THE ROLE OF ROS-MEDIATED ERK AND JNK ACTIVATION IN THE INDUCTION OF AUTOPHAGY AND APOPTOSIS IN TUMOR CELLS BY A NOVEL SMALL MOLECULE COMPOUND

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SUMMARY

It is now well established that chemotherapy-induced reduction in tumor load is a function of apoptotic cell death, orchestrated by intracellular caspase proteases. However, the effectiveness of some of these therapies is blunted by mutations affecting specific effectors genes controlling and/or regulating apoptotic signaling. Therefore, there has been a surge of activity around identification of novel pathways of cell death, which could function in tandem with or in the absence of efficient apoptotic machinery. In this regard, recent evidence has highlighted the existence of a novel, caspase-independent cell death pathway, termed autophagy, which is activated in response to growth factor deprivation or upon exposure to genotoxic compounds. It should be noted that autophagy has been described as a cell survival mechanism as well as a death execution pathway. Using a novel small molecule 1,3-dibutyl-2-thiooxo-imidazolidine-4,5-dione (C1), which is a strong inducer of intracellular hydrogen peroxide (H_2O_2), this work demonstrated the simultaneous induction of non-canonical autophagy and apoptotic cell death in human colorectal carcinoma cells. It was later discovered that the ability of C1 to induce autophagy is not limited to a single cell line. Of importance, this study supported the existence of non-canonical autophagy induced by C1. Whereas, silencing of the integral mediator of autophagy, *beclin1* did not provide protection against autophagy or cell death, *Atg 7* or *Ulk1* knock-down significantly abrogated C1-induced autophagy. In the current study, the induction of autophagy and apoptosis was found to be mutually exclusive. Despite this fact, early H_2O_2 production was critical in controlling the induction of autophagy and apoptosis via activation of extracellular regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). Inhibition of ERK and JNK virtually completely

blocked drug-induced autophagy and apoptosis. Interestingly, inhibition of JNK activity reversed C1-induced increase in Atg7 expression, indicating that JNK may regulate the autophagic pathway by activating Atg7. On the other hand, this study also uncovered a novel role of ERK in mediating p53 suppression which is integral in the induction of autophagy. This work implicates ERK and JNK in the induction of non-canonical autophagy and apoptotic cell death by a small molecule compound, and underscores the plausibility of these proteins as targets in cancer therapy. In particular, the ability of ERK inhibitor to attenuate cell death in metastatic and highly malignant tumor cells presented an alternative mechanism for tumor eradication.

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Abbreviations

2-ME	2-methoxyestradiol
3-MA	3-Methyl adenine
AIF	Apoptosis inducing factor
AD	Actinomycin D
AP-1	Activator protein 1
Apaf-1	Apoptotic protease-activating factor-1
ASK1	Apoptosis signal-regulating kinase 1
ATF-2	Activating transcription factor 2
ATG	Autophagy related genes
ATP	2-adenosine 5'-triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma protein 2
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 interacting mediator
BPB	Bromophenyl blue
CAD	Caspase activated Dnase
CARD	Caspase recruitment domain
Caspase	Cysteine-dependent aspartate-specific protease
CAT	Catalase
CED	Caenorhabditis elegans genes defective
Cdk	Cyclin dependent kinase
Chk2	Checkpoint kinase 2
CHX	Cyclohexamide
CK2	Protein kinase 2
Cu/Zn SOD	Copper/zinc superoxide dismutase
Cyt. *c*	Cytochrome c

DCFDA	5-(and-6)-chloromethyl-2',7' -dichlorofluorescein diacetate
DD	Death domain
DDC	Diethyldithiocarbamate
DED	Death effector domain
DEVD-AFC	N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPI	Diphenyliodonium
DR	Death receptor
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethyleneglycotetraacetic acid
EndoG	Endonuclease G
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
EtBr	Ethidium Bromide
ETC	Electron transport chain
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain-containing protein
FBS	Fetal bovine serum
fmk	Fluoromethylketone
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GADD45	Growth arrest and DNA damage 45
Gate-16	Golgi-associated ATPase enhancer of 16 kDa
H_2O_2	Hydrogen peroxide
Hdm2	Human double minute 2
HE	Hydroethidine
HER2	Human epidermal growth factor receptor 2

IAP	Inhibitor of apoptosis protein
iCAD	Inhibitor of caspase-activated Dnase
IETD-AFC	N-Acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin
JNK	c-Jun N-terminal kinase
LEHD-AFC	N-Acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MAP1LC3	Microtubule associated protein 1 light chain 3
MEK	Meiosis-specific serine/threonine protein kinase
Mitosox^{TM}	Hexyl triphenylphosphonium cation (TPP^{+})-HE
MKP	MAPK phosphatase
MNK	Map kinase interacting kinase
MSK	Mitogen and stress-activated protein kinase
MnSOD	Manganese superoxide dismutase
MOMP	Mitochondrial outer membrane permeabilization
mTOR	Mammalian target of rapamycin
Myc	v-myc myelocytomatosis viral oncogene homolog (avian)
MTT	3-[4,5-dimethyl-2-hiazolyl]-2,5-diphenyl tetrazolium bromide
NAC	N-Acetyl cysteine
NAD	β-nicotinamide adenine dinucleotide
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NHE	Na^{+}/H^{+} exchanger
$NO^{\bullet}$	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\bullet -}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical
PARP	Poly(ADP-ribose) polymerase
PAK	p-21 activated kinase

PBS	Phosphate buffered saline
PE	Phycoerythrin
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol diphosphate
PIP3	Phosphatidylinositol triphosphate
PKC	Protein kinase C
PUMA	p53-upregulated mediator of apoptosis
pRB	Retinoblastoma
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNase	Ribonuclease
RNS	Reactive nitrogen species
ROCK1	Rho-associated kinase 1
ROS	Reactive oxygen species
RPMI 1640	Rosewell Park Memorial Institute 1640
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	Serine
siRNA	small interfering RNA
SMAC	Second mitochondrial activator of caspases
SOD	Superoxide dismutase
SOS	Son of sevenless
Sp1	Promoter specificity protein 1
tBid	Truncated Bid
TEMED	N,N,N',N'-tetramethlethylenediamine
Thr	Threonine
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing factor
Tyr	Tyrosine
ULK1	UNC-51 like kinase

UVRAG	UV irradiation resistance-associated gene
VEGF	Vascular endothelial growth factor
VDAC	Voltage dependent anion channel
XIAP	X-linked inhibitor of apoptosis protein
zVAD	Benzyoxycarbonyl valanyl alanyl

INTRODUCTION

PART I: PROGRAMMED CELL DEATH

Programmed cell death (PCD) was discovered in the 19th century, first described during amphibian metamorphosis (Reviewed in (Jacobson et al., 1997). Since its discovery, tremendous progress has been made in understanding PCD. To date, PCD has been linked to various physiological and pathological states. Its definition has also been modified to refer to any form of cell death that is mediated by an intracellular death program (Jacobson et al., 1997). Therefore, though apoptosis represents a well established form of cell death, other cell death pathways have gained prominent interest over the years. These non-apoptotic cell death pathways include necrosis, autophagy and mitotic catastrophe.

1.1 Necrosis

Necrosis has been viewed as a passive, uncontrolled and accidental form of cell death. Recent advancements in the field, however, propagated the notion that necrosis is a more defined and well controlled cell death. Necrotic cell death is characterized by several morphological distinctions, such as cytoplasmic swelling and loss of plasma membrane integrity (Zong and Thompson, 2006). Accompanying these necrotic phenotypes include biochemical alterations in terms of mitochondrial depolarization, activation of DNA repair protein poly (ADP-ribose) polymerase (PARP) and intracellular production of reactive oxygen species (ROS) (Zong and Thompson, 2006).

Identification of key molecular players in the regulation of necrosis has unravelled the complexity and intricacy of the programmed necrotic network. In many experimental systems, exposure of cells to tumor necrosis factor- α (TNF-α) culminates in necrotic phenotypes only in the background of caspase inhibition (Grooten et al., 1993; Vercammen et al., 1998). Moreover, the presence of the pan-caspase inhibitor, benzyoxycarbonyl valanyl alanyl-fluoromethylketone (zVAD-fmk) invariably potentiates the necrotic death pathway, indicating that necrosis serves as a back up death effector mechanism in the event of caspase inhibition or deficiency (Vercammen et al., 1998). Loss of function experiments of receptor interacting protein 1 (RIP1) have also confirmed the role of RIP1 as a central initiator of necrosis (Harper et al., 2003; Holler et al., 2000). In addition, it was discovered that full length RIP1 is essential in the induction of necrosis, while cleaved fragment of RIP1 by caspase-8 sensitizes the cells to apoptosis (Lin et al., 1999). These results placed RIP1 at the cross-road of apoptotic and necrotic signaling, indicating that RIP1 is a key molecule in mediating the cross-talk between these two prominent cell death pathways.

Stimulation of cells with excessive amount of ROS is invariably linked to necrotic cell death. ROS mediates cellular damage such as lipid oxidation or DNA damage, and is also capable of promoting necrotic influx of calcium (Ca^{2+}), further potentiating the necrotic response (Golstein and Kroemer, 2007; Morgan et al., 2008). DNA damage due to excessive ROS trigger may lead to hyperactivation of PARP, and in this process β-nicotinamide adenine dinucleotide (NAD), the substrate for poly(ADP-ribosyl)ation is depleted (van Wijk and Hageman, 2005).

Concomitantly, cellular adenosine triphosphate (ATP) level is depleted, as aerobic glycolysis is dependent on NAD (D'Amours et al., 1999). In relation to this, massive Ca^{2+} influx could also lead to enhanced nitric oxide synthase (NOS) activity (Dawson et al., 1991), as well as activating intracellular phospholipases and proteases (Festjens et al., 2006), invoking damage to cellular constituents and compromising membrane integrity.

1.2 Mitotic Catastrophe

Strictly speaking, mitotic catastrophe could not be categorized as a form of cell death; rather, it represents an irreversible trigger for cell death. Morphologically, mitotic catastrophe is characterized by enlarged cells, multiple micronuclei and decondensed chromatin (Roninson et al., 2001; Swanson et al., 1995). Mitotic catastrophe is a result of aberrant chromosome segregation, culminating in the failure to undergo mitosis (Castedo et al., 2004a). Accordingly, mitotic catastrophe is frequently induced by chemotherapeutic agents such as vincristine and daunorubicin, causing DNA damage and deranged spindle formation (Castedo et al., 2004b). Certainly, inability of the cells to activate proper DNA damage check points and ensuing DNA repair mechanisms contribute to the culmination of mitotic catastrophe. This is particularly relevant to cancer cells as they generally exhibit deficiencies in cell cycle check point control.

It is thus unsurprising that the cell cycle regulators and kinases have been implicated in mitotic catastrophe. The Cdk1/cyclin B1 complex activation is necessary for cell cycle progression from G2 to M phase and their degradation by

anaphase promoting complex (APC) is essential for progression into anaphase (Nigg, 2001). Premature entry of active Cdk1/cyclin B1 complex into the nucleus has been found to be associated with premature chromatin condensation (Fotedar et al., 1995; Porter et al., 2003). Indeed, various cases of mitotic catastrophe were linked to an increase in nuclear cyclin B1 (Chan et al., 1999; Yoshikawa et al., 2001).

In human cells, checkpoint kinase 2 (Chk2) was found to be a major protector from mitotic catastrophe. Inhibition of Chk2 facilitated the induction of mitotic catastrophe and sensitized cancer cells to apoptosis (Castedo et al., 2004c). The negative regulator of caspase-dependent apoptosis, survivin has also been implicated in mitotic catastrophe. Survivin was found to be a substrate of Cdk1 as well as a binding partner of aurora B kinase (Bolton et al., 2002). Survivin-Aurora B complex is essential for the spindle assembly checkpoint by overseeing the chromosome segregation process (Lens and Medema, 2003).

1.3 Apoptosis

Apoptosis represents one of the most prominent PCD pathways and it was first described in the 1970s to illustrate a specific type of cell death which occurs during normal development and tissue homeostasis (Kerr et al., 1972). Apoptosis has since been discovered as an essential component in various physiological functions such as embryonic development and immunity (Zimmermann et al., 2001). The elimination of unwanted cells by apoptosis serves important functions, which includes sculpting structures, deletion of unwanted structures, control of cell numbers, removal of harmful or damaged cells and production of differentiated cells

(Jacobson et al., 1997). Therefore, deregulation of apoptosis has been linked to various disease pathologies, including neurodegenerative diseases, autoimmunity and cancer (Okada and Mak, 2004). Cells undergoing apoptosis frequently display classical morphological characteristics such as cellular shrinkage, chromatin condensation, DNA fragmentation and membrane blebbing (Kerr et al., 1972). In addition, apoptosizing cells usually form apoptotic bodies which are rapidly phagocytosed by neighboring macrophages without eliciting an inflammatory response (Jacobson et al., 1997).

1.3.1 Molecular mechanisms of apoptosis

The remarkably uniform morphological and biochemical hallmarks of apoptotic cell death across tissues and animal models suggest an existence of a highly regulated and controlled cell death programme within the cell. Central to the apoptotic mechanisms are a group of cysteine proteases called caspases, which are the principle executioners of apoptotic cell death. Caspases are high specificity proteases, cleaving substrates with tetra- or pentapeptide recognition sequences at the aspartic residues (Green and Evan, 2002). To avoid detrimental effects to the cell, caspases are synthesized as inactive zymogens called pro-caspases, which undergo proteolytic cleavage to form mature proteases. In mammalian cells, caspases are divided into two major groups. The first being the ‘initiator’ caspases, exemplified by caspases 8 and 9, are being activated via oligomerization and autoactivation in response to upstream death signals. The ‘executioner’ caspases, such as caspases 3, 6 and 7 are being activated by the initiator caspases by proteolytic cleavage. The

executioner caspases, in turn, cleave other downstream substrates and their activation is largely responsible for most of the morphological hallmarks associated with apoptosis. One of the well described substrate of caspase 3 is the nuclease inhibitor inhibitor of caspase-activated Dnase (iCAD). Cleavage and inactivation of iCAD by caspase 3 results in the liberation of caspase activated Dnase (CAD) and ultimately leads to DNA fragmentation (Enari et al., 1998). The effector caspases also mediate the membrane blebbing phenomenon by activating several adaptor proteins such as Rho-associated kinase 1 (ROCK1), p-21 activated kinase (PAK) and gelsolin. (Coleman et al., 2001; Kothakota et al., 1997; Rudel and Bokoch, 1997)

1.3.2 Extrinsic and intrinsic apoptotic pathways

Apoptotic cascade is mediated through two major pathways of apoptosis, namely the extrinsic and intrinsic cell death pathways. The extrinsic pathway is engaged by ligation of death ligands to the cell surface death receptors. Some ligands which have been identified to date include Fas ligand, TNF-related apoptosis inducing factor (TRAIL) and TNF, which binds to specific death receptors such as FAS, TNF Receptor (TNF R) and Death Receptors 4 (DR4) and 5 (DR5) (Thornberry and Lazebnik, 1998). Efficient ligand and receptor binding mediates the activation of Death Inducing Signaling Complex (DISC), via the recruitment of the adaptor protein Fas-associated death domain-containing protein (FADD) to the cytoplasmic tail of the death receptors (Budihardjo et al., 1999). FADD connects caspase 8 to the DISC through interaction of their respective death- effector domain

(DED). The induced proximity of caspase 8 molecules resulted in their autoactivation (Hengartner, 2000).

BH3 interacting domain death agonist (Bid), a BH3 domain containing member of the B-cell lymphoma protein 2 (Bcl-2) family, transmits the death signals from the extrinsic pathway to the intrinsic pathway of apoptosis. Bid can be cleaved by caspase 8 to form truncated Bid (t-Bid), which then translocates to the mitochondria to elicit mitochondrial outer membrane permeabilization (MOMP) (Li et al., 1998). Effects of t-Bid on the mitochondria are predominantly due to its ability to activate pro-apoptotic Bcl-2 family members Bcl-2 associated X protein (Bax) and Bcl-2 antagonist/killer (Bak), leading to their oligomerization on the mitochondrial membrane (Korsmeyer et al., 2000). These signals mediate the release of a critical apoptogenic factor, cytochrome c from the mitochondria to the cytosol (Wei et al., 2001). An array of biochemical events eventually leads to the formation of a large holoenzyme, apoptosome, which comprises of cytochrome c, dATP, apoptotic protease activating factor-1 (Apaf-1) and caspase 9. Association of caspase 9 with the apoptosome leads to its activation by mean of conformational change (Rodriguez and Lazebnik, 1999). Notably, catalytic processing of caspase 9 does not determine its activity, unlike other caspases (Stennicke et al., 1999).

The interaction between Bcl-2 family members governs the mitochondrial death signals. Based on structural and functional similarities, the Bcl-2 family members are divided into three classes. Class I consists of anti-apoptotic proteins such as Bcl-2, Bcl-x_L, Bcl-w and Mcl-1. Class II and III contains pro-apoptotic members, including Bax and Bak (Adams and Cory, 1998). Apart from neutralizing

the actions of anti-apoptotic Bcl-2 family members, Bax and Bak could also form heterodimers, which interacts with the mitochondrial permeability transition pore (PTP) to induce cytochrome c release. The anti-apoptotic Bcl-2 and Bcl-x_L block cytochrome c release by either binding directly to one of the subunits of the PTP, Voltage dependent anion channel (VDAC) or heterodimerize with Bax or Bak (Narita et al., 1998; Shimizu et al., 1999).

The existence of caspase-independent pathway in apoptosis reveals the importance of apoptotic regulation in cellular homeostasis. One of the key regulators of caspase-independent pathway is endonuclease G (endoG), which is a mitochondrial specific nuclease responsible for chromatin DNA fragmentation (Li et al., 2001). Similar to cytochrome c, second mitochondrial activator of caspases/ direct IAP-binding protein with low pI (SMAC/DIABLO) is also a potent apoptogenic factor which is released from the mitochondria to the cytosol upon induction of apoptosis. Pro-apoptotic activity of SMAC is mainly due to its inhibitory effect on inhibitor of apoptosis (IAP), preventing IAP from inactivating caspase 9 (Du et al., 2000). In addition, apoptosis inducing factor (AIF) could also translocate from the mitochondria to the nucleus during apoptosis induction. The action of AIF is independent of caspase activation and could trigger DNA fragmentation leading to nuclear apoptosis (Susin et al., 1999).

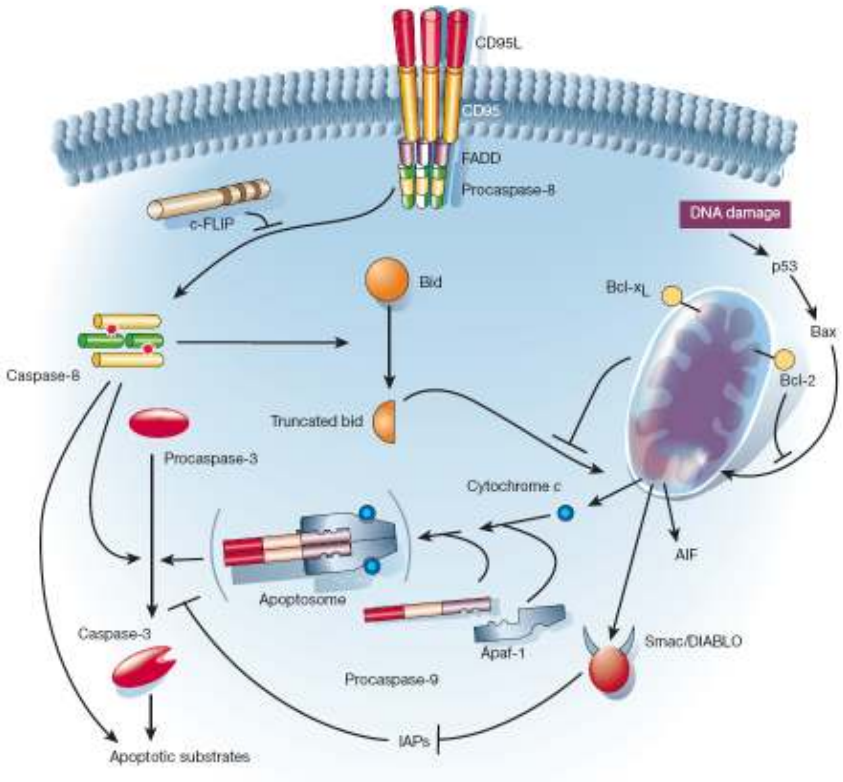


Figure 1: Two major apoptotic death signaling pathways in the cell. The extrinsic death pathway is exemplified by binding of CD95 ligand to CD95 death receptor, while the intrinsic or mitochondrial apoptotic signaling is usually a result of cellular insults such as DNA damage. The two pathways are interlinked by Bid, which could be cleaved by caspase-8 and subsequently promote mitochondrial cytochrome c release. (Hengartner, 2000)

1.3.3 Apoptosis: A barrier to cancer

Acquired resistance to apoptosis represents a hallmark of most, if not all types of cancer. The discovery of *Bcl-2* as a proto-oncogene further associates evasion of apoptosis to tumorigenesis. Various studies have lent support to this hypothesis. Bcl-2 is upregulated in follicular lymphoma following chromosomal translocation (Tsujimoto et al., 1987). In addition, overexpression of Bcl-2 prevents apoptotic cell death in hematopoietic cell lines following growth factor withdrawal (Vaux et al., 1988). High Bcl-2 level confers growth advantage to human B and T lymphoblasts (Tsujimoto, 1989). Moreover, mice overexpressing both Bcl-2 and v-myc myelocytomatosis viral oncogene homolog (avian) (myc) develop tumors much faster than myc-overexpressing mice alone (Strasser et al., 1990). It is now clear that instead of driving cell proliferation, Bcl-2 exerts its pro-survival effects by abrogating cell death responses (Johnstone et al., 2002).

The importance of apoptosis in circumventing tumor growth is further substantiated with the discovery of p53. Mutation of the tumor suppressor gene, *p53* results in loss of p53 protein in more than 50% of human cancers (Harris, 1996). To this end, p53 has been recognized as the 'guardian of the genome' as it plays a vital role in the regulation of cell cycle and apoptosis. p53 directly mediates apoptotic response in abnormally proliferating cells or damaged cells. The ability of p53 to transcriptionally activate an array of apoptotic genes contribute to its pro-apoptotic role. These 'p53-inducible genes' (PIGS) include *APAF1*, *BAX*, *FAS*, *NOXA* and p53-upregulated mediator of apoptosis (*PUMA*) (Polyak et al., 1997; Yu et al., 1999; Zhao et al., 2000). In addition, a transcriptionally independent role of p53 in

apoptosis has also been documented, which involves subcellular localization of p53 and its association with apoptotic proteins such as Bax and PUMA (Chipuk et al., 2008; Chipuk and Green, 2004; Schuler and Green, 2001)

1.4 Autophagy

1.4.1 Role of autophagy in human physiology and pathology

Evolutionarily, autophagy is a bulk degradation process which is highly conserved among eukaryotes. Since its discovery back in the 1960s, autophagy has been regarded as a non-selective cellular clearance mechanism. Nonetheless, interest in autophagy research has increased tremendously since a decade ago, and many important findings have been discovered in relation to autophagy in health and diseases.

Coined by Christian de Duve, who pioneers on lysosomal work, autophagy literally means 'self-eating' whereby cellular constituents and damaged organelles are being degraded by a sequential, dynamic process via the formation of autophagosomes (De Duve and Wattiaux, 1966). Autophagy is further divided into various subtypes, which includes chaperone-mediated autophagy, microautophagy and macroautophagy (Levine and Klionsky, 2004). Chaperone-mediated autophagy is a form of selective autophagy in which long-lived proteins with specific pentapeptide motif are being targeted for degradation. Microautophagy involves the direct engulfment of the damaged organelles by the vacuolar membrane through invagination and/protrusion. In contrast, macroautophagy involves the formation of a

double membrane vesicle called the autophagosomes, in which sequestration of long-lived proteins and organelles take place (Klionsky and Emr, 2000). During macroautophagy, dynamic rearrangements of the cytoplasmic membranes occur, and though not fully understood; the major molecular events have been uncovered and will be discussed in greater lengths in the following sections. In this study, the term "macroautophagy" is hereafter referred to as autophagy.

1.4.2 Molecular mechanisms of autophagy

In response to autophagic signals, including but not limited to nutrient starvation, growth factor withdrawal, genotoxic stress and chemotherapeutic drug treatments, autophagic process will be initiated. An isolation membrane, also known as the phagophore, is formed to enwrap cytoplasmic proteins and organelles to form the autophagosomal structures. The autophagosomes undergo a series of maturation process before its fusion with the lysosomes to form autolysosomes.

One of the key players involved in the regulation of autophagy is the Ser/Thr kinase mammalian target of rapamycin (mTOR) (Funakoshi et al., 1997; Kamada et al., 2000). mTOR is a major negative regulator of autophagy. Under nutrient-rich condition, mTOR is responsible for the hyperphosphorylation of Atg13, which would then dissociate from Atg1 kinase and Atg13 (Abeliovich et al., 2003; Matsuura et al., 1997). In contrast, when mTOR is inhibited, Atg13 remains hypophosphorylated, allowing its association with Atg1 and Atg17, thereby allowing the formation of a multiprotein complex to initiate the autophagic process.

The activity of the Atg13 – Atg1 multiprotein complex drives the vesicle nucleation step of the phagophore. A prominent feature in this step involves the formation of another multiprotein complex, comprising of Beclin1 (mammalian homologue of Atg6), UV irradiation resistance-associated tumour suppressor gene (UVRAG), p150 kinase and mammalian VPS34. This multiprotein complex aids in the activation of VPS34, a Class III PI3 kinase, resulting in the formation of phosphatidylinositol-3-phosphate (PtdIns3P) (Kihara et al., 2001; Nice et al., 2002).

During the vesicle elongation steps, a series of conjugation machineries take place leading to the formation of autophagosomes. The first conjugation pathway involves the conjugation of Atg12 to Atg5, via the intermediacy of Atg10 and the E1-like enzyme Atg7 (Mizushima et al., 1998; Ohsumi, 2001). This pathway resulted in the formation of an Atg12-Atg5-Atg16 homotetramer (Mizushima et al., 1999). The second conjugation system starts with the proteolytic activity of Atg4, which cleaves the C-terminal region of Atg8, allowing the action of Atg7 for the conjugation of Atg8 and Atg3. Finally, a lipid, phosphatidylethanolamine (PE), is being conjugated to Atg8 (Ohsumi, 2001).

Both Atg12-Atg5-Atg16 complex and Atg8-PE are found at the pre-autophagsomal structure (PAS), and are believed to play critical role in vesicle biogenesis (Kim et al., 2002). Atg12-Atg5- Atg16 complex are found at the phagophore, but not at the completed autophagosomes. Atg8-PE, however, are localized in both outer and inner membrane of the autophagosomes (Kabeya et al., 2000). This characteristic enables Atg8-PE to be utilized as a functional marker of autophagy. Consistent with this, Atg8 levels increase during starvation-induced

autophagy, and its amount are closely correlated with the number of autophagosomes (Kirisako et al., 1999). After the execution of autophagy, Atg8 in the outer autophagosomes membranes are being recycled to the cytosol, while those in the inner membranes are being degraded in the autolysosomes (Kirisako et al., 1999).

The whole process of autophagosome formation is elegantly described in yeast, the model organism for autophagy. However, the autophagic system in higher eukaryotes appears to be highly regulated and similar to its yeast counterparts. In the mammalian system, there are a few mammalian homologs of yeast Atg8, including gamma-aminobutyric acid receptor-associated protein (GABARAP), golgi-associated ATPase enhancer of 16 kDa (Gate-16) and microtubule associated protein 1 light chain 3 (MAP1LC3, hereafter referred to as LC3) (Kabeya et al., 2004). Among these proteins, LC3 is most extensively studied and its induction is highly correlative to autophagic signaling, similar to yeast Atg8.

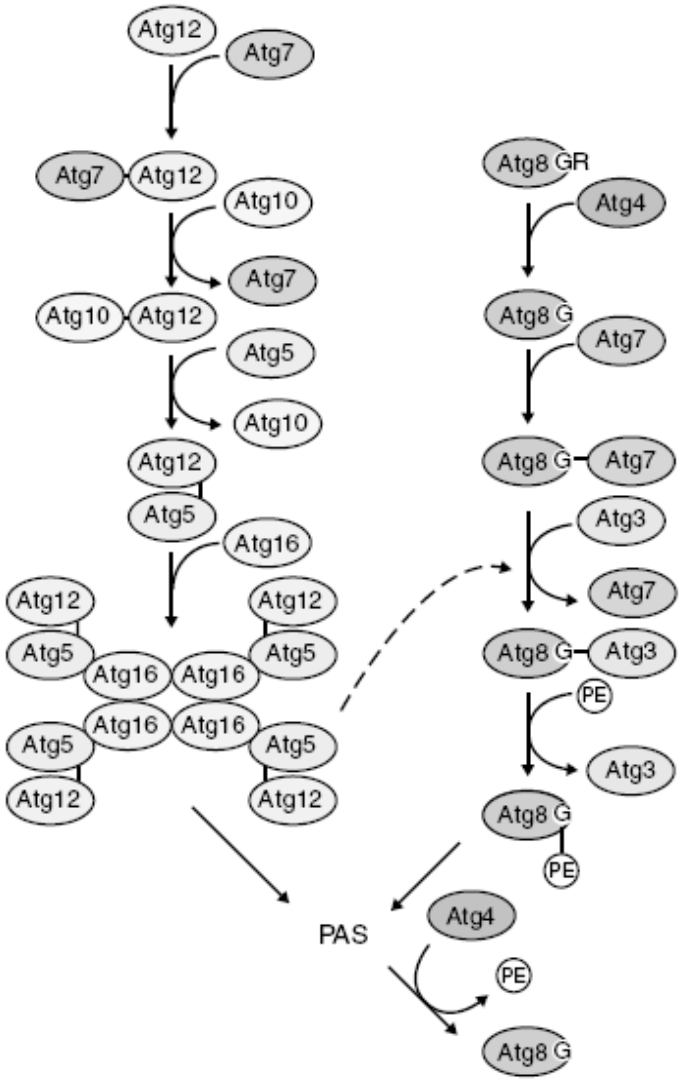


Figure 2: Two conjugation systems involved in autophagosome formation. The pathway on the left denotes Atg12-Atg5 conjugation, and on the other side, Atg8-PE conjugation pathway. The two pathways are both dependent on Atg7, which is an E1 ubiquitin-activating enzyme homolog. Both pathways eventually result in the formation of multiprotein conjugates, which localizes at the PAS. (Yorimitsu and Klionsky, 2005)

1.4.3 Autophagy in health and diseases

During normal cellular homeostasis, autophagy occurs at basal level to degrade abnormal cellular constituents, thereby contributing to the physiological adaptations of eukaryotes. Thus, it is unsurprising that various cellular stimuli, including nutrient depletion, hypoxia, high temperature and genotoxic stress invariably lead to autophagic induction. In fact, autophagy serves as an important cellular adaptation response in normal development and differentiation. In three different model organisms, *S. cerevisiae*, *D. discoideum* and *C. elegans*, *ATG* genes are essential in their differentiation process (Melendez et al., 2003; Otto et al., 2003; Tsukada and Ohsumi, 1993). During neonatal birth, autophagy is induced after trans-placental nutrient withdrawal to maintain cellular survival and in this situation, mice deficient in Atg5 die one day after delivery (Kuma et al., 2004). By employing GFP-LC3 staining in various tissues, autophagy is observed in skeletal muscle, liver, heart and podocytes in kidney upon nutrient withdrawal of 24 hours (Mizushima et al., 2004).

While normal autophagy is essential for homeostatic maintenance of a healthy individual, aberrations in autophagy have also been implicated in various disease pathologies.

1.4.4 Autophagy and cell death

While basal autophagy constitutes an important survival mechanism towards cellular insults, paradoxically autophagic induction has also been associated with cell death. Persistent autophagy in response to cellular stress states serves as a potent

death signal, as in the case of therapy-induced autophagy, a specific non-apoptotic death pathway triggered upon exposure to chemotherapeutic compounds (Amaravadi and Thompson, 2007). The latter forms the basis for the identification of type- II cell death, characterized by excessive autophagosome formation (Scott et al., 2007; Yu et al., 2006).

It is intriguing that a cytoprotective mechanism in one setting could mediate cell killing in other circumstances; hence involvement of autophagy in cell death has remained the controversial debates of current literature reviews. Therefore, it is of paramount importance to have proper guidelines for the definition of type-II cell death. Type-II cell death, or autophagic cell death is defined as a cell death mechanism where macroautophagy represents the only executioner of cell death, without any involvement of type-I apoptotic cell death and/ type-III necrotic cell death (Scarlatti et al., 2009). A mere observation of autophagic phenotype is not sufficient to attribute the cell death as autophagic cell death. In many instances, autophagososome formation alone may represent a cellular adaptation response to cellular insults, and should not be interpreted as an evidence of cell death (Maiuri et al., 2007). In addition, inhibitors of autophagy have also been shown to display pleiotropic, non-specific effects (Klionsky et al., 2008). It is thus, imperative; for any studies which aim to address a positive role of autophagy in cell death, to demonstrate that RNA-mediated silencing of *ATG* genes confers protection to cell death.

Though type-II cell death is classified as cell death devoid of caspase involvement, in many instances autophagy occur simultaneously with apoptosis. A

few possible scenarios may arise from this phenomenon. Autophagy may be accompanying the cell death but is not involved in the execution of cell death (Klionsky et al., 2008). In this instance autophagy may serve as the last desperate attempt by the cells to recover cellular viability. In other instances autophagy may serve as a signal to trigger the cell death cascade or contribute to the cell death response (Hippert et al., 2006). In the latter situation, the cell death could be classified as autophagy-dependent cell death.

In the *in vivo* setting, there are several examples showing a positive role of autophagy in cell death. In a *Drosophila melanogaster* larval salivary gland system, autophagic induction by over-expression of *Atg1* was shown to be sufficient to induce caspase-independent cell death (Berry and Baehrecke, 2007). Coincidentally, in another *in vivo* model, loss of *UNC-51* (*C. elegans* ortholog of *Atg1*) suppresses necrotic cell death (Samara et al., 2008). These two studies elegantly demonstrate that inhibition of crucial autophagy genes rescue cell death, rendering evidence that autophagy could indeed be a cell death executioner. Nevertheless, other *in vivo* evidence of autophagy regulation of cell death has been lacking, most likely due to limited number of studies involving in vivo models in autophagic cell death. This situation is of striking disparity with the pro-survival role of autophagy, which has been supported by various in vivo and in vitro studies.

Despite the scarcity of in vivo studies, there are more evidences in cultured mammalian cells which highlight the pro-death role of autophagy. In mouse L929 fibroblastic cells, gene silencing of *Atg7* or *Beclin1* reduces the extent of nonapoptotic cell death induced by the pan-caspase inhibitor, zVAD (Yu et al.,

2004). Similarly, caspase inhibition also leads to autophagic cell death due to autophagic degradation of catalase and ROS accumulation (Yu et al., 2006). Interestingly, several studies support the notion that excessive autophagy invariably leads to cell death. *Beclin1* mutants, which are defective in binding with Bcl-2, induce higher levels of autophagy than wild type *Beclin1*, and correspondingly, increase cell death in MCF-7 cells (Pattingre et al., 2005). Loss of cell viability induced by mutant *Beclin1* could be effectively rescued by siRNA against *Atg5*. Similarly, direct induction of *Atg1* results in high levels of autophagy in *D. melanogaster*. Activation of autophagic pathway then sets the stage for apoptotic cell death to occur, which could be blocked by mutant *Atg8a* (Scott et al., 2007).

1.4.5 Non-canonical autophagy

The activation of the canonical autophagy pathway is critically under the control of the BH-3 only Bcl-2 interacting protein, Beclin1 (Aita et al., 1999). *Beclin1* was identified as a haplo-insufficient tumor suppressor, monoallelically deleted in 40% to 75% of sporadic breast, ovarian and prostate cancers (Boya et al., 2005). Notably, recent evidence has unraveled a novel autophagic cell death pathway wherein Beclin1 is completely dispensible (Yano et al., 2007). This could be of paramount importance as the execution of non-canonical autophagy in cancer cells bearing a Beclin1 knockout phenotype, could represent a novel and effective strategy to induce cancer cell death (Scarlatti et al., 2008). Using a model of neurotoxin-induced cell death, Zhu *et al* showed that the existence of autophagic vacuolizations was independent of Beclin1 (Zhu et al., 2007). Similarly, in a breast cancer cell

model, the phytoalexin resveratrol was found to induce non-canonical autophagy, which was dependent on inhibition of mTOR signaling (Scarlatti et al., 2008).

1.4.6 Inducers and regulators of autophagy

1.4.6.1 Atg proteins

Advancement in the field of autophagy is greatly accelerated through the discovery of a series of autophagy-related (*ATG*) genes. To date, there are at least 31 *ATG* genes in yeast, and their gene products are mainly involved, though not exclusively, in the vesicle biogenesis pathway of autophagosome (Yorimitsu and Klionsky, 2005). Though the initial studies of the autophagic pathway was explored in yeast, the mammalian homologs of the yeast *ATG* genes has gradually been discovered, and this has led to tremendous understanding of the mammalian autophagic system (Meijer and Codogno, 2004). Interestingly, the two diverse systems have strikingly similar molecular machineries in autophagic regulation, suggestive of a highly conserved autophagic adaptation pathway which is essential for the well being of an organism. In the mammalian system, most of the *ATG* genes identified, and their products are crucial components of the autophagic system, mainly being involved in the regulation of autophagic induction, vesicle nucleation, vesicle expansion and the retrieval process (Maiuri et al., 2007). Most of the *ATG* genes which have been extensively studied were discussed in the previous section. Among the *ATG* genes being identified, Beclin1 (mammalian homolog of Atg6) has drawn heightened interest as it was identified as a haploinsufficient tumor suppressor in a variety of tumor cell lines, being monoallelically deleted in 40-75% of sporadic

human breast, ovarian and prostate cancers (Saito et al., 1993; Yue et al., 2003). Beclin1 was originally identified as an interacting partner of Bcl-2 in a yeast two-hybrid screening of adult mouse brain library (Liang et al., 1998). Following its discovery, several reports have linked Beclin1 induction to suppression of tumorigenesis with a correlating lower level of Beclin1 in activating human tumors (Liang et al., 1999; Qu et al., 2003). Interestingly, mammalian Beclin1 belongs to the BH3 only protein of the Bcl-2 family members (Oberstein et al., 2007). Apart from its canonical role in the vesicle nucleation step of autophagosome formation, Beclin1 was found to be the interacting partner of Bcl-2 and Bcl-x_L, and their interaction has intricately linked the Bcl-2 family members to the autophagic pathway (Oberstein et al., 2007; Pattingre et al., 2005).

1.4.6.2. PI3-Kinase-Akt-MTOR pathway

As discussed in the previous section, mTOR is a major negative regulator of autophagy. It controls the nucleation step of autophagosome formation. In the mammalian system, two classes of PI3-kinase complexes are implicated in autophagy. Class I PI3-kinase is an upstream activator of mTOR, and its activation which subsequently leads to Akt hyperactivation, has been shown to downregulate autophagic response (Coward et al., 2009). Indeed, Akt inhibition has invariably linked to a heightened autophagic response (Degtyarev et al., 2009; Degtyarev et al., 2008). Conversely class III PI3-kinase was shown to be similar to yeast VPS34, it is involved in the crucial step of the formation of a multiprotein complex with Beclin1 and UVRAG. In accordance to this, class III PI3-kinase activity was demonstrated to

be important in autophagic induction (Petiot et al., 2000). Thus, it could be deduced that a substantial amount of PI3-kinase- Akt pathways on autophagy converged on the activity of mTOR. How then, does mTOR display such strong suppression on autophagy? Recent report highlighted a direct evidence of mTOR phosphorylation on mammalian Atg13 and ULK1 (mammalian homolog of Atg1), thereby inhibiting the binding of ULK proteins with FIP200 (Ganley et al., 2009; Jung et al., 2009). Importantly, mTOR was found to be incorporated into the ULK1-Atg13-FIP200 complex in a nutrient-dependent manner, thus highlighting its role as a major suppressor during the induction point of autophagy (Hosokawa et al., 2009).

PART II: REACTIVE OXYGEN SPECIES

Among the several effector mechanisms involved in the control and regulation of cell death pathways, including apoptosis and autophagy, is the cellular redox status. The redox status of the cell is determined by the balance between the rates of production and breakdown of reactive oxygen and/or nitrogen species (ROS; RNS) (Orrenius, 2007), such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$) and hypochlorus acid (HOCl) (Kamata et al., 2005). The term 'reactive' is relative, for example $O_2^{\bullet -}$ and H_2O_2 are more selective in their reactions with other biological molecules, while $OH^{\cdot}$ is highly reactive and reacts quickly with molecules surrounding its vicinity (Halliwell B, 2007).

The Free Radical Theory of Oxygen Toxicity states that the deleterious effect of oxygen is derived from the conversion of oxygen to partially reduced form of

oxygen, such as $O_2^{\bullet-}$ (Fridovich, 1995). Since the inception of this theory in 1954, numerous discoveries have been made towards understanding ROS. It is now widely recognized that ROS plays a pleiotropic role in mammalian physiology, exerting both beneficial and harmful effects to an organism in a context dependent manner. In normal circumstances, the generation of ROS is tightly regulated by enzyme complexes, such as nitric oxide synthase and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase complex (Halliwell B, 2007). ROS production has been shown to play critical role in cellular physiology. At physiological concentration, ROS mediates cellular defenses against pathogen infections and is involved in numerous signaling pathways (Sauer et al., 2001). On the contrary, excessive stimulation of ROS production could result in oxidative stress, leading to deleterious damage to cells and macromolecules (Buccellato et al., 2004). Damage to macromolecules such as proteins, lipids and DNA could result in perturbations of their normal cellular functions (Mates and Sanchez-Jimenez, 1999). Thus, overproduction of ROS has been implicated in various disease models. The delicate balance between amount of ROS produced and antioxidant defences in organisms affects the well being of an organism, and is brought about by various 'redox-regulation' mechanisms in the cells.

2.1 Superoxide anion

$O_2^{\bullet-}$ is produced by one electron addition to the ground state O_2 molecule (Halliwell B, 2007). In aqueous solution, $O_2^{\bullet-}$ does not react with most biological molecules (Halliwell B, 2007). In aerobic systems, $O_2^{\bullet-}$ can be produced in the cells

through many sources. The mitochondrial electron transport chain is suggested to be the most important source of $O_2^{\bullet-}$ production, through leakage of electrons from the ETC (electron transport chain) component to O_2 (Turrens, 2003). Phagocytic cells produce $O_2^{\bullet-}$ by utilizing the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex for reduction of O_2 during respiratory burst (Halliwell B, 2007). O_2 can also undergo auto-oxidation reactions with biologically important molecules to yield $O_2^{\bullet-}$ (Kirsch et al., 2003). Thus, $O_2^{\bullet-}$ has been regarded as one of the primary free radical formed from the reduction of O_2. Once $O_2^{\bullet-}$ is formed, it could react with other molecules to further generate other types of ROS, via various enzyme or metal-catalyzed reactions (Kamata and Hirata, 1999). $O_2^{\bullet-}$ undergoes dismutation reaction to yield H_2O_2 via the action of superoxide dismutase (SOD) enzyme, and SOD represents one of the main antioxidant defense mechanisms in mammalian cells.

2.2 Hydrogen peroxide

H_2O_2 is one of the most important non-radicals in the field of ROS. H_2O_2 is poorly reactive; it does not react with DNA, lipids and most proteins even at high concentration (Halliwell B, 2007). H_2O_2 is only a weak oxidizing or reducing agent. It is thus, unsurprising that H_2O_2 can act as a signaling molecule in the various signal transduction pathways. However, H_2O_2 readily diffuses across cell membranes and between organelles (Henzler and Steudle, 2000), and could react with iron and copper ions to form the deleterious $OH^{\bullet}$ (Spencer et al., 1995). At low levels, H_2O_2 has been reported to have proliferative effects on the cells (Burdon, 1995). When being administered at 10 to 100 μM range, H_2O_2 is toxic to certain cells by activating

apoptosis (Burdon, 1995). Higher concentrations would result in necrotic phenotypes of the cells.

In addition to SOD, H_2O_2 could also be generated by several different enzymes, including xanthine, urate and monoamine oxidases (Halliwell B, 2007). In addition, H_2O_2 could be generated from oxidation reactions of flavanoids and ascorbate (Long et al., 2000). One of the major organelle involved in producing H_2O_2 is the mitochondria, by dismutation of $O_2^{\bullet-}$ from the electron transport chain (Boveris and Cadenas, 2000).

Though $O_2^{\bullet-}$ and H_2O_2 are not particularly reactive, interaction between these two molecules yield the highly reactive $OH^{\bullet}$, which is responsible for the destructive effects on biological molecules such as DNA, proteins and lipids. Indeed, $OH^{\bullet}$ is responsible for most of the damage incurred in cells treated with H_2O_2 (Spencer et al., 1995). In addition, H_2O_2 could also react with metal ions such as iron or copper to generate $OH^{\bullet}$ via Fenton or Haber-weiss reactions (Freeman and Crapo, 1982).

2.3 Intracellular antioxidant defense mechanisms

Intracellular concentrations of ROS are determined by the rates of ROS production and the rates of ROS elimination by antioxidant defences. Therefore, redox regulation represents an important safeguard mechanism to protect living organisms from oxidative stress. *In vivo*, various systems are in place to ensure that defense mechanisms are being activated when harmful situation arises for the cells. These antioxidant mechanisms are broadly divided into enzymatic and non-enzymatic antioxidant defences (Halliwell, 1999). Some of the important enzymatic

antioxidants include SOD, gluthathione peroxidase (GPx) and catalase. Non-enzymatic antioxidants in the like of gluthathione, carotenoids, flavonoids, ascorbate, α-tocopherol are essential to minimize the adverse effects of ROS on cellular physiology.

The superoxide theory of oxygen toxicity states that $O_2^{\bullet-}$ is the main contributor of oxygen toxicity and thus, highlighting the importance of SOD in cellular defenses (Fridovich, 1995). SOD efficiently catalyses the removal of $O_2^{\bullet-}$ in the cells, by accelerating the dismutation of $O_2^{\bullet-}$ to H_2O_2. Copper- and zinc-containing superoxide dismutase (CuZn SOD) are mainly found in the cytosol of animal cells, while manganese SOD (MnSOD) are almost entirely located in the mitochondria (Okado-Matsumoto and Fridovich, 2001).

Dismutation of $O_2^{\bullet-}$ results in the formation of H_2O_2. H_2O_2 can be effectively converted into molecular oxygen and water through the enzymatic activity of catalase (Lardinois, 1995). All animal organs contain catalases, with the highest amount found in the liver (Lardinois, 1995). In animal cells, catalases are mainly found in the peroxisomes, which are also the sites of H_2O_2 production (Halliwell B, 2007).

Glutathione (GSH) is ubiquitously found in animals, plants and aerobic bacteria. It is also present in various cellular compartments including cytosol, nucleus and mitochondria and serves important antioxidant functions in these compartments. GSH is reduced to GSSG by the action of GPx, thereby facilitating the conversion of H_2O_2 to water (Kosower and Kosower, 1978). In addition, GSH could also directly scavenge $OH^{\bullet}$ and singlet oxygen (Halliwell B, 2007).

2.4 Reactive Oxygen Species and Cell Death

ROS has been widely regarded as an effector mechanism of cell death. Numerous studies have provided evidence that inducers of apoptotic cell death also turn out as generators of ROS (Haddad, 2004). Correspondingly, amelioration of ROS generation by antioxidants attenuates the cell death progression. In most experimental systems, ROS production does not directly lead to cell death. In turn, ROS serves as important signaling molecule in the activation of the apoptotic signaling cascade (Mates and Sanchez-Jimenez, 2000). In the *in vitro* models, exogenous addition of oxidants culminates in the accumulation of oxidative stress leading to cell death. Exposure to high level of oxidative stress results in cellular injury in the form of macromolecular damage. In addition, cellular antioxidant defenses are depleted following excessive oxidative insults, thereby lowering threshold for apoptosis to occur (Kong et al., 2000).

Ionizing and UV irradiation trigger apoptosis through induction of H_2O_2 and $OH^{\bullet}$ production. Cellular response to H_2O_2 is largely dependent on the concentration of oxidant. Low to medium H_2O_2 concentration frequently results in apoptosis, while excessive amount of H_2O_2 exposure invariably leads to necrotic cell death. It has been reported that H_2O_2-induced cell death is dependent on mitochondrial permeabilization and cytochrome c release, eventually resulting in caspase activation (Haddad, 2004; Kannan and Jain, 2000). Exogenous addition of H_2O_2 or drug-induced H_2O_2 production in cancer cells is frequently accompanied by a drop in pHi, thereby creating a permissive intracellular milleu for apoptotic cell death (Pervaiz and Clement, 2002b). Another mechanism which has been proposed for H_2O_2-

mediated cell death involves the activation of apoptosis signal-regulating kinase 1 (ASK1), which could undergo dimerization upon H_2O_2 treatment to the cells (Gotoh and Cooper, 1998).

Numerous findings have demonstrated a direct association between ROS and the death receptor signaling pathways. Exogenous addition of ROS or the utilization of ROS-generating compounds have been shown to upregulate CD95 and TRAIL death receptors. Arsenic trioxide, which could induce intracellular ROS production, activates nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) transcription factor which in turn facilitates the transcription of CD95 (Woo et al., 2004). In addition, ROS could signal for CD95 clustering at the cell membrane, which is inhibited by antioxidants treatment (Huang et al., 2003). Fas- stimulated ROS production is critical in apoptosome formation in Jurkat T cells, subsequently promoting caspase-9 and caspase-3 activation (Sato et al., 2004). Uncoupling of oxidative phosphorylation by using carbonyl cyanide m-chlorophenylhydrazone (CCCP) results in mitochondrial ROS formation, thereby sensitizing human colon carcinoma cells to TRAIL-induced release of Smac/DIABLO and caspase-3 activation (Izeradjene et al., 2005).

As discussed previously, mitochondria is the major site of ROS production in the cell (Jezek and Hlavata, 2005). Thus, it is logical to deduce that ROS generated within mitochondria could represent an important regulatory axis in mitochondrial-dependent cell death. Indeed, ROS generation could target the mitochondrial membrane permeabilization, thereby facilitating cytochrome c release and downstream cell death effectors activation. In addition, H_2O_2 has been shown to be

an important signaling molecule in triggering Bax translocation to the mitochondria, through enhancing cytosolic acidification of tumor cells (Ahmad et al., 2004b). Interestingly, H_2O_2-mediated Bax translocation results in increased H_2O_2 amount in the mitochondria, further amplifying the death signals. Acidification of the intracellular milleu provides a more conducive environment for efficient caspase activation (Hirpara et al., 2001). H_2O_2- induced apoptosis is ablated by overexpression of Bcl-2 in tumor cells (Clement et al., 2003). In correlation with the abovementioned studies, protective role of Bcl-2 in intracellular acidification-mediated cell death is due to a slight elevation of $O_2^{\bullet-}$ and intracellular pH (Pervaiz and Clement, 2002b). Conversely, by means of decreasing the intracellular concentration of $O_2^{\bullet-}$, Bcl-2- overexpressing cells are being sensitized to drug-induced apoptosis (Clement et al., 2003). These observations lend credence to a regulatory role of $O_2^{\bullet-}$ and H_2O_2 in determining sensitivity of tumor cells to drug-induced apoptosis.

PART III: MAPK SIGNALING PATHWAYS

Cellular response to environmental changes requires an intricate balance of a wide range of intracellular signaling networks. Cells have the ability to adapt to extracellular and intracellular alterations such as growth factors, cytokines, ligand binding, cellular matrix adhesion and nutritional cues (Davis, 2000). Cells also need to respond to physical fluctuations in temperature, pH and environmental stresses. Intracellular signaling pathways are interconnected and act in a cooperative manner to mount an appropriate response towards physiological demands of the cells. It is

thus not surprising that the integrated signaling networks control and regulate cellular physiology in terms of cell proliferation, differentiation and cell death.

Of the major intracellular signaling pathways, mitogen activated protein kinases (MAPK) is shown to be important in mitigating cellular responses. To this end, MAPK have been identified to be evolutionarily conserved among eukaryotes. Evolutionarily, MAPK are organized into three-tier kinase signaling module, comprising of Mitogen activated protein kinase kinase kinase (MAPKKK), Mitogen activated protein kinase kinase (MAPKK) and MAPK (Johnson and Lapadat, 2002). Signal transduction which relays through the MAPK signaling module eventually leads to the translocation of MAPK into the nucleus and activate transcription factor. Thus, MAPK activation plays an integral role in the regulation of gene expression. Activation of the MAPK cascade is achieved through sequential phosphorylation of the MAPK signaling components (Wada and Penninger, 2004).

Genetic studies in the yeast *Saccharomyces cerevisiae* have led to the identification of five MAPK modules which are involved in mating, osmoregulation, sporulation, cell wall remodeling and filamentation (Schaeffer and Weber, 1999). In mammals, three MAPK modules have been identified, and are named after the last kinase in the series. Each MAPK is activated by dual phosphorylation of a tripeptide motif, Thr-Xaa-Tyr (Johnson and Lapadat, 2002). Though each MAPK carries out unique biological functions, there is overlapping of the components of the three distinct MAPK modules.

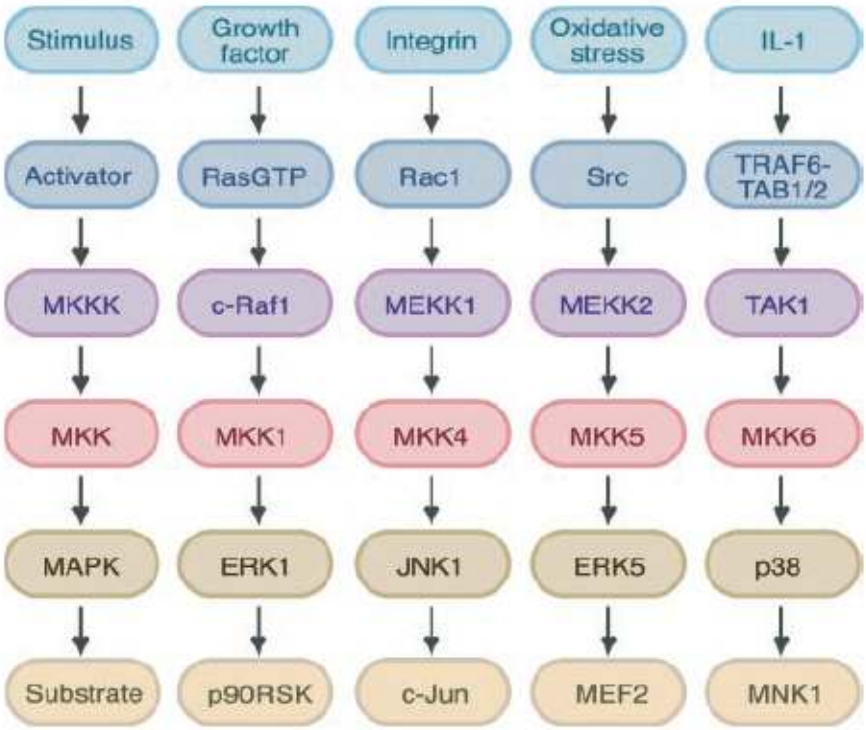


Figure 3: MAP kinase signaling modules. The MAPK are activated via dual phosphorylation of Thr and Tyr residues by MAPKK, which are in turn phosphorylated by MAPKKK. This linear diagram is a simplified representation of the signaling modules, and it should be noted that the complexities and cross-talk often exists between the various modules depending on the specific cellular context. (Johnson and Lapadat, 2002)

3.1 The ERK signaling cascade

ERK is encoded by 2 different genes, *ERK1* and *ERK2*, which give rise to two main proteins, p44 and p42 (Boulton et al., 1991). It was previously described that ERK1 and ERK2 shares 84% homology and have overlapping functions (Lloyd, 2006).

As a critical MAPK involved in the integration of cellular signaling network, it is unsurprising that ERK has been documented to play a diverse role in various cellular and physiological processes. ERK was shown to be functionally important in cell cycle control, differentiation, migration, proliferation, survival signaling, cellular senescense and migration (Rubinfeld and Seger, 2005). Given that ERK could mediate numerous physiological functions, it is unsurprising that ERK is implicated in multiple disease pathologies, such as cancer, diabetes and cardiovascular diseases.

ERK could be activated in response to a diverse range of extracellular factors, which includes growth factors, cytokines and neurotransmitters (Chang et al., 2003). These signals are transmitted via cell surface receptors, such as G- protein coupled receptors (GPCR), integrin receptors and tyrosine kinase receptors before being relayed through the ERK signaling cascade (Ramos, 2008). In a typical ERK signaling cascade, binding of extracellular ligands to the cell surface receptors would result in the receptor activation, followed by recruitment of adaptor proteins, such as Grb2, which would then bind to the Guanine exchange factors (GEF) of small GTP binding proteins. Activated GEF would in turn lead to activation of the small GTPases, such as Ras (Omerovic et al., 2007). Stimulation of Ras initiates the sequential activation of ERK signaling cascade, by transmitting the signals to

MAPKKK. MAPKKK, such as Raf-1, is then being recruited to the plasma membrane and mediates the activation of MEKs, including MEK1 and MEK2 (Fukuda et al., 1997). Activation of MEKs is mediated by phosphorylation at their signature Ser-Xaa-Ala-Xaa-Ser/Thr motif in the activation loop (Ramos, 2008). MEK activation represents a critical regulatory domain of ERK signaling as MEK1 and MEK2 display exceptionally high specificity towards native form of ERK (Seger et al., 1992). Another high degree of specificity lies in the fact that phosphorylation by MEK is the only upstream mechanism *in vivo* which can lead to ERK phosphorylation (Rubinfeld and Seger, 2005). MEKs are dual specificity protein kinases, being able to phosphorylate ERK at Thr and Tyr residues of the Thr-Glu-Tyr motif at their activation loop (McKay and Morrison, 2007). Once activated, ERK could undergo dimerization as well as translocation to the nucleus. Apart from activation of transcription factors residing in the nucleus, a multitude of intracellular targets have also been identified as ERK's substrates.

3.2 Substrates of ERK

ERK is known as 'proline-directed' kinase, for its ability to phosphorylate Ser/Thr residues that are followed by Pro. In general, ERK recognises the consensus sequence Pro-Leu-Ser/Thr-Pro, though other sequences have also been identified previously (Gonzalez et al., 1991). Therefore, ERK have been shown to have a broad range of substrates pool.

One group of the best studied substrates of ERK is the nuclear transcription factors. The ternary complex factors (TCFs), is a class of transcription factors with

members such as Elk-1, SAP-1 and SAP-2, which are known ERK substrates (Pearson et al., 2001). ERK-mediated phosphorylation of Elk-1 occurs on the C-terminus transactivating domain of Elk-1, and is able to potentiate the formation of ternary complexes (Gille et al., 1995).

In addition, another important class of transcription factors, namely activator protein-1 (AP-1) family of transcription factors were also identified to be ERK's targets. Among which, c-Jun, c-Fos and ATF-2 have all been shown to be phosphorylated by ERK (Morton et al., 2003; Murphy et al., 2002). Notably, phosphorylation of c-Jun by ERK occurs at the C-terminus inhibitory sites, preventing c-Jun from binding to the DNA, as opposed to transactivating domain in the case of JNK. This seems to suggest an opposing function for these Map kinases, at least in terms of regulation of c-Jun.

Apart from nuclear proteins, many cytosolic proteins have been identified as ERK targets. The kinases ribosomal S6 kinase (RSK), mitogen and stress-activated protein kinase (MSK) and Map kinase interacting kinase (MNK) are downsteam substrates of ERK and may serve to relay the propagating signals to mediate a wide array of cellular responses (Roux and Blenis, 2004). In addition, ERK also mediate the phosphorylation of cytoskeletal proteins, such as synapsin 1, vinexin and paxillin (Jovanovic et al., 1996; Ku and Meier, 2000; Mitsushima et al., 2004). Importantly, the evidences of ERK in mediating phosphorylation of its upstream activators such as Raf, MEKs and the guanine nucleotide exchange factor, son of sevenless (SOS) suggest that feedback inhibition is a regulatory mechanism in ERK signaling pathway (Dougherty et al., 2005; Eblen et al., 2004; Langlois et al., 1995).

3.3 ERK and oncogenesis

Activation of the canonical MEK-ERK pathway has been classically linked to proliferation and oncogenic transformation. Several lines of evidence are in support of this. Activation of the ERK cascade has been associated with tumor promotion, in both *in vitro* and *in vivo* models (Kyriakis et al., 1992; Sobczak et al., 2008). Conversely, inhibition of the ERK pathway could effectively contain tumorigenic progression (Murphy et al., 2006; Ouyang et al., 2006). MEK1, which directly activates ERK, has also been implicated in malignant transformation. The findings on the effect of constitutive activation of MEK1 in the induction of oncogenesis, coupled with dominant negative form of MEK1 which reversed tumor formation, have indicated a direct role of ERK in the regulation of neoplasia (Cowley et al., 1994; Manser et al., 1994; Seger et al., 1994). More importantly, ERK activation is often induced by mitogenic signals, and could lead to transcription factor activation. Thus, ERK signaling has been shown to integrate mitogenic signals to induce oncogenic transformation. For instance, activation of Elk-1 by ERK could lead to induction of c-fos, which is a transcription factor predominantly associated with cell growth and cell cycle progression (Marais et al., 1993; Whitmarsh et al., 1995).

3.4 Erk and cell death

While it is true that the current literature has attributed a pro- growth and proliferation role to ERK activation, it is not queer that in certain cellular systems, ERK has also been demonstrated to participate in cell death. It should be an important note, however, that the association between ERK and induction of

apoptosis is by no means as established as its role in proliferation and oncogenic transformation. There are many correlative evidences between ERK and cell death, but the molecular mechanisms detailing the interactions between the two remained poorly understood.

Regardless of the ambiguity in relation to dual roles of ERK in survival and death, it is at least certain that DNA damage-induced ERK activation invariably culminates in a cell death response. Various DNA damaging agents, such as etoposide, cisplastin and UV irradiation could stimulate ERK activity, and altering the state of ERK activation ultimately lead to a reduction or complete cessation of the apoptotic response, placing ERK as a central feature of DNA damage-induced cell death (Lee et al., 2000; Tang et al., 2002; Wang et al., 2000).

Interestingly, ROS production has been shown to be a critical event leading to DNA damage. Along the same line, the ability of various DNA damaging drugs in triggering apoptosis has been attributed to an overwhelming production of ROS (Kim et al., 2005b). In view of the fact that ERK inhibition could abrogate ROS-mediated cell death in many circumstances, it is not impossible that the DNA damage-induced signaling pathway is dependent on ERK status in certain cases (Dong et al., 2004; Wang et al., 2000).

Apart from DNA damaging agents, Fas was also shown to be an activator of ERK and expectedly, abrogation of ERK also attenuated Fas-mediated cell death signaling (Goillot et al., 1997; Holmstrom et al., 2000). Nevertheless, as mentioned previously, progress in cell death research involving ERK have been lagging due to the lack of molecular evidences supporting ERK's involvement in cell death.

3.5 ERK and autophagy

Though the involvement of ERK in autophagy is not as established as other major regulators of autophagy, such as JNK and Bcl-2, there are several lines of evidence indicating a relationship between ERK and autophagy.

Most of the current literatures have pointed to a positive regulatory role for ERK in regulating autophagy. The positive association linking ERK and autophagy have been demonstrated in studies involving TNF-α-induced autophagy (Cheng et al., 2008; Sivaprasad and Basu, 2008), as well as in neurotoxin-induced autophagic cell death (Zhu et al., 2007). These studies demonstrated a role for ERK in autophagic-mediated cell death. Similarly, ERK was implicated in starvation-induced autophagy leading to a cytoprotective response (Ogier-Denis et al., 2000). It is noteworthy that while consequences of ERK-elicited autophagy vary among different model systems, all of the studies mentioned above demonstrated an unequivocal role of ERK in autophagy by showing that pharmacological inhibition of ERK was found to be sufficient to impede autophagic progression.

At the molecular level, several mechanisms have been delineated with regards to ERK-mediated autophagy. One of the mechanisms involves phosphorylation and activation of Gα-interacting protein by ERK, which could then kick start the initiation process of autophagy (Ogier-Denis et al., 2000). Another recent publication has described a unique, dual role of ERK in mediating autophagy (Wang et al., 2009). The findings in this paper showed that moderate activation of ERK can lead to a moderately high level of Beclin1, which results in protective autophagy. On the

other hand, sustained activation of ERK causes high level of Beclin1 which is responsible for destructive autophagy (Wang et al., 2009).

3.6 The JNK/SAPK signaling cascade

The c-Jun NH_2-terminal kinase (JNK), otherwise known as the stress-activated protein kinase (SAPK) is activated by dual phosphorylation at specific Thr-Pro-Tyr residues (Davis, 2000). JNK is commonly stimulated by various cytokines and environmental stressors, including UV radiation, oxidative stress and osmotic stress (Weston and Davis, 2002; Weston et al., 2002). JNK was initially identified as a "p54- microtubule associated protein" which was activated following cyclohexamide treatment (Kyriakis and Avruch, 1990). Soon after, JNK was found to be a specific kinase responsible for the transcription factor, c-Jun activation by binding to the N-terminus activation domain of c-Jun (Hibi et al., 1993). The ability of JNK to activate c-Jun is due to the kinase activity of the protein, being able to phosphorylate c-Jun on Ser-63 and Ser-73 residues (Pulverer et al., 1991).

c-Jun belongs to the activator protein-1 (AP-1) transcription factor family. Phosphorylation of c-Jun by JNK on the N-terminus transactivation domain results in induction of c-Jun expression (Ip and Davis, 1998; Smeal et al., 1991). Apart from activating c-Jun, JNK also phosphorylates other AP-1 proteins, including JunB, JunD and activating transcription factor 2 (ATF-2) (Ip and Davis, 1998). Most of the cellular effects brought about by JNK have been attributed to its ability to transcriptionally activate AP-1.

JNK proteins are encoded by three different genes, *Jnk1*, *Jnk2* and *Jnk3*. *Jnk1* and *Jnk2* are ubiquitously expressed, while *Jnk3* expression is tissue specific, mainly being restricted to the brain, heart and testis (Kyriakis et al., 1994; Weston et al., 2002; Yang et al., 1997). Alternative splicing of these three genes results in ten isoforms of JNK (Gupta et al., 1996). JNK proteins are expressed as the short form (46 kDa) and long form (54 kDa) isoforms, with the latter having a COOH terminal extension (Pulverer et al., 1991). The different isoforms of *Jnk* genes appear to encode for different splice variants which differ in their substrate recognition and activation profile.

JNK mediates diverse roles in response to cellular stress. The exact biological outcome derived from JNK activation is often stimulus-specific and context-specific. Nevertheless, JNK has been implicated in various cellular processes such as apoptosis, autophagy, diabetes, metabolism and lifespan (Weston and Davis, 2007). In line with the scope of this study, the role of JNK in autophagy, cell death and tumorigenesis will be discussed in the following sections.

3.7 JNK and apoptotic cell death

Numerous experimental evidences have suggested that JNK can function as a pro-apoptotic kinase. Firstly, association studies with either dominant negative or gain-of-function components of the JNK pathway confirmed the contribution of JNK in promoting apoptosis (Xia et al., 1995; Yang et al., 1997). In addition, genetic studies involving *Jnk1*, *Jnk2* or *Jnk3* genes have established a stimulatory role for JNK in neuronal apoptosis (Kuan et al., 2003; Tournier et al., 2000).

One of the main discoveries with regard to JNK and apoptosis involved the interplay between JNK and NFκB pathway. TNF-α is a pro-inflammatory cytokine which could induce apoptosis or survival in a context dependent manner. It is generally suggested that TNF-α only induce apoptosis when NFκB activation is inhibited (De Smaele et al., 2001; Kamata et al., 2005; Sakon et al., 2003). The state of NFκB activation determines the temporal activation of JNK (Cavigelli et al., 1996; Liu et al., 1996). When the components of NFκB activation is deficient, in the case of IKK or Rel A deletion, JNK could be activated in a prolonged and sustained manner, thereby promoting apoptosis (Maeda and Karin, 2003). Though these studies demonstrated an integral role of JNK and NFκB in determining cell fate in TNF- α-induced apoptosis, the crucial players involved in this interplay are still at large. Several candidates have been suggested to mediate NFκB- induced JNK inhibition, including X-linked inhibitor of apoptosis protein (XIAP), growth arrest and DNA damage 45 (GADD45) and the MAPKK, MKK7 (De Smaele et al., 2001; Tang et al., 2001).

One way to determine the role of JNK in apoptosis was to investigate the interactions between JNK and the components of the apoptotic pathway. Thus, numerous studies were carried out to determine potential targets of JNK in the apoptotic signaling. Fas-L was shown to be one of the downstream targets of JNK and following its induction, apoptotic death signaling was enhanced (Faris et al., 1998; Kasibhatla et al., 1998; Lin et al., 1998). On a separate note, JNK has also been shown to phosphorylate murine p53 on Ser-34 residues in an *in vitro* model (Milne et al., 1995). In addition, human p53 was also identified as a JNK substrate (Fuchs et

al., 1998a; Fuchs et al., 1998b). In stress-induced apoptosis, p53 phosphorylation by JNK led to inhibition of p53 degradation, leading to p53 stabilization (Fuchs et al., 1998b). In another setting, interaction of JNK and c-Myc was found to contribute to apoptosis (Noguchi et al., 1999). JNK was previously shown to phosphorylate c-Myc on Ser-62 and Thr-71 residues (Noguchi et al., 1999). The involvement of JNK in c-Myc- induced apoptosis was further strengthened by dominant negative experiments of JNK.

In the exploration of mechanisms involved in JNK-mediated apoptosis, experimental evidences seemed to point to the mitochondria as a critical point of regulation. *Jnk* null MEFs were unable to induce MOMP or cytochrome c release, suggesting that JNK may be an intrinsic component of the mitochondrial death pathway (Tournier et al., 2000). Indeed, JNK activation has been linked to effective cytochrome c release from the mitochondria (Hatai et al., 2000). This phenomenon might be linked to c-Jun activation, as mutations of JNK phosphorylation sites on c-Jun partially impaired UV-induced apoptosis (Behrens et al., 1999). Another possible mechanism for JNK-induced cytochrome c release involved the action of JNK on the Bcl-2 family members. It was proposed that JNK was responsible for the phosphorylation of Bcl-2 and Bcl-xL in both *in vitro* and *in vivo* systems, and this was postulated to be inhibitory to their function as anti-apoptotic regulators (Maundrell et al., 1997; Yamamoto et al., 1999). Along similar lines, JNK's contribution in mitochondrial death pathway could be linked to the production of jBid, which is a novel cleaved fragment of Bid (Deng et al., 2003). jBid was

produced following prolonged activation of JNK, and was found to be effective in the release of mitochondrial SMAC, thereby activating the intrinsic death pathway.

Targeted disruption of *Jnk* genes represents a useful approach in detecting alterations in apoptotic components. Research involving *Jnk1*-/-*Jnk2*-/- mouse embryonic fibroblasts (MEFs) provided a powerful model in the investigations of JNK-induced apoptosis (Tournier et al., 2000). Jnk null MEFs were fully capable in the execution of Fas-induced cell death, indicating a dispensable role of JNK in death receptor-mediated apoptosis in MEFs. In contrast, Jnk null MEFs failed to promote stress-induced apoptosis and these cells could not execute efficient caspase activation. These studies provide useful insights on the apoptotic response mediated by JNK signaling circuitry.

3.8 JNK and autophagy

In addition to the many complexities in JNK-regulated pathways, many reports have associated an increase in JNK activity to autophagic regulation. JNK was first shown to be critical for zVAD-induced cell death in L929 cells, and JNK inhibition suppressed the extent of autophagosome formation and cell death (Yu et al., 2004). In addition, neurotoxin-induced autophagic death was associated with JNK and c-Jun activation (Borsello et al., 2003). In a different setting, JNK activity was found to be essential in ER stress-induced autophagy (Ogata et al., 2006). However, in this model, induction of JNK-dependent autophagy was shown to be important for cell survival.

While the earlier studies have unravelled a role for JNK kinase in the regulation of autophagy, the molecular mechanisms involving JNK was undetermined in these studies. Most of the studies presented an association of JNK and autophagy through the utilization of specific inhibition of JNK kinase activity via pharmacological inhibitors, such as SP600125 and U0126, or gene silencing technology targeted against *JNK 1/2*.

Recent data has pointed to Beclin1-Bcl2 complex as a putative JNK target. Under non-starvation condition, Beclin1 was found to be able to bind to Bcl-2, and their interaction was shown to be an important inhibitory function of Bcl-2 to keep the autophagic response in check (Pattingre et al., 2005). One of the earliest mechanisms of JNK identified in the modulation of autophagy was associated with its extra-nuclear function. Specifically, JNK1 was identified to be the kinase responsible for Bcl-2 phosphorylation, resulting in the disruption of Beclin1-Bcl-2 complex, thereby promoting starvation-induced autophagy (Wei et al., 2008a). Apart from the inactivation of protein complex from outside of the nucleus, transcriptional activity of the JNK signaling pathway was found to be crucial in increasing Beclin1 expression in the activation of autophagic cell death (Li et al., 2009; Park et al., 2009).

Despite the numerous studies linking JNK and autophagy, the mechanism of action involved in JNK signalling with regard to autophagy has been limited to Bcl-2 and Beclin1 regulation. Efforts to decipher the specific stimulus and context within which JNK activation was induced and the eventual purpose of JNK-mediated autophagic signaling remained an interesting biological function to be explored.

3.9 JNK and cell survival

Conventionally, JNK has been regarded as a pro-apoptotic kinase, supported by the bulk amount of data in the literature showcasing its pro-death activity. The literature provided in the previous section is definitely not an exhaustive list which demonstrated an association of JNK and cell death. In spite of the acceptance of JNK as a death-promoting protein, it is unsurprising that on the other hand, evidence supporting a pro-survival role of JNK has also been demonstrated. Consistent with this, many extracellular stimuli which activate JNK could also directly result in cell survival. For instance, TNF-α-induced JNK activation in fibroblasts was linked to cell survival and completely independent of the cell death pathway (Liu et al., 1996). JNK activation has also been demonstrated to be crucial for integrin- mediated cellular survival (Almeida et al., 2000). In addition, another convincing evidence linking JNK to anti-apoptosis came from the studies on *Jnk* deletion mutants. *Jnk1* and *Jnk2* null mice were prone to apoptosis in the forebrain and hindbrain regions, indicating that JNK could be involved in mitigating the survival response in these brain regions during development (Kuan et al., 1999; Sabapathy et al., 1999). In view of the pleiotropic roles of JNK in cell signaling, it should be borne in mind that the exact outcome associated with JNK activation is often dependent on the specific circumstances.

3.10 JNK and tumorigenesis

The activity of JNK is intricately linked to cell death and cell survival. Thus, studies which implicate JNK in carcinogenesis are also mainly based on these two cellular responses.

An important line of evidence in JNK-mediated oncogenic transformation comes from JNK's ability to phosphorylate the transcription factor c-Jun. Oncogenic transformation induced by Ras requires c-Jun and this phenomenon could be effectively ablated via c-Jun mutation on sites that are phosphorylated by JNK (Nateri et al., 2005). In addition to this, JNK have been implicated in various studies which employed tumor promoters and chemical carcinogens (Chen et al., 2001; Sakurai et al., 2006). These studies have proposed that JNK represents an ideal anti-tumor target as suppression of JNK activity could restrict tumor growth and proliferation.

In contrast, several other studies have established a tumor suppressor role for JNK. The rationale in search of a tumor suppressor gene in the JNK pathway lies in the argument that if JNK was implicated in apoptosis, then at least one of the components of the JNK pathway should be a putative tumor suppressor. The most realistic candidates would be the *Jnk1* or *Jnk2* genes; however, as these genes are largely functionally redundant, it is highly unlikely that they could act as tumor suppressors. In this regard, MKK4, which acts upstream of both JNK and p38 in the MAPK signaling module, was identified as a candidate tumor suppressor gene (Su et al., 1998; Teng et al., 1997). Loss of function mutations of MKK4 have been

identified in various cancers (Su et al., 1998; Su et al., 2002). In addition, epigenetic loss of MKK4 has also been associated with increased metastasis of ovarian and prostate carcinomas (Xin et al., 2004; Yamada et al., 2002). However, it should be clear that though MKK4 represents an ideal anti-tumor target, its effect should not be attributed solely to its link with JNK, as it could also mediate p38 activation.

MATERIALS & METHODS

In this study, the properties of C1 as an anti-tumor compound were being evaluated. In particular, the cellular response triggered by C1 was assessed. Various methods of detecting autophagy and apoptosis, as well as general cell proliferation were employed to determine the cellular processes involved in C1-treated cells. In addition, examinations of the cellular signaling pathways were carried out with inhibitors and RNAi-mediated silencing of the Map kinase family members, ERK and JNK. These experimental methods are summarized as below:

1 Synthesis and analysis of the small molecule compound C1:

The small molecule compound 1, 3-dibutyl-2-thiooxo-imidazolidine-4, 5-dione, herein referred to as C1, was synthesized as follows: Oxalyl chloride was added to 1, 3-dibutyl-2-thiourea (10 mM) in anhydrous ether in a round bottom flask under stirring. The reaction mixture was stirred for 1 to 2 hours at ambient temperature and then poured into saturated $NaHCO_3$. The product was extracted with 3X ethyl acetate. The ethyl acetate layer was then washed with distilled water and then brine water. Ethyl acetate was then dried with anhydrous Mg_2SO_4 and removed under reduced pressure. The purification through flash chromatography (ethyl acetate:hexane) afforded the yellow oil product. The oily product was solidified in a refrigerator. The compound was then analyzed by HNMR, C NMR and MS and results are presented as follows: *Name: 1,3-dibutyl-2-thiooxo-imidazolidine-4,5-dione; Color: Orange; FT-IR (in CH_2Cl_2): 2875-2960 cm^- (Aliphatic CH), 1770*

(C=O), 1410 (C=S); ^{1}HNMR (in $CDCL_3$): δ = 0.95 9 (t, J=7.3Hz, 6H, 4'-CH_3), 1.34 (sext, J=7.7 Hz, 4H, 3'-CH_2), 1.67 (quint, J=7.2 Hz, 4H, 2-CH_2) 3.93 (t, J=7.5 Hz, 4H, 1'-CH_2); C NMR (in $CHCl_3$): δ = 13.54 (C4'), 19.90 (C-3'), 29.72 (C-2'), 41.83 (C-1'), 155.35 (C-4.5), 180.63 (C-2); Mass m/z (%): 242 (100) [M^+], 209 (26) [M+ - HS], 187(22); MF $C_{11}H_{18}N_2O_2S$ calculated 242.34, Found 243.34.

Yield: 95%

2 Tumor cell lines:

HCT116 colorectal carcinoma cells were generously provided by Dr. Bert Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD) and maintained in McCoy 5A (Gibco Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum FBS), 1% L-glutamine, and 1% S-Penicillin (Hyclone, Thermo Scientific, Waltham, MA) in a 37°C incubator with 5% CO_2. HeLa cervical carcinoma, A549 small cell lung carcinoma, M14 melanoma, SHEP1 and SHSY5Y neuroblastoma cell lines were obtained from ATCC and maintained in DMEM (Hyclone) supplemented with 10% FBS. MCF-7, T47D, and MDA-MB-231 breast cancer cell lines were from American Type Culture Collection (ATCC) and cultured in RPMI (Hyclone) supplemented with 10% FBS. HK-1, C666-1 nasopharyngeal carcinoma cell lines, gifted by Dr. Lo Kwok-wai (The Chinese University of Hong Kong) and Dr. Hsieh Wen-son (Johns Hopkins Singapore), were maintained in RPMI supplemented with 10% FBS.

3 Reagents and chemicals:

The pan-caspase inhibitor, ZVAD-FMK, and the caspase 3 and 9 inhibitors (DEVD-fmk and VEHD-fmk) were obtained from Alexis Biochemicals, Lausen, Switzerland. The JNK inhibitor (SP600125), the ERK inhibitor (PD98059), bovine catalase, N-acetyl-cysteine, MG132, cyclohexamide, actinomycin D, crystal violet, and MTT were purchased from Sigma Aldrich, St. Louis, MO.

4 Plasmids and siRNAs:

The plasmid pGFP-rLC3 was a generous gift from Professor T. Yoshimori (National Institute of Genetics, Shizuoka, Japan). siRNAs for *Beclin1*, *Atg7*, *JNK1/2*, *ERK1/2* and *ULK-1* were obtained from Dharmacon Technologies (Thermo Scientific, MA). The pCINeoEV empty vector and pCINeo+Cat containing the full-length human catalase gene were a gift from Dr. Marie-Veronique Clément (Department of Biochemistry, National University of Singapore).

5 Amplification and purification of plasmids

Amplification of plasmids was carried out by using Nucleobond^{TM} DNA purification kit according to the manufacturer's manual. Briefly, 100 µl of *Escherichia coli* (*E.coli*) competent cells were incubated with 100 ng of each plasmid at 4 °C for 3 min before being subjected to heat-shock treatment for 1 min at 37 °C, then being incubated at 4 °C again for 5 mins. The cells were then added to 900 µl of super optimal catabolite repression (SOC) buffer and the mixture were incubated at 200

rpm for 30 mins in a 37 °C bacterial incubator with 5% CO_2. Next, 100 µl of the mixture were plated on lysogeny broth (LB) plates which was prepared with Ampicillin antibiotics, then being incubated at 37 °C for 16 hours. A mini culture was made by selecting a single cell colony from the agar plate and incubating it with 4 ml of LB medium containing ampicilin antibiotic for 16 hours at 37 °C. Following which, the mini culture was transferred to a maxi culture by adding 1 ml of mini culture to 250 ml of LB medium containing ampicilin antibiotic and the mixture broth was incubated for 16 hours at 37 °C. Next the maxi culture was being centrifuged at 2500 rpm for 30 min at 4 °C. The *E. coli* pellet was resuspended with 10 ml S1 buffer provided by the manufacturer and then lysed with 10 ml S2 buffer. The resultant supernatant was incubated for 10 min at 4 °C before being neutralized with 10 ml S3 buffer and subjected to centrifugation again at 12,000 rpm for 45 min at 4 °C. Next the supernatant was allowed to be filtered through Nucleobond™ ion-exchange column which was pre-equilibrated with 5 ml N2 buffer. When all volume has passed through the column, 12 ml of N3 buffer was being run through the column twice. The plasmids were then eluted with 12 ml of N5 buffer followed by precipitation with 8.4 ml of isopropanol before being centrifuged at 11,500 rpm for 30 mins at 4 °C. For the purification process, the plasmids were resuspended with 1:10 volume 5 M NaCl and 2 volume 95% vol/vol ethanol before being transferred to -80 °C for 30 mins. After incubation, the mixture was centrifuged at 14,000 rpm for 30 mins at 4 °C and the resulting pellet was washed with 70% vol/vol ethanol twice. The pellet was allowed to be air-dried before it was resuspended in 100 µl of sterile RNAse free water.

6 Analysis of DNA by Southern blotting

For nucleotide analysis, the plasmids were being digested with restriction endonucleases (RE) by incubating 25 µl of each plasmid with 2.5 µl of RE buffer and 0.5 µl of RE for 1 hr at 37 °C. Next 1 µl of DNA loading dye was added to 25 µl of RE digestion product before being subjected to 10% agarose gel with 1% ethidium bromide (EtBr). When the DNA fragments were well separated, the gel was visualized with an ultraviolet illuminator and the nucleotide fragments were analysed.

7 Transient transfection of plasmids:

For transient expression, HCT116 cells grown at 50% confluency in 6-well plate transfected with 8 µg of pGFP-LC3 or pCINeoEV or pCINeo+Cat plasmids in Optimem1^{TM} medium (Invitrogen Corporation, Carlsbad, CA) without serum using the Superfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, 10 µl of Superfect were mixed with 226 µl of plain Mc Coy's medium and being vortexed for 10 secs. Then 8 µg of plasmid was being added to the transfection mixture and being incubated at 37 °C for 20 mins. After that, the transfection mixture was added with additional 300 µl of plain Mc Coy's and 500 µl of each transfection mixture was being added drop-wise to each well. The plasmids were allowed to be incubated for 48 hours before being subjected to various treatments and analysis of protein expression by Western blots.

8 Transient silencing of messenger RNA:

For knockdown of gene expression, 50 nM siRNA (Beclin1 siRNA, or JNK1/2 siRNA, or ERK1/2 siRNA, or Atg7 siRNA, or ULK-1 siRNA) was transfected into cells in Optimem1^{TM} medium using the Dharmafect1 reagent (Dharmacon) according to the manufacturer's instructions. Briefly, 2 µl Dharmafect1 reagents was added to 200 µl of Optimem1 and the mixture was vortexed for 10 seconds before being incubated for 10 min at 37 °C. In another tube, 50 nM of siRNA was resuspended in 200 µl of Optimem1. Similarly, this mixture was also vortexed and incubated as the same condition as the previous mixture, and being added drop-wise to the first tube. Next, 400 µl of the final transfection mix was added drop-wise to the respective well. After transfection with the plasmid or siRNA, cells were cultured in 10% serum for 48 hours before the assessment of protein expression by Western blotting.

9 Drug treatments:

Various drugs and inhibitors were used in this study as useful tools to investigate relevant pathways involved as well as to explore the mechanisms related to C1-induced cellular responses. The timing and dosage for each drug has been optimized and subsequent experiments were repeated by following the exact condition for drug treatments to minimize variability in experimental setup. The procedures for drug treatments are summarized as follows:

C1 treatments at various doses were administered according to the methods described in the respective figure legends. Catalase, actinomycin D and cycloheximide were

added 1 hour while NAC and MG132 were pre-incubated for 2 hours prior to C1 treatment.

10 Cell viability and tumor colony forming assays:

Cell viability following drug exposure was determined by the MTT assay as described previously. Briefly, $1x10^6$ cells/well were seeded onto 6 well plates and exposed to C1 for 18-24 hours before being trypsinized, washed with cold PBS, and incubated with MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; Sigma Aldrich] for 2 hours. The formazan crystals were then being dissolved with Sorenson's glycine and DMSO (Sigma Aldrich, St. Louis, MO) and absorption was measured with a TECAN spectrophotometer at 570nm. For colony forming assays, 10,000 cells were plated in petri dishes and grown for 2 weeks. The plates were then stained with crystal violet solution (Sigma Aldrich, St. Louis, MO) and colonies were scored manually as described previously.

11 Western blotting:

Whole cell protein extracts were isolated using 1 X RIPA lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA and protease inhibitors (Calbiochem, San Diego, CA). Equal amounts of protein from the total cell lysates (30 to 120µg/lane) was separated by sodium dodecyl sulfate (10%, 12% or 15%) polyacrylamide gel electrophoresis gels (SDS-PAGE; BioRad Laboratories), transferred to PVDF membrane (BioRad Laboratories) using wet transfer (BioRad Laboratories). The membranes were blotted with 1:1000 dilution of

the primary antibodies specific for Bax, LC3, Caspase- 9, β-actin, GAPDH, ULK1 (Santa Cruz Biotechnology), Beclin1, ATG7, ATG12, ATG5, Cytochrome C, JNK, p-JNK, c-JUN, p-c-JUN, Bid (Cell Signaling Technology), Caspase 3 (Upstate, Millipore Corporation), Caspase 8, Smac, anti-poly(ADP-ribose) polymerase (BD Pharmingen) and Catalase (Calbiochem) overnight at 4 °C. After overnight incubation with the primary antibodies, the membranes were being washed with 1X TBS with 0.1% Tween-20 for 3 times and then blotted again with respective secondary isotype specific antibodies tagged with horseradish peroxidase (Thermo Scientific Pierce, Rockford, IL). Bound immuno-complexes were detected using WEST PICO Chemiluminescence substrate (Thermo Scientific Pierce, Rockford, IL) on Kodak films.

12 Immunoflurorescence for GFP-LC3:

Following transfection with pGFP empty vector or pGFP-rLC3, cells were incubated with C1 for 6 to 24 hours and visualized by a fluorescent microscope (Eclipse TE2000-S, Nikon) using excitation wavelength of 488nm and emission wavelength of 525nm.

13 Propidium Iodide staining for DNA fragmentation:

Briefly, $1x10^6$ cells/ml were fixed with 70% vol/vol ethanol, and stained with propidium iodide (Sigma Aldrich, St Louis, MO) for DNA content analysis. At least 10,000 events were analyzed by flow cytometry (Coulter EPICS Elite ESP) with the excitation set at 488 nm and emission at 610 nm. Histogram data indicating

percentage of cells with sub-diploid DNA are shown and are mean ± SD of three independent observations.

14 Flow cytometric analysis of intracellular ROS:

For determination of intracellular H_2O_2, cells were tripsinized, washed with 1 X PBS and loaded with 5 μmol/L of the redox sensitive dye 5-(and-6)-chloromethyl-2-,7-dichlorofluorescin diacetate (CM-H_2DCFDA) (Molecular Probes, Invitrogen Corporation) at 37°C for 15 minutes. Then the stained cells were washed again with 1 x PBS and resuspended with 500 ml of plain medium and analyzed by flow cytometry (Coulter EPICS Elite ESP) using an excitation wavelength of 488 nm and emission wavelength of 525 nm. CM-H_2DCFDA is a cell permeable dye which could be cleaved by intracellular asterases to CM-H_2DCF to prevent its backflow into the extracellular medium. It could be oxidized by intracellular oxidants to yield C-DCF which could be detected by flow cytometry.

15 $O_2^{\bullet -}$ measurement by MitoSox™ RED

Detection of intra-mitochondrial O_2^- was performed by loading cells with the cationic redox sensitive probe hexyl triphenylphosphonium cation (TPP^+)- HE, otherwise known as MitoSox™ RED MITOCHODNRIAL O_2^- INDICATOR (Molecular Probes, Invitrogen Corporation) at 37°C for 15 minutes and analyzed by flow cytometry (Coulter EPICS Elite ESP) using an excitation wavelength of 590nm and emission of 619nm. At least 10,000 events were analyzed. MitoSox™ RED is a cell

permeable dye which could be targeted to the negatively charged mitochondrial membrane whereby the HE moiety could then be oxidized by O_2^-.

16 Heavy membrane fractionation:

HCT116 cells treated with C1 for 6, 12 and 24hrs were suspended in 10 volume of Buffer A for 30 min and homogenized with a Dounce Homogenizer (Sartorius AG). Homogenates were centrifuged at 300g for 10mins at 4^{o}C. Supernatants were further centrifuged at 25,000g for 45mins at 4^{o}C to collect the mitochondrial pellets. The mitochondrial pellets were lysed in standard 1xRIPA lysis buffer and the supernatants were used as the cytosolic fractions. The resultant fractions would then be separated by SDS-PAGE followed by Western blotting to determine subcellular distribution of various proteins.

17 Subcellular fractionation for nuclear preparation:

Following drug treatments, HCT116 were harvested from petri dish by tripsinization and subjected to centrifugation at 1,500g for 3 min. The pellet was resuspended in 400 µl of Nuclear Buffer and incubated on ice for 15 min. After that, 25 µl of NP-40 was added to the cell suspension and vigorous vortexing was carried out for 10 sec. The cell suspension was then being centrifuged at 14,000 rpm for 30 sec at 4 °C. The supernatant (cytosolic fraction) was transferred to a clear centrifuge tube and stored at -80 °C. The pellet containing nuclear fraction was resuspended with 50 µl of ice cold Buffer C and incubated for 15 min at 4 °C before being kept at -80 °C. The

fractions were then subjected to SDS-PAGE and Western blotting as described in Materials & Methods.

18 Electron microscopy:

Cells were fixed overnight in 2.5% glutaraldelhyde in 0.1M phosphate buffer (pH 7.2), before being post-fixed in 1% OsO_4 for 1 hour. Next, cells were dehydrated in ethanol series and embedded in Spurr's resin. Ultra thin sections were stained with uranyl acetate and lead citrate and observed under a JEOL JEM-1230 transmission electron microscope.

19 Buffers and stock solutions used in the study:

Catalase
Catalase was freshly prepared by weighing out 7000 units/ml and dissolved in plain Mc Coy's medium.

NAC
NAC was prepared as 2 mM stock was prepared freshly prior to drug treatment.

SP600125
SP600125 was prepared as a stock solution of 20 mM by dissolving it with DMSO.

PD98059
PD98059 was prepared as a stock solution of 20 mM by dissolving it with DMSO.

MTT
MTT was freshly dissolved in plain Mc Coy's medium to a stock concentration of 5 mg/ml.

Propidium iodide dye
PI stock solution (50X) was dissolved in sodium citrate buffer (38 mM) to a stock concentration of 0.5 mg/ml and kept in 4 °C and prevented from light exposure.
PI: RNAse A solution was freshly prepared with 1/50 volume of PI and 1/40 volume of RNAse A stock solution in 38 mM sodium citrate buffer.

RNAse A
RNAse A was dissolved in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl to a stock concentration of 10 mg/ml and kept at -20 °C.

RIPA lysis buffer
RIPA lysis buffer was prepared with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% v/v Nonidet P40, 1% v/v deoxycholic acid, 0.1% v/v SDS, 1 mM EDTA. Before use, the buffer was supplemented with protease inhibitors (1 mM PMSF, 10 μg/ml Aprotinine, 20 μg/ml Pepstatin A) and phosphatase inhibitors (1 mM NaF, 1 mM Na_3VO_4).

Running Buffer for SDS-PAGE
Running buffer for SDS-PAGE was prepared by adding 60 g Tris-base 60, 288 g of Glycine, 20 g of SDS and dissolved in 2 L of dH_2O.

Transfer buffer for western blot: 1X (5L)
Transfer buffer was prepared by adding 12.1 g Tris-base, 57.84 g Glycine, 1 L methanol to a total volume of 5 L with dH_2O.

Laemmli loading buffer: 5X (10 ml)
Tris-HCl pH 6.8 3.1 ml (1 M), SDS 1 g (10%), glycerol 2 ml (20%), β-Mercapthoethanol 2.5 ml (25%), Bromophenol Blue (BPB) 0.01 g (0.1%), 2.4 ml dH_2O.

Buffer A for heavy membrane fractionation:
50 mM PIPES-KOH pH 7.4, 220 Mm mannitol, 68 mM sucrose, 50 Mm KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT. Before use, the buffer was supplemented with protease inhibitors (1 mM PMSF, 10 μg/ml Aprotinine, 20 μg/ml Pepstatin A) and phosphatase inhibitors (1 mM NaF, 1 mM Na_3VO_4).

Sorenson's Glycine Buffer
Sorenson's Glycine buffer was prepared as 0.1 mM NaCl and 0.1 M Glycine (pH 10.5) in distilled water and stored at 4 °C.

Buffer A for nuclear fractionation:
Buffer A for nuclear fractionation was prepared using 10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and resuspended with dH_2O. Before use, 0.5 mM of PMSF was added.

Buffer C for nuclear fractionation:
Buffer C was prepared with 20 mM Hepes pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and resuspended with dH_2O. Before use, 0.5 mM of PMSF was added.

TBS
TBS was prepared as 500 ml 1 M Tris HCl (pH 7.4) with 87.6 g of NaCl in 10L of distilled water and stored at room temperature.

TBST
TBST was prepared as 2 L TBS with 2 ml of Tween-20 and stored at room temperature.

20 Statistical Analysis:

Experimental differences were tested for statistical significance using Student's *t*-test. *P* value of <0.05 was considered as significant.

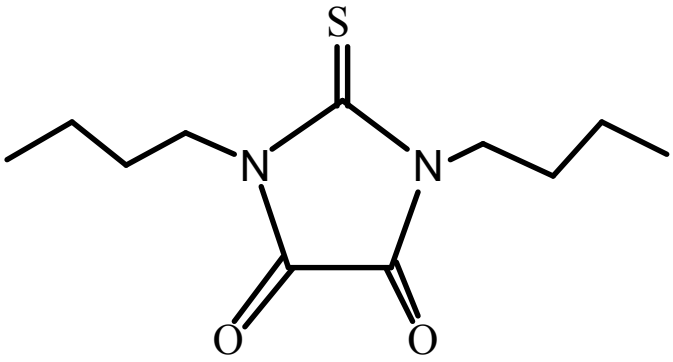
RESULTS

PART 1: C1 INDUCES CELL DEATH AND MOMP IN HUMAN TUMOR CELLS

1.1 C1 induces reduction of cell viability and colony formation in HCT116 cells

Merodantoin (Hereafter referred to as C1) is a small molecule compound with a molecular weight of 242. C1 was originally purified from the photooxidation of merocyanine 540. The chemical structure of C1 is *N,N*'-Dibutyl-thio-4,5-imidazolindion (Figure 4). To explore the death inducing ability of C1, a dose response curve against HCT116 human colorectal carcinoma cells was obtained. To do so, cells were treated with increasing concentrations of C1 for 24 hours and 48 hours, before being harvested for determination of cell viability. Cell viability is assessed by MTT assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a standard colorimetric assay, which capitalizes on the fact that live cells contain active mitochondrial reductase enzymes. These mitochondrial enzymes catalyze the reduction of MTT dye to an insoluble formazan. The purple formazan was solubilized with dimethylsulfoxide (DMSO) and Sorenson's Glycine buffer into a colored solution. Absorbance of this solution was then measured with a spectrophotometer at 570 nm. Cell viability was determined by percentage of absorbance value with regard to untreated control sample. Exposure of tumor cells to increasing concentrations (25-200 μg/ml) of C1 resulted in a dose-dependent

decrease in cell viability at 24 hours with the LD50 of ~100 µg/ml (Figure 5). At low doses of 25 and 50 µg/ml, there was minimal reduction in cell viability while at 200 µg/ml, more than 70% of the cells were not viable. The experiment was also performed at 48 hours to assess the effect of C1 drug at late time point. However, treatment of HCT116 cells by C1 at 48 hours was proven to be highly toxic and produced massive cell death regardless of the dose of C1. Therefore, treatment of HCT116 cells by C1 for 48 hours did not produce a dose response curve, unlike the treatment of 24 hours. This result is not unsurprising considering that cell death hallmarks were evident at 18 – 24 hours time point, which will be discussed in the next section. Having established the cell killing ability of C1, it is of paramount importance to determine whether C1 also retards the long term colony forming abilities of tumor cells. This is to ensure that anticancer drugs are not only effective in inducing cell death, but also impede the colony formation in long term cultures. Thus, cells were treated for 24 hours with 25-100 µg/ml of C1 and long-term colony forming ability was assessed after 14 days. Results showed a significant reduction in clonogenic ability at 25 and 50 µg/ml and a complete cessation of colony formation at 100 µg/ml (Figure 6).



MW 242.34

Figure 4: Chemical structure and molecular weight of the small molecule compound. 1,3- dibutyl-2-thio-oxo-imidazolidine-4,5-dione (C1)

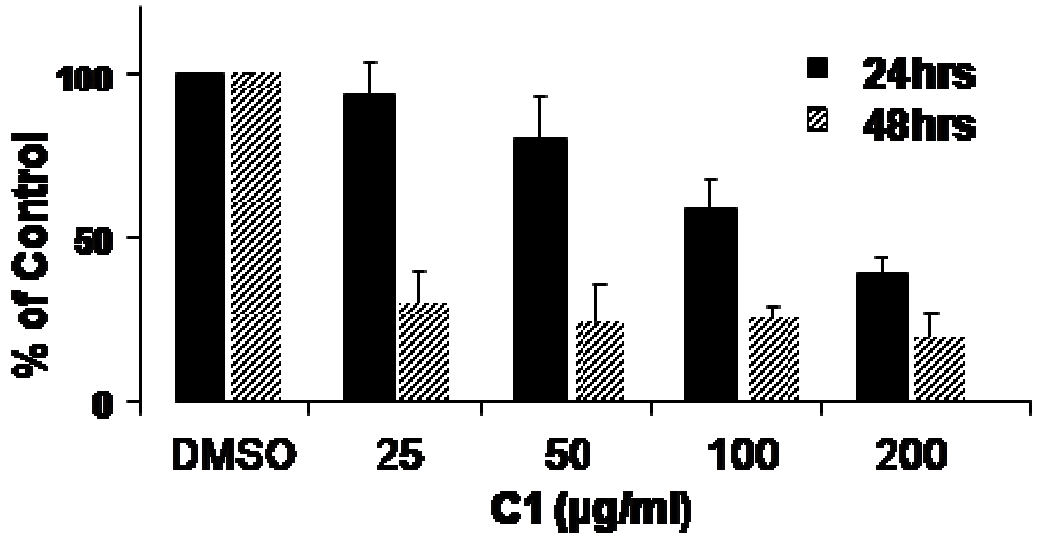


Figure 5: C1 induces reduction of cell viability in HCT116 colorectal cells.
HCT116 cells were treated with increasing concentrations of C1 (25 µg/ml to 100 µg/ml) for 18 hours and cell survival was assessed by the MTT assay as described in Materials and Methods.

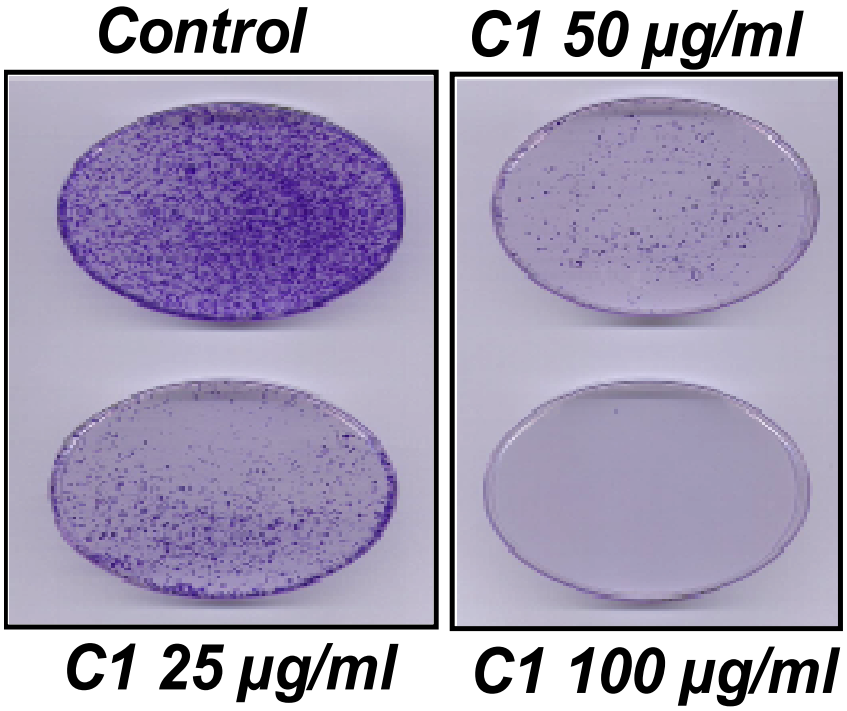


Figure 6: Exposure of HCT116 cells to C1 induces reduction in clonogenic ability of tumor cells. Following exposure to C1 for 18 hours, HCT116 cells were harvested and the number of cells in each sample was counted. 10,000 cells were seeded onto each well of 6-well plates for assessment of colony formation. 2 weeks later, wells were stained with crystal violet stain for 15 minutes before being washed away followed by colony counts.

1.2 C1 triggers MOMP in HCT116 cells

Having ascertained that C1 has a potent death inducing ability in tumor cells, the mechanisms of C1-induced cell death were investigated. Defects in apoptotic pathways have been recognized as one of the prime causes for cancer resistance. To assess whether C1 also activates the mitochondrial pathway, the subcellular translocation of apoptogenic factors were assessed. Upon incubation with C1 from 6, 12 to 24 hours, HCT116 cells were being harvested and subjected to homogenization and differential centrifugation. The resultant heavy membrane fractions, which are enriched with mitochondria as well as the cytosolic fractions were being assessed by Western blots. Results showed that incubation of cells with C1 triggered mitochondrial outer membrane permeabilization (MOMP) as evidenced by the translocation of cytochrome c and Smac, and the reciprocal translocation of Bax and Bid to the mitochondria in a time dependent manner (Figure 7).

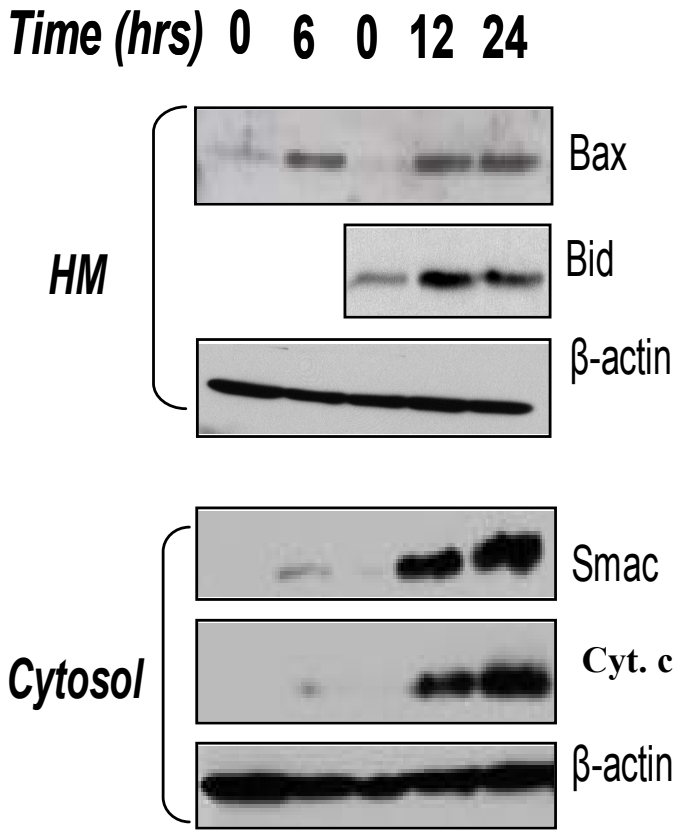


Figure 7: C1 triggers MOMP in HCT116 cells. Cells were treated with C1 (100 μg/ml) for 6, 12 and 24 hours, and sub-cellular fractions were obtained as described in Materials and Methods. Proteins from cytosolic and mitochondrial fractions were subjected to Western blot analysis using anti-Bax, anti-Smac, anti-Cytochrome C, and anti-Bid antibodies. Anti β-actin was used as a loading control.

1.3 C1 triggers efficient caspase processing in HCT116 cells

Processing of pro-caspases into active form are important mechanisms to ensure maximal activation of both initiator and executioner caspases (Rupinder et al., 2007). Indeed, efficient processing of caspases by oligomerization has been shown to enhance their proteolytic activity (Fuentes-Prior and Salvesen, 2004). Despite this fact, many apoptogenic factors may culminate in apoptotic cell death without the involvement of caspases. The existence of efficient caspase processing can be investigated through analysis of caspase cleavage by Western blot. To determine if the major executioner of apoptosis, caspases were being implicated in C1-induced cell death, caspases 3, 8 and 9 processing were investigated. Caspase 3 cleavage (p19, p17 fragments) was evident following 12 hours of C1 treatment, and peaks at 24 hours (Figure 8). Similarly, caspase 9 processing followed the same trend as caspase 3 (Figure 8). Intriguingly, caspase 8 processing was also detected, but its activation profile was similar for both 12 hours and 24 hours time point (Figure 8). Collectively, these data suggested an involvement of caspases in the activation of death signaling pathways by C1.

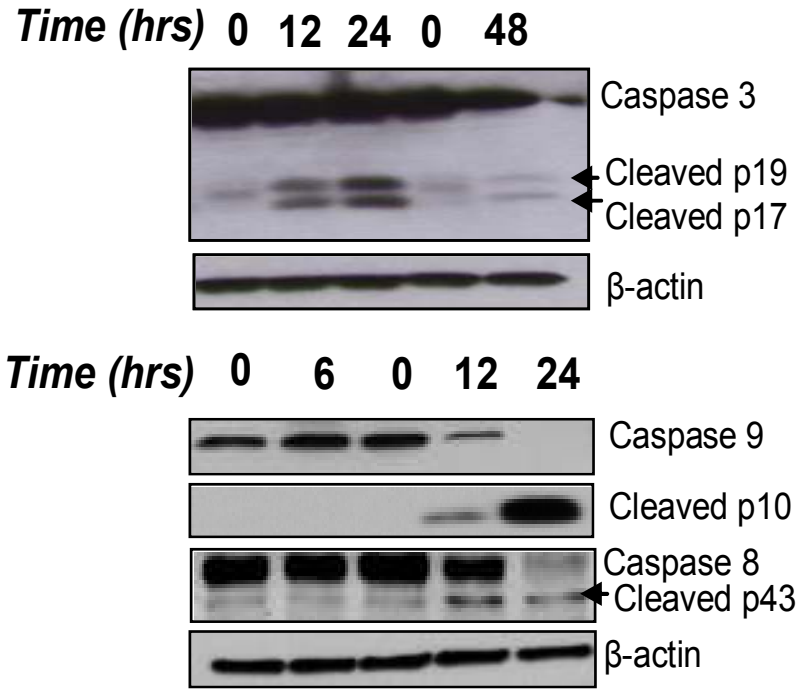


Figure 8: C1 triggers efficient caspase processing in HCT116 cells. Lysates from cells treated with C1 (100 μg/ml) for various time intervals were probed for processing of caspases 3, 9 and 8 via Western blotting.

1.4 Apoptotic cell death induced by C1 is caspase-dependent

To further substantiate the existence of apoptosis in these cells, poly (ADP-ribose) polymerase (PARP) cleavage was investigated. PARP has been shown to be primarily cleaved by caspase-3, one of the major executioner caspases responsible for various morphological hallmarks of apoptosis (Nicholson et al., 1995). PARP cleavage is also a central morphological hallmark of apoptosis as cleavage of PARP into 86 and 24 kDa fragments inactivates the enzyme and results in its loss of DNA repair function (Berger and Petzold, 1985). Exposure of HCT116 cells to C1 for 24 hours resulted in PARP cleavage at 89 kDa (Figure 9). To investigate the involvement of caspases in this model, cells were pre-incubated with the pan-caspase tetrapeptide inhibitor, zVAD-fmk for one hour before the addition of C1. Total cell lysates were then being analysed for PARP cleavage. As seen in Figure 9, zVAD-fmk treatment completely abolished the effect of PARP cleavage induced by C1.

In addition, cell cycle analysis was performed on C1-treated cells. Propidium iodide (PI) staining is commonly used to assess cell cycle profile by way of intercalation of the PI dye with DNA. The intensity of fluorescence emitted is proportional to the amount of dye being incorporated into the DNA and could be detected by flow cytometry analysis (Suzuki et al., 1997). Particularly relevant to apoptosis are the existence of sub-G1/ sub-diploid population of cells which falls at the left side of the G1 peak. The sub-G1 population consists of the cells with fragmented DNA. Thus, uptake of PI dye by these cells is much less than cells with normal DNA content. To perform cell cycle analysis, cells were being treated with or without zVAD-fmk for an hour, followed by C1 treatment for either 24 hours or 48

hours. Harvested cells were then being permeabilized and fixed, before being stained with PI. Analysis of cell cycle revealed 19% of the cells with sub-G1 population following C1 treatment for 24 hours, which was significantly increased to 52% upon incubation for 48 hours and blocked in the presence of the pan-caspase inhibitor (Figure 10). Taken together, these data demonstrated an existence of caspase-dependent apoptotic cell death elicited by the novel small molecule, C1, exemplified by caspase activation, PARP cleavage, DNA fragmentation and release of apoptogenic factors from the mitochondria.

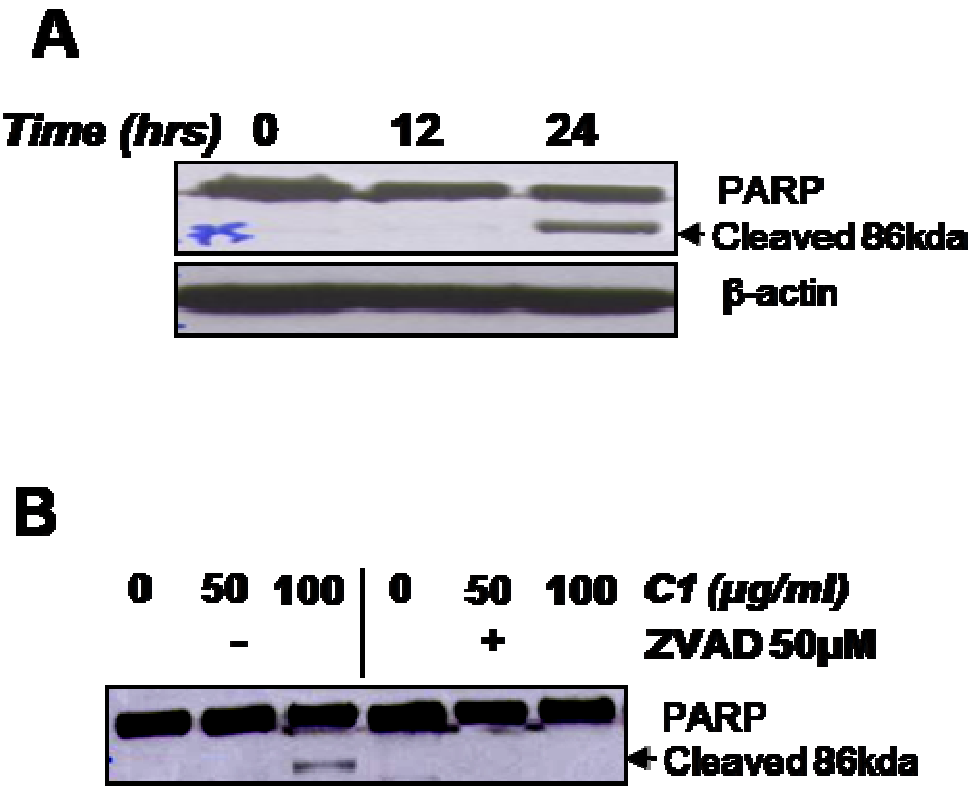


Figure 9: PARP cleavage induced by C1 is caspase- dependent. HCT116 cells were treated with (A) C1 (50 µg/ml or 100 µg/ml) or (B) in the presence or absence of zVAD-fmk (50 µM) for 12 and 24 hours and cleavage of PARP was evaluated by western blotting.

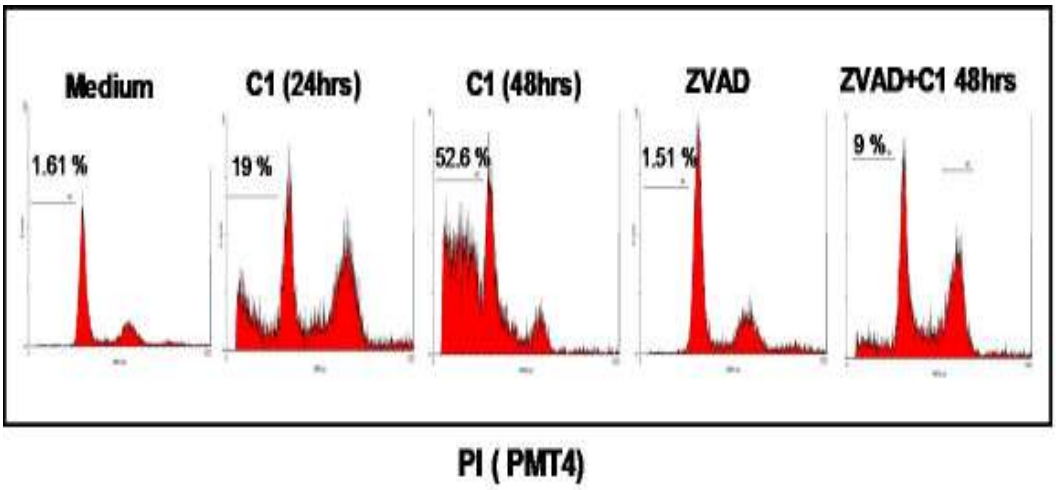


Figure 10: C1-induced DNA fragmentation is a caspase- dependent process. Cells were treated with C1 (100 μg/ml) in the presence or absence of zVAD (50 μM) and cell cycle profiles were obtained by PI staining as described in Materials and Methods. Data are representative of 3 different independent results.

1.5 zVAD-fmk, but not Necrostatin A, inhibits C1-induced reduction in cell viability

In this model, apoptotic cell death, as evidenced by PARP cleavage and increase in sub-G1 fraction, was dependent on caspase activation. Nonetheless, the reduction in cell proliferation may indicate multiple forms of cell death phenotypes. Apoptosis may constitute the sole pathway in C1-induced cell death, or it could co-exist with other modes of programmed cell death. To determine this, cell viability assay was carried out by incubating the pan-caspase inhibitor, zVAD-fmk prior to C1 treatment. Cells pre-treated with zVAD displayed higher survival rate than cells treated with C1 alone. Intriguingly, while zVAD inhibited the effect of C1 on PARP cleavage and DNA fragmentation (sub-G1), it only partially provided protection from C1-induced cell death (Figure 11A). These data indicate that C1-induced cell death might involve caspase-independent pathways, in parallel or distinct from apoptosis. To investigate that, the effect of the necrosis inhibitor, necrostatin A, on C1-induced cell death was evaluated. Necrostatin A was reported to inhibit necrosis by inhibiting the loss of mitochondrial membrane potential as well as blocking RIP1 activation. Results show that necrostatin A had no effect on C1-induced cell death, thereby ruling out the involvement of necrosis (Figure 11B).

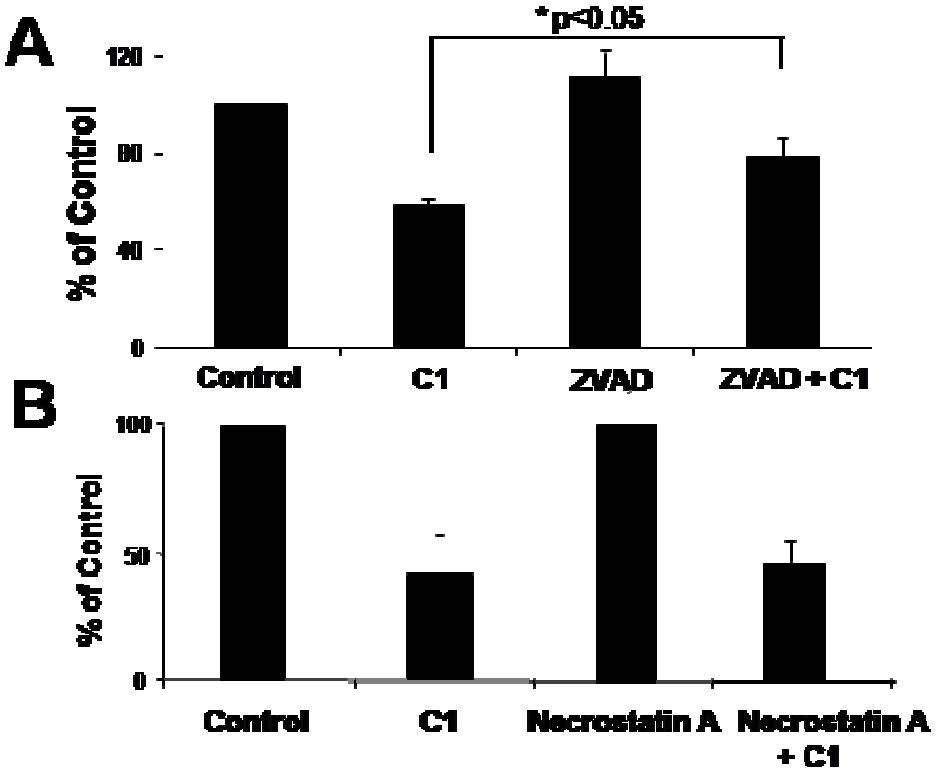


Figure 11: zVAD-fmk, but not Necrostatin A, inhibits C1-induced reduction in cell viability. (**A**) HCT116 cells were pre-incubated with zVAD (50 μM) for 1 hour before exposure to C1 (100 μg/ml for 18 hours) and survival was assessed by the MTT assay. (**B**) Cells were pre-incubated with necrostatin (50 μM for 1 hour) before exposure to C1 (100 μg/ml) and survival was assessed 18 hours later by the MTT assay.

PART II: INDUCTION OF NON-CANONICAL AUTOPHAGY BY C1

2.1 Autophagosome formation is a morphological hallmark of C1-treated cells

Having shown that zVAD and necrostatin A were unable to rescue cells completely from C1-induced cell death, electron microscopic analysis of cell morphology following exposure to C1 was performed. HCT116 cells were being treated with C1 (100 µg/ml for 12 hours and 24 hours) before being fixed, dehydrated and sectioned for EM analysis. Intriguingly, exposure of cells to C1 resulted in the formation of autophagosomes and autophagic vacuoles, reminiscent of autophagy (Figure 12). The appearance of autophagosomes, which are double membrane-bound vesicles of 300 – 900 nm in diameter, is one of the morphological characteristics of autophagy (Yorimitsu and Klionsky, 2005). As depicted in Figure 12, autophagosome formation can be detected upon 24 hours of incubation with C1.

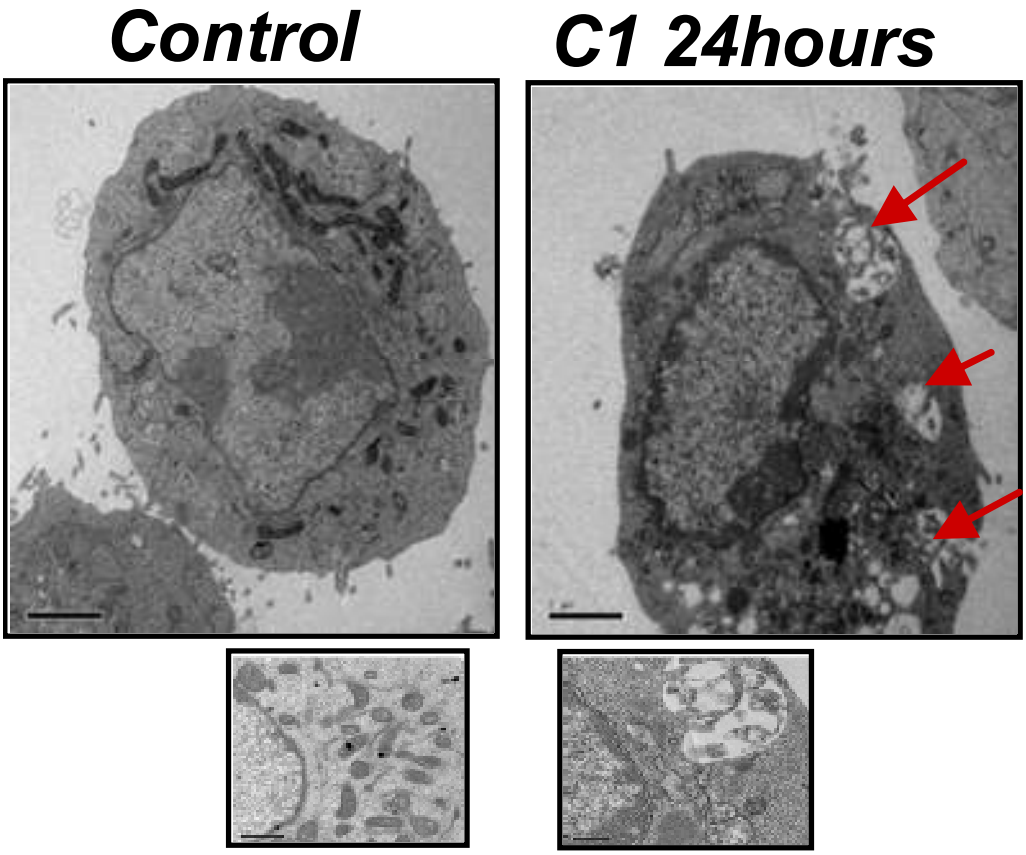


Figure 12: Autophagosome formation is a morphological hallmark of C1-treated cells. HCT116 cells were treated with C1 (100 µg/ml) for 24 hours, fixed and viewed under an electron microscope as described in Materials and Methods (Magnification X 40,000). Arrows indicate the presence of autophagosomes and autophagic vacuoles in C1-treated cells.

2.2 Detection of LC3II accumulation and translocation to the mitochondria in C1-treated cells

Several ways of autophagosome measurement exists, and is not limited to direct detection of autophagosomal structures via EM imaging. Other methods of autophagy analysis include detection of LC3 lipidation on Western blots and fluorescent GFP-LC3 imaging. Intrigued by the large amount of autophagosomes present in C1-treated cells, it is logical to probe into other mechanistic detail of autophagic induction in this system. To gain further insight into the autophagic pathway, the increased expression of MAP1 LC3II (herein referred to as LC3II), a bona fide marker of autophagy was evaluated. LC3II is the cleaved product of LC3I mediated by Atg4, and following the proteolytic cleavage, it is readily conjugated to the lipid phosphatidylethanolamine (PE) (Kabeya et al., 2000). In contrast to other protein complexes which also localizes to the pre-autophagosomal structures (PAS), LC3II accumulates at the intermediate vesicles and also the completed autophagosome (Kirisako et al., 1999). Thus, LC3II is widely used as an autophagic marker in mammalian system. To assess the lipidation of LC3, increase in LC3II protein level was being examined in a time kinetics experiment. Indeed, elevated accumulation of LC3II was observed following incubation with C1 in a time-dependent manner (Figure 13A). Interestingly, LC3II level diminished at 48 hours post treatment, which may indicate completion of the autophagic degradation process, with accompanying breakdown and recycling of the cargo. Of particular note, it was identified for the first time LC3II enrichment in the heavy membrane fractions following drug treatment, suggesting its localization to the mitochondria

(Figure 13B). This may indicate the presence of mitochondrial engulfment by the autophagic vacuoles.

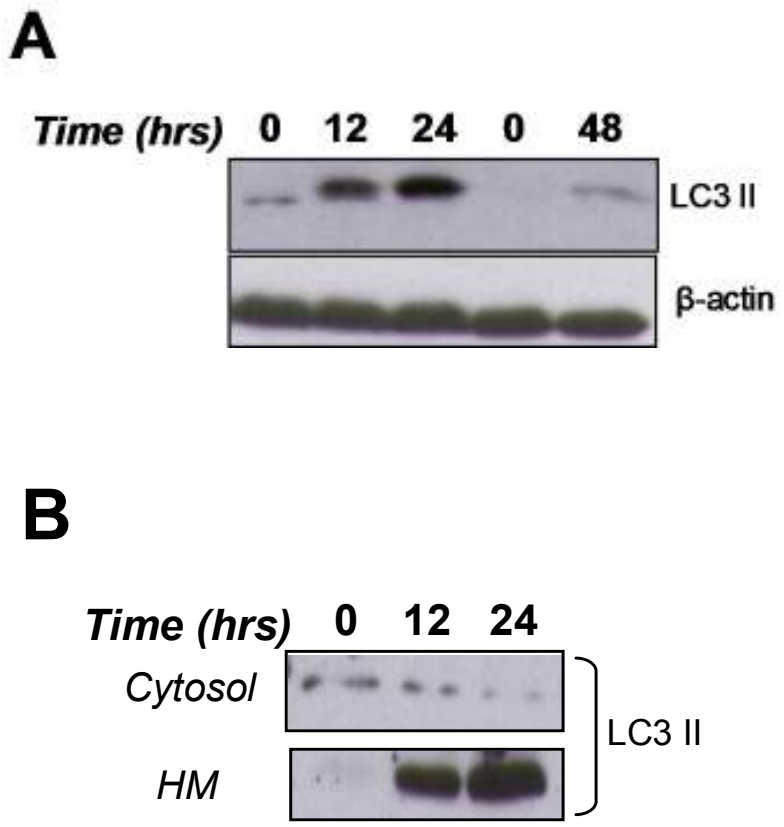


Figure 13: Detection of LC3II accumulation and translocation to the mitochondria in C1-treated cells. (**A**) Lysates of cells treated with C1 (100 µg/ml) for 12, 24 and 48 hours were probed for LC3II using a specific antibody (14 kDa) that has higher affinity for LC3II than the other LC3 forms. (**B**) Western blotting analysis of LC3II within cytosolic and mitochondrial fractions of cells following exposure to C1 for 12 to 24 hours.

2.3 C1-treated GFP-LC3 transfected cells display punctate staining reminiscent of autophagic phenotype

Next, HCT116 cells were transfected with a plasmid encoding GFP-LC3 and immunofluorescence was performed following exposure to C1. GFP-LC3 plasmid is a construct of LC3 protein with a fluorescent protein, GFP. GFP-LC3 has been widely used as a method to monitor autophagy via fluorescence microscopy. The cells which express GFP-LC3 exhibit diffuse green GFP staining, and the fluorescence pattern could be converted to punctate staining during the induction of autophagy, due to LC3II aggregation on the autophagosomes. To determine autophagosome formation, GFP-LC3 or GFP plasmid alone were being transfected into the cells with the cationic-lipid mediated delivery method. Drug treatment was administered for the indicated time points 48 hours post transfection. Whereas the distribution of LC3-GFP in non-treated cells displayed a diffuse pattern, exposure to C1 for 6 hours resulted in a green punctate staining, indicative of LC3II accumulation within the autophagosomal membranes (Figure 14).

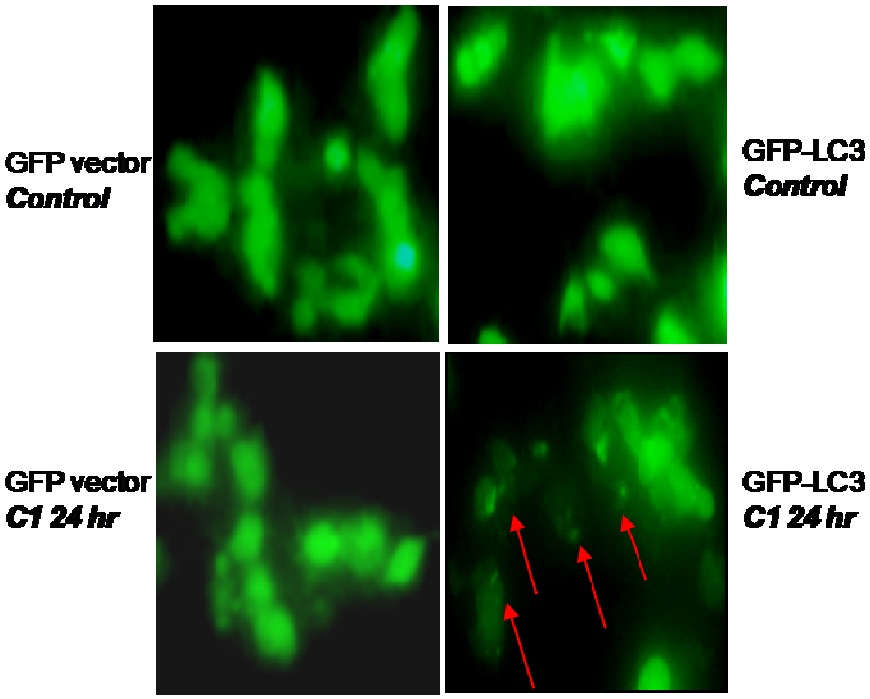


Figure 14: C1-treated GFP-LC3 transfected cells display punctate staining reminiscent of autophagic phenotype. Cells were transiently transfected with GFP-Vector or LC3-GFP for 48 hours before exposure to C1 for 24 hours and analyzed using a fluorescence microscope. Arrows point to the punctate staining indicating LC3 II aggregation into autophagosomes as opposed to diffuse staining in untreated cells. (Mag: 40,000X)

2.4 Treatment of HCT116 cells with C1 increases expression of Atg7 and conjugation of Atg12-Atg5

In order to further examine C1-induced autophagy in greater detail, protein expression levels of Atg7, 5, and 12 were also assessed in response to C1 treatment. The protein expression of Atg7, a critical E1-like enzyme responsible for the conjugation of Atg5 to Atg12 as well as partially involved in the conjugation of phosphatidylethanolamine (PE) to LC3 was evaluated. A significant increase in expression of Atg7 was observed upon exposure (6-24 hours) of cells to C1 (Figure 15). Atg12-Atg5 conjugation represents one of the two important ubiquitin-like conjugation systems involved in the generation of autophagosomes (Maiuri et al., 2007). In some systems where free Atg5 could be detected, the amount of conjugated Atg12-Atg5 increases in response to autophagic signals. Thus monitoring conjugation of Atg12-Atg5 is frequently used as a measurement of autophagy. In the current model, an increase in Atg5 expression in a time dependent manner from 12 hours to 24 hours, with a concomitant decrease in Atg12 level were being detected (Figure 15). The decrease in Atg12 expression at the late time points (12-24 hrs) when Atg5 expression was increased is in agreement with similar data indicating Atg5-Atg12 conjugation.

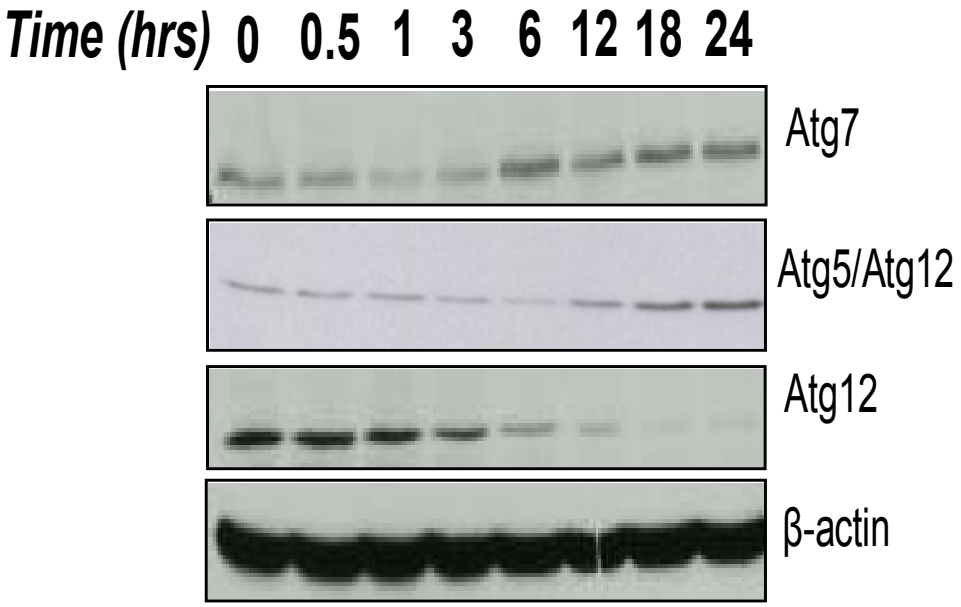


Figure 15: Treatment of HCT116 cells with C1 increases expression of Atg7 and conjugation of Atg12-Atg5. Following C1 exposure (100 μg/ml) for various time points, from 30 minutes to 24 hours, cell lysates were probed for Atg7, Atg5 and Atg12 by Western blotting. Note the decrease in Atg12 and the reciprocal increase in Atg5 indicating conjugation of Atg12 to Atg5.

2.5 Increase in LC3 Lipidation in response to C1 treatment is a general phenomenon across cell lines

To provide evidence that the induction of autophagy by C1 was not exclusive to HCT116 cells, 9 other cancer cell lines from different lineages were investigated. These cells include melanoma M14 cells, lung carcinoma A549, cervical cell HeLa, neuroblastoma SHEP-1 and SH-SY5Y, nasopharyngeal carcinoma C666-1, HK-1 and three different breast cancer cell lines MCF-7, T47D and MDA-MB231 cells. In all cell lines LC3II formation were observed in a dose-dependent manner, being exposed with C1 from 25 – 200 μg/ml (Figure 16). Notably most of the cell lines displayed observable LC3 lipidation when being treated with 100 μg/ml of C1, which was in agreement with the effective dose required to induce autophagy in HCT116 cells. These observations confirmed that C1 is a potent autophagic inducer in a variety of tumor cells. Interestingly, across the breast cancer cell lines there were varying degrees of LC3II formation with regard to C1 treatment. It appears that MCF-7 had the lowest level of LC3II accumulation, followed by T47D while MDA-MB-231 was most responsive to C1 treatment. This observation is intriguing as both T47D and MDA-MB-231 cells are both highly invasive and metastatic cancer cell lines, while MCF-7 is a non-invasive cancer cell line.

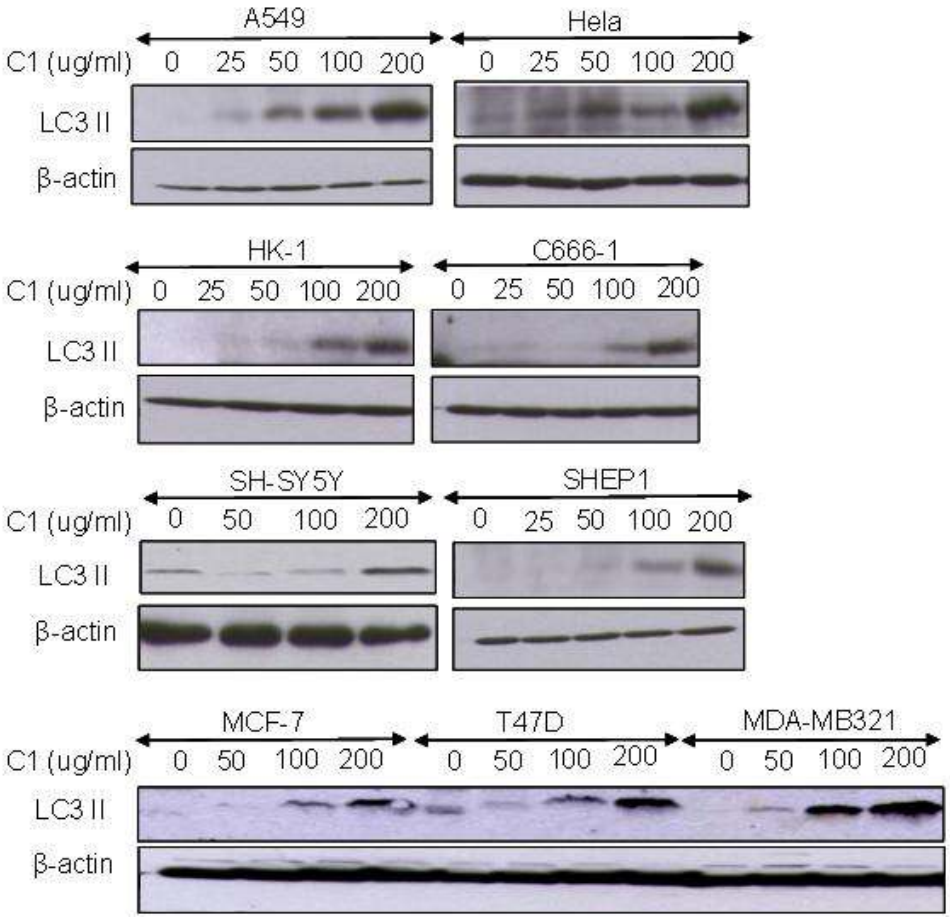


Figure 16: Increase in LC3 lipidation in response to C1 treatment is a general phenomenon across cell lines. Cervical cancer cells HeLa, nasopharyngeal carcinoma HK-1, C666-1, breast cancer cells MCF-7, T47D, MDA-MB-231, lung carcinoma A549, neuroblastoma cells SHEP1, SH5YSY and melanoma M14 cells were treated with various concentrations of C1 for 24 hours and total cell lysates were probed for LC3 II and β-actin by Western blotting.

2.6 Beclin1 is not involved in C1-induced autophagy

Beclin1 was shown to be one of the most important *ATG* genes, being involved in the initial steps of autophagosome formation. Intriguingly, the discovery of a non-canonical pathway in autophagy which is not dependent on Beclin1 has raised interesting question on the biological role of Beclin1 in the induction of autophagy (Scarlatti et al., 2008). In order to address this issue, Beclin1's role in C1-induced autophagy was determined. To do so, Beclin1 expression level in total lysates was first assessed. Interestingly, despite the formation of autophagosomes and increase in LC3II formation, the expression of Beclin1 did not increase significantly, but if anything, after 12 hours following drug exposure Beclin1 level was significantly reduced in whole cell lysates (Figure 17A). Since protein expression level may not always correlate with its activity, the involvement of Beclin1 was further investigated by evaluating the effect of siRNA-mediated gene silencing of *Beclin1* on C1-induced autophagy. Beclin1 mRNA was being silenced with 100 nM of siBeclin1 by using the DharmaFECT1 siRNA transfection reagent, after which the expression level of Beclin1 and the accumulation of LC3II were being assessed with western blotting. Beclin1 expression was efficiently silenced with 100 nm of siBeclin1; however knock down of *Beclin1* failed to inhibit LC3II accumulation induced by C1 (Figure 17B). Similarly when the cell lysates were being assessed by the MTT assay, silencing of *Beclin1* could not rescue cells from the death triggering activity of the small molecule compound (Figure 17C). Collectively, these data argue in favor of an existence of a non-canonical autophagy pathway whereby Beclin1 is completely dispensable.

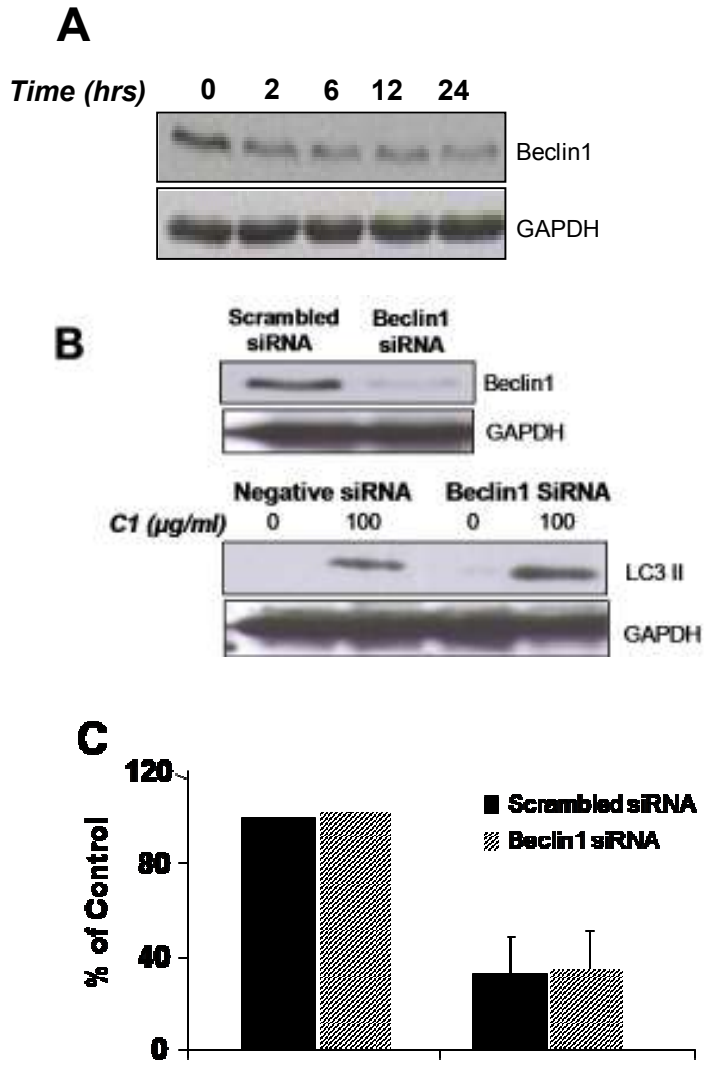


Figure 17: Beclin1 is not involved in C1-induced autophagy. (**A**) Lysates from cells exposed to 100 μg/ml of C1 for 2, 6, 12, and 24 hours were subjected to Western blot analysis using anti-Beclin1. Anti-β-actin was used as the loading control. (**B**) HCT116 cells were transiently transfected with siRNA against Beclin1 for 48 hours followed by exposure to C1 (100 μg/ml) for 24 hours. Lysates were then probed for LC3II using a specific antibody. Knockdown of Beclin1 was confirmed by immuno-blotting. GAPDH expression was used as a loading control. (**C**) Cells were transiently transfected with siRNA against Beclin1 for 48 hours and then treated with C1 for 18 hours. Survival was assessed by the MTT assay.

2.7 Increase in LC3II is dependent on *ATG* genes

To determine whether other crucial components of the autophagic pathway were involved in C1-induced autophagy, gene knockdown of *ATG7* was performed. As Atg7's presence is integral to two integral conjugation machineries in autophagy (Yorimitsu and Klionsky, 2005), silencing of *Atg7* is expected to suppress the autophagic response. Following 48 hours of si*ATG7* transfection of HCT116 cells, C1 (100 μg/ml) treatment was administered for 24 hours. The resultant cells were then harvested for Western blotting. In contrast to the redundancy of *Beclin 1*, the presence of *ATG7* siRNA significantly decreased the LC3 II accumulation induced upon C1 exposure while the apoptotic signal (PARP cleavage) remained unchanged (Figure 18). Corroborating these findings are results obtained with gene knockdown of the UNC-51 like kinase (*ULK1*; mammalian homolog of yeast *Atg1*), which is crucial in controlling the induction stage of autophagy. Similar to the results obtained with *ATG7* silencing, the presence of *ULK1* siRNA inhibited LC3II formation in this model but did not have any effect on the processing of PARP (Figure 19). Taken together, these data provided strong evidence that exposure of HCT116 cells to C1 triggered non-canonical autophagy, but involved the intermediacy of the ubiquitin E1-like enzyme Atg7 and ULK1. Interestingly, these findings also suggested that retardation of autophagic signals do not affect caspase-dependent apoptosis, indicating that these two pathways are mutually exclusive and may operate parallel to each other.

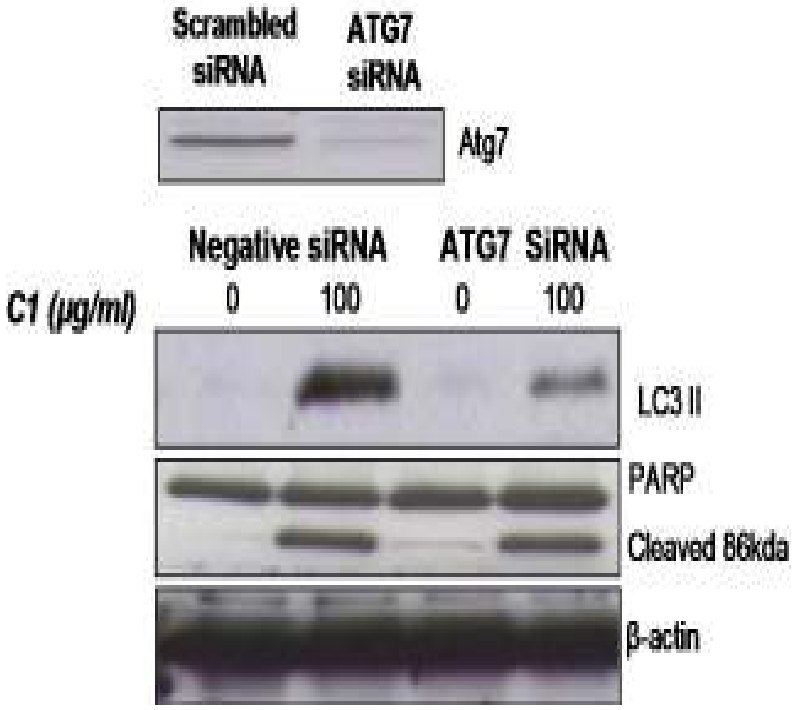


Figure 18: Increase in LC3II is dependent on *ATG 7* gene. HCT116 cells were transiently transfected with siRNA against *ATG7* for 48 hours followed by exposure to C1 (100 µg/ml) for 24 hours. Lysates were then probed for LC3II using a specific antibody. Knockdown of each gene was confirmed by immuno-blotting. GAPDH expression was used as a loading control.

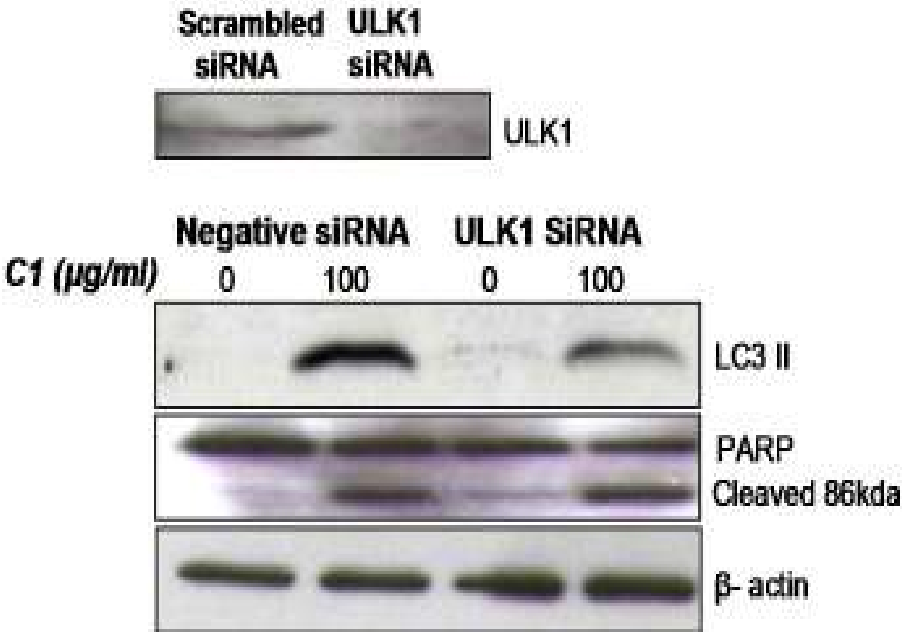


Figure 19: RNAi-mediated silencing of *ULK1* could revert the increase in LC3 lipidation in C1-treated cells. HCT116 cells were transiently transfected with siRNA against ULK1 for 48 hours followed by exposure to C1 (100 µg/ml) for 24 hours. Lysates were then probed for LC3II using a specific antibody. Knockdown of each gene was confirmed by immuno-blotting. GAPDH expression was used as a loading control.

2.8 Autophagy process mediated by C1 is insensitive to 3-MA treatment

The existence of a Beclin1- independent autophagy pathway was reported to be refractory to the Class III PI3 kinase inhibitor, 3-methyladenine (3-MA), routinely used as a pharmacological inhibitor of canonical autophagy (Yano et al., 2007). Class III PI3 Kinase, or hVPS34, is known to positively regulate autophagy by generating phosphatidylinositol-3-phosphate (PtdIns(3)P). 3-MA has been shown to be a general PI3 kinase inhibitor, in other words, it could inhibit both Class I and Class III PI3 kinase activity (Petiot et al., 2000). The two classes of PI3 kinase enzymes have vastly different roles, with Class I having a negative regulatory role in autophagic sequestration, while Class III actively promotes autophagic sequestration (Kondo et al., 2005). That being said, the reason why 3-MA is still being widely used as an autophagic inhibitor is because the class III enzymes are usually found to act downstream of Class I enzymes. To corroborate the data on non-canonical, Beclin1-independent autophagy, 3-MA pre-treatment for 1 hour (5 or 10 mM) was being used to inhibit the autophagic process, prior to addition of C1 for 18 hours. Indeed, *a priori* treatment of cells with 3-MA (5 or 10 mM) had virtually no effect on LC3II accumulation induced by C1 (Figure 20).

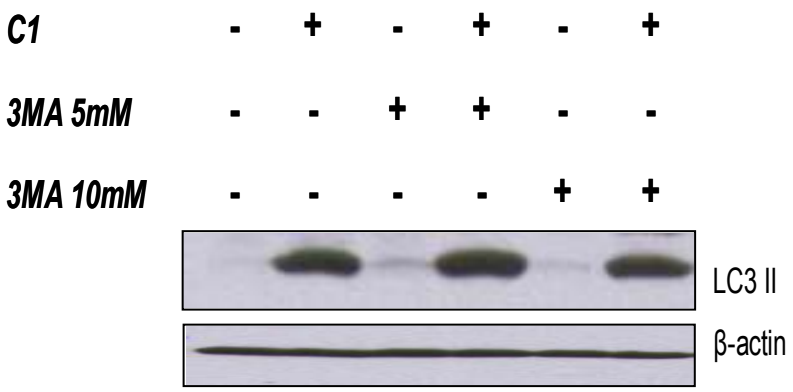


Figure 20: Autophagy process mediated by C1 is insensitive to 3-MA treatment. HCT116 cells were pre-incubated with 3-MA (5 or 10 mM) for 1 hour before exposure to 100 μg/ml of C1 for 18 hours. Lysates were then immuno-blotted with anti-LC3. β-actin expression was probed as a loading control.

2.9 Inhibition of caspase activity does not affect LC3 lipidation elicited by C1

The interrelationship between autophagy and apoptosis is a complex issue. Despite the many experimental endeavours made in addressing this issue, the existence of a link between autophagy and apoptosis remained controversial. Pertaining to this, the findings presented in the previous section suggested that inhibition of autophagy did not affect apoptotic outcome. In order to ascertain that apoptosis and autophagy is mutually exclusive, the reverse experiment was conducted. Inhibition of caspases by pharmacological inhibitor zVAD, caspase 3 or caspase 9 inhibitors was performed to investigate their effects on LC3II induction. Pre-treatment of HCT116 cells with these inhibitors did not affect the level of LC3 lipidation as assessed by Western blot, despite their effectiveness in the prevention of PARP cleavage induced by C1 (Figure 21). These results suggested that blocking the apoptotic pathway did not alter the autophagic response, and thus strongly recommend that the two integral cellular pathways are mutually exclusive to each other, specifically in the context of C1-induced cellular responses.

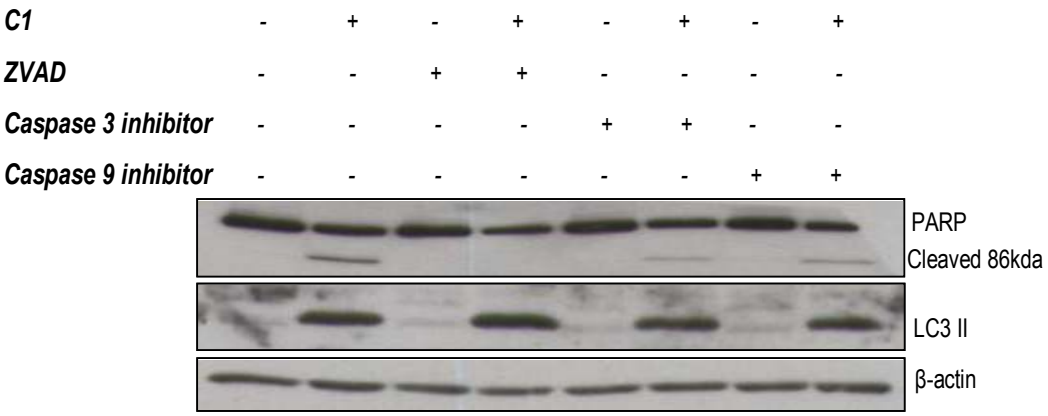


Figure 21: Inhibition of caspase activity does not affect LC3 lipidation elicited by C1. Cells were pre-incubated with zVAD (50 μM) or caspase 3 inhibitor (DEVD; 20 μM) or caspase 9 inhibitor (LEHD; 20 μM) followed by treatment with 100 μg/ml of C1 for 24 hours. Cell lysates were probed for cleavage of PARP and LC3II formation by Western blotting.

PART III: MODULATION OF AUTOPHAGY AND APOPTOSIS BY ROS

As discussed previously in the Introduction session, a complex relationship between ROS and various cellular processes existed. Unsurprisingly, the intracellular redox status has also been intricately linked to apoptosis and autophagy. Given the close interplay between ROS, autophagy and apoptosis, it was a logical progression to examine the role of ROS in the activation of cellular signaling pathways by C1.

3.1 C1 triggers mitochondrial superoxide production in HCT116 cells

In order to determine the relevance of ROS in this system, intracellular ROS production was being assessed with redox-sensitive dye. To do so, a fluorescent probe, MITOSOX^{TM} Red, was being used to measure mitochondrial $O_2^{\bullet-}$ level following incubation with C1 (15 min, 1hr, 3hr, 6hr). MITOSOX^{TM} Red selectively detects mitochondrial $O_2^{\bullet-}$ as it is specifically targeted to the mitochondria of live cells. MITOSOX^{TM} Red is cell permeable and once inside the mitochondria, it will be oxidized by $O_2^{\bullet-}$ and emits red fluorescence, with emission wavelength at 510 nm and excitation at 580 nm. This dye is also specific to $O_2^{\bullet-}$ as it is less prone to oxidation by other ROS molecules. Following incubation of MITOSOX^{TM} Red (5 μM) for 15 minutes, HCT116 cells are being analyzed with the Fluorescence Activated Cell Sorter (FACS). Results showed a significant increase in mitochondrial $O_2^{\bullet-}$ levels, as early as 15 minutes post-treatment, which was sustained for 1 hour and began to decrease at 3 hours (Figure 22), indicative of an existence of mitochondrial $O_2^{\bullet-}$ accumulation upon C1 treatment.

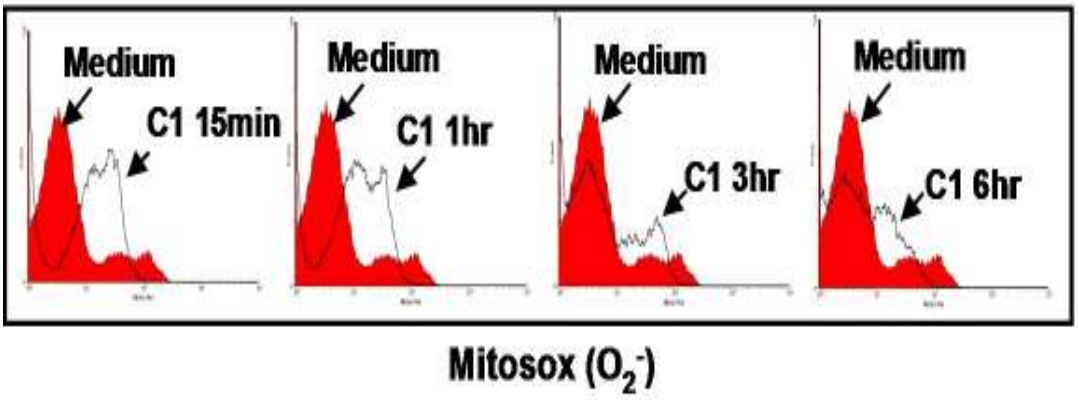


Figure 22: C1 triggers mitochondrial superoxide production in HCT116 cells. $1X10^6$ cells were incubated with 100 µg/ml of C1 for 15 min to 6 hours and intra-mitochondrial $O_2^{\bullet-}$ was determined using the fluorescent dye MitoSox™ RED Mitochondrial $O_2^{\bullet-}$ Indicator. Data are representative of 3 different independent results.

3.2 Increase in intracellular H_2O_2 production following C1 treatment in HCT116 cells

As $O_2^{\bullet-}$ does not readily cross membranes, it is highly unlikely that the mitochondrial $O_2^{\bullet-}$ detected by MITOSOX^{TM} Red could transverse from the mitochondria to the cytosol and thereby eliciting its downstream signaling effect. Moreover, the presence of MnSOD in the mitochondria could mediate the conversion of $O_2^{\bullet-}$ to H_2O_2. Being a polar molecule, H_2O_2 can diffuse within the cells *in vivo*, and has also been shown to transverse the membrane water channels, aquaporin (Henzler and Steudle, 2000). To confirm the presence of ROS in the cells, the redox-sensitive probe, CM-DCHF-DA is utilized in this study. CM-DCHF-DA is cell permeable and once it enters live cells, it can be hydrolysed by cellular esterases to form DCFH. Oxidation of DCFH by cellular oxidants leads to the formation of fluorescent DCF (Halliwell and Whiteman, 2004), which could be detected by flow cytometry. Incubation of HCT116 cells with C1 at various time points (15 min to 3 hours) resulted in the right-ward shift in fluorescence, indicating an increase in intracellular ROS production (Figure 23). Moreover, treatment of 100 μg/ml of C1 for 6 hours resulted in a less significant fluorescence shift, as compared to 3 hours of C1 treatment at the same dosage (Figure 23). These results were indicative of an early burst of ROS production, which could not be sustained for a prolonged time.

An important cautionary note in working with DCFDA probe is that it is a measurement of general ROS production, and is not exactly specific for any single type of oxidant. Many ROS/RNS such as H_2O_2, $OH^{\bullet}$ and $ONOO^{-}$ can oxidize DCFDA, and the extent of oxidation is also dependent on other factors, such as the

intracellular metal ions concentrations (Halliwell B, 2007). To circumvent this problem, catalase was used in conjunction with C1 treatment to check for the specificity of ROS produced. Catalase is a specific antioxidant which converts H_2O_2 to water and ground-state O_2 through the dismutation reaction. As shown in Figure 23, pre-incubation of catalase (7000 units/ml) for 15 minutes reverted the production of ROS induced by C1, indicating that the signal was due to H_2O_2 generation.

Apart from catalase, another important type of enzyme which could remove intracellular H_2O_2 is the peroxidase. N-acetyl-L-cysteine (NAC) was typically used as an H_2O_2 scavenger. NAC can be hydrolysed to cysteine, which is the precursor of gluthathione (GSH). In addition, the antioxidant properties of NAC has also attributed to its ability to scavenge H_2O_2 directly (Halliwell B, 2007). To test out the effect of removing H_2O_2 in the system, NAC (200 μM) was pre-incubated with the cells for 2 hours to remove the oxidants, followed by C1 treatment. Indeed, the existence of NAC suppressed the production of ROS triggered C1, similar to the effect of catalase pre-incubation (Figure 24).

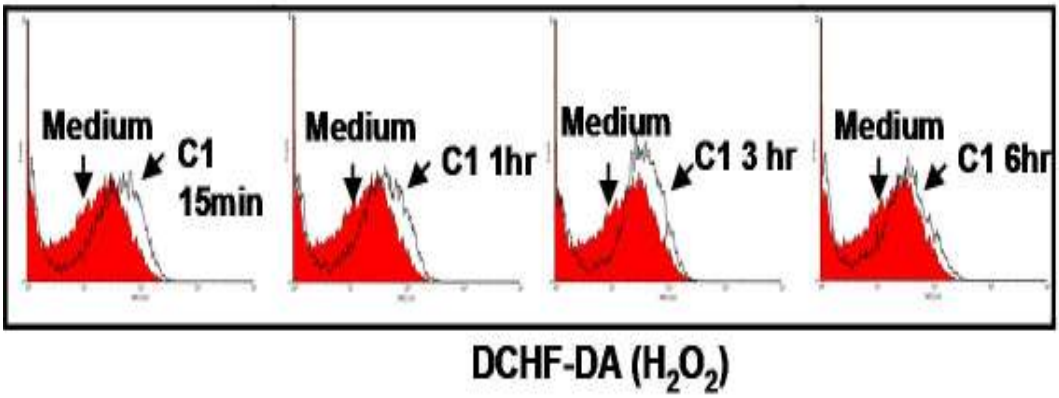


Figure 23: Increase in intracellular H_2O_2 production following C1 treatment in HCT116 cells. HCT116 cells ($1X10^6$) were treated with C1 (100 µg/ml) at varying time points. Subsequently intracellular H_2O_2 was detected by DCHF-DA loading and analyzed by flow cytometry as described in Materials and Methods. Data are representative of 3 different independent results.

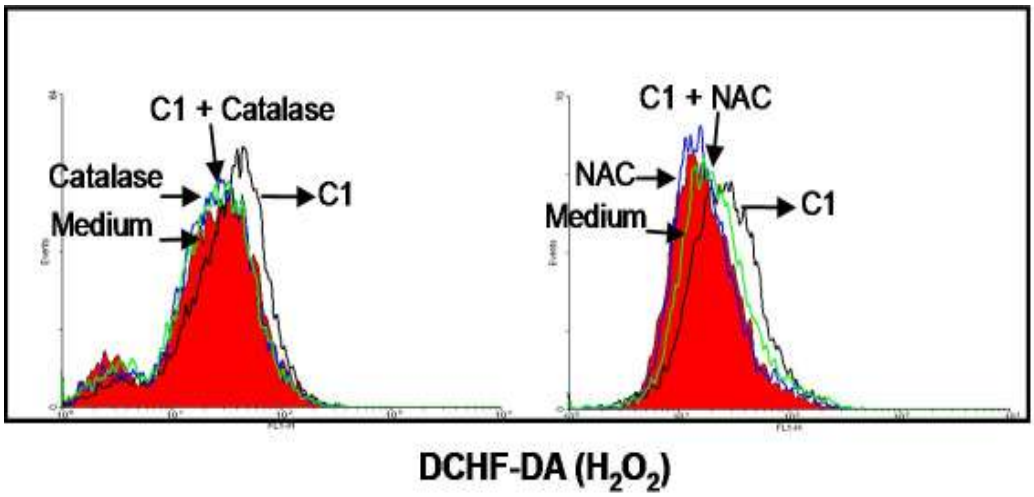
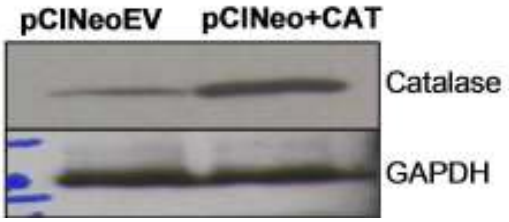


Figure 24: Exogenous addition of catalase and NAC abrogated C1-induced ROS production. HCT116 cells were pre-incubated with catalase (7000 units/ml) for 1 hour or NAC (200 μM) for 2 hours before treatment with C1 (100 μg/ml for 3 hours) and intracellular H_2O_2 was determined by flow cytomtery using DCHF-DA loading. Data are representative of 3 different independent results. (Figure legend: Solid fill: Medium; Black: C1; Green: C1 + NAC; Blue: NAC)

3.3 Transient overexpression of human catalase suppresses C1-induced ROS production

One of the important aspects of this thesis is based on the ability of C1 drug to generate intracellular ROS. Thus, multiple ways of scavenging ROS ought to be performed to show a direct correlation between the production of ROS and its role in modulating the cell death signaling pathways. While exogenous addition of catalase to the culture medium is a good way to check for H_2O_2 production, this method may not be the most effective way in scavenging H_2O_2. Catalase itself is a large protein of approximately 256 kDa and is unable to enter the cells to scavenge the intracellular H_2O_2. Thus, exogenous addition of catalase results in the localization of catalase in the extracellular milleu, and will only react with H_2O_2 which diffuses out from inside the cell, thereby lowering the intracellular amount of H_2O_2. Often, overnight incubation of catalase powder into the cells resulted in reduced activity of this compound due to its degradation. To circumvent this problem, an array of catalase doses have been tested out to synchronize the ideal dose for short term and long term assays, and the final dose has been fixed at 7000 units/ml of catalase concentration. In addition, in search of a more effective way in lowering intracellular H_2O_2 level, cells were transiently transfected with a plasmid encoding human catalase gene and exposed to C1. Cells transfected with pC1Neo empty vector exhibited an increase in ROS production, in both C1-treated and H_2O_2-treated cells as compared to the untreated controls. In contrast, catalase-overexpressing cells were markedly resistant to the increase in ROS triggered by C1, as assessed by CM-DCHF-DA staining (Figure 25).



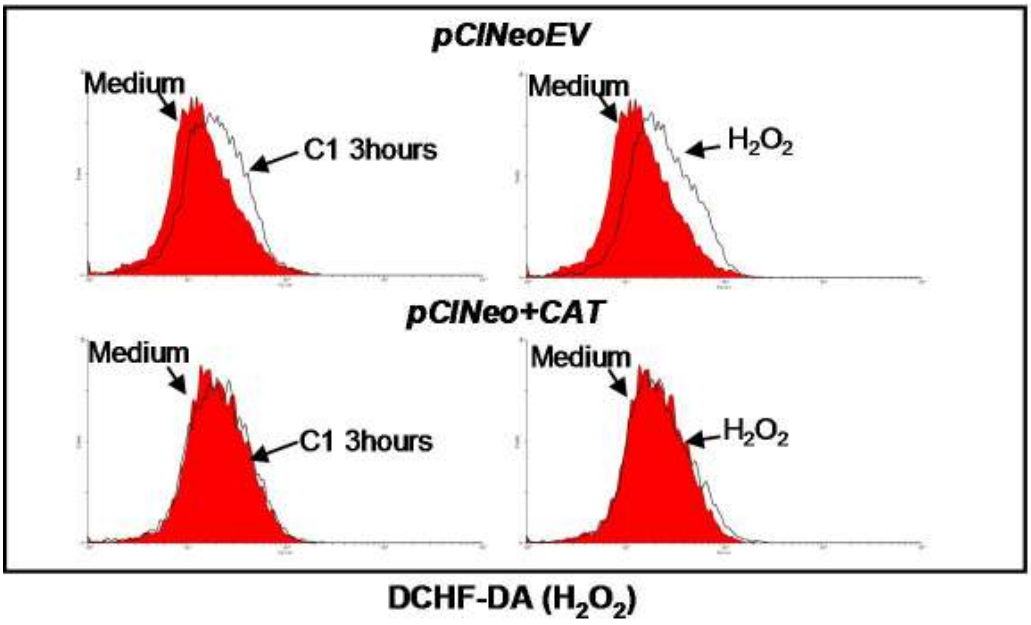


Figure 25: Transient overexpression of human catalase abrogated C1-induced ROS production.) HCT116 cells were transiently transfected with 8 µg of pCINeoEV or pCINeo+CAT for 48 hours and treated with C1 (100 µg/ml for 3 hours) and intracellular H_2O_2 was determined by flow cytometry analysis. The catalase protein expression level was shown on the top left panel and depicts successful catalase transfection. Data are representative of 3 different independent results.

3.4 Catalase pre-treatment or overexpression confers protection against C1-mediated PARP cleavage

After determining the ability of C1 in generating intracellular ROS, the next logical question is whether the production of ROS was integral in the execution of cell death. To do this, inhibition of ROS production was performed by way of an ROS scavenger to lower the intracellular ROS and also through a more specific method of lowering intracellular H_2O_2, by transient over-expression of catalase in the cells. Indeed, catalase pre-incubation as well as transient overexpression of plasmid containing human catalase gene inhibited C1-induced PARP cleavage, a marker of caspase 3 activation (Figure 26). These data demonstrated the critical role of ROS in the induction of apoptosis by C1, lending credence to an antitumor role of ROS by effectively inducing apoptotic cell death.

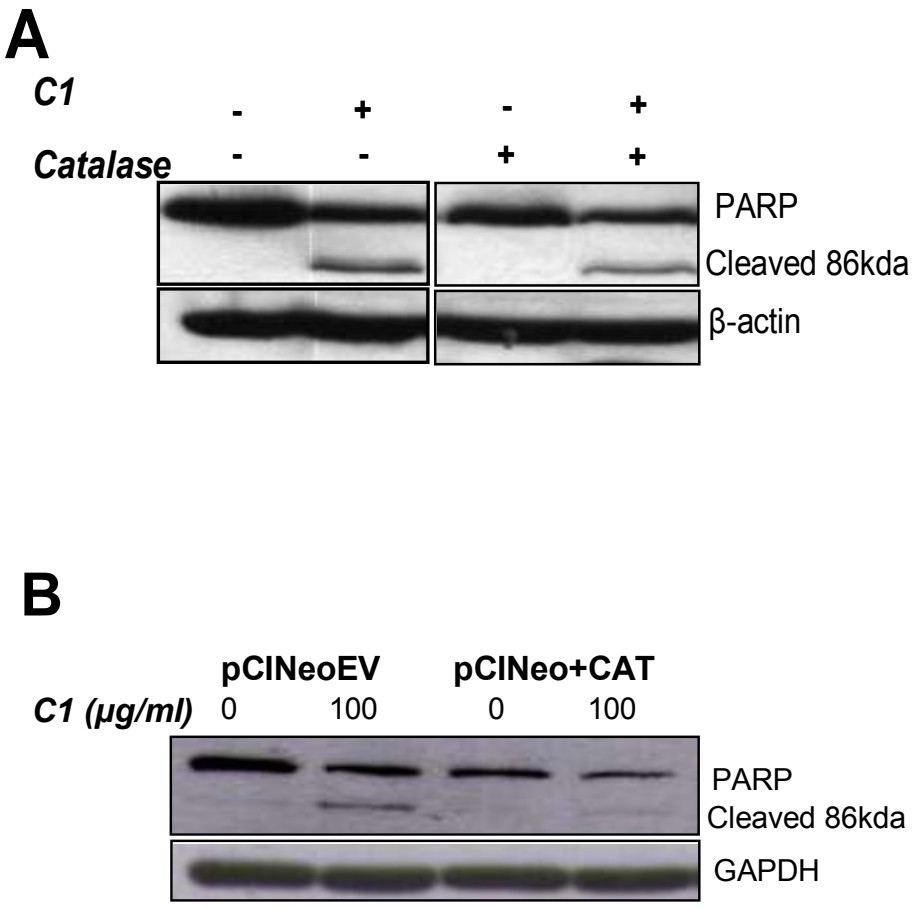


Figure 26: Catalase pre-treatment or Overexpression conferred protection against C1-mediated PARP cleavage. HCT116 cells were pre-incubated with catalase (7000 units/ml for 1 hour) or were transiently transfected with pCINeoEV or pCINeo+CAT before exposure to C1 (100 μg/ml for 24 hours), and total cell lysates were probed for PARP cleavage by Western blotting.

3.5 Antioxidants pre-treatment or catalase overexpression inhibits LC3II accumulation induced by C1

Having observed the involvement of ROS in C1- induced apoptotic signaling, the link between ROS and autophagy was investigated. To that end, the effect of scavenging ROS on C1-induced LC3II formation was assessed. Similar to apoptotic assays, pre-incubation of ROS scavengers, catalase and NAC were performed as described previously. Following these assays, catalase overexpression was also carried out in order to further affirm the role of ROS in terms of autophagic regulation. Results showed that addition of exogenous catalase or its transient overexpression as well as pre-incubation of cells with NAC significantly blocked LC3II accumulation induced by C1 (Figure 27). These data strongly suggest the involvement of ROS in C1-induced autophagy.

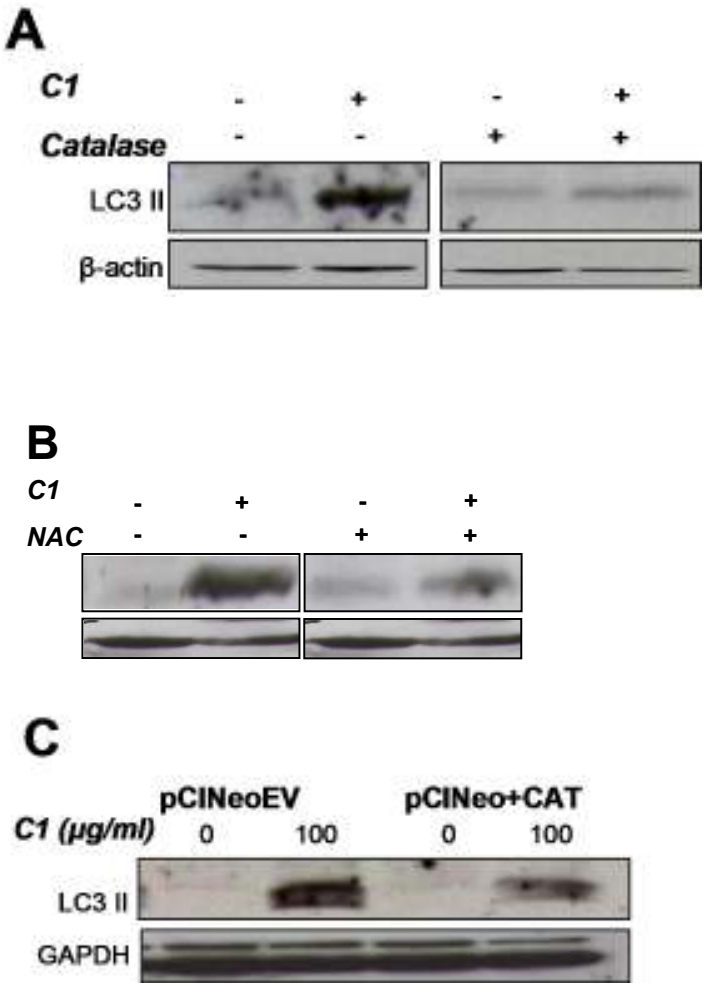


Figure 27: Antioxidants pre-treatment or catalase overexpression inhibits LC3II accumulation induced by C1. Cells were pre-incubated with catalase (7000 units/ml for 1 hour) **(A)** or NAC at 200 μM **(B)** or were transiently transfected with pCINeoEV or pCINeo+CAT **(C)** before exposure to C1 (100 μg/ml for 24 hours), and total cell lysates were probed for LC3II accumulation by Western blotting. GAPDH was assessed as a loading control.

3.6 C1 induces ERK and JNK phosphorylation with a concomitant decrease in MKP1 protein expression

ROS has been shown as a potent regulator of MAP kinase family members (Kamata et al., 2005; Temkin and Karin, 2007; Westwick et al., 1994). Intrigued by the robust activation of ROS in our system, activation of MAPK family members was investigated. Firstly, phosphorylation of ERK was detected in a time-dependent manner, which peaked at 3 hours and subsided at late time points (Figure 28A). In addition, JNK phosphorylation at Thr 183 and Tyr 185 was assessed in whole cell lysate from C1-treated cells (0.5 to 24 hours). Robust activation of JNK (phosphorylation) was detected as early as 30 minutes and was sustained for 24 hours after the stimulus, while no detectable difference in total JNK levels was observed (Figure 28A). Stimulated by these findings, the involvement of downstream effectors of JNK activation, in particular the transcription factor c-Jun was investigated. c-Jun is phosphorylated by JNK at Ser-73 and Ser-63 residues. Therefore, a kinetic analysis of c-Jun phosphorylation by Western blotting in total cell lysates was performed. Indeed, total c-Jun levels as well as phosphorylation at Ser-73 residue were detected as early as 30 minutes upon C1 treatment and persisted throughout the time course of 24 hours (Figure 28A). MKP1, the downstream inhibitory phosphatase of MAPK had been shown to be an important determinant in JNK-induced cell death (Kamata et al., 2005). Sustained JNK activation was suggested to be due to MKPs oxidation by ROS. It is thus highly likely that prolonged JNK activation induced by C1 is linked to a concomitant decrease in MKP1 protein level. Indeed, an examination on MKP1 level revealed a significantly lower MKP1 level in C1-treated cells (Figure 28B).

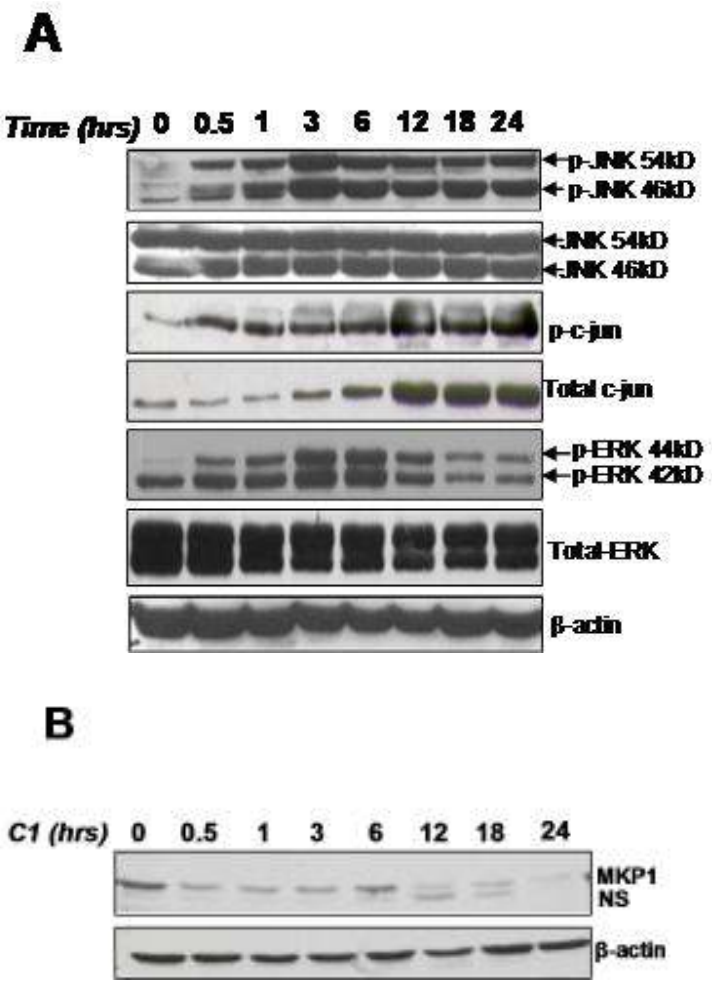


Figure 28: C1 induces phosphorylation of JNK, c-Jun and ERK kinases as well as decrease in MKP1 protein expression. HCT116 cells were exposed to 100 µg/ml of C1 for the indicated time points and total cell lysates were subjected to Western blotting for the detection of (A) phospho-JNK, total JNK, phospho-c-JUN, phospho-ERK, total ERK and (B) MKP1. β-actin was probed for equal loading. (NS:non-specific band)

3.7 Inhibition of ROS production decreases JNK and ERK phosphorylation

Having shown earlier that JNK and ERK were robustly activated by C1 and that ROS production is a critical signal for autophagy and apoptosis, the impending question is whether ROS production and MAPK activation is linked in any way. As ROS has been shown to be the activating signal for Map kinases, the connection between C1-induced ROS production and Map kinase activation was first investigated. To do so, the effect of scavenging ROS on C1-induced activation of MAP kinases and c-Jun was analyzed. Exogenous addition of antioxidants was first performed to determine the sensitivity of Map kinases to ROS inhibition. For catalase pre-incubation, the dose for catalase powder was fixed at 7000 units/ml which was the same for all other catalase experiments. Cell lysates were then harvested 3 hours post incubation with C1, which correlates to their maximal activation time point. Figure 5B showed that the presence of catalase (7000 Units/ml) abrogated C1-induced JNK, ERK and c-Jun phosphorylation (Figure 29A and 29C). Similar results were obtained upon pre-incubation of cells with NAC or upon transient transfection of cells with human catalase gene (Figure 29B). Taken together, these findings clearly highlighted the involvement of ERK and JNK in the biological activity of C1 and strongly implicated ROS in this signaling pathway.

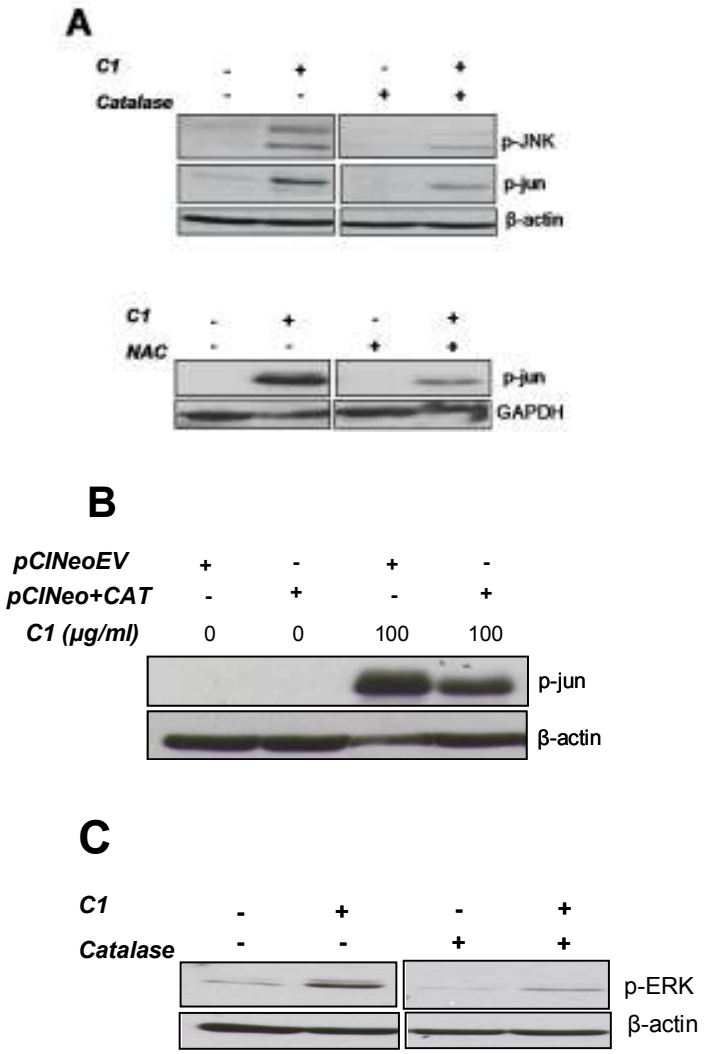


Figure 29: Inhibition of ROS production decreases JNK, c-Jun and ERK phosphorylation. Cells were pre-incubated for 1 hour with 7000 units/ml of catalase or 200 µM NAC **(A)** or transiently transfected with pCINeoEV or pCINeo+CAT **(B)** before exposure to C1 (100µg/ml for 3 hours). Total cell lysates were probed for the detection of phospho-JNK and phospho-c-JUN by Western blotting. GAPDH and β-actin were used, respectively as the loading control. **(C)** Cells were pre-incubated for 1 hour with 7000 units/ml of catalase before being assessed for phospho- ERK level. β-actin was used as the loading control.

PART IV: ERK AND JNK ACTIVATION ARE INTEGRAL IN THE MODULATION OF CELL DEATH AND AUTOPHAGY

4.1 The presence of ERK inhibitor and JNK inhibitor protect against C1-induced cell death

Having shown that ERK and JNK were downstream targets of ROS which were both activated following C1 exposure, the next logical step would be to dissect the physiological functions brought about by the activation states of these MAPKs.

One of the predominant effects of C1 on tumor cells was the execution of cell death signaling pathway. To investigate on this, the ability of pharmacological inhibitors of ERK and JNK in affecting the cell death read outs was first carried out. For the inhibition of ERK, a cell permeable inhibitor, PD98059 was being utilized in this system. PD98059 is a specific MEK1 inhibitor and has been shown to be selective in the suppression of ERK signaling pathway. MEK1 is the specific MAPKK for ERK activation by inducing phosphorylation of Thr and Tyr residues at the activation loop of the kinase domain. PD98059 exerts its inhibitory effect by binding to inactive form of MEK1 and thus, preventing MEK1 from being activated by upstream regulators (Rosen et al., 1994). A dose response analysis of PD98059 was first carried out to establish the effective dose for ERK inhibition. Though the recommended IC50 dose of PD98059 for the inhibitory activity against MEK1 are around 5-10 μM, a dose response experiment is critical due to cell line variations. To perform the dose response experiment, HCT116 cells were being incubated with various doses of PD98059 (5 – 20 μM) for one hour prior to C1 treatment of 3 hours.

It was determined that 20 μM of PD98059 for 1 hour is most effective in inhibiting ERK phosphorylation (Figure 30A).

Similarly, SP600125 was being selected as the pharmacological inhibitor against JNK. SP600125 (MW=220) is a reversible, competitive inhibitor of JNK with a high selectivity against JNK kinase (Bennett et al., 2001). It was shown to be a potent inhibitor of JNK which blocks the phosphorylation of various JNK targets, including c-Jun and Cox-2 (Bennett et al., 2001). To establish the effective dose for JNK inhibition, SP600125 at two different doses was incubated for one hour followed by C1 addition. c-Jun phosphorylation was used as a read out for JNK activation and 5 μM of SP600125 was selected for future experiments for JNK inhibition (Figure 30B).

Next, the effect of the respective ERK and JNK inhibitors on cell death was being investigated. Inhibition of JNK activity partially protected against C1-induced reduction in cell proliferation (Figure 31). Intriguingly, as compared to SP600125, PD98059 was found to be much more effective in the rescue of cell death (Figure 31). The conventional dogma has established that JNK and ERK mainly display opposing roles in terms of cell death regulation, with JNK being mainly involved in cell death while ERK generally have an anti-apoptotic characteristic. Nevertheless, the results in this study suggested that ERK could also act as a potent cell death mediator. In fact, the inhibitory effect brought about by ERK inhibition was much more pronounced than inhibition of JNK. To further study this, the effect of Map kinases inhibition on long term cultures were also assessed. Here, a similar trend was depicted; while JNK inhibition significantly rescued the reduction in colony

formation triggered by C1, ERK suppression resulted in a more dramatic effect on cellular proliferation (Figure 32). To ascertain that Map kinases not only affect general cell proliferation and colony formation but also specifically mediate apoptotic cell death, the same experiment setting were repeated and tested for PARP cleavage via western blotting. Indeed, JNK and ERK inhibition significantly reduced the extent of PARP cleavage induced by C1 treatment (Figure 33). Taken together, these results indicated an integral regulatory role of the Map kinases, ERK and JNK in inducing cell death.

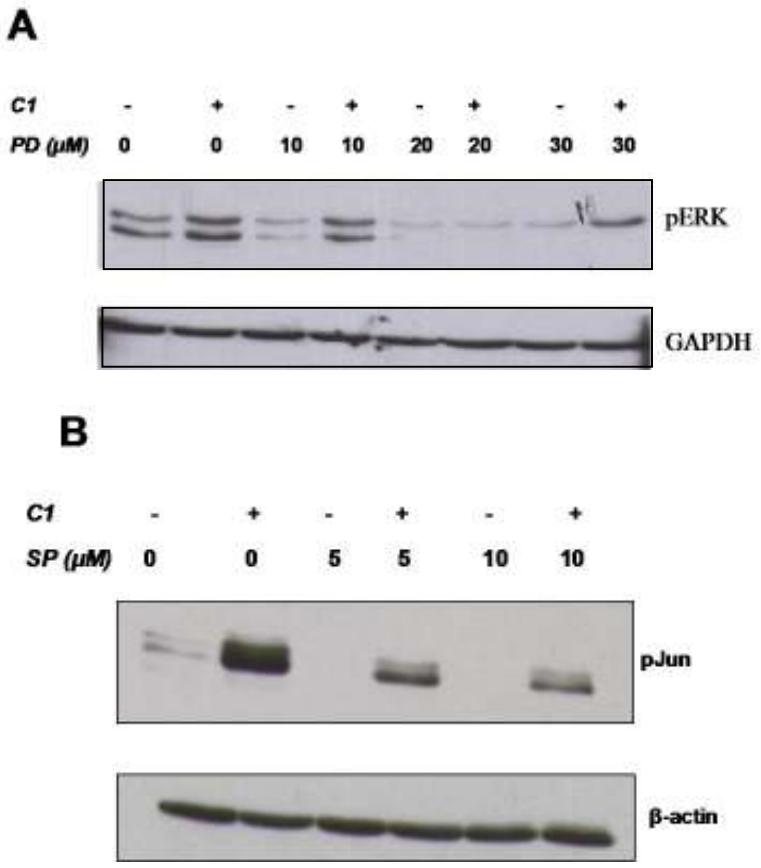


Figure 30: Establishment of effective dose for Map kinase inhibition. Cells were pre-incubated with (A) PD98059 (PD) or (B) SP600125 (SP) for 1 hour at various doses as indicated prior to C1 treatment (100 μg/ml for 3 hours). Activation profile of ERK and c-Jun was determined by Western blots.

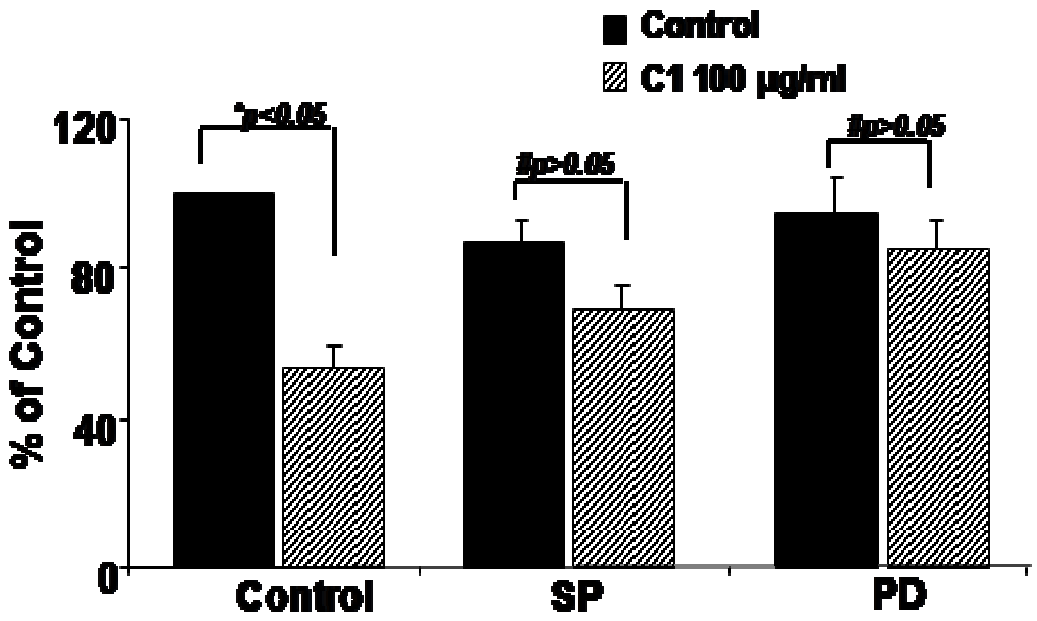


Figure 31: The presence of ERK inhibitor and JNK inhibitor protected against C1-induced reduction in cell proliferation. Cells were pre-incubated with SP600125 (SP; 5 µM) or PD98059 (PD; 20µM) for 1 hour before treatment with 100 µg/ml of C1 for 24 hours and cell survival (MTT assay) was determined as described in Materials and Methods.

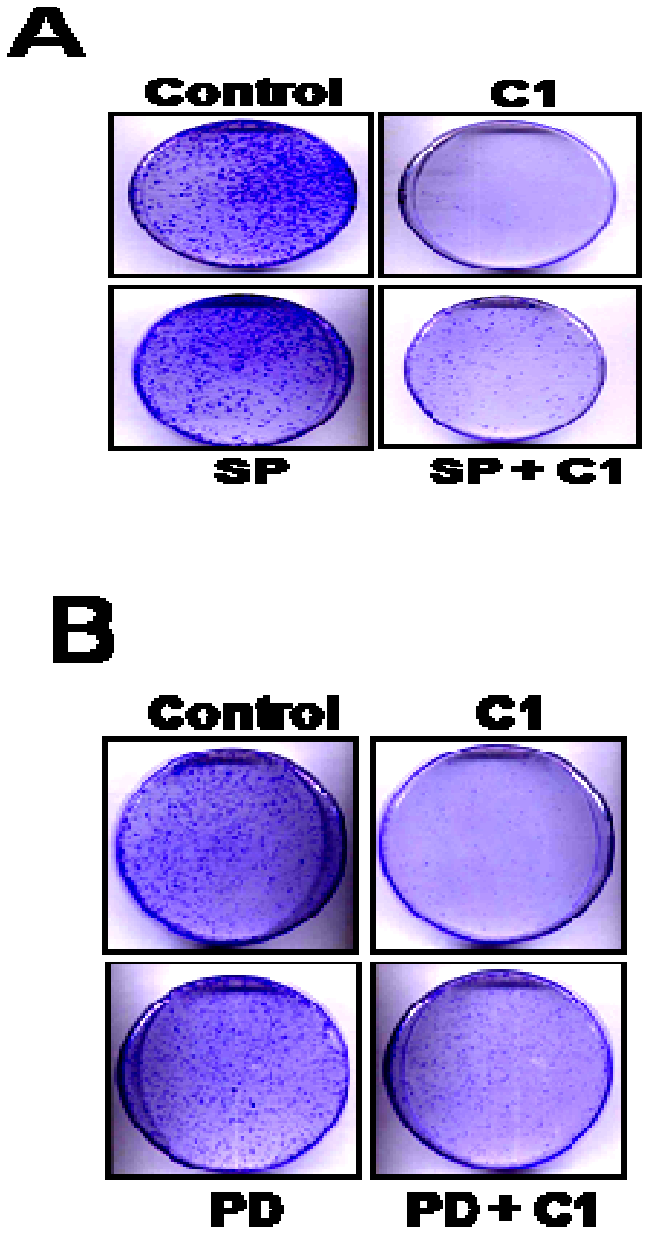


Figure 32: Inhibition of JNK and ERK effectively enhance clonogenic activity of C1-treated cells. HCT116 cells were pre-incubated with (A) SP600125 (SP; 5 μM) or (B) PD98059 (PD; 20 μM) for 1 hour before treatment with 100 μg/ml of C1 for 24 hours and tumor colony formation were determined as described in Materials and Methods.

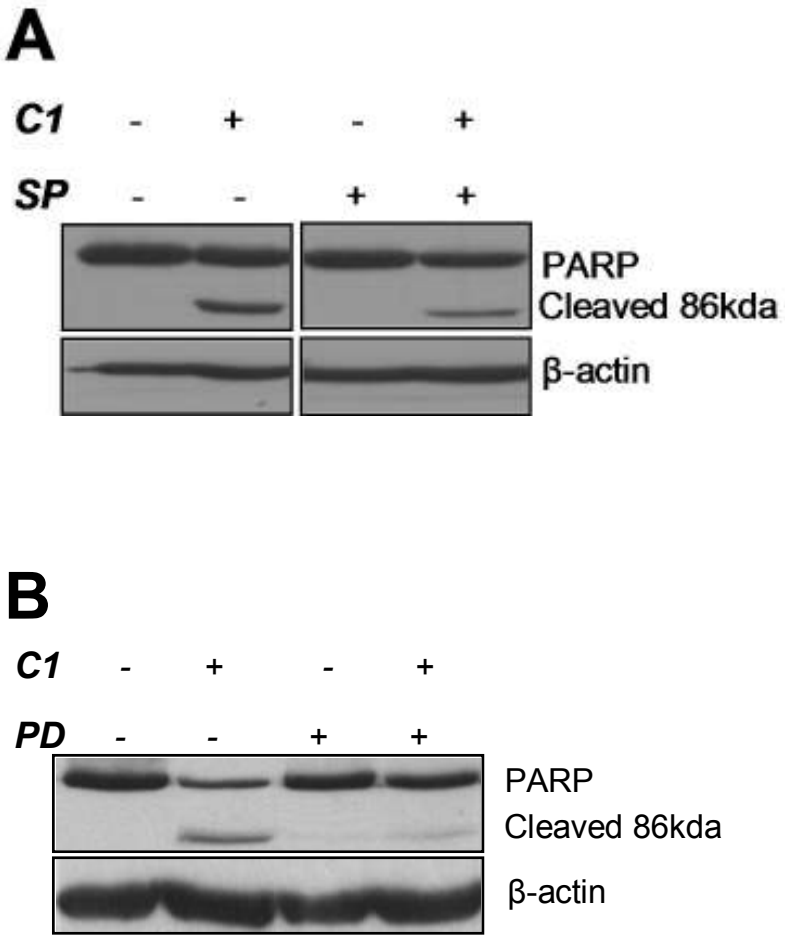


Figure 33: JNK and ERK inhibitors decrease the extent of PARP cleavage in C1-treated cells. HCT116 cells were pre-treated with JNK inhibitor, SP600125 (SP; 5 μM) or ERK inhibitor PD98059 (PD; 20 μM) for 1 hour before treatment with 100 μg/ml of C1 for 24 hours. Subsequently total cell lysates from these cells were probed for cleavage of PARP by Western blotting.

4.2 Pharmacological inhibition of ERK and JNK effectively suppress autophagic hallmarks in tumor cells

Given that exposure of cells to C1 resulted in simultaneous induction of autophagy and apoptosis, the next question is whether ERK and JNK activation were also central to the autophagic inducing activity of C1. To that end, recent evidence has implicated JNK activation in autophagy (Yu et al., 2004). Indeed, pre-incubation of cells with the JNK inhibitor SP600125, and ERK inhibitor PD98059, respectively, significantly blocked LC3II formation induced by C1 (Figure 34). Importantly, inhibition of JNK also reduced the increase in Atg7 expression (Figure 34). These data point to a central role for JNK and ERK activation downstream of ROS production in the induction of autophagy and apoptosis upon exposure of cancer cells to C1. In addition, the effect of JNK inhibition on autophagy was assessed by electron microscopy. Indeed, in addition to suppression of LC3 II, SP600125 also reduced the amount of autophagic vesicles (autophagosomes and autophagic vacuoles) induced by C1 (Figure 35).

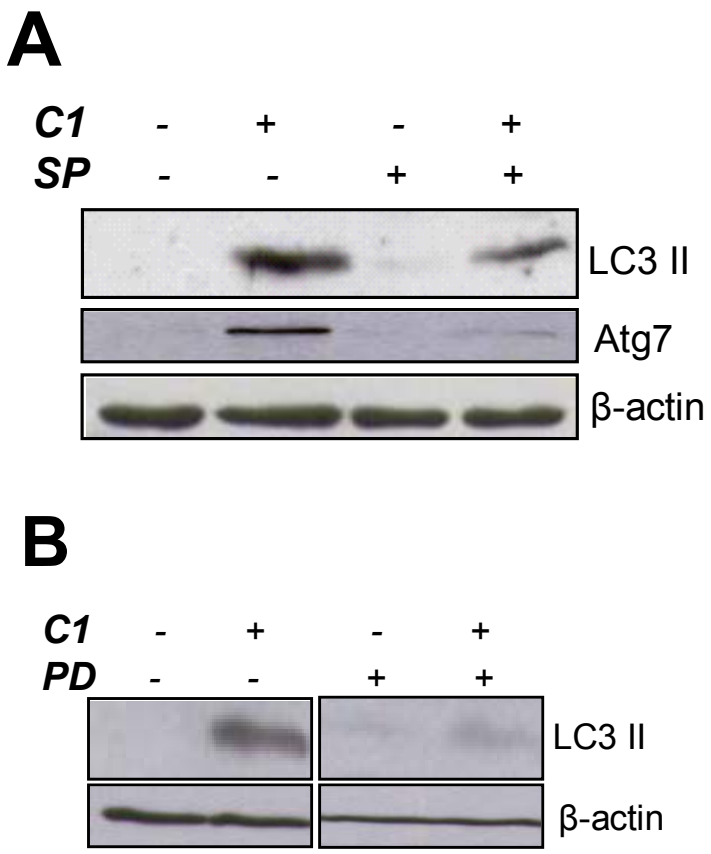


Figure 34: JNK and ERK inhibitors suppress LC3II accumulation induced by C1. HCT116 cells were pre-treated with (A) JNK inhibitor, SP600125 (SP; 5 μM) or (B) ERK inhibitor PD98059 (PD; 20 μM) for 1 hour before treatment with 100 μg/ml of C1 for 24hours. Subsequently total cell lysates from these cells were probed for LC3 and Atg7 expression level by immunoblotting.

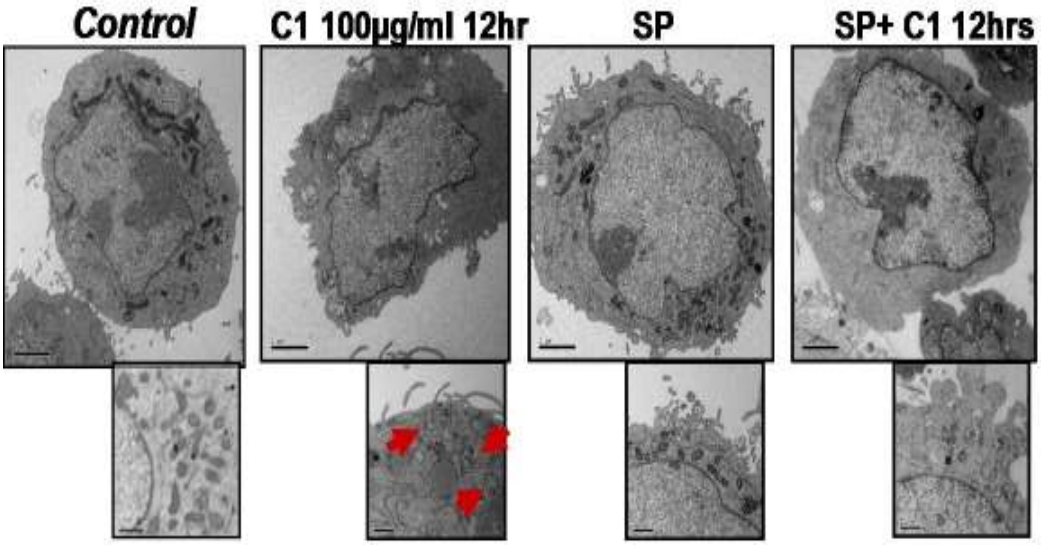


Figure 35: Pre-incubation of JNK inhibitor attenuates autophagosome formation in HCT116 cells. Cells were pre-incubated with SP600125 (SP; 5 µM for 1 hour) before exposure to C1 (100 µg/ml for 24 hours) and then were fixed and viewed by electron microscopy as described in Materials and Methods (Mag: 40,000X).

4.3 RNAi- mediated silencing of ERK inhibits PARP cleavage and LC3 lipidation in HCT116 cells

In view of the fact that pharmacological inhibition of a cellular target may not be the best experimental model to work with, it is integral to show inhibition of a certain pathway by way of RNAi-mediated gene silencing. In this regard, knockdown of ERK mRNA is not only able to corroborate the inhibitor studies; it will also provide greater insights into the molecular mechanisms of ERK-elicited cellular responses. In accordance to the inhibitor studies, silencing of both *ERK1* and *ERK2* genes proved to be effective in the suppression of PARP cleavage induced by C1 (Figure 36). These data highlight the essentiality of *ERK1* and *ERK2* genes in controlling the apoptotic cell death pathway. In addition, the effect of silencing ERK on LC3 lipidation was also investigated. The data suggested that *ERK2* silencing was able to abrogate LC3II accumulation, while silencing of *ERK1* had no appreciable effect on LC3II accumulation.

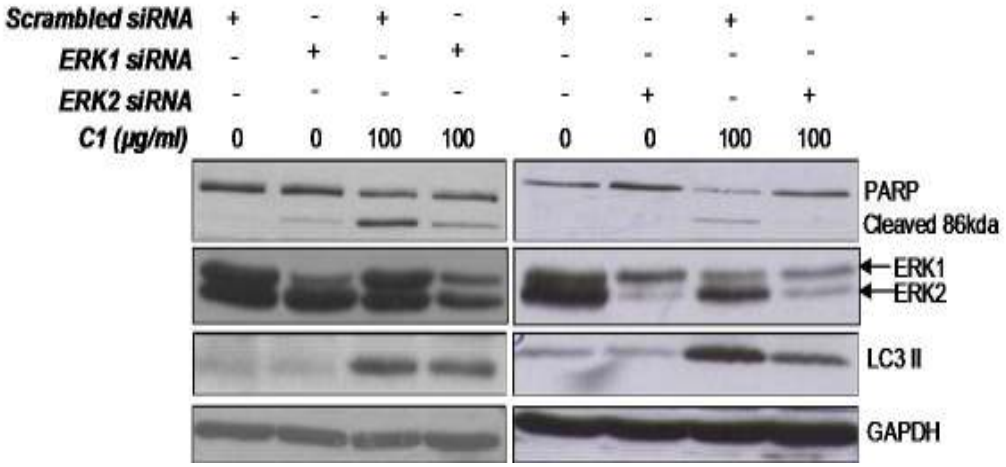


Figure 36: RNAi- mediated silencing of ERK inhibit PARP cleavage and LC3 lipidation in HCT116 cells. Cells were being silenced with ERK1, ERK2 or scrambled siRNA according to Materials & Methods, and allowed to recover for 48 hours post transfection. Cells were then subjected to C1 treatment for 18 hours before being harvested and analysed by western blot.

4.4 RNAi- mediated silencing of JNK also inhibit PARP cleavage and LC3 lipidation in HCT116 cells

Similarly, JNK silencing was also performed to further investigate its effect on apoptosis and autophagy. JNK silencing was carried out by using the DharmaFECT1 transfection reagent and C1 treatment (100 μg/ml for 18 hours) was administered 48 hours post-transfection. Successful knockdown of JNK was confirmed by expression level of total JNK protein (Figure 37). The findings suggest that silencing of *JNK* inhibited PARP cleavage, corroborating the effects of mitigating cell death via pharmacological inhibition of JNK (Figure 37). On the other hand, siJNK also rendered the cells more resistant to autophagy as assessed by LC3II accumulation (Figure 37).

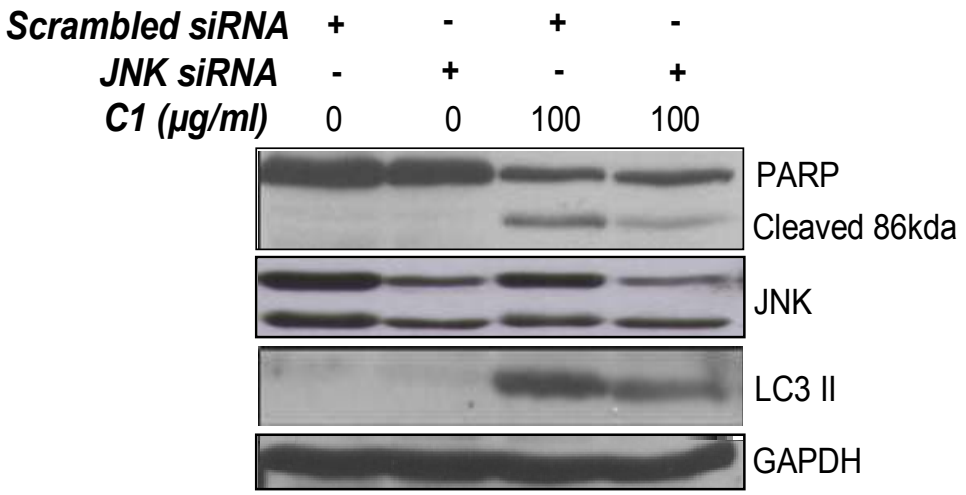


Figure 37: RNAi- mediated silencing of JNK inhibits PARP cleavage and LC3 lipidation in HCT116 cells. Cells were being silenced with *JNK* siRNA or scrambled siRNA according to Materials & Methods, and allowed to recover for 48 hours post transfection. Cells were then subjected to C1 treatment for 18 hours before being harvested and analysed by western blot by using PARP and LC3II specific antibodies. Expression of total JNK was probed to assess for successful silencing while GAPDH was used as loading control.

4.5 Inhibition of mRNA and protein synthesis attenuates LC3II accumulation

To provide further insights into the operational mechanisms on the autophagy pathway, the effect of transcriptional and translational inhibition was evaluated. To inhibit mRNA synthesis, actinomycin D was used to block the transcription of DNA. Actinomycin D is able to bind to DNA at the transcription initiation complex and thereby, preventing elongation of DNA strand by RNA polymerases (Sobell, 1985). Similarly, cycloheximide was utilized to block protein synthesis in the cells by way of inhibiting translational elongation. These inhibitors were pre-incubated in the cells for 1 hour to allow their inhibitory effect to take place prior to C1 addition for 18 hours. Results demonstrated that both actinomycin D and cycloheximide were able to suppress LC3II accumulation induced by C1 (Figure 38). This is suggestive of the involvement of protein synthesis in modulating an autophagic response in C1-treated cells. Furthermore, these data corroborated the earlier findings on the activation of a transcriptional pathway involving c-Jun activation.

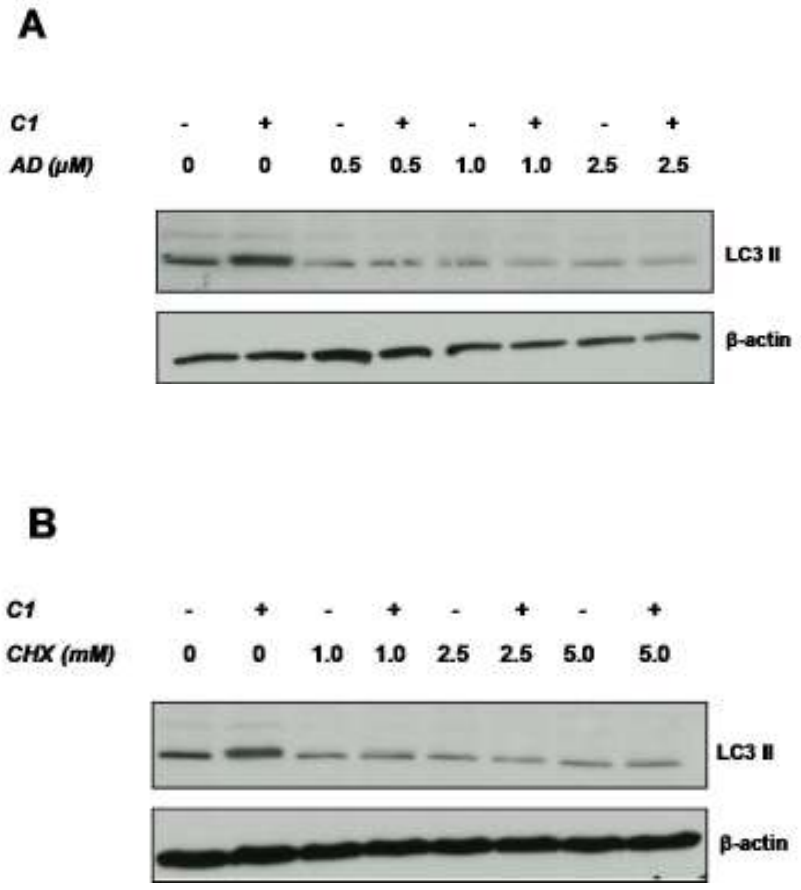


Figure 38: Inhibition of mRNA and protein synthesis attenuates LC3II accumulation. HCT116 cells were being treated with (A) mRNA synthesis inhibitor, actinomycin D (AD) or (B) protein synthesis inhibitor, cyclohexamide (CHX) for the indicated doses prior to C1 treatment at 100 μg/ml for 18 hours. Harvested cells were being assessed by Western blots for LC3II protein level.

4.6 C1 induces translocation of JNK to the nucleus

One of the major effects of JNK on cellular homeostasis is due to its ability to activate transcription factors. In accordance to this, c-Jun phosphorylation was detected in response to C1 treatment, implicating that JNK directly activates c-Jun. To test out whether JNK's ability in activating transcription factors is mediated by its translocation into the nucleus, subcellular distribution of JNK was being assessed by Western blot. In order to so, subcellular fractions of nuclear-rich extracts and cytosolic fractions were obtained by homogenization and differential centrifugation according to an established protocol, as described previously. As with most fractionation procedures, the purity of the individual fractions need to be stringently maintained to avoid contaminants carried over from different fractions. It is thus of utmost importance to optimize the existing standard protocols to better suit respective cell lines and model systems. In addition, the purity of the fractions was also assessed by using resident nuclear protein, PARP. Results showed that JNK's translocation from the cytosol to the nucleus was being detected in C1-treated cells, in a time dependent manner (Figure 39).

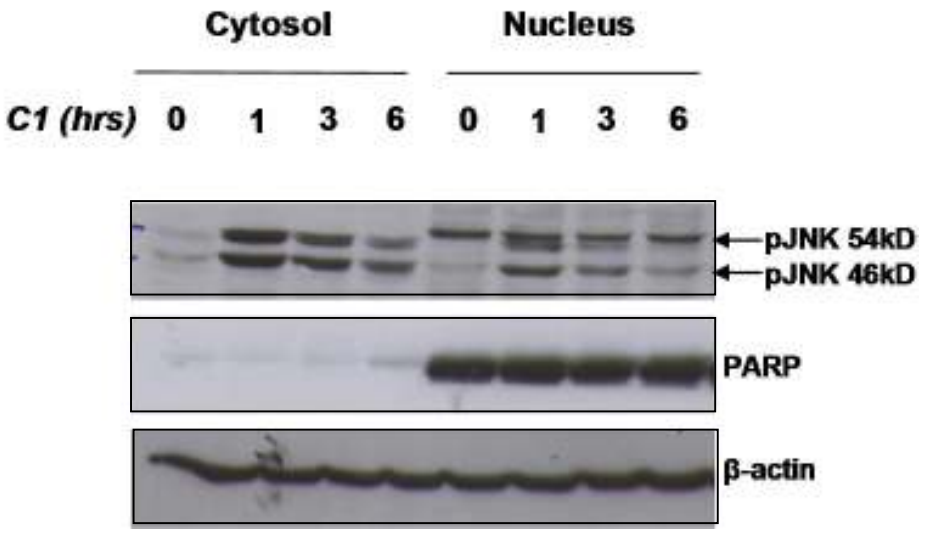


Figure 39: C1 induces translocation of JNK to the nucleus. HCT116 cells were treated with C1 (100 μg/ml) for various time points from 1 hour to 6 hours before being subjected to differential centrifugation and subcellular fractionation. The resultant cytosolic and nuclear fractions were assessed by Western blots to determine JNK's distribution in these compartments by using a specific antibody against phosphorylated JNK. Detection of PARP was performed as a purity control and β-actin was used as loading control.

4.7 PD98059 effectively inhibits cytochrome c and bax translocation in C1-treated cells

In the quest to explore the mechanisms of ERK-dependent apoptotic cell death, mitochondrial pathway of apoptosis was being investigated. A heavy membrane fraction, being enriched with mitochondria was being obtained following PD98059 pre-treatment and C1 exposure. This heavy membrane fraction, together with the cytosolic fraction were being analysed concurrently on Western blot to assess any changes in the subcellular distribution of bax and cytochrome c. It was observed that C1 induced efficient bax translocation from the cytosolic fraction to the mitochondria, with a reciprocal translocation of cytochrome c (Figure 40). More importantly, inhibition of ERK activity by PD98059 almost completely abolished the translocation of these apoptogenic factors. These data indicated that the effects of ERK on C1-elicited cell death may involve the intrinsic mitochondrial pathway.

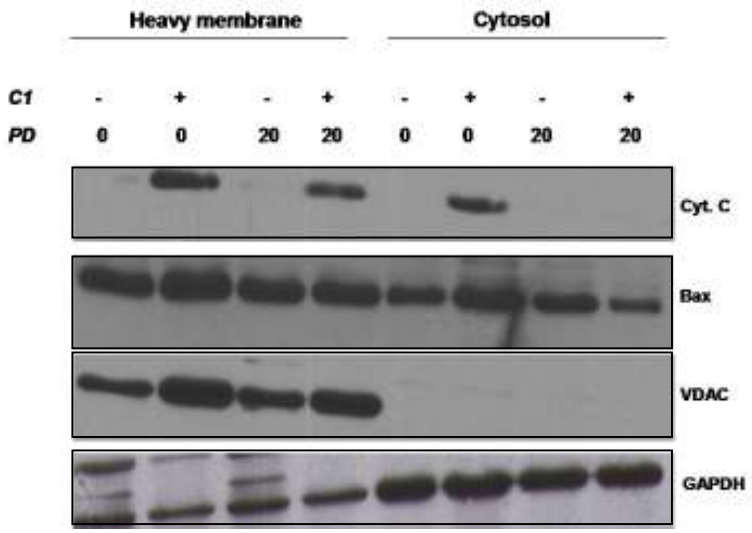
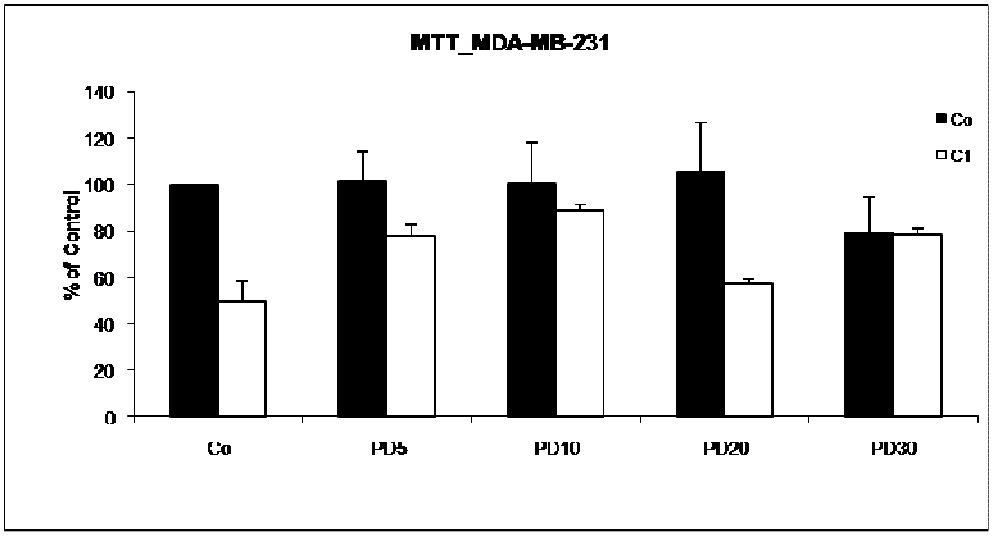


Figure 40: PD98059 effectively inhibits cytochrome c and bax translocation in C1-treated cells. PD98059 (PD, 20 μM) was added to HCT116 cells for an hour followed by C1 incubation for 18 hours. Cell lysates were being subjected to subcellular fractionation to obtain cytosolic fraction and heavy membrane fraction, which is enriched with mitochondria. These fractions were being used for Western blotting for the detection of cytochrome c and bax with their respective antibody as described in Materials & Methods. VDAC was used to assess the purity of the fractions, and GAPDH was used as loading control.

4.8 Pharmacological inhibition of ERK also rescued C1-induced reduction in cell viability in MDA-MB-231 cells, but not in MCF-7 cells

The major effects in inducing autophagy and apoptosis by C1 were mediated by ERK, and attenuation of ERK signaling effectively abrogated both signaling pathways. To confirm that the integral role of ERK in cell death is not limited to one specific cell line, two other breast cancer lines were being tested for the sensitivity to ERK inhibition by PD98059. In order to circumvent the probability of cell line variations, dose response analysis was carried out once again to select the effective dose for these breast cancer cells. In order to assess cell survival, PD98059 (5 μM, 10 μM, 20 μM) was pre-incubated for an hour before C1 incubation for 18 hours, followed by MTT assessment as per described in materials and methods. It was found that PD98059 effectively blocked cell death induced by C1 in MDA-MB-231 cells, while MCF-7 was completely refractory to PD98059 treatment (Figure 41). These results suggest that ERK inhibition could effectively inhibit cell death in tumor cells of different lineages; however, it raised an interesting question on the insensitivity of MCF-7 cells to the same treatment. An analysis of the three cell lines used in this study revealed an inherent difference of the Ras status. Interestingly, both HCT116 and MDA-MB-231 cells harbour oncogenic Ras mutation, while MCF-7 contains normal Ras gene. Moreover, the indication that PD98059 is only effective in systems with oncogenic Ras status could have direct implication in anticancer therapy.

A



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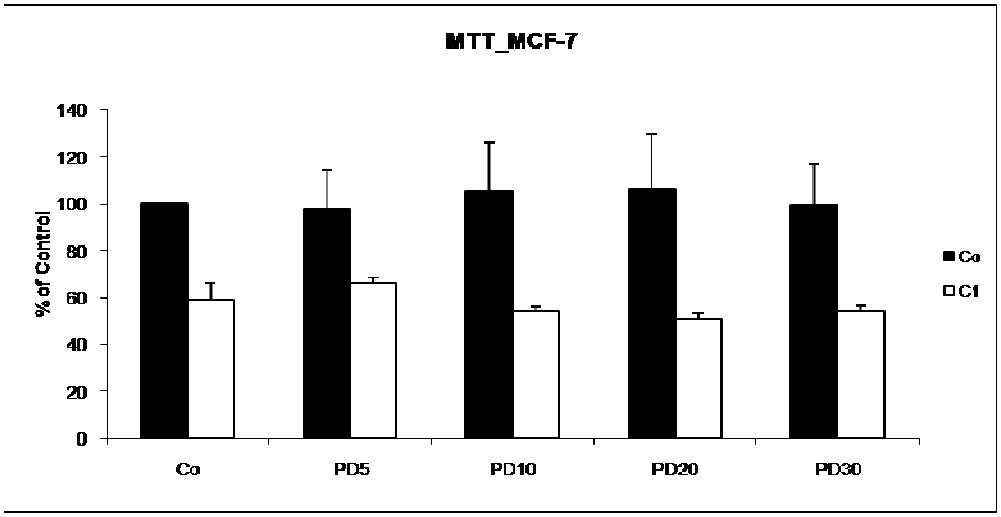

Figure 41: Pharmacological inhibition of ERK rescued C1-induced reduction in cell viability in MDA-MB-231 cells, but not in MCF-7 cells. (A) MDA-MB-231 and (B) MCF-7 breast cancer cells were pre-treated with various doses of PD98059 (PD; in μM) for an hour, before being exposed to C1 for 18 hours. Cell survival was then assessed by MTT assay.

4.9 A possible mechanism of ERK-mediated LC3 lipidation: Involvement of p53

4.9 C1 induces p53 downregulation in HCT116 cells

Since its discovery nearly 30 years ago, p53 has been unequivocally proven to be one of the most prominent tumor suppressors that are frequently mutated in human tumors. In order to assess the role of p53 in the current model, a protein expression profile of p53 was analysed following C1 treatment. It is intriguing to find out that p53 expression was reduced when the cells were exposed to C1, in a time-dependent as well as dose-dependent manner. Reduction in p53 protein expression was most prominent at late time points, ranging from 12 to 24 hours (Figure 42). As HCT116 harbours wild type p53 alleles, this result could indicate a dispensable role of p53 in terms of regulation of cell death and autophagy brought about by C1.

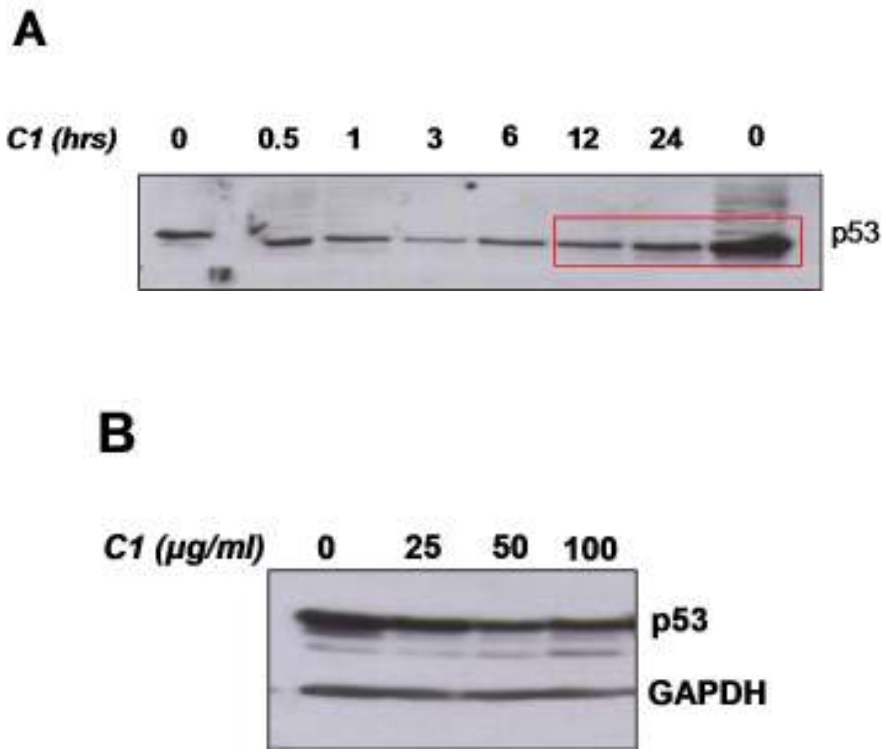

Figure 42: C1 induces p53 downregulation in HCT116 cells. p53 expression was verified by western blot following C1 treatment at various time points, (A) from 30 minutes to 24 hour at 100 µg/ml of C1 and (B) at various doses, from 25 µg/ml to 100 µg/ml.

4.10 Downregulation of p53 protein expression could be restored by pre-treatment of proteasomal inhibitor

Previously, depletion of p53 has been associated with the activation of murine double minute 2 (MDM2) , an E3 ubiquitin ligase which mediates p53 ubiquitination and subsequently, targeting p53 to the proteasomal degradation pathway. To verify that loss of p53 in C1-treated cells was attributed to proteasomal degradation, a specific proteasomal inhibitor, MG132, was utilized to investigate this. MG132 is a specific and cell-permeable inhibitor of proteasome, which could impair the degradation of ubiquitin-conjugated proteins. By pre-incubating the cells with two different doses of MG132 (1.25 μM, 2.5 μM) for 2 hours, loss of p53 expression induced by C1 was completely restored (Figure 43). This result indicated that downregulation of p53 protein level elicited by C1 was intricately linked to the proteasomal degradation pathway.

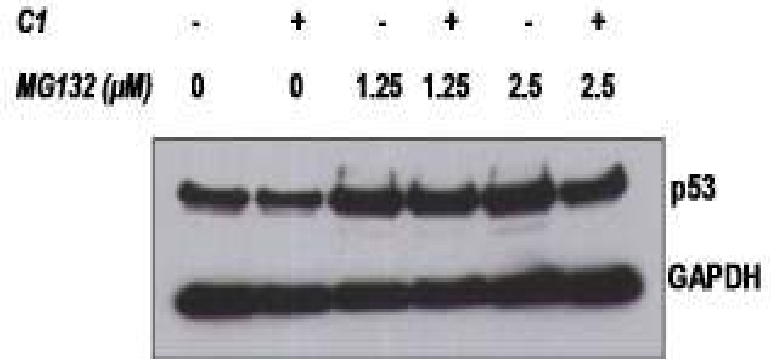

Figure 43: Downregulation of p53 protein expression can be restored by pre-treatment of proteasomal inhibitor. HCT116 cells were pre-incubated with the proteasomal inhibitor, MG132 (1.25 μM, 2.5μM) for 1 hour before being triggered with C1 (100 μg/ml) for 18 hours. Cell lysates were harvested by tripsinization before being subjected to SDS-PAGE and Western blotting. p53 expression level was assessed using an α-p53 antibody.

4.11 Inhibition of proteasomal degradation impedes LC3II accumulation induced by C1

Stimulated by the findings that proteasomal inhibition could affect p53 stabilization in the cells, it is interesting to know that whether this, in any way is linked to the autophagic induction in C1-treated cells. This is especially relevant according to a previous report highlighting a unique role brought about by p53 inhibition which promotes autophagy (Tasdemir et al., 2008). In the said study, depletion, deletion or inhibition of p53 serves as a signal for autophagy progression. More importantly, inducers of autophagy, such as nutrient deprivation and rapamycin invariably caused p53 depletion. It is thus likely that C1 may act in a similar manner as these autophagic inducing agents. Indeed, induction of LC3II accumulation by C1 was effectively blocked in cells pre-treated with MG132 (Figure 44). This data is negatively correlated with p53 status in the cells, and suggests correlative evidence between p53 depletion and autophagic induction.

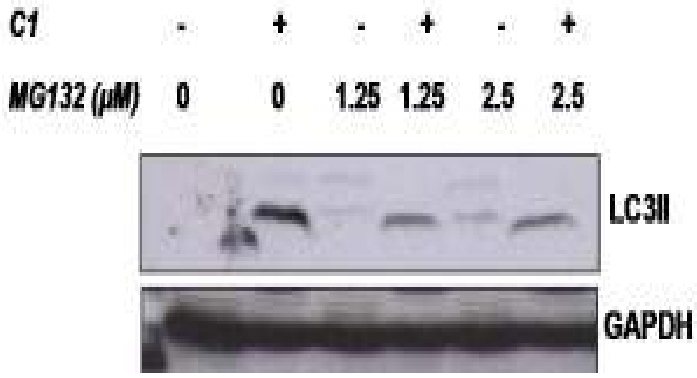

Figure 44: Inhibition of proteasomal degradation impedes LC3II accumulation induced by C1. Following MG132 pre-treatment for 1 hour at 1.25 μM or 2.5 μM, cells were treated with or without C1 (100 μg/ml) for 18 hours. Total lysates were collected for Western blot assessments, and LC3II protein expression was observed with α-LC3 antibody.

4.12 Decrease in p53 protein expression and increase in LC3 lipidation are controlled by ERK signaling

The Ras-Raf-MEK-ERK signaling pathway was shown to be a regulatory factor for p53-Hdm2 auto-regulatory feedback loop (Phelps et al., 2005). The MEK kinase, which directly acts upstream of ERK, was suggested to play a homeostatic role in maintaining the balance between p53 and human double minute 2 (Hdm2) by regulating the nuclear export of *hdm2* mRNA, thereby suppressing p53-induced cell cycle arrest and apoptosis (Phelps et al., 2005). This study provides evidence that ERK signaling pathway may regulate p53 level in a completely opposite way. siRNA targeted against ERK2 effectively restored downregulation of p53 brought about by C1 treatment at 18 hours (Figure 45). In contrast, LC3II expression was effectively inhibited following ERK2 silencing (Figure 45). As the depletion of total p53 level is important for autophagy progression, and ERK2 activation is a potent signal for autophagy, these data are suggestive of an integral role mediated by ERK-induced p53 downregulation in autophagy.

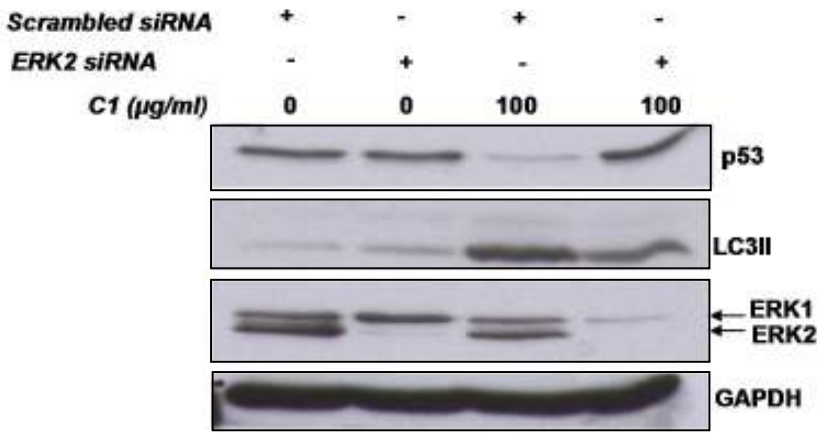

Figure 45: Decrease in p53 protein expression and increase in LC3 lipidation are controlled by ERK signaling. Cells were being silenced with ERK2 or scrambled siRNA according to Materials & Methods, and allowed to recover for 48 hours post transfection. Cells were then subjected to C1 treatment for 18 hours before being harvested and protein expression of p53, LC3II and ERK1/2 were analysed by Western blot. GAPDH level was used as loading control.

DISCUSSION

1 Cell death pathways activated by C1 in HCT116 cells

This is a report on a novel anti-cancer compound, C1, triggered cell death with hallmarks of apoptosis, and significantly inhibited clonogenic capacity of HCT116 cells. Intriguingly, pre-treatment of cells with the pan-caspase inhibitor, zVAD-fmk, failed to rescue colony formation, despite blocking apoptotic features. Similarly, the necrosis inhibitor, necrostatin, had no effect of C1-induced cell death, thereby suggesting the existence of alternative cell death mechanism(s) in conjunction with apoptosis. Electron microscopic and biochemical analysis revealed a morphological phenotype consistent with autophagy. Of note, neither the pharmacological inhibition of apoptosis (zVAD-fmk) nor autophagy (3-MA) was able to salvage cells from the effect of C1, suggesting that both pathways were essential in the regulation of cell death.

2 Autophagic signaling induced by C1 follows a non-canonical pathway

The tumor suppressor protein Beclin1 has been shown to play a critical role in autophagy execution and its knockdown blocks autophagic cell death (Yue et al., 2003). However, using a model of neurotoxin-induced cell death, Zhu *et al* showed that the existence of autophagic vacuolizations was independent of Beclin1 (Zhu et al., 2007). Similarly, in a breast cancer cell model, the phytoalexin resveratrol was found to induce non-canonical autophagy, which was dependent on inhibition of mTOR signaling (Scarlatti et al., 2008). The data presented here clearly indicate that

gene silencing of *Beclin1* neither inhibited autophagy nor rescued human cancer cells from C1-induced death. Although the functional relevance of autophagy as a cell survival response or a death execution mechanism is still being debated, recent evidence tends to favor the model whereby autophagy in the non-canonical settings is invariably associated with cell death (Scarlatti et al., 2008). Of note, Beclin1 expression was significantly lower in tumor cells undergoing C1-induced autophagy, which begs the question whether this is a general feature of non-canonical autophagy or an unrelated event exclusive to this system. In addition to the redundancy of Beclin1, the requirement of the class III PI3 kinase was also questionable as inhibition of its activity by 3-MA did not significantly rescue autophagic phenotype in C1-treated cells. This is also in line with the observations reported with resveratrol-induced autophagy (Scarlatti et al., 2008). Contrary to the effect of *beclin1* knockdown, silencing of *Ulk1* (homolog of yeast Atg 1) or *Atg7* impeded autophagic signaling, thus proving that these two proteins remained as essential mediators even in non-classical autophagy. However, it remains to be determined whether the multi-protein complex in the vesicle nucleation step is still being formed, regardless of the redundancy of Beclin1 in C1-induced autophagy.

3 Autophagy and apoptosis are independent of each other but controlled by upstream ROS production

In several settings, autophagic signaling sets the stage for apoptosis to occur, while in others inhibition of autophagy triggers an apoptotic cell death program (Boya et al., 2005; Gonzalez-Polo et al., 2005). It was shown here that C1-induced

cell death could be orchestrated through multiple signals that are independent of each other. Although apoptosis inhibitors effectively blocked caspase-dependent cell death, there was virtually no effect of these inhibitors on the autophagic pathway. Reciprocally, gene silencing of *Atg7* or *Atg1* reduced the extent of autophagic induction, but apoptotic signaling remained unaffected. While the downstream signaling for each pathway appears to be autonomous, the upstream trigger controlling the induction of each of these signals is an early increase in intracellular ROS production, which drives ERK and JNK activation.

4 Photoactivation as a cancer treatment modality

This study focused on the biological activity of a photoactivated product, Merodantoin, or Compound 1 (C1). Previously, photoactivation was used as a treatment modality for certain cancers (Hsi et al., 1999). Photoactivation is a therapy in which certain bacteria, viruses and cancer cells are targetted to photoactive compounds and at the same time, exposure to light (Oleinick and Evans, 1998). This treatment modality was termed photodynamic therapy (PDT). However, with time, PDT was proven to be a cumbersome process as it requires simultaneous exposure of the biological system to both photoactivated compound and light (Gulliya et al., 1990). Due to this limitation, PDT was only suitable for treatments of solid tumors and purging of bone marrow for transplantation (Hsi et al., 1999; Mulroney et al., 1994). To circumvent this problem, our group had devised a process to preactivate the compound by exposure to light before it was used as a therapeutic agent (Gulliya et al., 1990). In this way, the biological activity of the compound can be separated

from the photoactivation process. This method of activation of photodynamic compound was then termed 'preactivation' (Pervaiz et al., 1993).

To test out the concept of preactivation, a lipophilic polymethine dye merocyanine 540 (MC540) was used as a test compound. MC540 was initially discovered as a photosensitizer which showed high selectivity for tumor cells, especially useful in killing lymphoma and leukemic cells (Easton et al., 1978; Sieber, 1987). Preactivated MC540 (pMC540) was remarkably effective in targeting tumor cells, activating apoptosis in a variety of cancer cells while showing minimal toxicity in normal blood mononuclear cells (Gulliya and Pervaiz, 1989; Itoh et al., 1993; Sieber et al., 1987). *In vivo*, pMC540 was proven to be more effective than non-preactivated MC540 compounds in prolonging the lifespan of L1210 leukemic mice (Pervaiz, 2001).

Though preactivation solved the limitations of PDT, pMC540 had a short storage limit of 30 days and was prone to degradation (Moan and Berg, 1991). The activity of pMC540 was also reduced drastically by 50% upon light exposure. In view of the fact that pMC540 is not a pure compound but contains a mixture of photoactivated products, our group set out to purify and identify the components of the preactivated compounds. Three pure products, as analyzed by mass spectrometry and NMR analysis were being extracted. These compounds were termed C1, C2 and C5 (Pervaiz et al., 1999b).

Further analysis of these compounds revealed that C1 and C2 were potent activators of the classical apoptotic pathways in promyelocytic leukemia cell line HL-60 (Pervaiz et al., 1999b). Cell death induced by these purified products was

exemplified by phosphatidylserine exposure, caspase-8 and -3 activation and cytosolic translocation of cytochrome c. Interestingly, C1-induced cytochrome c release was dependent on MOMP while C2 treatment did not result in MOMP and mitochondrial swelling, suggesting that the release of cytochrome c by C1 and C2 were dependent on different mechanisms.

5 Intracellular generation of H_2O_2 as a novel effector mechanism of cell death

It is now well established that the redox status and changes in intracellular milleu are important determinants of cell fate. It was previously documented that the activity of the purified photoactivated compound, C2 is dependent on its ability to generate intracellular H_2O_2 (Hirpara et al., 2001). Intriguingly, H_2O_2 production induced by C2 originated from the mitochondria. More importantly, mitochondrial-derived H_2O_2 production subsequently resulted in intracellular acidification, which served as an effector mechanism for C2-induced apoptosis in HL-60 leukemic cells. In contrast, though C5 also induced cytochrome c release, it was unable to trigger cytosolic acidification and thus, elicited a marked reduced potency in caspase activation.

In search of a mechanistic explanation for cytosolic acidification, it was later discovered that H_2O_2 could act as an effector molecule in mediating Bax translocation in tumor cells (Ahmad et al., 2004b). By using either exogenous addition of H_2O_2 or drug-induced H_2O_2 production, H_2O_2-mediated cytosolic acidification was proven to be essential in the recruitment of mitochondrial pathway

in a variety of tumor cells (Ahmad et al., 2004b; Hirpara et al., 2001). Interestingly, in another study involving Resveratrol, the presence of low concentrations of Resveratrol reverted apoptotic hallmarks induced by anticancer drugs C2, vincristine or daunorubicin (Ahmad et al., 2004a). Low concentrations of Resveratrol resulted in elevation of intracellular $O_2^{\bullet -}$ dependent on the NADPH oxidase system, which blocked mitochondrial H_2O_2 production. Consequently, decreased intracellular H_2O_2 levels also inhibited cytosolic acidification thereby resulting in a non-permissive environment for apoptotic signaling.

In addition, the ability of small molecular compounds to generate intracellular H_2O_2 was shown to be an effector mechanism in the sensitization of tumor cells to death receptor-mediated apoptosis. LY294002, a phosphotidylinositol-3-kinase (PI3K) inhibitor, and its inactive analog, LY303511, were found to sensitize tumor cells to drug-induced apoptosis by an elevation of intracellular H_2O_2 level in cervical carcinoma HeLa (Poh and Pervaiz, 2005). The increase in intracellular H_2O_2 level induced by these two compounds is independent of PI3K activity. In a neuroblastoma model, LY303511 was also found to be capable in sensitizing tumor cells to TRAIL-induced apoptosis mediated by intracellular H_2O_2 generation (Shenoy et al., 2009). Notably, H_2O_2 elevation by the LY compound was an important signaling molecule in MAPK family members' activation, leading to upregulation of death receptors and enhanced cell death response.

Apart from the activation of cell death by targeting the executioners of the cell death circuitry, apoptosis could also be achieved by downregulation of survival factors. The involvement of H_2O_2 in this setting was elegantly shown by

downregulation of protein kinase 2 (CK2), a serine/threonine kinase involved in cell growth and proliferation (Ahmad et al., 2006; Wang et al., 2006b). Chemical inhibition or antisense-mediated knockdown of CK2 elevates H_2O_2 production which sensitized ALVA-41 and PC-3 prostate cancer cells to apoptosis (Wang et al., 2006a).

Collectively, these studies demonstrated a positive role for H_2O_2 in the modulation of cell death. Particularly, elevation of intracellular H_2O_2 level appears to be a common denominator in drug-induced apoptosis. The myriad ways of how H_2O_2 could mediate apoptotic induction may contribute to its potency as an upstream mediator of cell death.

6 Reactive oxygen species: A paradigm shift

As discussed previously in the Introduction section, the relationship between ROS and cell death is not always clear cut. Though it has been firmly established that high levels of ROS mediates cellular damage and apoptosis, in recent years there are emerging evidence to suggest a conflicting role of ROS with regard to oncogenesis.

The vast amount of evidence linking $O_2^{\bullet-}$ to growth and survival has led to a paradigm shift which involves a tight balance between $O_2^{\bullet-}$ and H_2O_2 in determining the cellular response (Pervaiz and Clement, 2002b). The ratio between H_2O_2 and $O_2^{\bullet-}$ is determined by the intracellular antioxidant defences and is maintained in a tightly regulated manner during normal homeostasis. A tilt of the balance to $O_2^{\bullet-}$ over H_2O_2 favors cell survival by promoting cell proliferation or inhibiting apoptosis, thereby facilitating the process of tumorigenesis (Clement et al., 1998). In contrast, a slight

elevation of H_2O_2 with a concomitant decrease in $O_2^{\bullet-}$ level is prohibitive for oncogenesis by promoting the execution of apoptotic signals (Pervaiz and Clement, 2002a; Pervaiz and Clement, 2002b). This is predominantly achieved via the effect of cytosolic acidification and suppression of $O_2^{\bullet-}$ accumulation. It is important to acknowledge that a slight tilt of the ratio between the two species is the key point of this theory; while in the case of overwhelming production of ROS, regardless of the species, will inadvertently be deleterious to the cells.

Currently, there is a huge body of evidence in support of the highly varied role of ROS in regulating cell metabolism. In particular, a mild elevation in $O_2^{\bullet-}$ level has been linked to cell proliferation and cell growth. This is mediated, in part, by the activity of $O_2^{\bullet-}$ in stimulating early growth-related genes such as *c-jun* and *c-fos* (Burdon, 1995). In addition, $O_2^{\bullet-}$ has also been linked to receptor tyrosine kinase (RTK) activation, which is a major proliferative signal involved in oncogenesis (Heffetz et al., 1990). Similarly, increase in $O_2^{\bullet-}$ was also found to be responsible for activation of transcription factors, such as AP-1 and NFκB, as well as in the activation of various ion channels such as Na^+/H^+ exchanger (NHE) membrane transport pumps (Droge, 2002; Sauer et al., 2001). The alterations in the intracellular $O_2^{\bullet-}$ level was invariably shown to lead to a mild "pro-oxidant" state in the cells, which is permissive for cell proliferation.

Interestingly, an increase in intracellular $O_2^{\bullet-}$ was shown to be critical in inducing the activity of Na+/H+ exchanger 1 (NHE1) gene promoter, leading to NHE1 expression, which strongly correlates to tumor cell's resistance to apoptosis (Akram et al., 2006). In a separate study, inhibition of $O_2^{\bullet-}$ production overrode the

protection conferred by Bcl-2 and sensitized Bcl-2 over-expressing cells to drug-induced apoptosis (Chen and Pervaiz, 2007). Along this line, Bcl-2 over-expression was able to generate a pro-oxidant state by constitutively increasing $O_2^{\bullet-}$ level in the cells leading to inhibition of cytosolic acidification. To further substantiate the role of $O_2^{\bullet-}$ in oncogenesis, constitutively active Rac-1 increased $O_2^{\bullet-}$ level and cytosolic pH, thereby conferring resistance of tumor cells to apoptosis (Pervaiz et al., 2001). Interestingly, treatment of cells with exogenous H_2O_2 concurrently led to a decrease in $O_2^{\bullet-}$ concentration accompanied by acidification of the intracellular milleu.

On the flip side, augmentation in intracellular $O_2^{\bullet-}$ level was also shown to be inhibitory for apoptosis. Over-expression of Cu/Zn SOD, which led to reduction of intracellular $O_2^{\bullet-}$ level, increased the sensitivity of tumor cells to chemotherapeutic drugs (Saito et al., 2003). The reverse experiment with SOD inhibitor, diethyldithiocarbamate (DDC), elevated $O_2^{\bullet-}$ concentration in the cells and reduced the extent of cell death induced by anti-cancer agents (Clement and Stamenkovic, 1996). Similarly, $O_2^{\bullet-}$ elevation in M14 melanoma cells inhibited drug-induced apoptosis by decreasing the activity of caspase-3 (Pervaiz et al., 1999a).

7 C1-induced ROS production is a signal for autophagy and apoptosis

The premise of this thesis centres on the ability of the small molecule compound, C1 in generating ROS as an intracellular messenger. In the current model, the outburst of ROS production occurred in a very early setting, within 15 minutes of incubation with C1. Utilizing a mitochondrial $O_2^{\bullet-}$ specific probe, $O_2^{\bullet-}$ was detected as one of the ROS species being produced. The burst of $O_2^{\bullet-}$ production increased

with time, which peaked at 1 hour time point and subsequently decreased over time. Similarly, ROS level was also significantly higher upon short time points of C1 incubation, from 15 minutes to 3 hours, as labeled by CM-DCFHDA and detected by flow cytometric analysis. Importantly, utilization of H_2O_2-specific scavengers, catalase and transient over-expression of the catalase gene confirmed the role of H_2O_2 in this model.

An interesting question which arises from these observations is the proportional contribution of these ROS species in the signaling of C1-activated pathways. In view of the fact that $O_2^{\bullet-}$ detected in this model originated from the mitochondria, it is highly unlikely that $O_2^{\bullet-}$, being a charged molecule, could efficiently translocate from the mitochondria to the cytosol and activate the downstream effectors, such as ERK and JNK, which are predominantly cytosolic proteins in their inactive form. $O_2^{\bullet-}$ generated from the mitochondrial electron transport chain is mainly released into the matrix, with a small proportion being released into the intermembrane space (Turrens, 2003). It is widely acknowledged that most of the $O_2^{\bullet-}$ being produced in the mitochondria undergoes dismutation reaction to H_2O_2 via the action of MnSOD (Halliwell B, 2007). Furthermore, the rapid decrease in $O_2^{\bullet-}$ level may suggest a heightened SOD activity in HCT116 cells. Therefore, it is most probable that H_2O_2 generated from $O_2^{\bullet-}$ dismutation was being translocated from the mitochondria to the cytosol. This is due to the fact that H_2O_2 could freely transverse membranes. That being said, it remained possible that the source of H_2O_2 produced by C1 is generated by other enzyme systems, such as xanthine oxidase and monoamine oxidase.

8 ROS is a critical signal for the activation of Map Kinases

This study highlighted the role of ROS by demonstrating its ability to activate two members of the MAP kinase family, JNK and ERK. Indeed, generation of ROS induced by C1 could be effectively scavenged by exogenous addition or transient overexpression of catalase, strongly suggesting an involvement of H_2O_2 in the current model. The identification of ROS production is an important component in this work mainly due to the fact that ROS signaling eventually resulted in the activation of MAPKs, specifically ERK and JNK kinases, which are central to C1-induced autophagy and apoptotic cell death.

Oxidative stress has been associated with MAPK through various mechanisms. Oxidative stress-induced ERK kinase signaling has been documented in a variety of cell lines including hepatocytes, cardiomyocytes, fibroblast, smooth muscles and epithelial cells (Blanc et al., 2003; Buder-Hoffmann et al., 2001; Conde de la Rosa et al., 2006; Kim et al., 2001; Xiao et al., 2002). In addition, the myriad ways of how ROS could lead to ERK activation provide substantial insights on the significance of this pathway. It appears that during most circumstances, ERK activation by free radicals is mediated by an upstream effector involved in ERK signaling pathway. For instance, ROS could enhance epidermal growth factor (EGF) signaling by inducing EGF receptor phosphorylation and downstream activation of the Ras-RAF-MEK-ERK signaling pathway (Knebel et al., 1996). In addition, platelet-derived growth factor (PDGF) receptor and SRC kinases are known targets of oxidative stress, and are both capable of activating ERK (Knebel et al., 1996; Zou

et al., 1996). Moreover, nitric oxide may lead to ERK activation by causing nitrosylation of a critical cysteine residue in Ras protein (Lander et al., 1996). Pharmacological inhibitors of MEK1 and MEK2, such as U0126 and PD98059 effectively inhibited ROS-induced ERK activation, rendering evidence that ROS acts via upstream mediators of ERK in inducing ERK activation (Lee et al., 2006; Lee et al., 2005). Interestingly, the data presented here coincide with these observations as pre-incubation of PD98059 completely abolished the extent of ERK phosphorylation induced by C1.

On the other hand, JNK activation was also shown to be mediated by various oxidants and ROS-inducing drugs including H_2O_2, arsenite trioxide, UV radiation and cadmium chloride (Conde de la Rosa et al., 2006; Dent et al., 2003; Leonard et al., 2004; Meier et al., 1996). Similar to ERK, a surge in JNK activity induced by ROS could be mediated by upstream modulators in the MAP kinase signaling pathway. Apoptosis signal-regulating kinase 1 (ASK1) is a MAPKKK which mediates the activation of JNK and p38 Map kinases (Ueda et al., 2002). ASK1 is in turn regulated by the binding of reduced thioredoxin during normal states which, upon oxidative stress, becomes oxidized and dissociated from ASK1 (Saitoh et al., 1998). The critical role of ASK1 in ROS-mediated apoptosis has been shown in both *in vitro* and *in vivo* settings (Yasinska et al., 2004). Mouse embryonic fibroblasts (MEFs) deficient in ASK1 activity was shown to be more resistant to oxidative stress-induced apoptosis while exhibiting a significantly lower level of JNK activation as compared to wild type MEFs (Matsukawa et al., 2004; Matsuzawa et al., 2002). One of the most potent activator of ASK1 is H_2O_2, and H_2O_2 production is

the mechanism of activation for various anti-cancer drugs in the regulation of ASK1-mediated cell death (Goldman et al., 2004; Machino et al., 2003; Tobiume et al., 2001). In this study, though the upstream mediator involved ROS-induced JNK activation is not known, ASK1 remained a highly probable target of ROS. Moreover, H_2O_2 was identified as one of the major species responsible for C1-induced cell death. ASK1 could represent a possible link between ROS and JNK activation, leading to downstream cellular responses.

To conclusively delineate the pathways involved in the anti-tumor activity of C1, ROS scavengers and transient over-expression of catalase were utilized in this study. Robust activation of ERK and JNK were dramatically reduced following inhibition of ROS production, indicating that ROS is the upstream mediator of MAPKs activation in this system.

Previously, various studies have demonstrated the role of inactivating phosphatases in the regulation of Map kinases. $OH^{\bullet}$ could lead to attenuation of the MKP activity, thereby resulting in a surge in ERK activity (Whisler et al., 1995). In another model involving TNF-α- induced cell death, MKPs oxidation mediated by H_2O_2 was identified as the main mechanism of JNK-mediated cell death (Kamata et al., 2005). While the treatment of HCT116 cells by C1 also led to a reduction in MKP1 protein level, it remained to be investigated whether oxidation of MKPs is indeed responsible for ERK and JNK activation brought about by C1.

9 The role of ROS in modulating cell death: Involvement of ERK and JNK

The earlier part of the Discussion section had illustrated the myriad ways of utilizing ROS as an effector mechanism in inducing MAPKs activation. While the connection between ROS and MAPKs activation had been extensively studied, more importantly the physiological outcomes of their interaction need to be deciphered.

The effects of ROS production in carcinogenesis are manifested in many ways, depending on the stimulus and the rate of ROS production. ROS could play a stimulatory role in oncogenesis, and this is mainly propagated through ROS-stimulation of the Ras-Raf-Mek-ERK pathway (Jiang et al., 2005). One of the downstream targets of this pathway is the transcription factor promoter specificity protein 1 (Sp1), which has been shown to be phosphorylated by ERK (Yagoda et al., 2007). Following its activation, Sp1 mediates the upregulation of vascular endothelial growth factor (VEGF) which, in turn, contributes to angiogenesis (Banan et al., 2001). In addition to this, the Ras signaling pathway also contributes to oncogenesis by activation of the PI3 kinase- Akt axis, the major survival factors in the cells (Shelton et al., 2004; von Gise et al., 2001).

In contrast to the widely known cancer promoting effects in oxidative stress-induced Ras-ERK signaling pathway, a tumor suppression role of ROS has also been reported. In these settings it is generally believed that the anti-tumor activity of ROS is attributed to its ability to engage JNK and p38 signaling pathways with the eventual execution of apoptosis. The p38 Map kinase pathway negatively regulates tumorigenesis by its activating role in replicative senescence, contact inhibition, DNA damage responses and apoptosis (Han and Sun, 2007; Ren et al., 2009). In the

current study, the presence of p38 is ruled out as its activation was not significantly observed in C1-treated cells. In contrast, ROS-induced JNK activation turned out to be an important signaling mechanism in the modulation of cell death. In addition, abrogation of ROS production by exogenous ROS scavengers and catalase over-expression protected HCT116 cells against C1-induced cell death. A large body of evidence in the literature is in line with this finding. In a study utilizing pancreatic beta cells, synergistic activation of JNK was triggered by TNF-α and interferon gamma (IFN-gamma), and apoptosis induced in this system is dependent on JNK-mediated p53 activation (Kim et al., 2005a). In exploring mechanisms of ROS accumulation-induced cell death, the apoptotic response was further amplified by c-FLIP downregulation, accompanied by prolonged JNK activation (Nakajima et al., 2008). H_2O_2- induced non-apoptotic cell death was mediated by JNK1 via its regulation on PARP-1 activation and phosphorylation (Zhang et al., 2007).

Evidence presented here suggested that ROS-mediated JNK activation is in line with the current literature in its ability to induce apoptosis. On the other hand, although ROS-mediated ERK activation has often been linked to transformation and malignancy, the data in this study clearly showed otherwise. This study demonstrated a crucial role of ROS in modulating the ERK-mediated cell death pathway. Activation of ERK demonstrated an anti-tumor role of ROS which positively regulates cell death.

10 ROS controls autophagic signals through MAPK

Classically, autophagy is described as a cellular clearance mechanism to remove damaged organelles and protein aggregates and thus serves as a cytoprotective mechanism to counteract oxidative stress in cells (Levine, 2005). In yeast, mitophagy occurs as a response to nitrogen starvation and is mediated through a regulator of oxidative stress, *Uth1* gene (Kissova et al., 2004). On the other hand, reports have also shown that ROS could serve as signaling molecules that directly or indirectly activate autophagy. To that end, it has been shown that induction of autophagy resulted in selective degradation of catalase, leading to accumulation of mitochondrial ROS and ultimately cell death (Yu et al., 2006). In a separate study, TNF-α was shown to increase the expression of Beclin1 by a ROS-dependent mechanism (Djavaheri-Mergny et al., 2006). By using mitochondrial electron transport chain inhibitors, Chen *et al* demonstrated the importance of mitochondrial ROS in the regulation of autophagic cell death. The existence of autophagic cell death in transformed and cancer cell lines was salvaged by the presence of tiron and over-expression of *SOD2*, indicating a role of $O_2^{\bullet-}$ to act as a second messenger in autophagic signaling (Chen et al., 2007). The findings reported here lend support to these observations by demonstrating the involvement of mitochondrial-derived ROS in C1-induced autophagy, as well as the inhibitory effect of H_2O_2 scavenger, catalase. To provide a link between C1-induced ROS production and autophagy, its ability to activate two members of the MAP kinase family, JNK and ERK, was highlighted. Indeed, generation of ROS induced by C1 could be effectively scavenged by exogenous addition or transient overexpression of catalase, strongly pointing to the involvement of H_2O_2 in the current model. Furthermore, LC3II accumulation could

also be abolished through the exogenous addition of or transient transfection with human catalase, suggesting a major signaling role of H_2O_2 in autophagy. This is consistent with a previous report on TNF-α induced accumulation of H_2O_2, which was shown to be responsible for autophagic cell death (Djavaheri-Mergny et al., 2006). Indeed, H_2O_2 was also found to be an important mediator in starvation-induced autophagy, through its activity in regulating Atg4 protease (Scherz-Shouval et al., 2007). Post-translational modification of Atg4 by oxidation greatly enhanced autophagosome formation in starvation-induced autophagy (Scherz-Shouval et al., 2007). H_2O_2 and ROS-inducing agents, 2-methoxyestradiol (2-ME) was shown to trigger autophagic cell death in transformed and cancer cell lines, while it failed to induce autophagy in non-transformed cells (Chen et al., 2008). Therefore, it appears that redox-regulation of autophagy is largely dependent on the magnitude and the rate of ROS accumulation. Whether ROS involvement in autophagy represents a general mechanism in autophagic induction remains to be investigated. Nevertheless, this study highlighted the novel role of ROS in mediating MAPK activation, specifically in the signaling of ERK and JNK kinase pathways in the modulation of autophagy.

11 ERK is a major mediator in C1-induced signaling pathways

This study reported the importance of the ERK signaling cascade in mediating C1-induced autophagy and apoptosis. Of note, ERK activation occurred in a transient manner. It was activated rapidly within a short span (30 minutes) of C1 incubation, and the extent of ERK phosphorylation increased with time, peaked at 3

hours before it was gradually reduced. Despite the fact that ERK phosphorylation did not occur in a sustained and prolonged manner, ERK activation was able to exert important biological functions in the modulation of two critical physiological processes. It would be interesting to investigate further on the relationship between this mode of transient ERK activation and the biological impacts it transmits, if there is any. Furthermore, the ability of the MEK inhibitor, PD98059 to inhibit phosphorylation of ERK revealed that ERK activation by C1 was dependent on the sequential activation of the ERK signaling module, and suggested that ERK was not a direct target of ROS in this system. In the next section, the role played by ERK in controlling two different axes of cellular modalities, namely autophagy and apoptosis, will be discussed in detail.

12 Modulation of apoptosis by ERK signaling

A general dogma surrounding the MAPK signaling cascade entails that ERK activation is associated with cell survival, whereas JNK and p38 control cell death. This dichotomy arises from the large body of data linking ERK activation to Ras-induced transformation, cell proliferation and cell cycle progression (Dhillon et al., 2007). This is no doubt an oversimplification on the roles of MAPKs, as the cell fate upon extracellular stimulus often depends on cell type and the specific cellular context.

With regard to the activation of cell death by C1, ERK activation was found to be indispensable. Inhibition of ERK phosphorylation by PD98059 virtually completely blocked various hallmarks of apoptosis. Furthermore, pharmacological

inhibition of ERK also impaired cytochrome c and Bax translocation, suggesting that the mitochondrial pathway could be a major target of ERK activation. More importantly, PD98059 treatment conferred significant protection against C1-induced reduction of colony formation, which underscored the tremendous implication for ERK as a novel target for anti-tumor therapy. Notably, gene-mediated silencing of *ERK* further corroborated the inhibitor studies, and highlighted the critical involvement of both *ERK1* and *ERK2* in the modulation of apoptosis.

In addition to HCT116 cells, the metastatic breast cancer cell line MDA-MB-231 was also sensitive to inhibition of ERK. Serendipitously, both HCT116 and MDA-MB-231 cells harbour oncogenic K-Ras mutation. In contrast, MCF-7, which is a non-metastatic breast cancer cell line with normal Ras status, is refractory to PD98059 treatment. These results suggested a possible link between Ras status and sensitivity of cells to ERK activation. Furthermore, activating mutation of K-Ras is associated with 30% of human cancers, including colorectal carcinomas (Andreyev et al., 2001; Schubbert et al., 2007). It is thus promising that ERK activation by ROS-dependent mechanisms in these Ras oncogene- transformed cells could specifically target these tumors to self destruction by activating their intrinsic programmed cell death.

Interestingly, activation of cell death by C1 is associated with a transient activation profile of ERK. On the other hand, oncogenic transformation and accelerated proliferation induced by ERK has been shown to be dependent on a constitutively activated signal of ERK (Oka et al., 1995). This is partly mediated through the promoting effects of ERK on cell cycle progression (Pages et al., 1993).

It remains a possible hypothesis that the transient activation of ERK induced by C1 precludes the possibility of prolonged oncogenic induction, but is sufficient to activate cell death machineries for efficient apoptotic execution. Nevertheless, the feasibility of this proposal would need to be substantiated with more conclusive experimental evidences.

13 Modulation of autophagy by ERK signaling

Although the advancement in autophagy research has accelerated in recent years, the involvement of ERK in this field has not been studied in great depths. In particular, the mechanisms of ERK involvement in autophagy have not been extensively studied. Most of the studies in the current literature have pointed to a positive regulatory role of ERK in autophagy, though there are other studies which contradict this role. In a neuroblastoma model, ERK 1/2 activation was reported to be critical in neurotoxin-induced autophagic cell death (Zhu et al., 2007). The activity of ERK and JNK were also found to be important in TNF-α induced apoptotic and autophagic cell death in L929 cells (Cheng et al., 2008). Curcumin, a natural compound, induces autophagic cell death in an ERK-dependent manner (Aoki et al., 2007). These studies have established a causal relationship between ERK activation and autophagy. However, in contrast to the interplay between JNK and autophagy, the exact role of ERK in autophagy has not been clearly defined. In this study, the association of ERK in autophagy was firmly established. Pharmacological inhibition of ERK almost completely abrogated the autophagic response triggered by C1, as determined by LC3II accumulation and EM analysis, implicating ERK in the

induction of autophagy. Genetic silencing of *ERK* also corroborated the inhibitor studies, further confirming a positive regulatory role of ERK in the induction of autophagy. Serendipitously, only *ERK2* silencing could abrogate C1-induced LC3 expression, excluding the role of *ERK1* in controlling autophagy. This specificity in controlling autophagy indicated that, at least with regard to C1-induced autophagy, ERK1 and ERK2 are not exactly functionally redundant. This is consistent with a recent study on the mechanism of ERK2 localization to the mitochondria which was indispensable for mitophagy and autophagic cell death (Dagda et al., 2008). To date, it is as yet unclear as to whether the association of ERK activation with autophagy is a function of its ability to activate downstream transcription factors or an extra-nuclear function of ERK. This study provided new insights into the molecular mechanisms of ERK-induced autophagy by identifying an association between ERK and p53. Previously, degradation of p53 was linked to an increased autophagic response, and p53 was suggested be a repressor of autophagy (Tasdemir et al., 2008). Here, a more upstream mechanism was identified in the heightened interest of p53-mediated suppression of autophagy. C1 activation mediated a decrease in p53 protein expression, indicating redundancy of p53 in C1-induced cell death. Silencing of *ERK2*, which inhibited autophagy, also attenuated p53 downregulation. In addition, p53 level was restored by inhibition of proteasomal degradation. Similarly, proteasome inhibitors also decreased the extent of autophagy in C1-treated cells. Taken together, these data suggested that the activation of autophagy by C1 was in part, due to ERK activation and subsequent ERK-mediated p53 degradation by the proteosomal degradation pathway.

14 Targeting the ERK signaling pathway for cancer therapy

Over the years, the ERK signaling cascade has gained prominent interest as attractive targets for cancer therapy, due to the critical involvement of its components in the regulation of cell proliferation and tumor progression. Virtually all, if not most of the key components of the ERK MAPK pathway have been found to be either overexpressed in human cancers or have been identified as putative oncogenes. Receptor tyrosine kinases (RTK), which includes members such as epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), is one of the most upstream signal which activates the Ras-Raf-MEK-ERK signaling module. EGFR was found to be mutationally activated in many tumors (Baselga and Arteaga, 2005), and developments of monoclonal antibodies and small molecule inhibitors against aberrant hyperactivated EGFR are currently underway (Roberts and Der, 2007), with some of them already in clinical use for the treatment of EGFR-detectable cancers.

Downstream of the receptor tyrosine kinase pathway, aberrant Ras signaling has also been detected in multiple malignancies. Ras has been proposed to be mutated in 30% of human cancers, with high prevalence in lung and colon cancers (Malumbres and Barbacid, 2003). In addition, germline mutations and genetic alterations of Raf have also lend credence to the efforts devoted into the inhibition of this particular pathway (Davies et al., 2002).

The findings generated from this study, however, cautioned against an over-simplified approach in devising ERK signaling pathway as target-based therapies.

This study demonstrates that instead of propagating cell death, MEK inhibitors could be potent death suppressors. This observation suggests a novel feature of ERK away from its conventional dogma of anti-death and pro-survival roles. In particular, evidence presented here supports the notion that ROS-mediated ERK signaling could represent an attractive chemotherapeutic approach for cancer elimination, especially relevant to highly metastatic cancers harbouring oncogenic Ras mutations. Nevertheless, this study adds complexities to the seemingly straightforward properties of ERK-related therapies in cancer and suggests that any target based therapies should be carefully examined before its procession.

15 Kinetics of JNK activation could determine the sensitivity of tumor cells to apoptosis

JNK activity alone may not be sufficient to determine the cell death response. It has been suggested that kinetics of JNK activation play a role in determining its biological function. Transient and modest JNK activation is required for liver regeneration (Schwabe et al., 2003), while sustained JNK activation caused by cytotoxic drugs or cellular insults has been shown to trigger apoptosis (Lin, 2003). Inhibition of dual specificity phosphatases, the MKPs, via oxidation of the critical cysteine residues by ROS, resulted in sustained JNK activation which was responsible for apoptotic cell death (Kamata et al., 2005). In contrast, NFκB could prevent sustained JNK activation via upregulation of ferritin heavy chain, a primary iron storage factor in the cells, leading to attenuated apoptosis (Pham et al., 2004). The data presented here is in agreement with recent reports highlighting the

importance of temporal activation of JNK in inducing apoptosis. In this study, sustained JNK activation was demonstrated following C1 incubation. A time kinetics analysis revealed that, although JNK phosphorylation was triggered almost immediately, within 30 minutes of drug treatment, the activation profile persisted throughout the time course of analysis. Correspondingly, MKP1 protein level was significantly downregulated at the time points when JNK phosphorylation was detected. It is hitherto unclear as to how different ROS levels could augment the extent and duration of JNK activation. Possibly, low levels of ROS production did not alter the MKP activity sufficiently resulting in transient JNK activation. In contrast, higher levels of ROS or a specific type of ROS is more efficient in mediating inhibition of MKP activity, leading to sustained JNK activation and apoptosis (McCubrey et al., 2006). In a separate study, c-FLIP downregulation was proposed as the mechanism responsible for prolonged JNK activation (Nakajima et al., 2008). This observation was also consistent with cleavage of MEKK1 in c-FLIP knockdown cells, providing an explanatory mechanism for prolonged JNK activation.

16 Modulation of Apoptosis by JNK signaling

The involvement of MAPKs in apoptosis have been demonstrated in a variety of model systems including cytokines activation, oxidative stress and radiation (Bode and Dong, 2003; Kyriakis and Avruch, 2001; Rincon et al., 2001). Apart from transcriptional activation of c-Jun leading to induction of apoptotic genes such as Fas-L (Faris et al., 1998), a transcriptional independent role of JNK in apoptosis has

also been documented. This is largely documented via phosphorylation of pro or anti-apoptotic proteins such as p53 and Bcl-2 (Maundrell et al., 1997; Milne et al., 1995). In this study, inhibition of JNK activation also attenuates apoptotic cell death. This was shown by inhibition of sub-G1 population, colony formation and cell death by a JNK inhibitor as well as by siRNA directed against JNK. Thus, we provide evidence that JNK represents an important mediator in controlling the apoptotic signaling cascade. Interestingly, it has been shown that JNK phosphorylation of Bcl-2 serves to promote autophagy and cell survival during early time points while delayed activation of JNK is a signal for apoptosis to occur (Wei et al., 2008b). In accordance with these findings, our findings demonstrate sustained and prolonged JNK activation, which may account for the differential cellular response brought about by C1. It is likely that early JNK activation signals for autophagy as an adaptive response to cellular stress while at the later time point, when the cells are overwhelmed by cytotoxic stimuli, JNK activation serves to switch the cells towards apoptotic cell death.

17 Modulation of autophagy by JNK signaling

In this thesis the integral role of the Map kinases, ERK and JNK in mediating autophagy was highlighted. Activation of the JNK signaling pathway by ROS represents a novel mechanism of autophagic induction. Inhibition of JNK by its pharmacological inhibitor, SP600125, resulted in the reduction of LC3II protein expression. By gene-mediated knock-down experiments, JNK was found to be crucial for the regulation of C1-induced autophagy and apoptosis. Previously, JNK1

activity was demonstrated to be the key factor in starvation-induced autophagy, through phosphorylation of Bcl-2 and displacement of the Beclin1-Bcl-2 complex (Wei et al., 2008a). In contrast, this study did not implicate JNK1 in both autophagy and apoptosis, adding evidence to the JNK1/2 isoform specific roles in mediating cellular response (Bogoyevitch, 2006). It is hitherto unclear as to the cause of this disparity brought about by different JNK isoforms; however, it is apparent that the mode of actions and the effect of autophagic induction mediated by the different JNK isoforms were in stark contrast. To begin with, the extent of autophagic induction via drug-induced autophagy and starvation mediated autophagy may not be of the same magnitude. In addition, induction of c-Jun phosphorylation was found to be dependent on JNK activity. Moreover, pre-treatment of cells with cyclohexamide and actinomycin D reverted the increase in LC3II accumulation in C1-treated cells, indicating that the increased accumulation of LC3II might involve protein synthesis. A novel mechanism for JNK in autophagy was demonstrated in this study by showing that JNK inhibition could effectively augment Atg7 expression. This implies that autophagy-inducing activity of JNK was due to induction of the crucial mediator in autophagosome formation. Collectively, these data indicated that the regulation of autophagy by JNK could implicate the transcriptional activity of c-Jun.

JNK has been implicated in various models of autophagy in response to serum starvation, cytokines, growth factor withdrawal and neurotoxic drugs (Borsello et al., 2003; Jia et al., 2006; Li et al., 2006; Wei et al., 2008a). The myriad ways of JNK induction in autophagy may indicate that JNK is a core component in the autophagic signaling pathway.

CONCLUSION

This is a novel report describing the ability of a small molecule compound to simultaneously activate autophagic as well as apoptotic signaling pathways in the same cell, and where autophagy appears to be fueling rather than abetting death signaling. Interestingly, the fact that this small molecule compound functions in a manner independent of Beclin1, further testifies to its therapeutic potential in the clinical settings where the tumor suppressor *Beclin1* is non functional or down-regulated. In contrast, the involvement of two critical Atg genes, *Atg7* and *Ulk-1* was integral in C1-induced autophagy. This unique cell death odyssey of tumor cells, i.e., simultaneous autophagy and apoptosis, is not restricted to a particular cell type as evidenced by the induction of autophagy in a variety of tumor cell types. Furthermore, our work highlights the critical involvement of early ROS production and downstream ERK and JNK activation in the dual signaling triggered by C1. ROS represents a central initiator of the C1-elicited cellular signaling pathway, which kick-starts the autophagy and apoptosis process by simultaneously activating two critical members of the Map kinase family, ERK and JNK. A novel function of JNK in autophagy was linked to its involvement in the regulation of Atg7 expression. Similarly, ERK may constitute a major initiator of autophagy by mediating p53 degradation, though further studies are required to delineate the role of ERK in autophagy. While JNK inhibition appears to be sufficient to suppress the death signals, ERK was found to be a greater factor in the determinacy of life and death. This is most intriguing in view of the fact that the ERK signaling pathway has been

largely associated with oncogenesis and cellular transformation. Suppression of ERK activity almost completely abolished cell death hallmarks, suggesting that ERK is a major pathway which integrates the signals transmitted by ROS. The data also implicate ERK in mediating the intrinsic mitochondrial pathway which could further amplify the death signals. The potency of ERK as a cell death mediator is not limited to one cell lineage and, more importantly, ERK was shown to be critical in cell lineages with active Ras mutation. These data portrayed a novel role of ERK in the modulation of cell fate. While ERK activation normally contributes to proliferation and malignancy, on the flip side ROS-mediated ERK stimulation could specifically target oncogenic Ras-associated tumor cells, placing ERK as an attractive target for circumventing highly malignant cancers. In addition, this study underscores the feasibility of ERK and JNK as novel targets of cancer therapy. Taken together, these data underscore the tremendous potential of this small molecule compound for enhancing our understanding of the intricate complexities between different networks of cell death, as well as for the therapeutic induction of cell death in tumors that are responsive to autophagic and/or apoptosis stimuli.

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APPENDIX

LIST OF PUBLICATION

Wong C.H., Iskandar K., Yadav S.K., Pervaiz, S. Simultaneous Induction of Autophagy and Apoptotic Cell Death in Cancer Cells by ROS-dependent ERK and JNK activation. (Manuscript under revision)

CONFERENCE PAPERS

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