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REVIEW

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Regulation of Apoptosis by Autophagy to Enhance Cancer Therapy

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In cancer therapy, a principle goal is to kill cancer cells while minimizing death of normal cells. Traditional cytotoxic therapies and the newer agents that target specific signaling proteins that are critical for cancer cell growth do this by activating a specific type of programmed cell death – apoptosis. However, it has been well established that cancer cells have varying levels of responses to apoptotic stimuli, with some being close to an "apoptotic threshold" and others being further away and that this ultimately determines whether cancer therapy is successful or not. In this review, we will highlight how the underlying mechanisms that control apoptosis thresholds relate to another important homeostatic process in cell survival and cell death, autophagy, and discuss recent evidence suggesting how inhibition of autophagy can enhance the action of anti-cancer drugs by modulating the apoptotic response.

INTRODUCTION

Cell fate decisions, including perhaps the most basic decision of whether to live or die, are important in both health and disease. A complex system of checks and balances regulates these decisions, ensuring that cells are viable and healthy when needed, but undergo cell death when their function has been met or they have been damaged beyond repair [1]. The appropriate timing of cell death is therefore a crucial component of healthy biological function. As tissues develop and mature, cell death pathways are recruited to maintain normal homeostasis. Premature activation of cell death pathways can lead to degenerative conditions such as Alzheimer's dementia [2] or cirrhosis of the liver [3]. Conversely, loss of activity of cell death pathways can lead to diseases of excess cells such as cancer or autoimmune disease [4,5]. Cell death is also important during treatment of disease. For example, in cancer treatment, the main goal is to kill a patient's cancer cells without killing too many of their normal cells.

Multiple cell death pathways have been characterized. In some cases, an environmental insult or exposure to a toxic substance promotes unexpected or accidental

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†Abbreviations: AMBRA, Activating molecule in BECN1-regulated autophagy protein 1; AMPK, 5'AMP Activated Protein Kinase; APAF1, apoptotic protease activating factor-1; ATG, Autophagy related genes; BCL2, B Cell Lymphoma; BH, BCL-1 homology; CNS, central nervous system; EGF, Epidermal growth factor; FIP200, Focal Adhesion Kinase Family Interacting Protein of 200kd; FHRE, Forkhead Response element; FOXO, forkhead homeobox type O; FOXO3a, Forkhead box class O 3a; GABARAP, Gamma-aminobutyric acid receptor associated protein; HCQ, Hydroxychloroquine; MOMP, Mitochondrial Outer Membrane Permeabilization; MTOR, mammalian Target of Rapamycin; PUMA, p53 upregulated modulator of apoptosis; STX17, syntaxin 17; RUBICON, Run domain Beclin-1 interacting and cysteine-rich domain containing protein; TFEB, transcription factor EB; TRAIL, Tumor Necrosis Factor Related Apoptosis Inducing Ligand; ULK1, Unc51-like kinase-1; VPS34, Vacuolar protein sorting-associated protein.

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cell death. However, the vast majority of cell death events in normal biology, disease and the treatment of disease occurs by one or another type of programmed cell death. In these processes, specific machinery within the cell is recruited to terminate its existence in an orderly, controlled fashion. The best described type of programmed cell death is apoptosis [6].

Autophagy (from the Greek for "self-eating") is another important homeostatic process important in health and disease. Autophagy serves as the cell's recycling system by delivering cellular material to the lysosome for degradation, especially damaged intracellular proteins and organelles, with subsequent reuse of the constituent macromolecules for other processes [7]. The machinery regulating autophagy interacts closely with the cell death machinery allowing for significant and dynamic interplay between these processes [8]. In this review, we focus on how the interplay between apoptosis and autophagy can be leveraged to improve cancer therapy.

OVERVIEW OF APOPTOSIS AND ITS RELATIONSHIP TO CANCER THERAPY

Apoptosis is usually divided into intrinsic and extrinsic pathways, which ultimately converge to activate effector caspases, most importantly caspases 3 and 7 [9]. The extrinsic pathway is dependent upon the binding of death receptors of the Tumor Necrosis Factor receptor family to their ligands, which triggers intracellular cascades driven by various protein interactions that activate caspase 8 then ultimately caspase 3 and 7, the final "executioners" of apoptosis. The intrinsic pathway is controlled by a complex interplay of signals that ultimately cause Mitochondrial Outer Membrane Permeabilization (MOMP) and release of cytochrome c (and other proteins) into the cytoplasm. Cytochrome c binds to apoptotic protease activating factor-1 (APAF1). This leads to conformational change and oligomerization of APAF-1 into a heptameric structure known as the apoptosome. The apoptosome promotes aggregation and cleavage of proscaspase 9 into active caspase 9. Caspase 9 in turn activates caspase 3 and 7 which execute the irreversible final steps of apoptosis. MOMP is usually the rate limiting step that determines whether the cell commits to die.

The decision to undergo MOMP is driven by interactions between the B Cell Lymphoma 2 (BCL-2) family of proteins [10]. Family members are characterized based on their degree of homology with domains first identified in BCL-2 called BH (BCL-2 homology) domains [11]. Anti-apoptotic BCL family proteins share all four characteristic BH domains and include BCL-2, BCL-xL, BCL-w, MCL-1, and BFL-1. The pro-apoptotic proteins are divided into two subfamilies. One contains only the BH3 domain and include BIM, BIK, BID, NOXA, and PUMA. These BH3-only proteins serve as activators of the intrinsic apoptotic pathway by detecting signals that tell the cell to commit to die. The third subfamily consisting of BAX, BAK, and a less well studied protein called BOK are the effectors of apoptosis and are defined by the presence of BH1-3 domains. The BH3-only proteins have a consensus sequence that binds to complementary hydrophobic regions in BAX and BAK [12]. This binding promotes homo-oligomerization of BAX and BAK, forming a pore complex in the outer mitochondrial membrane and thus MOMP. These events are countered by the anti-apoptotic members of the family, which generally exhibit their effects by inhibiting the interactions between the BH3-only proteins and BAX/BAK [13].

The balance of interactions of pro- and anti-apoptotic factors within a cell determines how close it is to its apoptotic threshold. Why might this be important in cancer therapy? Most cancer therapies (traditional cytotoxic agents such as DNA damaging agents and so-called "targeted therapies" like kinase inhibitors) ultimately work by inducing tumor cell apoptosis following MOMP. A successful therapeutic agent is one that efficiently kills cancer cells while sparing that of normal cells, a concept that relies on the feature that different cell types have disparate apoptotic thresholds [14]. The ideal therapy is able to push cancer cells over their apoptotic threshold while avoiding this same toxicity in healthy, normal cells. In pharmacology, this is the concept of the therapeutic window, *i.e.* the ability of a drug to have a beneficial effect without an unacceptable level of toxicity. In cancer treatment, the therapeutic window is the difference between apoptotic thresholds for normal cells versus cancer cells and this, in turn, is defined by the overall interplay between the pro- and anti-apoptotic members of the BCL2 family. These interactions can be directly measured in cancer cells using a technique called BH3 profiling [15]. Indeed, the relative level of so-called BH3 priming (*i.e.* how close cancer cells are to their apoptotic threshold as measured by BH3 profiling) can predict clinical responses to anti-cancer treatments [16,17] and explain different levels of normal tissue toxicities in cancer therapy [18].

This concept also explains an often misunderstood question in cancer pharmacology– why is it ever possible to successfully treat cancer, particularly with treatments that target molecules such as DNA? Cancer is defined by several, well characterized hallmarks such as failure to respond to growth suppressing signals, ability to undergo continuous cell division and invade other tissues, etc. One such hallmark is resistance to cell death [4]. A common misunderstanding is that this hallmark means that cancer cells must be harder to kill than normal

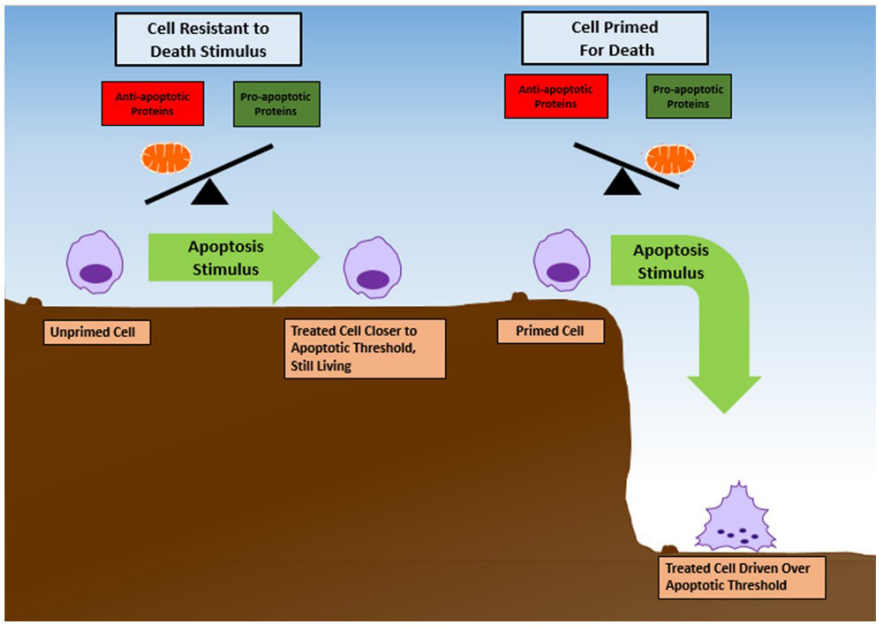

Figure 1. Schematic representation of the effects of apoptosis inducing stimuli, such as cytotoxic chemotherapy, on cancer cell death. Some cells are "primed" for apoptosis (right side). These cells have a balance of bcl-2 proteins that favor apoptosis. In the face of a stimulus, these cells are driven over the apoptotic threshold and are committed to cell death. Another population of cells is more resistant to apoptosis inducing stimuli. These cells have a balance of bcl-2 proteins that favors protection against apoptosis. In the face of apoptotic stimuli, these cells are moved closer to the threshold, but remain viable in response to treatment.

cells. In fact, the opposite is true. Pathways commonly implicated in oncogenesis, such as Myc or Epidermal Growth Factor (EGF), can promote proliferation and tumor growth, but also prime the apoptotic machinery, making the cell more vulnerable to death signals [19]. Additionally, the accumulation of damaged cellular components and environmental stressors that cancer cells are commonly exposed to further push those cells closer to their apoptotic threshold. The net result is a dysregulated cell that may be growing in a tumor but is also teetering on the edge of death. This is born out in BH3 profiling studies that have shown that cancer cells are generally closer to their apoptotic threshold than corresponding normal cells [14] (Figure 1). Thus, even drugs that target molecules that are equally important in both normal cells and cancer cells (such as DNA) can have a useful therapeutic window and make for a successful therapy. These mechanisms raise an obvious question – is there a way to manipulate these processes and thus improve cancer treatment by moving the apoptotic threshold so it's even easier to induce tumor cell apoptosis? One way to do this is with drugs that mimic the actions of the BH3 proteins [11]. However, there may be another even more general way to do this – by manipulating another important homeostatic process, autophagy.

AUTOPHAGY AND CELL DEATH

Autophagy is the process that delivers cellular material to the lysosome resulting in the degradation of intracellular proteins and organelles, and subsequent release of constituent macromolecular precursors such as amino acids for reuse in metabolism or to make new macromolecules [7,20]. Several subtypes of autophagy exist, including macroautophagy (herein referred to as "autophagy"), microautophagy, and chaperone mediated autophagy. Autophagy was originally described morphologically in the 1950s and the term "autophagy" was first coined to describe this process in the 1960s, though for decades the molecular nature and physiological role of the process remained elusive [21]. It was not until the 1990s, that Dr. Ohsumi *et al*. began to characterize the genetic machinery involved in autophagy. Studying autophagy deficient mutants in yeast, he was able to characterize the first autophagy related genes (ATGs). He also identified the important phenotype that autophagy deficient yeast survived in normal conditions, but perished in the presence of nitrogen starvation [22]. This led to an important understanding of one of the most important physiological roles of autophagy that has persisted over years and subsequently expanded to mammalian systems: autophagy is crucial in promoting survival in stress conditions.

As our understanding of the complexity of the genetic basis of autophagy has grown over the years, so has our understanding of the complexity of its physiological roles. Autophagy plays an important role in homeostatic functions of healthy cells, occurring at a basal level in all mammalian cells [23]. This is evident by the antineoplastic effects of autophagy in protecting against cancer development. Thus, by promoting clearing of damaged organelles or proteins, it is thought that the cell reduces the likelihood of DNA damage and subsequent neoplasia [24]. Consistent with this, mouse knockouts where autophagy genes are inactivated show increased development of liver and pancreatic pre-cancerous lesions *in vivo*, illustrating the protective nature of autophagy in certain situations [25,26].

Nevertheless, it is also well established that autophagy can be maladaptive in certain diseases, especially cancer [23]. As mentioned above, autophagy often helps promote cell survival in the presence of nutrient scarcity. Cancer cells hijack this process to allow them to grow and thrive in the harsh, nutrient deplete tumor microenvironment [24]. Certain cancer types, such as pancreatic cancer, are highly dependent on autophagy for their growth and survival [27]. Furthermore, autophagy is implicated in the development of a metastatic phenotype [28]. Thus, the current consensus is that, while autophagy may serve to prevent tumor initiation and early steps in cancer development, in many cases, fully developed tumors may rely on autophagy to promote their continued survival and growth.

A similarly complex picture is seen when we consider the relationship between autophagy and cell death pathways, with both pro- and anti-death effects of autophagy. The term "autophagic cell death" has been used in the biomedical literature for years. However, this is primarily a descriptive term, used to describe a morphological phenotype where cells are dying but lack the chromatin condensation typical of apoptosis, instead having extensive cytoplasmic vacuolization and formation of structures similar to autophagosomes [29]. Historically, this label has often been used to reference this phenotypic appearance, rather than the specific involvement of autophagic machinery in the death process. This often leads to misinterpretation and confusion [30]. Current recommendations suggest the term "autophagic cell death" should only be used if it can be shown that specific targeting of the autophagy pathway, whether genetic or pharmacologically, abrogates the cell death response in question and that death occurs without involving other programmed death mechanisms such as apoptosis [29].

There are limited examples of true "autophagy dependent" cell death. One type is "autosis." First characterized in 2013, this describes a morphologically distinct type of death that occurs in the presence of high degrees of autophagic stimulation. At a molecular level, modulation of apoptosis or necrosis pathways did not affect death, though knockdown of autophagy genes did mitigate cell death [31]. In normal physiology, autophagy is essential for cell death and involution during organogenesis in *Drosophila* [32]. Still, even in this context, autophagy may often be working by enhancing other types of death rather than by killing cells on its own. For example, in some developmental contexts, autophagy is responsible for the degradation of the inhibitor of apoptosis dBruce, thus making the cell more vulnerable to apoptotic stimuli [33]. However, in most examples, the interplay of autophagy and the cell death machinery still remains poorly understood.

In summary, the landscape of autophagy is quite broad and variable especially as it relates to cancer. However, in many cancers, autophagy is a maladaptive process that helps the cancer cells survive. And, although this is not universal and the context that autophagy is occurring in must always be considered when evaluating it, in the right circumstances, autophagy may serve as a unique therapeutic target. To understand how this is achieved it is first necessary to consider how autophagy works.

THE MACHINERY OF AUTOPHAGY

Autophagy is a complex, stepwise process involving numerous protein complexes encoded by over 30 different evolutionarily conserved autophagy related genes. Extensive work has been performed to better understand the process in mammalian cells [7]. Autophagy originates when membranes donated by various intracellular organelles facilitate development of an incomplete vesicular membrane known as the phagophore. The autophagic machinery then promotes elongation of this membrane into the fully enclosed, double-membraned autophagosome. In the final step of autophagy, the autophagosome fuses with the lysosome to form the autophagolysosome. Therein, contents are degraded by the acid dependent hydrolases of the lysosome and the component macromolecules are exported to the cytosol for reuse by the cell [34] (Figure 2).

Each step is carried out by a characteristic complex of proteins. In mammalian cells, initiation occurs primarily around the endoplasmic reticulum, though other locations can also serve as initiation sites for autophagosomes leading to the use of alternate membrane sources and potentially causing different cargos to be targeted. The initiation complex in mammalian cells involves Unc51-like kinase-1 (ULK1) and ULK2, two highly homologous protein kinases along with Focal Adhesion Kinase Family Interacting Protein of 200kd (FIP200),

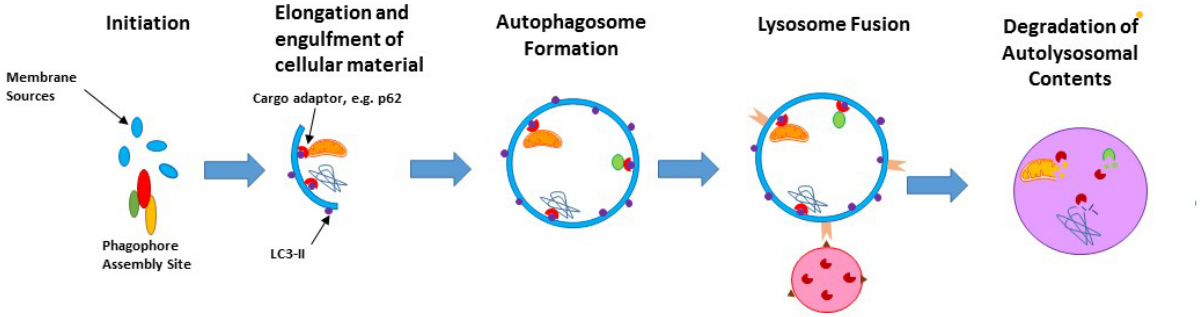

Figure 2. Diagram illustrating the essential steps in the autophagy pathway. Initiation begins with the formation of the phagophore membrane, receiving membrane from various donor sources within the cell. The membrane continues to elongate, recruiting cellular material marked for consumption as it grows. Adaptor proteins, such as p62, are responsible for binding material marked for degradation. These adaptor proteins also bind to proteins embedded within the forming phagophore, such as LC3-II, promoting sequestration of this material. Eventually the membrane fuses to form a completed autophagosome, containing cargo marked for degradation. The lysosome then fuses with the autophagosome, forming the autophagolysosome. The acidic hydrolases degrade the luminal contents of the autophagolysosome, and the constituent macromolecules are exported to the cytosol for reuse by the cells.

ATG13, and ATG101 [35], to create a complex that integrates signals from various pathways and initiate autophagosome formation. For example, in states of glucose or nitrogen (amino acid) deprivation, the 5'AMP Activated Protein Kinase (AMPK) pathway is activated. Active AMPK phosphorylates and activates ULK1 to stimulate autophagy initiation.[36] Conversely, in states of nutrient abundance, the mammalian Target of Rapamycin (MTOR) inhibits the interaction between AMPK and ULK1, thus inhibiting autophagy [36].

The ULK complex recruits the Vacuolar protein sorting-associated protein (VPS34) complex, which is itself responsible for the trafficking of intracellular membrane sources that lead to development and elongation of the phagophore [37]. The VPS34 complex contains VPS34, a class III PI-3 kinase, as well as several regulatory proteins including Beclin-1, ATG14, activating molecule in BECN1-regulated autophagy protein 1 (AMBRA) and Run domain Beclin-1 interacting and cysteine-rich domain containing protein (RUBICON). This complex orchestrates delivery of intracellular membranes to promote elongation into the eventual autophagosome with delivery of membrane material mediated by ATG9. Two protein conjugation systems are also involved in autophagosome formation, the ATG12 and ATG8 systems [38]. In mammalian cells the relevant ATG8s are members of the Gamma-aminobutyric acid receptor associated protein (GABARAP) family of proteins and microtubule-associated protein light chain 3 (LC3) family. The ATG12 conjugation system ligates ATG5 and ATG12 using ATG7 (which serves as an E1 enzyme for the conjugation) and ATG10 (the E2 enzyme) in a manner that is similar to the way that Ubiquitins are conjugated to proteins. The ATG12~ATG5 complex then interacts with ATG16, forming the ATG12~ATG5-ATG16 complex. ATG8/LC3/GABARAP conjugation involves proteolysis of the target protein by a specific protease (ATG4) followed by attachment of a lipid, phosphatidylethanolamine (PE). Again, the mechanism is similar to ubiquitin conjugation [39] with ATG7 again serving as an E1 like enzyme, ATG3 as the E2 and the ATG12~ATG5-ATG16L complex functions as an E3 like enzyme to catalyze coupling of PE to the ATG8 protein. Conjugation of the LC3/GABARAP family is often used as a way to assess and measure autophagy [40] and is functionally important for the process. LC3 and GABARAP proteins help in sequestering organelles and macromolecules that are selectively targeted via adaptor proteins (*e.g.* Sequestom 1, SQSTM 1, commonly known as p62) for degradation by autophagy [41]. PE-conjugated LC3 proteins are also critical in autophagosome trafficking and fusion with the lysosome [42] and necessary for degradation of the inner autophagosome membrane to allow access of lysosomal hydrolases to the material that was engulfed in the autophagosomes [38]. Fusion with the lysosome is also controlled by other proteins including ATG14, the SNARE protein syntaxin 17 (STX17), and the Rab family of GTPases.

As noted above, this complicated cellular machinery is controlled by acute signaling events – *e.g.* when the MTOR pathway is altered to affect phosphorylation of the components of the ULK complex. For the most part, this explains how cellular stresses lead to acute changes in the amount of autophagy – *e.g.* in response to starvation or exposure to specific drugs. In addition, autophagy is controlled by various transcription factors including the transcription factor EB (TFEB) family and

members of the forkhead homeobox type O (FOXO) family [43]. Regulation of the rate of autophagic flux by these transcriptional mechanisms is important but is not as responsive to rapid changes as the acute signaling events discussed above. Instead, alterations in the transcriptional regulation of autophagy are thought to be responsible for stable differences in the basal rate of autophagy between cells types or in different tissues. For example, sustained activation of the TFEB family of transcription factors causes high levels of autophagy in pancreatic cancer cells [44].

AUTOPHAGY AS A THERAPEUTIC TARGET IN CANCER

The modern era of cancer therapy is defined by the idea that we should have a rational basis for our therapies. Thus *in vitro* and *in vivo* preclinical models have elucidated specific signaling pathways that are overactive in specific tumor types and which are then targeted as a treatment for the disease. In this paradigm, so called "targeted" agents directed against the underlying biology of the tumor can be used to sensitize cancer cells to apoptosis induction from traditional cytotoxic chemotherapies.

In the past few years has shown that certain tumors are highly dependent on autophagy. Chloroquine (CQ), originally marketed as an antimalarial drug, is an inhibitor of lysosome function and treatment with CQ can thereby prevent fusion of the autophagosome with the lysosome [45]. This drug and a closely related molecule Hydroxychloroquine (HCQ) has been used clinically for decades to treat infectious and rheumatologic conditions and is well tolerated. The availability of a well-tolerated autophagy inhibitor poses an opportunity to test if autophagy inhibition is useful in cancer. Indeed, based on extensive preclinical studies showing that autophagy inhibition can enhance cancer treatment, especially in combination with other agents, dozens of clinical trials have been developed to target autophagy in cancer by testing CQ and HCQ [46] (Table 1). These trials (and the pre-clinical studies that led to them) have tested many kinds of drugs including standard cytotoxic chemotherapy that ultimately targets DNA, the cytoskeleton or core metabolic pathways, as well as various "targeted" agents including kinase inhibitors, epigenetic regulators, proteasome inhibitors, and many others [47-49]. In addition to the quinacrine derivatives already in use, such as chloroquine, more specific inhibitors of lysosome function have been identified [50]. Inhibitors of other components of the autophagy machinery such as ULK1 [51] and ATG4B [52] are also currently being used preclinical studies.

A beneficial effect of autophagy inhibition has been observed in several models. One important example is KRAS mutated pancreatic cancer. KRAS mutations are present in > 90 percent of pancreatic adenocarcinomas and cause significant dysregulation of a number of intracellular signaling pathways including the MEK/ERK pathways as well as the PI3/AKT/mTOR pathways [53]. It has been well described that KRAS mutated pancreatic cancer is associated with higher levels of autophagy, which is particularly important for tumor cell survival through a variety of mechanisms [27,54,55]. Despite significant evidence that KRAS and MEK activity are pathogenic in these tumors, there has been minimal response to MEK inhibition. Recently it was identified that MEK inhibition leads to increased activity in the LKB1/AMPK/ULK1 pathway, which upregulates autophagic activity [56]. Indeed, autophagy is further increased following treatment with MEK inhibitors, and combination therapy with MEK inhibitors and HCQ was recently shown to have significant impact on pancreas cancer *in vitro* and *in vivo* xenografts as well as in a patient [56,57].

Encouraging results have been seen on other tumor types too. For example, a subset of pediatric central nervous system (CNS) tumors are characterized by BRAF V600E mutations. Vemurafenib is a BRAF inhibitor used clinically to treat BRAF mutated malignancies including melanoma and thyroid cancer and has also been used to treat CNS tumors with the mutation as well. However, as is common with kinase inhibitors, the efficacy of the drug is limited by development of resistance. Initial *in vitro* data suggested that human CNS tumors with a BRAF V600E mutation were more reliant on autophagy compared to wildtype tumors [58]. Thus, treatment with CQ or genetic inhibition of autophagy by knocking down different ATGs had no significant effect on survival and viability on BRAF wildtype cells, but had a dramatic impact on survival in BRAF mutated cells. Moreover, both CQ treatment and genetic inhibition of autophagy could increase tumor cell killing by other drugs including both standard cytotoxic chemotherapy drugs and vemurafenib. Importantly, this benefit translated into improved outcomes for a patient as well [58]. Our case study showed dramatic response to combination therapy with vemurafenib and CQ. The patient had a previous response to therapy with vemurafenib and vinblastine but eventually developed disease progression on therapy with increase in size of her CNS lesions and associated neurological deficit. Following this treatment failure, it was decided to use combination therapy with CQ to attempt to potentiate the effect of vemurafenib. The patient had rapid improvement in the neurologic deficits accompanied by decreased inflammatory signal and stabilization of intracranial CNS lesions. For a period of time, this patient had to stop

Table 1. Current active trials obtained from clinicaltrials.gov search on August 18, 2019 [67].

Tumor Type	NCT Number	Anticancer Therapy	Autophagy Inhibitor	Molecular Marker (if applicable)
Advanced Solid Tumor	NCT01266057	Vorinostat, sirolimus	HCQ	
Advanced Solid Tumor	NCT01023737	Vorinostat	HCQ	
Advanced Solid Tumor	NCT00813423	Sunitinib	HCQ	
Advancer Solid Tumor	NCT01480154	MK2206 (AKT inhibitor)	HCQ	
Breast Cancer	NCT03774472	Palbociclib, letrozole	HCQ	Estrogen Receptor +, HER2 Negative
Breast Cancer	NCT03400254	Gedatolisib	HCQ	
Breast Cancer	NCT03032406	Everolimus	HCQ	
Colorectal Cancer	NCT02316340	Vorinostat	HCQ	
Colorectal cancer	NCT03215264	Entinostat, regorafenib	HCQ	
Glioblastoma Multiforme	NCT03008148	Temozolomide, radiation	Siroquine	
Glioblastoma Multiforme	NCT03243461	Temozolomide, valproic acid	CQ	
Glioblastoma Multiforme	NCT02378532	Radiation, temozolomide	CQ	
Glioblastoma Multiforme	NCT02432417	Standard Treatment	CQ	
Hepatocellular Carcinoma	NCT03037437	Sorafenib	HCQ	
Hepatocellular Carcinoma	NCT02013778	Chemo-embolization	HCQ	
Melanoma	NCT03754179	Dabrafenib, trametinib	HCQ	BRAF V600E mutant
Melanoma	NCT02257424	Dabrafenib, trametinib	HCQ	BRAF V600E/V600K mutant
Melanoma	NCT03979651	Trametinib	HCQ	Activating NRAS mutation
Non-small Cell Lung Cancer	NCT01649947	Paclitaxel, carboplatin, bevacizumab	HCQ	
Non-small Cell Lung Cancer	NCT00977470	Erlotinib	HCQ	EGFR mutation
Osteosarcoma	NCT03598595	Docetaxel, gemcitabine	HCQ	
Ovarian Cancer	NCT03081702	Itraconazole	HCQ	
Pancreatic Cancer	NCT01506973	Gemcitabine	HCQ	
Pancreatic Cancer	NCT01494155	Capecitabine, Radiation	HCQ	
Pancreatic Cancer	NCT03825289	Trametinib	HCQ	
Pancreatic Cancer	NCT01506973	Gemcitabine, abraxane	HCQ	
Prolactinoma	NCT03400865	Cabergoline	HCQ/CQ	
Prostate Cancer	NCT03513211	Itraconazole	HCQ	
Renal Cell Carcinoma	NCT01550367	IL-2	HCQ	
Small Cell Lung Cancer	NCT02722369	Gemcitabine, carboplatin, etoposide	HCQ	

taking vemurafenib but remained on CQ. This too led to increased tumor growth and disease progression. Most importantly, when she was again treated with the combination of vemurafenib plus CQ, her tumor regressed, neurological deficits were again reduced and continued tumor control was maintained for more than two years [58]. This case study has very interesting implications. It showed that the addition of an autophagy inhibitor, CQ, could make another drug (the BRAF inhibitor vemurafenib) more effective. Perhaps even more importantly, it implies that autophagy inhibition with CQ is not only capable of making an active drug better, it can

actually overcome the acquired resistance that occurs when a kinase inhibitor stops working. This last point was directly tested in a follow up paper [59] where we found that multiple, molecularly distinct, mechanisms of resistance to the BRAF inhibitor could be overcome by both genetic and pharmacological inhibition of autophagy. Again, these effects were also shown to lead to clinical improvement in patients [59]. Thus, in brain tumors and also now in pancreas cancer, we have evidence that an autophagy inhibitor especially in combination with kinase inhibitors of the RAS/RAF/MEK/ERK pathway can kill more cancer cells and can even overcome the acquired (also intrinsic) resistance that invariably limits the use of kinase inhibitors in people. These conclusions are also supported by numerous other studies, *e.g.* in mouse models of lung cancers where RAS-driven cancers also seem especially sensitive to autophagy inhibition [60-62]. An important question is why this works. Why does autophagy inhibition sensitize cells to other death-inducing stimuli like anti-cancer drugs? It seems unlikely that this could be a specific effect for particular drugs since the synergistic interactions are seen with many different kinds of drugs and even when we focus on just one drug like vemurafenib, molecularly distinct resistance mechanisms can all be overcome. Instead we propose that these data suggest that autophagy inhibition is affecting something much more fundamental – the apoptotic threshold, which as we described above, ultimately determines whether all cells, normal or cancer, die or not and explains the fundamental basis for why cancer therapy is possible at all.

A POTENTIAL MECHANISTIC EXPLANATION FOR WHY THESE STRATEGIES WORK

We recently discovered a specific mechanism by which autophagy can regulate the expression of the BH3-only protein p53 upregulated modulator of apoptosis (PUMA), thereby affecting the apoptotic threshold of the cells and "priming" the cell for apoptosis induction in response to cytotoxic agents. Our initial studies showed that when autophagy was inhibited, PUMA protein levels were selectively increased (there is little effect on other BCL family proteins) [63]. By studying a well-known activator of the extrinsic apoptosis pathway, Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL), we found that this effect was not killing cells on its own. Instead it enhanced the activity of another apoptosis stimulus. Thus, since PUMA works by altering the balance of BCL family proteins in favor of apoptosis, it effectively moves the cancer cells closer to their apoptotic threshold. Because we observed an increase in PUMA protein levels, and autophagy is a mechanism for degrading proteins, we initially thought that autophagy must be degrading PUMA protein. The actual mechanism is more interesting and provides an important insight into how all these pathways connect.

To test if autophagy degrades PUMA protein itself, we first performed a control experiment to test if PUMA mRNA increases when we inhibit autophagy. Surprisingly the answer was yes [63], and the question then became how this takes place. Autophagy increases the rate of transcription of the *PUMA* gene independently of p53, which is the best known transcription factor that controls PUMA [64]. As mentioned above, basal autophagy is regulated by several transcription factors [43]. One such transcription factor, Forkhead box class O 3a (FOXO3a) plays an important role in promoting expression of *ATG* genes in hematopoietic stem cells in the setting of cytokine deprivation [65]. FOXO3a can also control *PUMA* transcription [66] and, in the hematopoietic stem cells, PUMA transcription was enhanced at the same time as the ATG genes [65]. This led us to hypothesize that FOXO3a may not only be regulating autophagy, it may itself also be regulated by autophagy in a classic feedback mechanism. Indeed, both genetic and pharmacologic methods of autophagy inhibition led to accumulation of the FOXO3a protein without a corresponding change in FOXO3a mRNA expression. Moreover, we showed that FOXO3a protein is targeted to autophagosomes that fuse with lysosomes, and when we inhibited autophagy, FOXO3a translocated to the nucleus [64]. This implies that the basal rate of autophagy is controlled by a transcription factor, which is itself turned over by autophagy. Is this also linked to the apoptotic threshold? Chromatin immunoprecipitation was performed at the PUMA locus after autophagy inhibition. This showed enrichment of FOXO3a at a specific Forkhead Response element (FHRE) in an intronic region 1900 base pairs downstream from the *PUMA* transcription start site. We selectively mutated this single FHRE site using CRISPR genome editing, thus preventing FOXO3a binding at just one site in the genome. This mutation was sufficient to prevent *PUMA* activation by both genetic and pharmacological inhibition of autophagy. Thus, the specific ability of an autophagy inhibitor to enhance *PUMA* expression is blocked when only FOXO3 binding to the *PUMA* gene is prevented. Most important, mutation of just this one FHRE was also able to block the increase in apoptosis that is observed with autophagy inhibition in combination with cytotoxic chemotherapies. This was another surprising finding as FOXO3a is a ubiquitous transcription factor that has been shown to regulate expression of multiple genes including several other apoptosis regulators. Thus, it seems that the pro-apoptotic effects of autophagy inhibitors are achieved through a single transcription factor, FOXO3a, working at just one specific FHRE binding site

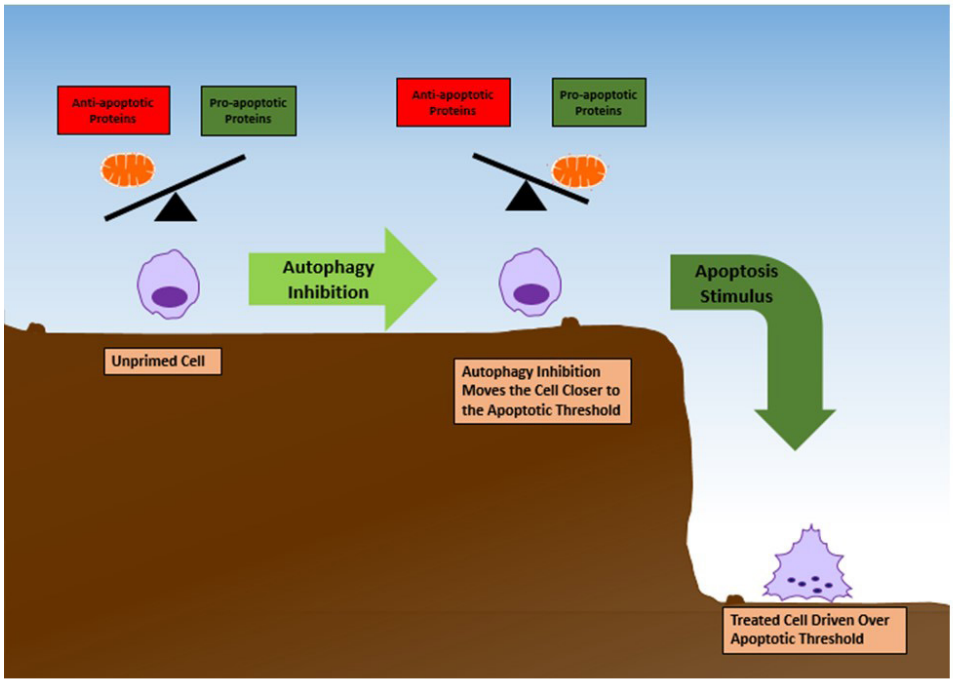

Figure 3. Autophagy inhibition is able to sensitize the cells to apoptotic stimuli due to the accumulation of FOXO3a and subsequent generation of the pro-apoptotic PUMA. Thus, cells that were previously resistant to apoptotic stimuli, such as cytotoxic chemotherapy, now have a balance of bcl-2 family of proteins that favor apoptosis. These cells are now "primed" and will now cross the apoptotic threshold in the face of treatment.

in the *PUMA* gene.

Taken together, these studies suggest that autophagy inhibition sensitizes tumor cells to anti-cancer therapy through a very specific mechanism that ultimately controls the proximity of the cancer cell to its apoptotic threshold. Because FOXO3a is degraded by basal autophagy, autophagy inhibition leads to increased FOXO3a as part of a feedback loop that maintains homeostatic regulation of autophagy. But the increased FOXO3a also leads to increased transcription of the proapoptotic gene *PUMA*. This skews the balance of pro- and anti-apoptotic BCL family proteins that determine how "primed" the cell is to undergo apoptosis. Thus, when cancer cells have lower levels of autophagy, there is a shift in the balance of those scales that makes it easier to induce apoptosis. While autophagy inhibition alone may not be sufficient to commit the cells to undergo apoptosis on its own, it brings them closer to the "edge of the cliff." Then, with the addition of another cytotoxic agent that provides an additional pro-apoptotic push, this mechanism is enough to force the cell over the edge of the cliff and commit to apoptosis (Figure 3).

CONCLUSIONS AND OUTLOOK

Manipulation of autophagy, and specifically the inhibition of autophagy is being pursued in dozens of clinical studies. As we describe above, we are now beginning to understand how this strategy works. When we inhibit autophagy, we make cancer cells more susceptible to other treatments because we alter the apoptotic threshold that determines whether a cell will live or die. This mechanism effectively takes advantage of what seems to be a core connection between two otherwise different cellular processes – autophagy and apoptosis. An autophagy homeostasis mechanism is directly controlling the cell death machinery. Perhaps this provides a way to ensure that when cells are under stress, and autophagy is working to protect against that stress, that cells are protected from apoptotic stimuli. Conversely, cells under stress that are not effectively utilizing autophagy are more susceptible to the death stimulus. We propose that by taking advantage of this mechanism in cancer therapy we can also explain why autophagy inhibition works with many different kinds of anti-cancer agent – it doesn't matter what the other drug is so long as it is capable of providing an extra pro-apoptotic push in that cancer cell. And, when we have a cancer cell that relies on a particular pathway, such as we see with our BRAF mutant CNS tumors or the KRAS mutant pancreas cancers, this is what makes the particular combination of an autophagy inhibitor with that pathway-specific drug so effective. Thus, using the framework of apoptotic priming, we can now propose a rational approach for the use of autophagy inhibitors with

anti-cancer agents.

Much remains to be further understood regarding the use of autophagy inhibitors as therapeutic agents in cancer. As mentioned above, autophagy is very context dependent. Therefore, dedicated study for individual tumor types needs to be done. What tumor types respond best to this therapy? What are the optimal combinations of drug therapies? Are there biomarkers that can be identified and measured in human subjects that will predict response to therapy?

Our knowledge of autophagy has grown substantially over the past decades. We now understand that autophagy is a complex homeostatic mechanism, interacting with numerous other cellular processes including the apoptotic machinery. And, by understanding the molecular interactions of autophagy and apoptosis, we may ultimately develop a better and more precise rationale for the use of combination of therapeutics that take advantage of these mechanism to better treat patients with cancer.

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