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Sildenafil and celecoxib interact to kill breast cancer cells

Brittany Binion
Virginia Commonwealth University

Sildenafil and Celecoxib Interact to Kill Breast Cancer Cells

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By

Brittany Binion
Bachelor of Science in Biochemistry, Virginia Tech 2012
Bachelor of Science in Psychology, Virginia Tech 2012

Paul Dent, Ph.D.
Department of Biochemistry

Virginia Commonwealth University
Richmond, Virginia
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List of Abbreviations

Activating transcription factor 4 ..... ATF4

Activating transcription factor 6 ..... ATF6

Apoptosis inducing factor ..... AIF

Apoptotic protease-activating factor 1 ..... Apaf-1

Autophagy related gene ..... ATG

B-cell lymphoma 2 ..... Bcl-2

B-cell lymphoma –extra long ..... Bcl-xL

Bax interacting factor Bif-1

Bcl-2 associated X protein ..... Bax

Bcl-2 homologous antagonist/killer ..... Bak

Bcl-2 interacting mediatior of cell death ..... BIM

BH3 interacting domain death agonist ..... BID

Binding immunoglobin protein ..... BiP

Caspase-activated deoxyribonuclease ..... CAD

Cluster of differentiation 95 ..... CD95

Cyclic guanosine monophosphate ..... cGMP

Cyclooxygenase-2 ..... COX-2

Death domain ..... DD

Death effector domain ..... DED

Death inducing signaling complex ..... DISC

Dimethyl sulfoxide ..... DMSO

Endoplasmic reticulum ..... ER

Epidermal growth factor receptor ..... EGFR

ER-associated degradation ..... ERAD

Estrogen Receptor ..... ER

Eukaryotic translation initiation factor 2 α eIF2α

Fas-associated death domain..... FADD

FLICE-inhibitor protein ..... FLIP

Glucose regulated protein of 78 kDA GRP78

Glyceraldehyde-3-phosphate dehydrogenase ..... GAPDH

Guanosine triphosphate ..... GTP

Human epidermal growth factor receptor HER2

Inhibitor of CAD ..... ICAD

Inositol requiring enxyme 1 ..... IRE1

Mammalian target of rapamycin ..... mTOR

Multiplicity of infection ..... MOI

Myeloid cell leukemia-1 ..... Mcl-1

NADPH oxidase activator ..... Noxa

Nitric oxide synthase..... NOS

Non-steroidal anti-inflammatory drug ..... NSAID

p53 unregulated modulator of apoptosis ..... Puma

Phosphate buffered saline solution ..... PBS

Phosphatidylethanolamine ..... PE

Phosphatidylinositol ..... PI

Phosphatidylinositol3-posphate PI3P

Phosphodiesterase ..... PDE

Phosphoinositide 3-kinase complex PI3KC3

PKR-like eukaryotic initiation factor 2 α kinase ..... PERK

Progesterone receptor ..... PR

Prostaglandins ..... PG

Selective ER modulators ..... SERM

Selective ER downregulators ..... SERD

Triple negative breast cancer ..... TNBC

Truncated BID ..... tBID

Tumor necrosis factor ..... TNF

Ubituitin-like ..... UBL

Ultraviolet radiation resistance-associated gene ..... UVRAG

Unc51-like kinase 1 ..... ULK1

Unfolded protein response ..... UPR

X-box binding protein XBP1

Abstract

SILDENAFIL AND CELECOXIB INTERACT TO KILL BREAST CANCER CELLS

By Brittany Binion, MS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Major Director: Paul Dent, Ph.D. Department of Biochemistry

Breast cancer is the second most commonly diagnosed cancer among American women and is responsible for the second highest number of cancer-related deaths. Targeted therapeutic agents sildenafil, a phosphodiesterase type 5 inhibitor, and celecoxib, a cyclooxygenase-2 inhibitor, have been used individually in conjunction with other chemotherapeutic agents to enhance cell killing in a variety of cancers. Sildenafil when combined with traditional chemotherapeutic drugs, such as the taxanes and anthracyclines, or celecoxib combined with traditional hormone therapies have been used to increase cytotoxicity and cell killing. The data presented here demonstrates that the novel combination of sildenafil and celecoxib work together to enhance cell killing in both receptor positive and triple negative breast cancer through the induction of autophagy, ER stress, as well as both intrinsic and extrinsic apoptosis.

Introduction

Breast Cancer

Over 200,000 new cases of invasive breast cancer are diagnosed in the United States each year, and approximately 40,000 of the patients diagnosed will die from the disease.[1] From 2001 to 2010, the incidence of breast cancer in the United States remained level among women, and the mortality rate decreased by 2.0% per year[2]. It has been suggested that at least half of the reduction in breast cancer mortality that was observed over the last thirty years is attributable to the widespread use of adjuvant therapy.[3] It is common to classify the disease based upon nodal status, tumor grade and size, as well as other prognostic factors such as hormone receptor status.[4,5] These designations are useful, particularly the expression of receptors present in the cancer, as they are used to determine potentially effective treatments.

The breast cell lines utilized in experiments in this manuscript were ductal carcinomas, with differing receptor expression patterns, indicated in table 1. The BT 474 cell line is characterized mostly by the overexpression of human epidermal growth factor receptor 2 (HER2), as it retains functional estrogen receptors (ER) and progesterone receptors (PR).[6,7] However, the BT 549 cell line is referred to as triple negative breast cancer (TNBC), as it does not express any of the three major receptors used in breast cancer diagnosis.[8] The absence of ER and PR expression has been shown to be associated with early disease recurrence and poor survival, and 15% of breast cancers diagnosed worldwide are designated as TNBC .[1, 9, 10, 11, 12]

Table 1: Cell line receptor expression. A table listing the characteristic receptor expression status of the cell lines used in this study. For expression levels, (-) signifies absent, (+) indicates expression, (++) signifies overexpression.

Cell line	ER	PR	HER2
BT 474	+	+	++
BT 549	-	-	-

Conventional Treatment

The conventional treatment course for patients with breast cancer typically involve surgical removal and a neo-adjuvant (pre-surgery) or more commonly an adjuvant (post-surgery) therapy. These adjuvant therapies can include radiation, chemotherapeutics such as anthracyclines and taxanes, hormone therapy, targeted therapy, or a combination of treatments.

TNBC is highly aggressive, and is responsible for a disproportionate number of metastatic disease cases and breast cancer deaths.[13,14,15] It is typically treated with a combination of cytotoxic chemotherapy agents, including the anthracyclines and taxanes.[16] Commonly there is residual disease after treatment of early breast cancer, and these patients have a high risk of relapse, with a sharp decrease in survival in the first 3 to 5 years after treatment.[13, 17, 18, 19] Conventional treatments for relapsed patients are limited, particularly because standard chemotherapeutic regimens containing anthracyclines and taxanes have already been given in the adjuvant and neoadjuvant settings, opening up the potential for resistance to drugs already administered.[20]

Typically, conventional chemotherapy does not have the ability to discriminate between normal rapidly dividing cells and cancer cells and has low therapeutic efficacy. In contrast,

targeted therapies have specific molecular targets in tumor cells, which act with higher specificity and induce less overall toxicity.[21] Due to these aspects of targeted therapies, they represent a more promising approach based on the molecular understanding of tumorigenesis, which may potentially replace conventional cytotoxic chemotherapy in the future. The largest obstacle in targeted therapies comes from the potential for crosstalk between cellular survival pathways, commonly resulting in the activation of alternative pathways, ultimately leading to drug resistance. Therefore, it is becoming more evident that targeted therapeutics used in combination may provide a more rational strategy to increase the efficacy of drug treatments in cancer patients.

Hormone and Targeted Therapies

Both the ER and the PR play important roles in the physiology of the reproductive tract, and have effects on the normal growth of the breast as well as the progression of breast cancer.[22, 23] The ER is a hormone-regulated nuclear transcription factor that can induce the expression of a number of genes, including that of the PR.[24] Upon ligand activation, ER binds to estrogen response elements on target genes and regulates the transcription of these specific genes, including that of the PR.[25] Due to the complex nature of co-regulatory proteins and extra nuclear actions involved in ER signaling, there is a requirement for tight regulation of these factors, with dysregulation being implicated in the progression of cancer attributed to the importance of ER in growth and survival pathways.[23] For hormone receptor positive patients, therapeutic strategies are commonly directed at inhibiting the actions of ER using selective ER modulators (SERMs), targeting ER for degradation with selective ER downregulators (SERDs), or withdrawing estrogen via surgical methods (oophorectomy) or medically by aromatase inhibitors in post-menopausal women.[26]

Human epidermal growth factor receptor 2 (HER2) is a receptor tyrosine kinase gene that is amplified and causes overexpression of protein in 20-30% of metastatic breast cancer.[27] This amplification and overexpression is associated with reduced time to progression of the disease as well as a reduction in overall survival in breast cancer patients.[28] The HER2 gene does not need to be mutated for oncogenic function, simply the amplification of wild-type HER2 is sufficient to produce the oncogenic effects.

In HER2 overexpressing cells, excess expression can lead to spontaneous and constitutive ligand-independent dimerization, which activates the cytoplasmic kinase region of the receptor.[29, 30, 31] This kinase activation can stimulate autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors and initiate downstream signaling, primarily though the AKT and MAPK pathways, ultimately leading to increased proliferation, protein synthesis and cell survival.[27] The PI3K/AKT signaling pathway is a major effector of HER2 activity; PI3K blockade suppressed tumor growth in multiple models of HER2-overexpressing breast cancers.[32, 33] MAPK signaling appears to contribute to progression of HER2-positive breast cancer, with hyper activation of MAPK signaling and resistance to the ER modulator tamoxifen seen in HER2 and ER-positive cells.[31]

Post-surgery, adjuvant treatments for HER2-positive breast cancer patients typically involves long-term treatment with trastuzumab (Herceptin), a monoclonal antibody that attaches to HER2 receptors on breast cancer cells and blocks them from receiving growth signals. This blockade can slow or even stop cancer growth. In the event that the cancer metastasizes, a tyrosine kinase inhibitor called lapatinib is used to inhibit receptor signal processes by binding to the ATP-binding pocket of the HER2 protein kinase domain.[35] This prevents autophosphorylation and subsequent activation of downstream signaling mechanisms.

Taken together, most common therapies are geared towards directly blocking or eliminating receptor activation. However, there is evidence of crosstalk between ER, PR and growth factor receptor signaling pathways, especially the epidermal growth factor receptor (EGFR) family, as one of the mechanisms for resistance to endocrine therapy in breast cancer.[36, 37, 38] Bidirectional crosstalk between ER and growth factor pathways, particularly HER2, result in a positive feedback cycle of survival and cell proliferative stimuli.[38] This indicates that the current methods of blocking receptor signaling are no longer as effective, as resistances are becoming more commonplace. Drug resistance is also of particular concern for TNBC patients as therapies are quite limited for patients experiencing cases of metastasis. This is largely due to a lack of targeted therapies available as well as the prevalence for acquired resistance against chemotherapies previously administered.

Sildenafil

Phosphodiesterases (PDEs) are a family of enzymes which catalyze the hydrolysis of the cyclic nucleotides to their corresponding 5-monophosphate counterparts, leading to a decrease in levels of the cyclic nucleotides.[39] Sildenafil (Viagra) is an oral PDE inhibitor specific to isoform 5 (PDE5), with selectivity for cyclic guanosine monophosphate (cGMP).[40, 41] Nitric oxide (NO) stimulates the enzyme guanylate cyclase to convert guanosine triphosphate (GTP) to cGMP, with high levels of cGMP being responsible for the relaxation of smooth muscle.[42] Thus, sildenafil enhances the actions of the endogenous NO-cGMP pathway, by mediating the elevation of cGMP levels due to inhibiting its degradation by PDE5.[43, 44] Due to the localization of PDE5 in the corpus cavernosum, sildenafil is successfully used in the treatment of erectile dysfunction.

It has been demonstrated that hypoxia-induced inhibition of intracellular NO-cGMP signaling can lead to a more malignant phenotype in cancer cells, including chemoresistance and evasion of immune detection.[45, 46] PDE-specific inhibition decreased hypoxia-mediated chemo resistance, confirming the potential clinical utilization of enhancing NO-cGMP signaling to chemo sensitizes cancer cells.[45,46, 47] Thus, PDE5 inhibitors may be used as an anticancer therapy, due to their essential role in regulating cGMP, which as a second messenger causes a reduction in cell growth as well as the induction of apoptosis.[48, 49, 50]

It has also been demonstrated that when paired with standard of care chemotherapy treatments, such as doxorubicin, sildenafil mediates a cardio protective effect through the NO-cGMP pathway involving the enhanced expression of nitric oxide synthase (NOS).[51] This protective effect offsets one of the biggest concerns with doxorubicin treatment as well as enhancing doxorubicin induced cell death through apoptosis, particularly the extrinsic pathway through death receptors.[51, 52]

Sildenafil is generally administered as a single 50 milligram (mg) dose per day as needed, and can be adjusted up to 100 mg or down to 25 mg. It is well tolerated by patients, with the occurrence of mild side effects such as headache, flushing, dyspepsia, nasal congestion, urinary tract infection, abnormal vision, diarrhea, dizziness, and rash.

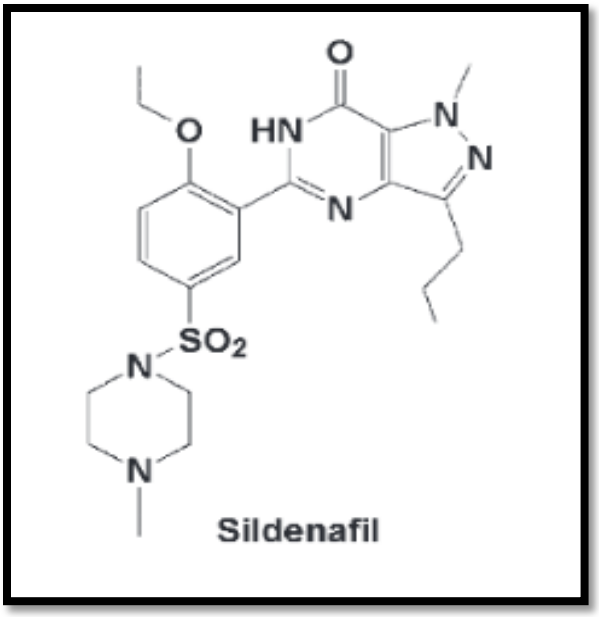


Figure 1: Chemical structure of sildenafil[72]

Celecoxib

Celecoxib (Celebrex) was the first specific inhibitor of cyclooxygenase-2 (Cox-2) approved by the US Food and Drug Administration (FDA) in December of 1998 to treat patients with rheumatoid arthritis and osteoarthritis.[53] The purpose of Celecoxib was to produce a non-steroidal anti-inflammatory drug (NSAID) that had little to no effects on the gastrointestinal system or the kidneys, which are commonly negatively affected by NSAIDS.

Patients taking celecoxib have an initial recommended dosage of 200mg daily for osteoarthritis and 400 mg daily for rheumatoid arthritis.[54] Common side effects are relatively mild, and include abdominal pain, diarrhea, dyspepsia, flatulence, dizziness, upper respiratory tract infection and rash, with a potential for increased cardiovascular risks.[55]

The COX enzymes catalyze the synthesis of prostaglandins (PGs) from arachidonic acid, which are important messengers involved in the process of inflammation.[56] There are two

isoforms of COX, with COX-1 being expressed in most tissues and producing PGs that are responsible for the control of normal physiological functions such as the maintenance of gastric mucosa.[56] Thus the gastric side effects caused by traditional NSAIDs that non-selectively inhibit both isoforms of COX are likely through the inhibition of COX-1, which mediates gastroprotective PGs. In contrast, COX-2 is not found in most normal tissues and is induced by inflammatory stimuli, which leads to enhanced synthesis of PGs in neoplastic and inflamed tissues.[56] The selectivity of COX inhibitors arises from a single substitution in the NSAID binding site, which produces a void volume to the side of the central active-site channel; compounds that bind in this additional space inhibit COX-2 selectively.[57]

It was noted that long term use of NSAIDs appeared to reduce the risk of developing cancer.[56, 58] COX2 is commonly observed to be upregulated in cancer, including breast, and administration of celecoxib caused a greater suppression of the incidence of malignant breast tumors when compared to the administration of traditional NSAIDs.[59, 60] It was indicated that the increase in tumorigenic potential by COX2 overexpression was associated with a resistance to apoptosis through the overproduction of PGs, and celecoxib was therefore inducing apoptosis.[61]

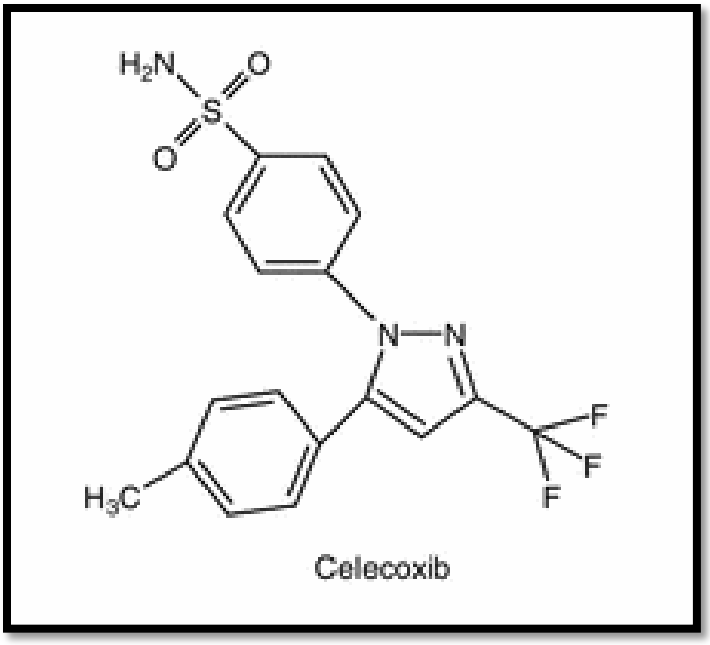


Figure 2: Chemical structure of celecoxib.[73]

Apoptosis: Intrinsic and Extrinsic Pathways

Apoptosis is a form of programmed cell death that is intimately involved in the development and homeostasis of normal tissues.[62] Most notably, apoptosis is characterized by morphological changes that take place, such as cell shrinkage, membrane blebbing, nucleus fragmentation, chromatin condensation and DNA degradation.[63, 64, 65] Within the designation of apoptosis there are two distinct pathways: the extrinsic, or death receptor pathway, and the intrinsic, or mitochondrial pathway (figure 3). It is suggested that these two pathways are connected, and have the ability influence each other.[63] Both the intrinsic and extrinsic pathways ultimately converge on the activation of specific intracellular proteases, known as the caspase family, that are responsible for cleaving proteins adjacent to aspartate residues. These proteases are commonly categorized as initiators or executioners and are synthesized as inactive zymogens that become activated through cleavage by their upstream modulators.[63, 66] Caspase 3 is the most

important executioner, as it is the link between the two pathways; it is activated by any of the initiator caspases (8, 9 and 10). Executioner caspases (3, 6 and 7) cleave and affect a multitude of substrates, including cytoskeletal and nuclear proteins, as well as activating other proteases and endonucleases involved in protein degradation and DNA fragmentation.[63,66]

The intrinsic pathway is strictly regulated by the B-cell lymphoma-2 (Bcl-2) family of proteins.[63, 66] This family contains three different classes of proteins: the anti-apoptotic group I, the pro-apoptotic group II, and group III proteins that are responsible for binding and regulating the activity of anti-apoptotic group II proteins. The group I family members such as Bcl-2, B-cell lymphoma-extra large (Bcl-xL) and myeloid cell leukemia-1 (Mcl-1) bind directly to and inhibit pro-apoptiotic group II family members, that include Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak). The group III family members that include p53 unregulated modulator of apoptosis (Puma), NADPH oxidase activator (Noxa), BH3 interacting domain death agonist (BID) and Bcl-2 interacting mediator of cell death (BIM) all interact with the group II pro-apoptotic members as well, however they induce the insertion of the group II proteins into the mitochondrial membrane.[63, 66, 67] Recently studies have demonstrated that the tumor suppressor p53 may be implicated in the synthesis of Puma and Noxa, providing a link between DNA damage and apoptotic cell death.[68, 69]

Various stimuli, including viral infection, DNA damage and the absence of particular growth factors such as hormones and cytokines lead to the activation of the intrinsic pathway. Upon exposure to these stimuli, Bax and Bak are inserted in to the mitochondrial outer membrane, leading to membrane permeabilization via the formation of pores. The formation of these pores leads to the release of cytochrome-c and other various pro-apoptotic proteins that include caspase-activated deoxyribonuclease (CAD), apoptosis-inducing factor (AIF) and

endonuclease G, from the inter-membrane space in the mitochondria to the cytosol.[63, 66] Once in the cytosol, cytochrome-c binds to apoptotic protease-activating factor-1 (Apaf-1), which then binds pro-caspase 9 to form a complex called the apoptosome that includes multiple Apaf-1 and procaspase 9 molecules.[70] The binding event induces a conformational change and leads to the activation of caspase 9, that goes on to proteolytically activate the executioner caspase 3.[63, 66] Once activated, caspase 3 goes on to activate caspase 6 as well as CAD through cleavage of its inhibitor (ICAD).[70] CAD, with AIF and endonuclease G, translocates to the nucleus where they all contribute to DNA fragmentation.[63, 65, 66]

Binding of ligands from the tumor necrosis factor (TNF) family to death receptors leads to the activation of the extrinsic pathway. These TNF family receptors consist of a cysteine-rich extracellular domain for ligand binding and a cytoplasmic domain of 80 amino acids, termed the death domain (DD), which is involved in transducing the signal into the cell.[63, 65, 67]

The best characterized member of this receptor family is the Fas receptor, also called cluster of differentiation 96 (CD95). CD95 is a 45 kDa trans-membrane protein that upon binding with its ligand, FasL, induces a conformational change allowing for the recruitment of an adaptor protein called Fas-associated death domain (FADD). FADD itself contains another key motif, the death-effector domain (DED) that binds complementary DED domains on initiator caspases 8 and 10.[63, 65, 67] This entire intracellular complex is titled the death-inducing signaling complex (DISC) and its formation leads to the auto-proteolytic cleavage and activation of caspases 8 and 10.The activation of these initiatior caspases then causes the activation of the executioner caspases 3 and 7, that are responsible for the induction of the apoptotic response.[63,65, 67] This pathway can be inhibited by FLICE-inhibitory proteins (FLIP), which bind to the DISC and inhibit the activation of caspase 8.[65,67] The extrinsic pathway may also cause the release of

cytochrome-c and the induction of the intrinsic pathway through activation of BID, which serves as a substrate for caspase 8. Upon the activation of the DISC, truncated BID (tBID) translocates to the mitochondria and induces the release of apoptotic proteins from the inter membrane space into the cytosol.[63, 65, 71]

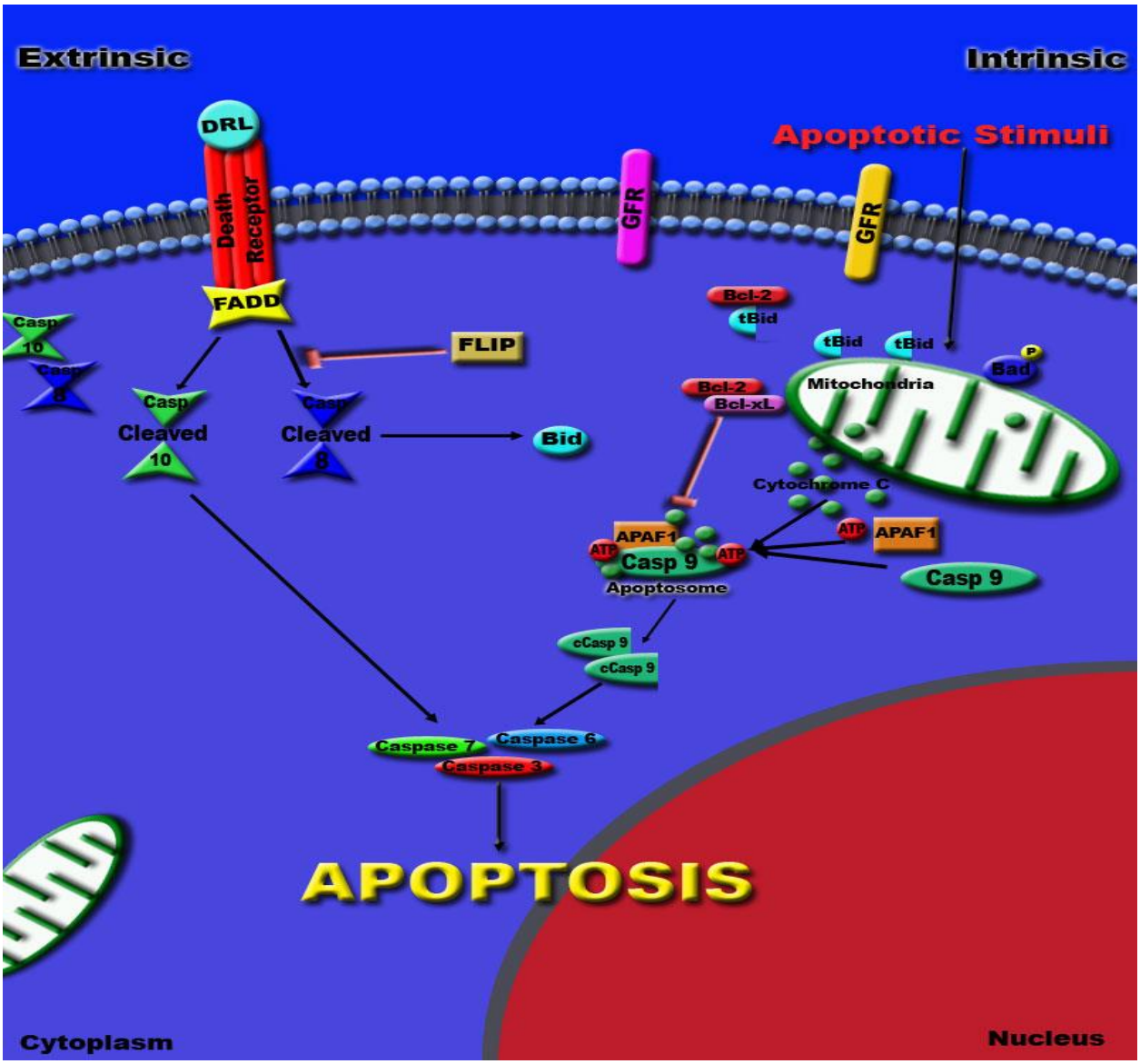


Figure 3: The intrinsic and extrinsic apoptotic pathways.[74] The intrinsic pathway is controlled by the Bcl-2 family of proteins that induce the release of cytochrome-c from the inter-membrane space of mitochondria in to the cytosol. Cytochrome-c causes Apaf-1 to bind and activate initiator caspase 9. Cleaved caspase 9 then activates executioner caspase 3. The extrinsic pathway is initiated by binding of TNFs to TNFRs that recruit adaptor proteins that can bind and activate initiator caspases 8 and 10. These initiator caspases in turn activate caspases 3 and 7. Caspase 8 also activates BID, which can translocate to the mitochondria and induce the release of cytochrome-c.

Autophagy

Autophagy is another mechanism that is tasked with maintaining cellular homeostasis, and this happens through the regular recycling and turnover of cytoplasmic components. This process is a multifunctional pathway associated with not only the removal of damaged organelles, but as a method of programmed cell death and various physiological and pathological processes that include: normal development, aging, cancer, and neurodegenerative diseases.[75, 76, 77, 78, 79] Where apoptosis is an irreversible form of cell death, autophagy can lead to either cell death or, paradoxically, it can allow cells to escape death and therefore can be considered a protective mechanism at times.[80]

An organelle called the lysosome contains hydrolytic enzymes that give it the ability to degrade cellular components and whole organelles.[75] The process of getting these cellular components to the lysosome is collectively referred to as autophagy. There are three distinct categories of autophagy: microautophagy, chaperone-mediated autophagy and macroautophagy. Microautophagy involves the direct engulfment of cytoplasmic components by the lysosome through an invagination of the lysosomal membrane.[75, 76, 81] Cytoplasmic proteins that are to be degraded may contain specific motifs that are recognized by lysosomal receptors, leading to chaperone-mediated autophagy.[75, 75, 81] Macroautophagy, henceforth referred to simply as autophagy (figure 4), is the main method of turnover for cytoplasmic components, such as long-lived macromolecules and organelles. The process of autophagy begins with sequestration of cytoplasm into the isolation membrane, that goes on to become a double-membrane vesicle called the autophagosome. The autophagosome fuses with the lysosome and at this point the inner membrane of the autophagosome and its contents are degraded by the hydrolases present within the lysosome.[82,83]

To elucidate the molecular mechanisms that underlie the process of autophagy, mutagenesis-based genetic screens have been performed in yeast. These studies have revealed a host of genes not only in yeast, but in mammals that are responsible for the regulation of the autophagic response, termed autophagy-related genes (ATG).[75, 76] It has been observed that autophagy is a highly conserved evolutionary process and there are many homologues with similar functionality in the mammalian system.[75, 76]

Three different signaling complexes and pathways are integral to the development of an autophagic response: including the mammalian target of rapamycin (mTOR) signaling pathway, the ATG1 complex and the class III phosphoinositide 3-kinase (PI3KC3) complex. mTOR is a serine/threonine kinase that is responsible for sensing changes in nutrient conditions to control multiple cellular processes. In respect to autophagy, mTOR is responsible for causing changes in the phosphorylation of ATG13. Under nutrient-rich conditions, mTOR activation causes hyperphosphorylation of ATG13, preventing its association with the mammalian orthologue of ATG1 known as ULK1 (Unc51-like kinase 1).[75, 77] However, under starvation conditions mTOR's inhibitory affects are inhibited, causing hypophosphorylation of ATG13, leading it to interact with ULK1. Activated ULK1 is recruited by ATG14L to directly phosphorylate Beclin-1 (ATG 6) and induce activation of the PI3KC3 complex. The PI3Ks represent a family of enzymes that are implicated in an array of diverse cellular processes, such as intracellular trafficking, proliferation and assembly of cytoskeletal elements.[84] Activated PI3KC phosphorylates phosphoatidylinositol (PI) to produce phosphatidylinositol 3-phosphate (PI3P) that serves as an anchor for PI3P-binding proteins, such as ATG18 to bind and form phagophores.[85] Studies show that there are two different PI3KC complexes: complex one is involved in the formation of phagophores and includes PI3KC, p150, Beclin-1 and ATG14L

while complex two contributes to the maturation of the autophagosome and contains UVRAG (ultraviolet radiation resistance-associated gene) in place of ATG14L.[86, 75, 76]

Phagophores, also known as isolation membranes, are crescent-shaped membranes that are extended to form double-membrane autophagosomes through a process that requires two ubiquitin-like (UBL) conjugation systems. These systems function similarly to the ubiquitylation process involved in protein degradation, and requires three enzymes: ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase enzyme (E3) (Marino, Mizushima). In the first UBL system ATG12 is activated by an E1-like enzyme ATG7, and forms a thioester intermediate before being transferred to ATG10, an E2-like enzyme. The last step involves covalently linking ATG5 and ATG12, and this conjugate non-covalently interacts with ATG16L to form the final complex. This complex dissociates from the membrane when autophagosome formation is completed.[75, 87]

The second UBL system involves the modification and incorporation of microtubule-associated protein 1 Light Chain 3 (LC3) into the autophagosome membrane. The C-terminal region of LC3 is cleaved by ATG4 to form LC3-I and the E1-like enzyme ATG7 activates it. Upon activation, LC3-I is transferred to ATG3, an E2-like enzyme, and finally covalently bound to phosphatidylethanolamine (PE) to form the lipid-protein conjugate LC3-II. Upon formation, autophagosomes are fused with lyosomes to complete the protein degradation process.[75, 87]

As well as having the major role of recycling macromolecules and organelles during times of nutrient deprivation, autophagy can also take part in the degradation of misfolded proteins. This process is mediated by the adaptor molecule p62, which has domains that bind to both the ubiquitin moiety on poly-ubiquitinated misfolded proteins, as well as the LC3 on the autophagosome membrane. Lysosomal degradation of autophagosomes results in a decrease in

p62 levels, which makes p62 another suitable marker for tracking autophagy in mammalian cells.[88]

Autophagy can induce two opposing responses in cancer cells: protection leading to cell survival and cytotoxicity resulting in cell death. Although toxic effects of autophagy had been proposed to be accompanied by apoptosis, it has been demonstrated that knockdown of the anti-apoptotic protein Bcl-2 induced caspase-independent autophagic cell death, by increasing the expression of Beclin1.[89] This study ultimately suggested that autophagy can directly induce cell death without activating apoptotic pathways.

It has been documented that there is a link between autophagy and the unfolded protein response (UPR). Both processes are closely related as some of the signaling routes activated during the ER stress response are involved in stimulating autophagy.[90] There is indication that the phosphorylation of eukaryotic initiation factor α (eIF2α) in the UPR is linked to the induction of autophagy.

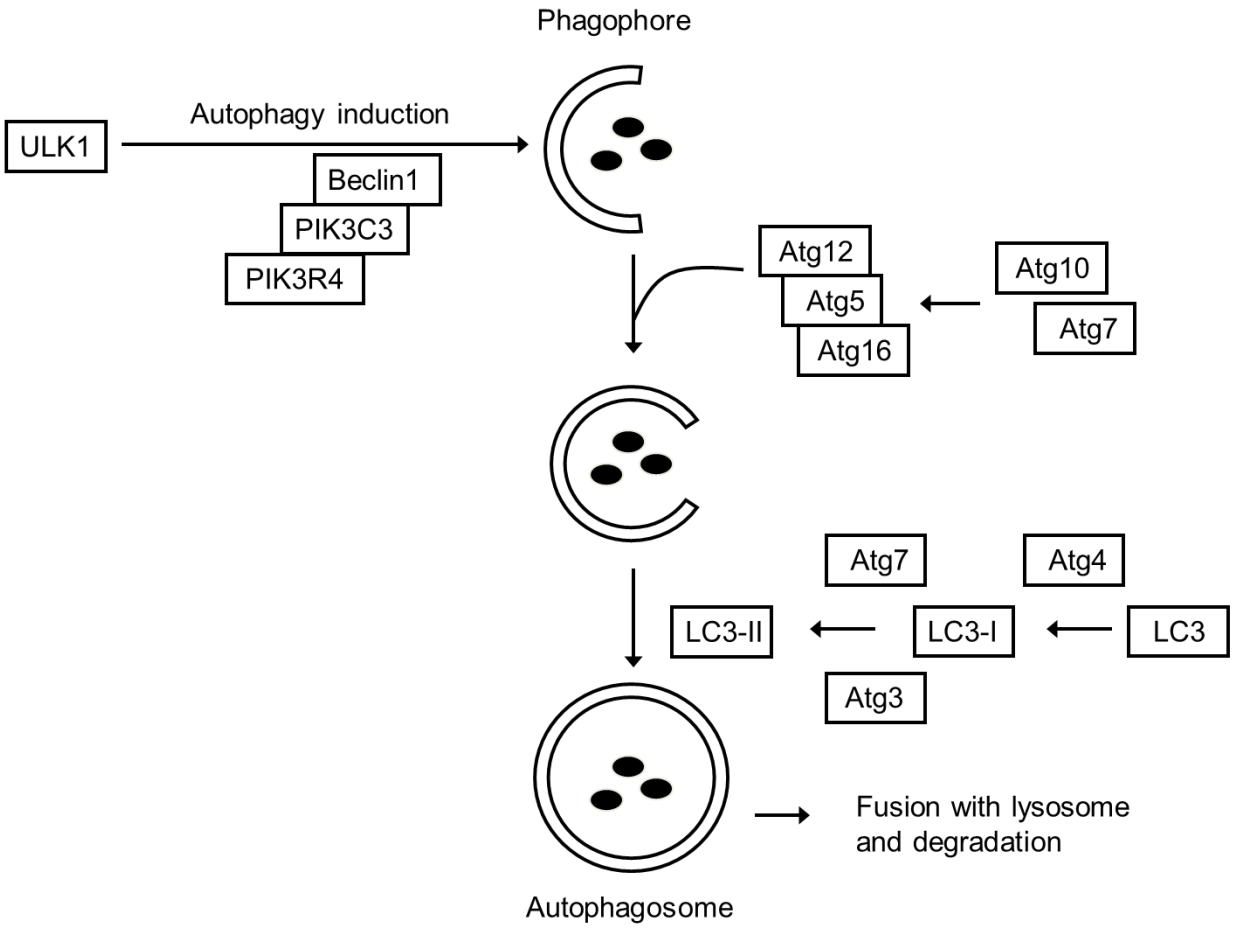


Figure 4: An overview of the autophagic pathway.[91] Upon release of inhibitory effects by mTOR, ULK1 is activated and causes the phosphorylation of Beclin1, which activates the PI3KC3 in complex I and induces autophagy. Autophagosome formation requires the two UBL conjugation systems. UBL system 1 produces ATG5-ATG12-ATG16 conjugates that attach the isolation membranes and facilitate membrane nucleation. UBL system 2 modifies LC3 and incorporates the final product, LC3-II into the autophagosome membrane. The final step in this process is the fusion of the lysososmes with the autophagosomes, which leads to complete degradation of autophagosome contents.

Endoplasmic Reticulum Stress

Proteins that are targeted for the secretory pathway are folded in the lumen of the endoplasmic reticulum (ER) by chaperones before being transported to the golgi apparatus for final modification and secretion. If there is an interruption in this process, there is an accumulation of unfolded proteins in the lumen of the ER that causes stress on the system and is the trigger for the unfolded protein response (UPR) illustrated in figure 5. The UPR is a series of actions that collectively reduce the rate of protein synthesis and activates transcription factors that enhance the function of the ER.[92, 93] There are three transmembrane proteins in the membrane of the ER that sense the accumulation of misfolded proteins and trigger the UPR: PKR-like eukaryotic initiation factor 2α kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). This sensory mechanism is mediated by the chaperone protein glucose regulated protein of 78 kDa (GRP78), also known as binding immunoglobulin protein (BiP), present in the lumen of the ER. Under normal conditions, GRP78 is bound to the luminal domains of PERK, IRE1 and ATF6, effectively inhibiting their function. When ER stress occurs, GRP78 is released to bind to the unfolded protein, leading to the activation of the three stress sensors. Upon activation, ATF6 is proteolytically cleaved and directly translocated into the nucleus to induce the expression of genes required for the UPR. However, activation of PERK and IRE1 is associated with the dimerization and subsequent autophosphorylation of specific residues on their cytoplasmic kinase domains.[92, 93] Activated IRE1 induces the formation of the transcription activator spliced X-box binding protein (XBP1) through splicing of the XBP1 messenger RNA whereas PERK phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2α). Normally, GTP-bound eIF2α binds to methionyl-transfer RNA and enhances recognition of the start codon and is released from ribosomal machinery when GTP is

hydrolyzed. Phosphorylation of the α subunit of eIF2 inhibits the exchange of GDP and GTP; thus, reducing protein synthesis. Furthermore, activated PERK translationally controls the expression of activating transcription factor 4 (ATF4) that induces the expression of variable UPR-related genes involved in amino acid metabolism, regulation of oxidative stress and apoptosis.[93]

To prevent aggregation of misfolded proteins in the lumen of the ER during ER stress, XBP1 and ATF6 increase expression of proteins that facilitate ER-associated degredation (ERAD). ERAD is accomplished by retrotranslocation of misfolded proteins into the cytosol followed by ubiquitination and proteasomal degredation. ER stress can also induce autophagy as an alternate route for protein degredation.[92] As previously stated, this process is regulated by p62, which has the proper domains to bind the ubiquitin moiety of the misfolded proteins as well as the LC3 on the autophagosomes.[88]

Severe ER stress can also induce apoptosis by increase the expression of the group III Bcl-2 family of proteins including Puma, Noxa, BIM and BID which induce the insertion of proapoptotic proteins Bax and Bak in the mitochondrial membrane, and consequently results in the release of cytochrome c.[92] It has also been suggested that ER stress-induced apoptosis occurs through cleavage of caspase 4, a member of caspase 1 subfamily that localizes to the ER membrane.[94]

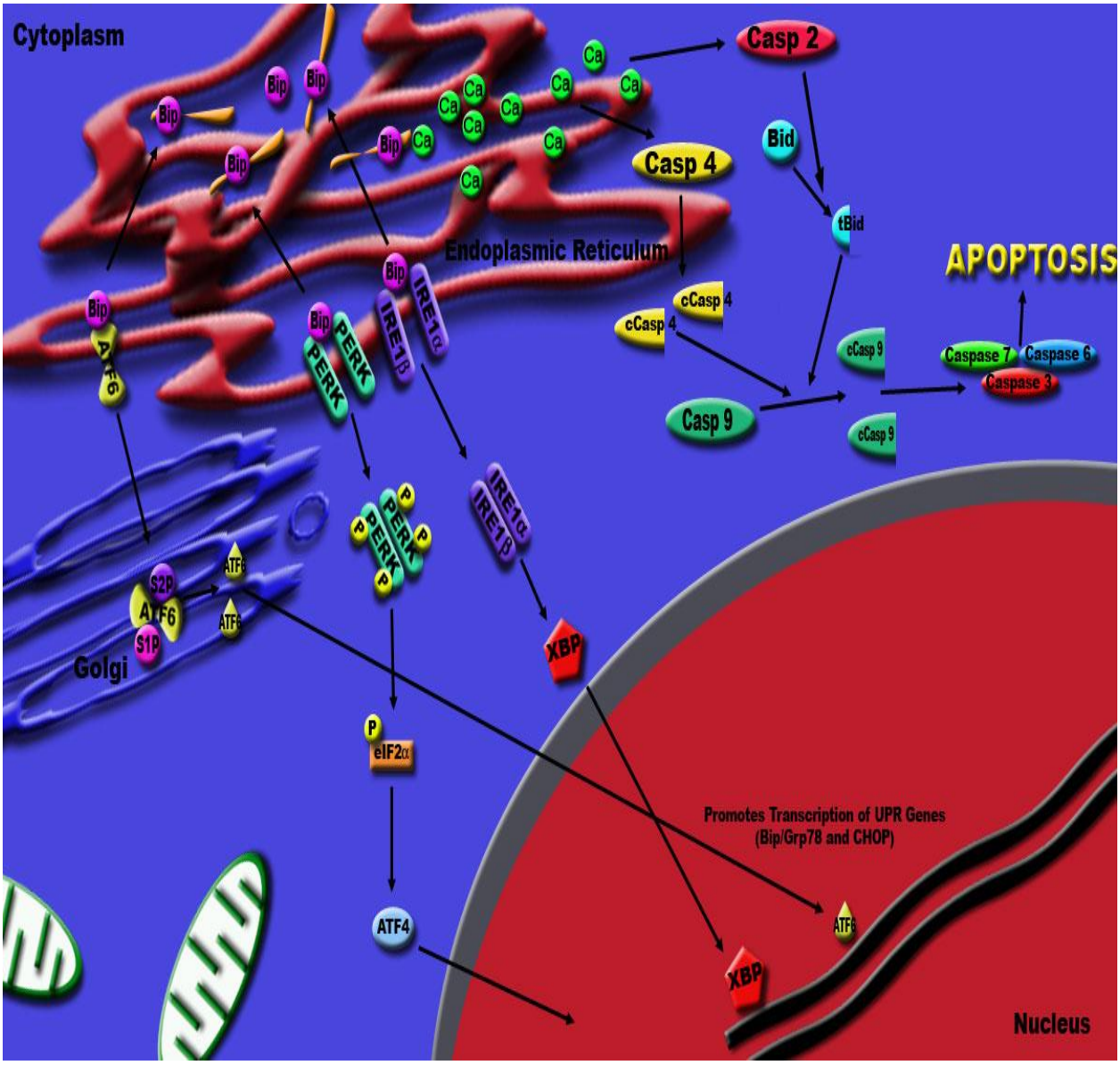


Figure 5: The mechanism of the unfolded protein response.[74] Upon accumulation of unfolded proteins in the lumen of the ER, the chaperone GRP78 (BiP) is released from the luminal domains of PERK, ATF6 and IRE1. These activated transmembrane proteins then trigger a cascade of events that collectively result in expression of UPR-related genes. Severe ER stress can also induce apoptosis through cleavage of ER membrane-bound caspase 4 and inducing caspase 9.

Materials and Methods

Materials

For cell culture, RPMI 1640, penicillin/streptomycin, trypsin-EDTA and phosphate-buffered saline solution (PBS) were all purchased from GIBCO (Invitrogen Life Technologies, Waltham, MA), and the fetal bovine serum was from HyClone Laboratories, Inc. (Thermo Scientific HyClone, South Logan, UT). Trypan blue solution and dimethyl sulfoxide (DMSO) were both obtained from Sigma Chemical (St. Louis, MO). The drugs Sildenafil and Celecoxib, as well as the inhibitor

Recombinant adenoviruses to express constitutively activated c-FLIP-s and Bcl-xL, as well as dominant negative (DN) caspase 9 and DN AKT were purchased from Vector Biolabs (Philadelphia, PA). The DNp38 adenovirus was purchased from Cell Biolabs, Inc. (San Diego, CA). Validated siRNA were all purchased from QIAGEN (Valencia, CA). The opti-MEM reduced serum medium and the lipofectamine 2000 transfection reagent used for transfections was purchased from GIBCO (Invitrogen Life Technologies, Waltham, MA).

Antibodies were all purchased from either Cell Signaling Technologies (Worchester, MA) or Santa Cruz Biotechnology (Santa Cruz, CA) and were diluted 1:1000 in Odyssey infared imaging system blocking buffer obtained from LI-COR Biosciences (Lincoln, NE). anti-FasL, anti-CD95, anti- eIF2α, anti-Bcl-xL and anti-GAPDH were all purchased from Santa Cruz Biotechnology. The anti-ATG5, anti-Beclin 1, anti-ATF4, anti-ATF6, anti-CHOP, anti-caspase 9, anti-FLIP, and anti-p38 (MAPK) were all purchased from Cell Signaling Technologies. Secondary antibodies used were IRDye 680LT Goat anti-rabbit IgG and IRDye 800CW Goat anti-mouse IgG, both purchased from LI-COR Biosciences (Lincoln, NE).

Methods

Cell Culture

BT-474 and BT-549 ductal carcinoma cells were acquired from ATCC (Manassas, VA) and cultured in RPMI 1640. The medium was supplemented with 10% (v/v) FBS and 100 µg/mL (1% v/v) penicillin/streptomycin. Cells were maintained in an incubator with 5% CO_2 at 37ºC.

Drug Treatments

Plated cells are given a 24 hour period to establish before being treated with indicated concentrations of Sildenafil and/or Celecoxib. Both drugs were taken from stock solutions and diluted in DMSO to reach the desired concentrations. In all treatments, the maximal concentration of solvent did not exceed 0.02% (v/v).

Western Blot Analysis

Cells were plated in 60 x 15 mm dishes and treated with the desired concentration of each drug for 24 hour. After incubation, cells were lysed and scraped using whole-cell lysis buffer (0.5 M Tris-HCL, ph 6.8 2% (v/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, 0.02% (v/v) bromophenol blue). Collected samples were boiled for 10 minutes followed by loading onto 8-12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Proteins were electrophretically separated and transferred onto 0.45 µm PVDF membrane using the Bio-Rad Trans-Blot Turbo system. Membranes were blocked in Odyssey infared imaging system blocking buffer obtained from LI-COR Biosciences (Lincoln, NE) for an hour. Primary antibody exposure took place overnight at 4ºC. After washing with TBST, membranes were incubated in the correct corresponding secondary antibody for a minimum of 1 hour at room temperature. Membranes were then washed again with TBST before being visualized using the Odyssey Infared Imager (LI-COR Biosciences, Lincoln, NE).

Infection with Adenovirus

Cells were plated in 12-well plates and left to establish for 24 hours. Media was removed and replaced with 1 mL of 5% (v/v) RPMI. Recombinant adenoviruses for caBcl-xL, dnCasp9, C-FLIP, dnp38, dnAKT and empty vector were added at a multiplicity of infection (MOI) of 50. Cells were incubated for 24 hours before changing the medium to 10% (v/v) RMPI, followed by the addition of the indicated concentrations of each drug for 24 hours before being subjected to trypan blue exclusion assay.

Plasmid and siRNA Transfections

Cells were plated in 12-well plates and left to establish for 24 hours. Media was removed and replaced with 800 µL of 5% (v/v) RPMI. For transfections, 1 µg of plasmid or 1 µL of siRNA was added to 100 µL of OPTIMEM per transfection and allowed to incubate for 5 minutes at room temperature. Concurrently, 1 µL of lipofectamine in 100 µL of OPTIMEM per transfection was also incubated for 5 minutes at room temperature. Both solutions were then combined and allowed to incubate for 20 minutes at room temperature. After incubation, 200 µL of the solution was added to the cells, bringing the total volume per well to 1 mL. The plates were incubated for 24 hours before changing the medium to 10% (v/v) RPMI, followed by the addition of indicated concentrations of each drug for 24 hours before being subjected to trypan blue exclusion assay.

Plasmids and siRNA included the scrambled control (SCR), FASL, FADD, ATG5, Beclin1, ATF4, ATF6, CHOP, eIF2α, dnPERK, and caAKT.

Trypan Blue Exclusion Assay

The media and any unattached cells from each well of a 12-well plate were transferred to a 15 mL conical tube. Attached cells were collected via trypsinization with trypsin/EDTA for 2 minutes at 37ºC. After centrifugation at 1,000 rpm for 5 minutes, most of the supernatant was removed, leaving approximately 50 µL to which 50 µL of trypan blue was added. The pellet was resuspended in the mixture and counted using a hemocytometer and a light microscope. Cell death was determined as a percentage of dead cells from the total number of cells counted.

Fluorescence Cell Viability Assay

Cells were plated at a density of $1x10^4$ cells per well on a 96-well plate. After 24 hours they were drug treated and incubated for an additional 24 hours. A working solution of 2 µM calcein AM and 4 µM Ethidium homodimer-1 were added to the wells and the plate was centrifuged at 1,000 rpm for 5 minutes. The plate was then visualized using the Hermes WiScan (IDEA Bio-Medical LTD Atlanta, GA) to quantify cell death and for imaging.

Colony Formation Assay

Varying numbers of cells were plated on 12-well plates, between $5x10^2$ and $4x10^3$, and the next day were drug treated with the specified concentration of drugs. The media was then changed after an additional 24 hours, in which the cells were left to grow. Once the control plate produces visible, distinct colonies, the media is carefully removed from the wells and is gently washed with 3 mL of PBS. The PBS is carefully aspirated, and 3 mL of methanol is added to each well for 10 minutes to fix the cells. The methanol is removed, and the plates are again washed with 3 mL of PBS. Crystal violet stain is then added for a minimum of 30 minutes. The stain is removed and the plates are washed with water and left overnight to dry. Upon drying, the colonies were counted.

Data Analysis

The effects of the various treatments were analyzed using one-way analysis of variance and a two-tailed Student's *t*-test. Results with a *P* value of <0.05 were considered statistically significant.

Results

The drugs sildenafil (referred to as sil) and celecoxib (referred to as cel) have been previously used in the context of anticancer therapies. They have been utilized separately, each to enhance the killing capacity of standard of care treatments already in wide use. Initial experiments of this study were performed to demonstrate the toxicity of the combination of 2 μM sildenafil with 10μM celecoxib in both BT 474 and BT 549 cells lines after twenty-four hours of drug exposure. In each experiment the control group (referred to as vehicle or veh) is treated with DMSO and serves as a measure of solvent effects for comparison, as the drugs were dissolved in DMSO for delivery.

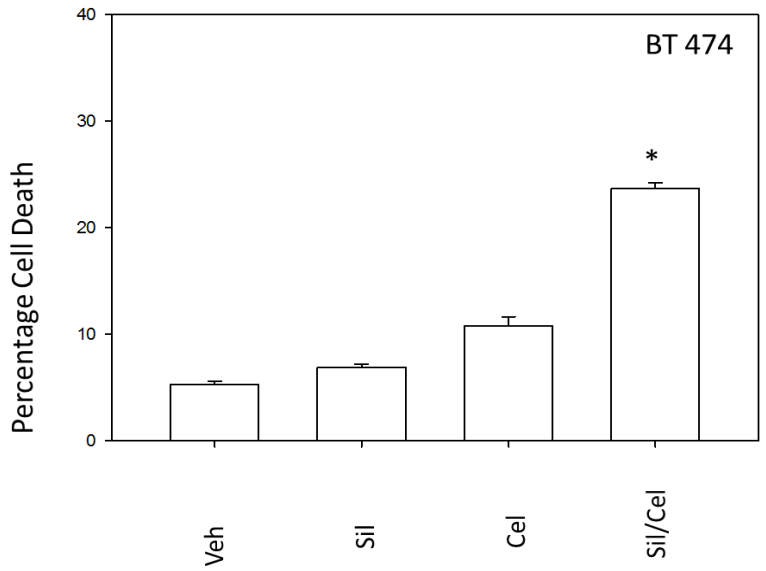
The cytotoxic effects of the drugs singularly and in combination were determined by using the trypan blue exclusion assay, which is considered a measure of cell viability. As seen in figure 6, sildenafil alone caused a minimal increase in cell death when compared to the vehicle, yet celecoxib caused an increase in cell death as a singular agent from the vehicle at an average of 8% to an average of 12% in both cell lines. There was a significant increase in cell death ($P<0.05$ compared to vehicle) in the drug combinations of both cell lines, with an even larger response witnessed in the BT 474 cells at about 22% cell death in comparison to the response seen in the BT 474 cells at 18%.

It was observed that there was an increase in blebbing and cell debris in treated cells when compared to control cells, present in both cell lines during their assessments of cell viability using trypan blue. To better visualize the physical characteristics of cell death, morphological changes induced by the drugs were characterized by utilizing the fluorescence cell viability assay. In the BT 474 cells shown in figure 7 and the BT 549 cells in figure 8, there was an increase in cell death across the treatments, as indicated by the increase in red stained cells.

Also of note was the decrease in total cells visualized upon treatment, suggesting a potential cytostatic effect. The combination of 2µM sildenafil and 10 µM celecoxib in the BT 474 cells in 7D illustrated cell elongation and large amounts of cell fragmentation. The BT 549 cells in figure 8D did not illustrate as much cell fragmentation, in agreement with the decreased percentage of cell death upon comparison with the combination treated BT 474 cells.

For an assessment of a longer term response to the sildenafil and celecoxib combination, a colony assay was performed to determine long term cell survival following twenty-four hours of drug treatment. Colony formation illustrates the ability of the cells to survive for a period of time following exposure to the drugs, with cells that survive the treatment continuing to grow and produce colonies. As seen in figures 9 and 10, both BT 474 and BT 549 cells responded to treatment in a similar fashion, with a decrease in cell survival upon increasing concentrations of both sildenafil and celecoxib, as single agents and in combination. The BT 474 cells illustrated greater toxicity to both sildenafil and celecoxib overall in comparison to the BT 549 cells; cell death increased from 24% or 12% with 2 µM sildenafil to 40% or 32% with 10 µM celecoxib, and the combination of the two caused an even further increase in death to 47% and 36%, respectively. Taken together with the previous cell death assays performed, this combination of 2 µM sildenafil and 10 µM celecoxib induced a significantly (* $P<0.05$ compared to vehicle) increased amount of cell death, working more effectively together than as independent agents.

A



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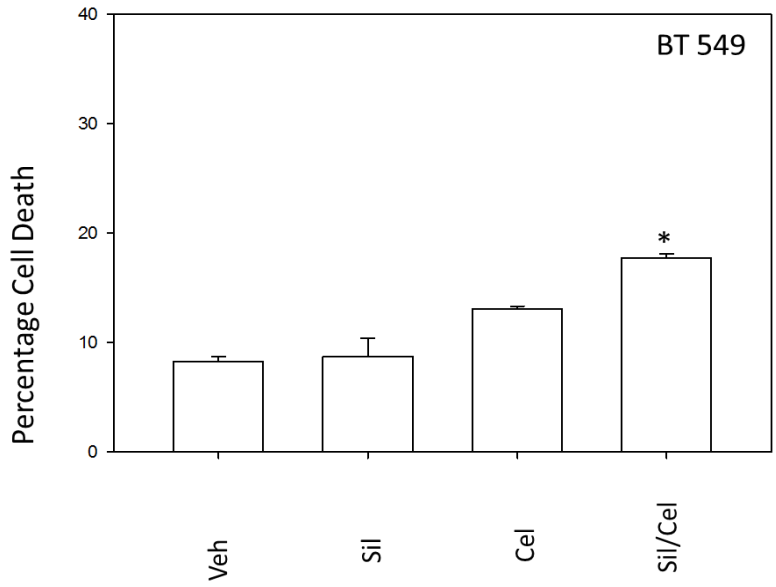

Figure 6: Assessment of cell viability in BT 474 and BT 549 cells treated with sildenafil and celecoxib. Cells were treated with DMSO, sildenafil (2μM), celecoxib (10μM) or combination for 24 hours and then subjected to trypan blue exclusion assay to determine cell viability.
* $P<0.05$ compared to vehicle.

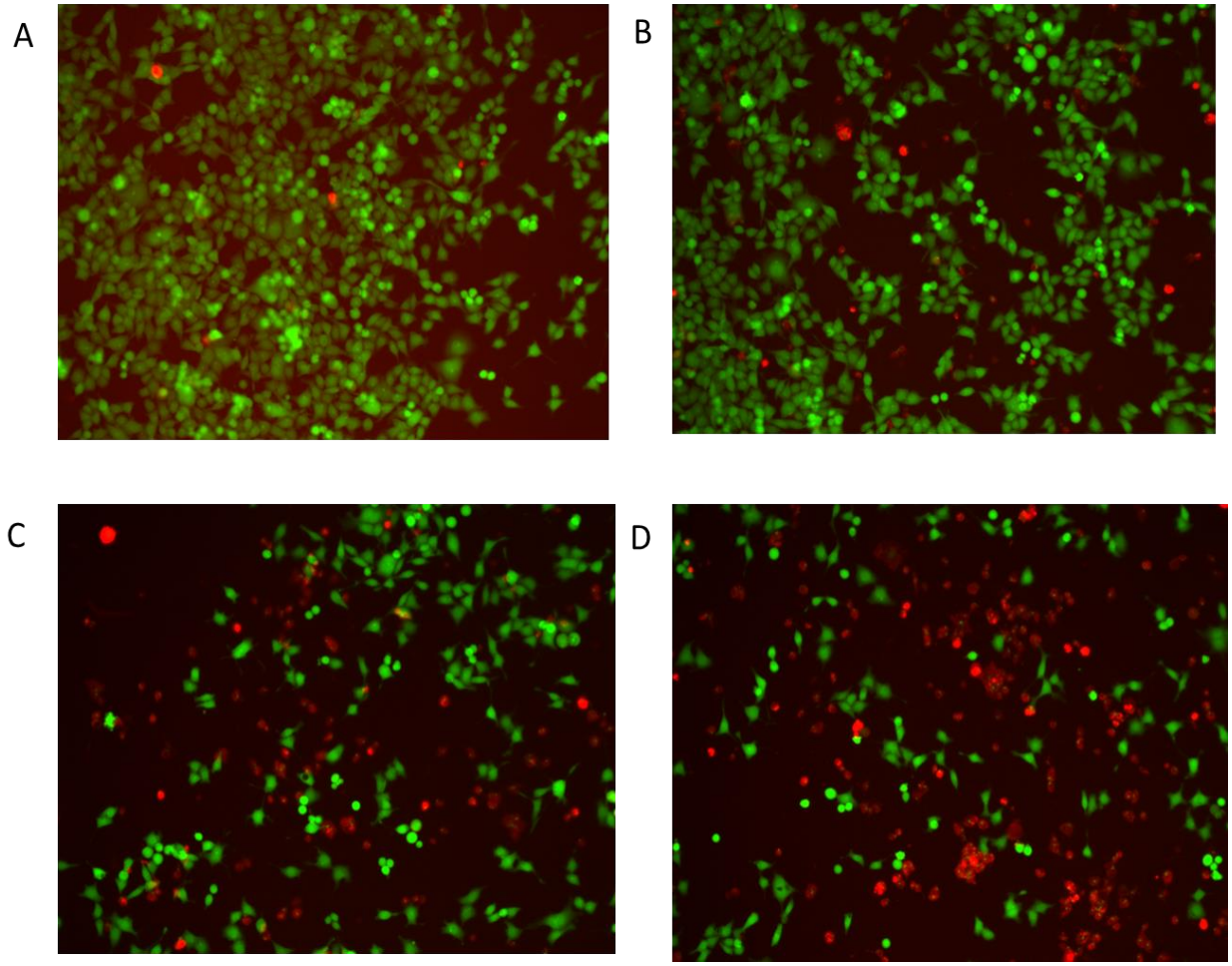

Figure 7: Sildenafil and celecoxib cell death visualization in BT 474 cells. BT 474 cells were treated with DMSO (panel A), sildenafil (2μM, panel B), celecoxib (10μM, panel C) or combination (panel D). After 24 hours they were visualized using the fluorescence cell viability assay protocol. Green is indicative of live cells (calcein), while red indicates dead cells (ethidium).

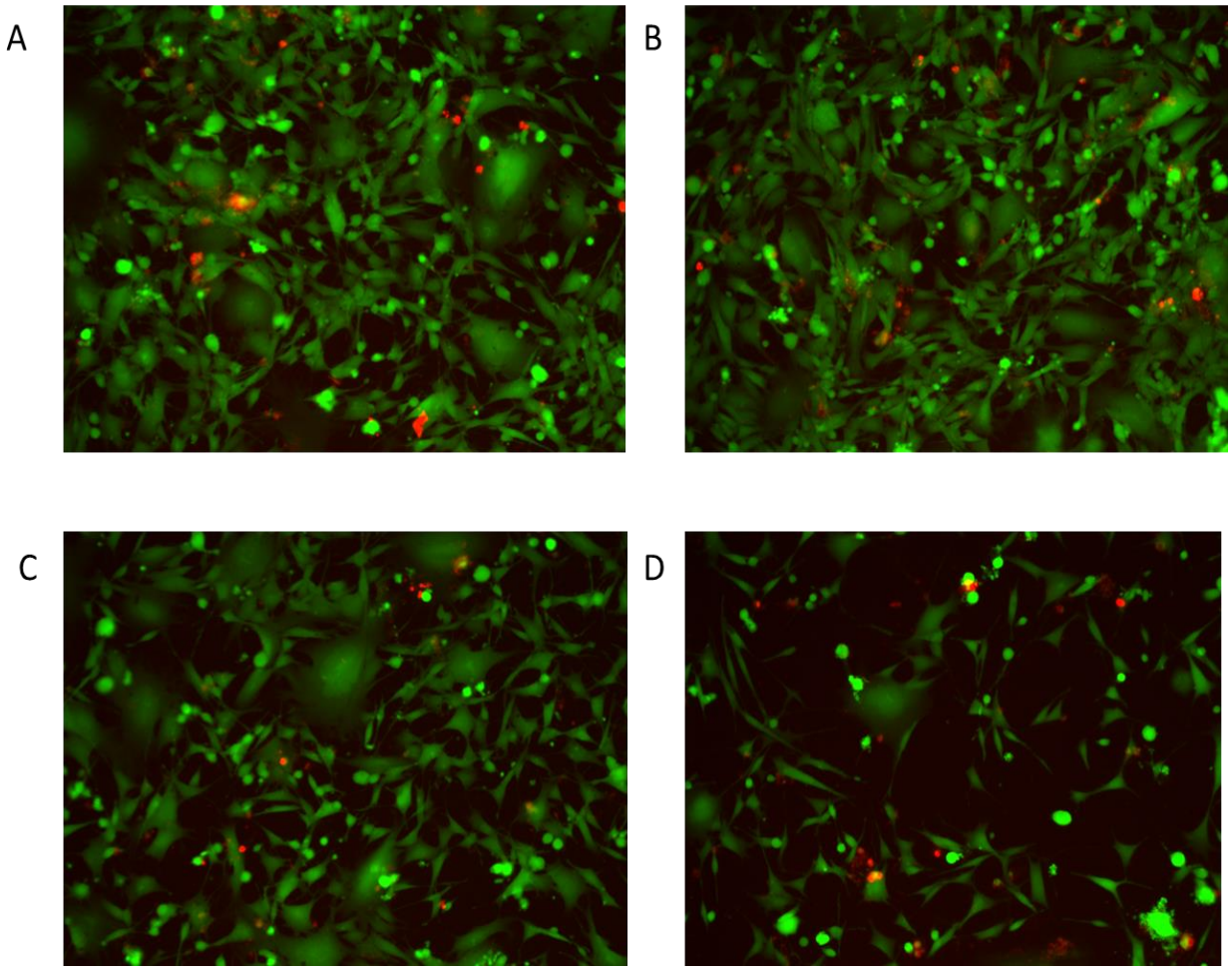

Figure 8: Sildenafil and celecoxib cell death visualization in BT 549 cells. BT 549 cells were treated with control DMSO (panel A), sildenafil (2µM, panel B), celecoxib (10µM, panel C) or combination (panel D). After 24 hours they were visualized using the fluorescence cell viability assay protocol. Green is indicative of live cells (calcein), while red indicates dead cells (ethidium).

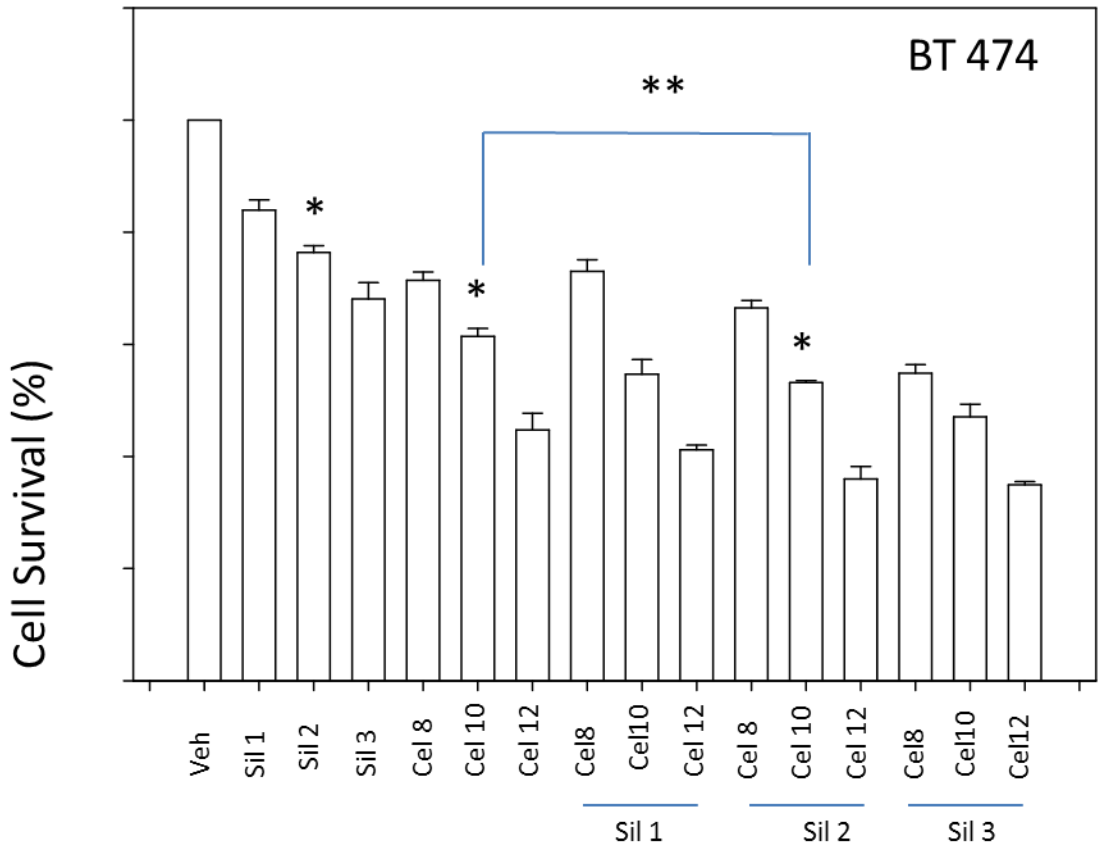

Figure 9: Colony formation assay in BT 474 cells. Cells were treated with indicated µM concentrations of sildenafil or celecoxib once for a twenty-four hour period and allowed to grow colonies for seven days before fixing and staining with crystal violet to asses cell survival. * $P<0.05$ compared to vehicle. ** $P<0.05$ comparison between celecoxib at 10 µM and the combination of 2µM sildenafil and 10 µM celecoxib.

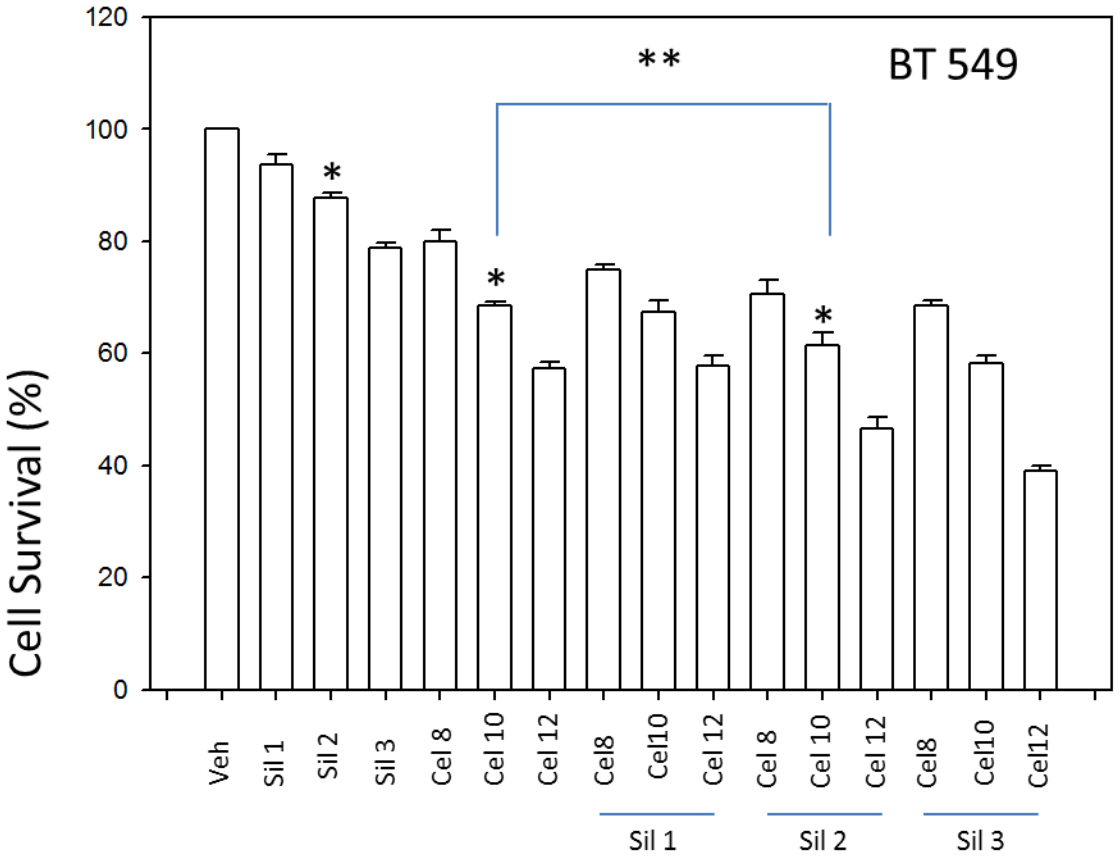

Figure 10: Colony formation assay in BT 549 cells. Cells were treated with indicated µM concentrations of sildenafil or celecoxib once for twenty-four hours and allowed to grow colonies for a week before fixing and staining with crystal violet to asses cell survival. * $P<0.05$ compared to vehicle. ** $P<0.05$ comparison between celecoxib at 10 µM and the combination of 2µM sildenafil and 10 µM celecoxib.

Mechanistic Determination of Induced Cell Death

To investigate the mechanism of action of cell death seen in the combination of 2 µM sildenafil and 10 µM celecoxib, the expression of various proteins involved in the three major cell death pathways previously mentioned were modulated to assess their involvement in the sildenafil and celecoxib-mediated cell death observed in both BT 474 and BT 549 cell lines.

For the determination of the possible invovement of apoptosis, some key components of both the intrinsic and extrinsic pathways were modulated. For intrinsic apoptosis, cells were infected with adenoviruses carrying an empty vector (CMV) or constructs designed to either up-regulate Bcl-xL (caBcl-xL) through consititutive activation or downregulate caspase 9 via dominant negative mutation (dn Casp9). Cells were infected and then treated with 2µM sildenafil and/or 10 µM celecoxib for twenty-four hours before they were subjected to trypan blue exlusion assay to assess cell death. Both cell lines demonstrated a decrease in cell death upon infection of both intrinsic mediators as seen in figures 11 and 12. The control in both the BT 474 and BT 549 cells illustrated an average of 21% cell death in the combination, while both cell lines produced an average of 15% cell death in the combination in both caBcl-xL and dnCasp9.

Involvement of the extrinsic aspect of apoptosis was assessed by siRNA mediated downregulation of FasL and CD95, with a scrambled nonspecific siRNA as control (siSCR), as well as viral up-regulation of FLIP (c-FLIP). Cells were infected or transfected and then treated with 2µM sildenafil and/or 10 µM celecoxib for twenty-four hours before they were subjected to trypan blue exlusion assay to assess cell death. As seen in figures 11, 12 the increase in FLIP expression caused a decrease in cell death from an average of 21% in the control to 15% in both BT 474 and BT 549 cells. As illustrated in figure 13, the knockdown of both FasL and CD95

resulted in decreased cell death. The combination treatment in the siSCR transfected BT 474 cells had cell death averaged at 27% while cell death was measured to be 23% in the BT 549 cells, and both produced significant decreases (* $P<0.05$ compared to vehicle) in the combination treatments after the knockdown of both FasL (21% and 19%, respectively) and CD95 (both 15%).

Cell death through autophagic response was explored by using siRNA downregulation of ATG5 and Beclin1. Cells were transfected with the SCR control, ATG5 or Beclin1 and then treated with 2μM sildenafil and/or 10 μM celecoxib for twenty-four hours before they were subjected to trypan blue exlusion assay to assess cell death. As illustrated in figure 14, downregulation of ATG5 and Beclin1 decreased cell killing in both BT 474 and BT 549 cells. The cell death in the siSCR transfected cells upon treatment with the combination was 24% in both cell lines, yet illustrated slightly different decreases in cell death. There was an overall decrease in death seen with both ATG5 and Beclin1 knockdown, yet the BT 474 cells produced 17% and 19% changes, respectively, while treatment of the BT 549 cells gave 21% and 17% cell killing in ATG5 and Beclin1 knockdowns.

For assessing the involvement of the ER stress response, ATF4, ATF6, CHOP and eIF2α were all downregulated through siRNA-mediated knockdown. Cells were transfected and then treated with 2μM sildenafil and/or 10 μM celecoxib for twenty-four hours before they were subjected to trypan blue exlusion assay to assess cell death. In both BT 474 and BT 549 cell lines, all transfections showed an overall decrease in cell death, as illustrated in figures 15 and 16. The siSCR transfected cells in both cell lines had an average of 20% cell death, with the combination treatment in every siRNA-mediated knockdown producing a combination cell death at an average of 15%. Another protein implicated in the ER stress response, p38 MAPK, was

investigated through viral infection of the dominant negative mutation of p38 MAPK (dnp38).This protein is an upstream regulator of CHOP, and was illustrated to have caused a decrease in cell death, as demonstrated in figures 11 and 12.The CMV infected cells for both BT 474 and BT 549 cell lines upon treatment with the combination had on average 21% cell death, and the combination treatment in both cell lines upon knockdown of p38 MAPK produced an average of 15% cell death.

The AKT signaling pathway is implicated in cell proliferation, growth and survival, as well as regulating apoptosis. Using either plasmid or viral infection, AKT was downregulated using the dominant negative mutation, and upregulated by mutating it to be constitutively active. Cells were infected or transfected and then treated with 2μM sildenafil and/or 10 μM celecoxib for twenty-four hours before they were subjected to trypan blue exlusion assay to assess cell death. Increasing the expression of AKT caused a protective effect and downregulating it caused an increase in cell death as seen in figure 17. The control combination treatment in both cell lines produced cell death of about 18%, with caAKT causing a decrease in death to about 15% and dnAKT increasing cell death to an average of 27% in both BT 549 and BT 474 cell lines.

All experiments performed illustrated the same characteristic response profile that was observed in figure 6. It was observed that 2μM sildenafil is seen to have a marginal increase in cell death that was not significantly different from control, celecoxib as a single agent causes a significant increase in cell death, with the combination of 2μM sildenafil and 10 μM celecoxib producing the highest amount of cell death in all experiments, comparatively.

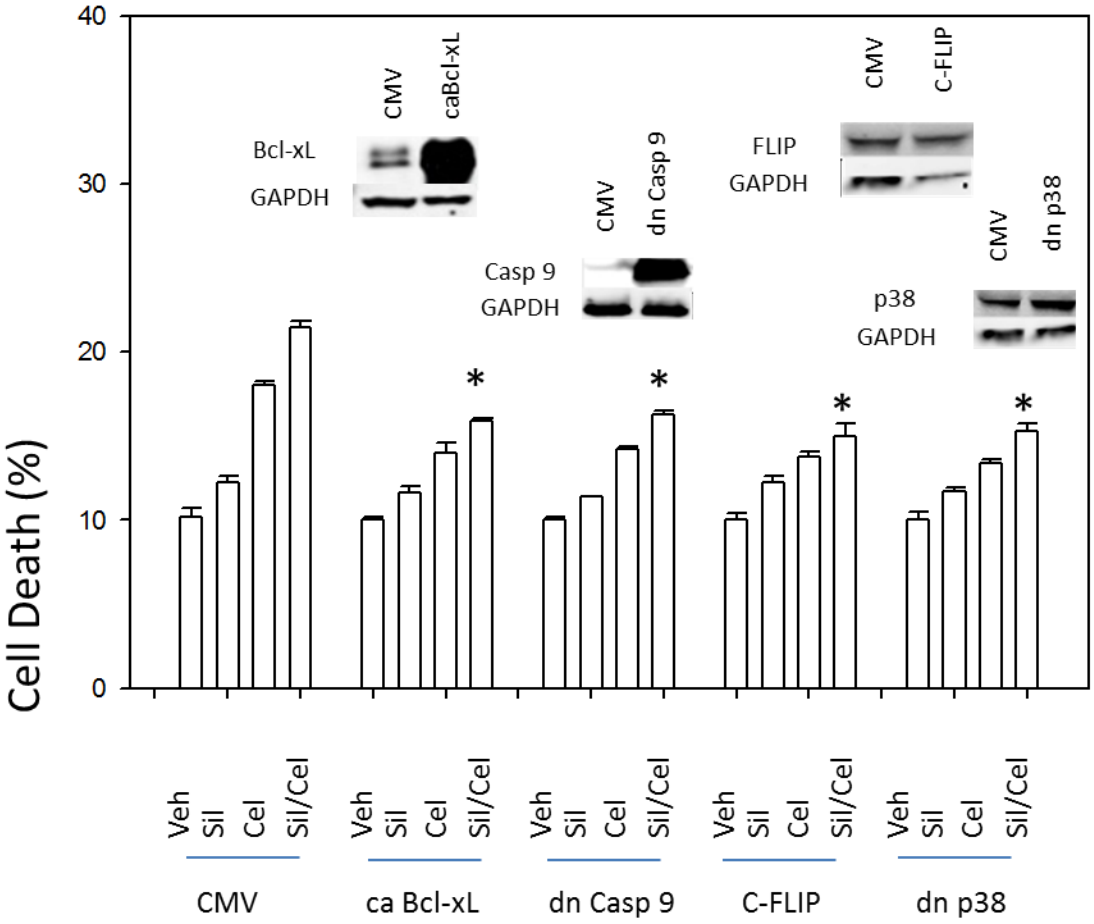

Figure 11: Viral regulation of Bcl-xL, caspase 9, C-FLIP and p38 downregulated drug combination-mediated toxicity in BT 474 cells. Cells were first infected with their respective virus and then treated with vehicle, 2μM sildenafil, 10 μM celecoxib, or combination. Cell viability was determined by trypan blue exclusion assay 24 hours after treatment. *P <.005 less than corresponding value of CMV cells.

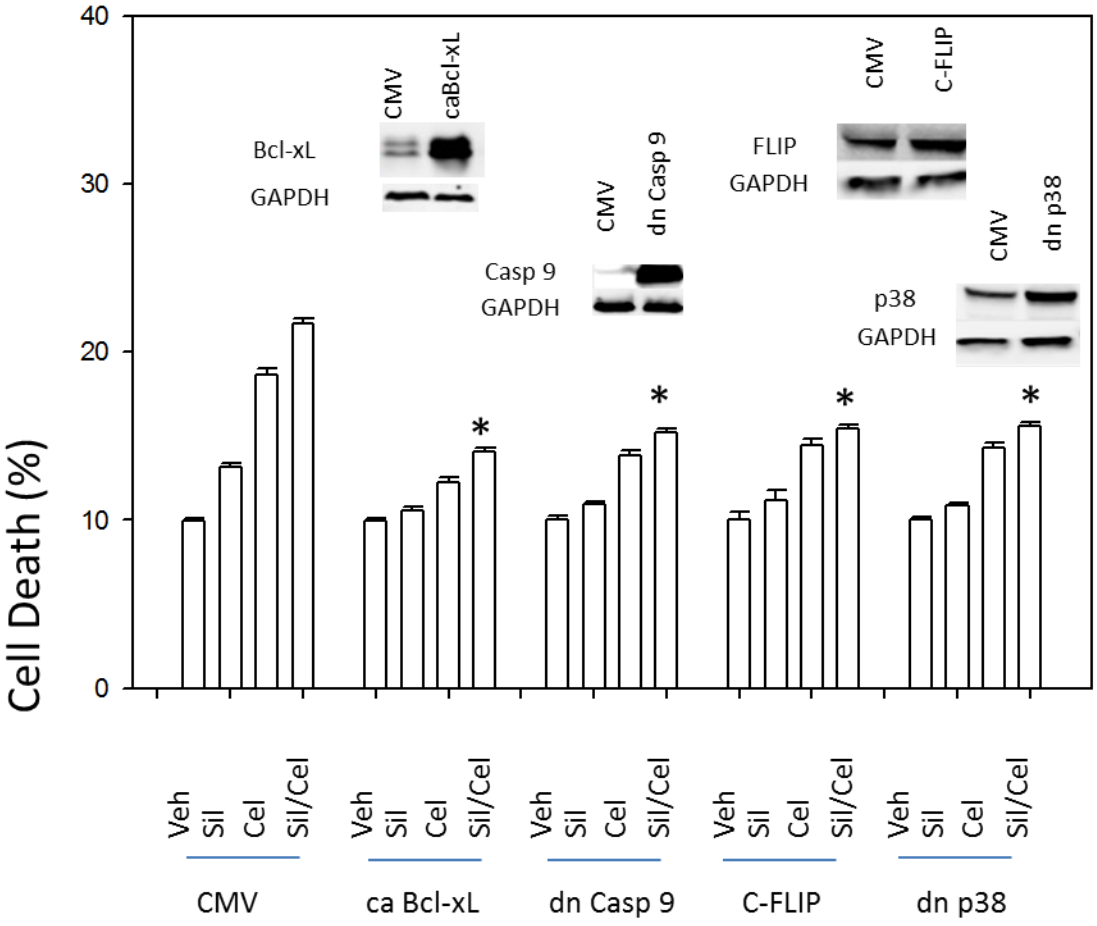

Figure 12: Viral regulation of Bcl-xL, caspase 9, C-FLIP and p38 downregulated drug combination-mediated toxicity in BT 549 cells. Cells were first infected with their respective virus and then treated with vehicle, 2μM sildenafil, 10 μM celecoxib, or combination. Cell viability was determined by trypan blue exclusion assay 24 hours after treatment. **P* <.005 less than corresponding value of CMV cells.

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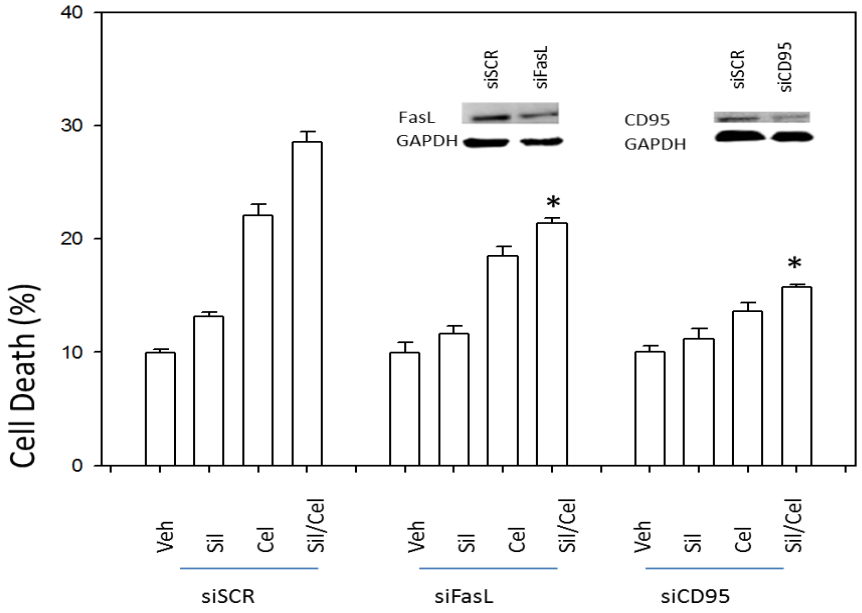

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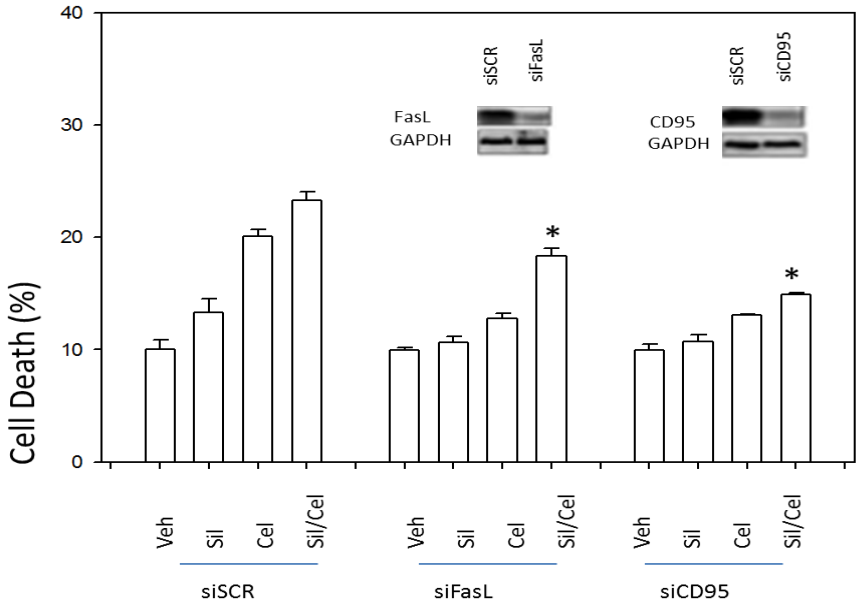
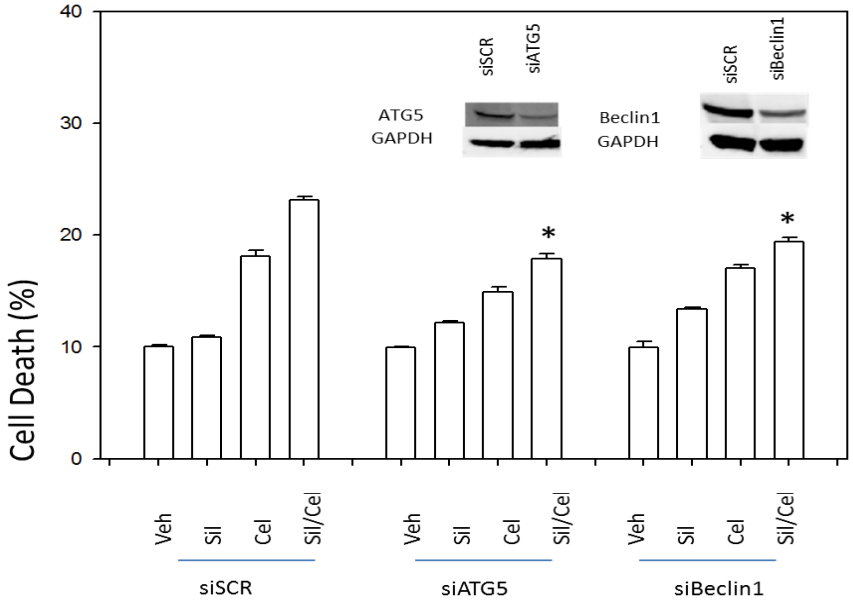


Figure 13: Knockdown of FasL and CD95 reduced drug combination-mediated toxicity. Cells were first transfected with either siFasL or siCD95 and then treated with vehicle, 2μM sildenafil, 10 μM celecoxib, or combination. Cell viability was determined by trypan blue exclusion assay 24 hours after treatment, in (A) BT 474 and (B) BT 549 cells. **P* <.005 less than corresponding value of siSCR cells.

A



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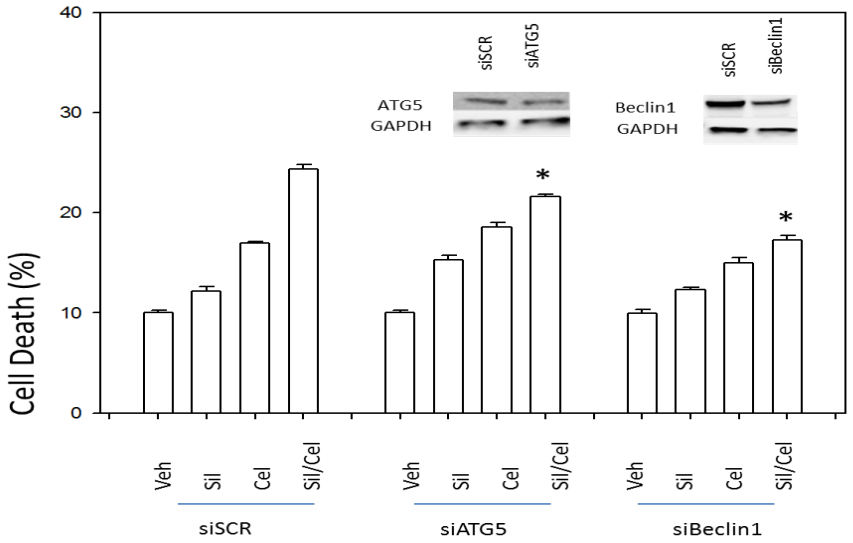
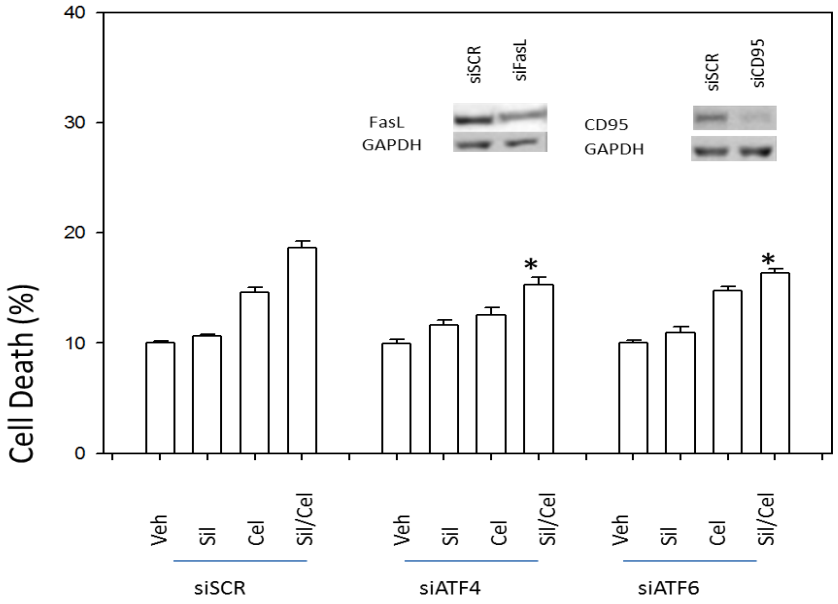


Figure 14: Knockdown of ATG5 and Beclin1 reduced drug combination-mediated toxicity. Cells were first transfected with siATG5 or siBeclin 1and then treated with vehicle, 2μM sildenafil, 10 μM celecoxib, or combination. Cell viability was determined by trypan blue exclusion assay 24 hours after treatment, in (A) BT 474 and (B) BT 549 cells. **P* <.005 less than corresponding value of siSCR cells.

A



B

BT549 – 2 uM Sil, 10uM Cel

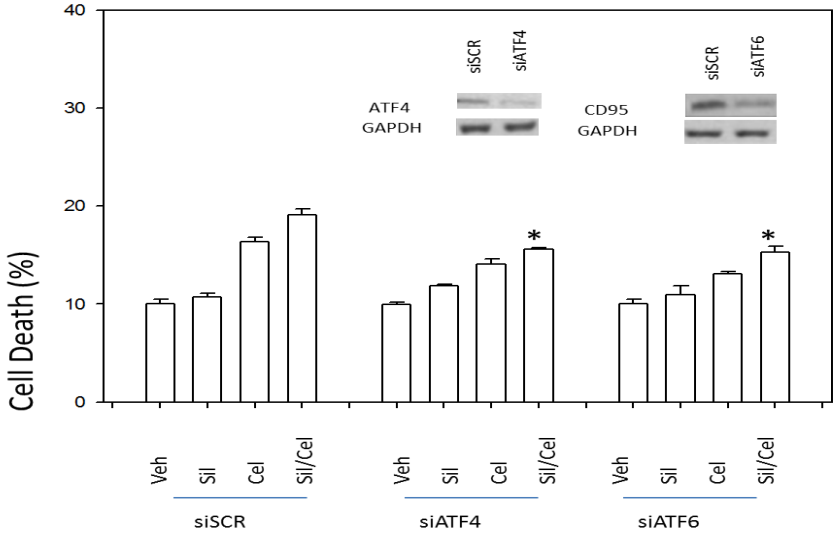
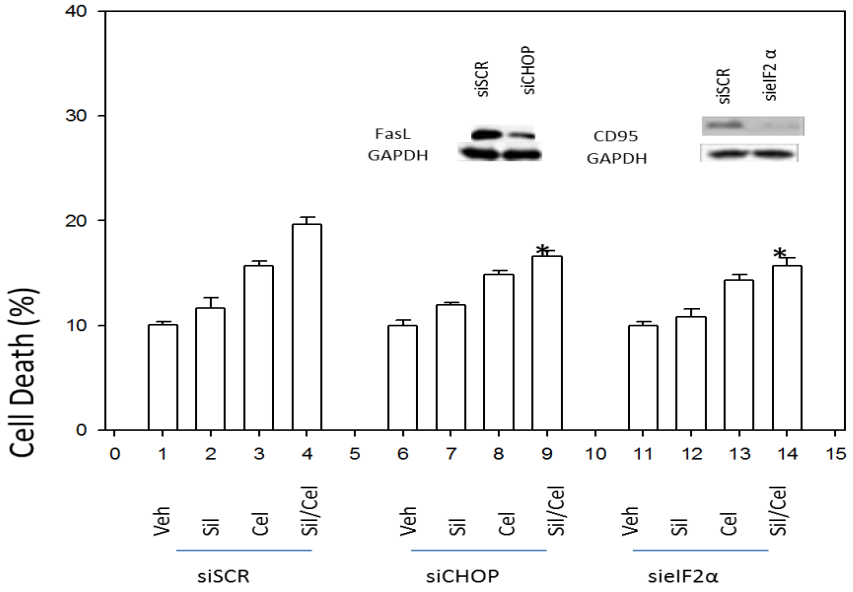


Figure 15: Knockdown of ATF4 and ATF6 reduced drug combination-mediated toxicity. Cells were first transfected with either siATF4 or siATF6 and then treated with vehicle, 2μM sildenafil, 10 μM celecoxib, or combination. Cell viability was determined by trypan blue exclusion assay 24 hours after treatment, in (A) BT 474 and (B) BT 549 cells. **P* <.005 less than corresponding value of siSCR cells.

A



B

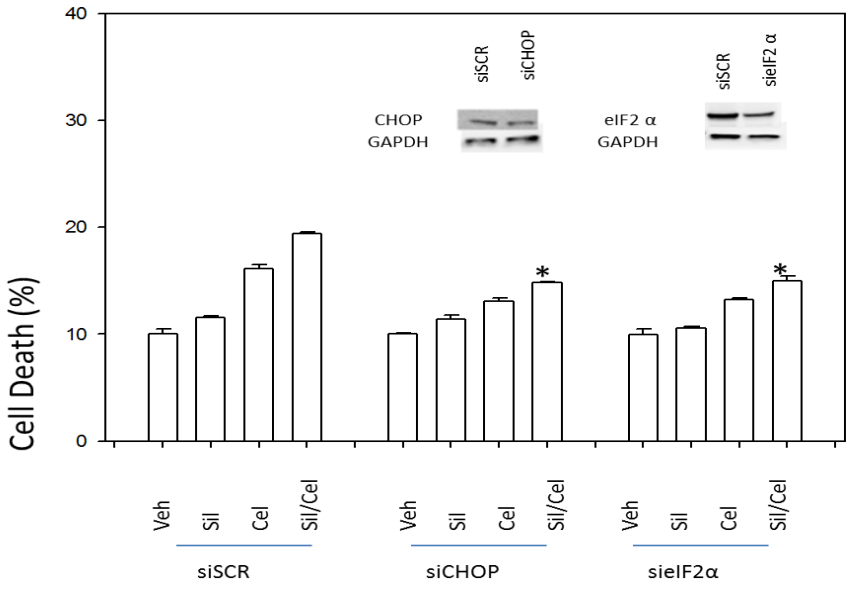
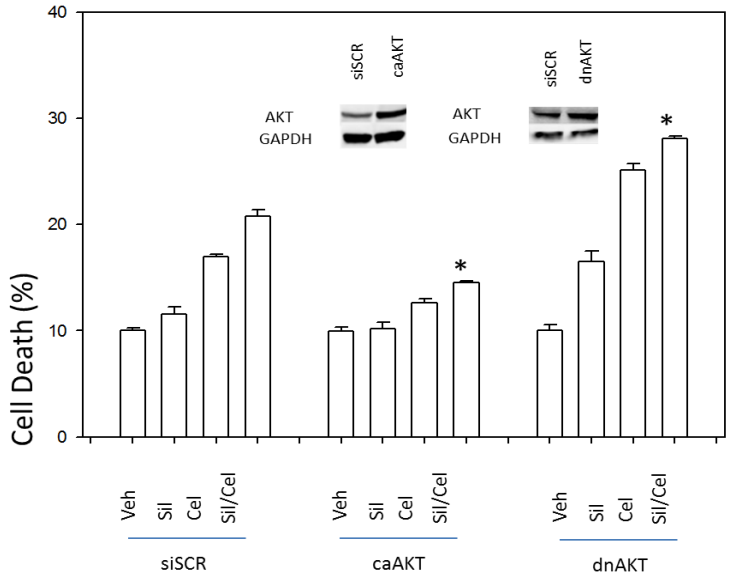


Figure 16: Knockdown of CHOP and eIF2α reduced drug combination-mediated toxicity. Cells were first transfected with either siCHOP or sieIF2α and then treated with vehicle, 2μM sildenafil, 10 μM celecoxib, or combination. Cell viability was determined by trypan blue exclusion assay 24 hours after treatment, in (A) BT 474 and (B) BT 549 cells. **P* <.005 less than corresponding value of siSCR cells.

A



B

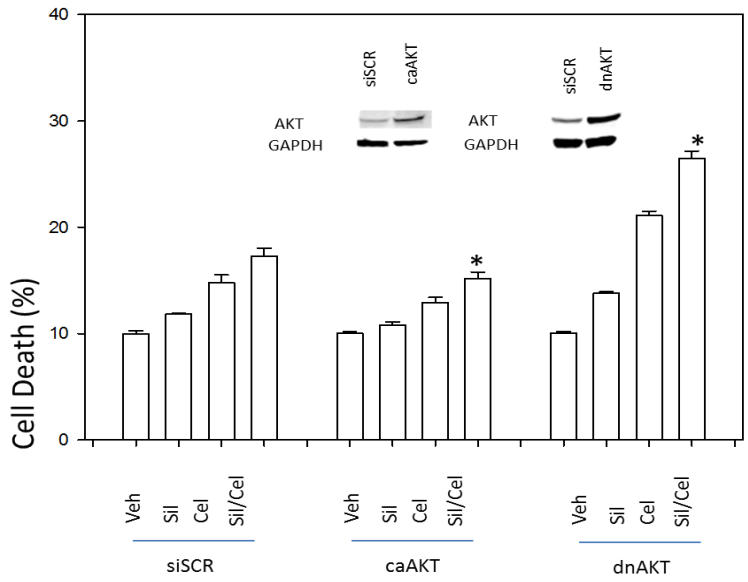


Figure 17: Modulation of AKT casused changes in levels of cell death. Cells were first infected or transfected and then treated with vehicle, 2μM sildenafil, 10 μM celecoxib, or combination. Cell viability was determined by trypan blue exclusion assay 24 hours after treatment, in (A) BT 474 and (B) BT 549 cells. *P <.005 less than corresponding value of siSCR cells.

Discussion

Targeted therapies common in treating breast cancer have been generally focused on hormone and growth receptor pathways, due to the changes in regulation of the receptor pathways in different types of breast cancers. However, it has been demonstrated that it is common for resistances to these treatments to form, thus alternative treatment options are necessary.

Doxorubicin, like many chemotherapeutic agents causes cardiotoxic effects, and upon supplementation with 10 µM of sildenafil there appeared to be attenuation of this severe side effect.[51] However, upon further investigation sildenafil was shown to enhance cell death by apoptosis through caspase 9 activity in prostate cancer when paired with doxorubicin treatment.[51] It was later demonstrated that sildenafil facilitates cytotoxic chemotherapy killing dependent on the activation of apoptosis through the extrinsic pathway via death receptors in pediatric CNS tumors.[52]

The tumorigenic potential of COX-2 overexpression has frequently been associated with a resistance to apoptosis, due to the upregulation of AKT signaling.[95] It was believed that celecoxib treatment can inhibit AKT signaling, and thus increase apoptosis through its inhibitory effects on COX2.[61, 96, 97, 98, 99, 100,101] However, a drug called OSU-03012 was developed using celecoxib as the chemical backbone, yet lacking COX-2 inhibitory activity.[100] Cell death upon treatment with OSU was found to be linked to the AKT pathway, with OSU supressing phosphorylation of AKT.[100] Yet other studies have shown that the toxicity produced by OSU was not closely correlated with the suppression of AKT signaling, indicating other methods of killing may be involved.[102] It was later elucidated that both autophagy and ER stress played a role in OSU lethality, with knockdown of ATG5 and Beclin1 suppressing cell killing as well as the

supression of BiP, that causes a PERK-dependent autophagic response.[103, 104] This demonstrated that celecoxib may be killing through apoptosis via the AKT pathway since it is indicated both in cells treated with celecoxib as well as OSU, yet also regulating autophagy and ER stress in a COX-independent manner.

Further studies demonstrated that the intrinsic pathway is involved in cell death upon treatment with celecoxib.[105] It has been observed that there is a decrease in expression of antiapoptotic proteins such as Bcl-2 and Bcl-xL, and expression of the proapoptotic protein Bad increases upon treatment, causing the release of cytochrome c from the mitochondria and inducing caspases 3, 8, and 9.[105, 106, 107, 108]The extrinsic pathway was noted to be activated as well, through FasL and CD95signaling in celecoxib treated cervical carcinoma cells.[109, 110, 111] Celecoxib treatment also increases ceramide levels in mammary tumor cells, and increases in ceramide levels are associated with the induction of apotosis.[112, 113, 114, 115] It has been shown that ceramide is important for the generation of receptor clusters, including CD95 clustering, and it constitutes an important prerequisite for receptor signaling.[116]

Results of the studies performed in this manuscript indicated that 2 µM sildenafil and 10 µM celecoxib worked better in combination than individually to promote cell killing in both BT 474 and BT 549 breast cancer cell lines, with a higher toxicity illustrated in BT 474 cells (figures 6-10). Through various modulations of key players in cell death pathways, it is suggested that the extrinsic, receptor-mediated pathway of apoptosis may be the major effector of cell death in this drug combination. The downregulation of FasL and CD95 in figure 13 illustrates the production of a larger protective effect from cell death when compared to other knockdowns. The over-expression of FLIP, an inhibitor that blocks the activation of caspase 8, also shows a protective

effect (figure 11), further indicating the role of the extrinsic apoptotic pathway in cell death via the combination of 2 µM sildenafil and 10 µM celecoxib.

There was a less pronounced change in cell death present through the changes in autophagy and ER stress, yet the downregulation of proteins involved in these pathways suggested that they too are playing a role in cell death mediated by the drug combination of sildenafil and celecoxib. The decrease in cell death seen after knockdown of ATG5 and Beclin1 in figure 14 coincides with the literature produced on OSU, as well as the results on the induction of ER stress. While PERK itself was not used in these experiments, the protective effects of siRNA-mediated knockdown of eIF2α, a down stream target of PERK, and ATF4 (figures 15 and 16) which is acted on by eIF2α may implicate a dependence on PERK signaling in the ER stress response as well as the induction of ATF6 and p38 MAPK to act on CHOP. Modulation of AKT signaling also followed what is expected of celecoxib treatment, in that further reduction in AKT signaling through dominant negative downregulation increased cell death, while increasing its activation decreased cell killing.

It is commonly seen in the literature that these drugs are used at non-clinically relevant doses, such as 10µM sildenafil and 50 µM celecoxib.[51, 60, 100] The concentrations used in these experiments are within the clinically acheiveable range, and still induced cell death. This was further verified by unpublished data that has since been produced in the lab, when 2µM silenafil and 10µM celecoxib was used *in vivo* and the combination was successful in slowing the progresssion of tumor growth in athymic nude mice. Our lab has also recently produced results that indicated the combination of 2µM sildenafil and 10 µM celecoxib is capable of killing in a number of other cancers, including brain and colon. Ceramide assays have also been performed,

and results indicate that ceramide levels do indeed increase with celecoxib treatment as expected, and the effect is even further enhanced when sildenafil is added to the treatment in combination.

Taken together, the results presented in this manuscript agree with the literature on the potential mechanisms of cell death induced by celecoxib, and indicates that the addition of 2 µM sildenafil upon treatment with 10 µM celecoxib enhances toxicity through the induction of ER stress, autophagy, and both apoptotic pathways, with a larger emphasis on the extrinsic pathway. It is a novel combination with a multi-pronged approach to the treatment of breast cancer specifically, with implications for future work in multiple types of cancer.

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