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*CIS*-3, 4', 5-TRIMETHOXY-3'-AMINOSTILBENE (STILBENE 5C) INDUCES  
APOPTOSIS AND PROTECTIVE AUTOPHAGY IN B16F10 MELANOMA CELLS.

A thesis submitted in fulfillment of the requirements for the degree of M.S. in Pharmacology and  
Toxicology at Virginia Commonwealth University

by

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## Abbreviations

D: day

SS : serum starved

baf: bafilomycin A1

IR: irradiated (10gy)

PARP: Poly(ADP-ribose) polymerase

LC3: Light chain microtubule

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

DAPI: 4',6-diamidino-2-phenylindole

AO: acridine orange

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

DMSO: dimethyl sulfoxide

ADR: adriamycin

combo: stilbene 5c + bafilomycin A1

## Abstract

### *CIS*-3,4', 5-TRIMETHOXY-3'-AMINOSTILBENE (STILBENE 5C) INDUCES APOPTOSIS AND PROTECTIVE AUTOPHAGY IN B16F10 MELANOMA CELLS

By Betelehem Mulu Asnake

A thesis submitted in fulfillment of the requirements for the degree of M.S. in  
Pharmacology/Toxicology at Virginia Commonwealth University

Virginia Commonwealth University, 2011

Major Director: Dr. David Gewirtz  
Professor in Pharmacology and Toxicology

The weak selectivity of chemotherapeutic drugs against tumors has sustained efforts to develop better chemotherapeutic agents that are more potent and selective at destroying tumor cell populations versus normal tissues. This project focuses on evaluating the cell killing effects of the microtubule inhibitor, stilbene 5c, against melanoma cancer. We utilized an *in vitro* murine melanoma model to study the effects of stilbene 5c on tumor proliferation and survival, as well as growth arrest and cell death. Our findings indicate that stilbene 5c promotes dose dependent cell death in melanomas with the induction of apoptosis and autophagy. The role of autophagy was further assessed using the pharmacological autophagy inhibitor, bafilomycin A1. It was concluded that autophagy was partially cytoprotective as inhibition of autophagy was shown to induce extensive cell death through an increase in apoptosis. Residual surviving cells were shown to be in a state of growth arrest characterized to be senescence. These findings indicate that stilbene 5c could potentially be developed for the treatment of melanoma.

# **Chapter 1: Introduction**

## **I. Cancer**

Millions of cells in our body have differentiated to perform specific functions. For instance, nerve cells control the transmission of messages, muscle cells coordinate contraction and relaxation while white blood cells defend our tissues from foreign microorganisms. The processes of cell growth and cell division, which are essential components of replacing aged cells by new ones, are tightly controlled. Specific genes work in coordination with one another to determine the fate of the cell, its survival or its programmed death. Cancer develops when these various regulating mechanisms are disrupted, resulting in the unresponsiveness of the cell to regulatory signals and thus uncontrolled division of the cells. Cells that become cancerous grow, invade, erode and destroy surrounding normal tissue. If left untreated, cancer cells become autonomous and develop their own signaling pathways that maintain their uncontrolled proliferation. At a later stage, most cancer cells undergo metastasis and invade distant tissues causing serious, often fatal consequences to the patient.

## **II. Statistics**

Cancer is a serious health problem in the United States as well as the rest of the world. There has been an estimate of over 11 million cases of cancer prevalence in the US in the past 3 years (Altekruse, 2011). These cancers are often initiated by natural sources of radiation

(cosmic, radon), chemical mutagens in the environment (such as benzpyrene in cigarette smoke), viral transformation (HPV in cervical cancer) and spontaneous carcinogenesis (failure of the immune system). While breast cancer affects mostly women over the age of forty and prostate cancer affects men fifty years or older, melanoma skin cancer has a prevalence rate of 793,000 per year in the United States, with an equivalent rate between men and women (Altekruse et al. 2011).

### **III. Skin Cancer: Melanoma**

The skin is the largest organ in our body. It is thus not surprising that cancer of the skin is the most common of all cancers. Melanoma is the most serious form of skin cancer. While melanoma is the least common of skin cancers, it causes the most deaths. If recognized and treated early, it is almost always curable, but if not, the cancer can advance and spread to other parts of the body, where it becomes difficult to treat and can be fatal. The American Cancer Society estimates that, at present, about 120,000 new cases of melanoma in the US are diagnosed every year. In 2010, about 68,130 of these were invasive melanomas, with about 38,870 in males and 29, 260 in women. Melanoma originates in melanocytes, the cells which produce the pigment melanin that colors our skin, hair, and eyes. Melanocytes are located in the basal layer of the epidermis and represent about 5-10% of cells of the basal layer. The majority of melanomas are black or brown, but they can also be skin-colored, pink, red, purple, blue or white. Risk factors for melanoma include moles, UV exposure, suppressed immune system and age. (The Skin Cancer Foundation, 2010)

#### **IV. Current treatments against melanoma**

Treatment options for melanoma are limited despite advances in immunotherapy and targeted therapy. Standard treatment for melanoma patients with thick ( $\geq 2.0$ mm) primary melanoma with or without metastasis is surgery followed by adjuvant therapy. Chemotherapy is an accepted therapy for stage IV metastatic disease with the alkylating agent, dacarbazine being the most widely used single chemotherapeutic agent for the treatment of metastatic melanoma.(Lens and Eisen, 2003) Although dacarbazine treatment has yielded positive responses in 25% of patients in phase II clinical trials, drug responses to the agent are transient, with only 1-2% of the patients achieving a durable long-term response. Temozolomide, an oral pro-drug which generates dacarbazine, has been shown to be as effective as dacarbazine in phase III clinical trials. Patients who progress to stage IV metastatic melanoma have a median survival rate of less than 1 year. Treatment with chemotherapy yields low response rates, of which few are durable. Another approved drug for the treatment of melanoma is interferon- $\alpha$  (IFN- $\alpha$ ). (Molife and Hancock, 2002) However, the limited benefit of disease free survival and the small potential improvement in overall survival make IFN- $\alpha$  treatment very controversial.(Wheatley et al. 2003) Other types of treatments include cytokine therapy with IL-2 which achieves durable benefits in a larger population; however, most other treatments produce severe toxicities such as edema, hypotension and decrease in renal function, which require patient's hospitalization. Therefore, new treatments approaches with low drug toxicity are needed in order to increase patients' survival rates.

## **V. Microtubule inhibiting agents**

Targeting tumor vasculature is a new approach in treating cancer. Microtubules form the core component of the cell's cytoskeleton. They serve as structural elements within cells and are involved in many cellular processes including mitosis, cytokinesis and vesicular transport. Microtubules are polymers of  $\alpha$ - and  $\beta$ -tubulin dimers. The tubulin dimers polymerize end to end and bundle into hollow cylindrical filaments, enabling them to serve their various functions. There are three types of microtubule inhibiting agents discussed in the literature. Vinca alkaloids, such as vincristine and vinorelbine, bind between the heterodimers. Taxanes which include paclitaxel and docetaxel bind on the surface of the microtubule. Both of these classes of drugs with specific binding sites on microtubules have been used clinically against various types of cancers. (Cao et al. 2008) Colchicine site inhibitors (CSIs) such as Combrestatin A4-phosphate and ZD6126 represent the last group of the microtubule inhibitors. CSIs induce growth suppression through the inhibition of the cell's capacity to form mitotic spindles that pull chromatids to either side of the cell, thus causing cell cycle arrest. Both Combrestatin and ZD6126 can suppress tumor perfusion and have been identified as vascular disrupting agents (VDAs). (Pettit et al. 1989) However, these drugs have not been approved by the FDA not only because of their neurotoxicity, which is common for most microtubule inhibiting compounds but due to severe cardiotoxicity. (Dowlati et al. 2002)

## VI. Stilbenes

Stilbene-derived compounds are widely found in nature and have been of interest to the scientific community due to their wide range of biological activities including cell growth suppression and anti-vascular properties. While functional stilbenes do not occur in nature, hydroxylated forms are natural compounds. (Simoni et al. 2009) A well-known example of a hydroxylated stilbene is *trans*-resveratrol (*trans*-3, 4', 5-trihydroxystilbene) which is present in grapes and which plays an important role in the prevention of coronary artery disease associated with red wine consumption. (Pace-Asciak et al. 1995) Another natural *cis*-stilbene, combretastatin A-4 (CA-4) isolated from the South African tree *Combretum caffrum* possesses potent antitumor activity. (Chaudhary et al. 2007) This drug works by binding to tubulin, which results in tumor endothelial cell damage and neovascular disruption. (Holwell et al. 2002) Recently, Simoni et al. (2009) used structure activity relationships (SAR) to synthesize series of drugs that have similar structures to resveratrol in both *cis* and *trans* orientations by adding or removing -OH, -NH<sub>2</sub> or -OCH<sub>3</sub> functional groups on the structural ring of the compound. Several active stilbenes have been identified varying in potency and among them stilbene 5c and stilbene 6c are the two most active compounds.

*Cis*-3, 4', 5-trimethoxy-3'-aminostilbene (Stilbene 5c), is highly potent and is particularly interesting because it has no bone marrow or cardiac toxicity in mice. (Durrant et al. 2009) Stilbene 5c is effective against various tumor cells and blocks cell cycle progression in the G2/M phase at low dose while inducing cell death at high dose. (Durrant et al. 2008) It has been shown that stilbene 5c is tolerated in mice up to 100mg/kg while no bone marrow toxicity, a major side effect of most cancer drugs, has been observed. Moreover, mice treated with 5 daily injections of

stilbene 5c did not show any cardiac toxicity, suggesting that stilbene 5c may be a better agent than existing VDAs due to its low cardiac toxicity. (Cao et al. 2008)

Several mechanisms have been reported for the suppression of tumor growth by stilbenes. Resveratrol, a widely studied stilbene derivative, has been shown to suppress tumor growth *in vitro* by inhibiting cell proliferation and *in vivo* by inhibiting the formation of new blood vessels. (Larrosa et al. 2004) It has also been shown that resveratrol enhances TRAIL-induced apoptosis through G<sub>1</sub> cell-cycle arrest and depletion of survivin. (Fulda, Debatin 2005) Other stilbene derivatives such as 3,5,4'-trimethoxy-*trans*-stilbene induces the activation of caspases as well as microtubule disassembly by depolymerization of tubulin in endothelial cells, leading to the disruption of tumor vasculature. (Belleri et al. 2005) Similarly, stilbene 5c has been shown to induce apoptosis at nanomolar concentrations (IC<sub>50</sub> = 30nM). (Roberti et al. 2003) In *in-vitro* assays using propidium iodide staining and clonogenic survival, stilbene 5c has also been shown to induce cell death through apoptosis in HL60 and U937 leukemia cells at concentrations of 30nM to 1000nM. (Cao et al. 2008) In the same study, *in vivo* data indicate that stilbene 5c injected IV at 10, 50, and 100mg/kg showed 22, 62 and 58% apoptosis respectively. While H&E staining of untreated tumors showed features of rapid tumor proliferation, tumors treated with 100mg/kg had extensive cell death in the central portion of the tumor with the peripheral rim remaining viable. (Durrant et al. 2009) Given the anti-proliferative and anti-neovasculture properties of stilbene 5c, it could be a promising agent for the chronic treatment of melanoma.



## **VII. Responses to Drug Treatment**

### **A. Apoptosis**

Programmed cell death or apoptosis is important for maintaining cellular homeostasis. In adult animals, the total number of cells is maintained constant through this mechanism. Cell death through apoptosis is very important in human embryonic development because intermediate structures must be removed at every stage of development; apoptosis is also critical during the course of neural development in the formation of the brain and organs such as the eye. While another mode of cell death, specifically necrosis, also exists to eliminate damaged cells, apoptosis is a unique programmed cell death pathway.

Necrosis occurs as a result of an external insult while apoptosis is a planned cellular death coordinated by the cell but which can also occur in response to external stress. The necrotic mode of cell death occurs when cells are exposed to extreme variance in physiological conditions which may result in damage to the plasma membrane. It begins with an impairment of the cell's capacity to maintain homeostasis that leads to the influx of water and extracellular ions. Intracellular organelles such as the mitochondria swell and the cell ruptures. Due to the breakdown of the cell membrane, cytoplasmic organelles are digested by lysosomal enzymes and released from the cells, resulting in tissue damage and an intense inflammatory response. (Ashkenazi et al. 1998) In contrast, during apoptosis, the cell shrinks and the chromosomes condense, leading to membrane blebbing and the subsequent fragmentation of the nucleus. The remnants of apoptotic cells are rapidly taken up by phagocytic cells, thereby inhibiting the induction of any inflammatory response.

The process of apoptosis is divided into three parts which includes the initiation, effector and degradation phases. The morphological changes in apoptosis described above occur once the cell is committed and has entered the degradation phase. The degradation phase of apoptosis is controlled by the cleavage of proteins that activate caspase proteases. Pro-apoptotic signals activate upstream caspases 2, 8, 9 and 10 which lead to the activation of downstream effector caspases 3, 6 and 7 by proteolytic cleavage. This signal cascade leads to the cleavage of vital cellular proteins by the caspases and subsequent DNA cleavage by endogeneous DNases. These endogeneous DNases cut the double stranded DNA into 180-200 base pair fragments, also known as DNA laddering, a common feature of apoptosis. The 3' overhang cuts made by the endonucleases are detected in the TUNEL assay using microscopy. (See Fig. 1)

Apoptosis is known to occur through two different mechanisms: the extrinsic death receptor pathway (Ashkenazi, Dixit 1998) and the intrinsic mitochondrial pathway (Kroemer, Zamzami & Susin 1997). The *extrinsic* or *death receptor mediated* pathway involves the binding of a ligand to a membrane receptor that will ultimately lead to the demise of the cell. Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as APO-1/Fas or TNF-related apoptosis-inducing ligand (TRAIL) receptor and TRAIL ligands result in receptor recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Upon recruitment, caspase 8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases such as caspase 3 and 7. The *intrinsic mitochondrial* pathway is generally initiated by stress signals through the release of cytochrome c, apoptosis inducing factor (AIF) or Smac/DIABLO from the mitochondrial inner membrane space. The release of cytochrome c into the cytoplasm triggers caspase 3 activation through the formation of the cytochrome c/Apaf-1/caspase-9 containing apoptosome complex. Smac/DIABLO promotes

caspase activation by removing inhibitory effects to apoptosis while AIF causes DNA condensation. The receptor mediated and mitochondrial pathways can be interconnected at different levels, for example, by Bid, a BH3 domain-containing protein of the Bcl-2 family which assumes cytochrome-c releasing activity upon cleavage by caspase-8. Activation of caspases is negatively regulated at the receptor level by FLIP, which blocks caspase-8 activation, at the mitochondria level by Bcl-2 family proteins and by inhibitor of apoptosis proteins (IAPs).

Apoptosis plays a critical role in cancer and in cancer therapy since apoptosis can prevent a cell from being transformed while chemotherapeutic drugs usually kill the cancer cells by inducing apoptosis with the most dangerous forms of cancer being the ones that have become resistant to apoptosis. (Brown, Attardi 2005) Recent evidence suggests that some degenerative diseases may also be due to non-physiological apoptosis. (Guenette, Tanzi 1999)

## **B. Autophagy**

Another major pathway for cellular constituent degradation is autophagy. This pathway is particularly important during development and under certain environmental stress conditions. (Klionsky, Emr 2000) Cellular death and resorption are critical during periods of development that require extensive cellular remodeling such as postpartum luteal cell regression (Tian et al. 2010) as well as in preventing disease states including some types of cancer. Autophagy promotes cellular survival by enabling cells to maintain the energy homeostasis in the cell during nutrient deprivation and other forms of cellular stress. The disruption of autophagy has been implicated in a wide variety of diseases including cancer, neurodegenerative diseases and cardiac diseases. (Levine, Kroemer 2008) Autophagy is a complex process that is divided into three distinct,

biochemically different pathways: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). (Fimia, Piacentini 2010)

Macroautophagy is the most heavily studied mechanism of autophagy. During this process, cytoplasmic components of the cell undergo massive degradation that involves the formation of a phagophore which leads to the sequestration of the cellular components into double membrane vesicles called autophagosomes. The induction of autophagy requires the recruitment of autophagy-related gene (atg) proteins such as Atg 5 and Atg 7 that become integrated in the autophagosomal cell membrane. In eukaryotic cells, the lysosome is a major degradative organelle. It contains a wide range of hydrolases that are able to degrade proteins, lipids, nucleic acids and carbohydrates. The autophagosome, with the recruitment of more Atg proteins as well as the conversion of microtubule light-chain protein I, LC3-I to LC3-II, fuses with the lysosome resulting in the degradation and subsequent recycling of cellular constituents. (Klionsky, Emr 2000) Microtubule associated protein light-chain or LC3 is generally utilized as a marker of autophagy. During autophagy, LC3-I is cleaved by Atg4 and linked to phosphatidylethanolamine, Atg 3 and Atg7 to be converted to LC3-II which is incorporated in the autophagosomal membrane. The conversion of LC3-I to LC3-II as well as the degradation of LC3-II are ideal markers for the time-dependent progression of autophagy. (See Fig. 2)

Microautophagy takes place when lysosomes directly engulf cytoplasmic components by invaginating their membranes. It involves the pinocytosis of the cytosol directly by the lysosome. In chaperone-mediated autophagy or CMA, only proteins with a particular consensus sequence are recognized by the binding of the HSC70-containing chaperone complex. (Massey, Zhang & Cuervo 2006) The chaperone complex and its substrate move to the lysosome where the protein is unfolded and translocated across the lysosome membrane. CMA differs from the other modes of

autophagy because the substrates are transported across the lysosomal membrane on a one-by-one basis, whereas in the macroautophagy and microautophagy, the substrates are engulfed or sequestered in bulk. CMA is very selective as it degrades only single proteins and not organelles.

One of the most studied mammalian autophagy associated proteins that regulates autophagy is Beclin-1. Beclin-1 is one of the early proteins that are responsible for the nucleation of the phagosome. Beclin-1, Atg6 and the class III phosphatidylinositol 3-kinase (PI3K) and Vps34 form the core complex in the vesicle nucleation process. (Levine et al. 2004) Beclin-1 has also been shown to bind to Bcl-2 family proteins and act as a tumor suppressor. There is an ongoing controversy on the role of autophagy; some believe that autophagy is a process that generates energy for the cell thereby referring to it as a cytoprotective autophagy usually in response to nutrient starvation. (Levine, Klionsky 2004, Andrzejak, Price & Kessel 2011, Davids et al. 2009) There is also evidence suggesting that autophagy can function as a mode of cell death in normal cellular development as well as in response to chemotherapy. (Levine, Yuan 2005, Wang et al. 2011, Suk et al. 2011)

Another key protein that regulates autophagy is the mammalian protein kinase mTOR which is the target of the drug rapamycin. mTOR is a serine/threonine kinase that is active under favorable growth conditions and promotes initiation of translation, protein synthesis and nutrient import. When mTOR is inactivated during conditions of nutrient deprivation, cells undergo autophagy. (Yang, Klionsky 2009) Rapamycin also has been shown to increase autophagy via mTOR inactivation but it is unknown through what pathways other drugs increase autophagy. Other pathways upstream of mTOR involved in autophagy include PI3K, AMPK pathways and certain G-protein coupled receptor pathways. Although the PI3K and AMPK pathways are

known to be activated by nutrient deprivation and not by conventional chemotherapy, it could be worthwhile investigating the effects of microtubule inhibitors such as stilbene 5c in the activation of these pathways.

### **C. Cell-cycle independent and dependent growth arrest**

Senescence was first described by Hayflick and colleagues when they observed that normal cells can only divide for a finite number of times. Cells would go into a permanent growth arrest state after serial passage in culture. (Pazolli, Stewart 2008) Senescent cultures remained viable and metabolically active for long period of time. In addition to irreversible growth arrest, senescent cells undergo distinctive morphological changes and become flattened, enlarged and granular(Dimri et al. 1995).<sup>34</sup> These cells are usually characterized in the literature by positive staining for  $\beta$ -galactosidase activity at pH 6. (Dimri et al. 1995)

The two major types of senescence are replicative senescence and stress-induced senescence. During replicative senescence, cells undergo a certain number of cell divisions and enter a permanent state of growth arrest due to the shortening of telomeres, the ends of chromosomes. (Hayflick 1974) The telomeres of chromosomes are made of repeating sequence of TTAGGG that extend from 5-15kb pair long. The major purpose of telomeres is to allow the replication machinery to transcribe the ends of chromosomes as far out as possible, preventing loss of genetic material. The mechanisms by which shortened telomeres are sensed and the senescence program is activated remain unclear. Several hypotheses have been proposed to account for the effects of telomere shortening on senescence including induction of the DNA

damage response pathway through ATR/ATM, p53, p21 and Rb dephosphorylation. (Harley, Futcher & Greider 1990) The progressive shortening of the telomeres results in cell cycle arrest.

Telomerase is the enzyme responsible for the *de novo* synthesis of telomeric repeats at the end of chromosomes. In the vast majority of human somatic cells, there is no detectable activity of telomerase. In the absence of telomerase, telomeres get progressively shortened during successive cell division because DNA polymerase cannot replicate the very ends of chromosomes. (Harley, Futcher & Greider 1990) Telomerase is upregulated in highly proliferating cancer cells and overcomes senescent growth arrest by extending TTAGGG repeats. (Counter et al. 1992)

Senescence is also used to describe other growth arrest events unrelated to telomere shortening. A senescence-like state also known as stress-induced senescence can be induced by a variety of stress stimuli. These stresses include overexpression of certain oncogenes, inadequate culture conditions, radiation damage and chemotherapy. (Di Leonardo et al. 1994) It has also been shown that stress-induced senescence involves telomeric dysfunction rather than telomere shortening and involves p53, p21, different cyclins and Rb dephosphorylation in response to chemotherapy including microtubule inhibiting agents. (Roninson 2002)

Senescence presents characteristics distinct from normal cells. Senescent cells exhibit a flattened and enlarged morphology as they grow in size without dividing. The cells also have a pH 6 dependent  $\beta$ -galactosidase activity which is used as a marker for senescence. (Dimri et al. 1995) Another consequence of senescence is the upregulation of the negative cell cycle regulator p16 which inhibits cyclin dependent kinase (CDK) 4 and 6. CDK 4 & 6 are needed for cell cycle progression from G1 to S phase. Therefore, upregulation of p16 inhibits this progression and

leads to the accumulation of the cells in G1 phase. Although senescence has been thought as an irreversible process, there has been some data in the literature that suggests otherwise where the inactivation of p53 and other mechanisms have been reported to allow cells to escape G1 arrest. (Gudkov et al. 1993, Beausejour et al. 2003, Elmore et al. 2005, Roninson et al. 2001)

Growth arrest is not always a senescent response. Another type of growth arrest is called cell-cycle dependent growth arrest which inhibits cells progression into the next phase. The cell cycle is composed of a G1 phase in which most cells prepare for the replication/synthesis phase (S phase). The G2 phase occurs before the mitosis or M phase in which cells prepare for mitosis. The cell cycle is regulated by cyclins and cyclin-dependent kinases (CDKs) that regulate the progression of cells from one phase to the next. DNA damage through radiation has been shown to arrest cells in G1 phase via the ATM/ATR, p53, p21, Rb/E2F pathway. However, most cancer cells have evolved in acquiring mutated forms of p53 which enables them to progress into the S phase without cell cycle arrest thus leading to uncontrollable proliferation with damaged DNA. (Beausejour et al. 2003)

Microtubule inhibiting agents such as Taxol and stilbene 5c, which interfere with the depolymerization and polymerization of microtubules, also induce cell cycle arrest by preventing cells from undergoing mitosis. This is due to the disruption of microtubules which activates the mitotic spindle assembly checkpoint and inhibits the separation of sister chromatids, thus interfering with the completion of mitosis. Sister chromatids are held together by cohesin proteins. The enzyme responsible for cleaving these proteins is separase. The activity of separase is inhibited by a protein called securin which is normally ubiquitinated for degradation by the anaphase promoting complex (APC) in cycling cells. Once securin is ubiquitinated, separase is released and cleavage of cohesion proteins followed by sister chromatid separation



occurs. However, in cells treated with microtubule inhibiting agents, securin is not degraded resulting to the continued inactivation of separase and the inhibition of anaphase entry. In this case, cells cannot undergo mitosis and arrest in the G2 phase.

## **Chapter 2: Materials and Methods**

### **I. Materials**

RPMI 1640 medium containing L-glutamine were obtained from Invitrogen (Eugene, OR). Trypsin-EDTA (0.25% trypsin, 0.53mM EDTA-4 Na) was obtained from Fisher. Fetal Bovine Serum was purchased from Hyclone Laboratories (Logan, UT). TUNEL assay reagents (terminal transferase, reaction buffer,  $\text{CoCl}_2$  and fluorescein-dUTP) were purchased from Roche. Annexin V and propidium iodide (PI) were purchased from BD Biosciences. Trypan blue dye, acetic acid, 6-diamidino-2-Phenylindole (DAPI), formaldehyde, albumin bovine, DMSO, and acridine orange were purchased from Invitrogen.

### **II. Cell lines**

B16F10 murine melanoma cells were obtained from Dr. Kimber White's laboratory at Virginia Commonwealth University and kept frozen under liquid nitrogen in 10% DMSO (Sigma Chemical, St. Louis, MO) with Fetal Bovine Serum (FBS) until use.

### **III. Drug preparation and treatment**

The stilbene compound has been kindly provided by Dr. Ray Lee's lab at Virginia Commonwealth University. Stilbene 5c has been diluted in autoclaved DMSO (Sigma Chemical, St. Louis, MO) into stock solutions of 30mM and 100mM aliquots. B16F10 cells were kept frozen under liquid nitrogen in 10% DMSO (Sigma Chemical, St. Louis, MO) with Fetal Bovine Serum (FBS). Cells were quickly thawed and cultured in T75 flasks (Cellstar) in RPMI

1640 medium. RPMI 1640 used for all experiments with B16F10 cells was supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1.5% HEPES (GIBCO), 5% sodium bicarbonate diluted in PBS (7.5g in 100ml PBS) and penicillin/streptomycin (1ml/500ml medium) at 37°C under a humidified 5% CO<sub>2</sub> atmosphere. Cells were passed at 80% confluence after washing one time with 1X PBS (GIBCO) and harvested with 0.25% Trypsin-EDTA (GIBCO), then deactivated with complete RPMI medium. Cells were collected and centrifuged at 15,000 rpm for 5 minutes. Medium was aspirated and fresh medium added to the cell pellet; cells were re-suspended in medium and 100µl of the re-suspended cells was placed into a fresh T75 flask with 10ml RPMI medium. For all culture experiments, cells were plated and allowed to adhere overnight before drug treatment. Unless otherwise indicated, all treatments involved continuous drug exposure.

#### **IV. Assays and techniques**

##### **a) MTT Assay**

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced by enzymes in living cells to purple formazan. A solubilization solution, usually dimethyl sulfoxide (DMSO) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of the colored solution can be quantified by measuring at a specific wavelength by spectrophotometer. For this experiment, cells were plated in a 96 well plates (3 wells per sample) at a density of 4000 cells per well in 100µl of RPMI medium. Cells were allowed to adhere to plate overnight and treated the next day (Day 0) with stilbene 5c diluted in medium. After a 72 hour long incubation period, medium was aspirated and MTT solution (2mg/ml in PBS prepared in the dark) was added to each well. Cells were allowed to incubate for 3 hours at 37°C, medium

was aspirated and 100µl of autoclaved DMSO was added to each well. An absorbance reading was taken at 490 nm (KC Junior software, EL 800 Universal Microplate Reader).

### **b) Clonogenic Survival**

In order to better understand the cells' capacity of forming colonies with or without treatment, clonogenic survival assay was performed. Cells were plated in triplicates in 6-well plates using RPMI medium and incubated overnight for treatment on the next day. Drug was removed at the end of 72 hours and cells were incubated in fresh medium until cells formed colonies of at least 50 cells. Once colony formation was established through microscopy, cells were washed with 1X PBS prior to fixation with 100% methanol for 10 minutes. After removal of methanol, plates were stained with crystal violet dye (1mg/100ml) in deionized water for 10 minutes. Wells were washed with deionized water and stained colonies were counted manually. Each condition received 100-150 cells per well. Formed colonies were counted and plotted relative to control cells.

### **c) Time course for cell viability using the trypan blue exclusion assay**

Trypan blue is a dye that stains dead cells due to their compromised cell membranes. Cell counting was performed to assess cell killing of drug by excluding live cells from dead ones. Cells were plated in triplicates in 6-well plates at  $1-2 \times 10^5$  cells per well and allowed to adhere overnight. At indicated time points, drug was aspirated and cells were washed with 1X PBS. Cells were then harvested using trypsin and centrifuged at 15,000 rpm for 5mins. The

supernatant was aspirated and cell pellet was re-suspended in 1 ml of medium. Cells were stained with trypan blue at a 1:1 dilution and cells were counted using the automated Countess (Invitrogen) apparatus. The displayed viable number of cells per ml has been used to generate the time course datas.

#### **d) Acridine Orange Staining**

3,6-Acridinediamine or acridine orange is cell permeable and enters cellular acidic compartments such as lysosomes and becomes protonated and sequestered. In low pH conditions, the dye will emit an orange stain when excited by blue light. For this experiment, cells were plated at a density of  $4 \times 10^5$  per well and allowed to adhere overnight. Cells were treated with stilbene 5c the next day and incubated at 37°C until the indicated time points. After treatment, drug was removed and cells were washed once with 1X PBS. Cells were then stained with acridine orange (1 µg/ml) for 10 minutes. Stain was removed and fresh media was added to each well. Cells were immediately taken to the microscope for visualization. Photographs were taken using an Olympus IX 70 microscope and an Olympus SC 35 type 12 camera.

#### **e) DAPI staining**

DAPI or 4',6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to A-T rich regions in DNA and serves as a nuclear marker to detect nuclear fragmentation such as in apoptosis. Floating cells and adherent cells were collected at the specific time points and centrifuged at 15,000 rpm for 5 minutes. Supernatant was aspirated and fresh media was added

to resuspend the pellet. Cells were counted using 1:1 dilution of trypan blue exclusion dye. The automated Countess (Invitrogen) apparatus was used to count the total cell number. A dilution of 50,000 cells in 200  $\mu$ L of RPMI medium per slide was prepared and cells were spun at 10,000 rpm for 5mins (Shandon Cytospin 4, Thermal Electron Corp). Slides were refrigerated until ready for staining. When ready for staining, slides were washed with 1X PBS twice for 5 minutes at room temperature. A 1:2 dilution of acetic acid and ethanol was used to fix cells at 20°C for 5 minutes. Slides were then washed twice with 1X PBS for 5 minutes at room temperature. A 1:1000 dilution of: DAPI:Vectashield was prepared and each slide was mounted with 10 $\mu$ L of the solution. Coverslips were sealed with clear nail polish and photographs were taken using an Olympus 1X 70 microscope and an Olympus SC 35 type 12 camera.

#### **f) TUNEL assay**

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids. It is used as a marker for cells that have suffered severe DNA damage such as in apoptosis. For this experiment, cells were harvested at indicated time points and centrifuged at 15,000 rpm for 5 minutes. Medium was aspirated and fresh media was added to re-suspend the pellet. Cells were counted using 1:1 dilution of trypan blue exclusion dye. The automated Countess (Invitrogen) apparatus was used to count the total cell number. A dilution of 50,000 cells in 200  $\mu$ L of RPMI medium per slide was prepared and cells were spun at 10,000 rpm for 5 mins (Shandon Cytospin 4, Thermal Electron Corp). Slides were refrigerated at 4°C until ready for staining. When ready to stain, slides were washed with 1X PBS twice for 5 minutes at room temperature. Cells were then fixed

with 4% formaldehyde in PBS for 10mins at room temperature. Slides were washed twice with PBS for 5 mins at room temperature and fixed with a 1:2 dilution of acetic acid and ethanol for 5 mins at 20°C. After two consecutive washes with PBS for 5mins at room temperature, cells were marked with ImmunoPen (BD Biosciences) in a tight circle to contain solutions. Slides were blocked with 1mg/ml of bovine serum albumin (BSA) in PBS for 30mins at room temperature. After 2 washes with PBS, cells were stained with enzyme mix for 60 mins at 37°C. Enzyme mix (Roche) consists of 5X buffer, terminal transferase, 25mM CoCl<sub>2</sub>, fluorescein-12dUTP and deionized water. Cells were then washed twice with PBS for 5 mins at room temperature and cover slips were placed and slides visualized under the microscope. If also staining for DAPI cells would be mounted with Vectashield: DAPI (1:1000) before cover slips are placed then visualized using fluorescent microscopy.

#### **g) Beta galactosidase staining**

X-gal (also abbreviated BCIG for bromo-chloro-indolyl-galactopyranoside) is the substrate of the  $\beta$ -galactosidase enzyme which can cleave X-gal to release a blue color. Senescent cells upregulate their  $\beta$ -galactosidase enzyme and thus an extensive bluish color is detected upon the addition of X-gal in the medium. To setup this experiment, cells were washed once with 1X PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde for 5 min. The cells were washed again with PBS and stained with the staining solution which consists of 20mg/ml X-gal in dimethylformamide, 0.2M citric acid/Na phosphate buffer pH 6.0, 100mM potassium ferrocyanide, 100mM potassium ferricyanide, 5M NaCl, 1M MgCl<sub>2</sub> and distilled water. Following overnight incubation at 37°C, cells were washed twice with 1X PBS and images were captured using light microscopy.

#### **h) Western immunoblotting analysis**

In order to assess for the presence, induction or degradation of proteins, western immunoblotting was conducted for the analysis of specific cellular proteins. For this experiment, cells were washed once with PBS, harvested with 0.25% trypsin and deactivated with complete RPMI medium. The solution is then centrifuged at 15,000 rpm for 5 mins. Supernatant was aspirated and pellet ( $\sim 5 \times 10^5$  cells) were lysed using 50-100  $\mu$ l of M-PER Mammalian Protein Extraction reagent (ThermoScientific) containing protease (1:50) and phosphatase (1:100) inhibitors. Lysed cell extract was transferred into 1.5ml tubes and sonicated. Protein concentration was determined using the Bradford protein assay (BioRad) and 40  $\mu$ g of total cell lysate was separated using 10%, 12% or 15% gel using SDS-PAGE. Proteins were transferred onto nitrocellulose membrane (BioRad) for 1:30 hours and washed 3 times with PBS containing 0.01% Tween. Membranes were blocked with the Odyssey Blocking Buffer (LICOR) and immunoblotted with specific primary antibodies for 15 minutes on the SNAP- i.d system (Millipore). Membranes were washed three times with PBS containing 0.01% Tween and incubated in the dark with immunofluorescent secondary antibody for 15 minutes using the SNAP-i.d. Membranes were washed three times and bands were detected using the Odyssey infrared scanner (LICOR). Primary antibodies used include mouse anti-LC3, rabbit anti- $\beta$  actin, rabbit anti-PARP and mouse anti-caspase 3. Secondary antibodies include goat/mouse anti-rabbit IRDye 800CW and goat/rabbit-anti-mouse IRDye 680CW. (LICOR)



### **i) Annexin V-PI staining**

During early apoptosis, phosphatidyl serine (PS) is translocated from the inner cell membrane to the outer cell membrane. Annexin V coupled with a fluorescent chromophore (FITC) binds to PS and serves as a marker of early apoptosis. Propidium iodide (PI) stains cells with compromised cell membrane as in cells in late apoptosis or necrosis. For this assay, cells were plated and incubated overnight to adhere to plate. Cells were treated and cell pellets collected using 0.25% trypsin at indicated time points. Cells were centrifuged at 15,000 rpm and washed twice with cold 1X PBS and resuspended in 100ul of 1X binding buffer (BD Biosciences) per tube; 5µl of Annexin-FITC (BD Biosciences) and 5µl PI at 10ug/ml (BD Biosciences) were added per tube. Cells were gently vortexed and incubated for 15 min at room temperature in the dark. 400µl of 1X binding buffer was added to each tube and analyzed by flow cytometry within 1 hour.

## **V. Statistical Analysis and Quantification**

Statistical analysis was performed using Tukey-Kramer followed by post-hoc analysis. Groups with a p-value <0.05 were considered to be significantly different from compared conditions.

## Chapter 3: Results

### **I. Effect of stilbene 5c on cell viability and colony formation**

Treatment of B16F10 melanoma cells with 10nM, 30nM, 100nM, 300nM and 1000nM stilbene 5c for 72 hours resulted in a dose dependent decrease in viable cell number. While 10nM produced no significant reduction in cell viability, 30nM and 300nM of drug reduced viable cell number by approximately 25% and 50%, respectively. (Fig. 3A) The drug was dissolved in DMSO, which was used as a solvent control in this experiment. Effects on colony formation of similar doses of stilbene 5c were also investigated by conducting a clonogenic survival assay where B16F10 cells were treated with 3nM, 30nM, 300nM and 1000nM stilbene 5c for 72 hours; colony formation was evaluated after 2 weeks. (Fig. 4) The results of the clonogenic survival assay closely correlated with the MTT assay. Subsequent studies were performed using stilbene 5c at 30nM and/or 300nM concentrations in order to understand the effects of a low dose and high dose treatment.

### **II. Cell death, senescence arrest and lack of proliferative recovery**

In order to distinguish between growth arrest and cell death effects of stilbene 5c, a time course study of cell viability was performed where cells were treated with 300nM stilbene 5c over a period of 5 days. At this concentration of drug, extensive cell death is observed over a period of three days and the cells enter an apparent state of growth arrest on days 4 and 5. (Fig 5B) The possibility that this growth arrest reflects senescence was investigated using the  $\beta$ -galactosidase

assay and by assessing alterations in cell morphology. Cells in senescence remain viable and metabolically active and become flattened, enlarged and granular. Cells treated with 300nM of stilbene 5c clearly appear to have entered senescence based on the  $\beta$ -galactosidase staining as well as by the enlarged and granular phenotype. (Fig. 6)

One key desirable element for the action of cancer chemotherapeutic agents in addition to cell killing or anti-proliferative effects is the ability to prevent tumor cells from recovering growth upon drug removal. In order to assess proliferative recovery, B16F10 cells were treated continuously with 300nM of stilbene 5c for 5 days. Fewer than 6% of cells survived after 5 days and fewer than 1.5% remained after 22 days; clearly, there was no evidence of proliferative recovery over this extended time period. (Fig 7)

### **III. Minimal apoptosis in B16F10 cells treated with 300nM of stilbene 5c**

Apoptosis was assessed as a potential cause of cell death in cells treated with 300nM of stilbene 5c using DAPI staining and the TUNEL assay. Only a small percentage of the DAPI stained nuclei showed the shrinking and distortion associated with apoptosis in cells treated with stilbene 5c. Similarly, there was little evidence of fluorescence that would indicate an increase in DNA fragmentation using fluorescent dUTP. (Fig. 8) Our observations were confirmed using Annexin V-PI staining. (Fig. 9) Cells treated with 300nM of stilbene 5c did not exhibit early apoptosis for 5 days post-treatment. A small increase in late apoptosis is observed on day 3 which correlates with the pronounced cell death seen in the time course study in figure 5.

Apoptosis was further assessed by investigating cleavage of the DNA repair protein, Poly (ADP-ribose) polymerase, PARP as well as caspase-3 activation. Cells treated with 300nM of stilbene

5c showed minimal PARP cleavage. Irradiated B16F10 cells (10 Gy) were utilized as a positive control. (Fig. 10) As with PARP cleavage, treatment with stilbene 5c resulted in minimal caspase 3 cleavage. Taken together, these data suggest that apoptosis is unlikely to play a significant role in promoting the cell death observed in B16F10 melanoma cells after treatment with 300nM of stilbene 5c.

#### **IV. Induction of autophagy in cells treated with 300nM stilbene 5c**

Autophagy involves the programmed breakdown of cellular components which can ultimately lead to cell death. (Levine, Klionsky 2004) Since apoptosis could not account for the extensive cell killing effects seen with 300nM of stilbene 5c, subsequent experiments were designed to investigate the promotion of autophagy. During autophagy, autophagosomes become acidified which leads to the fusion with lysosomes. We used acridine orange to stain the acidic compartments in cells that are actively undergoing autophagy. In Figure 11, acridine orange staining is evident in one localized area, suggesting that a basal level of autophagy exists in B16F10 melanoma cells. Cells treated for 1, 2, 3, 4 and 5 days with 300nM of stilbene 5c clearly exhibit extensive distributed punctuate staining of acidic vesicles indicating the induction of autophagy. Moreover, at all time points, the treated cells are observed to be significantly enlarged compared to controls, confirming autophagic morphology.

The induction of autophagy was further confirmed by western immunoblotting analysis of the cytosolic microtubule-associated protein light chain 3 (LC3-I) conversion to the autophagosome-incorporated microtubule-associated protein light chain 3 (LC3-II). During autophagy, the autophagosome is formed from the phagophore and in this process LC3-I is conjugated to

phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II) which is recruited to the autophagosomal membrane. Autophagosomes fuse with lysosomes to form autophagolysosomes where components of the autophagosomes are degraded by lysosomal hydrolases. In Figure 12, control cells demonstrated some conversion of LC3-I to LC3-II which corresponds to the basal level of autophagy previously detected with acridine orange. However, there was a significant increase in LC3-I to II conversion 2 days post-treatment with 300nM of stilbene 5c suggesting the induction of autophagy. As serum starved cells are known to undergo autophagy, three days serum starved B16F10 cells were used as our positive control.

## **V. Effect of pharmacological inhibition of autophagy**

In order to assess the role autophagy plays in the antiproliferative and cytotoxic actions of stilbene 5c, cells were pre-treated with 10nM of Bafilomycin A1, which prevents lysosomal acidification and fusion with the autophagosome, then exposed to 300nM of stilbene 5c.

Bafilomycin, at 10nM, did not significantly inhibit cell growth. Cells treated with 300nM of stilbene 5c alone demonstrated pronounced cell death over a period of 7 days; pre-treatment with the autophagy inhibitor, bafilomycin A1, resulted in increased cell death (Fig 13A)

Similarly, while cells treated with stilbene 5c alone showed a significant decrease in colony formation, pre-treatment with 10nM bafilomycin resulted in an additive inhibition in colony formation compared to stilbene 5c alone. (Fig 13B)

Apoptosis was assessed in order to evaluate the nature of cell death that occurs when autophagy is inhibited in cells exposed to 300nM of stilbene 5c. The TUNEL assay revealed a significant increase in fluorescent staining in cells undergoing the combination treatment especially on days

5 and 7 post-treatment. (Fig. 14A) Furthermore, apoptotic cell death was confirmed through western immunoblotting analysis. Minimal PARP cleavage is detected with 300nM of stilbene 5c treatment while complete PARP cleavage is detected when cells are treated with both the autophagy inhibitor bafilomycin A1 and 300nM of stilbene 5c. (Fig. 14B) These data suggest that extensive apoptotic cell death occurs in response to 300nM of stilbene 5c when autophagy is inhibited, suggesting cytotoxic autophagy at this dose.

## **VI. Delayed cell death, lack of proliferative recovery and induction of autophagy in B16F10 melanoma in response to low dose stilbene 5c**

As seen in figure 13A, 300nM of stilbene 5c produced such robust cell death by itself that the combination treatment could not significantly enhance the cell killing effects. Therefore, in order to better understand what role autophagy might play in cells treated with stilbene, we conducted a series of studies using 30nM of stilbene 5c. Figure 15A shows a delay in cell death in cells treated with 30nM stilbene 5c where cells continue to proliferate for 2-3 days. Cell death occurs at later time points starting days 3 and 4. Moreover, low level of apoptotic cell death is observed in cell treated with 30nM stilbene 5c. (Fig. 20) B16F10 melanoma cells treated with colchicine at 30nM showed a similar profile of cell death where cells initially continue to proliferate and then undergo delayed cell death (data not shown). Most importantly, despite the low dose of stilbene 5c used, cells supplemented with fresh medium on day 5 resulted in no proliferative recovery up to 2 weeks after drug removal. (Fig. 15B)

In order to understand the role of autophagy, acridine orange staining assay was used to monitor the formation of acidic vesicular organelles. Cells treated with 30nM of stilbene 5c showed an

increase in acridine orange staining indicative of autophagy (Fig 16) Moreover, treatment with 30nM induced a significant level of LC3-I to LC3-II conversion, which is consistent with autophagy being induced in these cells. Serum starved B16F10 cells were used as a positive control (Fig. 17)

## **VII. Effect of pharmacological inhibition of autophagy in B16F10 cells treated with a low dose of stilbene 5c**

With the aim of understanding the role of autophagy, cells were treated with 30nM of stilbene 5c which resulted in an initial cell proliferation but subsequent cell death starting on day 3. More importantly, combination treatment of bafilomycin A1 with 30nM of stilbene 5c resulted in a significant increase in cell death starting day 2 post-treatment. (Fig 18A) This suggests that autophagy may play a cytoprotective role in these cells. We further assessed the substantially high cell killing seen with the combination treatment using clonogenic survival assay, which is a more sensitive assay. Control cells and cells treated with 10nM of bafilomycin A1 alone did not show any significant decrease in colony formation. However cells treated with 30nM of stilbene 5c and bafilomycin 1A showed a significant decrease in colony formation compared to stilbene 5c alone treatment. (Fig 18B) Lastly the nature of the extensive cell death induced with the combination treatment was evaluated by assessing for apoptotic cell death. Using western immunoblotting, we detected low levels of PARP cleavage in cells treated with 30nM of stilbene 5c alone. However, cells treated with the combination treatment showed complete PARP cleavage indicating that an extensive level of apoptosis is occurring when autophagy is inhibited. (Fig. 19)

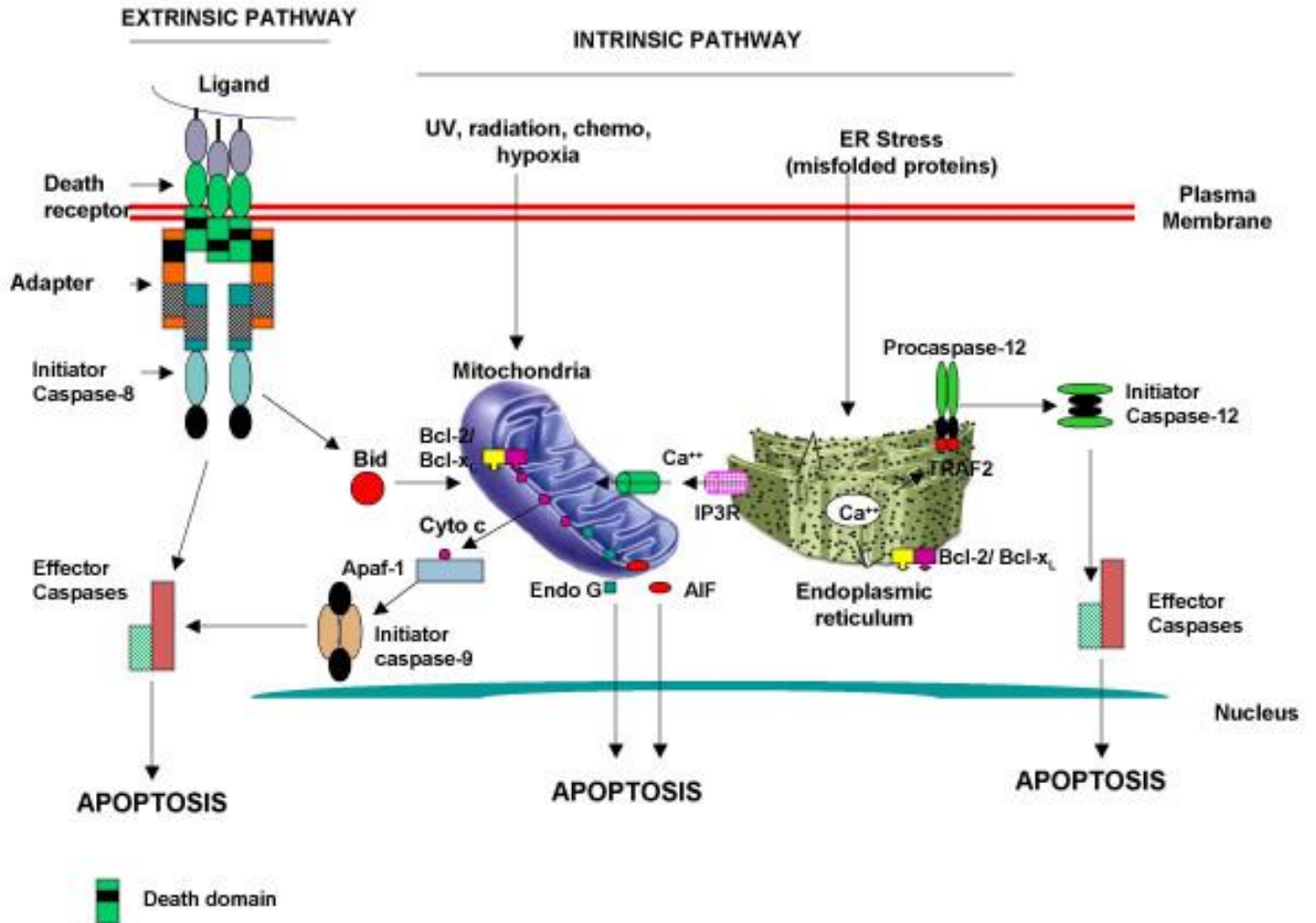
### **VIII. Tumor cell killing delay with 30nM of stilbene 5c due to protective autophagy and lack of apoptosis**

In order to further understand the initial tumor cell death delay observed with 30nM stilbene 5c in Figure 15, we investigated if any modes of cell death were activated at early time points. Apoptosis was assessed using Annexin V–PI staining. (Fig. 20A) Cells treated with 30nM of stilbene 5c for 1, 2 and 3 days did not show any significant early or late apoptotic cell death, which is consistent with the previous cell viability data. Treatment with 30nM of stilbene 5c for 4 days showed a slight increase in late apoptotic cells which can account for some of the cell death observed on day 4 in the cell viability time course study in figure 15A. In general, similar to the cells treated with 300nM of stilbene 5c, treatment with 30nM exhibited a very low level of apoptosis compared to the rest of the cellular population. (Fig 20B) This indicates that apoptosis is induced minimally in these cells at a 30nM and 300nM treatment. However, cells treated with 30nM showed increased cell death when treated with 10nM of bafilomycin, suggesting that autophagy is protective. Cells treated with 300nM stilbene 5c presented an additive cellular death and an extensive induction of apoptosis when autophagy is inhibited suggesting cytotoxic autophagy.



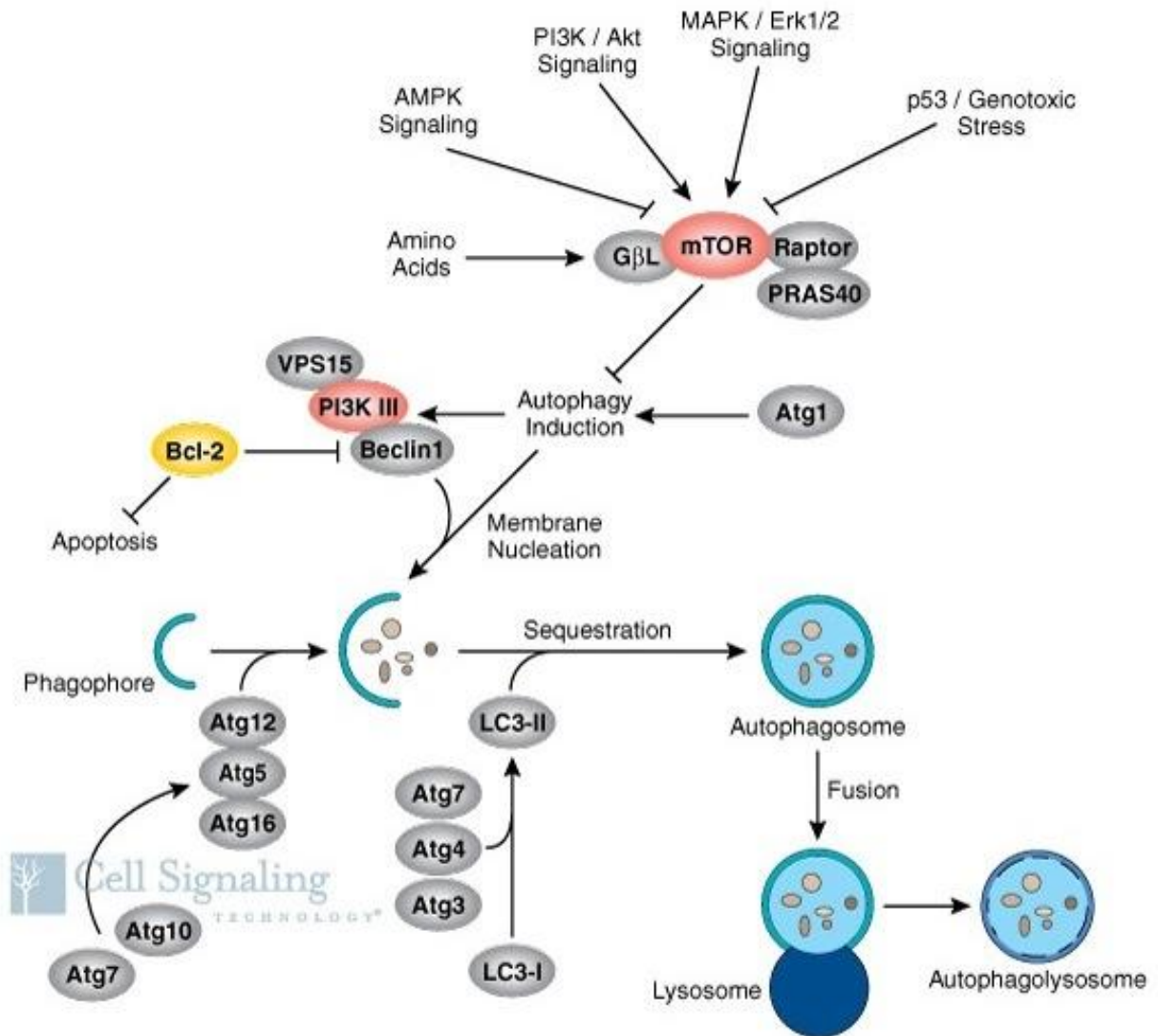
**Figure 1. The intrinsic and extrinsic pathways of apoptosis**

The extrinsic or death receptor mediated pathway involves the binding of a ligand (eg. Fas) to a death receptor (eg. TRAIL), the recruitment of adaptor proteins such as FADD and the subsequent cleavage of caspase 8 which leads to downstream activation of effector caspases. The intrinsic apoptotic pathway is initiated by stress signals such as radiation, chemotherapy and the presence of misfolded protein via ER stress which leads to the permeabilization of the mitochondria and the subsequent release of cytochrome c. Cytochrome c combines with adapter protein Apaf-1 and caspase 9 and leads to the activation of downstream effector caspase 3 and 7. These pathways are interconnected at different levels, for example, by Bid, a BH3 domain-containing protein of the Bcl-2 family which assumes cytochrome-c releasing activity upon cleavage by caspase-8.



**Figure 2. Regulation of autophagy and its pathways**

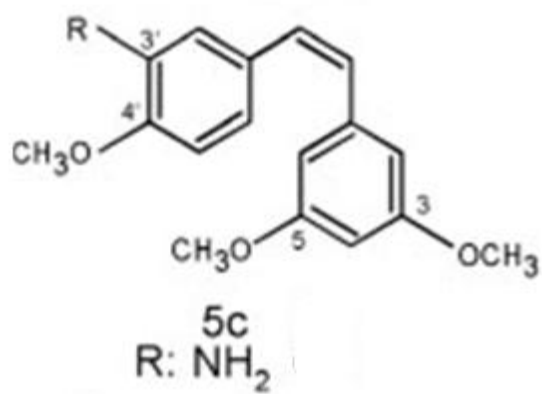
The induction of autophagy leads to the Beclin-1, Atg6 and the class III phosphatidylinositol 3-kinase (PI3K), Vps34 complex which initiates the vesicle nucleation process. Autophagy-related gene (atg) proteins such as Atg 5 and Atg 7 are recruited and become integrated in the autophagosomal cell membrane. LC3-I is cleaved by Atg4 and linked to phosphatidylethanolamine, Atg 3 and Atg7. After the linkage, LC3-I is converted to LC3-II and incorporated in the autophagosomal membrane. The autophagosome then fuses to the lysosome and forms the autophagolysosome which degrades cellular components. Autophagy can be induced by serum starvation or pharmacologically by Rapamycin which inhibits mTOR. This process can also be inhibited by the PI3K/Akt and MAPK/Erk1,2 signaling pathways or by pharmacological agents such as Bafilomycin A1, Chloroquine and Wortmanin.



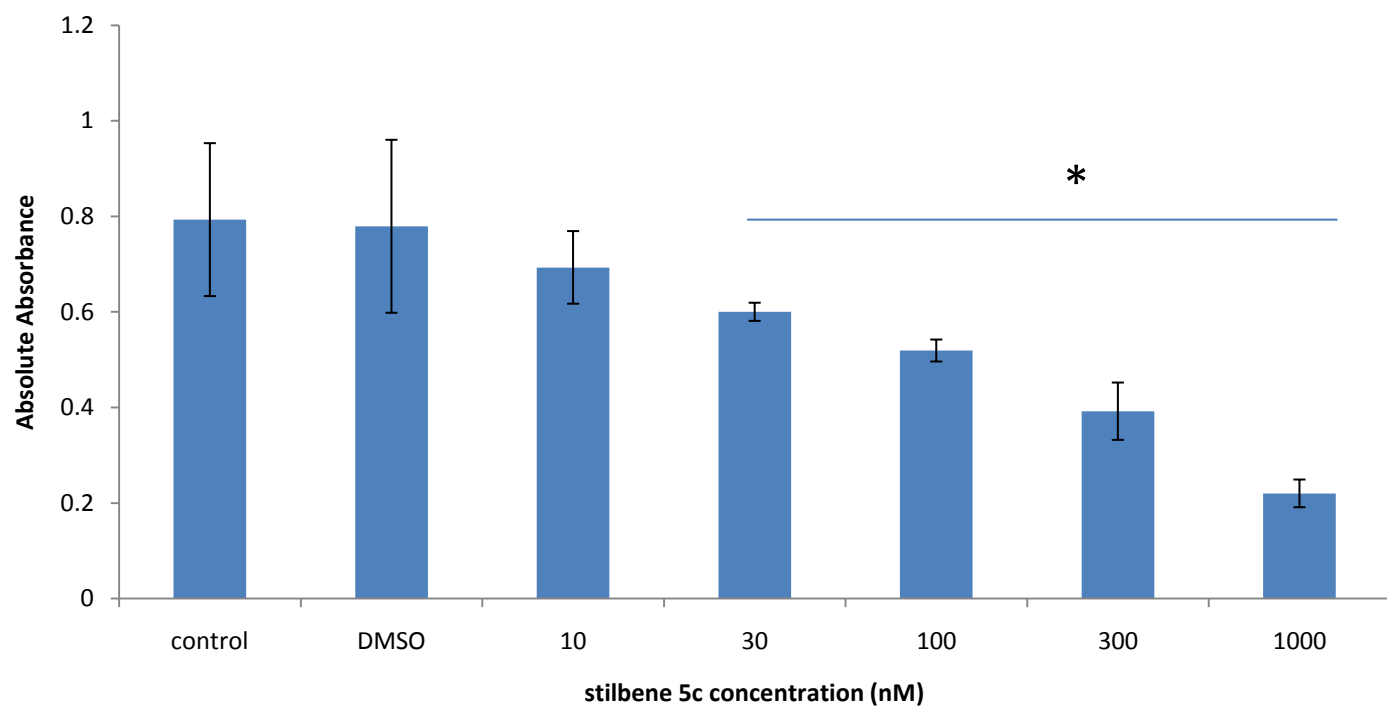
**Figure 3. Effects of increasing dose of stilbene 5c on B16F10 melanoma cells.**

**A.** Structure of stilbene 5c. Stilbene 5c has an amine  $\text{-NH}_2$  group on the 3' carbon. **B.** Cell viability after stilbene 5c treatment was measured using the MTT assay. Cells were plated in triplicates in 96-well plates and treated with increasing concentrations of stilbene 5c. After 72 hours of incubation, 2mg/ml of MTT was added to each well for an additional 3 hours incubation period. The blue MTT formazan precipitate was then dissolved in 100 $\mu$ l of DMSO. The absorbance at 490nm was measure on a multi-well plate reader. Cell viability was expressed using the absolute absorbance recorded ( $A_{490\text{nm}}$ ). (\*  $p < 0.05$  compared to control)

A.

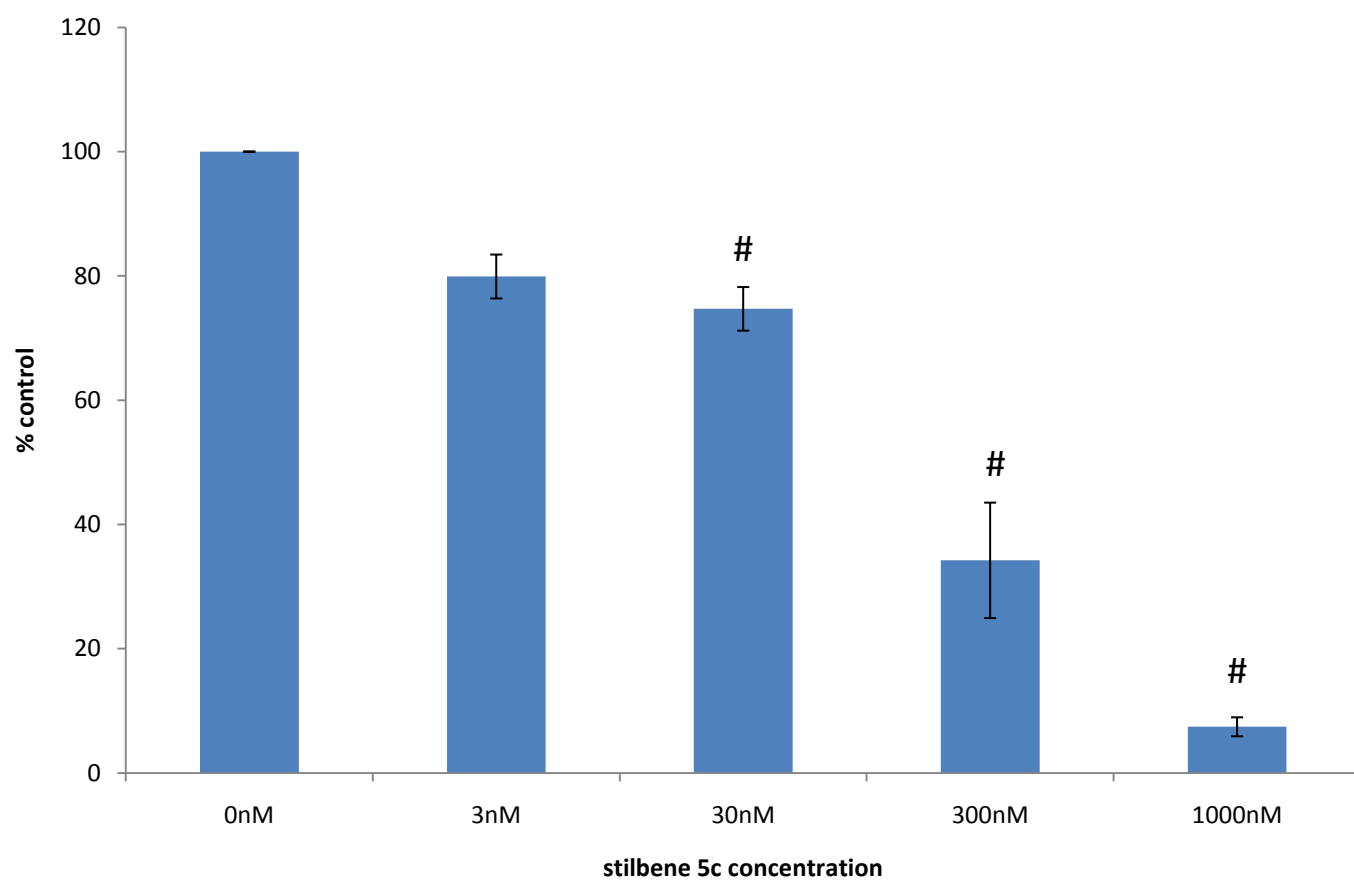


B.



**Figure 4. Inhibition of colony formation in melanoma cells treated with 300nM stilbene 5c**

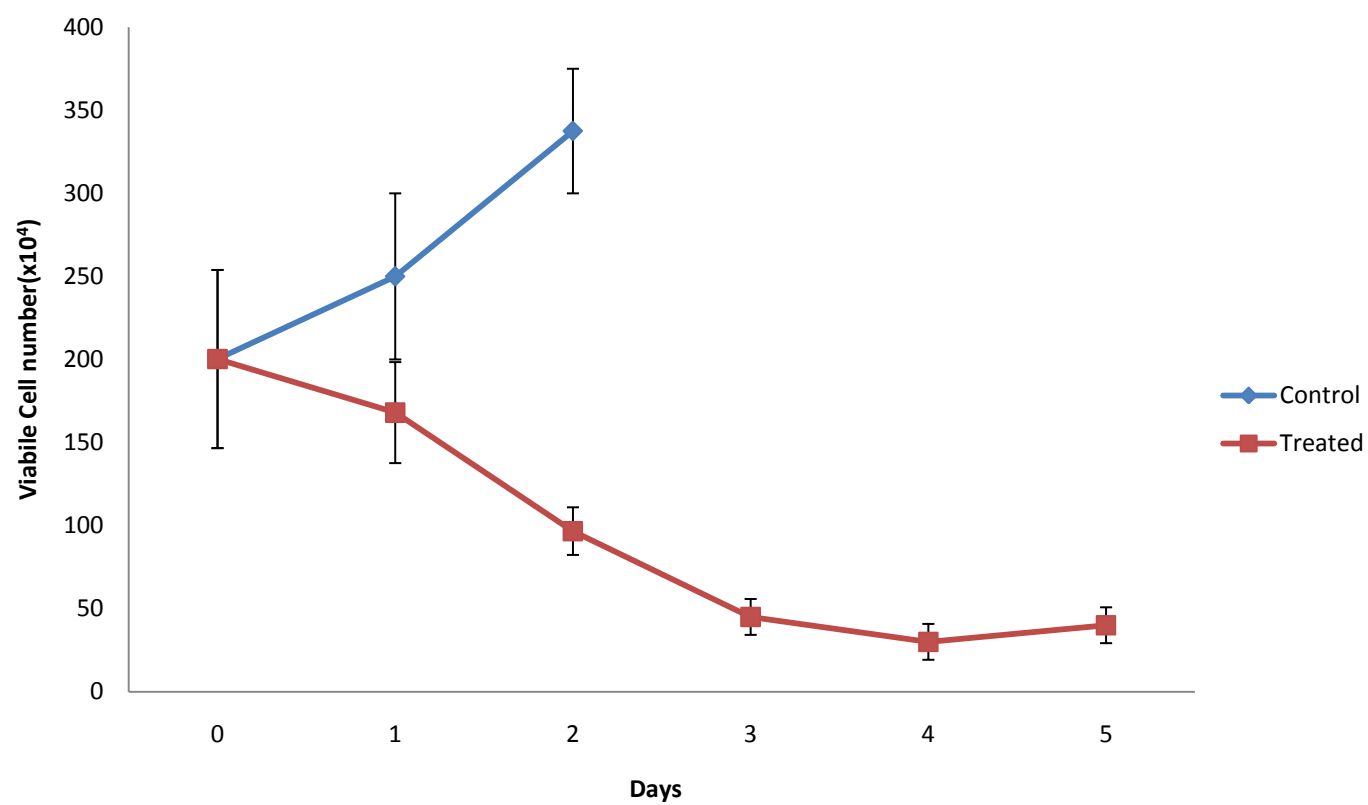
B16F10 cells were plated in triplicate in 6 well plates at a confluence of 150 cells per well. Control cells were seeded at the same density and allowed to incubate in drug free medium while cells treated with 3nM, 30nM, 300nM and 1000nM stilbene 5c were incubated for 72 hours. Drug containing medium was removed at the end of the 72 hours and replaced with fresh medium. When colonies of at least 50 cells are visible to the naked eye (~2weeks), cells were stained with crystal violet and counted manually. The number of colonies formed decreased in a dose-dependent manner in cells treated with stilbene 5c compared to control cells. (#  $p < 0.05$  compared to control)





**Figure 5. B16F10 melanoma cell viability timecourse study**

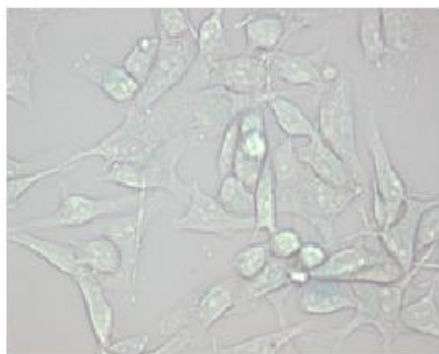
Cells were plated in triplicate using 6 well plates and treated on day 0 with 300nM stilbene 5c. At the indicated time points, cell were trypsinized and collected by centrifugation. Cells were counted using the trypan exclusion assay and the automated Countess cell counting apparatus. Cells showed a decrease in cell number up to day 3 post-treatment and growth arrest at later time points.



**Figure 6. Chronic exposure of 300nM stilbene 5c induces senescence in melanoma cells**

Cells were plated and treated with 300nM stilbene 5c. At indicated time points, cells were incubated with staining medium and X-gal. After one day of incubation, electron microscopy was used to analyze morphological changes in the cells as well as the appearance of blue stain which is a hallmark of senescence. B16F10 cells treated with 1 $\mu$ M adriamycin were used as positive control. All images were taken at 20X.

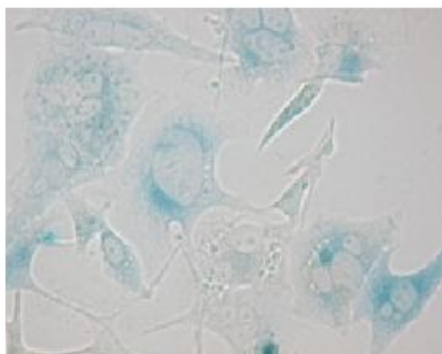
Control



20X

Day 5

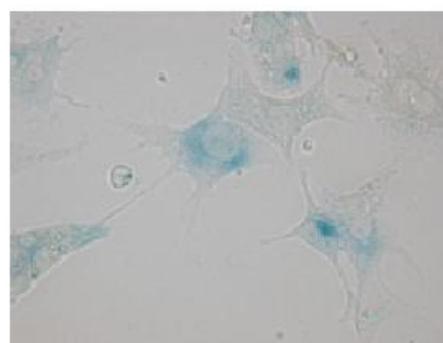
Day 3



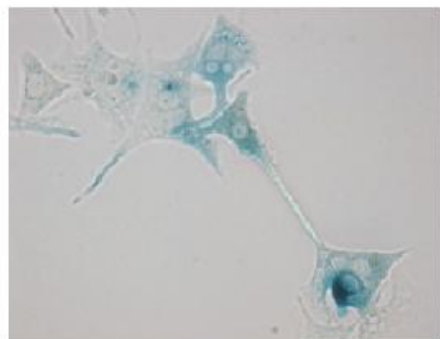
20X

ADR (1uM)

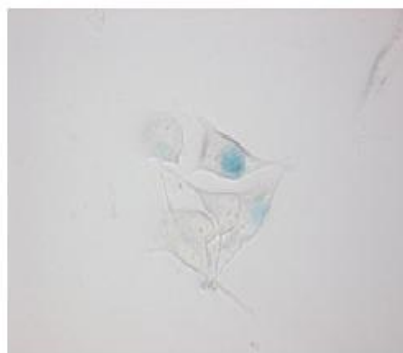
Day 4



20X



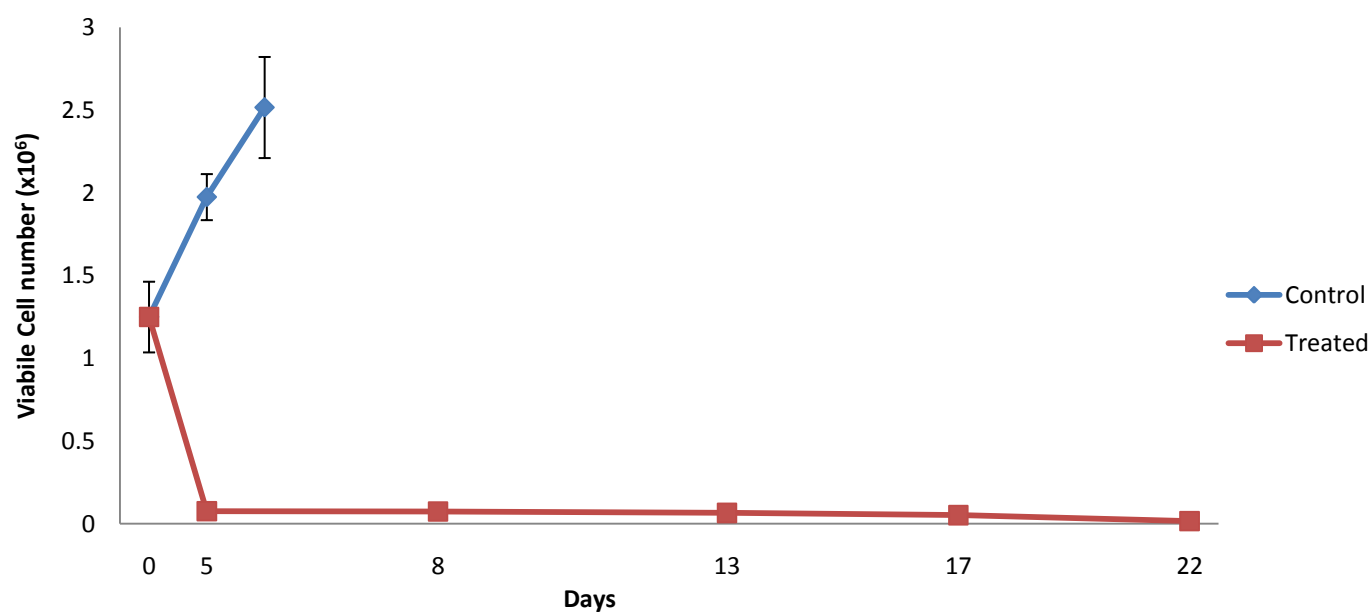
20X



20X

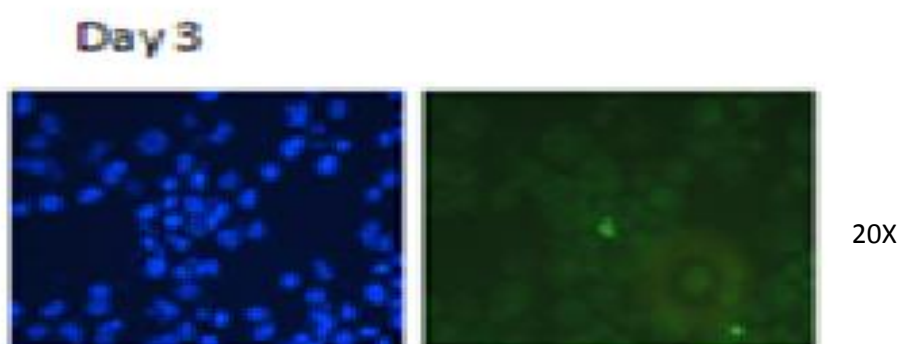
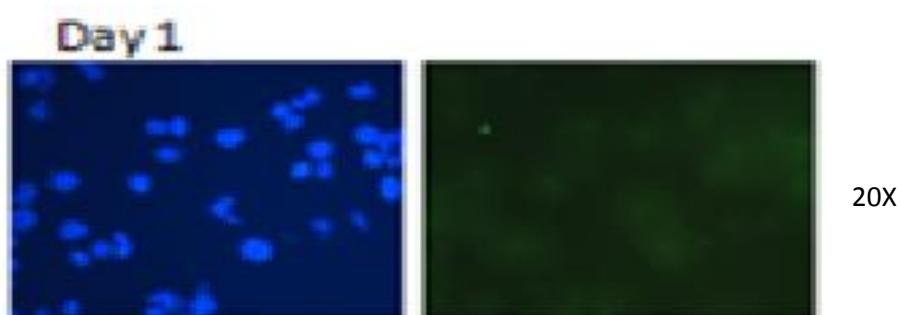
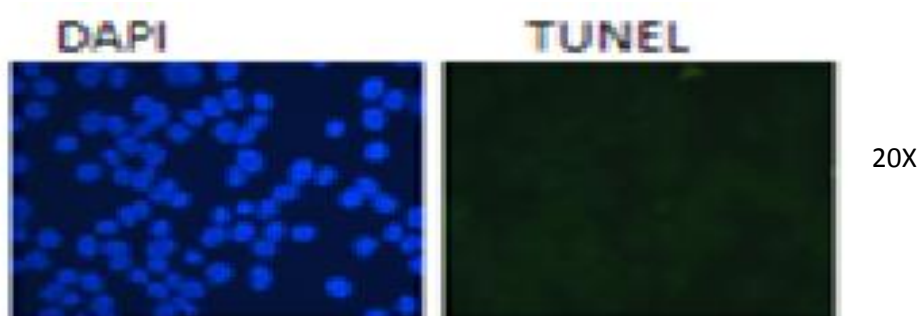
**Figure 7. Lack of proliferative recovery following continuous stilbene 5c exposure**

B16F10 cells were plated in triplicate in 6-well plates and treated with 300nM of Stilbene 5c continuously for 5 days. Drug was then removed and replaced with fresh medium on day 5. At indicated time points, cells were harvested with trypsin and counted using the trypan blue exclusion assay. Drug was removed on day 5 and replaced with fresh medium. Cells were monitored for proliferative recovery for 2 weeks post drug removal and cells were counted at indicated time points



**Figure 8. Apoptotic cell death in B16F10 melanoma cells exposed to 300nM stilbene 5c**

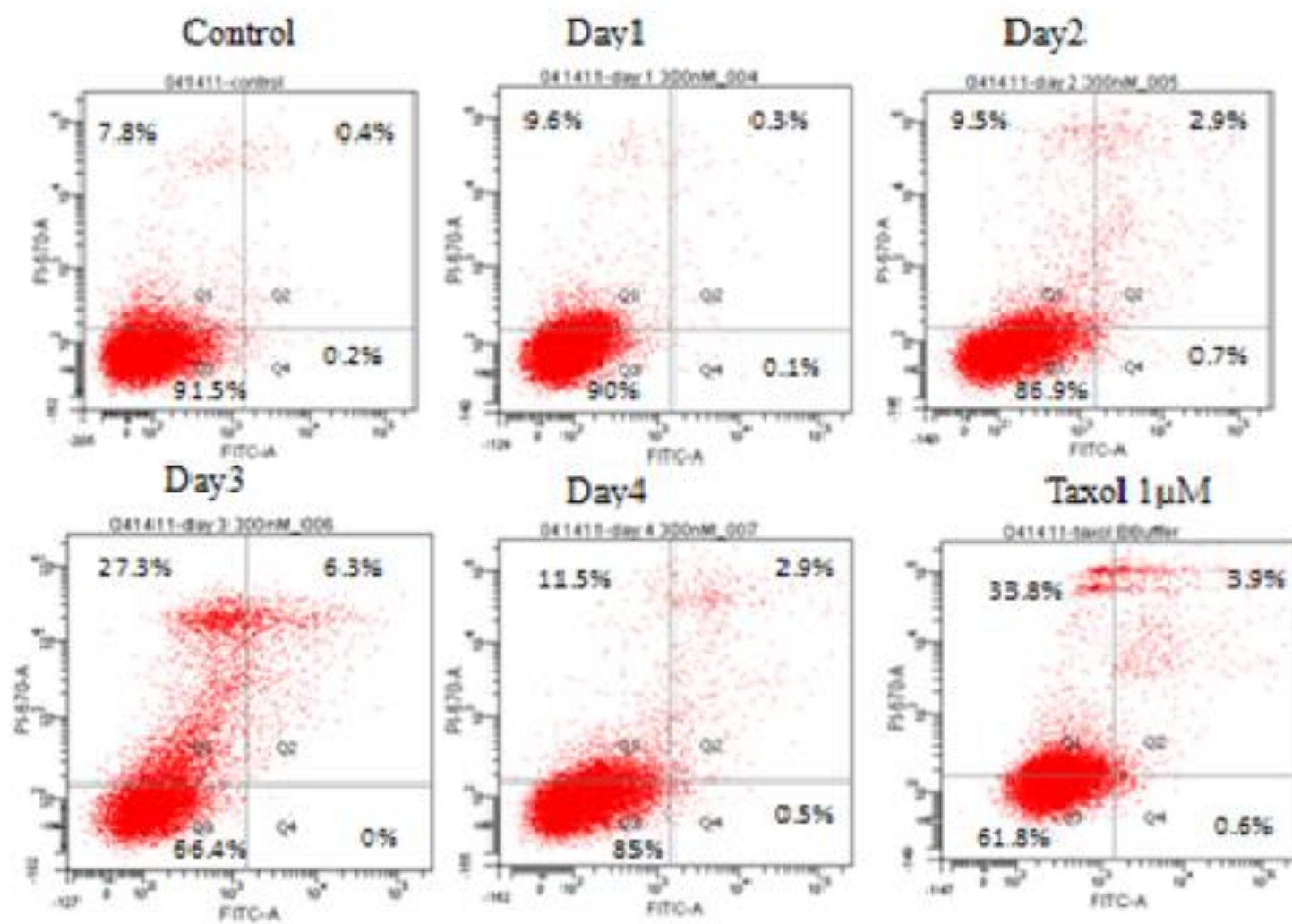
B16F10 cells were treated with 300nM stilbene 5c and fixed on slides on days 1, 2, 3, 4 and 5. Cells were stained with the nuclear marker DAPI and apoptosis was evaluated based on nuclear morphology. Some nuclear fragmentation is apparent on day 3 post-treatment while at day 1 cells do not appear to be undergoing apoptosis. TUNEL staining was performed simultaneously to assess for apoptosis associated DNA breaks. No significant level of apoptosis is apparent on day 1 while some fluorescence appears on day 3, suggesting minimal level of apoptosis.





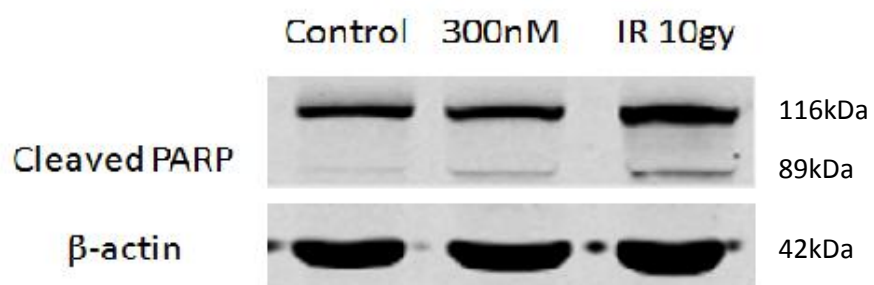
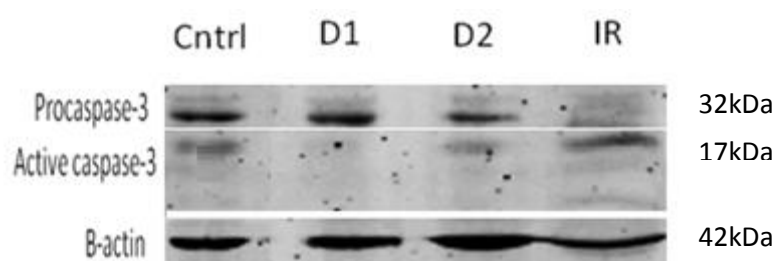
**Figure 9. Low level of apoptotic cell death in B16F10 melanoma cells exposed to 300nM stilbene 5c**

During early apoptosis, phosphatidyl serine (PS) is translocated from the inner cell membrane to the outer cell membrane. Annexin V coupled with a fluorescent chromophore (FITC) binds to PS and serves as marker of early apoptosis. Propidium Iodide (PI) stains cells with compromised cell membrane as in cells in late apoptosis or necrosis. Cells treated with 300nM stilbene 5c demonstrated no early apoptotic cells at indicated time points while a subset of cells undergo late apoptosis. Taxol was used as a positive control.



**Figure 10. Minimal PARP and caspase 3 cleavage in B16F10 cells treated with stilbene 5c**

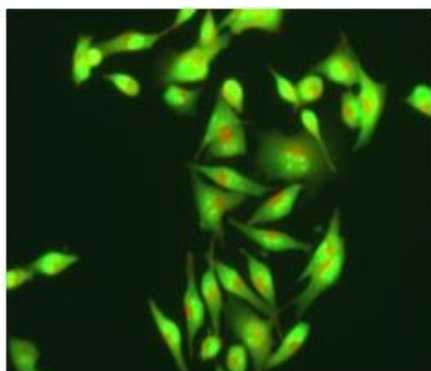
**A.** PARP cleavage using western immunoblotting was used to assess for apoptosis. Minimal PARP cleavage was seen 2 days post-treatment with 300nM stilbene 5c. B16F10 cells radiated with 10Gy were used as a positive control. **B.** Similarly, caspase 3 cleavage was assessed in cells treated with 300nM stilbene 5c for 1 and 2 days. Cells were collected, lysed and protein levels were analyzed on days 1 and 2 using western immunoblotting. Minimal caspase 3 cleavage was detected in cells treated with 300nM stilbene 5c, indicating that cells undergo a low level of apoptotic cell death. Cells radiated with 10 Gy were used as a positive control.

**A.****B.**

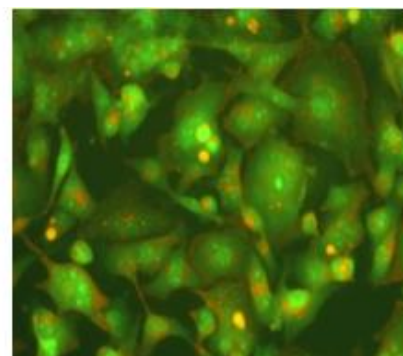
**Figure 11. Induction of autophagy in cells treated with 300nM stilbene 5c**

Acridine Orange staining was used to assess the induction of autophagy in B16F10 cells treated with 300nM stilbene 5c. Cells were plated, incubated overnight and treated the next day with 300nM stilbene 5c. Acridine orange was diluted in PBS (1:1000) to stain acidic compartments. Acridine Orange images were taken using the inverted fluorescent microscope. Distributed orange punctuate staining is apparent starting 1 day post-treatment, indicative of autophagy.

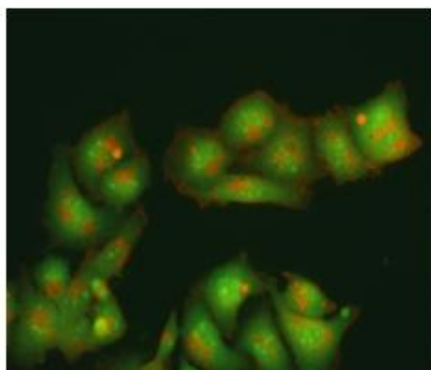
Control



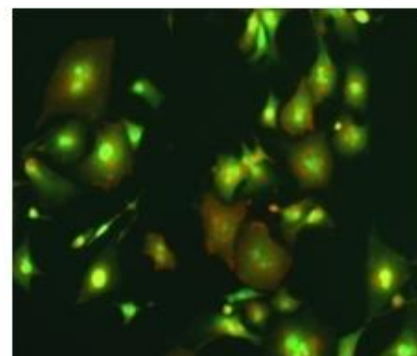
Day 3



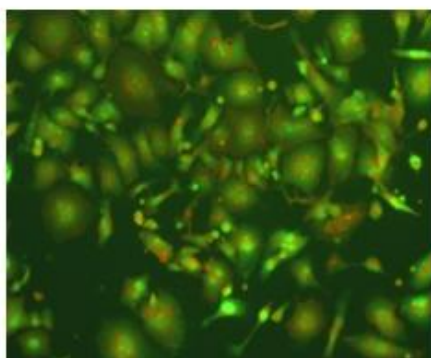
Day 1



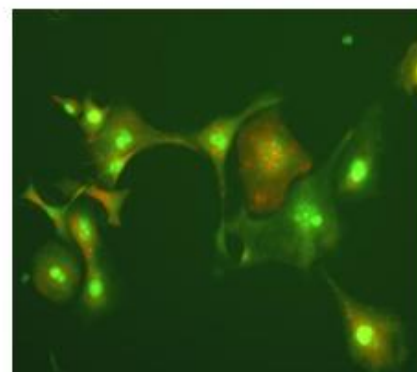
Day 4



Day 2



Day 5

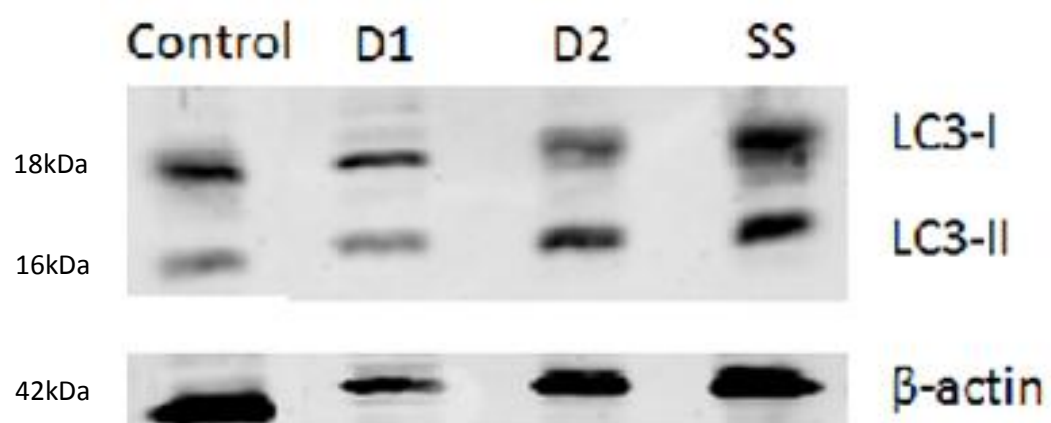


20X

20X

**Figure 12. LC3-I to LC3-II conversion in B16F10 cells treated with 300nM stilbene 5c**

Induction of autophagy was further investigated using western immunoblotting for the microtubule associated light chain protein (LC3-I) conversion to LC3-II. Cells treated with 300nM stilbene 5c for 1 and 2 days were collected, lysed and proteins were probed after gel electrophoresis. Conversion of LC3-I to LC3-II is noted on day 2 confirming induction of autophagy.

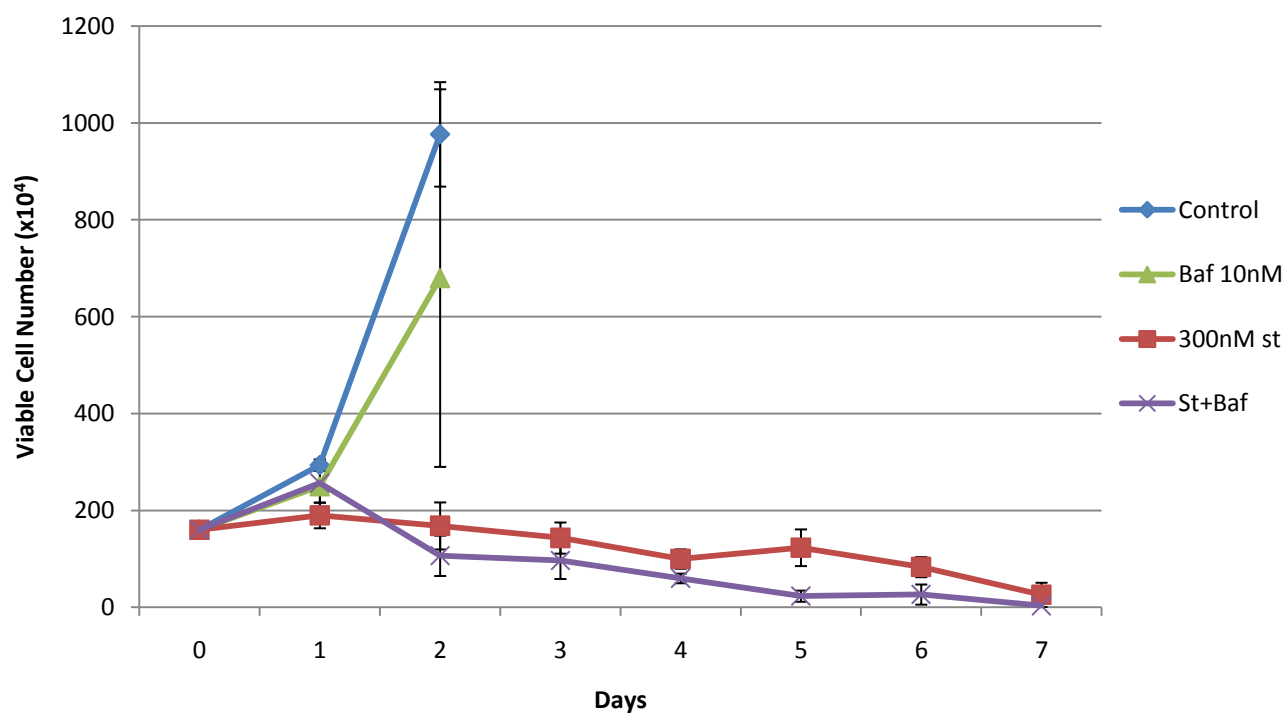




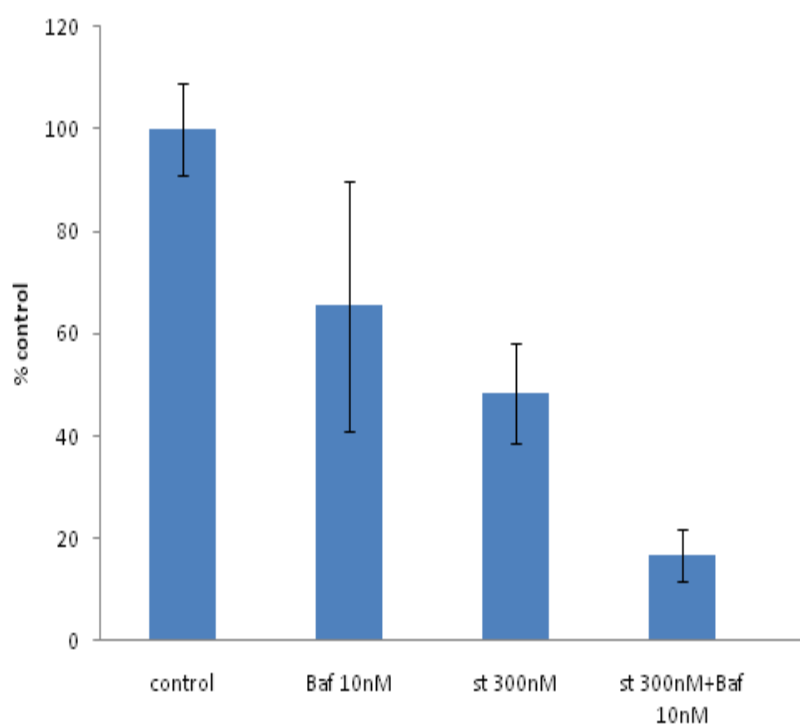
**Figure 13. Increased cell death and low colony survival when autophagy is pharmacologically inhibited**

**A.** Cells were pre-treated with 10nM Bafilomycin A1 to inhibit autophagy and then treated with stilbene 5c to a final concentration of 300nM. Control cells and cells treated with 10nM bafilomycin presented no cell death. Cells treated with 300nM stilbene alone resulted in pronounced cell death over of a period of 7 days. More importantly, cells pre-treated with bafilomycin A1 and treated with 300nM stilbene5c presented increased cell death as early as 2 days after treatment. **B.** Colony formation was evaluated with stilbene 5c alone as well with the combination treatment. While control and bafilomycin treated cell showed no significant change in colony formation, cells treated with stilbene 5c alone presented decrease colony formation. Cells treated with bafilomycin and stilbene 5c resulted in an additive decrease in cell number compared to control.

A.



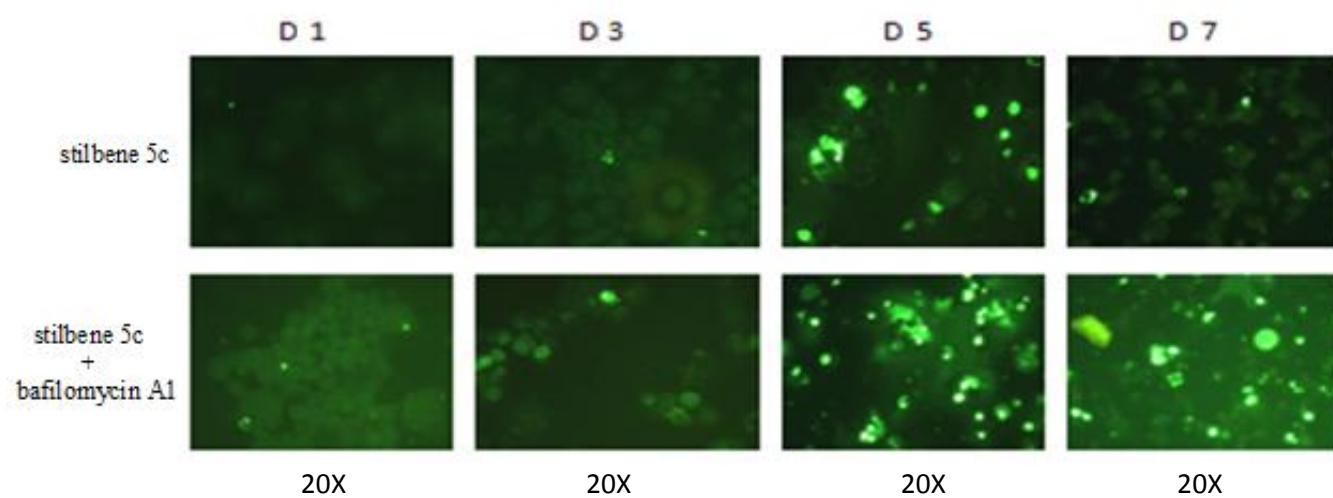
B.



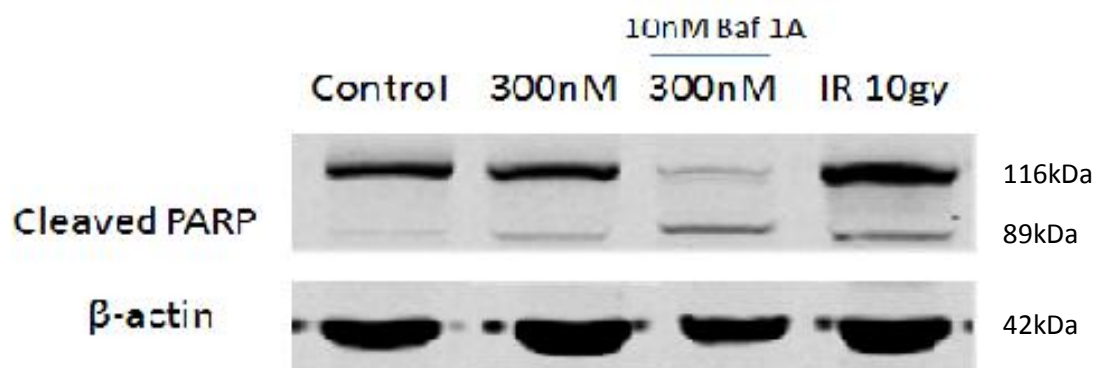
**Figure 14. Extensive apoptotic cell death when autophagy is pharmacologically inhibited**

**A.** The TUNEL assay was conducted in B16F10 cells treated with 300nM stilbene 5c. Increased TUNEL positive stain is observed for the combination treatment with bafilomycin and stilbene 5c indicative of apoptosis. **B.** Minimal PARP cleavage is observed with 300nM stilbene 5c alone, while combination treatment with bafilomycin 1A leads to complete PARP cleavage suggesting that extensive apoptosis is occurring when autophagy is inhibited. B16F10 cells irradiated with 10 Gy were used as a positive control.

A.



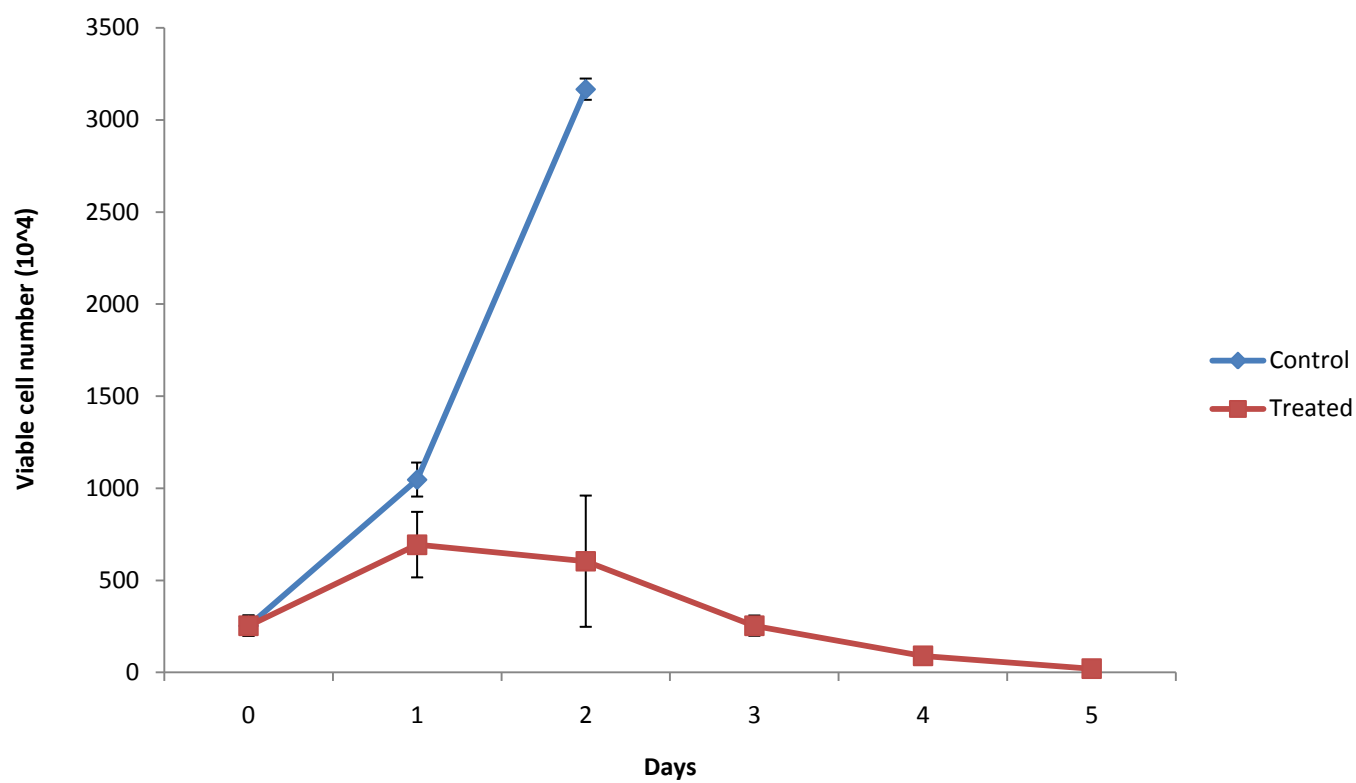
B.



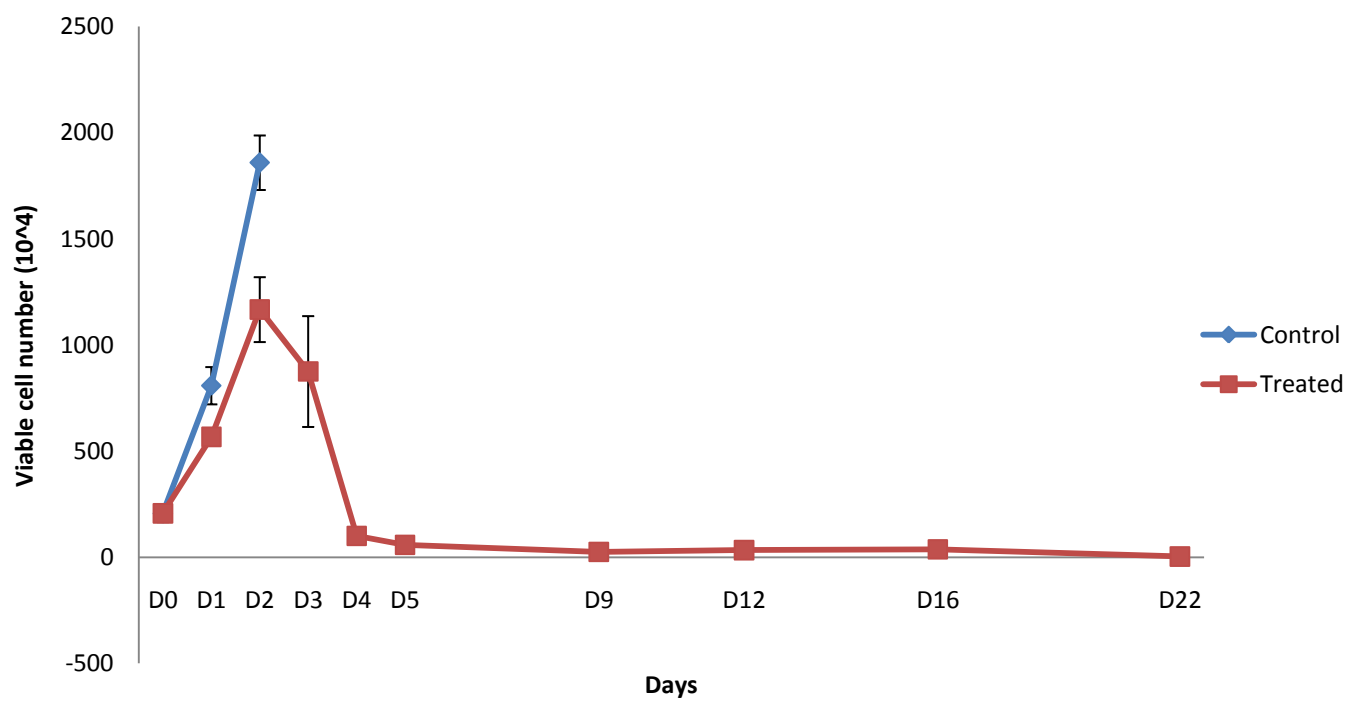
**Figure 15. Delayed cell death and lack of proliferative recovery following continuous low dose exposure**

**A.** B16F10 cells were plated in triplicates and treated with 30nM Stilbene 5c continuously for 5 days. At indicated time points, cells were harvested with trypsin and counted using the trypan blue exclusion dye. Cells treated with 30nM displayed an initial increase in cell number but a subsequent decrease and growth arrest by day 5. **B.** Drug was removed on day 5 and replaced with fresh medium. Cells were monitored for proliferative recovery for 2 weeks post drug removal and counted at indicated time points.

A.

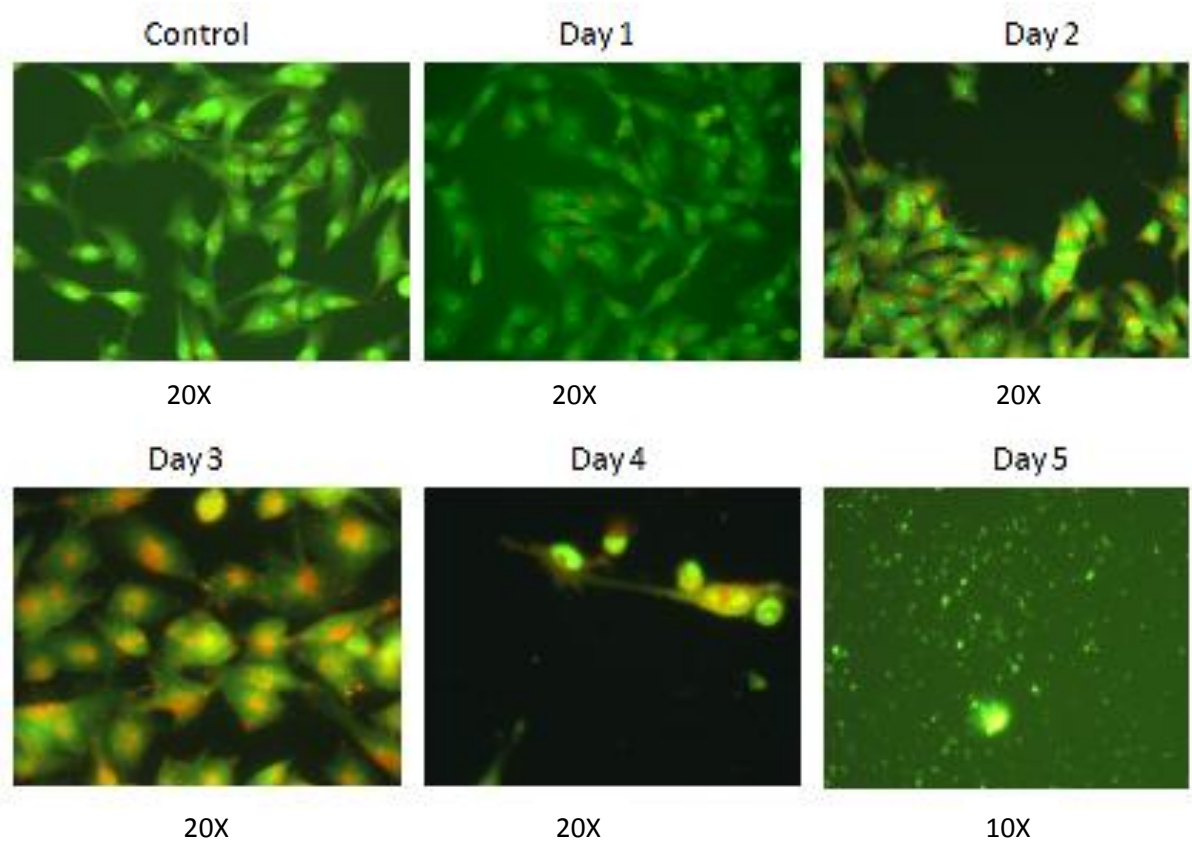


B.



**Figure 16. Induction of autophagy in cells treated with a low-dose of stilbene 5c**

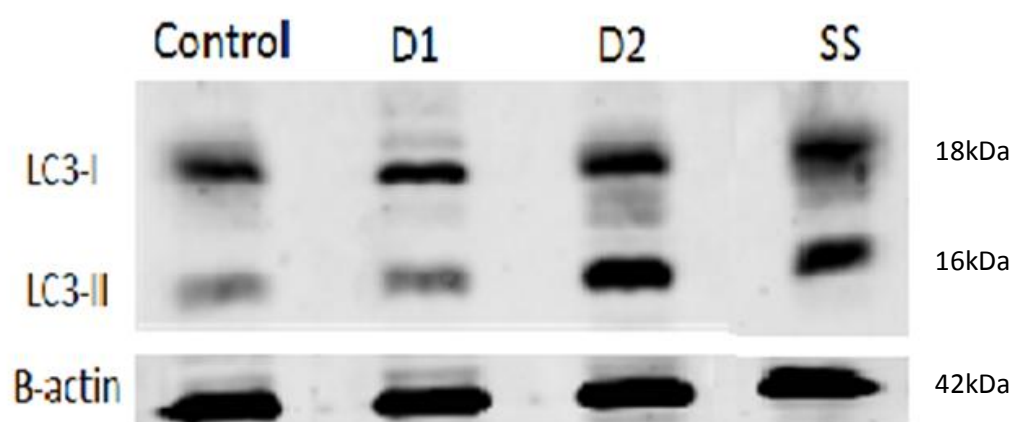
Cells were plated and left overnight to adhere. Cells were treated the next day with 30nM stilbene 5c and stained at indicated time points. Acridine orange staining is used to visualize acidic compartments in cells undergoing extensive autophagy. Cells treated for 1, 2, 3, 4, and 5 days with a low dose of stilbene 5c have distributed punctate orange staining suggestive of autophagy.





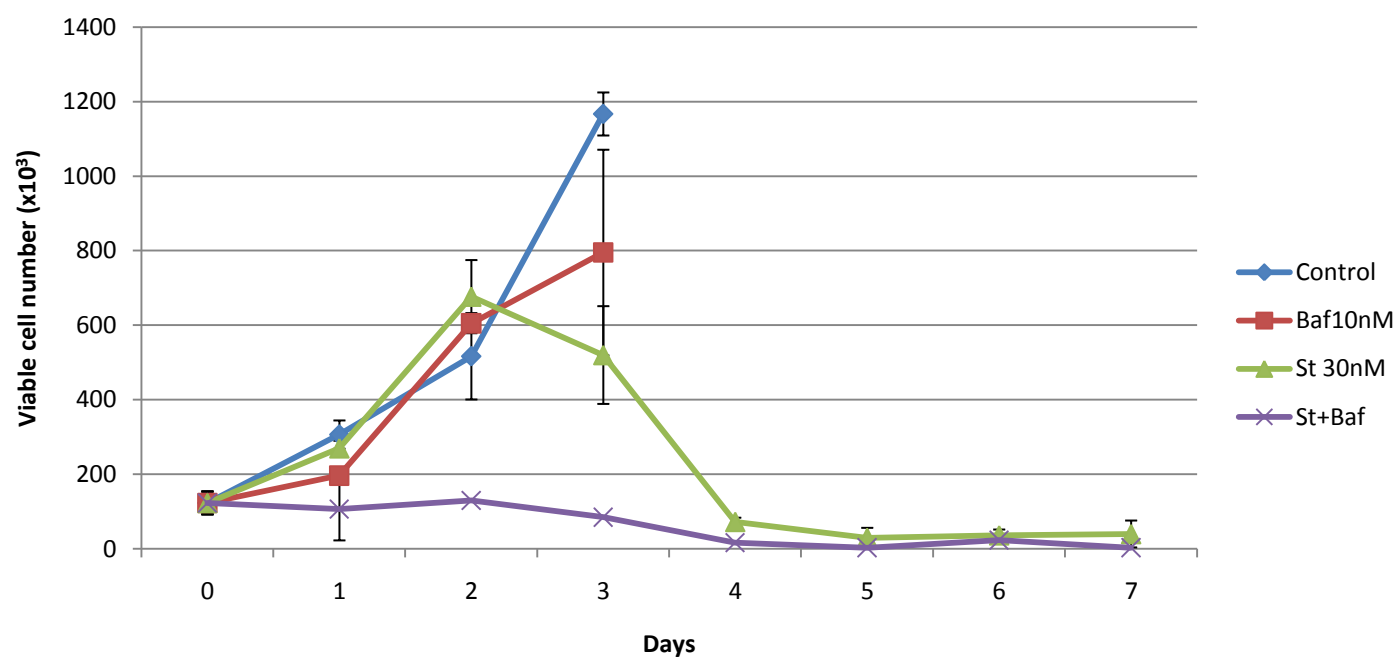
**Figure 17. Induction of autophagy detected through the conversion of LC3-I to LC3-II**

Cells were collected, lysed and protein concentration determined. Samples were run using a 15% gel and probed for LC3. Conversion of LC3-I to LC3-II was detected, which further confirms induction of autophagy. Cells treated with 30nM stilbene 5c for 2 days were shown to undergo autophagy. Serum starved B16F10 cells were used as our positive control.



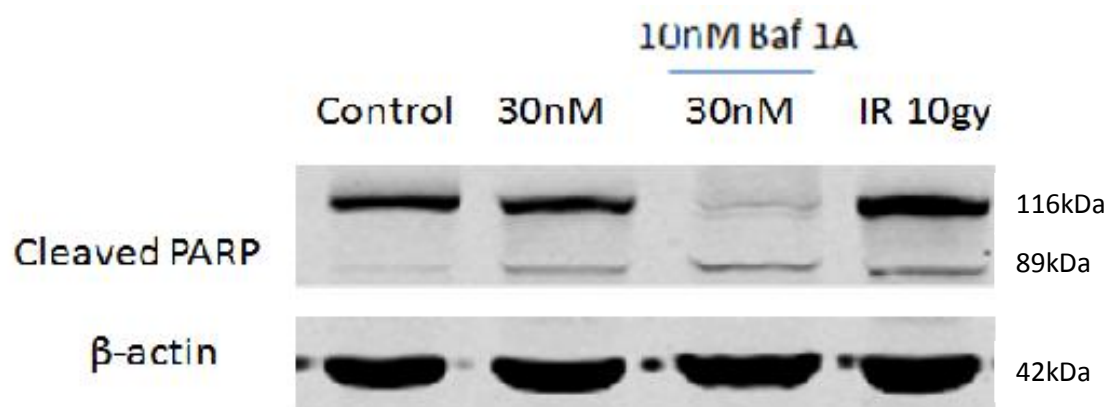
**Figure 18. Cytoprotective role of autophagy in cells treated with 30nM stilbene 5c**

Cells were plated in triplicate wells and treated with stilbene 5c and/or bafilomycin A1. Cells treated with 30nM stilbene 5c showed an initial increase in cell number followed by cell death on day 4. Cells pre-treated with 10nM bafilomycin for 3 hours followed by 30nM of stilbene 5c showed a significant decrease in cell number.



**Figure 19. Switch to apoptosis in response to drug treatment when autophagy is inhibited**

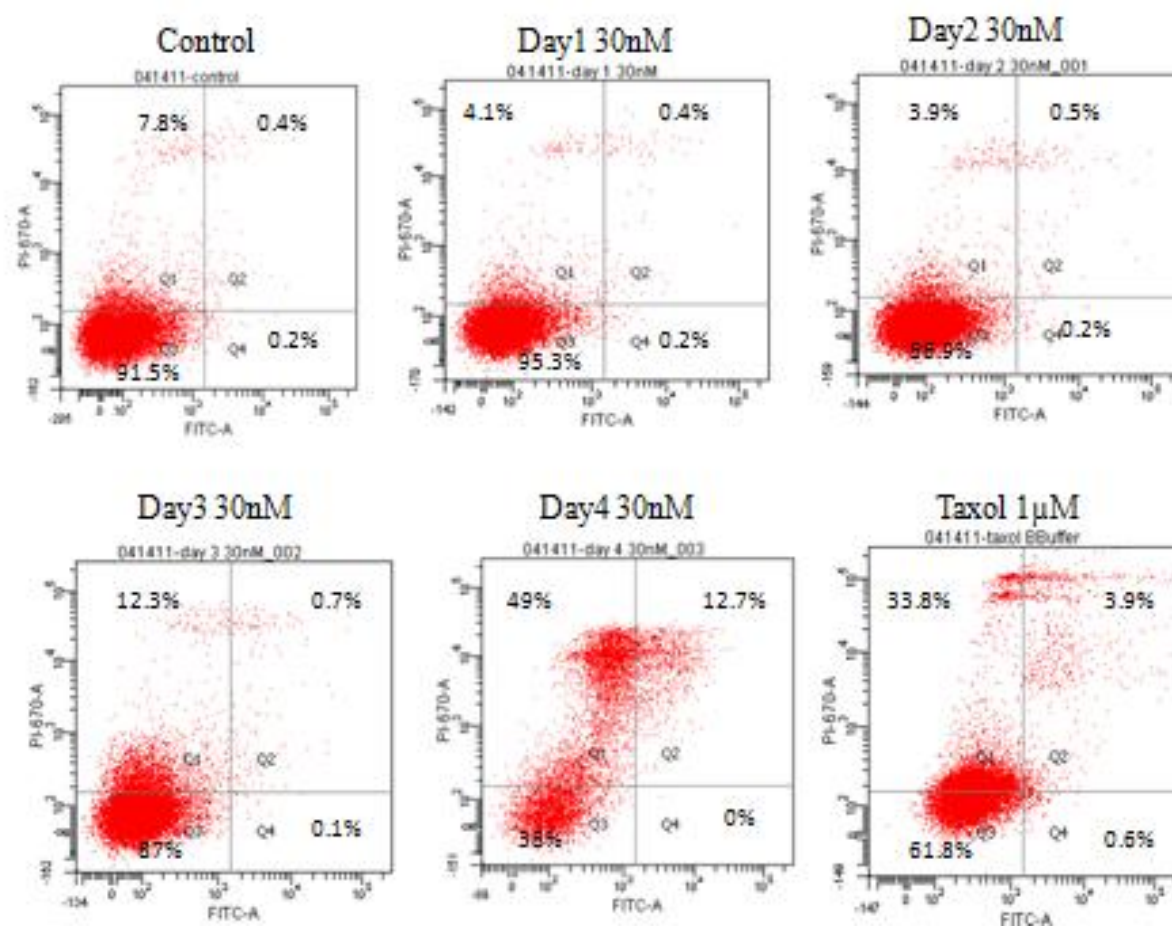
Western immunoblotting was used to assess the mode of cell death in the combination treatment of 30nM stilbene 5c with the autophagy inhibitor, bafilomycin A1. Cells were treated, lysed and probed with anti-PARP antibody. Minimal PARP cleavage is observed with 30nM of stilbene 5c alone, while the combination treatment with 10nM bafilomycin A1 led to complete PARP cleavage suggesting that extensive apoptosis is occurring when autophagy is inhibited. B16F10 cells radiated with 10 Gy were used as a positive control.



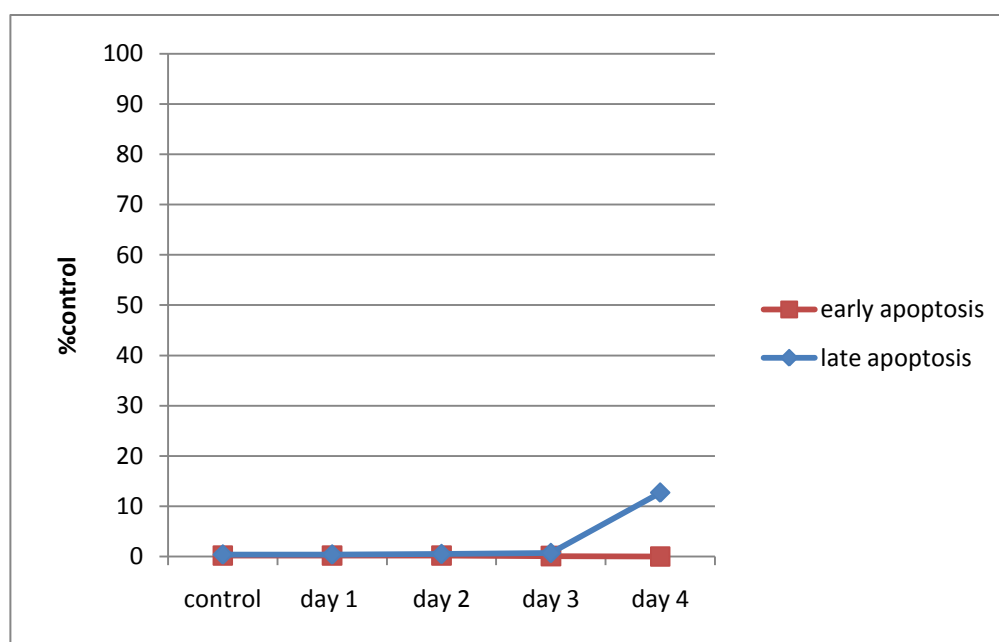
**Figure 20. Low level of apoptosis occurs in cells treated with 30nM stilbene 5c**

**A.** Annexin V-PI staining is used to detect the level of apoptosis occurring with 30nM stilbene 5c alone. Cells in early apoptosis will be annexin+/PI-, while cells in late apoptosis will be annexin+/PI-. No early apoptosis is detected while a slight increase in late apoptosis occurs in cells treated with 30nM stilbene 5c for 4 days. B16F10 cells treated with 1 $\mu$ M of Taxol were used as a positive control. **B.** Apoptotic cells in figure A were quantified, confirming the low level of apoptosis present in cells treated with 30nM stilbene 5c.

A.



B.





## Chapter 4: Discussion

Microtubules present a promising target for cancer therapy. Stilbenes are known to inhibit microtubules by binding to the colchicine binding site and thus are called colchicine site inhibitors (CSIs). Although they have structural similarity to other CSIs, they do not have the 4-methoxyl group of the trimethoxyphenyl moiety characteristic of others CSIs. We studied a stilbene derivative, *cis*-3, 4, 5-trimethoxy-3-aminostilbene (stilbene 5c) which is also a colchicine site inhibitor of microtubules that functions by promoting microtubule dissociation. Stilbene 5c was shown to be highly potent against various tumor cells with no major organ toxicity.(Durrant et al. 2009) Most importantly, the compound achieves barely detectable levels in the heart (Durrant et al. 2009), making it a more desirable VDA with negligible cardiac toxicity. In the present study, we evaluated the cell killing ability of stilbene 5c, the modes of cell death and the role that autophagy plays in B16F10 murine melanoma cells treated with stilbene 5c.

### I. Cell viability

Previous studies with ovarian cancer cells have shown significant cell killing effects with 30nM and 100nM of stilbene 5c. (Durrant et al. 2008) In our studies, stilbene 5c was effective against B16F10 melanoma cells at similar concentrations and shown to inhibit cellular proliferation at a concentration as low as 30nM with the highest effect seen at 1μM. While 10nM showed no

significant cell killing compared to control, 30nM exhibited about 25% cell killing by the third day of treatment. In order to further investigate the cytotoxicity of stilbene 5c, cells were treated with 3nM, 30nM, 300nM and 1000nM for 3 days and colony formation was monitored. As expected, cells treated with the highest concentration of stilbene 5c showed a greater than 95% reduction in colonies formed. Cells treated with 300nM of stilbene 5c had about a 40% decrease in colony formation compared to cells not treated with drug. The concentration dependent decrease in cell number, similar to the colony formation assay, reflects a dose-response curve with the ED 50 of the drug being around 300nM. A similar vascular disrupting agent, Combrestatin A-4 (CA-4), capable of disrupting the abnormal tumor vasculature, binds to tubulin within the colchicine binding site, altering endothelial structure and causing vascular permeability and rapid destruction of tumor vasculature. CA-4 used against B16F10 melanoma cells had an IC 50 of 16.1 $\mu$ M, a significantly higher dose compared to stilbene 5c. (Lee et al. 2011)

Stilbene 5c at a 300nM concentration was used for subsequent studies as it resulted in a robust inhibition of cellular proliferation. Cells were also treated with 30nM of stilbene 5c to understand the cell killing effects of a low dose stilbene 5c treatment. There is an initial delay in cell death 24 hours post treatment with 30nM but subsequently extensive cell death occurs by day 3. A similar growth delay was observed with 30nM of colchicine. Studies in cerebellar granule cells treated with a low dose of colchicine also show a similar delay in apoptosis where cell killing is observed as late as 24hrs post treatment. (Bonfoco et al. 1995) The delay in cell death seen on days 1 and 2 post-treatment may be due to the fact that stilbene 5c needs a longer incubation period to start inhibiting microtubules that are present extensively throughout the cell. This concentration of the drug may be just sufficient to inhibit colchicine binding sites on microtubules, which result in cells not undergoing mitosis. However, as the cells are incubated

with the drug for a longer period of time, all of the microtubules present are inhibited, which resulted in cell cycle arrest and a subsequent demise of the cell. (Cao et al. 2008) Cells treated with both a low and high dose of stilbene 5c seem to be in state of growth arrest on days 4 and 5 post-treatment. The  $\beta$ -galactosidase staining confirmed senescence as early as day 1 and up to day 5 post-treatment. Further investigation has revealed that cells do not recover upon drug removal but instead a subset of the senescent cells undergoes apoptotic cell death as shown by DAPI/TUNEL staining.

## **II. Apoptosis**

Cells treated with 300nM of stilbene 5c showed minimal levels of apoptosis. TUNEL staining confirmed that apoptosis increases by day 5, which correlates with the lowest number of viable cells during the time course experiment. However, we did not detect a significant increase in TUNEL positive staining on day 3 when extensive cell death is actively occurring. This may be due to dead cells that have not adhered to the slides during centrifugation for TUNEL analysis and thus could not be evaluated. Annexin V-PI staining revealed that cells undergo late apoptosis while early apoptosis is not detected. The increase of apoptotic cells on day 3 correlates with the increase in cell death noted on the time course for cell viability. During early apoptosis, cells undergo a specific conformational change where the phospholipid phosphatidylserine (PS) is translocated from the inner plasma membrane to the outer membrane. Late apoptotic cells have compromised cell membranes and thus are PI positive in addition to having translocated PS. The absence of early apoptosis may be due to the precipitated entry of the cell into apoptosis that

leads to immediate cell membrane disruption. Cells treated with 30nM and 300nM of stilbene 5c did not show any increase in early apoptosis as well as little increase in late apoptosis, suggesting late apoptosis alone cannot account for the extensive level of cell killing observed. This led to the conclusion that apoptosis is not the only mode of cell death at play as it does not account for the 90% decrease in cell number seen in the time course studies. The increase in cells over time that are PI positive confirms that necrosis occurs probably following some type of programmed cell death other than apoptosis. The minimal cleavage of PARP and caspase 3 again confirmed that cells undergo apoptosis to a minimal extent. Both PARP and caspase 3 are late events in the apoptosis signaling cascade, which supports the observed Annexin-PI data. The reason why apoptosis is not occurring to a higher extent may be because the cells are “fighting” to survive using a cytoprotective mechanism, which can perhaps be autophagy, in order to overcome the drug effect. Moreover, the drug’s concentration may not be strong enough to initiate cell killing at early stages during treatment. The decrease in cell death observed on day 4 with 30nM and 300nM of stilbene 5c treated can be attributed to cells going into senescence, which is known to be a permanent stage of cell cycle arrest.

### **III. Autophagy**

The melanoma tumor model shows relatively high levels of autophagy detected using acridine orange. Acridine orange staining indicates the induction of autophagy as early as day 1 post treatment. However, it is unclear to what extent autophagy is responsible for the cytotoxicity of stilbene 5c. One of the important controversies currently undergoing debate

relates to the importance of functional microtubules for the successful induction and progression of autophagy. Studies by Kochl et al. (2006) have shown that microtubules facilitate the formation of autophagosomes present in cells. However, in the same study, vinblastine, a microtubule inhibitor, stimulated autophagosome formation more than two fold before any discernable change in the microtubule network. (Kochl et al. 2006) In contrast, drugs such as paclitaxel which inhibits microtubule depolymerization, had no effect on acidic vesicular organelle formation. On the other hand, nocodazole leads to disassembly of microtubules within a cell, and this resulted in decreased autophagosome formation. (Kochl et al. 2006) These findings suggest that microtubules may not be necessary for the formation of autophagosomes but that some destabilizers reduce the amount of autophagosomes while others do not. The increase of autophagic vacuoles may provide support for our findings that autophagy is up-regulated after perturbation of microtubule dynamics. This suggests that stilbene 5c might be directly stimulating the induction of autophagy through the increase of autophagosome formation.

Induction of autophagy was confirmed with the conversion of the microtubule light chain protein LC3-I to LC3-II as early as 2 days post-treatment. Autophagy being a multi-step process, conversion of LC3-I to II only supports the induction of autophagy and not the completion of the process. Increased levels of autophagosomes may accumulate in the cell and may result in LC3 conversion without the lysosomal fusion. Generally, levels of the cytoplasmic p62 protein can be monitored for a better understanding of the autophagic flux as degradation of p62 is used as a hallmark of autophagic flux. (Yang et al. 2009) Unfortunately p62 is not detectable in the B16F10 melanoma cells. Additional studies using RFP-LC3 and the use of a microscope to visualize acidic vesicular organelles can be conducted to confirm autophagy in this system.

Bafilomycin A1, which was used to assess the role of autophagy, showed a cell growth inhibitory behavior as early as day 2. B16F10 cells have basal level of autophagy as demonstrated by the localized round stain detected using acridine orange and through the cleavage of LC3 in control cells. Autophagy can thus be a cellular process vital for the survival of these cells. This can explain why an extended incubation of two days or longer with the autophagy inhibitor bafilomycin led to growth inhibitory effects. Treatment of B16 cells with 300nM of stilbene 5c in combination with bafilomycin showed an initial increase in cell number suggesting that cytotoxic autophagy was induced by stilbene 5c. The initial cytotoxic role of autophagy is not evident in cells treated with 30nM stilbene 5c in combination with bafilomycin. Cells undergo extensive cell death as early as day 1 in the combination treatment with a low dose treatment with stilbene 5c. The initial increase in cell number seen with 300nM in combination with bafilomycin may be due to the high toxicity of the drug at the level of the microtubules, which made it impossible for cells to divide properly and undergo mitosis; this initiated cytotoxic autophagy to eliminate the cells. As the combination treatment is incubated for a longer period of time, the dependence of the cells on autophagy to normally survive coupled with autophagy now altered to be cytotoxic, could account for the overall decrease in cell number. On the other hand, 30nM stilbene 5c in combination with bafilomycin resulted in immediate cell death because the cytoprotective autophagy which is normally needed by these cells to survive has been compromised and is no longer functioning.

There have been reports where microtubule poisons such as paclitaxel have differential effects on cells that are concentration dependent due to alterations to microtubule dynamics. (Goncalves et al. 2001) When the cytoprotective autophagy is inhibited pharmacologically with bafilomycin A1, apoptosis which was occurring at a lower extent, is “allowed” to fully take place

resulting in extensive cell killing. This suggests that when autophagy, which is a vital mechanism for these cells, is inhibited, cells not only are destined for death, but the programmed apoptotic machinery is turned on.

The exact mechanism at play between autophagy and apoptosis is unclear but a link between autophagy and apoptosis has been demonstrated in several studies. In mice and humans, Beclin-1 constitutively interacts with the anti-apoptotic proteins of the Bcl-2 family: Bcl-2, Bcl-xL, Bcl-w and Mcl-1. Beclin-1 was originally identified as a Bcl-2 interacting protein where Bcl-2 inhibits the autophagic function of Beclin-1. Maiuri et al. showed in 2007 that Beclin-1 possesses a Bcl-2 homology 3 domain (BH3-only) which is necessary for its binding to the BH3 receptor domain of Bcl-2 or Bcl-xL. (Maiuri et al. 2007) Mutations in either of the BH3-only domains within Beclin-1 or within the BH3 binding domain in Bcl-2 or Bcl-xL will disrupt the Beclin 1-Bcl 2 complex resulting in the stimulation of autophagy.

When autophagy is inhibited with bafilomycin, the fusion of the autophagosome and the lysosome is inhibited. One can assume that such inhibition will lead to a decreased activation of the upstream signaling protein Beclin-1. In other words, the decrease in autophagy may be due to the increased binding of the anti-apoptotic Bcl-2 to Beclin-1. Moreover, studies have shown that caspases can cleave Beclin-1 during apoptosis thereby rendering it inactive during autophagy. For instance, caspase 3 and 8 cleavage of Beclin 1 generates N- and C-terminal fragments that lose their ability to induce autophagy. (Kang et al. 2011) More importantly, the C terminal fragment translocates to the mitochondria and sensitizes the cell to apoptotic signals. (Djavaheri-Mergny, Maiuri & Kroemer 2010) This process represents a positive feedback for inducing massive apoptotic cell death which can explain the extensive cell killing observed using TUNEL and PARP cleavage when autophagy is inhibited with bafilomycin A1.

#### IV. Senescence

Cellular senescence was first noted in the 1960s as a permanent arrest of cell cycle of non-malignant cells, which undergo limited number of cell division before entering a permanent and irreversible proliferation arrest. (Hayflick 1974) Normal cells that are in senescence undergo irreversible growth arrest and are unable to resume cellular division but remain metabolically active. Tumor cells that undergo senescence in response to chemotherapy may undergo apoptosis after a finite period of growth arrest or resume proliferation. (Gewirtz, Holt & Elmore 2008) The lack of proliferative recovery in B16F10 melanoma cells is accompanied by a senescence response. This growth arrest was evident in the timecourse studies, proliferative recovery studies as well as the  $\beta$ -galactosidase assay. Bypassing cellular senescence is thought to be an important step in tumorigenesis. (Zhang 2007) In this regard, stilbene 5c induced senescence in melanoma cells could represent a potential novel attractive therapy for melanoma, which currently lacks effective treatment.

Recent studies have indicated that autophagy may play a role in senescence. Studies by Lu et al. showed that autophagy promoted cell death in xenograft models then led to growth arrest. Interestingly, the growth arrest was followed by a slow proliferative recovery which was taken to reflect tumor dormancy. (Lu et al. 2008) Furthermore, when the stress was removed, cells recovered their ability to replicate. The fact that autophagy is needed before senescence was further investigated by the finding that blocking autophagy with chloroquine into tumor-bearing animals prevented re-growth. This observation is further supported with studies of Young et al. which suggest that autophagy is a prerequisite for senescence. (Young et al. 2009) Taking into account these findings, the initial response of B16 cells to stilbene 5c is a cytoprotective



autophagy; when the drug is incubated longer and stress is excessive, autophagy is insufficient to maintain the cells in a prolonged growth arrested and protected state and the cells consequently undergo apoptosis. Autophagy can also be followed by a second type of protective response that of senescence which occurs at a higher extent on days 3, 4 and 5 post-treatment with stilbene 5c. Whether protection is conferred by autophagy or senescence or both, studies have shown that a subset of the tumor population might regain proliferative recovery and re-enter cell cycle. (DeMasters et al. 2004, Elmore et al. 2005) The proliferative recovery assays showed that cells do not regain their capability to re-enter cell cycle after a period of two weeks post-drug removal. This suggests that stilbene 5c can be a promising drug that prevents proliferative recovery which would otherwise be the basis for disease recurrence.

## **V. Summary**

Our findings indicate that stilbene 5c induces cytotoxic effects in B16F10 murine melanoma cells. We have shown that this compound can induce apoptosis, autophagy and senescence, but it is still uncertain what the exact mechanism of cell death of the compound is. Our hypothesis is that autophagy is cytoprotective in this model and functions to rescue tumor cells from toxic effects of the drug. Moreover, senescence is induced at the same time as autophagy to provide the same protective mechanism the cells need to overcome the cytotoxic effects of the drug. However, as the stress becomes excessive with incubation for five consecutive days, tumor cells start undergoing apoptosis. In order to confirm the cytoprotective role of autophagy, bafilomycin A1 was used to inhibit the formation of autophagolysosomes, a critical step for autophagy to occur. Treatment with high dose stilbene (300nM) resulted in extensive cell death as early as day

1 and thus the addition of Bafilomycin did not result in significant cell death. Treatment with 30nM of stilbene 5c alone resulted in a delay in cell death which is also observed in B16F10 cells treated with 30nM of colchicine. However, the combination treatment with Bafilomycin A1 resulted in extensive cell killing as early as day 2 confirming the cytoprotective role of autophagy. Furthermore, the extensive cell killing when autophagy is inhibited has been shown to be apoptosis which occurs at a significantly higher level than in cells treated with stilbene 5c alone. Overall, this study indicates that autophagy plays a cytoprotective role in melanoma cells as treatment with stilbene 5c led to an extensive level of apoptosis when autophagy is inhibited.

It is also important to mention that bafilomycin A1 alone showed a growth inhibitory effect two days post-treatment. As acridine orange staining and LC3 western immunoblotting experiments have indicated, B16F10 melanoma cells have a high level of basal autophagy. The extended inhibition of this cytoprotective autophagy with bafilomycin showed growth inhibitory effects. This might suggest that these cells might be “autophagy addicted” and thus heavily rely on this mechanism to undergo cellular division and continue their life cycle. Therefore, the extensive cell death observed with stilbene 5c and bafilomycin might be additive effects of the two drugs which work by inhibiting microtubules and, at some extent by inhibiting the cytoprotective autophagy. Either way, the net effect of autophagy inhibition and treatment with stilbene 5c is an increase in apoptotic cell death which can potentially be pursued in designing an approach to successfully inhibit melanoma cell proliferation.

## VI. Future studies

The next aim of this study will be to confirm the role of autophagy by using siRNA to genetically knock down autophagy related proteins such atg5 and atg7. It would also be advisable to use one other pharmacological autophagy inhibitor to confirm current results. Agents such as chloroquine and 3-methyladenine (3MA) can inhibit specific stages of autophagy. Chloroquine is the commonly used autophagy inhibitor; it prevents the lysosomal degradation of autophagosomes. On the other hand, 3MA is part of the class III PI3K inhibitor that suppress the earlier step in autophagy, the formation of autophagosomes. Chloroquine is currently used in the treatment of malaria and therefore its side effects and adverse effects are known. For this reason, it is the commonly used agent to inhibit autophagy. However, in studies done in our lab, chloroquine induced cell death in nanomolar concentration in B16F10 which was supported in studies that chloroquine is toxic to pigmented melanoma cells because it binds to melanin and induces cell death. (Inoue et al. 1993) For this reason, studies of stilbene in combination with chloroquine have been discontinued. (Inoue, S. 1993) Moreover, in order to differentiate the cytotoxic effects of autophagy from that of apoptosis, the use of a broad caspase inhibitor such as ZVAD-FMK would enable us to understand to what extent apoptosis is induced in this model and the role it plays in promoting cell death.

Another major aim for this study will be to identify other modes of cell death that might be involved. There have been recent reviews on molecular pathways operating in a non-apoptotic or autophagic pathways. Mitotic catastrophe is used to describe cell death that occurs during or after a faulty mitosis, usually ending in polyploidy together with the formation of cells that contain multiple nuclei. (Ianzini, Mackey 1997) It has been described as the principal form of cell death induced by ionizing radiation (Ianzini, Mackey 1997) and has been identified as the main

response to several antitumor drugs. (Roninson, Broude & Chang 2001) During mitotic catastrophe, a defective mitosis results in a missegregation of chromosomes and aneuploidy, followed by cell division. Nuclear envelopes form around individual chromosomes or groups of chromosomes, resulting in the presence of large cells with multiple micronuclei. Some of the detection methods include microscopy for cells with two or more nuclei; cytometry for detection of G2/M phase arrest and polyploidy. (Portugal, Mansilla & Bataller 2010) It has been reported that ovarian cancer cells treated with 100nM of stilbene 5c arrested in G2/M phase which is consistent with the fact that stilbene 5c inhibits microtubule polymerization and disrupts mitotic spindles to arrest cells in early mitosis. (Durrant et al. 2008) Similar studies need to be performed on melanoma cells in order to obtain a better understanding on the role mitotic catastrophe and to evaluate the cell cycle regulation of stilbene 5c on melanoma cells.

Finally, it would be vital to assess the effects of stilbene 5c in *in vivo* mouse models. It has been shown that stilbene 5c is tolerated in mice up to 100mg/kg and no bone marrow toxicity, a major side effect of most cancer drugs, has been observed. Moreover, mice treated with 5 daily injections of stilbene 5c did not show any cardiac toxicity, suggesting that stilbene 5c may be a better agent than existing VDAs due to its low cardiac toxicity. (Cao et al. 2008) In the same study, *in vivo* studies with HL60 and U937 leukemia cells at concentrations of 30nM to 1000nM of stilbene 5c, suggest that stilbene 5c injected IV at 10, 50, and 100mg/kg resulted in 22, 62 and 58% apoptosis respectively. (Cao et al. 2008) While H&E staining of untreated tumors showed rapid tumor proliferation, tumors treated with 100mg/kg had extensive cell death in the central portion of the tumor with the peripheral area remaining viable. (Durrant et al. 2009) Given the positive results as an anti-proliferative and anti-neovasculature agent, stilbene 5c could be a promising drug for the chronic treatment of melanoma.

## VII. Final thoughts

The stilbene derivative, *cis*-3, 4', 5-trimethoxy-3'-aminostilbene (Stilbene 5c) was shown to induce apoptosis, autophagy and senescence in murine B16F10 melanoma cells. This compound shows immediate cell killing effects at 300nM and delayed cell death at 30nM. However, both concentrations resulted in over 90% cell death after a period of 5 days of drug exposure. Moreover, no proliferative recovery was detected two weeks post-drug removal. This compound has also shown significant cell killing effects in ovarian as well as leukemia cancer cells. (Durrant et al. 2008) With a better understanding of autophagy, senescence and apoptosis in this model, inhibitors or stimulators of these pathways could be used to achieve a net effect that will result in a significant and permanent decrease in tumor cells. Studies conducted on various mouse models have already confirmed the cell killing ability of this drug *in vivo* which bears merit for future experiments with *in vivo* melanoma mouse models.

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## VITA

Betelehem Mulu Asnake is 23 years old and was born in Addis Ababa, Ethiopia in 1988. She attended the French School, Lycee Guebre Mariam in Addis Ababa, since she was three years old which led to her being fluent in French and Amharic, her native tongue. At the end of grade 9, her family was fortunate to win the Diversity Lottery that gave them legal residency in the United States. At the age of fifteen, Beti and her family moved to Los Angeles in July 2003 and started a new life, in a new country. Although her family had to endure lots of struggle including financial problems as well as her father's severe health problems, Beti excelled in her studies and graduated with honors from Westchester High School. In 2005, she was accepted into the University of California Los Angeles where she majored in French. During this time, Beti gathered team teaching experiences in the form of tutoring services for UCLA as well as tutoring underprivileged high school students. She also spared an extensive part of her time doing volunteer work at the UCLA Vascular Center and giving out free blood pressure screenings in Los Angeles' underserved communities as part of the Black Hypertension Project. In June of 2008, Beti graduated from UCLA in three years with a B.A. degree in French. After spending a year with family, Beti decided to move to Richmond to be closer to her current husband and joined the VCU Pharmacology & Toxicology graduate program the same year. In addition to her thesis, Beti's work will lead to an upcoming publication. Betelehem graduated with her M.S. in Pharmacology and Toxicology in June 2011. She will be pursuing her medical doctor degree education at the University of Virginia.