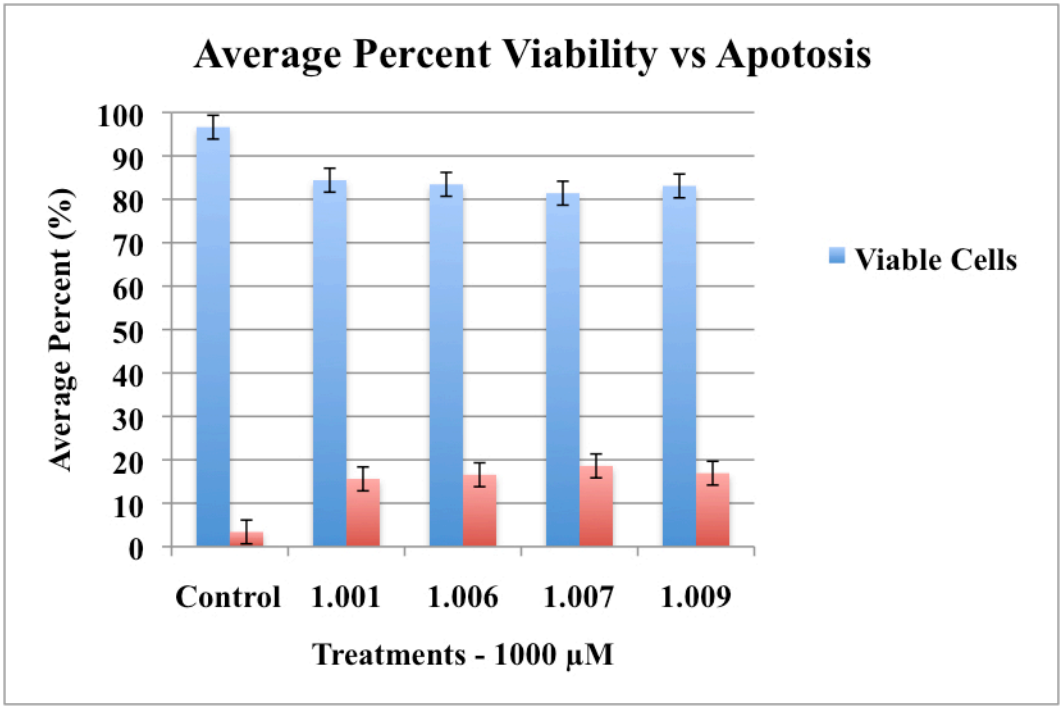

Figure 20. Quantitative Analysis of HCC1806 at 1000 µM

From here, we were able to identify the working concentration in both triple negative cell lines. For 70, the working concentration was 100 µM and for 1806, 10 µM.

An MTT assay was then performed with both cell lines at 100 µM. Results indicate that compounds 1.007 (HCC70) and 1.009 (HCC1806) yield the greatest amount of apoptosis at the 100 µM concentration (as seen in Figures 21 & 22).

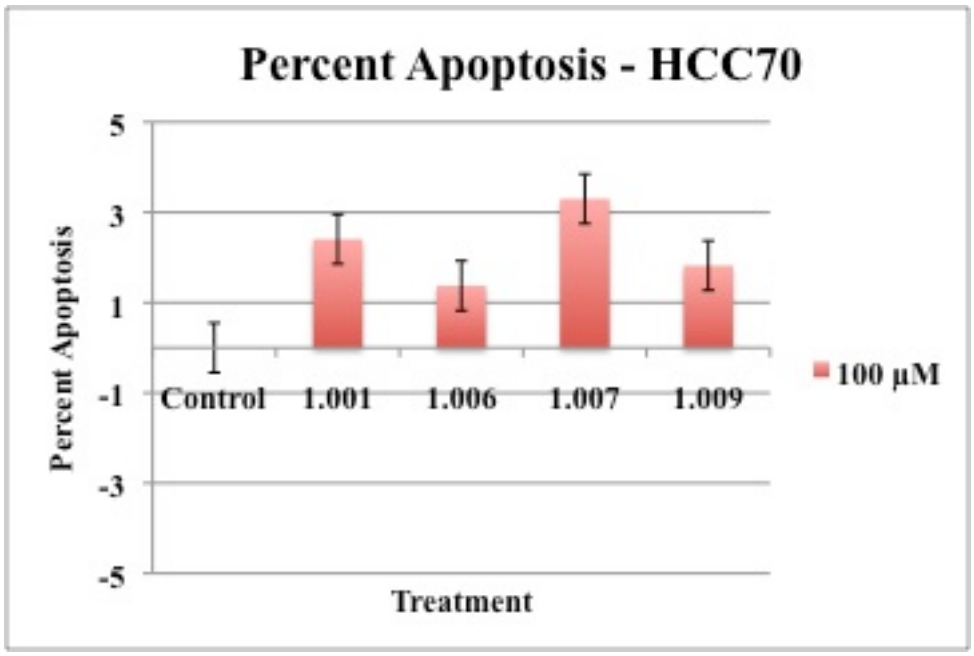

Figure 21. HCC70 MTT Assay at 100 µM

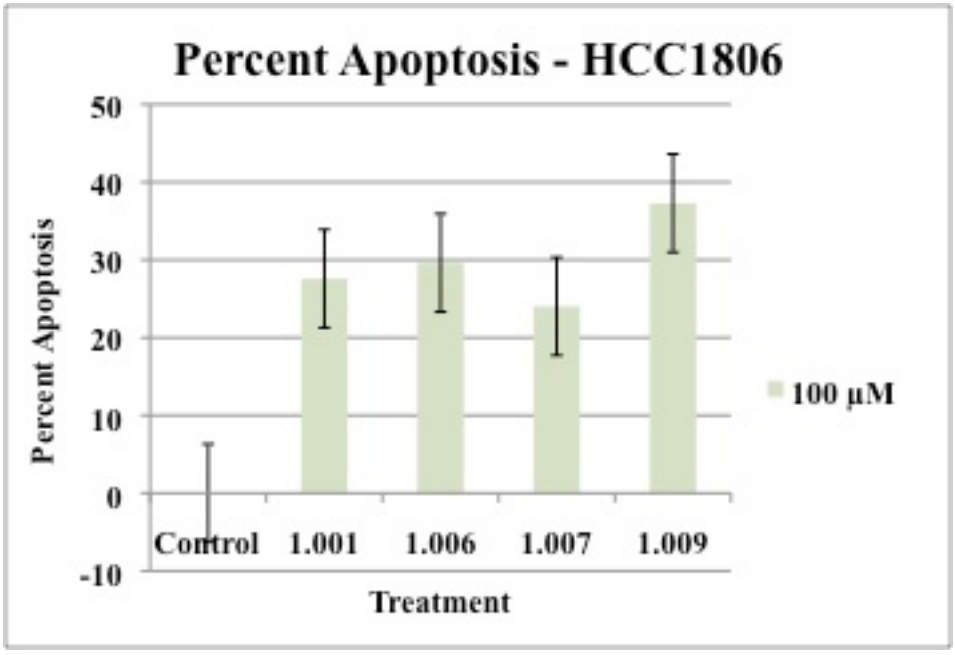

Figure 22. HCC1806 MTT Assay at 100 µM

4.2 To identify PARP Inhibition potential of each styrene compound.

HFHPIA was used to determine whether these compounds were PARP inhibitors. Results determine all compounds at various concentrations (5, 15 & 25x) are PARP inhibitors (Figure 23).

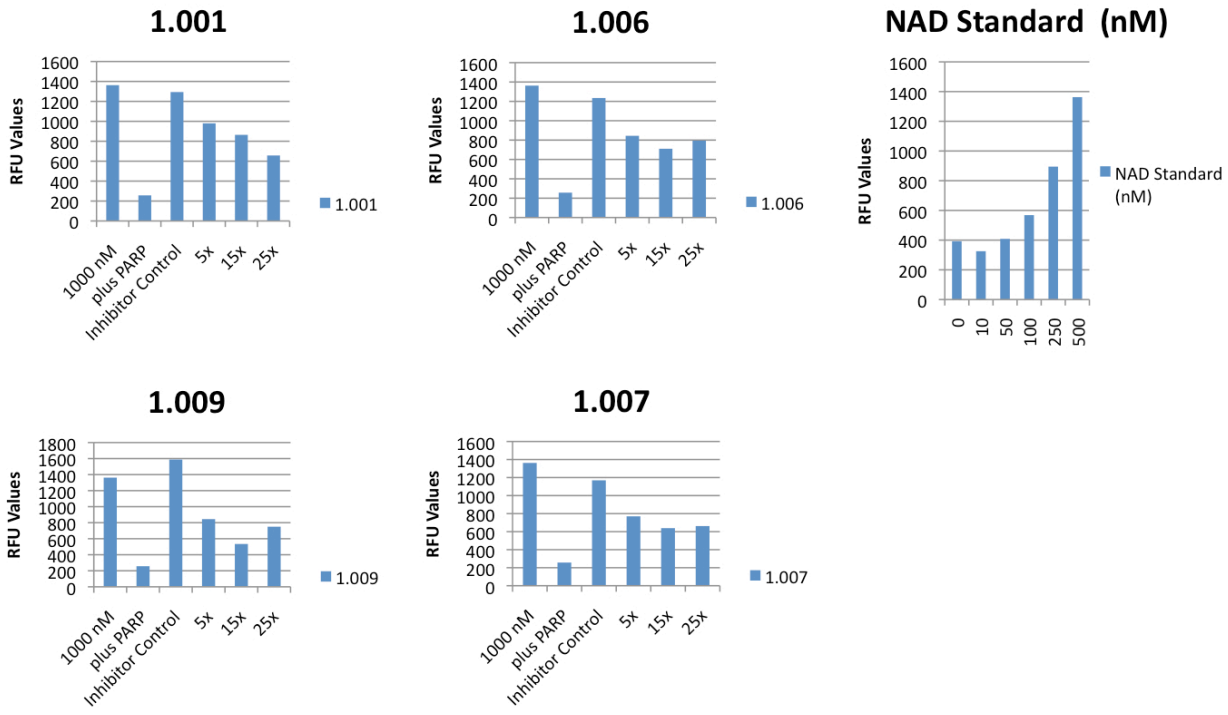

Figure 23. HT Fluorescent Homogeneous PARP Inhibition Assay

CHAPTER 5

Discussion

Experimental data have shown these styrene compounds to have an apoptotic effect on triple negative breast cancer cell lines, HCC1806 and HCC70. Although the results vary per compound and concentration, there is a significance seen in the cell death versus viable cells (Table 2).

Table 2

Summary of Styrene Compounds that produces the greatest amount of apoptosis

	HCC70	HCC1806
10 μM	1.006	1.001
100 μM	1.009	1.007
1000 μM	1.009	1.007

Although we do not see one common styrenyl compound yielding a great amount of apoptosis in both cell lines throughout this study, we see that each compound induces apoptosis within these TNBC cell lines. We can also determine at which concentration we produce the best results within a 24-hour period (Table 3).

Table 3

Summary of the best working concentration for cell lines HCC70 & HCC1806

(Numbers indicate Average Percent Viability)

	HCC70			HCC1806		
	10μM	100μM	1000μM	10μM	100μM	1000μM
1.001	76.2	**67.8**	76.2	**65.9**	93.4	84.4
1.006	75.3	**73.7**	77.7	**69.4**	93.6	83.4
1.007	80.3	**73.3**	75.9	**74.1**	92.5	81.4
1.009	76.5	**66.2**	71.6	**69.7**	93.7	83.1

5.1 Styrene compounds produce apoptosis in both HCC1806 and 70 cell lines.

Compounds 1.007 and 1.009 resulted in the greatest amount of apoptosis at a molar concentration of 100 μM in HCC70 and 1806, respectively. We verify this with a Cell Viability and MTT assay. The cell viability quantified the amount of apoptosis seen within each cell line and at various concentrations. It was expected that the styrene compounds would cause death in these cells within a given time. When identifying the working concentration the results we expected were not potrayed. In many drug development studies, the lower concentration is used most often because lower treatments are often used in in-vitro studies to caution the effect of the drug on normal healthy cells. We saw this in the HCC1806 cell line, where the most apoptosis was seen with each styrene compound at 10 μM. HCC1806 is a well-known TNBC cell line, with a different p53 status than HCC70. The p53 protein in HCC1806 contains a frame-shift mutation, where an insertion of 2 base pairs occurs at exon 7 codon 256. A difference is seen in

the p53 status of HCC70, where there is a missense (or point) mutation, where one single nucleotide is changed, at exon 7 codon 248 from GAC→CAC (Lacroix, 2006). The difference in p53 status could have an effect on the apoptotic effect of these compounds on these TNBC cell lines, since we know p53 play a major role as a regulator in the intrinsic apoptotic pathway. After a certain amount of time all cells will go into apoptosis due to other stress factors. This can be further analyzed by treating tells at different time points (ex. 8, 12, or 48 hours) other than the 24 hours period used here.

The focus then turned to the 100 μM concentration for further testing on apoptosis with these compounds. This concentration was chosen for further testing because it played the median between the other two concentrations initially used. Through observation we were able to direct the attention to certain styrene compounds that can be used in further testing. At 100 μM, compounds 1.007 and 1.009 exhibit the greatest amount of apoptosis after performing a MTT Assay (Figures 21 & 22). Both styrene compounds, 1.007 and 1.009, are the most mentioned in Table 2 with both cell lines at the higher concentrations.

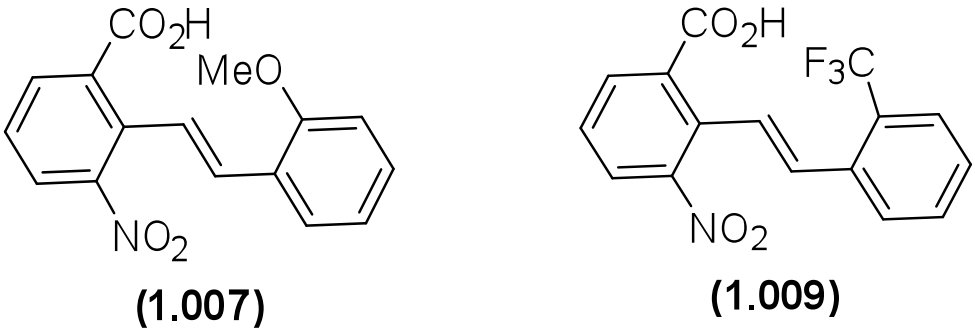

Figure 24. Styrene Compounds 1.007 & 1.009

Both of these compounds differ from the other 2 compounds by the ortho positioning of the substituents on the second benzene ring. However, each (1.007 and 1.009) has a different substituent at this position, methoxy and trifluoromethyl. (as seen in Figure 26) Each of these

functional groups could also play a role in its effect on these TNBC cell lines. Although each of these theories has not been analyzed in this study, what we do know is that each of these compounds, along with the other two, has an apoptotic effect on HCC70 and 1806.

5.2 Styrene compounds are identified as PARP inhibitors based upon the HT Fluorescent Homogenous PARP Inhibition Assay.

In this assay, inhibitors are identified by an increase in fluorescent signal when PARP mediated NAD^+ depletion is inhibited. The level of NAD^+ is coupled to a cycling assay involving alcohol dehydrogenase and diaphorase. Each time NAD^+ cycles through these coupled reactions, a molecule of highly fluorescent resorufin is generated (from the non-fluorescent substrate, resazurin). In the presence of the PARP enzyme (plus PARP) the NAD^+ fluorescent signal vastly decreases, in comparison to the inhibitor control where the signal increases. The known inhibitor used is able to block the impact of PARP. The assay interprets the data collected from the fluorescence plate reader (at 590 nm) as a median between the plus PARP and inhibitor control. Therefore results from this assay show each styrene compound at various concentrations (5, 15 and 25x) are PARP inhibitors. It is also shown with this assay that the lower concentrations of these compounds have a greater PARP inhibitor potential than the higher concentrations. This is the same trend that was seen with the apoptotic effect of these styrene compounds at the various molar concentrations previously. However this assay was not affected by the difference in cell lines. HFHPIA is solely based on the styrene compounds composition, structure, and concentrations.

This study successfully identified the apoptotic effect and the PARP inhibitor potential of these styrene compounds. Future directions would include 1) setting time points 2) performing titrations at lower concentrations 3) comparing compounds to known PARP inhibitors 4) analyze

the effect on these compounds in different breast cancer cell lines as well as healthy cell lines. Analyzing these cells at different time points will acquire the point at which the cells first begin to degrade and progress into cell death. We can also examine the effect these compounds will have in treating cells for an extended amount of time (i.e. 48 hours). Since most drug development uses the lowest concentration in clinical settings, titration of these drugs to lower concentrations to see if they still have an apoptotic effect is the next step in developing a novel chemotherapeutic. We can then test these compounds next to known PARP inhibitors to compare and contrast the effects with more knowledgeable drugs. The effect these compounds have on different breast cancer cell lines as well as normal healthy cells is important in understanding the cytotoxity to normal cells and the potential therapies used in other cancers.

In summary, this study was preliminary work for the treatment of TNBC with these styrene compounds. Results revealed these compounds as a possible novel chemotherapeutic in TNBC. Identifying a novel target therapy for TNBC will initiate clinical studies and create a significant impact in the African-American and breast cancer research community.

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