University of Alberta

Paclitaxel Inhibits Autophagy in Breast Cancer Cells

by

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Abstract

Taxanes are used for the treatment of breast, ovarian, and lung cancer. Unfortunately, taxane based therapy—the current treatment for metastatic breast cancer—has substantial shortcomings including myelosupression, neurotoxicity, and frequently acquired resistance. Our present understanding of taxane cytotoxicity is incomplete and prevents rational approaches to taxane improvement. Autophagy is a celluar process that digests portions of the cytosol to provide metabolic support in times of stress. This process is capable of promoting survival or conversely promoting cell death, depending on the context. The relationship between paclitaxel and autophagy is unclear. In this study, we show that paclitaxel causes inhibition of autophagy in breast cancer cells, both by decreasing autophagosome formation and by altering autophagosome trafficking and localization. Autophagy inhibition protects breast cancer cells against paclitaxel induced cell death, suggesting that manipulation of autophagy may represent a therapeutic target for improving breast cancer treatment options.

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-Christopher Hitchens

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List of Abbreviations

3MA	3-methyladenine
ATG	autophagy related gene
Baf	bafilomycin A1
BCA	bicinchoninic acid
BSA	bovine serum albumin
Cdk	cyclin dependent kinase
CMLE	classic maximum likelihood estimation
DAPI	2-(4-amidinophenyl)-1H -indole-6-carboxamidine
DFCP1	double FYVE-containing protein 1
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DPBS-M	Dulbecco's phosphate buffered saline (containing 1mM $MgCl_2$)
E1	ubiquitin-activating
E2	ubiquitin-conjugating
E3	ubiquitin ligase
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescence protein
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
HRP	horse radish peroxidase
LC3-B	microtubule-associated proteins 1A/1B light chain 3B
MTOC	microtubule organizing center
MTOR	mammalian target of rapamycin
NDS	normal donkey serum

NFSM	non-fat skim milk
NRK	normal rat kidney
PAGE	polyacrylamide gel electrophoresis
PAS	pre-autophagosomal structure
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PI3K	phosphoinositide 3-kinase
PI3P	phosphatidylinositol (3)-phosphate
PVDF	polyvinylidene difluoride
QMLE	quick maximum likelihood estimation
RNAi	ribonucleic acid interference
SAC	spindle assembly checkpoint
SSB	SDS-PAGE sample buffer
SDS	sodium dodecyl sulfate
TBS-T	tris buffered saline containing 0.1% Tween-20
TMRE	tetramethylrhodamine ethyl ester perchlorate
tris	tris(hydroxymethyl)aminomethane
Ulk	unc-51-like kinase
Vps	vacuolar protein sorting

Chapter 1: Introduction

1.1 Paclitaxel treatment of breast cancer

Paclitaxel was identified in a National Cancer Institute screen designed to identify antitumor agents in plant samples (Wall and Wani 1995). The isolation and purification of the compound occurred in the late 1960s, followed shortly by the first report describing its structure and antitumor activity (Wani, Taylor et al. 1971; Suffness 1993). Paclitaxel was initially shown to arrest cells at the G2-M phase of the cell cycle (Jordan, Toso et al. 1993), but in contrast to other known drugs with similar activity (vinca alkaloids, colchicine, etc.), it arrested cells by stabilizing microtubules, rather than disassembling them (Schiff, Fant et al. 1979). Paclitaxel and its analogue Docetaxel showed enough potential as chemotherapeutic agents to justify numerous clinical trials, and this led to their integration into standard treatment regimens for both early and advanced breast cancer (Lyseng-Williamson and Fenton 2005; Nabholtz and Gligorov 2005; Bedard, Di Leo et al. 2010).

While the taxanes (paclitaxel and docetaxel) have improved treatment outcomes for many patients, they suffer from several shortcomings. First, as both a monotherapy and as a combinational therapy, taxanes show significant myelosuppression and neuropathy (Tang 2009). Secondly, resistance to taxane based chemotherapies is common (McGrogan, Gilmartin et al. 2008), and refractory tumors have few treatment options (Perez 2009). Finally, there is no validated clinical marker available to predict taxane sensitivity (Noguchi 2006), though multi gene profiles may offer some insight

(Oakman, Bessi et al. 2009) and a recent report identified the protein Bad as a potential predictor (Craik, Veldhoen et al. 2010). Strategies to overcome these shortcomings will require detailed mechanistic knowledge of taxane induced cytotoxicity, but the mechanism is currently unclear (Gascoigne and Taylor 2009).

1.2 Paclitaxel induced cell death

Paclitaxel induced cell cycle arrest is well characterized: paclitaxel binds to a region of β-tubulin termed the 'taxol binding site,' and this binding considerably stabilizes the microtubule (Lowe, Li et al. 2001; Noguchi 2006). Microtubules are dynamic cytoskeletal structures that contribute to cell shape, intracellular transport, motility, and mitosis (Wade and Hyman 1997). These structures undergo rapid growth and shrinkage, termed 'dynamic instability,' and this instability is necessary for successful division of the chromosomes during mitosis (Rieder, Schultz et al. 1994; Wilson and Jordan 1995). By stabilizing the microtubules during mitosis, paclitaxel activates a signaling complex known as the spindle assembly checkpoint (SAC) (Rieder and Maiato 2004). The SAC is a control mechanism that prevents cell cycle progression from metaphase to anaphase in the presence of unattached kinetochores. In mitotically arrested cells treated with paclitaxel, at least one kinetochore is unable to properly attach to a microtubule, preventing satisfaction of the SAC (McEwen, Heagle et al. 1997; Waters, Chen et al. 1998). Prolonged mitotic arrest can lead to multiple cellular fates including death in mitosis, mitotic exit followed by death, viable mitotic exit without

continued cell cycle progression, and viable mitotic exit followed by further division (Rieder and Maiato 2004). Blajeski *et al.* first proposed the model that during prolonged mitotic arrest, cyclin B levels eventually decrease enough for the cell to escape mitotic arrest, and the cell enters a multinucleated, tetraploid, G1 state before dying (Blajeski, Kottke et al. 2001).

Different concentrations of paclitaxel elicit very different cellular responses. Very low concentrations (as low as 8nM) are sufficient to suppress microtubule instability and induce mitotic arrest and cell death in HeLa cells, while a higher concentration (1 μM) induces bundling of microtubules (Jordan, Toso et al. 1993; Jordan, Wendell et al. 1996). Clinically relevant concentrations of paclitaxel are in the low nanomolar range (~25nM) (Huizing, Vermorken et al. 1995; Derry, Wilson et al. 1998). It is yet unclear what cellular factors dictate paclitaxel response. A recent study utilizing high-throughput live-cell microscopy followed the individual cellular fates of seven different cancer cell lines treated with paclitaxel, and found extensive variation in cell fate upon exit from mitotic arrest (Gascoigne and Taylor 2008). The variation existed both between and within cell lines. They proposed a model in which cyclin B1 degradation is occurring simultaneously with activation of cell death pathways. Diminishing cyclin B1 levels cause exit from mitotic arrest (Brito and Rieder 2006) while increasing cell death activity causes the cell to die. Their relative rates dictate the fate of the cell. In an effort to determine the genetic contribution to anti-mitotic treatment, Gascoigne *et al.* observed cells originating from the same progenitor (Gascoigne and Taylor 2008). They showed that genetically identical sister

cells frequently undergo different response to paclitaxel treatment, indicating that cell response to paclitaxel is not solely dependent on genetic factors.

Paclitaxel has been shown to cause cell death through induction of apoptosis, a regulated cellular death pathway (Woods, Zhu et al. 1995; Jordan, Wendell et al. 1996). This is supported by several studies demonstrating that effective induction of cell death by paclitaxel requires caspase activity (Panvichian, Orth et al. 1998; Gascoigne and Taylor 2008; Shi, Orth et al. 2008). Caspases are the effectors of apoptosis, and cleave key cellular substrates leading to dismantling and destruction of the cell (Pop and Salvesen 2009).

The cellular signaling events that result from paclitaxel treatment and lead to caspase activation are still elusive (Rieder and Maiato 2004; Gascoigne and Taylor 2009), though the Bcl-2 family of proteins play an important role (Willis and Adams 2005). Members of the Bcl-2 family of proteins regulate apoptosis and contain both pro-death and pro-survival members (Youle and Strasser 2008). Bim, a pro-death member of the family, has been shown to contribute to paclitaxel induced apoptotic signaling in some cell types (Li, Moudgil et al. 2005; Tan, Degenhardt et al. 2005), though its importance is unclear in human breast cancer cells (Sunters, Fernández de Mattos et al. 2003; Czernick, Rieger et al. 2009). The pro-death protein Bad has also been shown to contribute to paclitaxel sensitivity, and clinical samples showed that Bad is a prognostic indicator of docetaxel response in breast cancer patient (Craik, Veldhoen et al. 2010). The pro-survival family members Bcl-2 and Bcl-xL oppose the effects of the pro death members, and they are associated

with resistance to paclitaxel. RNAi depletion of Bcl-2 and Bcl-xl sensitizes breast cancer cells to paclitaxel induced apoptosis (Simoes-Wust, Schurpf et al. 2002; Tanabe, Kim et al. 2003). Yet of all the members of the Bcl-2 family, only Bad has shown prognostic clinical potential, suggesting the existence of other paclitaxel responsive death pathways.

1.3 Autophagy

Autophagy is a catabolic cellular process that degrades double-membrane bound portions of the cytosol through fusion with lysosomes (Fig. 1.1) (Ravikumar, Sarkar et al. 2010). This process occurs constitutively at basal levels to support cellular homeostasis by providing energy through the breakdown of cellular components, degrading long lived proteins, and is uniquely capable of clearing large protein aggregates and disposing of damaged organelles (Yang and Klionsky 2010). Defects in autophagy are implicated in many pathologies and dysfunctional clearance of autophagic substrates can contribute to tumorigenesis (Mathew, Karp et al. 2009), neurodegeneration (Iwata, Riley et al. 2005), and premature aging (Juhasz, Erdi et al. 2007).

Autophagosomes begin as an isolation membrane, also known as a phagophore, at the pre-autophagosomal structure (PAS). The phagophore is a double membrane that expands, encloses the cytosol, and eventually develops into an autophagosome. The site of phagophore formation is well defined in yeast, occurring near the vacuole (Suzuki, Kirisako et al. 2001).

There appear to be several sites of autophagosome formation in mammalian cells, and they have been observed to originate from the ER, mitochondria, and plasma membrane (Axe, Walker et al. 2008; Hayashi-Nishino, Fujita et al. 2009; Yla-Anttila, Vihinen et al. 2009; Hailey, Rambold et al. 2010; Hayashi-Nishino, Fujita et al. 2010; Ravikumar, Moreau et al. 2010). The phagophore then expands, engulfs a portion of the cytoplasm, and finally closes, forming an autophagosome. This autophagosome is then trafficked along microtubules bi-directionally, with a bias toward the MTOC, putting it in close proximity with the Golgi apparatus, the site of lysosomal biogenesis. Finally, the autophagosome will dock and then fuse with a lysosome, and lysosomal hydrolases will degrade the contents of the autophagosome releasing metabolic substrates. At any point prior to final fusion with the lysosome, the autophagosome may interact with other autophagosomes through fusion, or by transferring cargo via membrane protrusions. Endosomes may also fuse with autophagosomes en route to lysosomal fusion (Jahreiss, Menzies et al. 2008).

1.3.1 The autophagic molecular machinery

Our understanding of the molecular machinery regulating autophagy is developing rapidly, but is still incomplete. The core autophagic machinery is composed of two kinase complexes that function in formation of the phagophore (Vps34 and Ulk) and two ubiquitin-like conjugation systems that promote phagophore elongation and closure (Atg12-5 and LC3-PE), see Fig. 1.2) (Yang and Klionsky 2010). Analysis of autophagy protein assembly at the

PAS shows a hierarchy: the Ulk complex is the most upstream, followed by the Vps34 complex, then the Atg12-5 conjugate system, and finally the LC3-PE conjugate system (Suzuki, Kubota et al. 2007; Itakura and Mizushima 2010). Each of these protein systems is necessary for autophagosome formation and a defect in one system will lead to improper function of downstream systems. Autophagy is regulated at each step in this process and allows fine tuning of autophagic flux in response to diverse stimuli.

1.3.2 Autophagic kinase complexes

The Ulk complex is composed of Ulk1, mATG13, Atg101, and FIP200 (Hara, Takamura et al. 2008; Chan, Longatti et al. 2009; Mercer, Kaliappan et al. 2009). ULK1 is a serine\threonine protein kinase necessary for autophagy (Kuroyanagi, Yan et al. 1998; Chan, Kir et al. 2007). Ulk complex activity is necessary to recruit downstream autophagy proteins to the PAS (Itakura and Mizushima 2010). Ulk1 activity is regulated through opposing phosphorylations by mTOR and AMPK; mTOR has an inhibitory effect on Ulk1 activity while AMPK has a stimulatory effect (Kim, Kundu et al. 2011; Shang and Wang 2011). mTOR can also phosphorylate Atg13, and this phosphorylation is associated with a decrease in autophagic activity (Jung, Jun et al. 2009). Ulk1 is also capable of autophosphorylation. Ulk1 autophosphorylation causes a conformational shift that promotes autophagic induction (Chan, Longatti et al. 2009). mATG13 and FIP200 are also Ulk1 substrates and are phosphorylated when autophagy is induced, further linking

Ulk1 kinase activity to increased induction of autophagy (Chan, Longatti et al. 2009; Jung, Jun et al. 2009).

The second autophagic kinase complex includes Vps34, a class III PI3K that is essential for mammalian autophagy (Volinia, Dhand et al. 1995). Vps34 phosphorylates phosphatidylinositol to create phosphatidylinositol (3)-phosphate (PI3P) (Schu, Takegawa et al. 1993; Volinia, Dhand et al. 1995), and its kinase activity is essential for induction of autophagy (Obara, Noda et al. 2008). PI3P is generated at the site of autophagosome formation (Axe, Walker et al. 2008), though the mechanism by which PI3P enrichment promotes autophagy is not clear (Yang and Klionsky 2010). Recently, proteins have been discovered that promote autophagy and possess the ability to bind PI3P, representing a mechanistic link between PI3P enrichment and autophagosome formation (Burman and Ktistakis 2010). WIP1\2 (orthologues of Atg18 in yeast) have been shown to bind PI3P (Proikas-Cezanne, Waddell et al. 2004; Proikas-Cezanne, Ruckerbauer et al. 2007). WIP2 localizes to the phagophore and promotes LC3-PE conjugation (see 1.3.3), and its depletion by RNAi causes the accumulation of immature autophagosomes (Polson, de Lartigue et al. 2010). DFCP1 is another PI3P binding protein that positively regulates autophagy (Derubeis, Young et al. 2000). It localizes to PI3P enriched punctate structures on the ER and seemingly functions by constricting portions of nascent autophagosomal membrane, causing them to separate from the ER (Axe, Walker et al. 2008). While specific mechanistic roles for PI3P in autophagy are still emerging, it is clear that Vps34 activity is essential for the formation of autophagosomes.

The core of the Vps34 complex is composed of three members: the class III PI3K Vps34 and its two binding partners P150 and Beclin 1, both of which increase its lipid kinase activity and stimulate autophagy (Volinia, Dhand et al. 1995; Petiot, Ogier-Denis et al. 2000; Kihara, Kabeya et al. 2001). The activity of the Vps34 complex can be regulated in several ways. Vps34 itself can be phosphorylated by Cdk1 and Cdk5. These phosphorylations disrupt binding of Vps34 to its positive regulator Beclin 1, causing decreased Vps34 activity (Furuya, Kim et al. 2010). Beclin 1 interacts with several proteins that modulate autophagy. Beclin 1 dependent autophagy is positively regulated by Atg14, Ambra1, and UVRAG (Fimia, Stoykova et al. 2007; Itakura, Kishi et al. 2008). Rubicon and the pro-survival members of the Bcl-2 family negatively regulate autophagy (Maiuri, Le Toumelin et al. 2007; Oberstein, Jeffrey et al. 2007; Matsunaga, Saitoh et al. 2009; Zhong, Wang et al. 2009). Pro-survival Bcl-2 family members can sequester Beclin 1 through interaction with its BH3 domain, preventing it from stimulating Vps34 activity. Consequently, pro-death Bcl-2 family members can induce autophagy by displacing Beclin 1 from the pro-survival family members (Maiuri, Zalckvar et al. 2007). The wide variety of Vps34 complex members allows fine control of Vps34 activity.

1.3.3 Autophagic ubiquitin-like conjugation systems

Two ubiquitin-like conjugation systems are required for phagophore elongation and closure. The first system conjugates Atg12 to Atg5 (Mizushima, Sugita et al. 1998; Yang and Klionsky 2010). Atg7 acts as an E1 enzyme, forming a conjugate with Atg12 (Tanida, Tanida-Miyake et al. 2001).

Next, Atg10 acts as an E2 enzyme and replaces Atg7, forming an Atg12-Atg10 conjugate (Shintani, Mizushima et al. 1999; Mizushima, Yoshimori et al. 2002; Nemoto, Tanida et al. 2003). The Atg12-Atg10 conjugate can then interact with Atg5, conjugating Atg12 to Atg5. Finally, The Atg12-Atg5 conjugate can then non-covalently interact with Atg16L, forming an Atg12-Atg5\Atg16L trimer. Knockout of Atg5 in murine cells prevented formation of mature autophagosomes. Rare autophagosome-like structures were observed in $Atg5^{-/-}$ cells, but these structures did not accumulate LC3-II, which is an indicator of maturing and completed autophagosomes (Mizushima, Yamamoto et al. 2001). Atg5 knockout also prevents localization of Atg16L to the PAS (Mizushima, Kuma et al. 2003).The Atg16L coiled coil domain allows oligomerization with other trimers, forming a homotetramer known as the Atg16L complex (Mizushima, Kuma et al. 2003). This complex localizes to the phagophore (primarily the outer leaflet) and functions in membrane elongation and closure (Mizushima, Yamamoto et al. 2001).

The second ubiquitin-like conjugation system conjugates LC3 to PE (Yang and Klionsky 2010). LC3 is the mammalian homologue of Atg8 in yeast. Atg8 was originally found to be associated with the autophagosome and is essential for autophagosome formation in yeast (Kirisako, Baba et al. 1999). Shortly afterwards, LC3 was shown to perform a homologous role in mammalian cells (Kabeya, Mizushima et al. 2000). LC3 is initially translated as a full length protein (proLC3) and is then immediately cleaved between glycine 120 and threonine 121 by Atg4 (Tanida, Sou et al. 2004). The cleaved form of LC3 is designated as LC3-I, and has an exposed C-terminal glycine

that is then conjugated to PE. The lipidated form of LC3 associates with the autophagosome and is known as LC3-II (Kabeya, Mizushima et al. 2000). LC3-PE conjugation requires Atg7 and Atg3 (Yang and Klionsky 2010). Atg7 functions as an E1-like enzyme in mammalian cells, and forms a conjugate with LC3-I (Tanida, Tanida-Miyake et al. 2001). The Atg7-LC3 conjugate then interacts with Atg3, which functions as an E2-like enzyme and forms an Atg3-LC3 conjugate (Tanida, Tanida-Miyake et al. 2002). Finally the Atg16L complex functions as an E3-like enzyme, specifying the location of LC3 lipidation and conjugating LC3 to PE, forming LC3-II (Hanada, Noda et al. 2007; Fujita, Itoh et al. 2008). LC3-II localized on the cytoplasmic face of the autophagosomal membrane can be recycled before degradation of the autophagosome. Atg4 delipidates the external LC3-II by cleaving off the PE moiety (Tanida, Sou et al. 2004), but LC3-II within the autophagosome is degraded after fusion with the lysosome, along with the autophagic cargo (Tanida, Minematsu-Ikeguchi et al. 2005).

Aside from the role of the Atg16L complex in LC3 PE conjugation, additional links between the two conjugation systems exist. Atg3 (the E2 like protein in the LC3-PE conjugation system) can interact with Atg12 and promotes formation of the Atg12-5 conjugate, but only in the presence of Atg7 (Tanida, Tanida-Miyake et al. 2002). Similarly, Atg10 (the E2 like protein in the Atg12-5 conjugation system) can interact with LC3 and promote its lipidation, but only in the presence of Atg7 (Nemoto, Tanida et al. 2003). Through these interactions, the E2-like proteins in one conjugation system support the second, and *vice versa*.

Functional studies of yeast Atg8 have provided insight into the function of LC3. Atg8 localizes to both sides of the phagophore membrane, and is required for phagophore elongation and maturation. Using an *in vitro* liposomal system and a combination of electron and fluorescent microscopy Nakatogawa *et al.* demonstrated that Atg8 promotes membrane tethering and hemifusion, and that PE conjugation alters these activities (Nakatogawa, Ichimura et al. 2007). They proposed that PE conjugation significantly changes the conformation of Atg8, and exposes regions of the protein that allow multimerization and subsequent tethering/hemifusion. Supporting the role of LC3 in autophagosome maturation, disruption of the LC3 conjugation system in mice through knockout of Atg3 led to an accumulation of incomplete autophagosome like structures. These structures were smaller than the autophagosomes in wild type cells, and were frequently unclosed and cuplike, supporting a role for LC3 in autophagosome expansion and closure. (Sou, Waguri et al. 2008).

1.4 Autophagosome trafficking

The mature autophagosome must be brought into the proximity of and fuse with a lysosome to degrade its cargo. Cytoskeletal components have long been known to support autophagy (Aplin, Jasionowski et al. 1992), and microtubules are specifically implicated in autophagosome formation, trafficking, and fusion. Autophagosomes move along microtubules in a dynein dependent manner (Iwata, Riley et al. 2005; Pandey, Nie et al. 2007; Lee, Koga et al. 2010), with LC3 serving as an autophagosomal anchor for

attachment (Kimura, Noda et al. 2008). Microtubules also support trafficking of lysosomes toward the MTOC, moving them into the proximity of autophagosomes (Matteoni and Kreis 1987). The significant functional relationship between autophagosomes and microtubules links autophagy to paclitaxel, and suggests that paclitaxel treatment may have pronounced effects on autophagic flux.

It is important to note that the specific role of microtubules in supporting autophagy is controversial (Monastyrska, Rieter et al. 2009), with one study showing that microtubules are required for autophagosome formation but not fusion with the lysosome (Fass, Shvets et al. 2006), and another study showing that it played a role in both formation and fusion (Kochl, Hu et al. 2006). A third study suggests that microtubules have some role in formation of autophagosomes and acetylated microtubules are especially important for autophagosome trafficking (Xie, Nguyen et al. 2010).

These studies primarily focus on the effects of microtubule depolymerization using drugs like nocodazole or vinblastine. Fass *et al.* primarily studied the effects of nocodazole treatment, and suggested that the depolymerization of microtubules caused redistribution of both lysosomes and autophagosomes throughout the cell, allowing diffusion to bring them together (Fass, Shvets et al. 2006). Kochl *et al.* found that high concentrations of paclitaxel (5μM) had minimal effects on both autophagosome formation and trafficking, and in contrast to Fass *et al.* found that depolymerization diminishes autophagosome\lysosome fusion (Kochl, Hu et al. 2006). Interestingly, their

study shows that depolymerization of microtubules by different drugs had different effects on autophagosome formation. Nocodazole decreased autophagosome formation while vinblastine strongly increased it. These differing effects may be better understood when considered in the context of microtubule acetylation. Xie *et al.* report that nocodazole decreases levels of acetylated tubulin while vinblastine increases them, but causes them to accumulate in large foci (Xie, Nguyen et al. 2010). Acetylated tubulin may then be important for the formation of autophagosomes. Paclitaxel was also shown to increase acetylated tubulin levels, but was used at a clinical unachievable concentration (10μM) (Xie, Nguyen et al. 2010).

Therefore, the effect of paclitaxel on autophagosome trafficking in breast cancer cells is difficult to predict. Firstly, the above studies focus on depolymerizing agents. Secondly, the high concentrations of paclitaxel used may make the data inapplicable to clinically relevant concentrations (5-20μM compared to 25nM) (Jordan, Wendell et al. 1996; Derry, Wilson et al. 1998). Finally, the studies by Fass *et al.* and Kochl *et al.* use rodent cell lines, while Xie *et al.* uses HeLa. None of these are a relevant model for paclitaxel treatment of breast cancer. Additionally, rodent cells have been shown to be significantly more resistant to nocodazole and other spindle poisons than human cells, raising a question about the applicability of rodent studies to human cell lines (Rieder and Maiato 2004).

1.5 Autophagy and paclitaxel

Predicting the relationship between paclitaxel treatment and autophagy is difficult because previous studies have used a variety of cancer cell lines, and concentrations of paclitaxel that are clinically unachievable (Jordan, Wendell et al. 1996; Derry, Wilson et al. 1998). Additionally, these studies did not report the effects of autophagy on survival. Gorka *et al.* treated MCF-7 breast cancer cells with paclitaxel and observed that the cells showed features of autophagy during death (Gorka, Daniewski et al. 2005). The authors defined this as autophagic cell death, and concluded that paclitaxel induces autophagy in breast cancer cells. Hayashi *et al.* observed that paclitaxel induced autophagy in endothelial cells, potentially contributing to cell death (Hayashi, Yamamoto et al. 2009). Again the study by Gorka *et al.* uses clinically unattainable concentrations of paclitaxel and both studies show that paclitaxel induces autophagy. Importantly, both studies neglect the use of lysosomal inhibitors when they observe accumulation of the autophagosomal marker LC3. This is significant, as an accumulation of LC3 may represent either increased formation or decreased degradation, corresponding to increased and decreased autophagic flux, respectively. Lysosomal inhibitors would prevent degradation of LC3 in the lysosome, and would allow unambiguous interpretation of the LC3 accumulation (Tanida, Minematsu-Ikeguchi et al. 2005).

One body of evidence, primarily from cytoskeletal studies, suggests that paclitaxel will inhibit autophagy by blocking autophagosome maturation.

Another smaller body of evidence suggests that paclitaxel induces autophagy. All of the evidence (excepting the study by Hayashi *et al.*) uses concentrations of paclitaxel that induce hyperstabilization of microtubules, and only one study uses breast cancer cells, the relevant model for this study (Hayashi, Yamamoto et al. 2009). Another line of evidence comes from study by Furuya *et al.* and shows that during mitotic arrest Cdk1 and Cdk5 can phosphorylate Vps34, inhibiting its activity by diminishing its binding with Beclin 1, and leading to an inhibition of autophagy (Furuya, Kim et al. 2010). The study used nocodazole to cause mitotic arrest. So while that report cannot directly address the relationship between paclitaxel and autophagy, a similar effect may be observed during paclitaxel treatment of breast cancer cells. Cdk1 activity depends on cyclin B1 to stimulate its activity, and the study by Furuya et al. showed that accumulation of cyclin B1 coincides with Vps34 phosphorylation (Furuya, Kim et al. 2010). Paclitaxel also induces a prolonged mitotic arrest, and maintenance of arrest requires persistence of cyclin B1 (Gascoigne and Taylor 2008). Clinical concentrations of paclitaxel induce mitotic arrest in breast cancer cells (Czernick, Rieger et al. 2009; Craik, Veldhoen et al. 2010), so it is possible that these cells will also have inhibited autophagosome formation. Ultimately, conflicting evidence and varied experimental models prevent confident prediction of paclitaxel's effects in a breast cancer model, necessitating further research.

1.6 Autophagy and cell death

Autophagy is an important survival response to many different stresses including oxidative stress (Yang, Wu et al. 2008), accumulation of protein aggregates (Iwata, Riley et al. 2005; Pandey, Nie et al. 2007; Lee, Koga et al. 2010), and ischemia (Degenhardt, Mathew et al. 2006). It also sustains life in many developmental contexts such as fertilization (Tsukamoto, Kuma et al. 2008), neonatal starvation (Kuma, Hatano et al. 2004) and T-cell development (Stephenson, Miller et al. 2009). Conversely, autophagy has also been shown to promote death in diverse cases such as inhibition of caspases (Yu, Alva et al. 2004), etoposide treatment of apoptosis deficient murine fibroblasts (Shimizu, Kanaseki et al. 2004), anti-estrogen treatment of breast cancer cells (Bursch, Ellinger et al. 1996), NGF or serum/potassium withdrawal from neurons (Xue, Fletcher et al. 1999; Canu, Tufi et al. 2005), and in a Myc-induced murine lymphoma model (Amaravadi, Yu et al. 2007). Consequently it is impossible to accurately predict the relationship between cell survival and autophagy *a priori*.

It is unclear whether autophagy can directly cause cell death in mammalian cells (Kroemer and Levine 2008). Developmental cell death requiring autophagy has been demonstrated in *Drosophila melanogaster* and in *Dictyostelium discoideum.* Autophagy deficient *drosophila* embryos mutant cannot properly degrade larval salivary glands (Berry and Baehrecke 2007). Additionally, during programmed remodelling of the larval *drosophila* midgut, autophagy is necessary for cell death while apoptosis is not (Denton,

Shravage et al. 2009). Dictyostelium undergoes developmental cell death while differentiating into stalk cells, and inactivation of autophagy through Atg1 disruption abolishes this death (Kosta, Roisin-Bouffay et al. 2004).

Presently, there is no *in vivo* evidence for autophagic cell death (ACD) in mammalian cells (Scarlatti, Granata et al. 2009). Several mammalian studies (outlined above) have reported the observation of prominent autophagic features coinciding with cell death, but it is technically challenging to separate cell death with the features of autophagy from cell death caused by autophagy. An alternative explanation is that autophagy alters the rate of cell death, or functions in concert with other cellular death pathways (Kroemer and Levine 2008). This is an appealing hypothesis, owing to the dearth of *in vivo* evidence of ACD in mammalian cells.

1.7 Purpose of study

The primary aim of this study is to establish the relationship between paclitaxel, autophagy, and cell survival in a breast cancer cell culture model. It shows that autophagy contributes to paclitaxel cytotoxicity, yet paclitaxel inhibits autophagic flux. Finally it explores the mechanism of paclitaxel inhibition of autophagy.

1.8 References

Amaravadi, R. K., D. Yu, J. J. Lum, T. Bui, M. A. Christophorou, G. I. Evan, A. Thomas-Tikhonenko and C. B. Thompson (2007). "Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma." J Clin Invest **117**(2): 326-336.

Aplin, A., T. Jasionowski, D. L. Tuttle, S. E. Lenk and W. A. Dunn, Jr. (1992). "Cytoskeletal elements are required for the formation and maturation of autophagic vacuoles." J Cell Physiol **152**(3): 458-466.

Axe, E. L., S. A. Walker, M. Manifava, P. Chandra, H. L. Roderick, A. Habermann, G. Griffiths and N. T. Ktistakis (2008). "Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum." J Cell Biol **182**(4): 685-701.

Bedard, P. L., A. Di Leo and M. J. Piccart-Gebhart (2010). "Taxanes: optimizing adjuvant chemotherapy for early-stage breast cancer." Nat Rev Clin Oncol **7**(1): 22-36.

Berry, D. L. and E. H. Baehrecke (2007). "Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila." Cell **131**(6): 1137-1148.

Blajeski, A. L., T. J. Kottke and S. H. Kaufmann (2001). "A multistep model for paclitaxel-induced apoptosis in human breast cancer cell lines." Exp Cell Res **270**(2): 277-288.

Brito, D. A. and C. L. Rieder (2006). "Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint." Curr Biol **16**(12): 1194-1200.

Burman, C. and N. T. Ktistakis (2010). "Regulation of autophagy by phosphatidylinositol 3-phosphate." FEBS Letters **584**(7): 1302-1312.

Bursch, W., A. Ellinger, H. Kienzl, L. Torok, S. Pandey, M. Sikorska, R. Walker and R. S. Hermann (1996). "Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy." Carcinogenesis **17**(8): 1595-1607.

Canu, N., R. Tufi, A. L. Serafino, G. Amadoro, M. T. Ciotti and P. Calissano (2005). "Role of the autophagic-lysosomal system on low potassium-induced apoptosis in cultured cerebellar granule cells." J Neurochem **92**(5): 1228-1242.

Chan, E. Y., S. Kir and S. A. Tooze (2007). "siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy." J Biol Chem **282**(35): 25464-25474.

Chan, E. Y., A. Longatti, N. C. McKnight and S. A. Tooze (2009). "Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism." Mol Cell Biol **29**(1): 157-171.

Craik, A. C., R. A. Veldhoen, M. Czernick, T. W. Buckland, K. Kyselytzia, S. Ghosh, R. Lai, S. Damaraju, D. A. Underhill, J. R. Mackey and I. S. Goping (2010). "The BH3-only protein Bad confers breast cancer taxane sensitivity through a nonapoptotic mechanism." Oncogene **29**(39): 5381-5391.

Czernick, M., A. Rieger and I. S. Goping (2009). "Bim is reversibly phosphorylated but plays a limited role in paclitaxel cytotoxicity of breast cancer cell lines." Biochem Biophys Res Commun **379**(1): 145-150.

Degenhardt, K., R. Mathew, B. Beaudoin, K. Bray, D. Anderson, G. Chen, C. Mukherjee, Y. Shi, C. Gelinas, Y. Fan, D. A. Nelson, S. Jin and E. White (2006). "Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis." Cancer Cell **10**(1): 51-64.

Denton, D., B. Shravage, R. Simin, K. Mills, D. L. Berry, E. H. Baehrecke and S. Kumar (2009). "Autophagy, not apoptosis, is essential for midgut cell death in Drosophila." Curr Biol **19**(20): 1741-1746.

Derry, W. B., L. Wilson and M. A. Jordan (1998). "Low potency of taxol at microtubule minus ends: implications for its antimitotic and therapeutic mechanism." Cancer Res **58**(6): 1177-1184.

Derubeis, A. R., M. F. Young, L. Jia, P. G. Robey and L. W. Fisher (2000). "Double FYVE-containing protein 1 (DFCP1): isolation, cloning and characterization of a novel FYVE finger protein from a human bone marrow cDNA library." Gene **255**(2): 195-203.

Fass, E., E. Shvets, I. Degani, K. Hirschberg and Z. Elazar (2006). "Microtubules support production of starvation-induced autophagosomes but not their targeting and fusion with lysosomes." J Biol Chem **281**(47): 36303-36316.

Fimia, G. M., A. Stoykova, A. Romagnoli, L. Giunta, S. Di Bartolomeo, R. Nardacci, M. Corazzari, C. Fuoco, A. Ucar, P. Schwartz, P. Gruss, M. Piacentini, K. Chowdhury and F. Cecconi (2007). "Ambra1 regulates autophagy and development of the nervous system." Nature **447**(7148): 1121-1125.

Fujita, N., T. Itoh, H. Omori, M. Fukuda, T. Noda and T. Yoshimori (2008). "The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy." Mol Biol Cell **19**(5): 2092-2100.

Furuya, T., M. Kim, M. Lipinski, J. Li, D. Kim, T. Lu, Y. Shen, L. Rameh, B. Yankner, L. H. Tsai and J. Yuan (2010). "Negative regulation of Vps34 by Cdk mediated phosphorylation." Mol Cell **38**(4): 500-511.

Gascoigne, K. E. and S. S. Taylor (2008). "Cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs." Cancer Cell **14**(2): 111-122.

Gascoigne, K. E. and S. S. Taylor (2009). "How do anti-mitotic drugs kill cancer cells?" J Cell Sci **122**(Pt 15): 2579-2585.

Gorka, M., W. M. Daniewski, B. Gajkowska, E. Lusakowska, M. M. Godlewski and T. Motyl (2005). "Autophagy is the dominant type of programmed cell death in breast cancer MCF-7 cells exposed to AGS 115 and EFDAC, new sesquiterpene analogs of paclitaxel." Anticancer Drugs **16**(7): 777-788.

Hailey, D. W., A. S. Rambold, P. Satpute-Krishnan, K. Mitra, R. Sougrat, P. K. Kim and J. Lippincott-Schwartz (2010). "Mitochondria supply membranes for autophagosome biogenesis during starvation." Cell **141**(4): 656-667.

Hanada, T., N. N. Noda, Y. Satomi, Y. Ichimura, Y. Fujioka, T. Takao, F. Inagaki and Y. Ohsumi (2007). "The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy." J Biol Chem **282**(52): 37298-37302.

Hara, T., A. Takamura, C. Kishi, S. Iemura, T. Natsume, J. L. Guan and N. Mizushima (2008). "FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells." J Cell Biol **181**(3): 497-510.

Hayashi-Nishino, M., N. Fujita, T. Noda, A. Yamaguchi, T. Yoshimori and A. Yamamoto (2009). "A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation." Nat Cell Biol **11**(12): 1433-1437.

Hayashi-Nishino, M., N. Fujita, T. Noda, A. Yamaguchi, T. Yoshimori and A. Yamamoto (2010). "Electron tomography reveals the endoplasmic reticulum as a membrane source for autophagosome formation." Autophagy **6**(2): 301-303.

Hayashi, S., A. Yamamoto, F. You, K. Yamashita, Y. Ikegame, M. Tawada, T. Yoshimori, S. Shimizu and S. Nakashima (2009). "The stent-eluting drugs sirolimus and paclitaxel suppress healing of the endothelium by induction of autophagy." Am J Pathol **175**(5): 2226-2234.

Huizing, M. T., J. B. Vermorken, H. Rosing, W. W. ten Bokkel Huinink, I. Mandjes, H. M. Pinedo and J. H. Beijnen (1995). "Pharmacokinetics of paclitaxel and three major metabolites in patients with advanced breast carcinoma refractory to anthracycline therapy treated with a 3-hour paclitaxel infusion: a European Cancer Centre (ECC) trial." Ann Oncol **6**(7): 699-704.

Itakura, E., C. Kishi, K. Inoue and N. Mizushima (2008). "Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG." Mol Biol Cell **19**(12): 5360-5372.

Itakura, E. and N. Mizushima (2010). "Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins." Autophagy **6**(6): 764-776.

Iwata, A., B. E. Riley, J. A. Johnston and R. R. Kopito (2005). "HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin." J Biol Chem **280**(48): 40282-40292.

Jahreiss, L., F. M. Menzies and D. C. Rubinsztein (2008). "The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes." Traffic **9**(4): 574-587.

Jordan, M. A., R. J. Toso, D. Thrower and L. Wilson (1993). "Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations." Proc Natl Acad Sci U S A **90**(20): 9552-9556.

Jordan, M. A., K. Wendell, S. Gardiner, W. B. Derry, H. Copp and L. Wilson (1996). "Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death." Cancer Res **56**(4): 816-825.

Juhasz, G., B. Erdi, M. Sass and T. P. Neufeld (2007). "Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila." Genes Dev **21**(23): 3061-3066.

Jung, C. H., C. B. Jun, S. H. Ro, Y. M. Kim, N. M. Otto, J. Cao, M. Kundu and D. H. Kim (2009). "ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery." Mol Biol Cell **20**(7): 1992-2003.

Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi and T. Yoshimori (2000). "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing." EMBO J **19**(21): 5720-5728.

Kihara, A., Y. Kabeya, Y. Ohsumi and T. Yoshimori (2001). "Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network." EMBO Rep **2**(4): 330-335.

Kim, J., M. Kundu, B. Viollet and K. L. Guan (2011). "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1." Nat Cell Biol **13**(2): 132-141.

Kimura, S., T. Noda and T. Yoshimori (2008). "Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes." Cell Struct Funct **33**(1): 109-122.

Kirisako, T., M. Baba, N. Ishihara, K. Miyazawa, M. Ohsumi, T. Yoshimori, T. Noda and Y. Ohsumi (1999). "Formation process of autophagosome is traced with Apg8/Aut7p in yeast." J Cell Biol **147**(2): 435-446.

Kochl, R., X. W. Hu, E. Y. Chan and S. A. Tooze (2006). "Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes." Traffic **7**(2): 129-145.

Kosta, A., C. Roisin-Bouffay, M. F. Luciani, G. P. Otto, R. H. Kessin and P. Golstein (2004). "Autophagy gene disruption reveals a non-vacuolar cell death pathway in Dictyostelium." J Biol Chem **279**(46): 48404-48409.

Kroemer, G. and B. Levine (2008). "Autophagic cell death: the story of a misnomer." Nat Rev Mol Cell Biol **9**(12): 1004-1010.

Kuma, A., M. Hatano, M. Matsui, A. Yamamoto, H. Nakaya, T. Yoshimori, Y. Ohsumi, T. Tokuhisa and N. Mizushima (2004). "The role of autophagy during the early neonatal starvation period." Nature **432**(7020): 1032-1036.

Kuroyanagi, H., J. Yan, N. Seki, Y. Yamanouchi, Y. Suzuki, T. Takano, M. Muramatsu and T. Shirasawa (1998). "Human ULK1, a novel serine/threonine kinase related to UNC-51 kinase of Caenorhabditis elegans: cDNA cloning, expression, and chromosomal assignment." Genomics **51**(1): 76-85.

Lee, J. Y., H. Koga, Y. Kawaguchi, W. Tang, E. Wong, Y. S. Gao, U. B. Pandey, S. Kaushik, E. Tresse, J. Lu, J. P. Taylor, A. M. Cuervo and T. P. Yao (2010). "HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy." EMBO J **29**(5): 969-980.

Li, R., T. Moudgil, H. J. Ross and H. M. Hu (2005). "Apoptosis of non-small-cell lung cancer cell lines after paclitaxel treatment involves the BH3-only proapoptotic protein Bim." Cell Death Differ **12**(3): 292-303.

Lowe, J., H. Li, K. H. Downing and E. Nogales (2001). "Refined structure of alpha beta-tubulin at 3.5 A resolution." J Mol Biol **313**(5): 1045-1057.

Lyseng-Williamson, K. A. and C. Fenton (2005). "Docetaxel: a review of its use in metastatic breast cancer." Drugs **65**(17): 2513-2531.

Maiuri, M. C., G. Le Toumelin, A. Criollo, J. C. Rain, F. Gautier, P. Juin, E. Tasdemir, G. Pierron, K. Troulinaki, N. Tavernarakis, J. A. Hickman, O. Geneste and G. Kroemer (2007). "Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1." EMBO J **26**(10): 2527-2539.

Maiuri, M. C., E. Zalckvar, A. Kimchi and G. Kroemer (2007). "Self-eating and self-killing: crosstalk between autophagy and apoptosis." Nat Rev Mol Cell Biol **8**(9): 741-752.

Mathew, R., C. M. Karp, B. Beaudoin, N. Vuong, G. Chen, H. Y. Chen, K. Bray, A. Reddy, G. Bhanot, C. Gelinas, R. S. Dipaola, V. Karantza-Wadsworth and E. White (2009). "Autophagy suppresses tumorigenesis through elimination of p62." Cell **137**(6): 1062-1075.

Matsunaga, K., T. Saitoh, K. Tabata, H. Omori, T. Satoh, N. Kurotori, I. Maejima, K. Shirahama-Noda, T. Ichimura, T. Isobe, S. Akira, T. Noda and T. Yoshimori (2009). "Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages." Nat Cell Biol **11**(4): 385-396.

Matteoni, R. and T. E. Kreis (1987). "Translocation and clustering of endosomes and lysosomes depends on microtubules." J Cell Biol **105**(3): 1253-1265.

McEwen, B. F., A. B. Heagle, G. O. Cassels, K. F. Buttle and C. L. Rieder (1997). "Kinetochore fiber maturation in PtK1 cells and its implications for the mechanisms of chromosome congression and anaphase onset." J Cell Biol **137**(7): 1567-1580.

McGrogan, B. T., B. Gilmartin, D. N. Carney and A. McCann (2008). "Taxanes, microtubules and chemoresistant breast cancer." Biochim Biophys Acta **1785**(2): 96-132.

Mercer, C. A., A. Kaliappan and P. B. Dennis (2009). "A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy." Autophagy **5**(5): 649-662.

Mizushima, N., A. Kuma, Y. Kobayashi, A. Yamamoto, M. Matsubae, T. Takao, T. Natsume, Y. Ohsumi and T. Yoshimori (2003). "Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate." J Cell Sci **116**(Pt 9): 1679-1688.

Mizushima, N., H. Sugita, T. Yoshimori and Y. Ohsumi (1998). "A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy." J Biol Chem **273**(51): 33889-33892.

Mizushima, N., A. Yamamoto, M. Hatano, Y. Kobayashi, Y. Kabeya, K. Suzuki, T. Tokuhisa, Y. Ohsumi and T. Yoshimori (2001). "Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells." J Cell Biol **152**(4): 657-668.

Mizushima, N., T. Yoshimori and Y. Ohsumi (2002). "Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method." FEBS Lett **532**(3): 450-454.

Monastyrska, I., E. Rieter, D. J. Klionsky and F. Reggiori (2009). "Multiple roles of the cytoskeleton in autophagy." Biol Rev Camb Philos Soc **84**(3): 431-448.

Nabholtz, J. M. and J. Gligorov (2005). "The role of taxanes in the treatment of breast cancer." Expert Opin Pharmacother **6**(7): 1073-1094.

Nakatogawa, H., Y. Ichimura and Y. Ohsumi (2007). "Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion." Cell **130**(1): 165-178.

Nemoto, T., I. Tanida, E. Tanida-Miyake, N. Minematsu-Ikeguchi, M. Yokota, M. Ohsumi, T. Ueno and E. Kominami (2003). "The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification." J Biol Chem **278**(41): 39517-39526.

Noguchi, S. (2006). "Predictive factors for response to docetaxel in human breast cancers." Cancer Sci **97**(9): 813-820.

Oakman, C., S. Bessi, E. Zafarana, F. Galardi, L. Biganzoli and A. Di Leo (2009). "Recent advances in systemic therapy: new diagnostics and biological predictors of outcome in early breast cancer." Breast Cancer Res **11**(2): 205.

Obara, K., T. Noda, K. Niimi and Y. Ohsumi (2008). "Transport of phosphatidylinositol 3-phosphate into the vacuole via autophagic membranes in Saccharomyces cerevisiae." Genes Cells **13**(6): 537-547.

Oberstein, A., P. D. Jeffrey and Y. Shi (2007). "Crystal structure of the Bcl-XL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein." J Biol Chem **282**(17): 13123-13132.

Pandey, U. B., Z. Nie, Y. Batlevi, B. A. McCray, G. P. Ritson, N. B. Nedelsky, S. L. Schwartz, N. A. DiProspero, M. A. Knight, O. Schuldiner, R. Padmanabhan, M. Hild, D. L. Berry, D. Garza, C. C. Hubbert, T. P. Yao, E. H. Baehrecke and J. P. Taylor (2007). "HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS." Nature **447**(7146): 859-863.

Panvichian, R., K. Orth, M. L. Day, K. C. Day, M. J. Pilat and K. J. Pienta (1998). "Paclitaxel-associated multimininucleation is permitted by the inhibition of caspase activation: a potential early step in drug resistance." Cancer Res **58**(20): 4667-4672.

Perez, E. A. (2009). "Impact, mechanisms, and novel chemotherapy strategies for overcoming resistance to anthracyclines and taxanes in metastatic breast cancer." Breast Cancer Res Treat **114**(2): 195-201.

Petiot, A., E. Ogier-Denis, E. F. Blommaart, A. J. Meijer and P. Codogno (2000). "Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells." J Biol Chem **275**(2): 992-998.

Polson, H. E., J. de Lartigue, D. J. Rigden, M. Reedijk, S. Urbe, M. J. Clague and S. A. Tooze (2010). "Mammalian Atg18 (WIPI2) localizes to omegasome-

anchored phagophores and positively regulates LC3 lipidation." Autophagy **6**(4).

Pop, C. and G. S. Salvesen (2009). "Human caspases: activation, specificity, and regulation." J Biol Chem **284**(33): 21777-21781.

Proikas-Cezanne, T., S. Ruckerbauer, Y. D. Stierhof, C. Berg and A. Nordheim (2007). "Human WIPI-1 puncta-formation: a novel assay to assess mammalian autophagy." FEBS Lett **581**(18): 3396-3404.

Proikas-Cezanne, T., S. Waddell, A. Gaugel, T. Frickey, A. Lupas and A. Nordheim (2004). "WIPI-1alpha (WIPI49), a member of the novel 7-bladed WIPI protein family, is aberrantly expressed in human cancer and is linked to starvation-induced autophagy." Oncogene **23**(58): 9314-9325.

Ravikumar, B., K. Moreau, L. Jahreiss, C. Puri and D. C. Rubinsztein (2010). "Plasma membrane contributes to the formation of pre-autophagosomal structures." Nat Cell Biol **12**(8): 747-757.

Ravikumar, B., S. Sarkar, J. E. Davies, M. Futter, M. Garcia-Arencibia, Z. W. Green-Thompson, M. Jimenez-Sanchez, V. I. Korolchuk, M. Lichtenberg, S. Luo, D. C. Massey, F. M. Menzies, K. Moreau, U. Narayanan, M. Renna, F. H. Siddiqi, B. R. Underwood, A. R. Winslow and D. C. Rubinsztein (2010). "Regulation of mammalian autophagy in physiology and pathophysiology." Physiol Rev **90**(4): 1383-1435.

Rieder, C. L. and H. Maiato (2004). "Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint." Dev Cell **7**(5): 637-651.

Rieder, C. L., A. Schultz, R. Cole and G. Sluder (1994). "Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle." J Cell Biol **127**(5): 1301-1310.

Scarlatti, F., R. Granata, A. J. Meijer and P. Codogno (2009). "Does autophagy have a license to kill mammalian cells?" Cell Death Differ **16**(1): 12-20.

Schiff, P. B., J. Fant and S. B. Horwitz (1979). "Promotion of microtubule assembly in vitro by taxol." Nature **277**(5698): 665-667.

Schu, P. V., K. Takegawa, M. J. Fry, J. H. Stack, M. D. Waterfield and S. D. Emr (1993). "Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting." Science **260**(5104): 88-91.

Shang, L. and X. Wang (2011). "AMPK and mTOR coordinate the regulation of Ulk1 and mammalian autophagy initiation." Autophagy **7**(8).

Shi, J., J. D. Orth and T. Mitchison (2008). "Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5." Cancer Res **68**(9): 3269-3276.

Shimizu, S., T. Kanaseki, N. Mizushima, T. Mizuta, S. Arakawa-Kobayashi, C. B. Thompson and Y. Tsujimoto (2004). "Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes." Nat Cell Biol **6**(12): 1221-1228.

Shintani, T., N. Mizushima, Y. Ogawa, A. Matsuura, T. Noda and Y. Ohsumi (1999). "Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast." EMBO J **18**(19): 5234-5241.

Simoes-Wust, A. P., T. Schurpf, J. Hall, R. A. Stahel and U. Zangemeister-Wittke (2002). "Bcl-2/bcl-xL bispecific antisense treatment sensitizes breast carcinoma cells to doxorubicin, paclitaxel and cyclophosphamide." Breast Cancer Res Treat **76**(2): 157-166.

Sou, Y. S., S. Waguri, J. Iwata, T. Ueno, T. Fujimura, T. Hara, N. Sawada, A. Yamada, N. Mizushima, Y. Uchiyama, E. Kominami, K. Tanaka and M. Komatsu (2008). "The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice." Mol Biol Cell **19**(11): 4762-4775.

Stephenson, L. M., B. C. Miller, A. Ng, J. Eisenberg, Z. Zhao, K. Cadwell, D. B. Graham, N. N. Mizushima, R. Xavier, H. W. Virgin and W. Swat (2009). "Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes." Autophagy **5**(5): 625-635.

Suffness, M. (1993). Chapter 32. Taxol: From Discovery to Therapeutic Use. Annual Reports in Medicinal Chemistry. A. B. James, Academic Press. **Volume 28:** 305-314.

Sunters, A., S. Fernández de Mattos, M. Stahl, J. J. Brosens, G. Zoumpoulidou, C. A. Saunders, P. J. Coffer, R. H. Medema, R. C. Coombes and E. W. F. Lam (2003). "FoxO3a Transcriptional Regulation of Bim Controls Apoptosis in Paclitaxel-treated Breast Cancer Cell Lines." Journal of Biological Chemistry **278**(50): 49795-49805.

Suzuki, K., T. Kirisako, Y. Kamada, N. Mizushima, T. Noda and Y. Ohsumi (2001). "The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation." EMBO J **20**(21): 5971-5981.

Suzuki, K., Y. Kubota, T. Sekito and Y. Ohsumi (2007). "Hierarchy of Atg proteins in pre-autophagosomal structure organization." Genes Cells **12**(2): 209-218.

Tan, T. T., K. Degenhardt, D. A. Nelson, B. Beaudoin, W. Nieves-Neira, P. Bouillet, A. Villunger, J. M. Adams and E. White (2005). "Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy." Cancer Cell **7**(3): 227-238.

Tanabe, K., R. Kim, H. Inoue, M. Emi, Y. Uchida and T. Toge (2003). "Antisense Bcl-2 and HER-2 oligonucleotide treatment of breast cancer cells enhances their sensitivity to anticancer drugs." Int J Oncol **22**(4): 875-881.

Tang, S. C. (2009). "Strategies to decrease taxanes toxicities in the adjuvant treatment of early breast cancer." Cancer Invest **27**(2): 206-214.

Tanida, I., N. Minematsu-Ikeguchi, T. Ueno and E. Kominami (2005). "Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy." Autophagy **1**(2): 84-91.

Tanida, I., Y. S. Sou, J. Ezaki, N. Minematsu-Ikeguchi, T. Ueno and E. Kominami (2004). "HsAtg4B/HsApg4B/autophagin-1 cleaves the carboxyl termini of three human Atg8 homologues and delipidates microtubule-associated protein light chain 3- and GABAA receptor-associated protein-phospholipid conjugates." J Biol Chem **279**(35): 36268-36276.

Tanida, I., E. Tanida-Miyake, M. Komatsu, T. Ueno and E. Kominami (2002). "Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p." J Biol Chem **277**(16): 13739-13744.

Tanida, I., E. Tanida-Miyake, T. Ueno and E. Kominami (2001). "The human homolog of Saccharomyces cerevisiae Apg7p is a Protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3." J Biol Chem **276**(3): 1701-1706.

Tsukamoto, S., A. Kuma, M. Murakami, C. Kishi, A. Yamamoto and N. Mizushima (2008). "Autophagy is essential for preimplantation development of mouse embryos." Science **321**(5885): 117-120.

Volinia, S., R. Dhand, B. Vanhaesebroeck, L. K. MacDougall, R. Stein, M. J. Zvelebil, J. Domin, C. Panaretou and M. D. Waterfield (1995). "A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system." EMBO J **14**(14): 3339-3348.

Wade, R. H. and A. A. Hyman (1997). "Microtubule structure and dynamics." Curr Opin Cell Biol **9**(1): 12-17.

Wall, M. E. and M. C. Wani (1995). "Camptothecin and taxol: discovery to clinic--thirteenth Bruce F. Cain Memorial Award Lecture." Cancer Res **55**(4): 753-760.

Wani, M. C., H. L. Taylor, M. E. Wall, P. Coggon and A. T. McPhail (1971). "Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia." J Am Chem Soc **93**(9): 2325-2327.

Waters, J. C., R. H. Chen, A. W. Murray and E. D. Salmon (1998). "Localization of Mad2 to kinetochores depends on microtubule attachment, not tension." J Cell Biol **141**(5): 1181-1191.

Willis, S. N. and J. M. Adams (2005). "Life in the balance: how BH3-only proteins induce apoptosis." Curr Opin Cell Biol **17**(6): 617-625.

Wilson, L. and M. A. Jordan (1995). "Microtubule dynamics: taking aim at a moving target." Chem Biol **2**(9): 569-573.

Woods, C. M., J. Zhu, P. A. McQueney, D. Bollag and E. Lazarides (1995). "Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway." Mol Med **1**(5): 506-526.

Xie, R., S. Nguyen, W. L. McKeehan and L. Liu (2010). "Acetylated microtubules are required for fusion of autophagosomes with lysosomes." BMC Cell Biol **11**: 89.

Xue, L., G. C. Fletcher and A. M. Tolkovsky (1999). "Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution." Mol Cell Neurosci **14**(3): 180-198.

Yang, J., L. J. Wu, S. Tashino, S. Onodera and T. Ikejima (2008). "Reactive oxygen species and nitric oxide regulate mitochondria-dependent apoptosis and autophagy in evodiamine-treated human cervix carcinoma HeLa cells." Free Radic Res **42**(5): 492-504.

Yang, Z. and D. J. Klionsky (2010). "Mammalian autophagy: core molecular machinery and signaling regulation." Curr Opin Cell Biol **22**(2): 124-131.

Yla-Anttila, P., H. Vihinen, E. Jokitalo and E. L. Eskelinen (2009). "3D tomography reveals connections between the phagophore and endoplasmic reticulum." Autophagy **5**(8): 1180-1185.

Youle, R. J. and A. Strasser (2008). "The BCL-2 protein family: opposing activities that mediate cell death." Nat Rev Mol Cell Biol **9**(1): 47-59.

Yu, L., A. Alva, H. Su, P. Dutt, E. Freundt, S. Welsh, E. H. Baehrecke and M. J. Lenardo (2004). "Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8." Science **304**(5676): 1500-1502.

Zhong, Y., Q. J. Wang, X. Li, Y. Yan, J. M. Backer, B. T. Chait, N. Heintz and Z. Yue (2009). "Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex." Nat Cell Biol **11**(4): 468-476.

Chapter 2: Materials and Methods

2.1 Chemicals

All chemicals were obtained from Fisher Scientific Company (Ottawa, ON, Canada) unless otherwise noted. 3-methyladenine, bafilomycin A1, and paclitaxel were obtained from Sigma-Aldrich (Oakville, ON, Canada). TMRE and Hoechst 33342 were obtained from Invitrogen (Carlsbad, CA, USA). FuGENE 6, Complete protease inhibitor and PHOSstop phosphatase inhibitor were obtained from Roche (Indianapolis, IN, USA).

2.2 Antibodies

Antibody sources and dilutions are summarized in table 2.1.

2.3 Centrifugation

Three centrifuges were used throughout this study:

1. Eppendorf 5810 R
    a. Rotor: Swinging bucket rotor (A-4-81)
2. Beckman Coulter Microfuge 16 Microcentrifuge
    a. Rotor: Fixed Angle FX241.5P
3. Eppendorf 5402 Refrigerated Centrifuge
    a. Rotor: Fixed Angle F-45-18-11

All centrifugations are described in parentheses in the following order: speed, duration, temperature, rotor.

2.4 Cell Culture Reagents and Materials

RPMI 1640 media, Penicillin-Streptomycin, 0.05% Trypsin-EDTA and G-418 were obtained from Invitrogen. Fetal calf serum was obtained from Sigma-Aldrich. Cell culture flasks, plates, dishes, and serological pipettes were obtained from BD Biosciences (Franklin Lakes, NJ, USA).

2.5 Cell Culture and Creation of GFP-LC3 Stably Expressing Cell Lines

MCF-7 and SK-BR-3 human breast cancer cells were obtained from Dr Gordon Mills (MD Anderson Cancer Center, University of Texas). Cells were cultured in RPMI 1640 media supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. Cultures were checked for mycoplasma using a Mycosensor PCR Assay Kit, according to the manufacturer's directions (Agilent Technologies, Mississauga, ON, Canada). Cells were passaged every two to three days to maintain subconfluent cultures. To passage the cells, growth media was removed, and the cells were washed once with 0.5% Trypsin-EDTA. The wash was removed, and additional 0.5% Trypsin-EDTA was added, followed by a 4 minute incubation at 37°C. Trypsinized cells were resuspended in an appropriate volume of growth media and then subcultured or plated for experiments.

To create MCF-7 cells stably expressing GFP-LC3, cells were transfected with pEGFP-C1 vector containing rat LC3-B cDNA (gift from Dr Gordon Shore, McGill University, Montreal, Canada). Transfection was performed using FuGENE 6, according to the manufacturer's directions. Stable transfectants were selected using 500mg/L G-418. The resulting polyclonal cell population was sorted into monoclonal cell lines using a Becton Dickinson FACSAria by the Faculty of Medicine and Dentistry Flow Cytometry Facility. Only cells showing moderate GFP-LC3 fluorescence were selected for sorting. Moderately expression was determined by selecting cells showing approximately 10 to 20 fold higher fluorescence than non-expressing cells. Individual colonies were expanded and then one cell line was chosen based on its continued moderate GFP-LC3 fluorescence and expected response to starvation.

2.6 Inhibitor Treatments

3-Methyladenine was resuspended in ~60 °C water to make a stock solution of 200mM. Before each use, the stock was heated as before to dissolve the solidified 3-methyladenine, and it was added to media to create a final concentration of 5mM.

Bafilomycin A1 was resuspended at a concentration of 8mM in anhydrous DMSO, and diluted to 100nM for usage in experiments. All bafilomycin treatments were performed for 4 hours.

2.7 Apoptosis Assays

Cells were subjected to experimental conditions then harvested, stained with TMRE, and analyzed by flow cytometry. Harvesting was accomplished by washing the cells once with DPBS (2.68mM KCl, 1.47mM KH_2PO_4, 136.9mM NaCl, 8.06mM Na_2HPO_4-$7H_2O$, pH 7.4), then lifted from the plate with 0.05% trypsin-EDTA. After addition of trypsin, plates were incubated at 37°C for 4 minutes followed by resuspension in complete media. All media and washes were collected and the cells were pelleted (500xg, 5 minutes, room temperature, A-4-81). The supernatant was removed and the cells resuspended in 100µL of 100nM TMRE diluted in growth media. The cells were then stained for 30 minutes at 37°C in growth conditions. After staining the cells were pelleted as above and resuspended in 200µL of DPBS. Resuspended cells were analyzed on a Becton Dickinson Biosciences FACScan or FACScalibur equipped with a 488nm laser line paired with a FITC (for EGFP) or phycoerythrin (for TMRE) emission filter. Fluorescent compensation was carried out using single fluorophore controls. Cell death was quantified by setting a gate in the FL2 channel that encompassed all events with lower fluorescence than the live cell population in the untreated control.

2.8 Shakeoff Assays

To collect mitotically arrested cells, plates were knocked to dislodge loosely adherent cells. After knocking, dishes were examined using a light

microscope, and knocked again if significant numbers of mitotic cells remained. The media containing these cells was collected along with a subsequent DPBS wash. These cell populations were denoted as 'Mitotic Fraction' in figures. The remaining cells were harvested using 0.05% Trypsin-EDTA and denoted as 'Adherent Cells' in figures. The cells were then analyzed as described in section 2.6 or lysed as described section 2.8.

2.9 Cell Lysis and Protein Quantification

Cells were subjected to experimental conditions and then harvested as separate mitotic and adherent populations as described in section 2.7, or as a total population by harvesting as in section 2.6. Cell suspensions were pelleted by centrifugation (500xg, 5 minutes, room temperature, FX241.5P). The pellets were washed twice by resuspension in DPBS followed by pelleting as above. The pellet was then lysed by resuspension in a buffer composed of 20mM tris pH 7.4, 150mM NaCl, 2% Triton X-100, 1X complete protease inhibitor, and 1x PHOSstop phosphatase inhibitor. Lysates were incubated for 15 minutes at 4°C. Cellular debris was pelleted by centrifugation (16000xg, 5 minutes, 4°C, F-45-18-11). Samples of the lysates were then taken and diluted to determine protein concentration using the BCA protein assay, following the manufacturer's instructions (Pierce, Rockford, IL, USA).

2.10 SDS-PAGE and Immunoblotting

Protein lysates were mixed with an equal volume of 2x SSB (0.1M tris pH 6.8, 16% glycerol, 3.2% SDS, 8% β-mercaptoethanol, 0.01% bromophenol blue) and loaded onto discontinuous SDS-PAGE gels. Stacking gels were composed of 4% acrylamide, 125mM tris pH 6.8, 0.1% SDS. Separating gels were composed of 375mM tris pH 8.8, 0.1% SDS, with the percentage of acrylamide denoted in figure legends. The lysates were then electrophoresed in a SDS running buffer (0.1% SDS, 24.8mM tris, 192mM glycine) for approximately 70 minutes (until the loading dye ran off) at 180V in a Mini-Protean 3 cell. The stacking gel was then removed, and the separating gel was transferred onto 0.22μm PVDF membrane in a transfer buffer composed of 192mM glycine, 24.8mM Tris, and 20% methanol. Proteins were transferred at 15V in the Mini-Protean 3 cell for at least 12 hours at 4°C. Non specific antibody interactions were prevented by blocking in 5% BSA in TBS-T (for antibodies from Cell Signaling) or in 5% NFSM/TBS-T (for all other antibodies) for one hour at room temperature. After blocking, the membrane was incubated with primary antibody diluted in 5% NFSM/TBS-T overnight at 4°C. The membrane was then washed in TBS-T for 10 minutes with gentle agitation at room temperature. The washing was repeated for a total of three times with fresh TBS-T. After washing, the membrane was incubated with the appropriate HRP conjugated secondary antibody diluted in NFSM/TBS-T for 1 hour at room temperature. The membrane was then washed three times as above and then incubated with ECL-Plus Western Blotting Detection Reagent for 5

minutes according to the manufacturer's directions. Protein bands were visualized by exposure of the membrane to GE Hyperfilm ECL.

2.11 Microarray Analysis

Microarray data from docetaxel resistant and sensitive cell lines (Chang, Wooten et al. 2003) was obtained from the Gene Expression Omnibus database (Edgar, Domrachev et al. 2002). The database was queried for each individual autophagy gene (ULK1, ATG12, ATG5, ATG4B, mATG13, ATG9A, ATG4A, BECN1, LC3B, ULK2, PIK3C3), and results were copied into Microsoft Excel for all further analysis. Using the complete data set, the average raw signal value was calculated. The raw values were then divided by the average value to create the normalized change in expression. The logarithm of each normalized expression to the base of 2 was compiled into a table. Conditional formatting was used to colour code the values in the table and create a heat map. The greatest upregulation on the scale was set to solid green, the lowest to solid red, and unchanged genes were set to black. The numbers in the table were hidden by entering three semicolons into the custom number formatting box. A Student's t-test was performed for each gene, using groups defined by docetaxel sensitivity.

2.12 Immunofluorescent and Fixed Microscopy

Cells were plated on #1.5 coverslips (Electron Microscopy Services, Hatfield, PA, USA) and exposed to experimental conditions. Media was

removed, and the cells were then washed once with DPBS-M and fixed in 4% paraformaldehyde/DPBS-M solution for 15 minutes at room temperature or 4°C overnight. Cells were then washed twice as above, and permeabilized with a 0.1% Triton X-100/DPBS-M solution for 2 minutes at room temperature. Cells were washed three times as above, then blocked in a solution of 4% NDS/DPBS-M (NDS) for 1 hour at room temperature. Cells were washed once as above then incubated with primary antibody diluted in 4%NDS/DPBS-M solution for 1 hour at room temperature. Cells were washed 3 times as above and then incubated with secondary antibody diluted in 4% NDS/DPBS-M containing 1μM Hoescht 33342 (Invitrogen) for 1 hour at room temperature. Cells were washed three times as above and then mounted on slides in Prolong Gold (Invitrogen).

2.13 Wide Field Live Cell Microscopy

Cells were plated on either Lab-Tek II Chambered Coverglass (Nunc, Rochester, NY, USA) or on 35mm glass bottom culture dishes (MatTek, Ashland, MA, USA). During live cell experiments cells were cultured in RPMI 1640 (without phenol red) supplemented with Penicillin (50U/mL), Streptomycin (50μg/mL) and 10% FCS, at 37°C in a humidified 5% CO_2 atmosphere. Wide field microscopy was performed on a Zeiss Axio Observer Z1 (Carl Zeiss Canada, Toronto, ON, Canada) equipped with an Axiocam MRm (Carl Zeiss Canada). Transmitted light was provided by a HAL 100 light source (Carl Zeiss Canada) and reflected light was provided by a HXP-120C lamp (Carl Zeiss Canada). Filter sets are listed in table 2.2.

Cells were imaged on a Heating Insert P S with a CO2-Cover PM (PeCon GmbH, Erbach, Germany). The 5% CO_2 atmosphere was provided by a TempModule S1 and a CO_2 Module S1 (Pecon GmbH).

2.14 Live Cell Spinning Disk Confocal Microscopy

Spinning disk confocal microscopy was performed on a UltraView VoX Confocal Imaging System (PerkinElmer, Woodbridge, ON, Canada) attached to a Leica DMI6000B microscope. Images were acquired on an ImagEM camera (Hamamatsu Corporation, Bridgewater, NJ, USA). Excitation radiation was provided by a 488nm laser line paired with a 527nm band pass (50 nm width) emission filter (PerkinElmer). The growth environment was maintained inside of a Universal ASI Stage Water Jacketed Incubator (Okolab, Ottaviano, NA, Italy). The atmosphere was kept at 19% O_2 and 5% CO_2 by paired DGTO2BX and DGTCO2BX gas mixers (Okolab). Temperature was measured by a TP00-1 thermometer (Okolab), maintained by a Ecoline Staredition E103 water bath (LAUDA, Lauda-Königshofen, Germany) controlled by TempControl Basic software (Okolab).

2.15 Deconvolution

Deconvolution was performed using Huygens Professional software (Scientific Volume Imaging B.V., Hilversum, Netherlands). Microscopic Z stacks were loaded into the software, then processed using either the CMLE or QMLE algorithm. The software used a theoretical point spread function,

an estimated signal to noise ratio, automatic background estimation, automatic bleaching correction, automatic brick mode, optimized iteration mode, automatic padding mode, a quality change threshold of 0.1%, for a maximum of 10 iterations during QMLE or 40 during CMLE.

2.16 GFP Puncta Counting and Quantification

After acquisition and deconvolution, image file names were randomized using Filename Randomizer (CodeUnit, Craig Lotter). Images were analyzed in a blinded fashion using Imaris x64 (Bitplane Scientific Software, Switzerland). Images were cropped to remove excess volume. To determine the number of puncta, a surface layer was created for the channel containing GFP-LC3 fluorescence. Mitotic cells were omitted using regions of interest. The surface layer was smoothed using a 0.1μm surface detail level, and thresholding was performed using background subtraction, using a largest sphere diameter of 0.1μm. The intensity threshold was set manually, so as to outline all visible puncta, while omitting false selections. Large clusters of continuous puncta were separated using the region growing function, using either a seed size of 0.3μm or 0.5μm for small and large puncta, respectively. Seed point thresholding was performed using a manually set quality threshold that included all visible puncta. Finally, if present, small erroneously generated puncta were eliminated using a voxel number filter, manually set between 1-10 voxels. A final surface layer was then calculated, and the software determined the number of puncta present. The total number of cells was counted manually, using the DAPI

fluorescence channel. The number of puncta/cell was quantified for three independent experiments, and the fold induction was calculated using the following formula: (Number of puncta/cell after four hours of Bafilomycin A1 treatment)/(Number of puncta/cell without Bafilomycin A1 treatment). This ratio was averaged for the three experiments and plotted.

2.17 GFP-LC3 Puncta Tracking

Live cell data for puncta tracking was acquired through live cell spinning disk microscopy (section 2.14). Three dimensional image stacks were acquired at maximum speed (5-15s/stack) for a total of 5 minutes. Data was then exported from Volocity (PerkinElmer, Woodbridge, ON, Canada). Pixel spacing and time metadata were reentered manually, and images were analyzed in Imaris x64. Puncta were tracked manually, and the statistics for each stack were exported, compiled, and analyzed in Excel (Microsoft, Redmond, Washington, USA).

2.18 Statistical Analysis

Data are presented as a mean of three independent experiments, with error bars indicating the standard deviation. Statistical significance was determined using a two-tailed Student's T Test for two means with equal variance.

2.19 References

Chang, J. C., E. C. Wooten, A. Tsimelzon, S. G. Hilsenbeck, M. C. Gutierrez, R. Elledge, S. Mohsin, C. K. Osborne, G. C. Chamness, D. C. Allred and P. O'Connell (2003). "Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer." Lancet **362**(9381): 362-369.

Edgar, R., M. Domrachev and A. E. Lash (2002). "Gene Expression Omnibus: NCBI gene expression and hybridization array data repository." Nucleic Acids Res **30**(1): 207-210.

Chapter 3: The Role of Autophagy During Paclitaxel Treatment of Breast Cancer Cells

3.1 Autophagy contributes to paclitaxel cytoxicity

To determine what role autophagy has on cellular response to paclitaxel, we decided to inhibit autophagy and assess whether the treatment affected the ability of paclitaxel to induce cell death. We treated MCF-7 and SK-BR-3 cells with paclitaxel for 48 hours in the presence or absence of the autophagy inhibitor 3-MA, followed by assessment of cell death by TMRE staining and analysis by flow cytometry (Fig. 3.1). In MCF-7 cells, paclitaxel induced 25% specific cell death (specific cell death will be used to refer to the percentage of TMRE negative cells with treatment, subtracted from the percentage of TMRE negative cells in the untreated sample). Adding 3MA to the cells for the duration of paclitaxel treatment decreased specific cell death from 25% to 14%. 3MA induced 6% specific cell death when used alone, so when this toxicity was taken into account, cell death specific to paclitaxel was reduced from 25% to 8%.

To ensure that these effects were not specific to MCF-7 cells, the experiments were repeated in SK-BR-3 cells. Paclitaxel caused 67% specific cell death in SK-BR-3 cells, and this was reduced to 47% in the presence of 3MA (Fig. 3.1). When 3MA toxicity was accounted for, 3MA reduced paclitaxel induced specific cell death from 67% to 41%. These data suggest that autophagy contributes to paclitaxel induced cell death in two independent breast carcinoma cell lines.

3.2 A subset of autophagy genes is down-regulated in docetaxel resistant breast tumors

Data from a breast cancer cell culture model indicate that autophagy contributes to paclitaxel induced cell death. To determine if the same relationship between taxanes, autophagy, and cell death existed in primary breast tumors, we queried a publically available database of microarray data, that includes breast tumors treated with four cycles of neoadjuvant docetaxel chemotherapy (Edgar, Domrachev et al. 2002; Chang, Wooten et al. 2003). The microarray samples are divided into docetaxel sensitive samples (from tumors that shrunk to less than 25% of their pre-treatment size) or docetaxel resistant samples (from tumors that did not shrink to at least 25% of their original size). After querying the microarray data for autophagy genes, we found that a subset were downregulated in the docetaxel resistant tumor samples (Fig. 3.2). Downregulated genes included ULK1, Atg12, Atg5, Atg4B, mAtg13, and Atg9A. These data support a functional link between autophagy and taxane cytotoxicity, suggest that autophagy may contribute to clinical efficacy of taxane chemotherapy, and further suggest that autophagy genes may be useful prognostic indicators of taxane responsiveness.

3.3 Paclitaxel decreases autophagic flux in breast cancer cells

Based on the observation that autophagy stimulates paclitaxel cytotoxicity, we wanted to determine if paclitaxel regulated autophagy. Therefore we

examined the accumulation of the autophagic marker LC3-II in untreated and paclitaxel treated cells, with starved cells as a control. LC3-II localizes to the autophagosome in response to autophagic induction, and remains associated with the autophagosome until it is degraded by fusion with a lysosome (Kabeya, Mizushima et al. 2000; Tanida, Minematsu-Ikeguchi et al. 2005). Therefore, to observe the accumulation of LC3-II, we treated cells with the lysosomal H^+-ATPase inhibitor bafilomycin A1 (Werner, Hagenmaier et al. 1984; Bowman, Siebers et al. 1988; Yoshimori, Yamamoto et al. 1991). Immunoblotting for LC3 showed that non-paclitaxel treated cells showed an increase in the intensity of the LC3-II band when treated with bafilomycin (Fig. 3.3). This difference is an indication of the basal level of autophagy in MCF-7 cells. Starved cells showed the largest increase in LC3-II intensity with the addition of bafilomycin, indicating the largest amount of autophagic flux. In contrast, paclitaxel treated cells accumulated a lower intensity LC3-II band with the addition of bafilomycin, indicating that paclitaxel treated cells have the lowest amount of autophagic flux.

These cells also stably expressed the fluorescent autophagosomal marker GFP-LC3. Non-paclitaxel treated cells showed an increase in GFP-LC3-II when treated with bafilomycin. Paclitaxel treated cells showed an increase in intensity of the GFP-LC3-II band compared to the untreated control, but the band showed a smaller increase with the addition of bafilomycin than the non-paclitaxel treated cells. Starved cells showed no clear GFP-LC3-II band in the absence of bafilomycin, but showed the largest band with its addition

(α-Tubulin was used as a loading control). These data indicate that paclitaxel inhibits autophagy in breast cancer cells.

To examine the mechanism of paclitaxel induced autophagy inhibition, we decided to examine autophagic flux at the single cell level. Therefore we generated MCF-7 cells that stably express GFP-LC3, a fluorescent autophagic marker that localizes to autophagosomes in response to autophagic induction. Briefly, cells were plated on glass coverslips, treated with paclitaxel for 24 hours, and then visualized along with untreated and starved controls (Fig. 3.4). Cells grown under normal conditions showed punctate GFP-LC3 fluorescence that was markedly increased in both quantity and intensity in the presence of bafilomycin. This indicates a robust basal level of autophagic flux. Paclitaxel treated cells showed two distinct patterns of GFP-LC3 fluorescence in two distinct subpopulations of cells. Paclitaxel induces mitotic arrest, so we observed both non-mitotic and mitotic cells (determined by chromosome condensation and indicated by arrowheads). Mitotic cells consistently show low number of GFP-LC3 puncta when compared to their non-mitotic counterparts. When treated with pac, mitotic cells show a smaller increase in the number of puncta than non-mitotic cells. This indicates that paclitaxel treated cells in mitotic arrest had diminished autophagosome production. This potential block in autophagosome formation may partially explain the paclitaxel induced inhibition of autophagy seen in figure 3.3.

In contrast, paclitaxel treated cells that were not in mitosis showed a modest increase in the number of observed GFP-LC3 puncta when compared to untreated control cells (Fig. 3.4A). While the observed increase in the number of autophagosomes suggests that paclitaxel induces autophagy, we also observed that starved cells showed fewer puncta than the untreated control cells where it is known that starvation is a strong inducer of autophagy. Therefore, assessment of autophagic induction cannot be performed by simply counting GFP-LC3 puncta in cells.

This is because successful fusion of an autophagosome with a lysosome results in the degradation of the autophagosomal cargo, with concomitant loss of GFP-LC3 signal. Since LC3-II (and GFP-LC3-II) are contained within the autophagosome, an increase in the number of GFP-LC3 puncta can indicate either increased autophagosome formation (induction of autophagy) or decreased autophagosome degradation (inhibition of autophagy) (Tanida, Minematsu-Ikeguchi et al. 2005; Mizushima and Yoshimori 2007). To prevent the clearance of autophagosomes and distinguish between the two possibilities, each treatment was therefore performed in duplicate, with one of each pair being treated with bafilomycin. Cells grown under normal conditions show a striking increase in the number of GFP-LC3 positive puncta in the presence of bafilomycin, illustrating the basal level of autophagy in MCF-7 cells. Starved cells showed a larger bafilomycin dependant increase in the number of GFP-LC3 puncta, indicating that starvation increased the autophagic flux in MCF-7 cells (Fig. 3.4A, 4h Baf). In comparison, non-mitotic paclitaxel treated cells show a small increase in the number of GFP-LC3

puncta, when comparing either non-bafilomycin or bafilomycin treated samples. These results suggest that paclitaxel inhibits autophagic flux. To quantify this increase, we counted the number of GFP-LC3 puncta/non-mitotic cell in each treatment and then determined the fold increase in the number of puncta/cell when the samples were treated with bafilomycin. This analysis demonstrated that the paclitaxel treated cells showed the least autophagic turnover (1.6 fold increase), the starved cells showed the most (4.5 fold increase), and the untreated cells showed an intermediate amount (3.3 fold increase) (Fig. 3.4B).

The above results suggest that paclitaxel may inhibit autophagy at two stages: by inhibiting autophagosome formation within mitotically arrested cells, and by inhibiting autophagosome maturation within non-mitotic cells. Therefore, we decided to examine these two distinct blocks.

3.4 Mitotic arrest inhibits autophagy in breast cancer cells

Mitotically arrested cells show lower numbers of GFP-LC3 puncta compared to neighboring non-mitotically arrested cells (Fig 3.4A). This observation, as well as a previous report, suggests that autophagy may be inhibited during mitosis (Furuya, Kim et al. 2010). As paclitaxel treatment induces widespread mitotic arrest, this may represent one mechanism of paclitaxel induced autophagy inhibition. Therefore, we decided to specifically examine mitotic cells for evidence of autophagic inhibition.

To isolate a population of mitotically arrested cells, we performed a mitotic shakeoff and collected the loosely adherent cells. These cells were then examined with a microscope. The collected cells showed low numbers of GFP-LC3 puncta compared to cells not in mitotic arrest (Figs 3.5 and 3.4A). The bafilomycin treated sample showed only a modest increase in the number of GFP-LC3 puncta when compared to paclitaxel treatment alone, showing that autophagy is inhibited in mitotically arrested cells (Fig. 3.5). These data also validate the ability of the mitotic shakeoff technique to isolate a population of mitotically arrested cells.

To confirm that autophagy is inhibited in mitotically arrested cells, we performed a paclitaxel treatment and mitotic shakeoff in two breast cancer cell lines, and collected cells for biochemical analysis. Each population (untreated, paclitaxel treated for 24 hours-adherent cells, paclitaxel treated for 24 hours-mitotic fraction; all with a paired bafilomycin treatment) was lysed separately and examined by immunoblot. Compared to their bafilomycin treated duplicates, the mitotic cell fraction showed the smallest increase in LC3-II band intensity, the adherent cells showed an intermediate amount, and the untreated cells showed a large increase (Fig. 3.6). To support the LC3 data, we also immunoblotted for p62, an autophagic substrate. Only the untreated cells showed an increase in p62 band intensity with bafilomycin treatment. Together, these data show that paclitaxel treatment inhibits autophagy, and that the inhibition is strongest in mitotically arrested cells.

To observe individual cells throughout the duration of paclitaxel treatment, we performed live cell microscopy on MCF-7 cells stably expressing GFP-LC3. As a cell entered into and remained in mitotic arrest, GFP-LC3 staining that localized to distinct puncta slowly disappeared and was replaced by a diffuse fluorescence (Fig. 3.7, 2-9h). Once the cell escaped mitotic arrest, fluorescent GFP-LC3 puncta staining reappeared (Fig. 3.7, 10-14h). To determine the amount of LC3 localized to autophagosomes, we determined the amount of punctate GFP-LC3 fluorescence as a percentage of total GFP-LC3 signal. The percentage of punctate GFP-LC3 decreased as the cells entered mitotic arrest, reaching a minimum one hour after arrest (Fig. 3.7, 5h). As the cell left mitotic arrest the percentage of punctate GFP-LC3 quickly increased, reaching a 6.5 fold increase over the initial percentage, 4 hours after exiting arrest (Fig. 3.7, 2h vs 14h). The live cell microscopy shows that autophagosome formation is strongly inhibited during mitotic arrest, and increases after exiting.

3.5 Inhibition of Autophagosome formation in paclitaxel mediated mitotic arrest is associated with Vps34 inhibition

Since we observed that autophagosome formation is blocked in cells undergoing paclitaxel-induced mitotic arrest, we decided to investigate possible causes of this inhibition. While there is some controversy as to whether autophagy is altered in mitotic cells, Furuya *et al.* showed convincingly that in HeLa, 293T and H4 cells, autophagy was blocked in

mitosis in response to nocodazole treatment. Furthermore, they identified that this block was dependent on cdk1 and cdk5 mediated inhibitory phosphorylation of Vps34 on T159 (Furuya, Kim et al. 2010). This phosphorylation caused decreased binding between Vps34 and Beclin 1, leading to lower Vps34 kinase activity. Therefore we decided to test whether the same phosphorylation occurred in breast cancer cells in response to paclitaxel. MCF-7 and SK-BR-3 cells were treated with paclitaxel for 24 hours, and then a mitotic shakeoff was performed. Western blot examination of the cell lysates using an antibody for Vps34 phospho-T159 showed the appearance of a band in the mitotic cell fraction (Fig. 3.8). This demonstrates that T159 of Vps34 is phosphorylated in paclitaxel treated, mitotically arrested cells, and that this may contribute to inhibition of autophagosome formation. On the other hand, adherent cells showed only low levels of Vps34 T159 phosphorylation in response to paclitaxel treatment. This suggests that as cells exit mitosis, the increased autophagosome formation that we observed (Fig. 3.8) is associated with, and may be the result of, increased Vps34 activity.

3.6 Paclitaxel disrupts autophagosome trafficking along microtubules

As mentioned previously, we observed two distinct blocks to autophagy in paclitaxel-treated cells. Not only did we observe a block in autophagosome formation in paclitaxel-induced mitotic arrest (Fig. 3.7), in non-mitotic cells we observed a block in autophagosome maturation (Fig. 3.4). Since correct

autophagosome trafficking towards lysosomes is central to autophagic flux, we decided to monitor autophagosome movement. Therefore, we plated MCF-7 cells stably expressing GFP-LC3 onto chambered coverglass and examined GFP-LC3 movement on a spinning disk confocal microscope. We acquired three dimensional image stacks to quantify the change in puncta speed (Fig. 3.9). In control cells the average speed was 0.104 μm/s, with speeds ranging from 0.014 to 0.423 μm/s. Many puncta were observed to move long distances during the observation period. By contrast, in cells treated with paclitaxel for 24 hours, the average speed was approximately half that of untreated cells (0.055 μm/s), with the speeds ranging from 0.011 to 0.244 μm/s. Puncta in paclitaxel treated cells moved comparatively short distances. This alteration in puncta dynamics indicates that paclitaxel inhibits puncta tracking and therefore likely affects autophagosome maturation.

3.7 Paclitaxel disrupts autophagosome localization

To determine whether the alteration in autophagosome dynamic movement affected autophagosome intracellular localization, we performed colocalizaion studies to assess the association of autophagosomes with the microtubules, microtubule organizing center (MTOC) and lysosomes. Autophagosomes are normally associated with and traffic along microtubules (Fass, Shvets et al. 2006). Analysis of bafilomycin treated GFP-LC3 expressing cells showed a distinct perinuclear localization. However, this association was disrupted by paclitaxel treatment (Fig. 3.4A). As paclitaxel induced stabilization of microtubules may contribute to aberrant trafficking of autophagosomes, we

performed immunofluorescent microscopy to examine the relationship between GFP-LC3 and microtubules in paclitaxel treated cells. In untreated and starved cells, GFP-LC3 puncta were associated with microtubules, and the microtubules appeared organized around a perinuclear microtubule organizing center (MTOC) as visualized by indirect immunofluorescence of pericentrin (Fig. 3.10). The GFP-LC3 puncta appeared to be localizing to the region of the cell surrounding the MTOC. In paclitaxel treated cells, GFP-LC3 puncta were associated with microtubules, but did not show a distinct cellular localization (Fig. 3.10). Additionally, the microtubule network appeared to be disorganized when compared to either the untreated or starvation control (Fig. 3.10).

One of the functions of autophagosome trafficking is to move the autophagosome into the proximity of a lysosome so that they may fuse and degrade the autophagic cargo. To examine the effect of the disrupted microtubule network we examined the relationship between the lysosomes and GFP-LC3 by fluorescent microscopy (Fig. 3.9). In both untreated and starvation controls, GFP-LC3 and LAMP1 colocalized to the same region of the cell (Fig. 3.9 and 3.10). The bafilomycin treated samples showed high levels of colocalization between GFP-LC3 and LAMP1 in the untreated and starved controls indicating that bafilomycin-treatment blocked autophagosome degradation after association with lysosomes. In contrast, paclitaxel treated cells did not show any clear colocalization with lysosomes as shown by LAMP1 immunofluorescence (Fig. 3.11). Furthermore, bafilomycin treatment showed a near absence of colocalization in paclitaxel

treated samples (Fig. 3.11). Together, these results show that paclitaxel significantly impacts autophagosome trafficking. Specifically, paclitaxel treatment causes disorganization of the microtubule network and disrupts the association of autophagosomes with lysosomes by causing mislocalization of both. This has the functional effect of preventing efficient degradation of autophagosomes and their cargo, and is one mechanism of paclitaxel inhbition of autophagy.

3.8 References

Bowman, E. J., A. Siebers and K. Altendorf (1988). "Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells." Proc Natl Acad Sci U S A **85**(21): 7972-7976.

Chang, J. C., E. C. Wooten, A. Tsimelzon, S. G. Hilsenbeck, M. C. Gutierrez, R. Elledge, S. Mohsin, C. K. Osborne, G. C. Chamness, D. C. Allred and P. O'Connell (2003). "Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer." Lancet **362**(9381): 362-369.

Edgar, R., M. Domrachev and A. E. Lash (2002). "Gene Expression Omnibus: NCBI gene expression and hybridization array data repository." Nucleic Acids Res **30**(1): 207-210.

Fass, E., E. Shvets, I. Degani, K. Hirschberg and Z. Elazar (2006). "Microtubules support production of starvation-induced autophagosomes but not their targeting and fusion with lysosomes." J Biol Chem **281**(47): 36303-36316.

Furuya, T., M. Kim, M. Lipinski, J. Li, D. Kim, T. Lu, Y. Shen, L. Rameh, B. Yankner, L. H. Tsai and J. Yuan (2010). "Negative regulation of Vps34 by Cdk mediated phosphorylation." Mol Cell **38**(4): 500-511.

Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi and T. Yoshimori (2000). "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing." EMBO J **19**(21): 5720-5728.

Mizushima, N. and T. Yoshimori (2007). "How to interpret LC3 immunoblotting." Autophagy **3**(6): 542-545.

Tanida, I., N. Minematsu-Ikeguchi, T. Ueno and E. Kominami (2005). "Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy." Autophagy **1**(2): 84-91.

Werner, G., H. Hagenmaier, H. Drautz, A. Baumgartner and H. Zahner (1984). "Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity." J Antibiot (Tokyo) **37**(2): 110-117.

Yoshimori, T., A. Yamamoto, Y. Moriyama, M. Futai and Y. Tashiro (1991). "Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells." J Biol Chem **266**(26): 17707-17712.

Chapter 4: Discussion

4.1 Paclitaxel inhibits autophagy through disruption of autophagosome localization and trafficking

The specific role of microtubules in autophagy is unclear (Monastyrska, Rieter et al. 2009). Using nocodazole to disrupt the tubulin network, several groups have shown that microtubules mediate autophagosome formation (Fass, Shvets et al. 2006; Kochl, Hu et al. 2006), trafficking (Kochl, Hu et al. 2006; Xie, Nguyen et al. 2010), and speed (Jahreiss, Menzies et al. 2008). The role of microtubule dynamics in this process, however, is less clear. Paclitaxel is a microtubule stabilizing agent that has multiple cellular effects depending on intracellular concentration. At high concentrations (greater than 200nM), paclitaxel bundles microtubules and initiates a cell-cycle independent cell death pathway, whereas at lower clinically achievable concentrations, paclitaxel initiates mitotic arrest-dependent apoptosis (Wang, Wang et al. 2000). With respect to autophagy, using high concentrations of paclitaxel (5-20μM) Xie *et al.* found that paclitaxel increased GFP-LC3 puncta formation specifically in mitotic cells (Xie, Nguyen et al. 2010), and Kimura *et al.* observed a modest diminishment of autophagosome speed (Kimura, Noda et al. 2008). On the other hand, Kochl *et al.* found that stabilized microtubules had no significant effect on autophagosome formation. Thus it is still not clear how paclitaxel regulates autophagy (Kochl, Hu et al. 2006).

This study shows that lower, clinically relevant doses of paclitaxel have an inhibitory effect on autophagic flux (see Fig. 4.1 for model). Biochemical examination of LC3-I/II conversion as well as immunofluorescent examination

of GFP-LC3 puncta formation demonstrated that autophagosomes accumulate in non mitotic, paclitaxel treated cells. While similar observations were initially interpreted as a paclitaxel-dependent stimulation of autophagy (Gorka, Daniewski et al. 2005), using the lysosomal inhibitor bafilomycin A1, we showed that the accumulation of autophagosomes was the result of inefficient autophagosome degradation, rather than an increase in autophagosome formation.

This observed block in autophagosome degradation led to examination of the underlying causes. We found that low-dose paclitaxel treatment altered autophagosome movement. Live cell microscopic examination showed that autophagosomes moved at lower speed in paclitaxel treated cells. In non-paclitaxel treated cells, autophagosome movement was not uniform, with some GFP-LC3 positive puncta moving very rapidly and for long distances, while others moved relatively slowly. In contrast, GFP-LC3 puncta in paclitaxel treated cells had a lower average speed, a lower maximum speed, and very few puncta that travelled long distances. Lysosomes are also trafficked along microtubules, presumably to the same final location of autophagosomes. So it is possible that diminished trafficking speeds and distances would delay the arrival of autophagosomes to areas enriched in lysosomes and therefore diminish autophagic flux. Therefore, we decided to evaluate autophagosome localization.

Paclitaxel treatment caused autophagosomes to change localization from primarily perinuclear to diffuse cytoplasmic localization. Perinuclear

autophagosomal localization coincides with the location of the Microtubule Organizing Center (MTOC), which is the eventual destination of autophagosomes (Matteoni and Kreis 1987; Jahreiss, Menzies et al. 2008). To assess the association of autophagosomes to the MTOC, we used the MTOC-specific marker, pericentrin. Immunofluorescent examination of GFP-LC3 and pericentrin showed that autophagosomes in both untreated and starved cells localize to the MTOC. On the other hand, paclitaxel treatment caused mislocalization of GFP-LC3 away from the MTOC.

The MTOC shares similar perinuclear localization to the Golgi apparatus, the site of lysosomal biogenesis (Anitei, Wassmer et al. 2010). Additionally, lysosomes are trafficked along the microtubules toward the MTOC (Matteoni and Kreis 1987). This may be biologically significant as a way of concentrating lysosomes and autophagosomes into the same region of the cell, allowing their efficient fusion. Therefore we assessed the effect of paclitaxel treatment on autophagosome-lysosome association. Immunofluorescent analysis revealed that during basal or starvation-induced autophagy, lysosomes and autophagosomes colocalized to the same perinuclear region. Paclitaxel treatment causes a relocalization of both the lysosomes and autophagosomes throughout the cytosol, with little co-association between the two organelles. As well, bafilomycin treatment shows diminished accumulation of LC3-II in paclitaxel treated cells versus non-paclitaxel treated cells. Taken together, this data indicates that paclitaxel treatment inhibits autophagosome-lysosome associations, resulting in a block in autophagosome maturation and turnover.

4.2 Mitotic arrest inhibits autophagy

Not only did we find that paclitaxel inhibits autophagosome traffic, we also found that paclitaxel inhibited autophagosome formation in mitotically arrested cells. Eskelinen *et al.* first described that autophagy was suppressed in mitotic NRK cells (Eskelinen, Prescott et al. 2002). However Liu *et al.* observed autophagy in mitotic HeLa cells (Liu, Xie et al. 2009). Whether these conflicting results reflect cell-type specific differences, it is obvious that extrapolation of results between different systems must be done with caution. This study shows that autophagy is inhibited in paclitaxel treated mitotically arrested breast cancer cells. This was demonstrated through LC3 blotting as well as through live cell analysis. Live cell microscopy revealed that when a cell entered mitotic arrest, it slowly degrades the autophagosomes present at the time of arrest. There was no obvious formation of new autophagosomes. This suggests that in mitotic cells, preexisting autophagosomes can still fuse with lysosomes and degrade their cargo, though it appears that new autophagosome formation is suppressed. Recently, Furuya *et al.* provided a mechanism for mitotic autophagy suppression by demonstrating that Cdk1 phosphorylates Vps34 on T159 during mitosis, decreasing its binding with Beclin 1 and inhibiting formation of autophagosomes (Furuya, Kim et al. 2010). Examination of paclitaxel treated, mitotically arrested cells showed phosphorylation of Vps34 on T159. To my knowledge, this is the first demonstration of Vps34-specific inhibition in response to paclitaxel treatment. Notably, this phosphorylation was nearly absent in the adherent fraction of paclitaxel treated cells, suggesting Vps34 phosphorylation was specifically

associated with mitotic arrest. Live cell examination of paclitaxel treated cells also revealed that as a cell left mitotic arrest, there was a rapid formation of autophagosomes, and the loss of Vps34 phosphorylation suggests a potential mechanism.

4.3 Autophagy as a therapeutic target during paclitaxel treatment

Manipulation of autophagy is currently being investigated as a treatment strategy to improve patient response to chemotherapy (Fleming, Noda et al. 2011). Determining whether a particular cancer treatment would benefit most from inhibition or induction of autophagy is of the utmost importance, as pharmaceuticals already exist for either approach. The autophagy inhibitor chloroquine has shown success in clinical trials for the treatment of glioblastoma (Sotelo, Briceno et al. 2006; Briceno, Calderon et al. 2007) and the autophagy inducer rapamycin is widely used to facilitate transplants (Campsen, Zimmerman et al. 2011). This study showed that inhibition of autophagosome formation with Vps34 inhibitor, 3MA, diminished paclitaxel-induced cell death *in vitro*. As well, a subset of autophagy genes was down-regulated in docetaxel-resistant tumors. Together, these data suggest that autophagy may enhance the effectiveness of taxane therapy in breast cancer. But does paclitaxel-inhibited autophagy limit cell death? If that were the case, then the rational approach would be to stimulate autophagy.

Yet it is unclear if traditional inducers of autophagy would be capable of increasing cell death, as paclitaxel inhibits autophagy at multiple steps.

Rapamycin induces autophagy (through inhibition of mTOR) and is known to be a well tolerated therapy. We did attempt to evaluate whether rapamycin could stimulate autophagy in the presence of paclitaxel. Preliminary data showed that rapamycin treatment has no effect on autophagosome formation or paclitaxel cytotoxicity in a breast cancer cell culture model. This may indicate that the duration of rapamycin treatment was insufficient to affect autophagosome formation, but this seems unlikely considering how quickly starvation (a potent inhibitor of mTOR) can affect autophagic flux. A more likely explanation is that autophagic induction is also inhibited downstream of mTOR. We found that Vps34 activity is likely to be inhibited during paclitaxel induced mitotic arrest. Inhibition of Vps34 would then prevent mTOR based induction of autophagy. Additionally, even if autophagosome formation can be enhanced, paclitaxel blocks downstream autophagosome trafficking and fusion with lysosomes through its effects on microtubules. Thus therapeutic induction of autophagy in combination with paclitaxel treatment is not possible until the molecular mechanisms of autophagosome trafficking are better understood.

Alternatively, these data are also consistent with the idea that blunted autophagy itself is a cell death signal. In this case, the rational approach would be to stimulate autophagosome-formation in combination with paclitaxel, which would result in an increased build-up of autophagosomes incapable of fusing with lysosomes. Potentially, this blunted autophagy then kills the cells. For the moment, we have shown that autophagy and taxane-

induced cell death are linked. How these diverse signaling pathways interact, will be the subject of future experiments.

4.4 Future Research

4.4.1 How does paclitaxel inhibit autophagy?

This study raises many significant questions about the relationship between paclitaxel, autophagy, and cell death. While the phenomenon of autophagy inhibition was well characterized, there are major uncertainties remaining about the regulation during paclitaxel treatment. Paclitaxel induced mitotic arrest causes an inhibitory phosphorylation on Vps34 that coincides with reduced autophagosome formation. The functional effect of Vps34 phosphorylation can be demonstrated by performing immunoprecipitations of Beclin 1, and determining if there is diminished binding to Vps34. This interaction is crucial for autophagic induction, so diminished binding would indicate a functional block in autophagosome formation. There may be additional levels of regulation that combine to create the observed inhibitory effect. Only an understanding of the complex regulation of autophagosome initiation will allow effective manipulation strategies.

mTOR is one of the best characterized regulators of autophagy. Under nutrient rich conditions, mTOR is active and phosphorylates multiple proteins within the Ulk complex, as well as downstream cell growth regulators (Yang and Klionsky 2010). The level of mTOR activity during paclitaxel treatment is

unknown, but can be assessed by examination of mTOR S2448 phosphorylation (Chiang and Abraham 2005) or by assessing the phosphorylation of its substrates p70SK and 4E-BP1 (Fang, Vilella-Bach et al. 2001). This pathway can be examined further downstream, at the level of the Ulk complex. The Ulk1 complex is a potentially important regulatory target, as it is essential for autophagosome formation (Yang and Klionsky 2010). Ulk1 has recently been shown to be regulated by AMPK and mTOR (Egan, Kim et al. 2011; Kim, Kundu et al. 2011; Loffler, Alers et al. 2011). The two kinases have opposing effects on Ulk activity: AMPK stimulates its activity and mTOR inhibits it. If paclitaxel induced autophagy inhibition involves suppression of Ulk complex kinase activity, this block could potentially be overcome through the use of mTOR inhibitors, leading to increased autophagosome formation and increased cytotoxicity.

4.4.2 How does autophagy promote cell death in paclitaxel treated cells?

The question of how autophagy potentiates paclitaxel cytotoxicity remains unanswered. One hypothesis is that autophagosome degradation is necessary to increase cytotoxicity. It is unclear why increased autophagy leads to increased cell death though it can be imagined that excessive autophagy could destroy key cellular components. An alternative hypothesis posits that autophagosome accumulation without degradation is toxic. In this scenario, autophagic cargo is sequestered within the autophagosome and provides no metabolic benefit due to downstream defects in trafficking. It is

challenging to distinguish between the two possibilities. Inhibiting autophagy with 3MA is protective, but that result supports either hypothesis. 3MA inhibits autophagosome formation, diminishing both the number of autophagosomes that accumulate within the cell as well as the number available to be degraded.

Effectively inducing autophagosome formation would allow discrimination between these possibilities. We attempted to address this using rapamycin treatment. The rationale was that if increased autophagosome formation lead to greater cell death in paclitaxel treated cells, it would indicate that autophagosome accumulation rather than degradation contributed to cell death, as any autophagosomes formed would traffic inefficiently. No change in cytotoxicity was observed. Although this result is inconclusive, as rapamycin did not increase autophagosome formation in the presence of paclitaxel. For this approach to work, it would require some additional treatment to induce autophagosome formation. The observed increase in T159 phosphorylation of Vps34 may represent a target for such a treatment. Furuya *et al.* identified Cdk1 and Cdk5 as the kinases responsible for this phosphorylation in mitotic cells (Furuya, Kim et al. 2010). Cdk5 is primarily associated with cells of the nervous system (Dhavan and Tsai 2001), so inhibition of Cdk1 to stimulate Vps34 mediated autophagosome formation may de-repress autophagosome formation. We predict that with Cdk1 inhibition, autophagosome formation of mitotically arrested cells will increase, yet autophagosome trafficking will still be blocked. If under these conditions, there was increased cell death, then this could indicate that accumulation of

undegraded autophagosomes contributes to cell death. These experiments will provide insight into how autophagy promotes death as well as how autophagy promotes cell death in paclitaxel treatment.

Another way to explore the mechanism of paclitaxel cytotoxicity would be to restore efficient autophagosome trafficking, though there is no obvious way to directly accomplish this. Monastrol, the small molecule inhibitor of Eg5 provides an alternative approach to study the effects of autophagy of cell death in the context of anti-mitotic treatment (Mayer, Kapoor et al. 1999). Eg5 is a mitotic kinesin, and is necessary for the formation of a bipolar spindle (Sawin, LeGuellec et al. 1992). Inhibition of Eg5 with monastrol causes mitotic arrest and eventually leads to apoptosis (Chin and Herbst 2006). As autophagosome trafficking is dynein dependent (Jahreiss, Menzies et al. 2008; Kimura, Noda et al. 2008), monastrol should theoretically induce mitotic arrest and cell death in the absence of an autophagy trafficking block. In this experimental system, existing modulators of autophagy can be used to determine the role of autophagy in cytotoxicity. With no downstream autophagosome trafficking block, inducers of autophagy such as rapamycin will increase autophagic flux. Then, by comparing the effects of an autophagy inducer in the presence or absence of a lysosomal inhibitor such as chloroquine or bafilomycin, we can determine whether cytotoxicity caused by mitotic inhibitors depends on autophagosome degradation or accumulation.

4.5 Summary

Considerable controversy and uncertainty exists regarding the interplay between paclitaxel, autophagy, and cell death. This study addressed the uncertainty using a breast cancer cell culture model and clinically attainable concentrations of paclitaxel. We determined that autophagy contributes to paclitaxel induced cell death, and that this may be a clinically relevant relationship as docetaxel sensitive primary breast tumors show upregulation of a subset of autophagy genes. We also found that paclitaxel treatment inhibits autophagy through two distinct mechanisms: disruption in autophagosome formation and trafficking. Paclitaxel's importance as a chemotherapeutic agent underscores the critical importance of understanding the relationship between paclitaxel, autophagy, and cell death.

4.6 References

Anitei, M., T. Wassmer, C. Stange and B. Hoflack (2010). "Bidirectional transport between the trans-Golgi network and the endosomal system." Mol Membr Biol **27**(8): 443-456.

Briceno, E., A. Calderon and J. Sotelo (2007). "Institutional experience with chloroquine as an adjuvant to the therapy for glioblastoma multiforme." Surg Neurol **67**(4): 388-391.

Campsen, J., M. A. Zimmerman, S. Mandell, M. Kaplan and I. Kam (2011). "A Decade of Experience Using mTor Inhibitors in Liver Transplantation." J Transplant **2011**: 913094.

Chiang, G. G. and R. T. Abraham (2005). "Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase." J Biol Chem **280**(27): 25485-25490.

Chin, G. M. and R. Herbst (2006). "Induction of apoptosis by monastrol, an inhibitor of the mitotic kinesin Eg5, is independent of the spindle checkpoint." Mol Cancer Ther **5**(10): 2580-2591.

Dhavan, R. and L.-H. Tsai (2001). "A decade of CDK5." Nat Rev Mol Cell Biol **2**(10): 749-759.

Egan, D., J. Kim, R. J. Shaw and K. L. Guan (2011). "The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR." Autophagy **7**(6).

Eskelinen, E. L., A. R. Prescott, J. Cooper, S. M. Brachmann, L. J. Wang, X. W. Tang, J. M. Backer and J. M. Lucocq (2002). "Inhibition of autophagy in mitotic animal cells." Traffic **3**(12): 878-893.

Fang, Y., M. Vilella-Bach, R. Bachmann, A. Flanigan and J. Chen (2001). "Phosphatidic acid-mediated mitogenic activation of mTOR signaling." Science **294**(5548): 1942-1945.

Fass, E., E. Shvets, I. Degani, K. Hirschberg and Z. Elazar (2006). "Microtubules support production of starvation-induced autophagosomes but not their targeting and fusion with lysosomes." J Biol Chem **281**(47): 36303-36316.

Fleming, A., T. Noda, T. Yoshimori and D. C. Rubinsztein (2011). "Chemical modulators of autophagy as biological probes and potential therapeutics." Nat Chem Biol **7**(1): 9-17.

Furuya, T., M. Kim, M. Lipinski, J. Li, D. Kim, T. Lu, Y. Shen, L. Rameh, B. Yankner, L. H. Tsai and J. Yuan (2010). "Negative regulation of Vps34 by Cdk mediated phosphorylation." Mol Cell **38**(4): 500-511.

Gorka, M., W. M. Daniewski, B. Gajkowska, E. Lusakowska, M. M. Godlewski and T. Motyl (2005). "Autophagy is the dominant type of programmed cell death in breast cancer MCF-7 cells exposed to AGS 115 and EFDAC, new sesquiterpene analogs of paclitaxel." Anticancer Drugs **16**(7): 777-788.

Jahreiss, L., F. M. Menzies and D. C. Rubinsztein (2008). "The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes." Traffic **9**(4): 574-587.

Kim, J., M. Kundu, B. Viollet and K. L. Guan (2011). "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1." Nat Cell Biol **13**(2): 132-141.

Kimura, S., T. Noda and T. Yoshimori (2008). "Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes." Cell Struct Funct **33**(1): 109-122.

Kochl, R., X. W. Hu, E. Y. Chan and S. A. Tooze (2006). "Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes." Traffic **7**(2): 129-145.

Liu, L., R. Xie, S. Nguyen, M. Ye and W. L. McKeehan (2009). "Robust autophagy/mitophagy persists during mitosis." Cell Cycle **8**(10): 1616-1620.

Loffler, A. S., S. Alers, A. M. Dieterle, H. Keppeler, M. Franz-Wachtel, M. Kundu, D. G. Campbell, S. Wesselborg, D. R. Alessi and B. Stork (2011). "Ulk1-mediated phosphorylation of AMPK constitutes a negative regulatory feedback loop." Autophagy **7**(7).

Matteoni, R. and T. E. Kreis (1987). "Translocation and clustering of endosomes and lysosomes depends on microtubules." J Cell Biol **105**(3): 1253-1265.

Mayer, T. U., T. M. Kapoor, S. J. Haggarty, R. W. King, S. L. Schreiber and T. J. Mitchison (1999). "Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen." Science **286**(5441): 971-974.

Monastyrska, I., E. Rieter, D. J. Klionsky and F. Reggiori (2009). "Multiple roles of the cytoskeleton in autophagy." Biol Rev Camb Philos Soc **84**(3): 431-448.

Sawin, K. E., K. LeGuellec, M. Philippe and T. J. Mitchison (1992). "Mitotic spindle organization by a plus-end-directed microtubule motor." Nature **359**(6395): 540-543.

Sotelo, J., E. Briceno and M. A. Lopez-Gonzalez (2006). "Adding chloroquine to conventional treatment for glioblastoma multiforme: a randomized, double-blind, placebo-controlled trial." Ann Intern Med **144**(5): 337-343.

Wang, T. H., H. S. Wang and Y. K. Soong (2000). "Paclitaxel-induced cell death: where the cell cycle and apoptosis come together." Cancer **88**(11): 2619-2628.

Xie, R., S. Nguyen, W. L. McKeehan and L. Liu (2010). "Acetylated microtubules are required for fusion of autophagosomes with lysosomes." BMC Cell Biol **11**: 89.

Yang, Z. and D. J. Klionsky (2010). "Mammalian autophagy: core molecular machinery and signaling regulation." Curr Opin Cell Biol **22**(2): 124-131.