Interplay of Apoptosis and Autophagy in Acute Lymphoblastic Leukemic Cells

A thesis submitted in partial fulfillment of the requirements
For the degree of Master of Science in Biology

By

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ABSTRACT

INTERPLAY OF APOPTOSIS AND AUTOPHAGY IN ACUTE LYMPHOBLASTIC LEUKEMIC CELLS

By

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Master of Science in Biology

Apoptosis and Autophagy are two processes that play key roles in the death and survival of cells. Apoptosis is a cell death mechanism, where autophagy is a cell survival as well as a cell death mechanism. In this study we investigated whether and how these two processes interact with each other in human lymphoblastic leukemic cells. Two cell lines were used: the first being CEM C1-15, resistant to dexamethasone-induced apoptotic death. The second cell line being CEM C7-14, sensitive to dexamethasone-induced death. The drug reagent rapamycin, an mTOR inhibitor, was utilized to induce autophagy. Dexamethasone, a synthetic glucocorticoid, was used to induce Apoptosis. Additionally, 3-Methyl Adenine (3MA) was employed to rescue cells from an autophagic fate caused by rapamycin. We show that apoptosis and autophagy collaborate in CEM C1-15 and C7-14 cells, and are able to take place simultaneously in the same cell. We also show that there is a synergistic effect when both processes are induced, resulting in a stronger death response. Moreover, we have used cell growth assay data in conjunction with fluorescent microscopy images to show that when autophagy is inhibited, apoptosis takes over as the primary cell death mechanism. In summary, our results add to the knowledge of apoptotic-autophagic interaction in leukemic cells and show that epifluorescence microscopy is a useful tool in aiding researchers to take specific and detailed images of cellular processes.
CHAPTER 1: INTRODUCTION

1.1 Mechanisms of cell death

The cell has many mechanisms in place to allow it to die before it can cause harm. Three main classifications of cell death exist; they are as follows: Apoptosis or type I, Autophagy or type II and Nonlysosomal Vesiculate Degradation or type III. Each one has very unique morphological features that can define them (Table 1.1). Apoptosis, or programmed cell death, shows symptoms of cell shrinkage, chromatin condensation, nucleosomal DNA degradation and fragmentation of the cell into apoptotic bodies. Autophagy, and more specifically macroautophagy, develops autophagosomes, which are double-membraned vesicles that engulf bulky cytoplasmic components in need of degradation. Type III, Nucleosomal Vesiculate Degradation, defines both nonlysosomal degradation and cytoplasmic type of degeneration.

1.2 Apoptosis

The term ‘Apoptosis’ comes from the Greek words απο (‘apo’, from) and πτοσις (‘ptosis’), meaning ‘falling’. Just like petals or leaves falling from trees, cells fall into programmed cell death. Programmed cell death refers to a process of cell death that is gene regulated. There are specific morphological and biochemical features that we expect to see when apoptosis is happening. As Table 1 shows, the plasma membrane blebs, the cell body shrinks, the nucleus condenses and fragments, and membrane bound cell bodies or apoptotic bodies form. Since these apoptotic bodies are membrane bound, they have the advantage of not eliciting an inflammatory response.

Major biochemical features of apoptosis include the cleavage of chromosomal DNA into fragments. During apoptosis, DNA breaks down by calcium and magnesium ion-dependent endonucleases, which results into fragments of DNA consisting of 180-200 base pairs. This allows the DNA when run on an agarose gel and run by gel electrophoresis under UV light to look like a very distinct “DNA ladder”. 1
Other biochemical features include the selective cleavage of cellular proteins and the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Studies have also shown that other proteins such as Annexin I and calreticulin are also shown on the cell’s outer cell membrane. Annexin V is a recombinant phosphatidylserine-binding protein that binds to phosphatidylserine residues and is used in this project to recognize if and when apoptosis is taking place. In the cultured cells used, the apoptotic cells are bound with fluorescein isothiocyanate (FITC) FITC-Annexin V and can later be visualized by epifluorescence microscopy. FITC derives from fluorescein, a synthetic organic compound that is a fluorophore.

The cell uses two main pathways to induce apoptosis. These are the intrinsic or mitochondrial pathway and extrinsic or death receptor pathway (Figure 1.1, 1.2). There is also a third pathway that relates to T-cell mediated cytotoxicity and perforin-granzyme dependent cell death. The third pathway induces death by granzyme A or granzyme B (Figure 1.1). The components that allow for apoptosis to happen are evolutionarily conserved and their protagonists are cysteiny1 aspartate-specific proteases, called caspases. Caspases are very specific proteases that cleave proteins at aspartic acid residues. Caspases are divided into three major categories depending on their roles: initiators, effectors or executioners and inflammatory. Initiator caspases include Caspase -2, -8, -9, -10. These have large prodoms and are the first to be recruited into large protein complexes. Effector or executioner caspases include -3, -6, -7 and inflammatory caspases are -1, -4, and -5.

The extrinsic pathway is also called the death receptor pathway, as it is triggered by the binding of extracellular death receptor ligands like the tumor necrosis factor (TNF), the Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) to their specific transmembrane receptors. When receptors aggregate in response to FasL binding, the death inducing signaling complex (DISC) starts forming and the adaptor molecule Fas-associated death domain (FADD) allows for the recruitment and activation of caspases -8 and -10. Then, execution caspases are triggered leading to apoptosis. DISC and the apoptosome complex are the two main scaffolds for proximity-induced autoactivation of initiator caspases. There is a protein called c-FLIP which binds to FADD and caspase-8, stopping their activity. Another protein called “Toso” is able to block the Fas-induced apoptosis in T cells by inhibiting caspase -8 function.
As Elmore\textsuperscript{1} and colleagues write, the intrinsic pathway of apoptosis involves stimuli that are not passed on by receptor binding, but signals that directly target the cell and are initiated by the mitochondrion. These stimuli may either be positive or negative, meaning that their presence or absence can initiate apoptosis. Positive stimuli comprise factors such as chemotherapeutic drugs, UV radiation, and microbial infections. Negative stimuli constitute the absence of growth factors, hormones and cytokines that suppress inhibition of death cascades, which leads to apoptosis being activated.

It is important to note that the bcl-2 protein family is instrumental in bringing in apoptosis or maintain cell survival. Under cellular stress conditions, the pro-apoptotic bcl-2 family effectors BAK (bcl-2 antagonistic killer 1) and BAX (bcl-2 associated X protein) are activated and cause mitochondrial outer membrane permeabilization (MOPM).\textsuperscript{5} Pro-survival bcl-2 family effectors bcl-2 and bcl-XL can inhibit Bax and Bak.\textsuperscript{6}

MOPM then leads to the release of apoptogenic proteins from the mitochondrial intermembrane space into the cytosol. The apoptogenic proteins include cytochrome c, SMAC/Diablo and HtrA2/Omi.\textsuperscript{7} Cytochrome c groups with the apoptosis protease activating factor-1 (Apaf-1) and forms the apoptosome complex. It is this protein complex that allows for the activation of caspase -9. Caspase-9 then activates the executioner caspases and apoptosis is on its way.

BAK and BAX in order to bring about MOPM need to interact with other bcl-2 family proteins including BID (BH3-interacting domain agonist). Anti-apoptotic bcl-2 protein availability is controlled by BH3-only proteins, like BAD (bcl-2 antagonist of cell death) or PUMA (p53 upregulated modulator of apoptosis).\textsuperscript{5}

Figure 1.2 shows that when Smac/Diablo as well as HtrA2/Omi are present they inhibit XIAP (X-linked inhibitor of Apoptosis), rendering it no longer able to inhibit the activity of the executioner caspases -3 and -7 which go on to cleave a group of cellular substrates and induce apoptosis. XIAP is an endogenous inhibitor of caspases.
Figure 1.1: The three main pathways of Apoptosis

The three main pathways through which apoptosis takes place are shown here: each pathway activates its own caspase, which then activates the executioner caspase 3, which begins the execution of apoptosis. The perforin/granzyme pathway can also work independently of caspase activity. DNA fragmentation is among many morphological changes, which take place leading finally to apoptotic body formation.
Figure 1.2: Extrinsic and Intrinsic Apoptotic Pathways

This figure shows the Extrinsic and Intrinsic Apoptotic pathways in detail. In the extrinsic pathway, FasL ligand binds to Fas that initiates the formation of the DISC complex that allows for the recruitment of procaspase -8 and -10. The activated caspase -8 helps cleave Bid, which helps connect these two pathways. In the intrinsic pathway, genotoxic stress and other factors help activate Bax and Bak, leading to cytochrome c, Smack/Diablo and HtrA2/Omi’s release. Cytochrome c associates with Apaf-1, which leads to the assembly of the apoptosome complex and then the activation of caspase -9. Caspase -9 leads to the processing of executioner caspases -3 and -7. Smack/Diablo and HtrA2/Omi inhibit XIAP, which leads to negating XIAP’s inhibition of caspases -3 and -7. Now, these executioner caspases are able to cleave a group of cell substrates and induce apoptosis.
1.3 Autophagy

Autophagy is a process used by the cell to engulf and “eat” internal proteins, organelles, debris and other substances. The term comes from the Greek words \(\text{αυτος} \) (‘autos’, self) and \(\text{φαγειν} \) (‘phagein’), meaning ‘eating’. Christian de Duve was the first to use this term in 1963 to describe mitochondrial degradation within lysosomes in rat liver perfused with glucagon.\(^8\)\(^9\)  Autophagy has a very important housekeeping function in the cell. It is responsible for degrading aging cytoplasmic proteins and damaged organelles like peroxisomes, mitochondria and endoplasmic reticulum. Not only does this allow for the cell to remain ‘healthy’, with no debris accumulating in its cytoplasm, but also provides a valuable resource for the basic building blocks of life such as amino acids, nucleic acids and lipids from the breakdown of aging proteins and organelles.\(^10\)

Genetic screens in yeast have found many genes that have been identified to regulate the process of autophagy. These are abbreviated as “Atg” genes. Figures 1.3 and 1.4 show a general schematic of how the mammalian target or rapamycin (mTOR), a serine/threonine kinase complex, negatively regulates autophagy. mTOR plays a vital role in nutrient sensing, cell proliferation and metabolism.\(^9\) When nutrients are present in the cell, mTOR is activated, and thus inhibits autophagy. However, when there are no nutrients available, mTOR is inactivated, hence autophagy can be initiated. As figure 1.4 shows, insulin and growth factors activate mTOR, suppressing autophagy. An activated mTOR obstructs autophagy through phosphorylating the ULK1 binding partner ATG13. In addition, elevated intracellular calcium in addition to cell starvation arrests mTOR and activates autophagy.
Rapamycin and its analogs as well as Torin 1 and PP242 also have the same effect of starvation and inhibit mTOR, allowing for autophagy to take place. Rapamycin is an antibiotic that has immunosuppressant and anticancer activity. It was firstly discovered in 1965 in a soil sample from the Easter Island as a product of the bacterium *Streptomyces hygroscopicus*. It forms a complex that binds to and inhibits mTOR. mTOR can function
as the catalytic component of two complexes; mTORC1 and mTORC2. Rapamycin has a greater inhibitory activity towards TORC1. Torin 1 unlike rapamycin is able to fully inhibit mTORC1. PP242 is a potent and selective mTOR inhibitor; it targets the ATP domain of mTOR (Sigma-Aldrich). As figure 1.4 shows, autophagy can be inhibited by many factors including 3-Methyl Adenine (3MA), via blocking autophagosome formation by inhibiting phosphoinositide 3-kinases (PI3Ks) and inositol. Wortmannin (WM), a metabolite from the fungi *Penicillium funiculosum*, is able to block autophagy by being a covalent inhibitor of PI3Ks as well. Additionally, when important Atg genes are knockout down or knockout out autophagy is inhibited. Transcriptional regulators like p53, eIF2α or FOXO3 also control autophagy by regulating the expression of many Atg genes. 

PI3Ks are components of the PI3K pathway, which is deregulated in most human cancers. As Vogt and colleagues write, PI3K signaling, affects many downstream targets, some of which are important for oncogenic transformation. The canonical signal cascade goes through the AKT, to the TSC (tuberous sclerosis complex), to RHEB (Ras homolog enriched in brain) to TOR and from here to more downstream targets. Regarding this pathway, there are two targets that are critical in oncogenicity, AKT and TOR. TOR as the authors describe functions as an integrator, receiving signals from many sources. Here it is important to keep in mind that the oncogenic effects of PI3K are delivered by TORC1, a target of rapamycin that strongly and specifically interferes with PI3K.

Beclin 1 is the first human protein discovered that was found to be necessary for autophagy. It has a BH3 domain, which helps it interact with bcl-2 and other homologs of bcl-2 including bcl-xL. Beclin 1 interacts with bcl-2, which inhibits the formation of the beclin 1- hVps34 PI3K complex, thus inhibiting autophagy; thus it plays a key role in autophagosome formation. Bcl-2 is able to regulate PI3K-Akt signaling; it is a mediator downstream of PI3K-Akt signaling, and is able to keep mTOR activated, which then will keep autophagy inhibited.

Autophagy is an important process during development, as it allows for cells to die, which gives way to tissue remodeling. Not only is it important in tissue formation, but also in single cell entity formation, as we see in erythrocytes. These cells have a very specific function of transporting oxygen as well as carbon dioxide to and from body tissues.
respectively. Given this, they lack many of the organelles other cells have including their mitochondria. The process responsible for this elimination is autophagy.\textsuperscript{12}

Autophagy also plays a role in the aging process. As the body ages, an accumulation of damaged proteins and organelles progresses, which predisposes the cell to also accumulate mutated proteins that are able to cause harm.\textsuperscript{17} This is especially important in cells that have reached their final differentiation state and do not divide. Another point to be made is that with age, the activity of macrophagy (discussed below in detail) and chaperone-mediated autophagy, CMA, (also discussed in detail below) decreases in almost all tissues of aging organisms. \textsuperscript{17} Hildebrandt \textsuperscript{35} and colleagues have shown that increased levels of the hormone glucagon in serum, allow for the upregulation of autophagy, and conversely the hormone insulin inhibits autophagy. Their hypothesis for this phenomenon is that possibly a higher content of reactive oxygen species in aging cells produce the persistently higher activity of the insulin receptor. The restriction of calories is the only method known currently to slow down the aging process and improve autophagy, possibly because of the lower levels of insulin present.

1.3.1 Autophagy in Disease

An interesting point brought up by Gozuacik \textsuperscript{10} and colleagues is that autophagy signaling pathways are deregulated during malignant cell formation. Meaning that autophagy in its natural state can act as a safeguard against uncontrolled cell development. They also add that in the use of some anticancer agents, specific malignant cells initiate autophagy, which shows the potential of autophagy in anticancer therapy. mTOR is found downstream of oncogenes like Akt, and for this reason rapamycin has been used in clinical trials for cancer therapy, where it is hypothesized that it might inhibit tumor growth by inducing autophagy and not allowing for proteins to be translated.\textsuperscript{13}

Figure 1.5 demonstrates that autophagy has been shown to be involved in disease affecting many organs including the liver, heart as well as muscle and nervous tissues.

Given that one of autophagy’s roles is cell housekeeping, specifically making sure older proteins and organelles are broken down, it is a process that is always maintained at a
basal level. This basal level varies between tissues. It is higher in tissues that are composed of cells that do not divide after they have differentiated, like neurons and myocytes.

According to Mizushima and colleagues, recent studies have shown that the degradation of disease-related mutant proteins is very much dependent on autophagy, as well as the ubiquitin-proteasome system. Some examples include the polyglutamine-containing proteins that cause neurodegenerative diseases like Huntington’s, as well as mutant forms of alpha-synuclein, which cause familial Parkinson’s disease.\textsuperscript{14,15}

Looking at autophagy in neural tissue and what its possible targets are, we see that they are diffused cytosolic proteins and not inclusion bodies. It is logical to say then, that firstly there is a general protein-recycling problem, which might lead to inclusion body formation.

To understand the role of autophagy in neurodegeneration, studies have been done where two Atg genes, \textit{atg5} and \textit{beclin 1}, have been knocked down specifically in neural tissue of mice. These mice show increasing motor deficits and have abnormal reflexes as well as ubiquitin-positive inclusion body accumulation in their neurons.

Autophagy has been shown to be a therapeutic target for treatment of polyglutamine disease models in Drosophila and mice. In using drugs like rapamycin, the regulatory protein kinase complex “TOR” or Target of Rapamycin, is inhibited and autophagy initiated. Autophagy clearly helps in the process of preventing neurodegenerative diseases, even though its role here is not completely illuminated.\textsuperscript{16}
As D. Gozuacik and A. Kimchi put it, the following question persists regarding autophagy: “Is this process used by the cell to degrade long-lived proteins and cytoplasmic organelles, or is it a legitimate form of cell death?”

One can argue that cell death can be the result of starvation after ischemia, or hypoxia and not from direct death signals to the tissue. The upside to this argument is that cases exist where cell death takes place that has morphological symptoms of autophagy, but not of apoptosis. As discussed earlier, these two processes have very different morphologies exemplified in the cell. In addition, these cases also show an autophagy response that is very much intensified in the cell, compared to the housekeeping autophagical response. These cases include the death of palatal epithelial cells during closure, regression of the Mullerian duct during male sexual development and regression caused by hormone deprivation in mammary glands. To further show evidence that autophagy has its own death signals, is the fact that 3-Methyl Adenine (3-MA) is able to rescue the cells from induced autophagy by means of amino acid deprivation.
<table>
<thead>
<tr>
<th></th>
<th>Type I Apoptotic</th>
<th>Type II Autophagic</th>
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<tbody>
<tr>
<td><strong>Nucleus</strong></td>
<td>Chromatin condensation</td>
<td>Partial chromatin condensation</td>
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<tr>
<td></td>
<td>Pyknosis of nucleus</td>
<td>Condensation</td>
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<tr>
<td></td>
<td>Nuclear fragmentation</td>
<td>Nucleus intact until late stage</td>
</tr>
<tr>
<td></td>
<td>DNA laddering</td>
<td>No DNA laddering</td>
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<tr>
<td><strong>Cytoplasm</strong></td>
<td>Cytoplasmic condensation</td>
<td>Increased autophagic vesicles</td>
</tr>
<tr>
<td></td>
<td>Fragmentation of apoptotic bodies</td>
<td>Increased autolysosome</td>
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<td>Caspases are active</td>
<td>Increased lysosomal activity</td>
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<td>Caspase-Independent</td>
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<td><strong>Cell Membrane</strong></td>
<td>Blebbing</td>
<td>Blebbing</td>
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<tr>
<td><strong>Corps Clearance</strong></td>
<td>Heterophagy by other cells</td>
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<td><strong>Detection methods</strong></td>
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<td>Caspase activation tests</td>
<td>lysotracker, MDC staining</td>
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<td>DNA laddering detection</td>
<td>Detection of LC3</td>
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<td>Nuclear/Cellular fragmentation detection</td>
<td>recruitment to autophagic membranes</td>
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Table 1.1: Apoptotic versus Autophagic Morphology

Autophagy and Apoptosis are two mechanisms that are interrelated. The exact characteristics of this relationship are still being elucidated today. This project’s goal is to add to the body of work already done to further define how these two processes relate to each other.

There are three defined types of Autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is a process where a double membrane-bound vesicle forms, called the autophagosome that fuses with the lysosome to form an autolysosome, where contents are broken down by lysosomal hydrolase. The origin of the sequestration membrane called the phagophore or isolation membrane is still unknown.

There is some evidence to support that the endoplasmic reticulum is the source of the membrane material for the formation of the autophagosome. In addition, the mitochondria and the plasma membrane are also seen as possible sources for the formation of the phagophore.

Mari and colleagues have proposed the “de novo” formation of the autophagosomal membrane by Atg 9 reservoirs. Another hypothesis is that a very specific region of the ER could be the site of autophagosome formation. This area is a cup-shaped structure called the omegasome (figure 1.6). This structure is called the ‘omegasome’ as it has a Ω-like shape.

Here after starvation of the cells, LC3 and ULK1 were recruited into the omegasome and Atg5 as well as LC3 and positive membranes developed from it. A very convincing argument for the omegasome being formed from ER membranes is the discovery that ER-targeted PI3P-binding proteins including trans-membrane proteins translocate to omegasomes in a PI3P-dependent fashion under starvation conditions. Otherwise, the origin of this material has not been confirmed yet.

The autophagosome forms by the elongation of a cup-shaped membrane as well as the help of two ubiquitin-like conjugation systems, keeping in mind that the ubiquitin/proteasome pathway is one the cell’s protein degradation pathways. The cell uses autophagy to degrade bulky cytosolic contents and recycle organelles.
**Figure 1.6: Hypothesized omegasome formation from the ER**

PI3P (red) delineates the site of omegasome formation on the ER membrane. The cisterna changes its form to create an invagination that looks like a cup, which allows for autophagic cargo to be isolated. Then the formed autophagosome fuses with a lysosome to create an autolysosome that allows for the autophagic cargo to be enzymatically degraded.

Figure 1.7 delineates the specific steps in Macroautophagy and Chaperone-mediated autophagy. The first step in macroautophagy begins with lipid-bilayers being donated by a structure that has not been confirmed yet. These bilayers form a pre-autophagosomal structure, (PAS) as it is named in yeast, or an analogous structure in mammalian organisms. The formation of a PAS needs a class III PI3K complex (in yellow) and we also see a MAPILC3 anchored to the membrane by a phosphoethanolamine anchor, LC3-II. In the second step, phagophore formation takes place, where the membrane invaginates to include organelles, debris, pathogens and proteins. This process can be both specific through the use of p62 and non-specific. The third step shows how the phagophore matures to being the autophagosome; here more LC3-II is recruited and the autophagosome has closed to form a double membrane vesicle. Now the autophagosome can fuse with multi-vesicular bodies or late endosomes to form the autolysosome in the fourth step. The more late endosomes it fuses with, the more acidic the lumen within the interior membrane becomes. These acid
hydrolases have a minimum of ~ 5.0 pH and include minimally five phosphatases, fourteen proteases and peptidases, two nucleases, six lipases, thirteen glycosidases and seven sulfatases. In this study LysoTracker Red DND-99, a red fluorescent dye, is used for labeling and tracking acidic organelles in live cells. This fluorescent dye is highly sensitive to acidic organelles and is used here to stain the increasing number of autophagolysosomes formed in the process of autophagy. As these vesicles are full of endosomes, their acidic content is high, thus it is a perfect target for LysoTracker Red DND-99. This fluorescent dye is a fluorophore linked to a weak base that is only partially protonated at neutral pH. This quality enables LysoTracker Red to easily go through cell membranes enabling it to label live cells.

All these hydrolases are able to break down the major biological macromolecules in the cell, and release amino acids, nucleic acids, lipids as well as carbohydrates that are now available to be re-utilized by the cell. This process is especially important in times of starvation. Autophagy is a clever way the cell uses its own resources to keep itself alive for as long as possible, until new resources are available.

Microautophagy (figure 1.8), in contrast with macroautophagy does not involve the creation of an autophagosome. Here, the lysosome invaginates and takes up any cellular content ready to be degraded. In chaperone-mediated autophagy, proteins get transported into the autolysosome from the cytosol with the help of chaperones. There is no vesicular trafficking taking place. The proteins to be degraded contain a pentapeptide protein motif, KFERQ). They bind to a cytosolic complex that includes the chaperone HSC70. Then this complex interacts with lysosomal membrane receptor protein LAMP2A, and is translocated into the autolysosome (step 6 in figure 1.7). This leads to the conclusion that unless a protein has the specific pentapeptide motif it does not get degraded through CMA. This motif or a biologically related motif to this exists in about 30% of all proteins found in the cytosol. The protein to be degraded is degraded with the assistance of l-HSC70, the luminal form of HSC70.
1.4 How do apoptosis and autophagy interact?

When it comes to the interaction of apoptosis and autophagy, the relationship is complex. There has been a recent wealth of studies on the subject, focusing on different proteins that have a role in both processes (figure 1.9, 1.10). Apoptosis as an individual process has been extensively studied for much longer than autophagy; however, the more autophagy is studied the more its complexity is revealed. As Zhou and colleagues put it,
not like the cell death pathway of apoptosis, autophagy is a complex cellular process with two roles: it can be the mechanism to respond to stress and keep the cell alive, and also a mechanism that responds to stress and leads the cell to death.

In apoptosis, one organelle that plays a protagonistic role is the mitochondrion. It lends its membranes and proteins to the extrinsic and intrinsic apoptotic pathways. This organelle connects apoptosis and autophagy. Autophagy is a process that allows for self-digestion of damaged or aging organelles including mitochondria. Since this is the case, through the autophagy of mitochondria or mitophagy, there is mitochondrial quality control that takes place in the cell. When extensive cellular damage is present, mitochondrial membrane permeabilization (MMP) takes place, which allows for pores to form in the mitochondrial membrane leading to apoptosis. However, if the damage is moderate, mitophagy can save the cell, by only eliminating the damaged mitochondrion, and not allowing for apoptosis to get initiated. An interesting fact is that if we define a mitochondrion to be healthy in terms of its energy supply, then the mitochondrial state in this respect can be a leading factor that determines whether the cell will go through autophagy or apoptosis.

Literature discussing the interaction of autophagy and apoptosis clearly shows that there is a significant overlap between the components that regulate these processes. As apoptosis is a cell-death mechanism and autophagy mainly a cell-survival mechanism, it can be concluded that these overlapping components are there to create a balance. This can further allow the cell to react to environmental conditions, either to try to save itself from dying, or to commit programmed cell death. Both apoptosis and autophagy give rise to an interesting interplay that keeps cellular homeostasis.

There are well-known proteins that have a dual role in both apoptosis and autophagy. These proteins belong to the Bcl-2 family. The Bcl-2 family encompasses proteins that contain at least one Bcl-2 homology (BH) region. In humans this includes anti-apoptotic proteins, pro-apoptotic proteins and the pro-apoptotic BH3-only protein group. Anti-apoptotic proteins include Bcl-2 and Bcl-XL and pro-apoptotic proteins include Bax and Bak. Beclin 1 is also under this family and was the first human protein to be discovered that is essential in the function of autophagy. Specifically, Beclin 1 is required for the autophagosome to be formed.
Taking into account our knowledge of Bcl-2 protein interaction in apoptosis, most likely this function is facilitated by the mitochondrial membrane. Multi-domain anti-apoptotic proteins such as Bcl-2 and Bcl-X\textsubscript{L} are found in mitochondria. Bak is normally found in the outer mitochondrial membrane and Bax is found in the cell cytosol. Bcl-2 regulates apoptosis by binding to the pro-apoptotic protein Bax. If the cell has a state of withstanding nutrient depletion, Bax gets released leading to apoptosis.

Bcl-2 also regulates autophagy by binding to the pro-autophagic protein Beclin-1. As Rambold et al put it, a state of depletion of nutrients firstly allows for the release of Beclin-1, which leads to the activation of PI3K and the initiation of autophagy. If it continues, apoptosis also occurs as mentioned above. An interesting note is that only ER-localized and not mitochondrial localized Bcl-2 or Bcl-xL inhibits autophagy\textsuperscript{46}. Pro-apoptotic Bcl-2 proteins have an important role in allowing cells that have been damaged to die and therefore to suppress tumor formation. Anti-apoptotic proteins on the other hand do not allow for damaged cells to die, therefore they have a potential to function as oncogenes. In many cancers, Bcl-2 and Bcl-xL are often overexpressed and bind to Bax and Bak, not allowing for apoptosis to take place.\textsuperscript{28} In a state where the cell is under metabolic or chemotherapy-associated stress, Bcl-2/ Bcl-xL can be overexpressed, inhibiting apoptosis, resulting in tumor cell survival.

Caspases, similarly to Bcl-2 proteins have a dual role in apoptosis and autophagy. They cleave Beclin-1 and the class III PI3K, destroying their autophagic function, leading them to move to the mitochondria where they can enhance apoptosis.\textsuperscript{29} In the case where caspases are inhibited, autophagy takes over.\textsuperscript{9} ATG5 is another protein that regulates both apoptosis and autophagy (figure 1.9, 1.10). In autophagy, ATG5 assists in extending the autophagosome, thus supporting autophagy. In apoptosis, under high cellular stress, it can be cleaved by calpains and translocated to the mitochondria. There it binds to Bcl-X\textsubscript{L} and initiates apoptosis.\textsuperscript{29}

Autophagy may be seen as an alternative cell death mechanism when compared to apoptosis. Experiments done by Shimizu et al.,\textsuperscript{31} show mice that cannot go through apoptosis. When mice deficient in the genes that code BAX and BAK are studied, they die through autophagy. However, apoptosis may also be seen as an alternative to autophagic cell death. When autophagy is inhibited by gene knockout of genes vital to autophagic function

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including Atg5 and Atg7, apoptosis causes cell death in nerve cells. Additionally, Lemasters and colleagues have shown that autophagy is able to prevent apoptosis in hepatocytes. Here, mitochondria that were depolarized are shown to be quickly degraded by autophagy in the cell. In this way, the damaged organelles are disposed from the cell, not allowing for the subsequent release of pre-apoptotic substances from them, which would lead to apoptosis. Therefore autophagy inherently provides the cell with a survival function.

Figure 1.9: Autophagy and Apoptosis Interaction
Figure 1.10: Detailed Interaction between Autophagy and Apoptosis

Here, we see how Rapamycin and 3MA (3-methyl adenine) (in red) affect autophagy. Rapamycin inhibits mTOR, which allows for autophagy to take place. The 3MA is able to inhibit the formation of the autophagosome, not allowing for autophagy to take place. Under short-term nutrient starvation, Beclin 1 is released from the Endoplasmic Reticulum (ER) and forms a complex with Vps34, a protein belonging to the class III PI3K. This complex communicates with Atg genes, which in turn regulate autophagy. Caspases cleave the Vps34-Beclin 1 complex, enhancing apoptosis. Beclin 1 regulates both autophagy and apoptosis.

Under high stress, calpains are shown to cleave Atg5 and form truncated Atg5 (tAtg5). The truncated version translocates from the cytosol into the mitochondrion and binds to Bcl-xL in the mitochondrion, which leads to cytochrome c release, caspase activation and apoptosis. Atg5 also regulates autophagy, as the truncated tAtg5 also inhibits Bcl-2 or Bcl-xL. When Bcl-2 or Bcl-xL are inhibited, they can no longer bind to Beclin 1 and inhibit autophagy.
This diagram shows how induction, vesicle nucleation and expansion of the autophagosome takes place in mammals. Rapamycin (in green lettering) has the ability to inhibit the inhibitor mTOR, thus initiate autophagy. Also, in red it can be seen how 3-Methyl Adenine (3-MA) as well as Wortmannin (WM) have the ability to inhibit Phosphatidylinositol 3-kinase (PI3K) activity, thus not allowing for the formation of the autophagosome. Quoting from the authors of this paper “Rapamycin inhibits mTOR and activates macroautophagy, while 3-methyladenine (3-MA) and wortmannin (WM) inhibit the PI3K activity and de-activate macroautophagy”.

1.5 Acute Lymphoblastic Leukemia and Glucocorticoids

Leukemia is defined as a disease of the bone marrow, where leukemic cells are resistant to apoptosis, cannot die and accumulate in the bone marrow. This tends to lead to overcrowding of leucocytes, which creates problems in the normal growth of other blood
cells including erythrocytes and platelets. Acute lymphoblastic leukemia (ALL) is the result of immature lymphoid cells (lymphoblasts) proliferating with no cell cycle control. Lymphoblasts give rise to leukocytes, a category of immune cells that includes T cells, B cells and NK (Natural Killer) cells. T cells are part of the adaptive immune response in the human body and are the cells responsible for the immune response that rejects transplanted organs. T cells come from the bone marrow and then mature in the thymus, thus are called “T cells”. B cells, differentiate into plasma cells, which are the cells responsible for antibody production.

Yearly, almost 15,000 adult and pediatric patients in the United States are diagnosed with having acute leukemia. Glucocorticoid hormones, specifically dexamethasone, play an essential part in treating ALL as seen in figure 1.12 below.

![Figure 1.12 Glucocorticoid Receptor Signaling](image)

The Glucocorticoid hormone enters the cell through the cell membrane and binds to its GR receptor. Then dissociation of the chaperone proteins takes place in the cytoplasm and the glucocorticoid hormone can either stay a monomer or dimerize into a homodimer. Then it is able to enter the nucleus and either bind to DNA, causing gene transcription, or stay a monomer and interact with transcription factors. In both scenarios, inhibition of cytokines results, as well as, cell cycle arrest and finally apoptosis.
The glucocorticoid receptor (GR) is a phosphoprotein that is present in low concentration in mostly all mammalian cells. This is a transcription factor that depends on its respective ligand(s) to bind and bring about change in growth, development, metabolism as well as stress responses. There is a nuclear receptor form as well as a cell membrane-associated receptor form. The nuclear receptor form binds as a homodimer to specific DNA sequences called GREs (Glucocorticoid Response Elements), where it can activate or repress gene transcription. The GR is found in the cytoplasm and after a ligand binds to it, it goes through a conformational change, that allows for the release of its chaperone proteins. Now the GR is able to translocate in the nucleus, where it binds to GRE and is able to repress or activate transcription. The exact mechanism of how glucocorticoid hormones bring about apoptosis is not known, however there are three main hypotheses that exist on this topic.

Medh and colleagues have shown that lymphoid cell apoptosis is very much dependent on the GR being fully occupied by its ligand. They used lymphoid CEM cells sensitive to dexamethasone-induced apoptosis and incubated them in a concentration of the steroid that was sufficient to occupy all of the GRs present in the cells for at least 24 hours before seeing any morphological evidence for apoptosis. They used a concentration of 1 µM for dexamethasone, the same concentration used in this study.

Glucocorticoids are good candidates for chemotherapeutic agents, as they are able to bring about death, through apoptosis, to cells and not create a systemic inflammatory response. Apoptosis is able to form apoptotic bodies, and destroy the respective cell without leaking any of its cytotoxic cellular contents.

We should mention that Rapamycin, also known as Sirolimus, is another immunosuppressive drug that has potential in being part of the therapy for acute lymphoblastic leukemia. We use rapamycin in this project to induce autophagy in cells. Rapamycin is an mTOR inhibitor, which has been approved by the FDA (Food and Drug Administration), to be used in solid organ transplantation. Given the knowledge that the PI3K/AKT/TOR signaling pathway helps contribute to many human cancers, including leukemia and lymphoma, additional
data shedding light on how rapamycin induced autophagy may interact with apoptosis in leukemic cells are valuable.

1.6 Acute Lymphoblastic Leukemia Cell Lines

Cells from two acute T-lymphoblastic cell lines (CCRF-CEM cells) were used in this examination as a model system to study the interaction of glucocorticoid induced apoptosis and autophagy.

Using a cell culture as a model system has many benefits, including the ability to study in detail how a defined population of human leukemia cells responds to: dexamethasone, rapamycin and 3-methyl adenine, specifically 6-amino-3-methyl-purine (3-MA). In addition, the effects of the drugs used can be isolated without many outside factors complicating the study. Two distinct sister cell lines are used that are clones of one original CEM cell line originating from a 3-year-old patient with late-stage acute lymphoblastic leukemia.

The first cell line used is CCRF-CEM C1-15 (abbreviated as CEM C1-15), which is resistant to dexamethasone, an artificial glucocorticoid. These CEM C1-15 cells are resistant to a dexamethasone concentration of up to $10^{-5}$ M. This is a 100-fold greater concentration than the one needed to completely saturate the glucocorticoid receptor. The equivalent natural glucocorticoid used by the body is cortisol. It is well established that glucocorticoids work well as therapies against many lymphomas and leukemias. They are able to prevent malignant cells from growing.

The second cell line used is CCRF-CEM C7-14 (abbreviated as CEM C7-14), which is susceptible to dexamethasone. CEM C7-14 cells that are treated with dexamethasone will die faster compared to the control. The use of these two cell lines is a great way to study the interactions of glucocorticoid evoked apoptosis and autophagy in acute lymphoblastic leukemia.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

CEM C1-15 and CEM C7-14 cells were kindly provided by Dr. E.B Thompson (UTMB, Galveston, TX). They have been derived from the parental line CCRF-CEM, obtained from a patient with acute lymphoblastic leukemia.

The cells were cultured in RPMI (Cellgro Cat # 50-020-PB), supplemented with 2.0 g/L sodium bicarbonate at pH 7.4 and 5% heat inactivated fetal bovine serum (FBS) (Atlanta Biological, Catalog #S11050). The cells were kept in an incubator with a constant 5% CO2 supply. Cells were maintained in log phase and passaged every two to three days.

2.2 Cell Counting

Trypan blue solution (0.4% w/v in normal saline Cellgro Cat # 25-900-CI) was used to differentiate between healthy and dead cells. Trypan blue is absorbed by nonviable cells and makes cells appear blue, while healthy cells do not absorb the dye and appear white. One hundred microliters were taken from each cell culture and mixed with 100 µl of Trypan Blue (Cellgro 25-900-CI), as well as 200 µl of 1x Phosphate Buffered Saline (PBS). This solution was mixed well and a sample was put on a hemocytometer (Hausser Scientific) for counting. Cells were counted using a light compound microscope.

2.3 Drug reagents employed

As this study focused on the interaction of apoptosis and autophagy, specific drugs were utilized to study each process. Dexamethasone was used to study the effects of apoptosis on the CEM C7-14 cells, as they are susceptible to it. Rapamycin (Sigma-Aldrich Cat No: R8781) was used to study autophagy, as it inhibits the inhibitor mTOR, resulting in the activation of autophagy. 3-Methyl Adenine (3-MA) (Sigma-Aldrich Cat No: M9281) was used as an inhibitor of autophagy. 3MA works by blocking the formation of the autophagosome through inhibiting type III phosphatidylinositol 3-kinases (PI-3K)\(^{40}\).
2.4 Cell Treatments for Growth Curves

Cells from both CEM cell lines were plated at a concentration of $4.0 \times 10^5$ /mL and treated over a period of four days with each drug or drug combination. Cells were counted on day 1 through day 4 and the cell number for each day was recorded. Day 0 was treatment day. There were eight different drug combinations that were utilized including the control. They were as follows: 1. Control: Ethanol and Dimethyl Sulfoxide (DMSO) 2. Dexamethasone 3. Rapamycin 4. 3-MA 5. Dexamethasone and Rapamycin 6. Dexamethasone and 3-MA, 7. Rapamycin and 3-MA and 8. Dexamethasone, Rapamycin and 3-MA combined. The control included 15 µl of 100% ethanol as well as 15µl of 100% DMSO. All treatments that included two drug reagents had 15µl taken from each drug reagent into a flask final volume of 15mL. Every single drug reagent treatment had 15µl of the designated drug taken into a flask final volume of 15mL. Each of the flasks had a total volume of 15ml. Treatment information is delineated in the following table:

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Final Concentration in Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>1 µM</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>50 nM</td>
</tr>
<tr>
<td>3-Methyl Adenine</td>
<td>200 µM</td>
</tr>
<tr>
<td>Ethanol (100%)</td>
<td>0.1 %</td>
</tr>
<tr>
<td>DMSO (100%)</td>
<td>0.1 %</td>
</tr>
</tbody>
</table>

Table 2.1: Cell Treatment Drug Concentrations Used

Cell growth curves allowed us to quantitatively see how each individual drug and each drug combination affected the growth of both cell lines over the course of four days.
2.5 Epifluorescence Microscopy

Epifluorescence microscopy was employed to provide visual evidence for the morphological changes that take place during apoptosis and autophagy separately, as well as when they were in combination. The fluorescence microscope used was the Accuscope 3025 series with fluorescence capability via a mercury lamp (NFP-1) house. A ProgRes MF camera (JENOPTIK, Germany) was used to capture the working images from the microscope and the 2.7 ProRes software used to view these images. iPhoto software was used (2012 version) to standardize all images with the same exposure, contrast, saturation and shadow settings. The specific settings are as follows: exposure -1.02, contrast -24, saturation 93 and shadows 10.2.

2.5.1 Cell Treatment for Epifluorescence Microscopy

There were eight different treatment combinations that were used: 1. Control Ethanol and DMSO 2. Dexamethasone 3. Rapamycin 4. 3-MA 5. Dexamethasone and Rapamycin 6. Dexamethasone and 3-MA, 7. Rapamycin and 3-MA and 8. Dexamethasone, Rapamycin and 3-MA combined. These treatments are the same ones employed in the cell growth curve section of this project. Cells from the CEM C7-14 cell line were plated at a concentration of 4.0 x 10^5 / mL and treated for two days. The CEM C1-15 cell line was not used in fluorescence imaging, as it is inherently resistant to dexamethasone, thus dexamethasone-induced apoptosis. Therefore it would not be a good model system to study apoptosis or the apoptotic-autophagic interaction.

The Hoechst 33342 dye binds DNA and was used to visualize it. It gets excited at 350 nm, and emits a blue/cyan fluorescent light at 461 nm. Annexin V-FITC dye binds to phosphatidylserine and was used here to visualize this protein. Annexin V-FITC gets excited at 488 nm and emits a green light at 520 nm. LysoTrackerRed is a dye that is a weak base, and therefore concentrates in acidic organelles. As lysosomes and autophagolysosomes are acidic, LysoTrackerRed will concentrate within them. It gets excited at 577 nm at emits at 600 nm.
Initially cells were harvested from both the 24-hour time point after treatment as well as the 48-hour time point after treatment. Cells were stained and prepared for fluorescent imaging. After seeing image results of both time points, it was concluded that the 24 hour time point was too early to show significant morphological changes for either process in the majority of cell images. Given this information, this thesis focuses on showing the morphological affects of treatment 48 hours after time zero on cells.

2.5.2 Preparation of Cells for Staining

After cells were plated at a concentration of 4.0 x10^5 / mL, they were treated with their designated drug or drug combination. Ten microliters of each drug were taken and pipetted into a 10mL cell culture suspension. Eight 25cm^2 cell culture flasks (Corning Catalog # 430639) were used, one for each treatment. Cells were stained and imaged 24 hours after treatment and 48 hours after treatment. To get the cells ready for staining, 2.5mL of cells were taken from each flask and put into 15mL falcon tubes. They were then spun at 1,500 rpm for 5 minutes. The supernatant was aspirated and the pellet washed with 500µl of 1x Phosphate Buffered Saline (PBS). The mixture was transferred to microcentrifuge tubes, and spun for 5 minutes at 1,500 rpm. The supernatant was aspirated.

2.5.3 Staining of Cells for Epifluorescence Microscopy

One mL of 1x stock binding buffer was made by taking 200µl of 5x Annexin V binding buffer (Biotium Catalog No: 99902) and mixing with 800µl of sterile water. Then, 750µl of 1x binding buffer was taken and mixed with the following: 15µl LysoTracker Red DND-99 (Invitrogen Catalog No: L7528), 37.5µl FITC-Annexin V (Biotium Catalog No: 99903) and 37.5µl Hoechst 33342 (Biotium Catalog No: 30018C). This total of 840µl was used to stain the prepared cell pellets. The following variations were used throughout the study, but variation 1 was used to take the images used and presented here.
Variations:

<table>
<thead>
<tr>
<th>Variation</th>
<th>1x Binding Buffer</th>
<th>LysoTracker Red DND-99 (100µM) (Biotium)</th>
<th>FITC - Annexin V (50µg/mL) (Biotium)</th>
<th>Hoechst 33342 (Biotium)</th>
<th>Annexin V-FITC (10µg/mL) (ebioscience)</th>
<th>1x Annexin V binding Buffer (ebioscience)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variation 1</td>
<td>750 µl</td>
<td>15 µl</td>
<td>37.5 µl</td>
<td>37.5 µl</td>
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<td>-</td>
</tr>
<tr>
<td>Variation 2</td>
<td>770 µl</td>
<td>15 µl</td>
<td>37.5 µl</td>
<td>18 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Variation 3</td>
<td>-</td>
<td>30 µl</td>
<td>-</td>
<td>15 µl</td>
<td>200 µl</td>
<td>595 µl</td>
</tr>
</tbody>
</table>

Table 2.2: Variations in Fluorescence Staining protocols

Each cell pellet received 100 µl of the 840µl staining solution. Each of the tubes was mixed by slightly shaking it. Then, all the tubes were placed in a dark place for 30 minutes (Variation 3 was stained for 45 minutes). Subsequently, each of the pellets was spun for 5 minutes at 1,500 rpm. The supernatant was aspirated and each pellet was washed with 500 µl of 1x PBS. The samples were spun again at 1,500 rpm for 5 minutes and the supernatant aspirated. Afterwards, each pellet was mixed with 10 µl of 1x binding buffer and was ready to be observed. Each sample was placed on a glass microscope slide, with a cover slip and was observed under the fluorescence microscope.
CHAPTER 3: RESULTS

In order to observe and build upon the existing knowledge of the relationship between apoptosis and autophagy two major techniques were employed in this study. The first was cell treatment with drug agents and subsequent counting of the cells to establish how each drug and drug combination affected the ability of cells to grow. The second was epifluorescence microscopy used to visualize specific morphologies that are hallmark features of apoptosis and autophagy.

The drug agents used in this study included: dexamethasone, rapamycin, 3-methyl adenine, ethanol and DMSO. Dexamethasone is an artificial steroid and is very well known for its potent anti-leukemic ability. It is a glucocorticoid, and as such, it has immunosuppressive properties. Dexamethasone is able to make the CCRF-CEM derived cell line, CEM C7-14, go into apoptotic death and is the inducer of apoptosis in this study. Rapamycin (known as Rapamune/ Sirolimus manufactured by Pfizer) is a drug that has been FDA approved for human use. It is an antibiotic that has immunosuppressant and anticancer activity. It has minimal side effects when used in high doses over a long period of time, and in combination with other immunosuppressant drugs. 37 3-methyl adenine (3MA) or 6-amino-3-methyl-purine, is a compound that is able to inhibit endogenous protein degradation via the autophagic/ lysosomal pathway, without negatively affecting the synthesis of proteins. 38

Each of the drugs was used individually to treat CEM C7-14 cells as well as in combinations with each other as outlined in section 2.4. The use of dexamethasone allowed us to study apoptosis in the CEM C7-14 cells. Rapamycin inhibits the inhibitor mTOR, thus initiates autophagy, hence the reason it was used in this study. 3-methyl adenine was used for its ability to rescue cells from a rapamycin-induced cell death. DMSO and ethanol were used as a control. They were used as the solvent where dexamethasone, rapamycin and 3-methyl adenine were dissolved in.
3.1 Growth Curves of Treated Cells

Figure 3.1: Modulation of CEM C1-15 Cell Growth mediated by Dexamethasone, Rapamycin and 3-Methyl Adenine drug reagents in eight combinations

The graph represents the averages for all eight treatment variations from four separate weekly trials. The dexamethasone-induced death resistant CEM C1-15 cells were treated over four days with the specified drug and drug combinations to elucidate the relationship of apoptosis versus autophagy. The graph represents the averages from four separate weekly trials.
Figure 3.2: Modulation of CEM C7-14 Cell Growth mediated by Dexamethasone, Rapamycin and 3-Methyl Adenine drug reagents in eight combinations

The graph represents the averages for all eight treatment variations from four separate weekly trials. The dexamethasone-induced death susceptible CEM C7-14 cells were treated over four days with the specified drug and drug combinations to elucidate the relationship of apoptosis versus autophagy. The graph represents the averages from four separate weekly trials.
Figure 3.3: The effect of Dexamethasone and Rapamycin on CEM C1-15 cells

Data show that CEM C1-15 cells are resistant to dexamethasone-induced death. Here, when cells cannot die through GR-induced apoptosis, they die through rapamycin-induced autophagy in the long run. The orange line, representing dexamethasone and rapamycin, shows a synergistic effect, where both of these drugs together cause faster cell death than either of them individually. The graph represents the averages from four separate weekly trials.
Figure 3.4: The effect of Rapamycin and 3-Methyl Adenine on CEM C1-15 cells
Data focus on the ability of rapamycin to induce autophagy. They show that the CEM C1-15 cells decrease in number when treated with rapamycin compared to the control, ethanol & DMSO. When 3-methyl adenine is paired with rapamycin, it is able to rescue some cells from the induced rapamycin death, compared to just rapamycin-treated cells alone. Cells that show the least growth and begin dying from treatment day two, are the ones treated with dexamethasone, rapamycin and 3-methyl adenine. In these dexamethasone resistant cells, it is curious to see that when dexamethasone is added to rapamycin and 3MA (green line) compared to rapamycin and 3MA alone (dark blue line), it has a clear effect on the cell number decreasing starting at day 2. This is evidence of a synergistic effect that is taking place between autophagy and apoptosis in these cells. The graph represents the averages from four separate weekly trials.
Dexamethasone is able to induce apoptosis in CEM C7-14 cells. The cell population for the purple line representing dexamethasone decreases from day 2 compared to the control (ethanol & DMSO). Rapamycin does not have a drastic effect on the cell population. The yellow line representing it, correlates with the control, however it is slightly decreased. Both dexamethasone and rapamycin have a stronger effect on the decrease of the cell population.

Here, we can see both apoptosis and rapamycin working together. It looks like dexamethasone affects rapamycin-induced autophagy in a way that results in a greater impact in affecting cell death compared to rapamycin alone. The graph represents the averages from four separate weekly trials.
Figure 3.6: The affect of Rapamycin and 3-Methyl Adenine on CEM C7-14 cells
Data focus on the ability of rapamycin to induce autophagy. Rapamycin treated cells
decrease in number compared to the ethanol and DMSO treated cells (control). Data show
that 3-methyl adenine is able to rescue rapamycin treated CEM C7-14 cells from death. The
graph represents the averages from four separate weekly trials.
3.2 Epifluorescence Imaging Results

3.2.1 Combined fluorescence images of the eight drug treatments at approximately 100 and 400 magnifications

<table>
<thead>
<tr>
<th>CEM C7-14 Cells</th>
<th>Phase Contrast</th>
<th>Annexin V-FITC</th>
<th>Hoechst 33342</th>
<th>Lysotracker Red</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol &amp; DMSO</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Dexamethasone</td>
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<tr>
<td>Rapamycin</td>
<td></td>
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</tr>
<tr>
<td>3MA</td>
<td></td>
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</table>

Figure 3.7: Staining of live CEM C7-14 cells treated with Ethanol & DMSO, Dexamethasone, Rapamycin and 3 Methyl-Adenine using Annexin V- FITC, Hoechst 33342 and LysoTracker Red at approximately 100 Magnification

Blue fluorescent staining of the cell nucleus (Hoechst 33342), green staining of the phosphatidylserine protein on the outer leaflet of the cell membrane (Annexin V- FITC) and red staining of the acidic cellular compartments (LysoTracker Red) are seen here. Each treatment has a series of five images from the same field of view, focusing on visualizing different cellular structures depending on the stain used.
Figure 3.8: Staining of live CEM C7-14 cells treated with combinations of Dexamethasone, Rapamycin and 3 Methyl Adenine as seen on the left of the figure at approximately 100 Magnification. Annexin V-FITC, Hoechst 33342 and LysoTracker Red stains were used. Blue fluorescent staining of the cell nucleus (Hoechst 33342), green staining of the phosphatidylserine protein on the outer leaflet of the cell membrane (Annexin V-FITC) and red staining of the acidic cellular compartments (LysoTracker Red) are seen. Images are from cells treated with the following combinations of drugs: dexamethasone & rapamycin, dexamethasone & 3-methyl adenine, rapamycin & 3-methyl adenine and dexamethasone & rapamycin & 3-methyl adenine. Each treatment has a series of five images from the same field of view, focusing on visualizing different cellular structures depending on the stain used.
Figure 3.9: Staining of live CEM C7-14 cells treated with Ethanol & DMSO, Dexamethasone, Rapamycin and 3 Methyl-Adenine using Annexin V- FITC, Hoechst 33342 and LysoTracker Red at approximately 400 Magnification

Blue fluorescent staining of the cell nucleus (Hoechst 33342), green staining of the phosphatidylserine protein on the outer leaflet of the cell membrane (Annexin V- FITC) and red staining of the acidic cellular compartments (LysoTracker Red) are seen here. Each treatment has a series of five images from the same field of view, focusing on visualizing different cellular structures depending on the stain used. The 400 magnification allows for the visualization in detail, of the changes in the stained cellular structures that occur during apoptosis and autophagy.
Figure 3.10: Staining of live CEM C7-14 cells treated with combinations of Dexamethasone, Rapamycin and 3 Methyl Adenine as seen on the left of the figure at approximately 400 Magnification. Annexin V-FITC, Hoechst 33342 and LysoTracker Red stains were used.

Blue fluorescent staining of the cell nucleus (Hoechst 33342), green staining of the phosphatidylserine protein on the outer leaflet of the cell membrane (Annexin V- FITC) and red staining of the acidic cellular compartments (LysoTracker Red) are seen. Images are from cells treated with the following combinations of drugs: dexamethasone & rapamycin, dexamethasone & 3-methyl adenine, rapamycin & 3-methyl adenine and dexamethasone & rapamycin & 3-methyl adenine. Each treatment has a series of five images from the same field of view, focusing on visualizing different cellular structures depending on the stain used. The 400 magnification allows for the detailed visualization of the changes in the stained cellular structures that occur during apoptosis and autophagy.
3.2.2 Fluorescence images of ethanol and DMSO treated C7-14 cells at approximately 400 magnification

Set I:

**Ethanol & DMSO**

![Fluorescence images of ethanol and DMSO treated C7-14 cells at approximately 400 magnification](image-url)
Set II:

**Ethanol & DMSO**

Figure 3.11 Fluorescent Images of live CEM C7-14 Cells 48 hours after being treated with Ethanol and DMSO approximately 400 Magnification

CEM C7-14 cells were fluorescently labeled with FITC conjugated to Annexin V targeting phosphatidylserine. The cells were also stained with LysoTracker Red, a red fluorescent dye, staining acidic cellular compartments. DNA was targeted with Hoechst 33342.
3.2.3 Fluorescence images of dexamethasone treated C7-14 cells at approximately 400 magnification

Set I:

Dexamethasone

[Images of fluorescence images showing phase contrast, Hoechst 33342, Annexin V-FITC, Lysotracker Red, Merged, and Magnified Merged]
Set II:

**Dexamethasone**

CEM C7-14 Cells at the **24 hour time point**

[Images of phase contrast, Hoechst 33342, Annexin V-FITC, Lysotracker Red, merged, and magnified merged images]

Set III:

**Dexamethasone**

CEM C7-14 Cells at the **48h time point**

[Images of phase contrast, Hoechst 33342, Annexin V-FITC, Lysotracker Red, merged, and magnified merged images]
Figure 3.12: Fluorescent Images of live CEM C7-14 Cells 24 and 48 hours after being treated with Dexamethasone at approximately 400 Magnification

Fluorescent staining of the cell nucleus (blue) and the phosphatidylserine protein on the outer leaflet of the cell membrane (green) is seen here. Only dexamethasone was used to treat these cells, therefore they are an excellent visual example of apoptotic morphology. Set I: (24 hour images) White arrowheads point to the stained condensed nuclei of the cells. Set II: (24 hour images) White arrowheads point out the condensed nuclei that are a result of dexamethasone induced cell death in CEM C7-14 cells. In the top right Annexin V- FITC image, green fluorescent formations can be seen, staining for the phosphatidylserine protein that has translocated from the inner to the outer layer of the cell membrane during the course of apoptosis. White arrowheads point out bright blue condensed nuclei undergoing apoptosis in the magnified merged image. Set III: (48h images) In the Hoechst 33342 image, many condensed nuclei are present, in bright blue color. The Annexin V-FITC image shows evidence of green fluorescent rings, which represent the phosphatidylserine stained by the Annexin V-FITC stain. The merged image is able to show exactly which cells are going through apoptosis by visualization of their bright blue nucleus combined with the green fluorescent ring (white arrows).

3.2.4 Fluorescence images of rapamycin treated C7-14 cells at approximately 400 magnification

Set I:

Rapamycin

Phase Contrast  Hoechst 33342  Annexin V-FITC

Lysotracker Red  Merged

Magnified Merged
Figure 3.13 Fluorescent Images of live CEM C7-14 Cells 48 hours after being treated with Rapamycin at approximately 400 Magnification

CEM C7-14 cells were fluorescently labeled with FITC conjugated to Annexin V targeting phosphatidylserine. The cells were also stained with LysoTracker Red, a red fluorescent dye, staining acidic cellular compartments. DNA was targeted with Hoechst 33342.

Set I: The arrows in the merged image point out two red fluorescence stain areas, thus the location of acidic compartments in two individual cells. Since rapamycin can induce autophagy, these red fluorescence stains can represent the existence of autophagolysosomes, a hallmark structure formed during autophagy. Set II: Red fluorescence staining is clearly seen in the LysoTracker Red image. The magnified merged image shows the location of possible autophagolysosomes marked by the red staining, pointed out by the white arrows.
3.2.5 Fluorescent images of 3-methyl adenine treated C7-14 cells at approximately 400 magnification

Set I:

3MA
Set II:

3MA

Figure 3.14: Fluorescent Images of live CEM C7-14 Cells 48 hours after being treated with 3MA at approximately 400 Magnification.

CEM C7-14 cells were fluorescently labeled with FITC conjugated to Annexin V targeting phosphatidylserine. The cells were also stained with LysoTracker Red, a red fluorescent dye staining acidic cellular compartments. DNA was targeted with Hoechst 33342. Set I & Set II: Both the Lysotracker Red and magnified merged images show minimal red staining, which infers that the 3MA is able to rescue cells from basal autophagic activity.
3.2.6 Fluorescent images of dexamethasone and rapamycin treated C7-14 cells at approximately 400 magnification.

Set I:

**Dexamethasone & Rapamycin**

<table>
<thead>
<tr>
<th>Hoechst 33342</th>
<th>Annexin V-FITC</th>
<th>LysoTracker Red</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Hoechst 33342" /></td>
<td><img src="image2" alt="Annexin V-FITC" /></td>
<td><img src="image3" alt="LysoTracker Red" /></td>
</tr>
</tbody>
</table>

Phase Contrast

Merged
Set II:

**Dexamethasone & Rapamycin**

Figure 3.15: Fluorescent Images of live CEM C7-14 Cells 48 hours after being treated with Dexamethasone and Rapamycin at approximately 400 Magnification.

CEM C7-14 cells were fluorescently labeled with FITC conjugated to Annexin V targeting phosphatidylserine. The cells were also stained with LysoTracker Red, a red fluorescent dye staining acidic cellular compartments. The blue fluorescent stain, Hoechst 33342, visualizes DNA. **Set I:** Green arrows point to the yellow fluorescence, which indicates colocalization of the red (LysoTracker) and green (Annexin-FITC) dyes. **Set II:** The green circles, are highlighting the areas that enclose a cluster of cells that are going through apoptosis and autophagy, as can been seen by the bright blue Hoechst staining as well as the red LysoTracker Red staining. Here, we can see results of both apoptosis (induced by dexamethasone) and autophagy (induced by rapamycin).
3.2.7 Fluorescent images of dexamethasone and 3-methyl adenine treated C7-14 Cells at approximately 400 magnification

Set I:

**Dexamethasone & 3-Methyl Adenine**

![Images of phase contrast, Hoechst 33342, Annexin V-FITC, Lysotracker Red, Merged, and Magnified Merged]
Figure 3.16: Fluorescent Images of live CEM C7-14 Cells 48 hours after being treated with Dexamethasone and 3-Methyl Adenine at approximately 400 Magnification.

CEM C7-14 cells were fluorescently labeled with FITC conjugated to Annexin V targeting phosphatidylserine. The cells were also stained with LysoTracker Red, a red fluorescent dye staining acidic cellular compartments. The blue fluorescent stain, Hoechst 33342, visualizes DNA. **Set I:** The orange arrows indicate the location of two condensed nuclei, showing evidence of apoptosis in the phase contrast, Hoechst 33342 and magnified merged images. The white arrows in the merged image indicate areas where condensed nuclei and the green ring of the phosphatidylserine stained protein exist, both indicating that apoptosis is taking
place. **Set II**: White arrows in both LysoTracker Red and the merged images point to six areas that show faint red staining, possibly indicating autophagosomal formation.

3.2.8 *Fluorescent images of rapamycin and 3-methyl adenine treated C7-14 cells at approximately 400 magnification*  

**Set I:**

**Rapamycin & 3-Methyl Adenine**

![Fluorescent images of rapamycin and 3-methyl adenine treated C7-14 cells at approximately 400 magnification](image-url)
Set II:

**Rapamycin & 3-Methyl Adenine**

Figure 3.17: Fluorescent Images of live CEM C7-14 Cells 48 hours after being treated with Rapamycin and 3-Methyl Adenine at approximately 400 Magnification.

CEM C7-14 cells were fluorescently labeled with FITC conjugated to Annexin V targeting phosphatidylserine. The cells were also stained with LysoTracker Red, a red fluorescent dye staining acidic cellular compartments. The blue fluorescent stain, Hoechst 33342, visualizes DNA.
3.2.9 Fluorescent images of dexamethasone, rapamycin and 3-Methyl adenine treated C7-14 cells at approximately 400 magnification

Set I:

Dexamethasone, Rapamycin & 3-Methyl Adenine

![Phase Contrast](image1)

![Hoechst 33342](image2)

![Annexin V-FITC](image3)

![Lysotracker Red](image4)

![Merged](image5)

![Magnified Merged](image6)
Set II:

Dexamethasone, Rapamycin & 3-Methyl Adenine

Figure 3.18: Fluorescent Images of live CEM C7-14 Cells 48 hours after being treated with Dexamethasone, Rapamycin and 3-Methyl Adenine at 400 Magnification

CEM C7-14 cells were fluorescently labeled with FITC conjugated to Annexin V targeting phosphatidylserine. The cells were also stained with LysoTracker Red, a red fluorescent dye staining acidic cellular compartments. The blue fluorescent stain, Hoechst 33342, visualizes DNA. **Set II:** Within the two circles presented in the above merged image nuclear pyknosis is seen. Red fluorescence staining, indicating possible autophagolysosomal formation, is also seen.
CHAPTER 4: DISCUSSION

4.1 Hypothesis

Autophagy is a process with dual roles. On one hand it can prolong cell survival in a nutrient-poor environment, and on the other hand, it can lead to cell death. Apoptosis however, is a programmed cell death mechanism. These two mechanisms as seen in figure 1.10 live in a constant state of interplay. Depending on the cell stress state and the nutrient availability, each can take over and either keep the cell alive or lead it to death. There are many common players in these processes, as can be seen in figure 1.10. Given the knowledge that many proteins are common to both of these pathways, our hypothesis states that these two processes collaborate together to lead a cell to death. If apoptosis misses its queue to initiate or the cell is resistant to it, autophagy will take over as the cell death mechanism. If autophagy is damaged, apoptosis will be the process through which the cell will die.

4.2 Methodology

We used three main tools to evaluate this relationship further. The first was two human leukemic cell lines, one resistant (CEM C1-15) and one sensitive (CEM-C7-14) to glucocorticoid hormones. The second tool used was three drug reagents, including rapamycin for its ability to induce autophagy, 3-methyl adenine (3MA) for its ability to prevent autophagosomal vesicle formation and dexamethasone for its ability to induce apoptosis in C7-14 cells. Dexamethasone binds to the GR cytoplasmic receptor, and after a conformational change, it translocates in the nucleus, where it arrests the cell cycle in the G0/G1 stage inducing apoptosis. The third tool used was two methodologies to study our cell culture system. These were a trypan blue cell growth assay, and fluorescent microscopy. The cell growth assay allowed us to distinguish between dead or alive cells, giving us an opportunity to quantitate cell growth. Epifluorescence microscopy was used to allow us to delve into the microscopic world of CEM C7-14 cells and help us visualize their morphology, during apoptotic and autophagic conditions.
In the trypan blue cell growth assay, we were able to quantify cell death in both CEM C1-15 and CEM C7-14 cell lines over a period of four days. Regarding cell treatments, we used the combination of ethanol and DMSO for our control as well as seven more combinations, outlined in section 2.4. Figures 3.1 and 3.2 combine data from four separate weekly treatments on CEM C1-15 and CEM C7-14 cells respectively. Averages for each day were taken from the four individual graphs and are shown here (3.1, 3.2). Standard deviations were calculated each day and are represented by vertical bars on each of the graphs. In order to focus on specific relationships that are of interest in this study, specific treatment curves are shown and compared (Figures 3.3-3.6).

In the epifluorescence microscopy assay we used three stains to visualize apoptotic and autophagic morphologies. The first was Hoechst 33342, a blue fluorescent stain, which visualizes DNA. Hoechst 33342 facilitates the visualization of nuclear condensation, apoptotic body formation and cell membrane blebbing. Table 1.1 describes the known morphologies of apoptosis and autophagy in the nucleus, cytoplasm and cell membrane of a cell and ways they are detected. The second stain was FITC-conjugated to Annexin V. This is a green fluorescent stain, which visualizes phosphatidylserine, a phospholipid found in the lipid bilayer of the cell membrane. Phospholipid serine translocates from the inner to the outer leaf of the cell membrane as a result of caspase activation in late apoptotic stages. FITC-Annexin V when binding to phosphatidylserine is seen as a green “halo” like structure. CEM C7-14 cells treated with dexamethasone (induced apoptosis) resulted in apoptotic morphologies we expected to see. Figure 3.12 shows off these apoptotic forms. When dexamethasone treated cell images are compared to the control, (Figure 3.11) there is a great difference between the number of cells that show evidence of apoptosis taking place. Comparing the control to the dexamethasone treated, it is clear that in the second case, cells are visibly going through apoptosis by their condensed nuclei, their cellular blebbing, as well as the green “ring-like” staining of the translocated phosphatidylserine. The third stain was LysoTrackerRed, a red fluorescent stain, which binds to acidic cellular compartments. In this study we used LysoTrackerRed to stain acidic vesicles in the cell to be able to detect and visualize autophagolysosomes (Figure 3.13). It is important to note that LysoTrackerRed is a stain that will stain all acidic compartments including lysosomes and autophagolysosomes. Given that autophagy degrades bulky cytosolic proteins as well as recycles organelles, it is
constantly taking place in the cell. To distinguish between basal autophagy and induced autophagy through rapamycin in this project, the following section is included.

4.3 Autophagosomal Vesicle Presence

When we compare fluorescent control images of CEM C7-14 cells (Figure 3.11), with fluorescent rapamycin-treated images of CEM C7-14 cells (Figure 3.13), it is evident that very little red staining shows up in the control images, meaning that there is a significant visual difference in the amount and intensity of red staining seen between these two treatments. This gives us reason to believe that the increase in red staining is the result of more autophagolysosomes being present, as a result of increased autophagy through the use of rapamycin, and not as a result of lysosomal presence alone. In autophagy, it is established that the autophagosomes fuse with lysosomes, which helps break down their contents by lysosomal enzyme action. The cellular debris formed as a product of the degradation is expelled from the cell by having these vesicles move to the cell periphery and merge with the cell membrane. A visual queue that can let us know that increased autophagy is taking place can be the existence of more of these autophagolysosomal vesicles. As seen in figure 4.1, these vesicles need to come close to the cell surface to be able to fuse with the plasma membrane and expel their contents. This observation has been shown very clearly via transmission electron microscopy, in kidney cells (Figure 4.2).
**Figure 4.1 Vesicular Exocytosis**

Exocytosis mechanism. A vesicle containing debris or other molecules to be secreted comes close to the cell membrane surface and fuses with the lipid bilayer, to allow for its contents to be secreted out of the cell.

**Figure 4.2 Transmission Electron Microscope Images of Autophagic Vesicles in Porcine Kidney Cells**

The black arrows point out lysosomal remnants that consist of multilamellar vacuoles and electron dense deposits. The white arrows indicate autophagic vacuoles containing cellular debris.
Figure 4.3 Autophagolysosomal Vesicles in Rapamycin Treated CEM C7-14 cells

Yellow arrows indicate the corresponding locations of possible autophagolysosomal vesicles in the Phase Contrast and Merged images (Merged image stained with Hoechst 33342, Annexin V-FITC and LysoTrackerRed stains).
Figure 4.4 Autophagolysosomal Vesicles in Rapamycin & 3MA Treated CEM C7-14 cells

Yellow arrows indicate the corresponding locations of possible autophagolysosomal vesicles in the Phase Contrast and Merged images (Merged image stained with Hoechst 33342, Annexin V-FITC and LysoTrackerRed stains).

In this study, we visually see the same observation taking place in figures 4.3 and 4.4 in rapamycin treated cells visualized with fluorescent microscopy. Each of the two white circles (Figure 4.3) encompasses corresponding cells on the phase contrast and fluorescent merged images. The yellow arrows (Figures 4.3 and 4.4) point to the exact location of the autophagolysosomal vesicles. These are examples of visual evidence that autophagolysosomal vesicles are found in the periphery of the cell, stained with the LysoTracker Red, and are observed in higher numbers in autophagy induced cell treatments versus the control treatments (Please also see figures 3.13, 3.15).
4.4 Autophagy and Apoptosis collaborate

The first clause of our hypothesis states that autophagy and apoptosis work together to lead a cell to death. In this study, when autophagy was induced and morphologies showing evidence for this process were seen, apoptotic morphologies were also present in the same cells. We see this in the co-localization of the red and green colors to form yellow fluorescence (Figure 3.15). In figure 3.15, CEM C7-14 cells were treated with dexamethasone and rapamycin, inducing apoptosis and autophagy respectively. The merged images combine the three individual fluorescent images of Hoechst 33342, Annexin V-FITC and LysoTracker Red staining. In Set I, the green arrows point to the yellow fluorescence, which is an indication of colocalization of the red (LysoTracker) and green (Annexin V-FITC) dyes. They show the combination of the condensed nuclei, an apoptotic morphology, with the red staining, an autophagic morphology. An interesting observation is that when CEM C7-14 cells are treated with rapamycin alone, red staining as well as apoptotic morphologies are seen (Figure 4.5). This confirms the clause in our hypothesis stating that these two processes collaborate to lead a cell to death.
Figure 4.5 Rapamycin Treated CEM C7-14 Cells

(a), (b) and (c) images are taken from rapamycin treated (50nM) cells on separate days. All demonstrate that cells that show visible signs of autophagy (Red staining for autophagolysosomes) also show apoptotic characteristics (Green staining for phosphatidylserine binding to Annexin V-FITC).

4.5 Synergistic Effect between Autophagy and Apoptosis

An observation that can be made from the epifluorescence microscopy images as well as the cell-viability treatment growth curves is that there is a synergistic effect that takes place between autophagy and apoptosis. From the images taken of CEM7-14 cells treated with dexamethasone and rapamycin (Figure 3.15) compared to dexamethasone only (Figure 3.12) or rapamycin only (Figure 3.13), we see that the effect of having both together has stronger phenotypic results. Keeping in mind that all images taken were normalized using the same exposure, contrast, saturation and shadow settings in the software used, when a cell is
fluorescing in blue/green (apoptosis) as well as red (autophagy) compared to cells that fluoresce in blue/green (apoptosis) or red (autophagy) alone, it has a stronger staining phenotype (Figures 3.7, 3.8). Similarly in results taken from the cell growth assays, where dexamethasone and rapamycin both are used in cell treatment (Figures 3.3, 3.5), we see the highest death rate. Wirawan and colleagues as well as Kang and colleagues\textsuperscript{44,45} have showed a pathway through which the enhanced apoptotic response can be explained. Specifically, caspases 3 7 and 8 help mediate cleavage of beclin 1, which generates N and C-terminal fragments. These fragments are no longer able to induce autophagy. Then the C-terminal fragments translocate to the mitochondria where they sensitize cells to apoptotic signals. This sensitization lends to an “amplifying loop” as described by the authors, which induces a greatly magnified apoptotic cell death. In this scenario, through the interaction of beclin 1 (a key autophagic player) and caspases (key apoptotic players) the apoptotic response is magnified.

4.6 When Autophagy is inhibited, Apoptosis takes over

To test the premise of the hypothesis, which states that apoptosis takes over the responsibility of cell death when autophagy is inhibited, we treated CEM C7-14, cells with rapamycin and 3MA simultaneously (Figure 3.17). Here, we induced autophagy (rapamycin) but at the same time inhibited it (3MA). We observed that cells in these treatments showed apoptotic morphologies. In this case, when 3MA is inhibiting autophagosome formation, aging and damaged organelles accumulate in the cell. This accumulation can result in damaged mitochondria releasing cytochrome c, Smack/Diablo as well as HtrA2/Omi, and initiating apoptosis (Figure 1.2). Data from our cell growth curves also show that rapamycin is able to induce autophagy in C7-14 cells (Figure 3.6), and 3MA is able to rescue this rapamycin-induced cell death.

Similarly, we know that nutrient and growth factor depletion will initiate autophagy (Figure 1.10). Given this, the fluorescent images were taken 48 hours after initial cell treatment, where a nutrient depletion would have been underway. However, since autophagy was inhibited (3MA), we see that apoptosis is taking a leading role in cell death. These observations lend evidence to show that apoptosis will take control of a cell that needs to die.
when autophagy is inhibited. Similarly, we also treated CEM C7-14 cells with 3MA to inhibit the basal autophagic activity, and see whether we see phenotypic evidence of apoptosis taking place in these cells (Figure 3.14). In set I of figure 3.14, there is one cell showing evidence of chromatin condensation and membrane blebbing, however most other cells in Set I and in Set II of this figure do not exemplify signs of apoptotic behavior. We expected to see more apoptotic signs in cells treated with 3MA compared to cells treated with rapamycin and 3MA. This is because 3MA when combined with rapamycin would have the role of stopping the formation of many more autophagosomes (induced by the inhibition of mTOR) compared to the housekeeping autophagic response. However, we see that 3MA was effective in diminishing the autophagic response when combined with rapamycin, leading the cell to accumulate damaged organelles including mitochondria, which can have initiated the intrinsic apoptotic pathway.

4.7 When Apoptosis is inhibited, Autophagy may take over

To test the premise of the hypothesis clause that writes that autophagy can take the responsibility of cell death when apoptosis is inhibited, we used CEM C1-15 cells to see whether this was true. Given that this cell line is resistant to dexamethasone-induced apoptosis, we treated cells with dexamethasone, rapamycin as well as both dexamethasone and rapamycin together (Figure 3.3) to see the emerging relationships. From these data we can see that dexamethasone does not cause apoptotic cell death, as expected. Rapamycin’s growth curve compared to the control shows a decrease in cell number, showing evidence of autophagy may be taking place and cells diminishing in number. From the data collected here, our hypothetical premise that autophagy takes over in a system where apoptosis cannot or is defective, may be supported. Future experiments can include taking fluorescent images of C1-15 cells, treated with dexamethasone, rapamycin, 3MA and combination of these to see possible phenotypic evidence of autophagy playing a protagonistic role in cells resistant to dexamethasone-induced apoptotic death.
4.8 Interesting role for 3MA

Figure 3.16 shows CEM C7-14 cells treated with both dexamethasone and 3MA. Here, we can see nucleic condensation and Annexin V-FITC staining, both being apoptotic morphologies. However, these phenotypes are not as pronounced as when dexamethasone is the only treatment drug. The implication being that 3-MA is inhibiting endogenous protein degradation (through inhibiting autophagy) and in parallel, also dimming the apoptotic response of dexamethasone on these cells. Interestingly, both the summary graphs from the cell growth treatment curves for CEM C1-15 and CEM C7-14 cells show that when 3MA is added to rapamycin and dexamethasone combined, the cell number decreases (Figures 3.1 and 3.2). Specifically in the CEM C1-15 cell cultures (Figure 3.1), all the growth curves have an upward trend, but the one that shows the least growth is the green line representing all three treatments (dexamethasone, rapamycin and 3MA). In CEM C7-14 cultures (Figure 3.2), we observe a more dramatic downward slope for the line representing all three treatments. However, when we include epifluorescence microscopy data for C7-14 cells treated with all of the above drug treatments (Figure 3.18), cells are morphologically in a very similar state between all three treatments used compared to rapamycin and dexamethasone alone.

3MA shows evidence of being able to increase the cell death rate when used in conjunction with dexamethasone and rapamycin. We know that 3MA is able to inhibit the PI3K activity of the Beclin 1-hVps34 complex, thus stopping autophagosomal formation. By doing this, autophagy through beclin 1 is inhibited, resulting in cells not being able to survive longer by breaking down organelles into needed building blocks. Thus, the cells would quickly die via dexamethasone-induced apoptosis. Another hypothesis can be that dexamethasone, being a glucocorticoid, might interfere with 3MA’s function as it can play a role in cytosolic signaling pathways, including the activation of PI3Ks. If it does interfere, 3MA is not able to inhibit autophagy and cells die both from induced apoptosis as well as autophagy.
4.9 Experimental Limitations

One of the goals of this project was to be able to have visual evidence that autophagy is taking place. Here, LysoTracker Red, a red fluorescent dye is used to stain the autophagolysosomal vesicles that formed. This stain is a general stain that is capable of staining any acidic organelle. Given this, it is not possible to distinguish a lysosome from an autophagosomal vesicle forming. In the future, it would be helpful to use a different system that allows for more specificity of staining between the acidic organelles.

A hemocytometer was used in this project to count cells between the various treatments within the different trials. The hemocytometer takes a sample of the original cell culture, and within this sample another smaller sample is taken and counted with the help of trypan blue. Error is inherent in the pipetting performed throughout the cell counting process. It is also inherent in the process of taking and counting a sample of the cell culture in the flask, and using that number to estimate the whole cell culture cell number. Human error also exists in the decision to define a cell as alive or dead by the use of color in this trypan blue cell counting assay. In the future, an automated cell counting method can be used to reduce human error, improve consistency, as well as reduce cell-counting time per counting trial.

4.10 Future Experiments

Experiments in the future should include the use of the confocal microscope to image the treated cells. This type of microscope has a much greater capacity in eliminating the out of focus glare in images and greatly improve their clarity. It also has an advantage, the ability to take a z-series of images, which is a sequence of optical sections collected at different levels that are perpendicular to the optical axis or z-axis within the specimen. This z-series can be further processed into a 3-D representation of the specimen using volume visualization techniques. The ability of the confocal microscope to perform this function gives it a great advantage in visualizing the 3-dimensional structure of the specimen. In the case of this study, this function could be used to give more depth and definition for the cell organelles, especially the autophagosomal structure, whose clear visualization is very important in this study.
This project used 3-MA to rescue cells from rapamycin-induced death through autophagy. In the literature 3-MA has been commonly used and characterized as an agent that is capable of this function. In the context of this project however, the data have not always shown that 3-MA is able to rescue cells from an autophagical phenotype. In the future, it will be helpful to use a higher concentration of 3-MA to 5mM to accomplish cell rescue from rapamycin or use an entirely different drug agent that has a similar function like Wortmannin and LY 294002.
REFERENCES:


2. Lamkanfi M and Dixit M.V. Manipulation of Host Cell Death Pathways during Microbial Infections. Cell Host & Microbe 8, July 22nd, 2010


5. Asciolla JJ, Renault TT, Chipuk JE. Examining bcl-2 family function with large unilamellar vesicles. J. Vis Exp. 2012 Oct 5; (68)


34. Fleming A, Noda T, Yoshimori T, Rubinszttein DC. Chemical modulators of autophagy as biological probes and potential therapeutics. Nature Chemical Biology 7, 9-17 (2011)


36. Alberto M. Martelli, Francesca Chiarini, Camilla Evangelisti, Alessandra Cappellini, Francesca Buontempo, Daniela Bressanin, Milena Fini and James A. McCubrey. Two hits are better than one: targeting both phosphatidylinositol 3-kinase and mammalian target of


autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. Cell Death and Disease Jan 2010 1, e18


46. Feifan Zhou, Ying Yang, Da Xing. Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis. The FEBS Journal Nov 2010. Review


