Novel small molecule modulators of autophagy in yeast and mammalian systems

A thesis submitted for partial fulfilment of degree of

MASTER OF SCIENCE

as part of Integrated PhD program

(Biological Sciences)

by

Somya Bats

Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

Jakkur, Bangalore

560064.

March 2015

DECLARATION

I hereby declare that the work described in this thesis entitled '**Novel small molecule modulators of autophagy in yeast and mammalian systems'** is the result of investigations carried out by myself under the guidance of **Dr. Ravi Manjithaya** at Autophagy Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India. This work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described has been based on findings of other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

March, 2015 Somya Bats

Jawaharlal Nehru Centre for Advanced Scientific Research
Jakkur Campus, Jakkur Post
Bengaluru 560 064, INDIA
Office Tel: +91 (80) 2208 2924
Office Fax: +91 (80) 2208 2766
+91 (80) 2208 2767
Email: ravim@jncasr.ac.in

Ravi Manjithaya PhD
Faculty Fellow
Molecular Biology and Genetics Unit

CERTIFICATE

This is to certify that the work described in this thesis entitled **'Novel small molecule modulators of autophagy in yeast and mammalian systems'** is the result of investigations carried out by Ms. Somya Bats at Autophagy laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, under my supervision.

March, 2015 Dr. Ravi Manjithaya

Acknowledgements

I hereby express my heartfelt gratitude to Dr. Ravi Manjithaya, my research supervisor for providing me a valuable opportunity to work in his lab and also for his constant guidance and encouragement without which this project would have been a difficult venture.

I would take this opportunity to thank Prof. Anuranjan Anand, Chairman, MBGU, Prof. Tapas Kumar Kundu, Prof. Uday Kumar Ranga, Prof. Hemalatha Balaram, Prof. Maneesha Inamdar, Prof. Namita Surolia, Dr. Kaustuv Sanyal, Dr. GR Ramesh and Dr. James Chelliah for guiding me during the course work.

I would like to express my gratitude to Dr. Aravinda Chavalmane for being an incredible teacher. My special thanks to my amazing seniors Piyush and Sunaina who were always there to help. I thank all my lab mates; Shashank, Suresh, Lalitha, Gaurav, Viswa, Sreedevi, Veena and Ramji for providing a great working environment and for their valuable suggestions.

Lastly, I would like to acknowledge the people around whom my life revolves; my parents, Raaghesh and my brother for their unconditional love, support and encouragement and for standing by me in my highs and lows.

Somya

List of Abbreviations

^{0}C	Degree Celcius
kDa	Kilo Dalton
µl	micro litre
µM	micro molar
Atg	Autophagy related proteins
Vps	Vacuolar protein sorting
NBR1	Neighbour of BRCA1 gene 1
LAMP	Lysosomal-associated membrane protein
AP	Autophagosomes
AL	Autolysosomes
GFP	Green Fluorescent Protein
EPC	Elaidylphosphocholine
RFP	Red Fluorescent Protein
RT	Room Temperature
h	hours
TOR	target of rapamycin
FIP200	FAK-family interacting protein of 200 kDa
HMGB1	High mobility group B1
MHC	Major Histocompatibility complex
SEM	Standard Error of Mean

List of Figures

Chapter 1

Chapter 3

Chapter 4

List of Tables

Table 1: Autophagy inhibitors in clinical trials

TABLE OF CONTENTS

SYNOPSIS

Macroautophagy (hereby Autophagy) is a cellular degradation pathway in which cytoplasmic components are captured in double membrane vesicles called 'autophagosomes' and delivered to lysosomes for degradation. The process of autophagy is evolutionarily conserved from yeast to mammals and has an indispensable role in maintaining cellular homeostasis. The rate at which the dynamic turnover of cellular components takes place via the process of autophagy is called autophagic flux. Deregulation of autophagic flux is involved in some of the major human diseases. The role of autophagy in cancer is complex and depends on the type, stage and genetic context of the tumor. Many scientific evidences support the tumor suppressor role of autophagy in early tumorigenesis while in established tumors, autophagy has been shown to protect tumor cells from metabolic stress induced necrosis and hence improve cell survival. Defective autophagy causes accumulation of protein aggregates and damaged organelles in neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). In addition to the role of autophagy in cellular homeostasis, it also has an intricate role in innate and adaptive immunity. Induced autophagy can assist in pathogen clearance in *Mycobacterium tuberculosis*, *Salmonella typhimurium* and Group A *Streptococcus* infections.

Given that autophagic defect is involved in some major human diseases, modulating i.e. enhancing or inhibiting autophagy can serve as a potential target in therapeutics. Autophagy modulation can be of two types either genetic or pharmacological modulation. We are interested in pharmacological modulation of autophagy using small molecules. Many autophagy inducers and inhibitors are already known but in order to target autophagy in cancer, the need of the hour is to develop more specific and potent autophagy inhibitors. Stronger autophagy inducers can be used to clear misfolded protein aggregates in neurodegeneration and to abrogate some viral, bacterial and parasitic infections. Moreover, developing new autophagy modulators will help in better understanding of the mechanism of autophagy and can also help unravel new molecular players in autophagy

In a high throughput based approach using a lab developed autophagy assay in yeast yielded several potential autophagy modulators. I validated some of these hits using secondary assays in yeast. Next, I tested these hits for modulation of autophagy in mammalian cells as well. In our study, we have found two small molecules Elaidylphosphocholine and AB-MECA which show potent, dose dependent autophagy inhibition and induction respectively. These results highlight the conserved nature of autophagy that can be utilized to discover promising novel autophagy regulating small molecules in a mammalian system employing yeast based autophagy assay. Future studies with them will entail delineating the mechanism through which the molecules act and structure-activity relationship studies with the molecules.

Chapter 1: Introduction

1.1 Autophagy

The term ‘Autophagy’ coined by Christian de Duve in 1963 derives its origin from the Greek words ‘auto’ and ‘phagy’ which literally translates to self-eating [1]. Macroautophagy (herein autophagy) is a cellular degradation pathway in which cytoplasmic components are captured in double membrane vesicles called ‘autophagosomes’ and delivered to lysosomes for degradation. The process of autophagy is evolutionarily conserved from yeast to mammals and has an indispensable role in maintaining cellular homeostasis. Around 38 autophagy related genes (ATGs) have been identified in *Saccharomyces cerevisiae* and *Pichia pastoris* and many of them are functionally conserved in higher eukaryotes [2]. Autophagy occurs at a basal rate in cells during normal growth conditions and is involved in degradation and removal of damaged or dead organelles and misfolded proteins [3]. The difference in levels of basal autophagy among different tissues was shown using transgenic mice expressing a fluorescent autophagosome marker. Basal autophagy levels were ranging from extremely low in brain, moderate in pancreatic acinar cells and relatively high in thymic epithelial cells [4]. Depending on how the cargo is sequestered, autophagy is of three types: Macroautophagy, microautophagy and chaperone mediated autophagy (CMA). Macroautophagy is the main autophagy pathway, in which cargo is sequestered in double membrane autophagosomes and taken to lysosomes. In microautophagy, the part of cytoplasm which needs to be degraded is directly engulfed in lysosome by invagination and folding of lysosomal membrane. In chaperone mediated autophagy (CMA), the protein cargo is recognised by the chaperone Hsc-70 which interacts with lysosomal membrane protein LAMP2A, unfolds the substrate protein, and transfers it across lysosomal membrane for degradation. Depending on the presence and absence of selectivity factors, autophagy is of two types: general and selective autophagy. Bulk degradation of long lived proteins and cytoplasmic components is called general autophagy. Selective autophagy involves specific recognition of cargo by autophagy receptors like p62, NBR1, Optineurin etc and their subsequent loading in autophagosomes [5].

1.2 Molecular mechanisms of Autophagy

The process of macroautophagy (hereafter autophagy) involves the biogenesis of the autophagosome which comprises of sequential sets of events beginning from induction of autophagosome formation at various membrane sources inside the cell, vesicle nucleation and elongation and eventual fusion of the autophagosome with the lysosome, degradation of the cargo and finally, efflux of the breakdown products such as amino acids, nucleotides, sugars and lipids back into the cytoplasm.

1.2.1 Induction of Autophagy

The most studied trigger of autophagy is nutrient starvation. Depletion of amino acids and growth factors forces a cell to induce autophagy for its survival. These signals converge through mammalian Target Of Rapamycin (mTOR) whose main role is to determine the nutritional status of a cell and thus negatively regulate autophagy [6]. Other inducers of autophagy are energy and oxygen stress which are sensed by AMP-activated protein kinase and stress induced kinases like C-jun N-terminal protein kinase (JNK). Upon induction of autophagy, Unc-51 like autophagy activating kinase 1(ULK1) complex comprising of ULK1, Atg13, FIP200 and Atg101 becomes active and gets targeted to the phagophore or isolation membrane. Hierarchical studies of autophagosomal proteins suggest that ULK1 complex activation is the most upstream step of the autophagosome biogenesis [7, 8].

1.2.2 Vesicle nucleation and elongation

In yeast, autophagosome synthesis begins at a single site where autophagy related proteins gather. This site is called as ‘pre-autophagosomal structure (PAS)’. In mammals, the autophagy initiation site i.e. phagophore is not well defined. Phagophores primarily appear to have arisen from endoplasmic reticulum (ER), mitochondria and other cytosolic membrane structures like trans-golgi and late endosomes [1, 9, 10]. ER-Mitochondria contact sites and plasma membrane also have been reported to have a role in autophagosome biogenesis [11, 12].

Mammalian phosphatidylinositol (PI) 3-kinases are proteins that phosphorylate phosphoinositides at the 3’-position of the inositol ring. They produce phospholipid

molecules that are involved in various cell signaling pathways. The class III PI3K complex is the best studied for its role in autophagy initiation and vesicle nucleation. The mammalian class III PI3K complex comprises of the class III PI3K Vps34, Beclin1 (mammalian homolog of yeast Atg6), p150 (mammalian homolog of Vps15) and Atg14L. Class III PI3K complex is needed for phagophore elongation and sequential recruitment of Atg proteins. The importance of PI3K complex is evident from the fact that autophagosomes are not formed on treatment with PI3K inhibitors like wortmannin or 3-Methyl Adenine (3-MA) [13, 14]. Beclin1, the mammalian homolog of Atg6 is well known to modulate the functions of other autophagy related proteins. In non starvation conditions, Beclin1 is bound to anti-apoptotic protein Bcl2. Upon starvation this interaction is disrupted and Beclin1 is free to induce autophagy [15]. The active ULK1 complex directly phosphorylates Beclin1 at Ser 14 and activates the pro-autophagy VPS34 complex to promote autophagosome biogenesis [16]. WD repeat domain phosphoinositide-interacting protein 2 (WIPI2, the mammalian homolog of Atg18) and Double FYVE domain containing protein (DFCP1) are PI(3)P binding proteins which are recruited on PI(3)P rich membranes called omegasomes and help in elongation of the isolation membrane [8, 14].

Elongation of the isolation membrane also involves two ubiquitin like systems which act at Atg12-Atg5 conjugation step and the Microtubule-associated proteins 1A/1B light chain 3A (LC3) processing step. In the Atg12-Atg5 conjugation step, Atg7 acts as E1 activating enzyme which activates Atg12 in an ATP dependent manner and transfers it to Atg10. Here, Atg10 which is an E2 like enzyme forms an Atg12-Atg10 intermediate and finally helps in linkage of Atg12 to Atg5 via covalent bonding. The Atg12-Atg5 conjugate forms a multimeric complex with Atg16L. This Atg12-Atg5-Atg16L complex helps in arching of the elongating phagophore by unequal recruitment of LC3-II protein on the membrane. The association of Atg12-Atg5-Atg16L complex with the phagophore is a transient one and the complex dissociates once autophagosome formation is complete. The second ubiquitin like system involved in the processing of LC3 is an ubiquitin like protein required for the formation of autophagosomes. LC3 is present as a full length protein in the cytoplasm which is cleaved by cysteine protease Atg4 to generate a C-terminal exposed glycine residue, the LC3-I form. LC3-I is then activated by E1 activating enzyme Atg7 in an ATP

dependent manner and transferred to Atg3. Atg3 which acts as E2 like enzyme attaches phosphatidylethanolamine (PE) to the exposed glycine residue of LC3-I and generates LC3-II. Lipidated LC3 goes and binds to inner and outer autophagosomal membranes. Since LC3 is the only known protein which is present on the autophagosome during and after its formation, it is studied as an autophagosomal marker. The recruitment of LC3-II on autophagosomal membranes depends on Atg12-Atg5-Atg16L complex and it is crucial for phagophore expansion as its absence can cause defects in autophagosome formation. It is also involved in cargo selection and in hemifusion of membranes [17, 18].

1.2.3 Lysosomal fusion and degradation

After completion, autophagosomes either directly fuse with lysosomes to form single membrane autolysosomes or initially with late endosomes to form amphisomes and later with lysosomes. The small G protein Rab7 is involved in autophagosome-lysosome fusion. Gene knockdown of Rab7 leads to disrupted lysosome biogenesis and accumulation of late autophagic vacuoles [19, 20]. The interaction of homotypic fusion and protein sorting (HOPS)–tethering complex with the autophagosomal SNARE syntaxin17 (STX17) is a prerequisite for autophagosome-lysosome fusion [21]. Microtubules are important for movement of autophagosomes towards lysosomes and disrupting them with vinblastine or nocadozole leads to massive buildup of autophagosomes. The completion of autophagic process requires degradation of autophagic cargo inside lysosomes. Lysosomes have several types of hydrolytic enzymes, most of which require an acidic pH maintained by proton pump vacuolar ATPases. Lysosomal acidification is necessary for autophagosome-lysosome fusion and vATPase inhibitors like Bafilomycin A1 and lysomotropic agents such as Chloroquine (CQ) inhibit fusion. Lysosomal membrane proteins like LAMP1 and LAMP2 which protect lysosomal membrane from self digestion and maintain lysosomal function are also important for autophagy and accumulation of early and late autophagic vacuoles are seen in LAMP2 deficient hepatocytes. Inactivation of lysosomal enzymes cathepsin B and D also leads to accumulation of late autophagic vacuoles [19, 22].

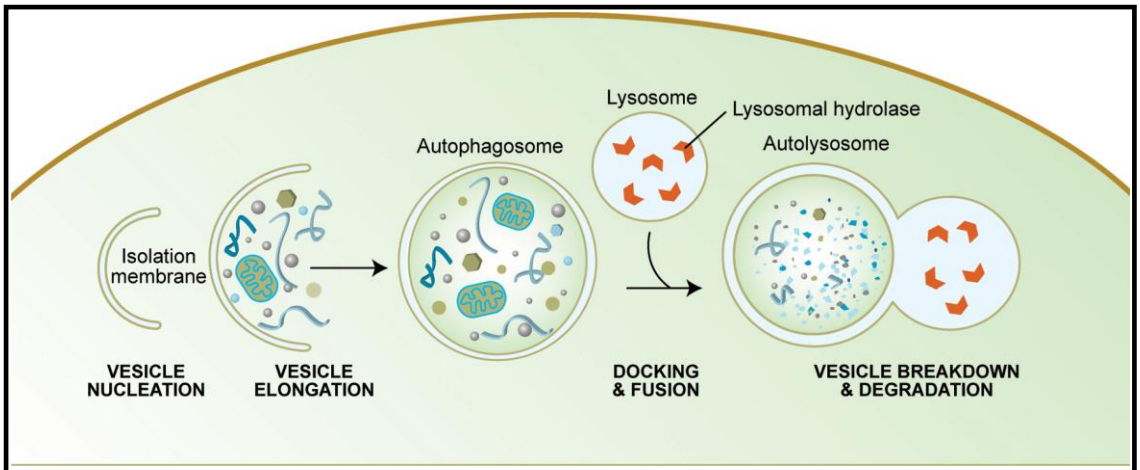


Figure 1: The process of autophagy (www.wormbook.org)

1.3 Signaling and regulatory mechanisms of autophagy

1.3.1 Nutrient stress

1.3.1.1 mTOR Signaling

Mechanistic target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that is a consolidating platform for signals from different stimuli like amino acids, energy levels, oxygen, growth factors and stress. In mammals, mTOR exists in the form of two complexes, mTORC1 and mTORC2 which are different from each other in terms of localization and function. In the presence of amino acid signaling, mTORC1 inhibits autophagy by phosphorylating ULK1 and thereby suppressing its kinase activity. It also phosphorylates Atg13 and prevents its interaction with ULK1 [23].This amino acid signaling through mTOR is mediated by Ragulator-Rag complex which targets mTOR to lysosome where it interacts with small GTPase Rheb which is necessary for mTOR activation. Rheb itself is inactivated by Tuberous sclerosis (TSC1/2) complex which is a GTPase activating protein for Rheb. In the presence of growth factors, the TSC1/2 complex is inactivated by Akt (Protein kinase B) mediated phosphorylation. Thus, mTOR activation involves collaborative signaling through amino acids as well as growth factors [24]. The effect of mTOR on ULK1 is seen in yeast too where Atg1, the yeast homolog of ULK1 is also phosphorylated and inhibited

by TOR [25]. In nutrient starvation conditions or in the presence of rapamycin, mTOR is inhibited which allows ULK1 to be activated by phosphorylation. ULK1 in turn phosphorylates Atg13 and FIP200 and autophagy is induced [7].

1.3.1.2 MEK/ERK Signaling

Mitogen activated kinases MEK and ERK form a signaling system that transfers signal from a cell surface receptor to the nucleus. Ras effector protein Raf-1 is an amino acid sensing protein that is activated by amino acid deprivation. Raf-1 in turn activates the Mitogen activated kinase signaling cascade. MEK and ERK, the downstream effectors of Raf-1, induce autophagy by up regulating Beclin-1 and by causing disassembly of the mTORC1 complex [26].

1.3.2 Energy stress

1.3.2.1 AMPK signaling

AMPK which stands for AMP-activated protein kinase is an enzyme which is important for energy sensing in a cell. The intracellular ratio of ATP to ADP and AMP is sensed by AMPK and low ATP to AMP ratio is a signal to induce energy stress inside a cell. AMPK has three subunits: α, β and γ. High concentration of AMP induces a conformational change in the γ subunit which exposes the Threonine 172 residue in the α subunit. Phosphorylation of this Thr-172 residue by LKB1 kinase is necessary for AMPK activation [27]. Autophagy induction upon glucose withdrawal is mediated by AMPK. AMPK can directly activate ULK1 kinase by an activating phosphorylation at Serine 555 [23]. It can also activate TSC1/2 complex which is a negative regulator of mTOR signaling. Phosphorylation of Raptor subunit of mTOR by AMPK inhibits downstream mTOR signaling [24].

1.3.3 Oxygen stress

Low levels of oxygen i.e. hypoxia have been shown to induce autophagy. The primary pathway involved in oxygen stress response is AMPK signaling pathway. Deficiency of oxygen leads to a decrease in intracellular ATP to AMP ratio which is sensed by AMPK which induces autophagy by inhibiting mTOR signaling [27]. Hypoxia-inducible factor-1 (HIF-1) is the primary transcription factor activated by oxygen deficiency. The downstream target of HIF-1, BNIP3 (Bcl-2 adenovirus E1a nineteen kDa interacting protein 3) induces autophagy by disrupting Bcl2-Beclin1 interaction. C-jun N-terminal protein kinase (JNK) is activated via protein kinase C pathway upon oxygen stress. JNK1 phosphorylates Bcl2 thereby disrupting Bcl2-Beclin1 interaction and inducing autophagy [28, 29].

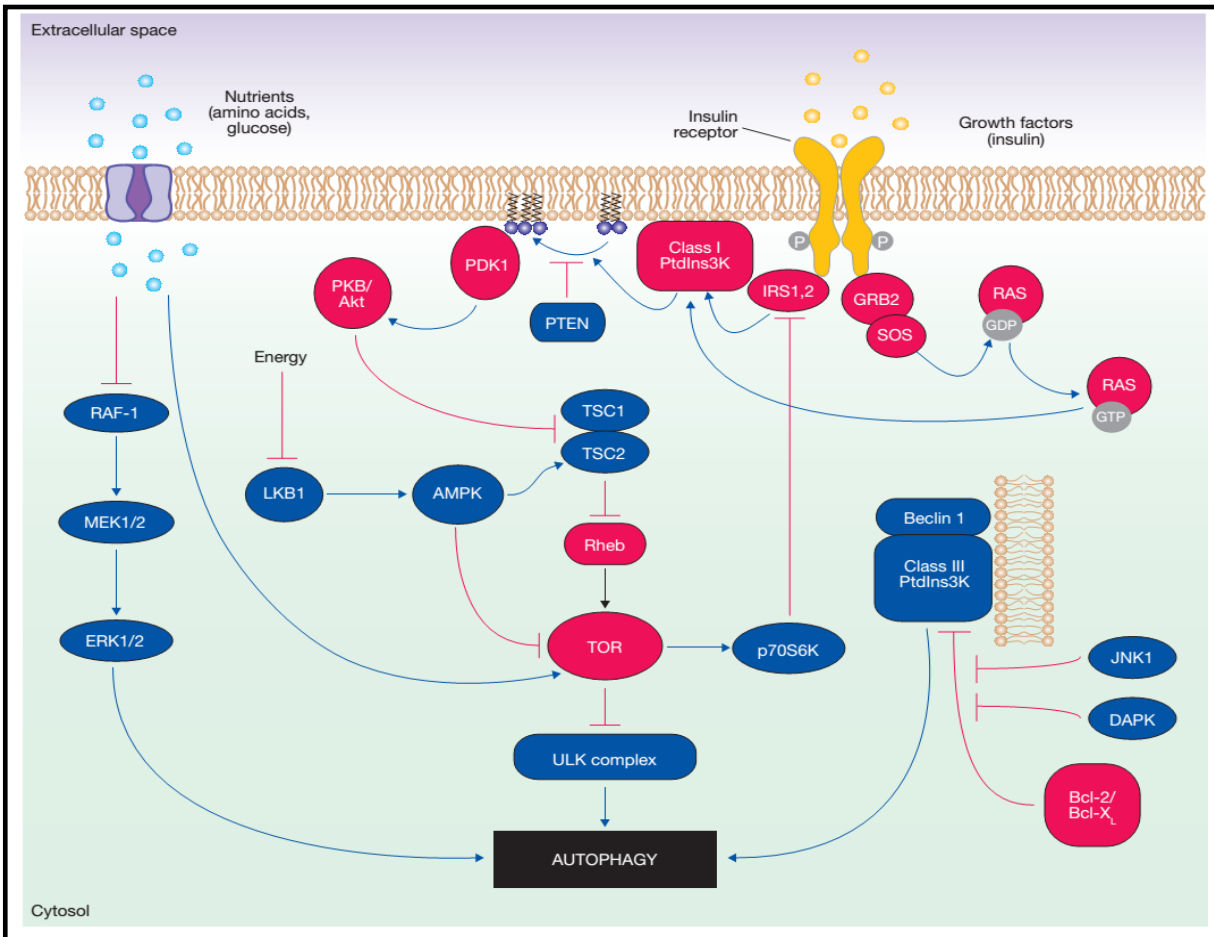


Figure 2: Signaling regulation of autophagy (With permission from Yang, Klionsky et al. 2010)

1.4 Why is autophagic flux important?

Autophagy is a cellular recycling process which has roles in cell growth, development, survival and death. The rate at which this dynamic turnover of cellular components takes place via the process of autophagy is called **Autophagic flux**. In detail, autophagic flux accounts for all the steps of autophagy, starting from the formation of autophagosome, its maturation and fusion with lysosome, degradation of cargo and the subsequent release of macromolecules in the cytosol [30]. Since deregulation of autophagy plays a key role in several human diseases, studying and understanding autophagic flux is of crucial importance. The role of autophagy in some of the major human diseases has been explained under the following subheadings.

1.4.1 Autophagy in cancer

The role of autophagy in cancer is complex and not clearly etched. It depends on the type, stage and genetic context of the tumor. Many scientific evidences support the tumor suppressor role of autophagy in early tumorigenesis. Activation of PI3K/Akt signaling pathway or Pten loss due to mTOR activation can be a cause of decreased autophagy in malignant cells [31]. Beclin1, a core autophagy protein and a member of PI3K/VPS34 complex has a clearly proven tumor suppressor role and loss of Beclin1 is found in 40 to 75% of breast, prostate and ovarian cancers. Mice with monoallelic deletion of *Becn1* are also shown to be predisposed to variety of tumors [31-34]. A constitutive level of basal autophagy is needed for cellular homeostasis and loss of autophagy leads to genomic instability and aneuploidy. Deletion of essential autophagy genes like Atg5 and Atg7 also results in liver tumors [33, 35, 36]. Mice lacking Atg4, a cysteine protease which mediates autophagosome formation have higher cases of chemically induced fibrosarcoma [31]. The accumulation of p62, a protein involved in selective autophagy, due to autophagy inhibition can make cells predisposed to tumor due to deregulation of NFκB signaling, DNA damage, ROS accumulation and insufficient clearance of damaged organelles and proteins [31, 37]. Another mechanism by which autophagy acts as a tumor suppressor is by inducing senescence in cells to prevent malignant transformation. It is also involved in protecting cells from chronic inflammation associated with release of proinflammatory HMGB1 [31, 38].

During metastasis, tumor cells need to overcome anoikis, a type of apoptotic cell death induced when cells lose attachment to the extracellular matrix. It is a protective mechanism against tumor invasion in secondary tissues [39]. Autophagy is induced following extracellular matrix detachment which helps cells escape anoikis. In established tumors, cells have high metabolic demand, low nutrient supply and hypoxic conditions prevail which leads to metabolic stress. Autophagy has been shown to protect tumor cells from metabolic stress induced necrosis and hence improve cell survival in established tumors. It can induce recycling of ATP and provide raw materials to promote cellular biosynthesis [40]. Hypoxia induced factor-1 alpha (HIF-1α) dependent and independent autophagy induction also contributes to tumor survival [29]. Autophagy inhibition in tumor cells by knockdown of essential autophagy genes leads to apoptotic cell death. Genetic studies in mice have shown that deletion of FIP200, an essential autophagy gene can inhibit growth of mammary tumors [41]. Some tumor cell lines having activating *H-ras* or *K-ras* mutations like human pancreatic cell lines are heavily dependent on autophagy and have high basal rate of autophagy [31, 42]. Inhibiting autophagy in these cells by RNAi or Chloroquine (CQ), a drug that inhibits autophagy by increasing lysosomal pH has shown tumor regression in pancreatic cancer xenografts and genetic mouse models [31, 38].

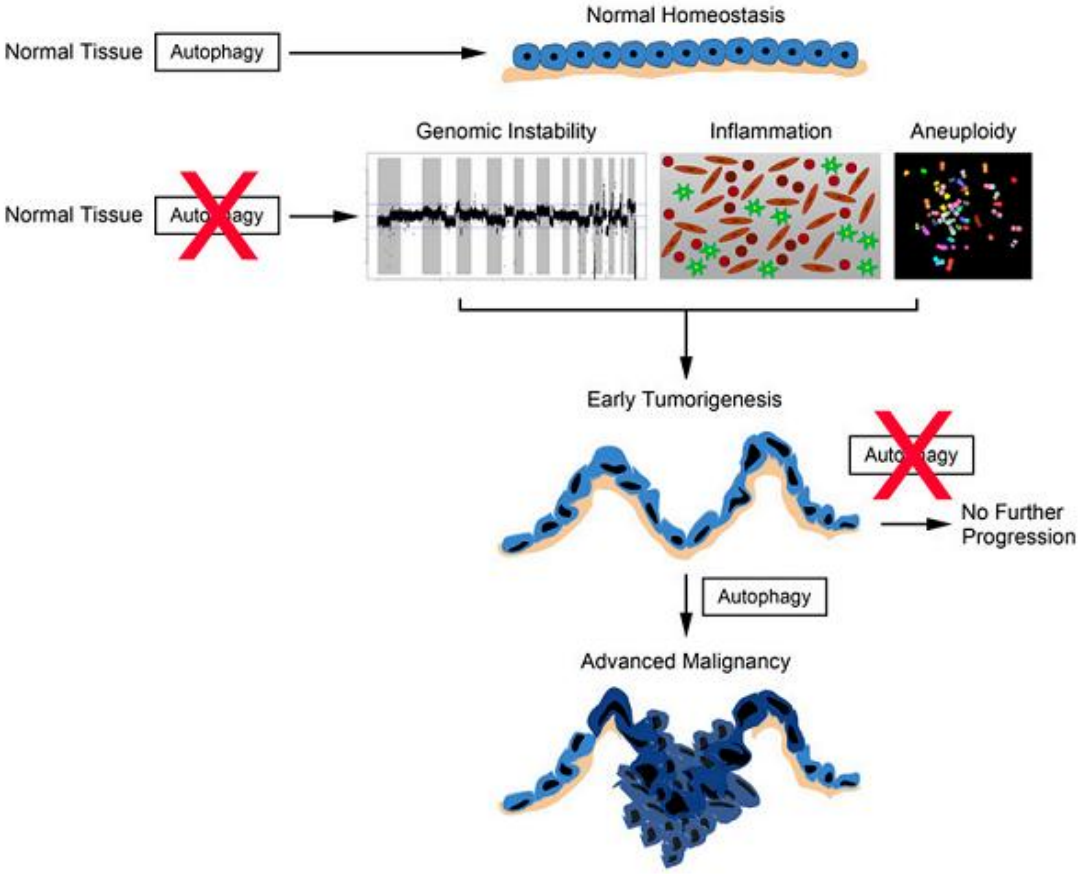


Figure 3: The role of autophagy in cancer (With permission from Kimmelman A, 2011)

1.4.2 Autophagy in neurodegeneration

Autophagy as a cellular homeostasis process is exceedingly important in neurons. This is evident from the fact that brain is the primarily affected organ in most of the lysosomal disorders and mutations in genes related to autophagy is very commonly found in neurodegenerative diseases. Accumulation of protein aggregates and damaged organelles is the primary feature of neurodegeneration seen in diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) which shines light on the hazards of improper cellular turnover. Major steps of autophagy include selection of cargo (such as aggregated/misfolded proteins in this context), sequestration and finally lysosomal

degradation of cargo. Defects in all of these steps of autophagy are seen in various neurodegenerative diseases [22, 43].

Parkinson's disease (PD) is a degenerative disease of the central nervous system which arises due the death of dopamine secreting neurons in the substantia nigra region of the midbrain. The pathology of this disease is characterized by the accumulation of aggregates of alpha-synuclein protein in form of inclusions termed as "Lewy bodies" in the neurons. Accumulation of alpha-synuclein aggregates in PD results in inhibition of small GTPase Rab1A. Rab1A has roles in regulation of vesicular protein transport and in the omegasome formation step of autophagosome biogenesis. Inhibition of Rab1A leads to Atg9 mislocalization and interferes with omegasome formation which in turn inhibits autophagosome biogenesis. In rare familial cases of Parkinson's disease, mutations have been identified in genes coding for alpha-synuclein (SNCA), parkin (PRKN), leucine-rich repeat kinase 2 (LRRK2 or dardarin), PTEN-induced putative kinase 1 (PINK1), and neuronal P-type ATPase ATP13A2. PINK and parkin mutations leads to defects in mitophagy, a selective autophagy of mitochondria. LRRK2 mutations results in attenuation of chaperone mediated autophagy (CMA). Mutations in genes coding for alpha-synuclein (SNCA) and neuronal P-type ATPase ATP13A2 hampers lysosomal function by causing an increase in lysosomal pH and inhibition of hydrolases which subsequently affects proper aggregate clearance [22, 44, 45].

Huntington's disease is a genetic neurodegenerative disease caused due to mutations in Huntingtin gene (HTT) which codes for Huntingtin protein (Htt). The mutation results in an expansion of the polyglutamine tract in the huntingtin protein which causes mHtt to accumulate in form of toxic oligomers in the absence of a proper clearance mechanism. The disease is characterized by lack of muscle coordination and behavioural problems due to gradual degeneration of nerve cells in the brain. The process of autophagosome formation and its clearance by lysosomal fusion appears normal in Huntington's disease but the autophagosomes are unusually empty which points towards a defect in substrate recognition and cargo loading. The reasons for the impaired ability to recognize cargo is not clear but it is proposed that this occurs due to abnormal association of the mutant htt protein with autophagy adaptors like p62 [22, 46, 47]. Mutations in p62 (SQSTM1) which affects selective recognition of autophagic

cargo has been identified in Amyotrophic lateral sclerosis (ALS). Dynactin mutations in ALS inhibit autophagosome transport and hence prevent its fusion with lysosome [22].

Alzheimer's disease is an irreversible and progressive brain disorder which is a major responsible factor for dementia and lack of cognition abilities in the elderly. It is marked by an accumulation of amyloid plaques and tau tangles in the brain which hampers efficient functioning of neurons and their ability to communicate with each other. The causes for familial Alzheimer disease known so far are mutations in one of these 3 genes: presenilin1, presenilin2, and amyloid precursor protein (APP). An allele of the APOE gene that encodes a protein variant ApoE4 is the strongest genetic risk factor for late onset Alzheimer's disease. Presenilin1 is required for proper lysosomal acidification and protease activity and mutations in this gene leads to an increase in lysosomal pH which affects the clearance of autophagosomal cargo. ApoE4 and mutant APP activate Rab5 and Rab7 hence causing a substrate overload in lysosomes resulting in damage of lysosomal membranes [22, 48, 49].

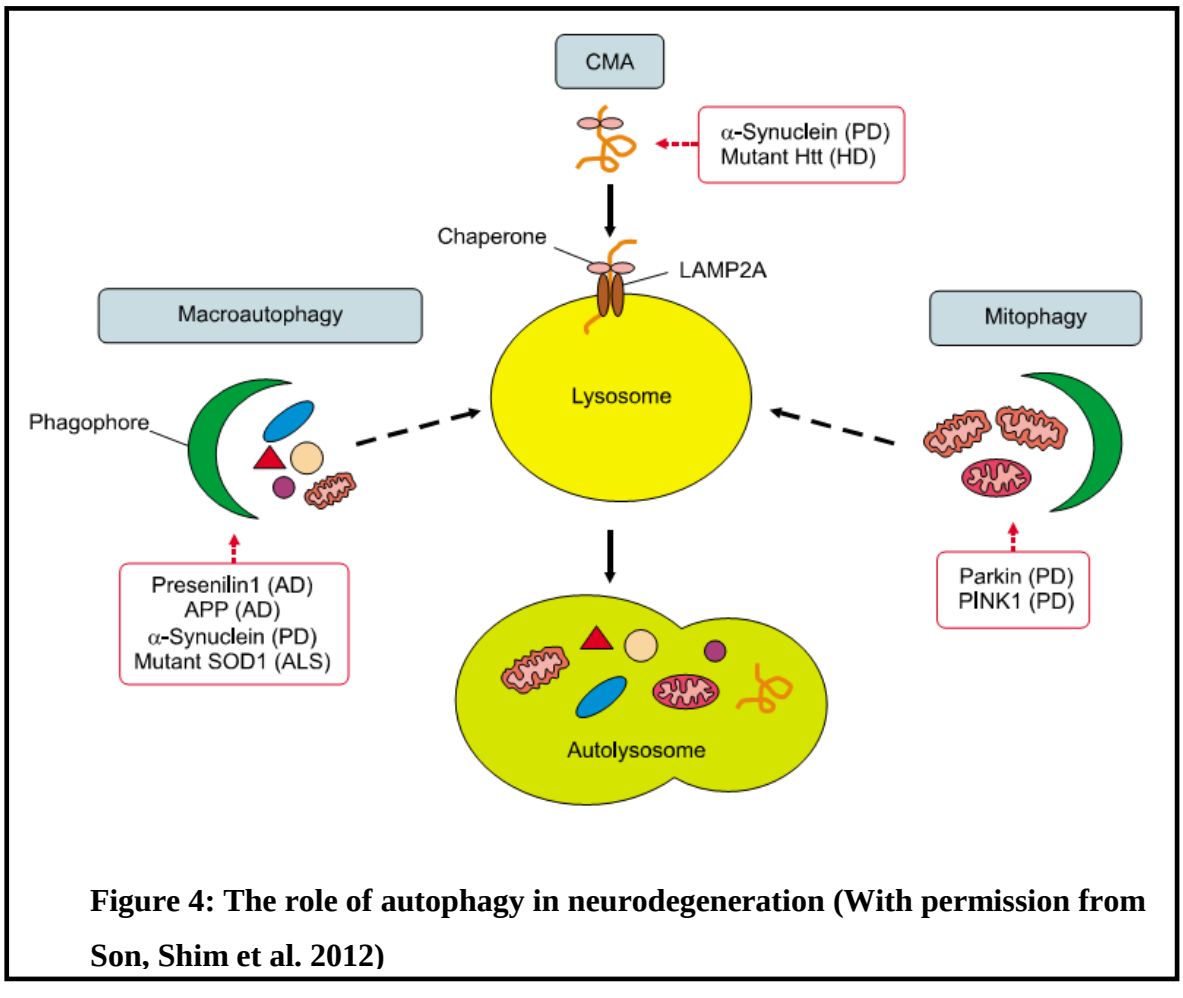


Figure 4: The role of autophagy in neurodegeneration (With permission from Son, Shim et al. 2012)

1.4.3 Autophagy in cellular defence

In addition to the role of autophagy in cellular homeostasis, it also has an intricate role in innate and adaptive immunity. The selective sequestration of intracellular pathogens by autophagic machinery is termed as Xenophagy. Invading pathogens like *Mycobacterium tuberculosis*, *Salmonella typhimurium* and Group A *Streptococcus* are captured by autophagy. The mechanism of xenophagy requires autophagic adaptors which can selectively recognise the microbes as cargo so that they can be delivered to autophagosomes. The importance of autophagy in containing intracellular pathogens is projected by the fact that *S. typhimurium* showed enhanced replication in ATG5 deficient mouse embryonic fibroblasts (MEFs). The same is shown for Group A *Streptococcus* which are also trapped in autophagosome like vacuoles and its degradation reduces in ATG5 deficient cells [50, 51].

The role of autophagy in pathogen clearance relies on a mechanism which can sense invading pathogens and elicit autophagy. Cells have surface and cytosolic pattern recognition receptors like Toll-like receptors (TLRs) and NOD-like receptors (NLRs) which recognise pathogen-associated molecular patterns (PAMPs). Recent studies propose a role of TLR and NLR signaling in autophagy induction. Toll-like receptor signaling increases interaction of TLR adaptor proteins MyD88 and trif with Beclin1. Activation of TLR signaling disrupts Beclin1-Bcl2 interaction and leads to subsequent autophagy induction [52-54]. Damage associated molecular patterns (DAMPs) are molecules that elicit immune response in non-infectious inflammation response. HMGB1, the most well studied DAMP, is a nuclear protein that regulates gene transcription but under stress conditions can be released by the cell to elicit inflammation response. HMGB1 induces autophagy by binding to Beclin1 and activating the Class III PI3K complex [55]. Th1 cytokines like IFN-γ and TNF-α induce autophagy by upregulating LC3 and Beclin1 levels through JNK signaling and by inhibiting Akt activation [52, 56].

In adaptive immunity, autophagy is involved in the delivering microbial antigens to MHC class II antigen-presenting molecules which results in activation of CD4 T lymphocytes. Several reports have suggested that autophagy enhances the presentation of endogenous viral antigens. The autophagy gene ATG5 has been shown to be

essential for dendritic cells to process and present antigens for MHC class II presentation [52, 57].

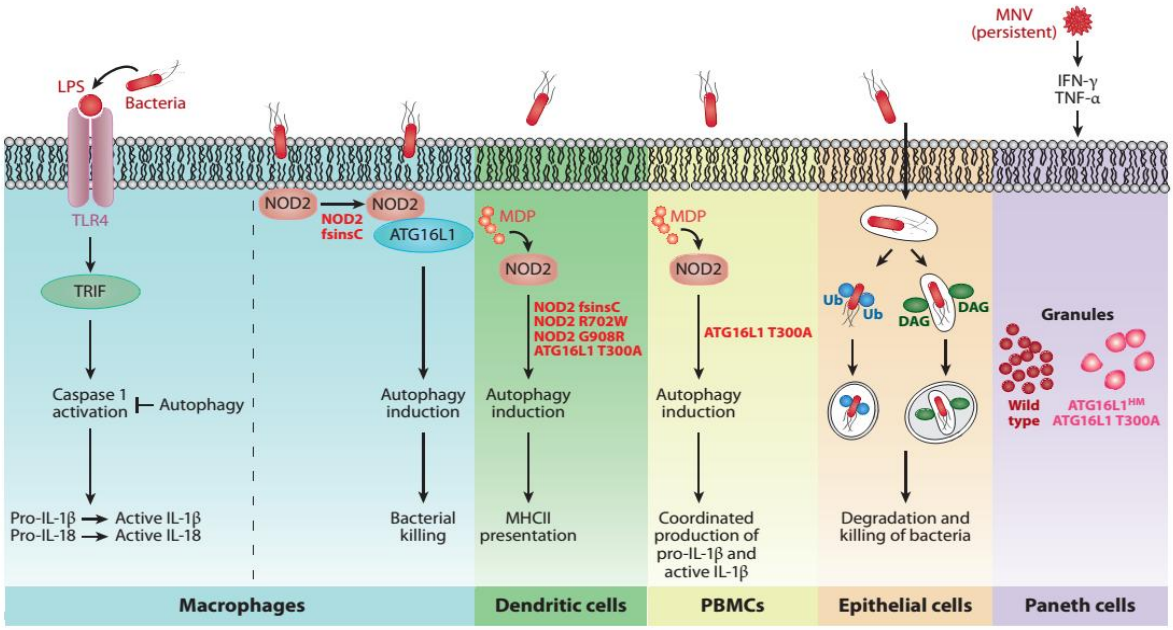


Figure 5: The role of autophagy in cellular defence (With permission from Kuballa P. et al. 2012)

1.5 Methods to monitor autophagic flux

A common misconception that arises while monitoring autophagy is the belief that an increase in number of autophagosomes means induction of autophagy. The accumulation of autophagosomes does not always indicate induction of autophagy and may also represent a block in later steps of autophagy i.e. the fusion of autophagosomes with lysosomes [30]. In order to get a clear picture on the status of autophagy, 'autophagic flux' measurement is needed. The most commonly used autophagic flux assays are discussed below:

1.5.1 LC3 Conversion Assay

LC3 is a protein that resides on the autophagosome membrane and is degraded by autophagy. Nascent LC3 is converted to LC3-I form by Atg4 mediated C-terminal cleavage which exposes a glycine residue. LC3-I is then converted to the autophagosome membrane bound LC3-II form by an ubiquitination like conjugation reaction that attaches phosphatidylethanolamine (PE) to LC3-I. LC3-II migrates more rapidly than LC3-I in SDS–PAGE despite having higher molecular weight due to higher hydrophobicity of the PE group. The amount of conversion from LC3-I to LC3-II form can be assessed using antibodies against LC3 which generally depicts the number of autophagosomes (Figure 6). However, LC3 conversion assay does not give a clear picture of autophagic flux because accumulation of LC3-II form can be either due to induction of autophagy or inhibition of later steps of autophagy i.e. fusion of autophagosomes with lysosomes [30].

1.5.2 LC3 Turnover Assay

Autophagic flux can be monitored by studying the dynamic turnover of LC3 in presence and absence of lysosomal inhibitors like BafilomycinA1 and Pepstatin which either increase lysosomal pH or inhibit lysosomal proteases. The difference in the amount of LC3-II in the presence and absence of inhibitors can give an idea about the amount of LC3-II being degraded by autophagy and whether or not autophagic flux is impaired (Figure 6) [30].

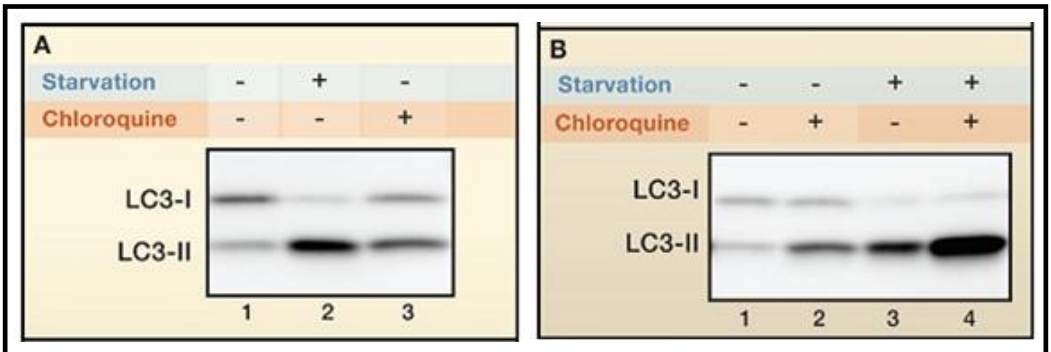


Figure 6: A. LC3 Conversion Assay B. LC3 Turnover Assay

(With permission from Mizushima N., Yoshimori T., Levine B., 2010)

1.5.3 Traffic Light Assay

The traffic light assay is useful in studying the maturation process of autophagosomes. In this assay, a tandem fluorescent tagged LC3 construct is used as a reporter which has LC3 tagged to mRFP and GFP. The idea behind this methodology is that due to the double tagging, autophagosomes will appear yellow but when they fuse with lysosomes, the GFP fluorescence will quench due to low pH of lysosome and hence autolysosomes will appear red (Figure 7). This reporter system gives a clear picture of the autophagic flux status. If autophagy is induced the number of both yellow and red dots i.e. autophagosomes and autolysosomes will increase. A block in the early steps of autophagy i.e. formation of autophagosomes will lead to decrease in number of both yellow and red dots, whereas a block in the fusion of autophagosomes with lysosomes will result in accumulation of yellow dots with no concomitant increase in red dots [30, 58].

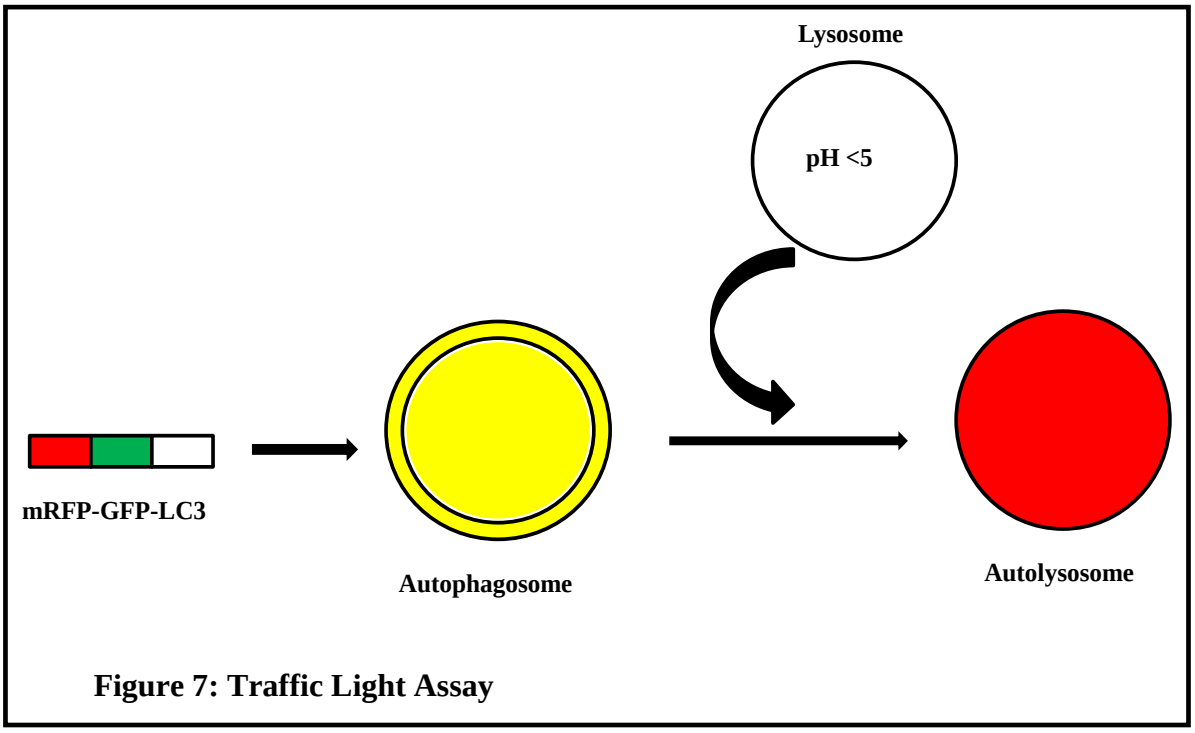


Figure 7: Traffic Light Assay

1.5.4 Degradation of selective substrates

Autophagy is generally considered a bulk degradation pathway but recently it has been reported that it targets some selected proteins for degradation which are called autophagy substrates by employing adaptor proteins. These are ubiquitin binding proteins like p62 (also called as Sequestome1/SQSTM1), Neighbour of BRCA1 (NBR1), Nuclear Dot protein (NDP52), Optineurin etc. These adaptor proteins bind to LC3 due to presence of **LC3 interacting region (LIR)** which is a conserved, hydrophobic W/YXXL sequence. They also bind to a variety of cargo like ubiquitinated proteins, damaged organelles and pathogens. One of the most studied selective substrate of autophagy is p62 whose levels inversely correlate with that of autophagy induction/activity. In autophagy deficient cells, p62 accumulates with ubiquitin containing aggregates while its levels decrease when autophagy is induced. Hence, p62 can be used as a marker to study autophagic flux [30, 59, 60].

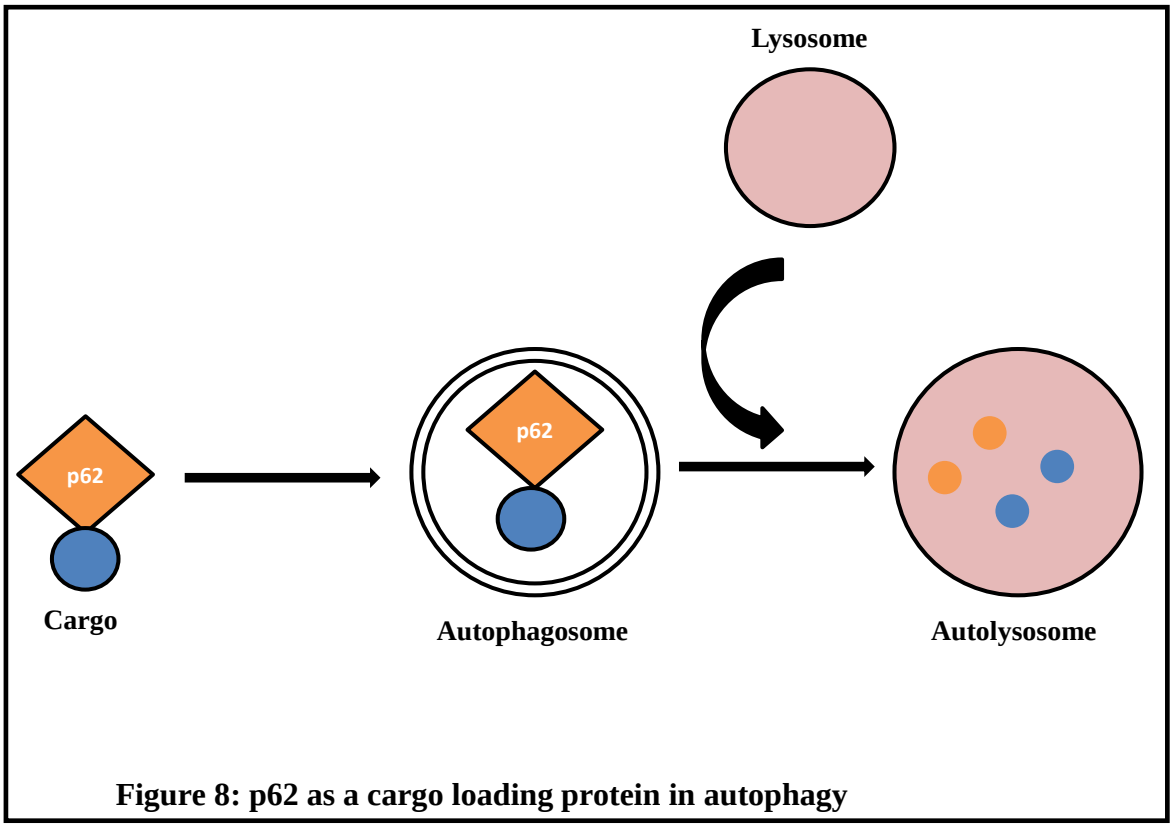


Figure 8: p62 as a cargo loading protein in autophagy

1.5.5 Degradation of long lived proteins

Studying degradation of long lived proteins is one the oldest methods of measuring autophagic flux. In this assay, cells are cultured with radiolabeled carbon or tritium for several days to label long lived proteins followed by a short incubation without isotope labeled amino acids to wash out short lived radiolabeled proteins. These cells are then treated with autophagy inducers and the release of degraded proteins is measured by quantitating trichloroacetic acid soluble radioactivity in the culture supernatant. In order to ensure that the degradation being measured is actually by autophagy, degradation rates of samples cultured in presence and absence of autophagy inhibitors is compared [30, 60].

1.6 Modulation of autophagy as a therapeutic approach

Deregulation of autophagy is crucial in development of some major human diseases as already described earlier. In cancer, autophagy has a tumor suppressor role in early tumorigenesis and largely cytoprotective function in established tumors. Impaired autophagy which leads to accumulation of misfolded protein aggregates is the major cause of most neurodegenerative diseases. Autophagy is also the effector in many adaptive and innate immunity pathways. Given these observations, modulating i.e. enhancing or inhibiting autophagy can serve as a potential target in therapeutics. Autophagy modulation can be of two types either genetic or pharmacological modulation.

1.6.1 Genetic modulation of autophagy

Knockdown of autophagy essential genes has shown to cause necrotic cell death in established tumors. *Becn1* loss showed block in macroscopic renal tumour formation in $Tsc2^{+/-}$ mice [61]. shRNA against essential autophagy genes like Atg5 inhibited tumour growth of human pancreatic cancer cells in a mouse xenograft model and siRNA against Atg5 or Atg7 also showed growth attenuation in K-Ras transformed malignant cells [42, 61, 62]. Atg5 knockdown in lymphoma cells revoked the pro-survival role of autophagy and caused tumor cell apoptosis. Genetic studies in mice showed that deletion of FIP200, an essential autophagy gene can inhibit growth of mammary tumors [41]

1.6.2 Pharmacological modulation of autophagy

Pharmacological modulation of autophagy entails usage of drugs, usually small molecules which can induce or inhibit autophagy.

1.6.2.1 Autophagy inducers

The most well studied autophagy inducer is rapamycin which selectively inhibits mTORC1 complex [63]. Rapamycin shows anti proliferative activity and inhibits the progression of dermal Kaposi's sarcoma [64]. Autophagy inducers can be used in neurodegenerative diseases to clear misfolded protein aggregates. Rapamycin decreased β-amyloid protein levels and brain lesions in mouse model of Alzheimer's [65]. Increase in nitric oxide levels which blocks autophagosome formation occurs in many neurodegenerative diseases. The levels of nitric oxide can be decreased by a compound, *N*-L-arginine methyl ester (L-NAME). L-NAME induces autophagy and decreases mutant huntingtin protein levels as seen in Huntington disease models [61]. Anti cancer drug like tamoxifen which is used in breast cancer treatment also induces autophagy [66]. Another autophagy inducer, Metformin which activates AMPK signaling decreases cancer risk in diabetic patients. The role of autophagy in pathogen clearance is known and hence autophagy inducers can be used to abrogate some viral, bacterial and parasitic infections [61].

1.6.2.2 Autophagy inhibitors

Inhibitors of autophagy can be classified as early- or late-stage inhibitors. Drugs like 3-methyladenine, wortmannin, and LY294002, which target the vesicle nucleation process by inhibiting class III PI3K (Vps34) are early stage inhibitors. Lysosomotropic drugs like monensin and chloroquine/hydroxychloroquine (CQ/HCQ) and vacuolar ATPase inhibitor BafilomycinA1 affect autophagosome-lysosome fusion and are late-stage inhibitors of the pathway. Microtubule disrupting agents like nocodazole and vinblastine also prevent autophagosome-lysosome fusion. The most well studied autophagy inhibitor is CQ which is an anti-malarial drug. Autophagy inhibition using these inhibitors has exhibited enhanced chemo-sensitivity and tumor regression in xenograft models. Autophagy inhibition by CQ showed tumor cell death in *Myc*

induced murine lymphoma model. Autophagy inhibition also enhanced the antitumor effect of chemotherapy drugs and hence combination of autophagy inhibitors like CQ with anticancer drugs are being used in phase I/II clinical trials in different tumor cell types (Table 1) [38, 61, 67-69]. CQ has a long half life and micromolar concentrations of it are needed to inhibit autophagy. These factors limit its efficiency in human trials [61].

Table 1. Preclinical and ongoing clinical studies using the autophagy inhibitors chloroquine and hydroxychloroguine in cancer treatment

Tumor type	Development status	Therapeutic combination
Colorectal cancer	*In vitro*, *in vivo*	CQ + bortezomib (63)
		CQ + vorinostat (56)
	Phase II	HCQ + XELOX + bevacizumab
Gastrointestinal stromal tumor	*In vitro*, *in vivo*	CQ + imatinib (75)
Prostate cancer	*In vitro*, *in vivo*	CQ + Src kinase inhibitors (57)
Vulvar cancer	*In vitro*	CQ + cetuximab (34)
Chronic myelogenous leukemia	*In vitro*	CQ + vorinostat (73)
	Phase II	HCQ + imatinib
Lymphoma	*In vivo*	CQ + cyclophosphamide (27)
Pancreatic cancer	Phase II	HCQ only
	Phase I/II	HCQ + gemcitabine
Prostate cancer	Phase II	HCQ + docetaxel
Lung cancer	Phase II	HCQ + erlotinib
Glioblastoma multiforme	Phase I/II	HCQ + temozolomide + radiation
Multiple myeloma	Phase I/II	HCQ + bortezomib
Renal cell carcinoma	Phase I	HCQ only
Breast cancer	Phase II	HCQ only
Chronic lymphocytic leukemia	Phase II	HCQ only
Advanced solid tumor	Phase I	HCQ + sirolimus or vorinostat
	Phase I	HCQ + temsirolimus
	Phase I	HCQ + sunitinib
	Phase I	HCQ + temozolomide

Abbreviations: CQ, chloroquine; HCQ, hydroxychloroquine.

Table 1: Autophagy inhibitors in clinical trials; with permission from Yang et al., 2011

In order to target autophagy in cancer, the need of the hour is to develop more specific and potent autophagy inhibitors. Stronger autophagy inducers can be used to clear misfolded protein aggregates in neurodegeneration and to abrogate some viral, bacterial and parasitic infections. Moreover, developing new autophagy modulators will help in better understanding of the mechanism of autophagy and can also help unravel new molecular players in autophagy.

Chapter 2: Materials and Methods

2.1 Cell culture

HeLa cells were maintained in growth medium comprising of Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, D5648) supplemented with 3.7 g/L sodium bicarbonate plus 10% fetal bovine serum (PAN, 3302-P121508) and 100 units/ml of penicillin and streptomycin (Sigma-Aldrich, P4333) at 5% CO2 and 37^0C.

2.2 Antibodies and reagents

The antibodies used were as follows: LC3B (L7543, Sigma-Aldrich), β-Tubulin (MA5-16308, Pierce), GFP (11814460001, Roche Diagnostics), Anti-rabbit IgG, HRP-linked antibody (7074, CST), Anti-mouse IgG, HRP-linked antibody (172-1011, Biorad).

Reagents used were: AB-MECA (A236, Sigma-Aldrich), Elaidylphosphocholine (EPC) (01505337, MicroSource Discovery Systems Inc.), and BafilomycinA1 (B1793, Sigma-Aldrich).

2.3 CellTiter-Glo cell viability assay

Toxicity of the compound was monitored by Cell titer glo cell viability assay (Promega, G7570). HeLa cells were counted and equal numbers (1500 cells/well) were plated in 384 well plate in growth medium. Different concentrations of AB-MECA and EPC ranging from 1nM to 100μM were added onto the cells and incubated for 72 hours. After 72 hours, CellTiter-Glo Reagent was added to each well, and luminescence measured using Varioskan Flash (Thermo Scientific)

2.4 Cell culture treatments (for western blotting)

Cells were grown in six well plates until 70-80% confluence. Dose response for AB-MECA and EPC was done by treating cells with various drug concentrations ranging from 500nM to 50μM. Starvation was induced by treating cells with Earle's balanced salt solution (EBSS) (Sigma-Aldrich, E7510) supplemented with 2.2 g/L sodium bicarbonate. Autophagosome to lysosome fusion was blocked by treating cells with BafilomycinA1 at a final concentration of 100nM in growth medium. All treatments were done for 2 hours until otherwise stated.

2.5 Western blotting

Following appropriate treatments, cells were washed with ice cold PBS. Cells were then lysed in 100 µl of sample buffer (10%w/v SDS, 10mM DTT, 20%v/v glycerol, 0.2M Tris-HCL pH 6.8, 0.05%w/v bromophenol blue) and then collected using a rubber cell scraper. The lysates were boiled at 99^0C for 15 minutes and stored at −20°C. Western blotting was performed using standard methods.

2.6 Traffic light assay

HeLa cells were grown on coverslips in 60mm dish until 60-70% confluency. Growth medium was removed and fresh growth medium (2.5ml) was added. The dish was kept in 37^0C incubator for few minutes to allow pre-warming of the medium. Cells were transfected with tandem RFP-EGFP-LC3 construct (Addgene plasmid #21074) using Lipofectamine2000 (11668-019, Invitrogen). 2.5µg of DNA and 5µl of Lipofectamine (1:2 ratio) was diluted in 100µl of OPTI-MEM (31985-070, Invitrogen) separately. After 5 minutes of incubation at room temperature, DNA and Lipofectamine solutions were mixed together and incubated again for 20 minutes (room temperature). Then, DNA-lipid complex was mixed properly and added to the cells. 6 hours after transfection, media containing DNA-lipid complex was removed, cells were washed with D-PBS and fresh growth medium (4ml) was added.

72 hrs after transfection cells were either left untreated or treatment with various concentrations of AB-MECA or EPC was done. Starvation was induced by treating cells with Earle's balanced salt solution (EBSS) and autophagosome to lysosome fusion was blocked by treating cells with BafilomycinA1 in growth medium. All treatments were done for 2 hours until otherwise stated. After treatment, cells were fixed in 4% paraformaldehyde and permeabilized using 0.25% Triton X-100. The coverslip was mounted with Vectashield antifade reagent (H-1000, Vector laboratories). Imaging was carried out using Delta vision microscope (Olympus 60X/1.42, Plan ApoN, excitation and emission filter FITC and TRITC, polychroic Quad, transmittance 10%, exposure 0.1 sec and 1 X 1 binning). On an average 20 stacks were taken for each image and distance between stacks was 0.4µm. Deconvolution was done using Enhanced Ratio algorithm and images were projected as Maximum Intensity Projection. Manual

counting was done using cell counter plugin of ImageJ fiji software. On an average, 15 cells were counted per treatment for each experiment.

2.7 Yeast strains and plasmids

Wild type *Saccharomyces cerevisiae*, BY4742 Pot1-GFP strain is a laboratory strain with genomically tagged GFP to the C terminus of Pot1 (HIS selection marker) obtained from Dr. Rachubinski. Wild type BY4741 was obtained from EUROpean *Saccharomyces cerevisiae* ARchive for Functional Analysis (EUROSCARF). *S. cerevisiae* shuttle vector pRS316 (URA) was obtained from Prof. Suresh Subramani.

2.8 Pexophagy Assay

Pot1-GFP positive strains were allowed to grow till the A_{600} reaches 0.8-1 in YPD (1% yeast extract, 2 % peptone, and 2% dextrose) medium. Peroxisome biogenesis was induced by growing these cells in YPG medium (1% yeast extract, 2% peptone and 3% glycerol) for 12 hours. Cells were harvested, washed twice to remove traces of YPG and transferred to SD-N (nitrogen starvation) medium, at inoculum density A_{600} =3, to induce pexophagy. Cells were collected at various time intervals after pexophagy induction and processed by TCA method (Jain et al. 2010).

2.9 TCA precipitation

All samples (A_{600} =3) were collected in 12.5% TCA final concentration and stored at -80°C for at least half an hour. Later, the samples were thawed on ice and centrifuged for 10 minutes at 16000 *g*, pellet was washed with 250µl of ice cold 80% acetone twice and air dried. This pellet was resuspended in 40µl of 1% SDS- 0.1N NaOH solution. Sample buffer (5X, 10µl) was added to the lysate and boiled for 10 minutes before loading; 0.3 A_{600} equivalent cells were loaded per well in SDS-PAGE. Western blotting was performed using standard methods.

2.10 GFP-Atg8 processing assay

S. cerevisiae strain containing the GFP-Atg8 (pRS 316 vector backbone) plasmid was grown in synthetic complete medium lacking uracil (SC-URA) under appropriate conditions (30^0C, 250 rpm). From this, a secondary culture was inoculated at A_{600}=0.2 and grown as above until A_{600} reached ~0.65. The cultures were transferred to SD-N (nitrogen starvation) medium at A_{600}=3, separately with and without 50µM of drugs

(AB-MECA or EPC), and time points (0, 1, 2, 4, and 6 h) were collected at A_{600} equivalent of 3. Sample preparation was done by the TCA precipitation method and western blotting was performed using standard methods.

Chapter 3

Small molecule inhibitor of autophagy: EPC

3.1 Background Information

Elaidylphosphocholine (EPC) is a mono-1-elaidyl ester of phosphocholine. It stands for (E)-octadec-9-enylphosphocholine; (9E)-octadec-9-en-1-ylphosphocholine. EPC has been found to have antibacterial activity against vancomycin-resistant Enterococcus and *Staphylococcus aureus* and antifungal activity against *Cryptococcus neoformans* and *Candida albicans* **(PubChem)**. EPC was identified as hit in a screen for autophagy inhibitors using a yeast high throughput assay by our laboratory.

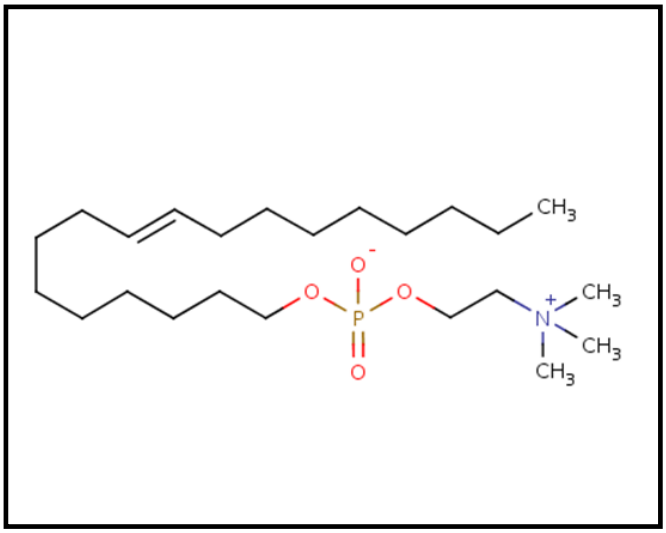


Figure 1: Structure of EPC (PubChem)

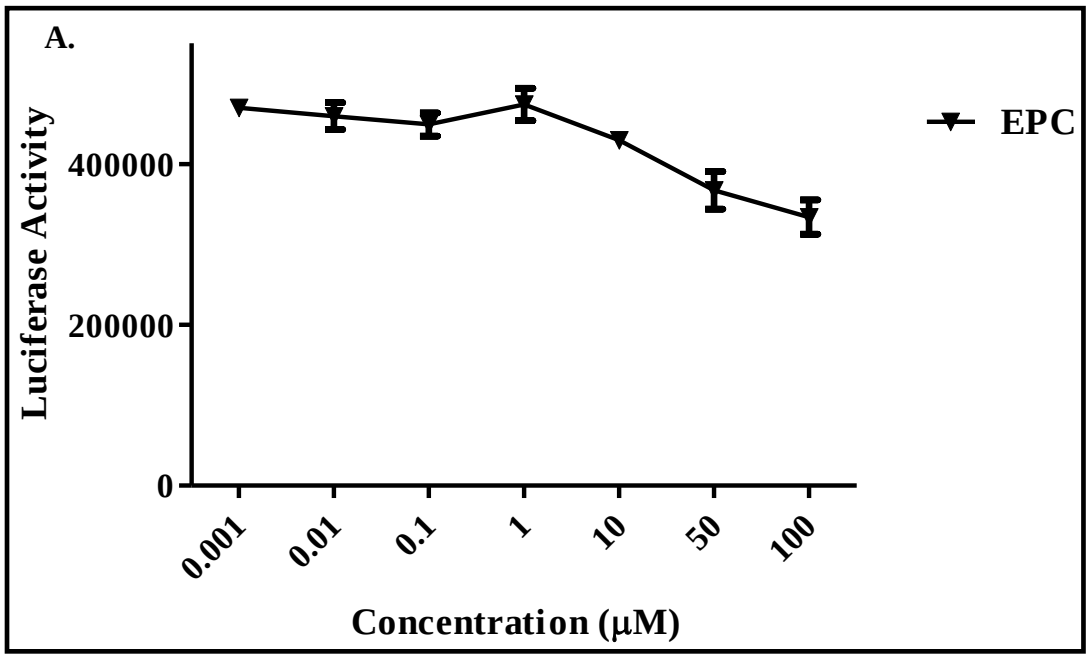
3.2 Results and discussions

As EPC was identified as hit in a luciferase based high throughput screen for autophagy inhibitors, further validation of this small molecule was carried out using secondary assays for autophagy in a mammalian cell culture system. These assays included toxicity assays of the molecule towards the cells, and western blotting and imaging based assays to characterise potential autophagy modulation by EPC.

3.2.1 CellTiter-Glo cell viability assay

The CellTiter-Glo Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present. Cell Titer Glo reagent is directly added onto the cells to lyse them and release ATP which reacts with luciferase and converts it to oxyluciferin. This generates a stable "glow-type" luminescent signal which can be read in a luminometer. This assay can be done performed in a high throughput manner to determine cytotoxic effects of small molecules.

This assay was employed to test the effect of EPC on the growth and viability of mammalian cells. HeLa cells were treated with concentrations of EPC ranging from 1nM to 100μM for 72 hours. After 72 hours, cells were lysed using Cell Titer Glo reagent and luciferase activity was measured. At the highest concentration of EPC (100μM) there was 40% decrease in cell viability. So, for further work EPC concentration of 50μM and below was used.



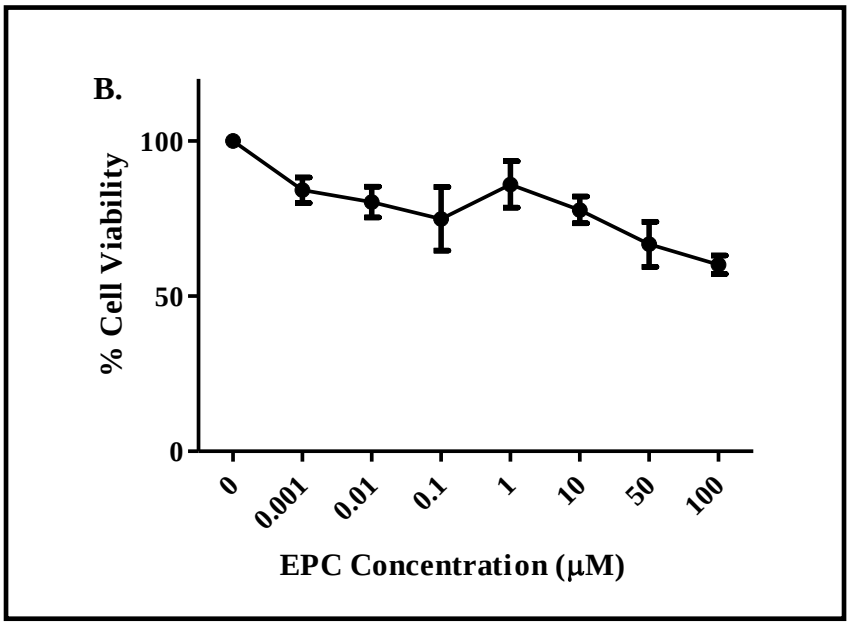


Figure 2: Cell viability assay; A. HeLa cells were treated with concentrations of EPC ranging from 1nM to 100μM for 72 hours and luciferase activity is plotted versus EPC concentration. B. Percentage cell viability is estimated using luciferase activity quantitation and plotted versus EPC concentration. Data represented here is from five replicates for the experiment.

3.2.2 Dose response for EPC in HeLa cells

We next wanted to test if the compound modulated autophagy in mammalian system in a dose dependent manner.

3.2.2.1 LC3 Conversion Assay

In order to check the effect of EPC in mammalian system, LC3 conversion assay (as described earlier) was performed by treating HeLa cells with increasing dose of EPC starting from 500nM to 50μM. EPC showed a dose dependent accumulation of LC3-II form (figure 3) which can be due to induction of autophagy or a block in fusion of the autophagosomes with that of lysosomes by the small molecule.

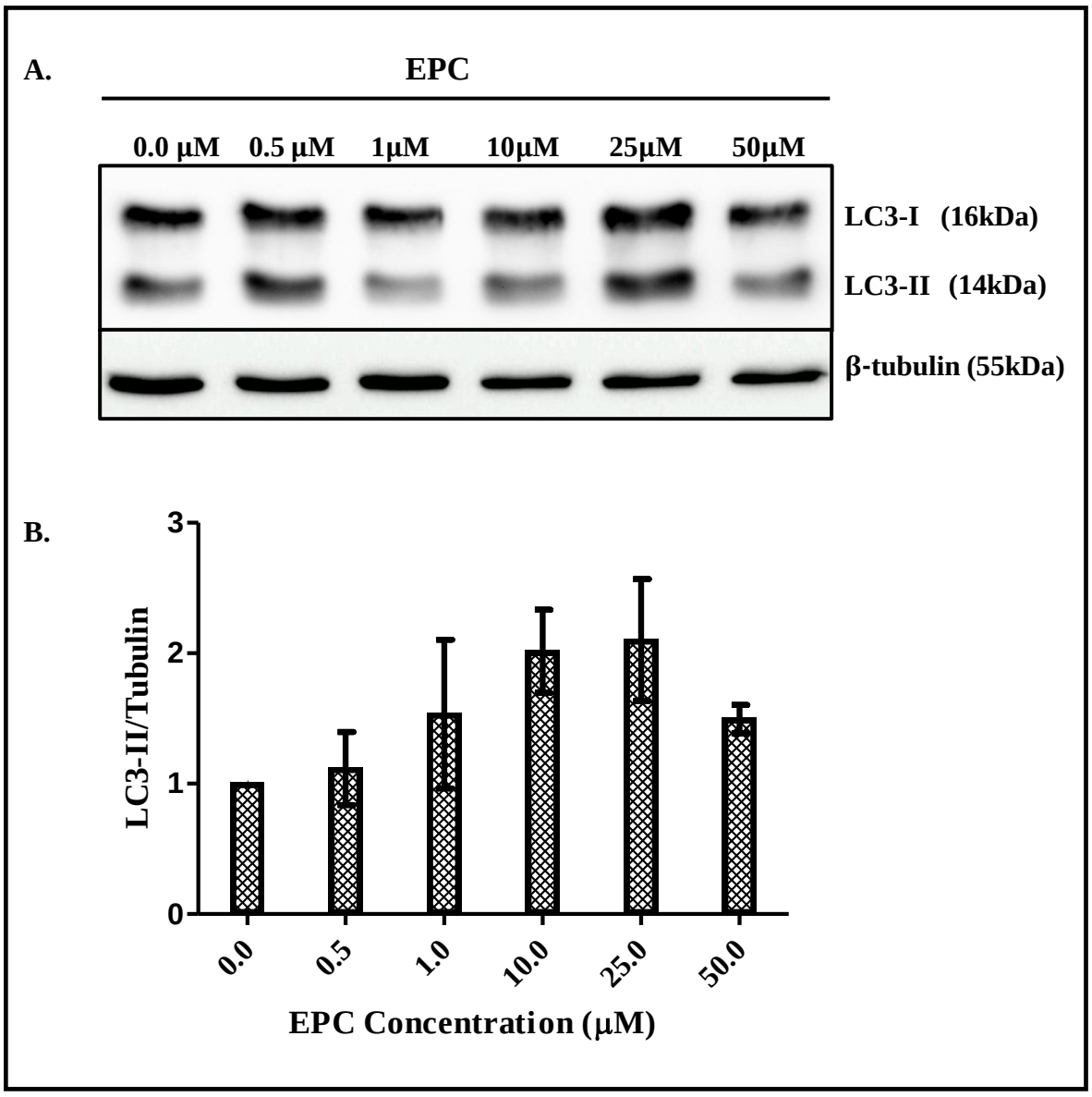


Figure 3:LC3 Conversion assay; A. HeLa cells were either left untreated (Control) or treated with increasing dose of EPC for 2 hours. B. Levels of LC3-II were normalized to β-tubulin for all treatments as above. Data are from three independent experiments; the fold change difference between LC3-II levels of Control and EPC is plotted.

3.2.2.2 Traffic light assay

The traffic light assay is useful in studying the autophagic flux. In this assay, a tandem fluorescent tagged LC3 construct is used as a reporter which has LC3 tagged to mRFP and GFP (ptfLC3). The idea behind this methodology is that due to the double tagging, autophagosomes will appear yellow but when they fuse with lysosomes, the GFP fluorescence will quench due to low pH of lysosome and hence autolysosomes will appear red. Thus, this assay gives a clear picture of the autophagic flux status of a cell. Induction of autophagy either due to starvation or a chemical inducer of autophagy will cause a significant increase in number of yellow and red dots (autophagosomes and autolysosomes respectively).

Traffic light assay was carried in presence of EPC to investigate further whether the accumulation of LC3-II form in the LC3 conversion assay (Figure 3) is due to induction of autophagy or a block in fusion of the autophagosomes with that of lysosomes by the small molecule. We tried various doses of EPC ranging from 1 to 25μM. Treatment with EPC (1μM) for 2 hours in ptfLC3 expressing HeLa cells showed slight increase in number of autophagosomes but no significant increase in number of autolysosomes (Figure 4). Treatment with EPC (10μM) for 2 hours in ptfLC3 expressing HeLa cells showed slight increase in number of autophagosomes and autolysosomes (Figure 5). EPC (25μM) treatment for 2 hours inhibited fusion between autophagosomes (yellow dots) and autolysosomes (red dots) in ptfLC3 expressing HeLa cells which is evident from the fold change difference between number of autophagosomes and autolysosomes (Figure 6B). We compared the number of autophagosomes and autolysosomes between BafA1, a known inhibitor of autophagosome-lysosome fusion and EPC which showed that EPC decreases number of autolysosomes over and above BafA1 treatment (Figure 6C). EPC (25μM) treatment for 2 hours showed almost 25% decrease in percentage of autolysosomes and a concomitant 25% increase in percentage of autophagosomes as compared to untreated control (Figure 6D,E).

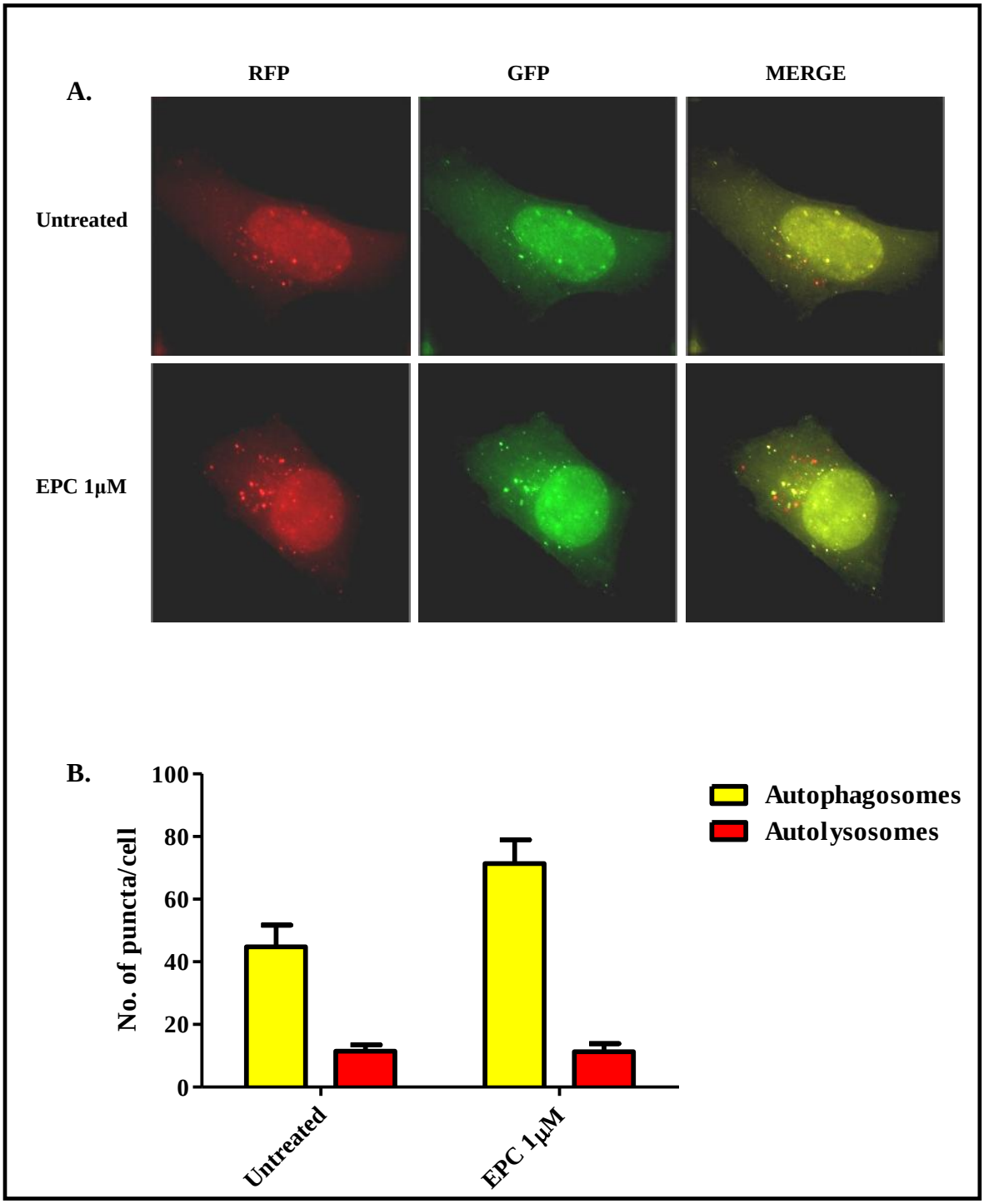


Figure 4: Traffic light assay with EPC 1 µM; A. Treatment with EPC (1µM) for 2 hours in ptfLC3 expressing HeLa cells. Scale bar: 25µm. B. Comparison of number of autophagosomes (yellow dots) and autolysosomes (red dots) in untreated control versus EPC (1µM) treatment. Data is expressed in terms of mean with SEM, n=15 cells.

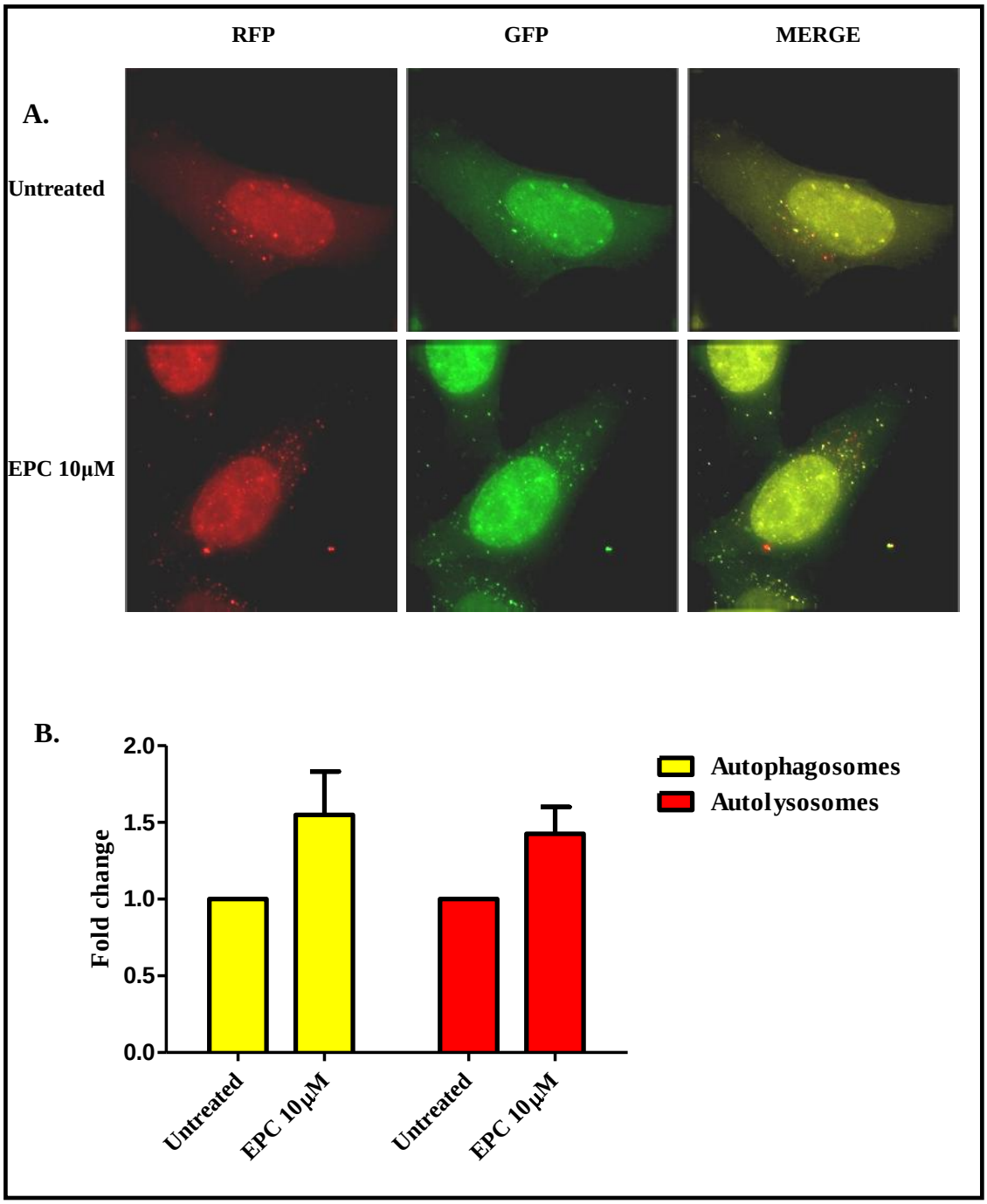
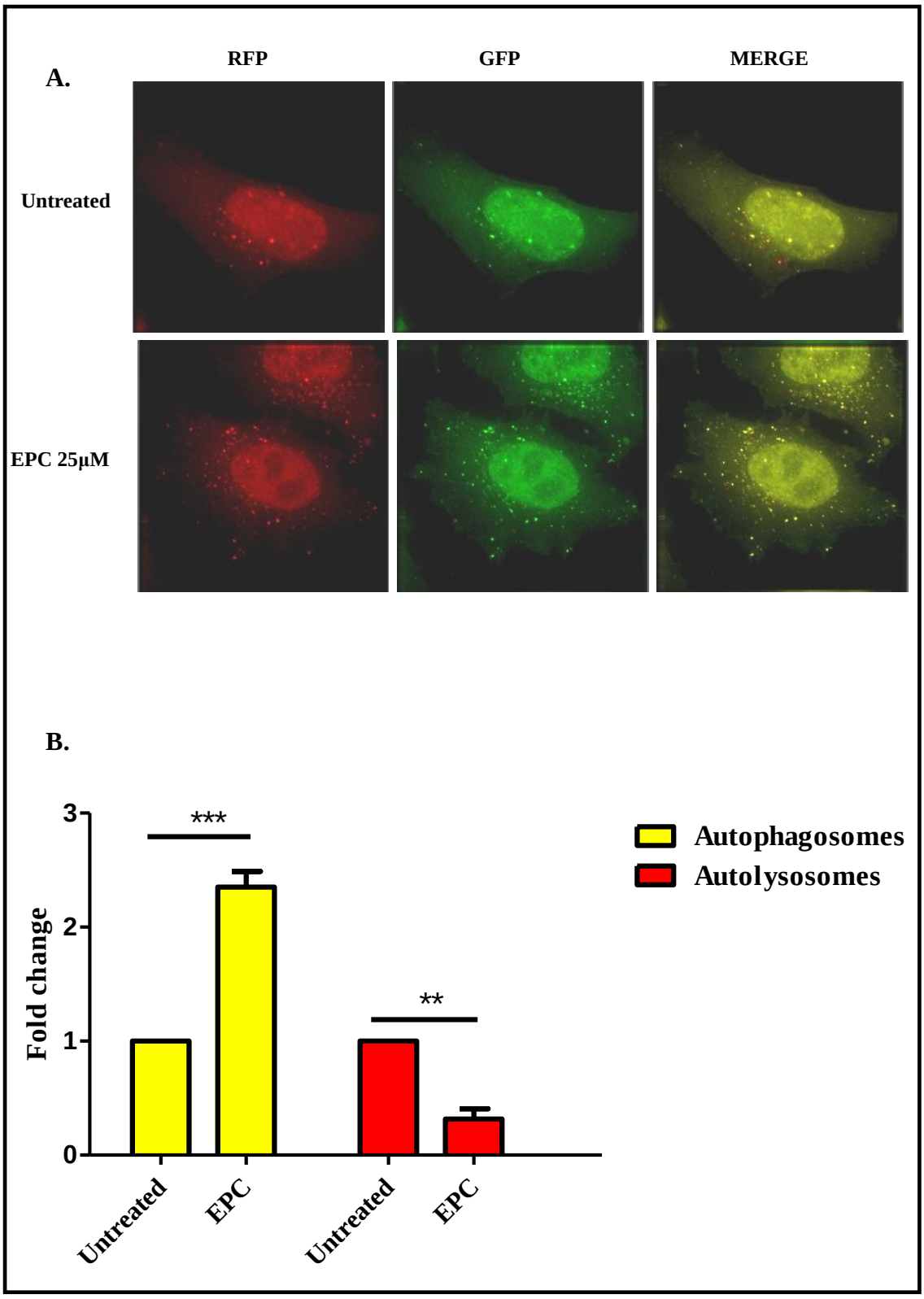
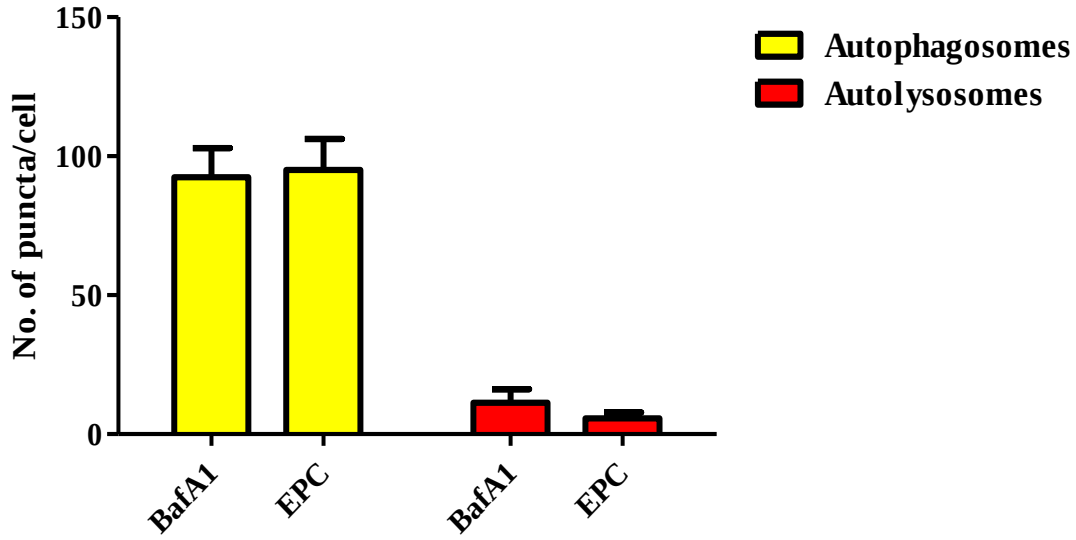


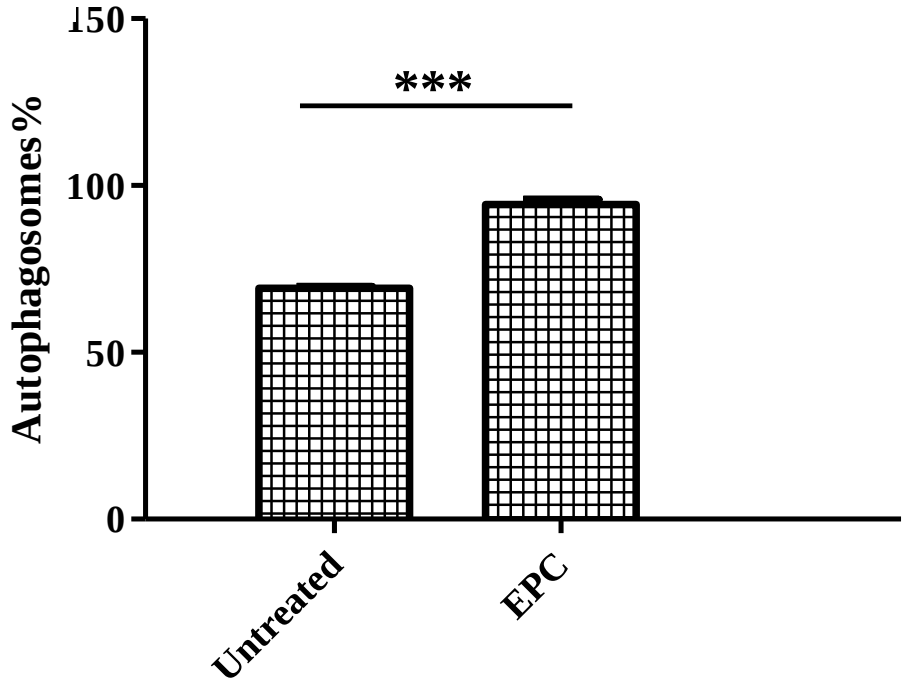
Figure 5: Traffic light assay; A. Treatment with EPC (10µM) for 2 in ptfLC3 expressing HeLa cells. Scale bar: 25µm. B. Quantification of fold change difference between number of autophagosomes and autolysosomes in untreated control versus EPC (10µM) treatment. Data is expressed in terms of mean with SEM, n=50 cells from three independent experiments.


A.
RFP
GFP
MERGE
Untreated
EPC 25µM
B.
Fold change
3
2
1
0
***
**
Autophagosomes
Autolysosomes
Untreated
EPC
Untreated
EPC

C.


150
100
50
0
No. of puncta/cell
Autophagosomes
Autolysosomes
BafA1
EPC
BafA1
EPC

D.


150
100
50
0
Autophagosomes %
***
Untreated
EPC

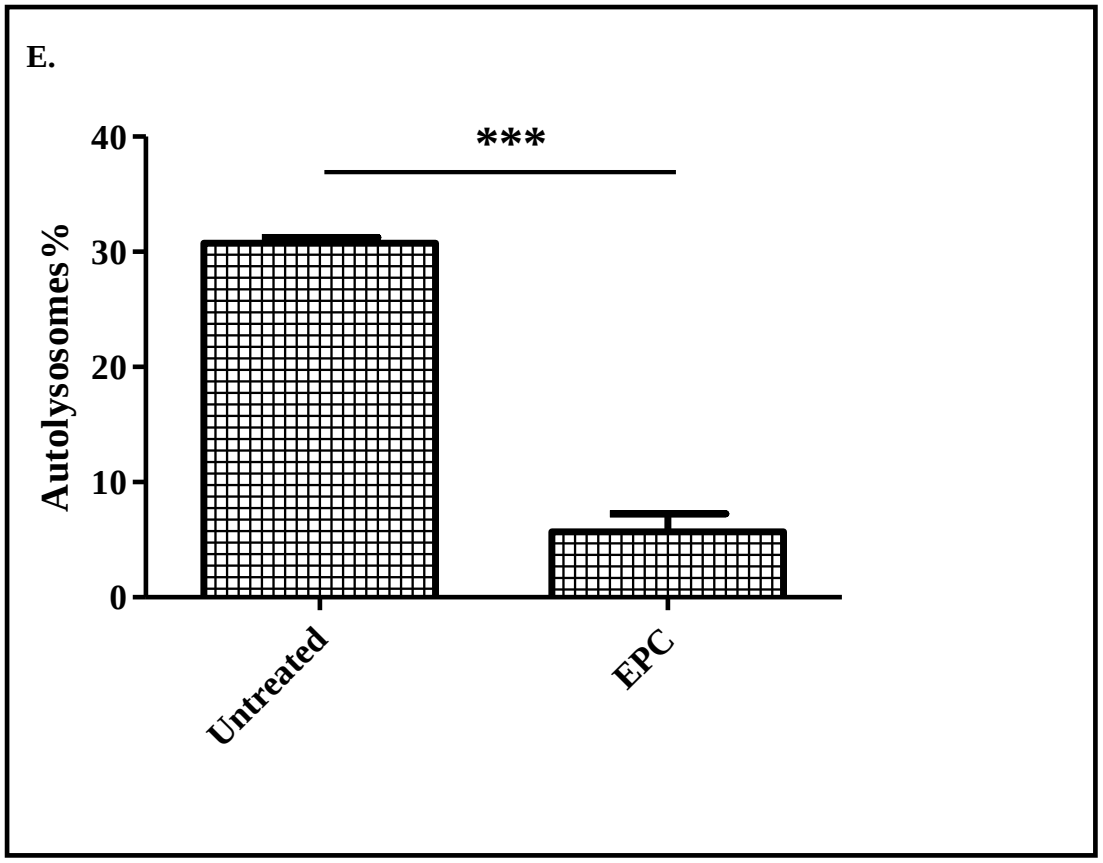


Figure 6: Traffic light assay A. Treatment with EPC (25µM) for 2 hours inhibits fusion between autophagosomes (yellow dots) and autolysosomes (red dots) in ptfLC3 expressing HeLa cells. Scale bar: 25µm. B. The fold change difference between number of autophagosomes and autolysosomes in untreated control versus EPC (25µM) treatment is statistically significant ($p<0.001$ and $p<0.01$ respectively using two-way ANOVA). C. Comparison of number of autophagosomes and autolysosomes between BafA1 and EPC (25µM) treatment. D. Difference in percentage of autophagosomes in untreated control versus EPC (25µM) treatment is statistically significant ($p<0.001$ using two-way ANOVA) E. Difference in percentage of autolysosomes in untreated control versus EPC (25µM) treatment is statistically significant ($p<0.001$ using two-way ANOVA). Data is expressed in terms of mean with SEM, n=50 cells from three independent experiments.

Chapter 4

Small molecule inducer of autophagy: AB-MECA

4.1 Background information

AB-MECA stands for N^6-(4-Aminobenzyl)-9-[5-(methylcarbonyl)-β-D-ribofuranosyl] adenine, N^6-(4-Aminobenzyl)-N-methylcarboxamidoadenosine. It is also supplied as a part of Sigma's Library of Pharmacologically Active Compounds (LOPAC). AB-MECA was identified as hit in a screen for autophagy modulators in our laboratory using a yeast high throughput assay. Adenosine is a purine nucleoside that has variety of physiological functions. Adenosine signaling occurs through adenosine receptors which are of four types: A1, A2A, A2B and A3. Adenosine receptors are a class of G-protein coupled receptors which have adenosine as their endogenous ligand. AB-MECA is a high affinity A3 adenosine receptor agonist. Known functions of A3 adenosine receptor include neuroprotective and neurodegenerative effects and involvement in both cell proliferation and cell death [70, 71]. Derivative of AB-MECA, thio-Cl-IB-MECA is known to inhibit cell proliferation in lung cancer cells through cell cycle arrest and apoptosis[72]. Another adenosine analog (IB-MECA) inhibits anchorage-dependent cell growth of various human breast cancer cell lines[73].

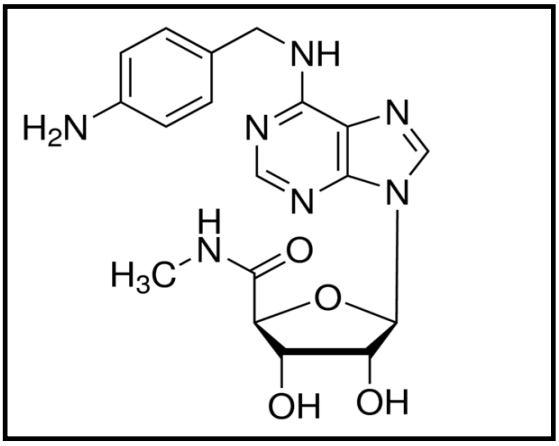


Figure 1: Structure of AB-MECA (PubChem)

4.2 Results and discussions

As AB-MECA was identified as hit in a luciferase based high throughput screen for autophagy modulators, further validation of this small molecule was carried out using secondary assays for autophagy in both yeast and mammalian systems.

4.2.1 Growth assay in yeast

Growth assay is performed in order to monitor any toxic effects of a given drug on *S. cerevisiae* cell viability and growth. Early exponential phase yeast culture (A_{600}=0.1) growing in YPD in a 96 well plate under normal growth conditions (30^0C, 300rpm) was used for growth assay. Cells were either left untreated or treated with AB-MECA (50µM). Absorbance reading at 600nm was taken every 30 minutes for 24 hours in plate spectrophotometer reader and graph was plotted. AB-MECA did not show any toxic effects on the growth of yeast as is evident from the growth curve (Figure 2).

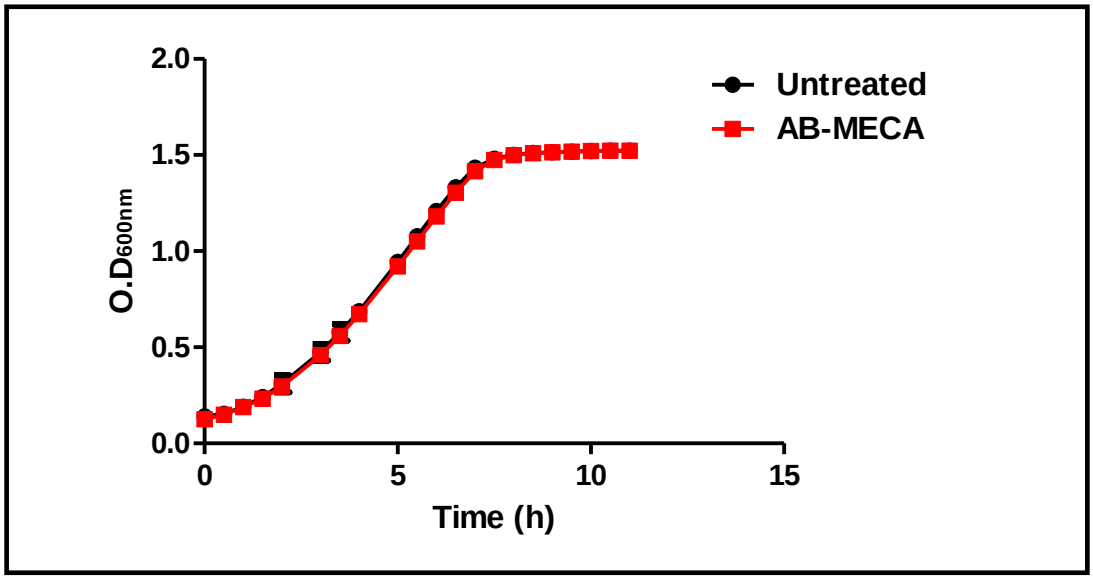


Figure 2: Growth assay in yeast. Early exponential phase yeast culture growing in YPD was seeded in a 96 well plate under normal growth conditions (30^0C, 300rpm). Cells were either left untreated or treated with AB-MECA (50µM). Absorbance reading at 600nm was taken every 30 minutes for 24 hours in plate spectrophotometer reader (Varioskan Flash, Thermo Scientific). Data from four replicates is expressed in terms of absorbance at 600nm plotted against time (in hours).

4.2.2 Pexophagy assay

In order to monitor selective autophagy, autophagic cargo is tagged with GFP and its degradation is monitored over time. For pexophagy assay, a peroxisomal resident protein Pot1 is tagged with GFP. Cells are grown in fatty acid or glycerol rich medium to induce peroxisome biogenesis. When the cells are moved to starvation medium (nitrogen starvation) Pot1 protein as a cargo is trapped in autophagosomes and delivered to vacuole. Pot1 gets degraded and free GFP appears in the vacuole which can also be observed as a free GFP band (around 26kDa) in a western blot. Decrease in the fusion band of Pot1-GFP and concomitant increase in the free GFP band shows induction of autophagy. Pexophagy assay was done with AB-MECA to test its effect as an autophagy inducer. Treatment of *S. cerevisiae* wild type cells expressing Pot1-GFP (sPM2 strain) with AB-MECA (50µM) increased release of free GFP and decreased the level of fusion protein over time as compared to the untreated control (Figure 3). This ascertains that AB-MECA (50µM) acts as an inducer of autophagy.

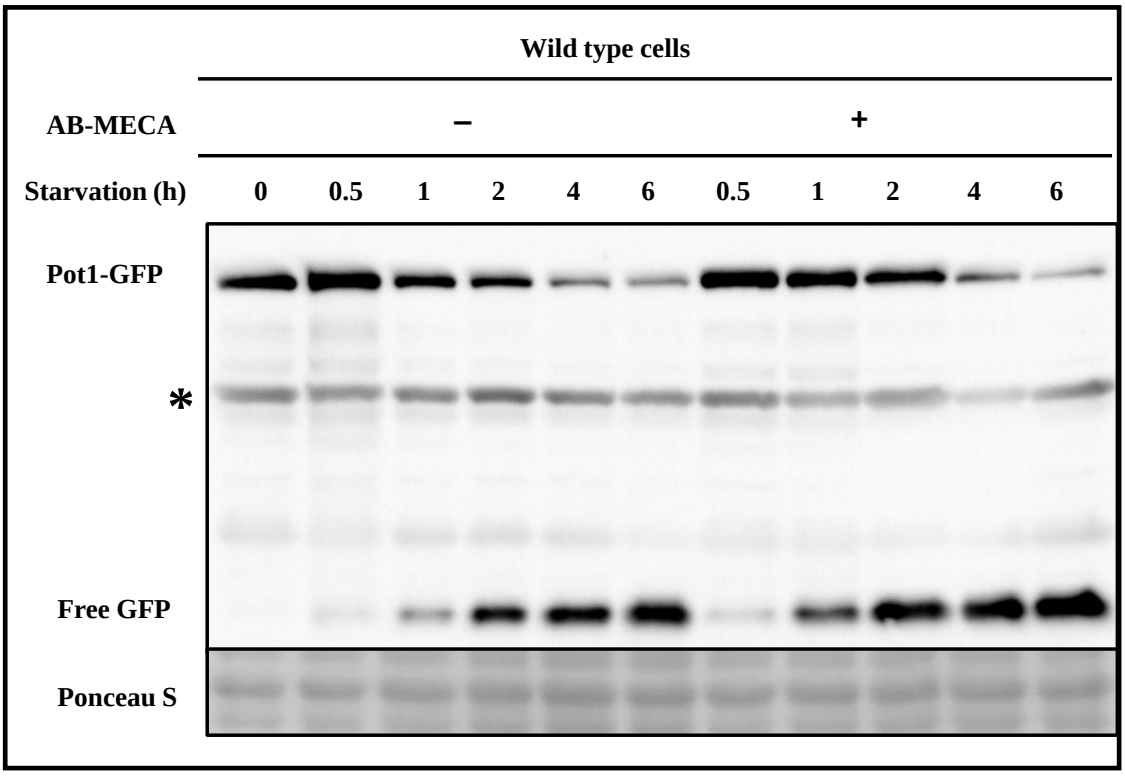


Figure 3: Pexophagy assay. AB-MECA increases the level of free GFP, as evident in 1, 2, 4 and 6h time points relative to that of untreated cells. Wild type cells expressing Pot1-GFP (sPM2) were grown to mid log phase in YPD medium and then transferred to nitrogen starvation medium (SD-N) with and without AB-MECA (50µM).

4.2.3 GFP-Atg8 processing assay

GFP–autophagy-related protein 8 (GFP-Atg8) processing assay is used to study general autophagy. In this assay, Atg8 protein which gets lipidated and attached to the autophagosomal membrane is tagged with N-terminal GFP. Upon induction of autophagy due to external cues such as starvation, autophagosomes are generated and they eventually fuse with vacuole and Atg8 along with GFP is released inside. Atg8 gets degraded by the action of vacuolar proteases while GFP which is resistant to protease action accumulates inside the vacuole. Increase in levels of free GFP over time shows induction of autophagy.

GFP-Atg8 processing assay was done with AB-MECA to ascertain its effect on autophagic flux. Treatment of *S. cerevisiae* wild type cells expressing GFP-Atg8 (pRS316) with AB-MECA (50µM) increased release of free GFP over time as compared to the untreated control (Figure 4). This result alsoshows that AB-MECA (50µM) acts as an inducer of autophagy and is able to stimulate autophagy over and above autophagy induction achieved by starvation.

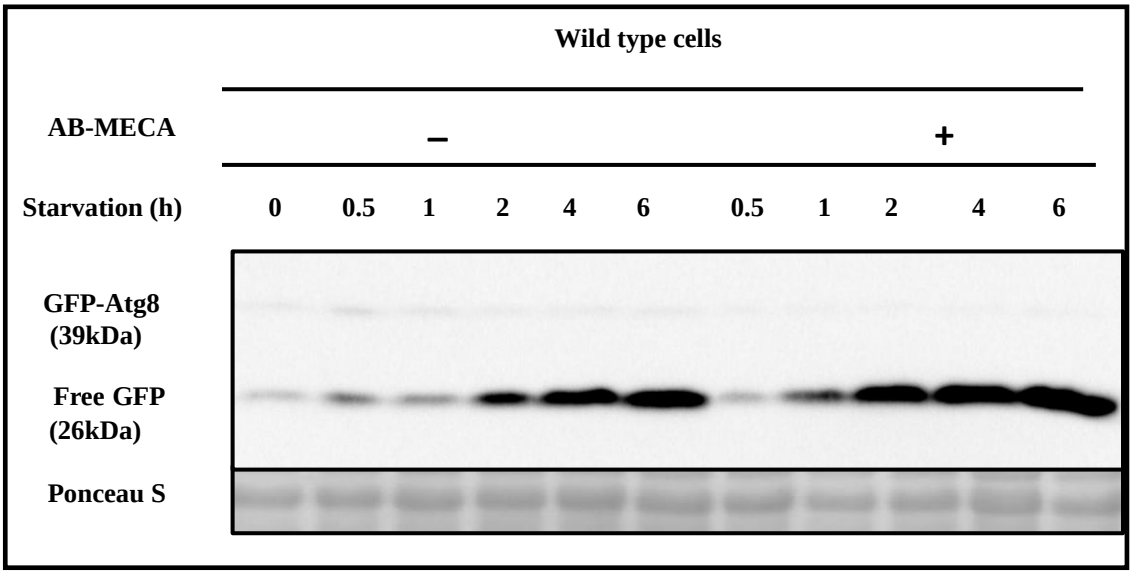
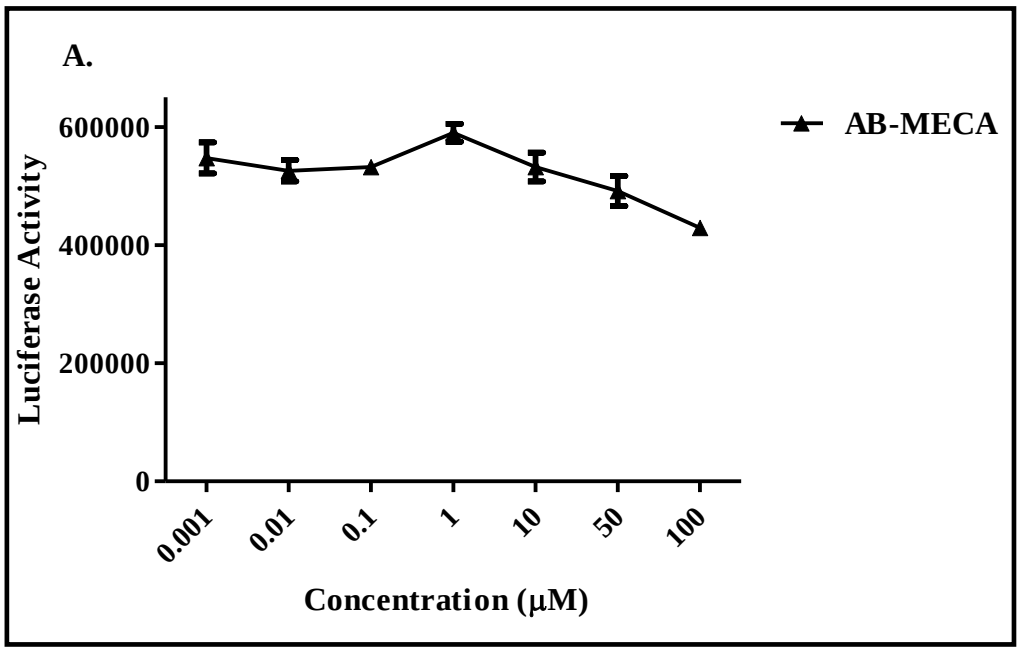


Figure 4: GFP-Atg8 processing assay. Wild type cells expressing GFP-ATG8 were grown to mid log phase in SD-URA medium and then transferred to nitrogen starvation medium (SD-N) with and without AB-MECA (50µM) and samples collected at indicated time points.

4.2.4 CellTiter-Glo cell viability assay

The CellTiter-Glo Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present. This assay can be done performed in a high throughput manner to determine cytotoxic effects of small molecules. Cell Titer glo reagent is directly added onto the cells to lyse them and release ATP which reacts with luciferase and converts it to oxyluciferin. This generates a stable "glow-type" luminescent signal which can be read in a luminometer

This assay was employed to test the effect of AB-MECA on the growth and viability of mammalian cells. HeLa cells were treated with concentrations of AB-MECA ranging from 1nM to 100µM for 72 hours. After 72 hours, cells were lysed using Cell Titer glo reagent and luciferase activity was measured. AB-MECA does not show toxicity at the tested concentrations in HeLa cells (Figure 5A and B).



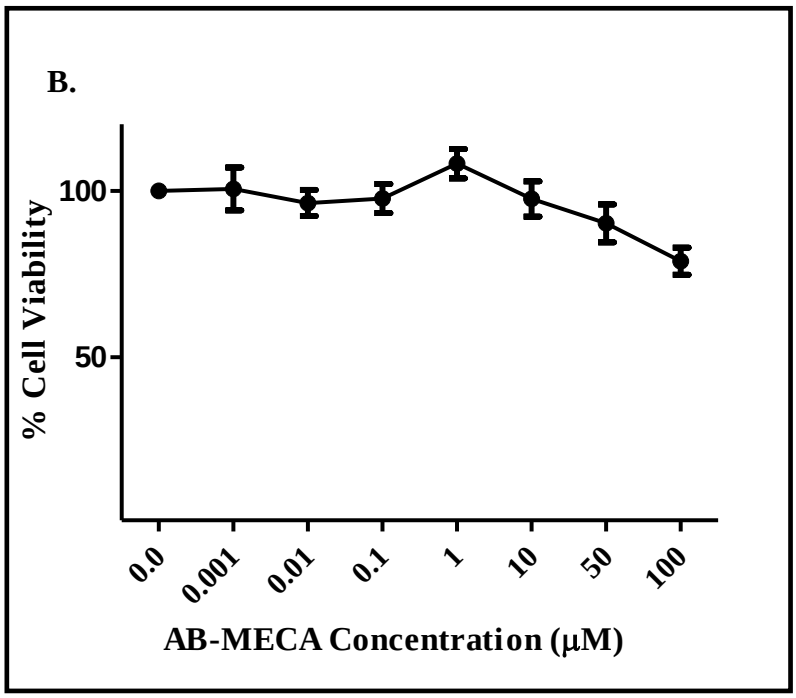


Figure 5: Cell viability assay; A. HeLa cells were treated with concentrations of AB-MECA ranging from 1nM to 100μM for 72 hours and luciferase activity was quantitated B. Percentage cell viability is estimated using luciferase activity quantitation and plotted versus AB-MECA concentration. Data represented here is from five replicates for the experiment.

4.2.5 Dose response for AB-MECA in HeLa cells

Next, we wanted to test if the compound induced autophagy in mammalian systems as well.

4.2.5.1 LC3 Conversion Assay

In order to check the effect of AB-MECA in mammalian system, LC3 conversion assay (as described earlier) was performed by treating HeLa cells with increasing dose of AB-MECA starting from 500nM to 50μM. AB-MECA showed a dose dependent accumulation of LC3-II form (figure 6A) which can be due to induction of autophagy or a block in fusion of the autophagosomes with that of lysosomes by the small molecule.

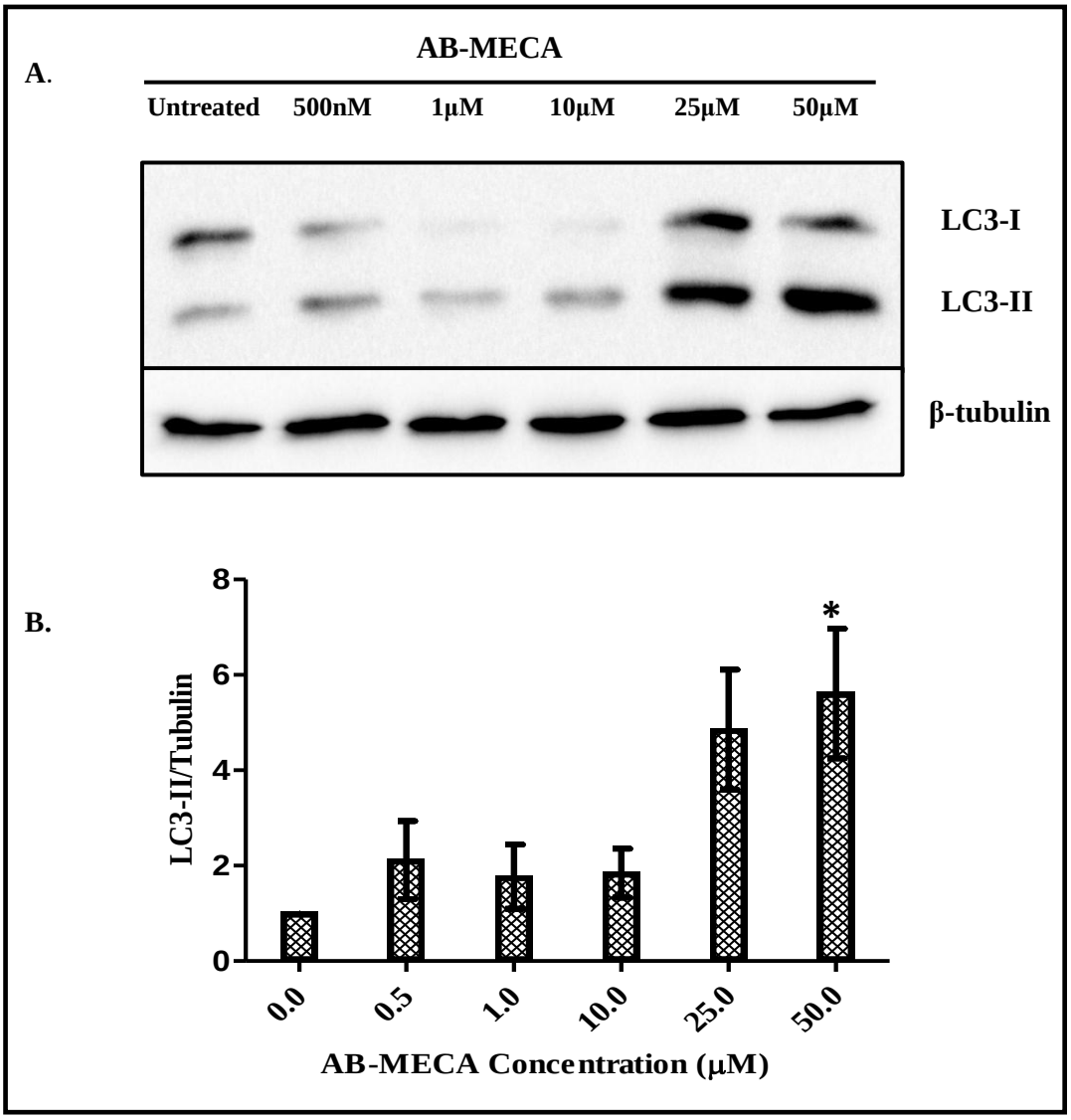


Figure 6: A. LC3 Conversion assay; HeLa cells were either left untreated (Control) or treated with increasing dose of AB-MECA for 2 hours. B. Levels of LC3-II were normalized to β-tubulin for all treatments as above. Data are from three independent experiments; the fold change difference between LC3-II levels of Control and AB-MECA (50µM) is statistically significant ($p<0.05$ using two way ANOVA).

4.2.5.2 Traffic light assay

The traffic light assay is useful in studying the autophagic flux. In this assay, a tandem fluorescent tagged LC3 construct is used as a reporter which has LC3 tagged to mRFP and GFP (ptfLC3). The idea behind this methodology is that due to the double tagging, autophagosomes will appear yellow but when they fuse with lysosomes, the GFP fluorescence will quench due to low pH of lysosome and hence autolysosomes will appear red. Thus, this assay gives a clear picture of the autophagic flux status of a cell. Induction of autophagy either due to starvation or a chemical inducer of autophagy will cause a significant increase in number of yellow and red dots (autophagosomes and autolysosomes respectively).

Traffic light assay was carried in presence of AB-MECA to validate the results obtained earlier that it induces autophagy in HeLa cells in a dose dependent manner. We tried two doses of AB-MECA (25 and 50 μM) which had shown accumulation of LC3-II form in the LC3 conversion assay (Figure 6). Treatment with AB-MECA (25μM) for 2 hours increased the number of autophagosomes (yellow dots) and autolysosomes (red dots) in ptfLC3 expressing HeLa cells (Figure 7A). However, the fold change difference between number of autophagosomes and autolysosomes in untreated control as compared to AB-MECA (25μM) treatment was not significant (Figure 7B).

Treatment with AB-MECA (50μM) for 2 hours induced autophagy by increasing the number of both autophagosomes and autolysosomes in ptfLC3 expressing HeLa cells (Figure 8A).The fold change difference between number of autophagosomes and autolysosomes in untreated control as compared to AB-MECA (50μM) treatment is statistically significant (Figure 8B). In order to test the potency of the inducer, we compared it with the induction as seen in starvation medium (EBSS). AB-MECA increased the number of autophagosomes over and above the starvation medium (EBSS) (Figure 8D).

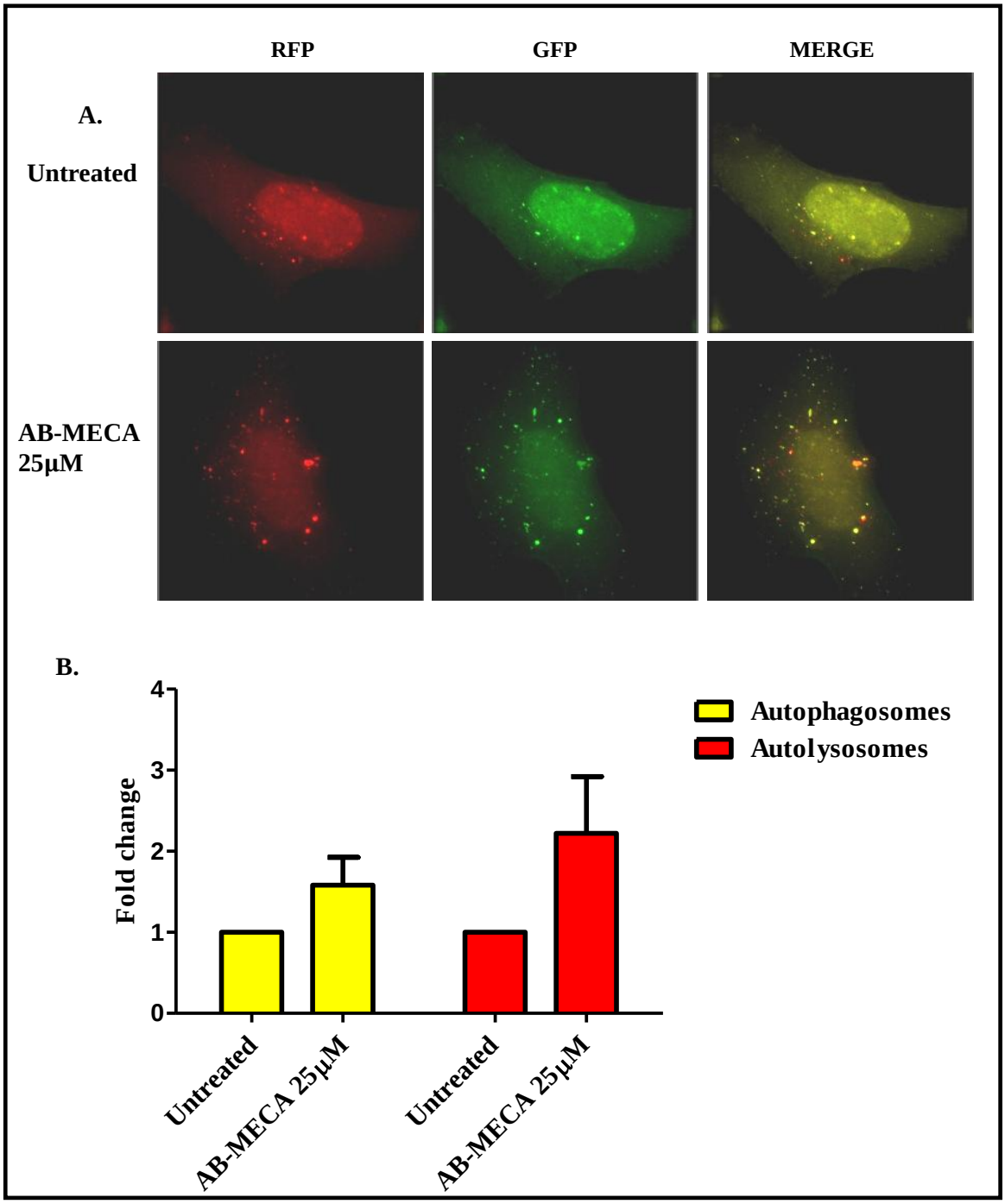
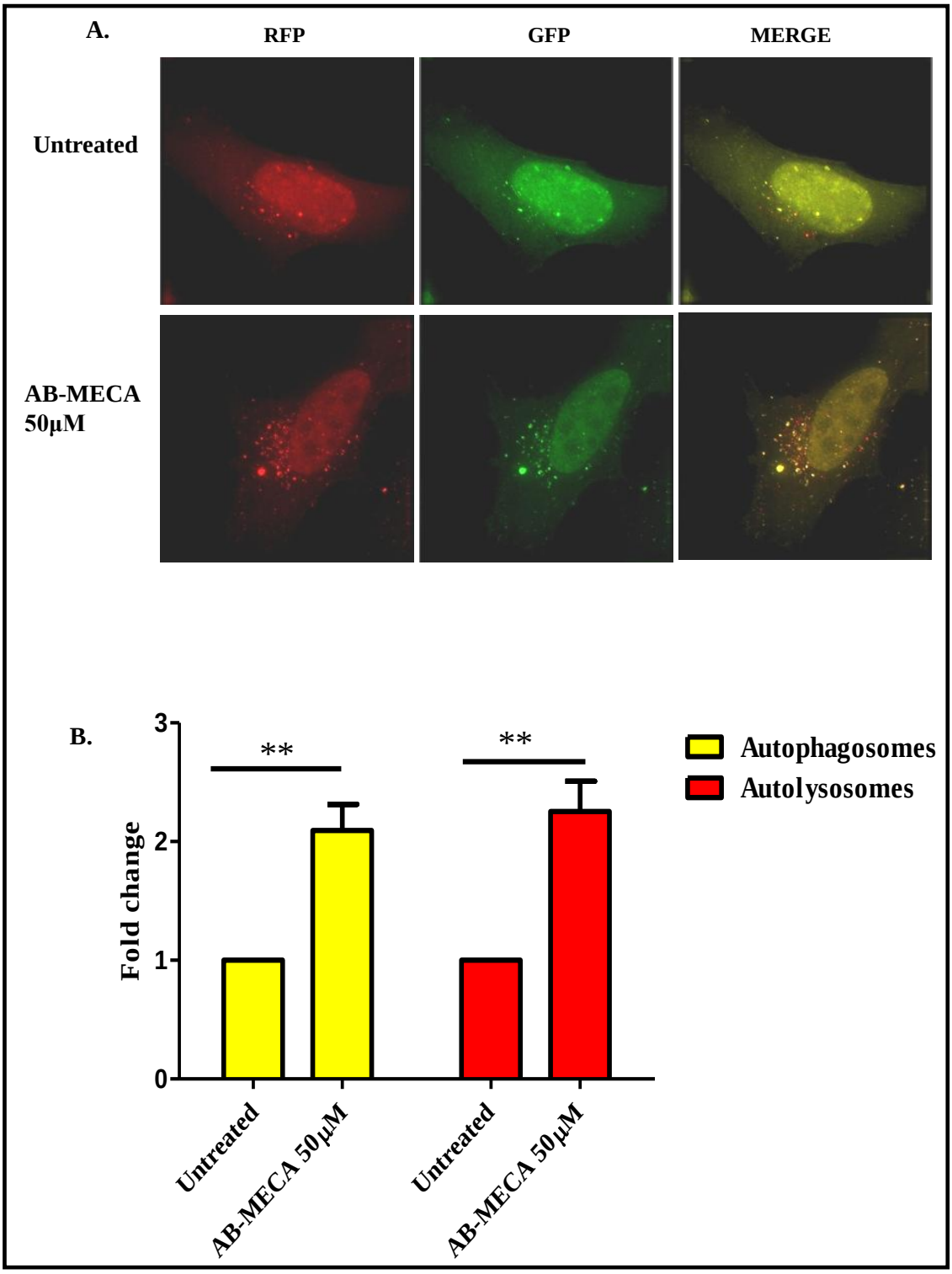


Figure 7: Traffic light assay A. Treatment with AB-MECA (25µM) for 2 hours increased the number of autophagosomes (yellow dots) and autolysosomes (red dots) in ptfLC3 expressing HeLa cells. Scale bar: 25µm. B. Quantification of fold change difference between number of autophagosomes and autolysosomes in untreated control versus AB-MECA (25µM) treatment. Data is expressed in terms of mean with SEM, n=50 cells from three independent experiments.


A.
RFP
GFP
MERGE
Untreated
AB-MECA 50µM
B.
Fold change
3
2
1
0
**
**
Autophagosomes
Autolysosomes
Untreated
AB-MECA 50µM
Untreated
AB-MECA 50µM

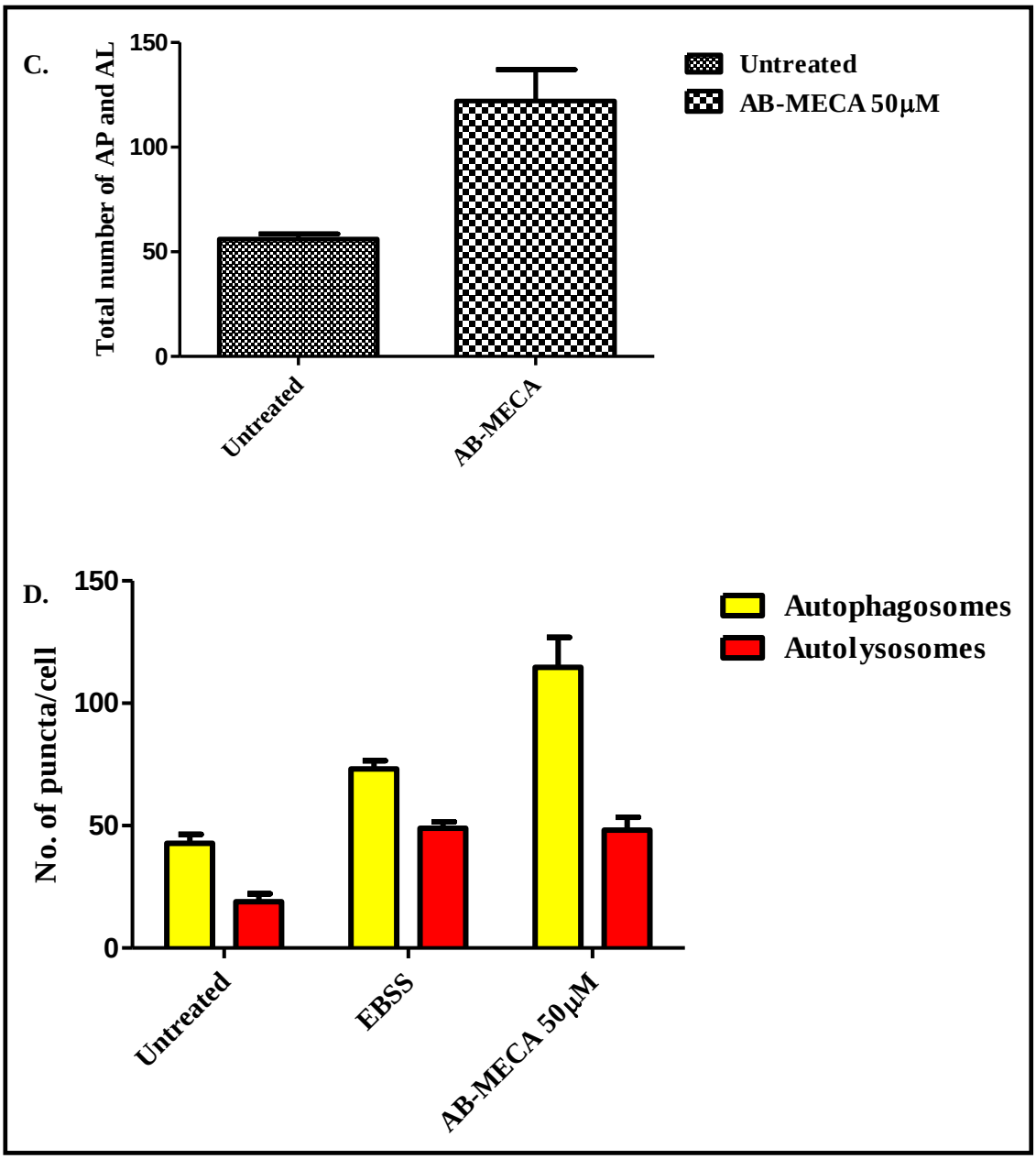


Figure 8: Traffic light assay A. Treatment with AB-MECA (50µM) for 2 hours induced autophagy by increasing the number of both autophagosomes (yellow dots) and autolysosomes (red dots) in ptfLC3 expressing HeLa cells. Scale bar: 25µm. B. The fold change difference between number of autophagosomes and autolysosomes in untreated control vs. AB-MECA (50µM) treatment is statistically significant ($p<0.01$). C. Comparison of total number of autophagosomes and autolysosomes between untreated control and AB-MECA (50µM) treatment. Data is expressed in terms of mean with SEM, n=65 cells from

three independent experiments. D. Comparison of number of autophagosomes and autolysosomes between untreated control, EBSS and AB-MECA (50μM) treatment. Treatment is for 2 hours.

Chapter 5: Discussions and Conclusions

Macroautophagy (herein autophagy) is a cellular degradation pathway which has an indispensable role in maintaining cellular homeostasis. Autophagy occurs at a basal rate in cells during normal growth conditions and is involved in degradation and removal of damaged or dead organelles and misfolded and long lived proteins proteins. In the past years, deregulation of 'autophagic flux' i.e. the rate at which the dynamic turnover of cellular components takes place via autophagy has been shown to play a major role in human diseases like neurodegeneration and cancer.

Given that autophagic defect is involved in some major human diseases, modulating i.e. enhancing or inhibiting autophagy can serve as a potential target in therapeutics. Autophagy inhibition has exhibited enhanced chemo-sensitivity and tumor regression in xenograft models. Autophagy inhibition also enhanced the antitumor effect of chemotherapy drugs and hence combination of autophagy inhibitors like CQ with anticancer drugs are being used in phase I/II clinical trials in different tumor cell types [38]. Many autophagy inducers and inhibitors are already known but there are hardly any that specifically target the core autophagy machinery while most of them act via affecting promiscous signaling cascades or affecting lysosomal function. Thus, the need of the hour is to develop more specific and potent autophagy inhibitors. Stronger autophagy inducers can be used to clear misfolded protein aggregates in neurodegeneration and to abrogate some viral, bacterial and parasitic infections that subvert autophagy for their survival and multiplication. Moreover, developing new autophagy modulators will help in better understanding of the mechanism of autophagy and can also help unravel new molecular players in autophagy.

Keeping the above stated rationale in mind, we were interested in discovering novel autophagy modulators in yeast and mammalian systems. AB-MECA and EPC were identified as hits in a luciferase based high throughput screen for autophagy modulators in our laboratory. I further validated that AB-MECA acts as inducer of autophagy by doing secondary assays for general autophagy and selective autophagy in yeast. Next, we wanted to test if the compounds modulated autophagy in mammalian systems as well. We started by establishing microscopy and western based autophagy assays to

study autophagy in mammalian cells. AB-MECA and EPC both showed a dose dependent accumulation of the autophagosome marker LC3-II form in LC3 conversion assay which can be due to induction of autophagy or a block in fusion of the autophagosomes with that of lysosomes. This was investigated next by performing the traffic light assay. Treatment with AB-MECA (50μM) for 2 hours induced autophagy by increasing the number of both autophagosomes and autolysosomes in ptfLC3 expressing HeLa cells (Figure 8). It showed more than two fold increase in total number of autophagosomes and autolysosomes as compared to the untreated control. Potency of the small molecule, AB-MECA as an inducer of autophagy was proved by the fact that it increased the number of autophagosomes over and above that of EBSS (starvation medium). EPC at 25μM concentration showed potent block in fusion of autophagosomes with lysosomes. It showed more than two folds increase in the number of autophagosomes and a concomitant two fold decrease in number of autolysosomes as compared to the untreated control (Figure 6B). We compared the number of autophagosomes and autolysosomes between BafA1, a known inhibitor of autophagosome-lysosome fusion and EPC which showed that EPC decreases number of autolysosomes over and above BafA1 treatment (Figure 6C).

These results highlight the conserved nature of autophagy that can be utilized to discover promising novel autophagy regulating small molecules in a mammalian system employing yeast based autophagy assay. As an autophagy inducer, AB-MECA can be tested in neurodegenerative disease models and/or xenophagy (intracellular pathogen killing by autophagy) to check if it can induce clearance of misfolded protein aggregates or pathogens. EPC as an autophagy inhibitor can be tested in xenograft models to check if it can sensitise tumor cells to chemotherapy drugs and cause tumor regression. It can also be tried in combination with anticancer drugs to ascertain its effect on the efficacy of tumor cell killing.

Future Plans

Future studies with the small molecules will entail delineating the mechanism through which the molecules act. TOR signaling is intricately involved in autophagy regulation. So, we would like to investigate if autophagy modulation by the molecules is due to any perturbations in TOR signaling. In order to investigate the mechanism by which EPC inhibits autophagosome-lysosome fusion, we would like to ascertain whether it affects lysosomal pH. We also plan to perform Structure-Activity Relationship (SAR) studies to improve potency of the molecules. For physiological studies, we want to test EPC in xenograft models to check if it can sensitise tumor cells to chemotherapy drugs and cause tumor regression. It can also be tried in combination with anticancer drugs to ascertain its effect on the efficacy of tumor cell killing.

REFERENCES

1. Tooze, S.A. and T. Yoshimori, *The origin of the autophagosomal membrane.* Nat Cell Biol, 2010. **12**(9): p. 831-835.
2. Mizushima, N., T. Yoshimori, and Y. Ohsumi, *The Role of Atg Proteins in Autophagosome Formation.* Annual Review of Cell and Developmental Biology, 2011. **27**(1): p. 107-132.
3. Mizushima, N., *Autophagy: process and function.* Genes & Development, 2007. **21**(22): p. 2861-2873.
4. Mizushima, N., et al., *In Vivo Analysis of Autophagy in Response to Nutrient Starvation Using Transgenic Mice Expressing a Fluorescent Autophagosome Marker.* Molecular Biology of the Cell, 2004. **15**(3): p. 1101-1111.
5. Boya, P., F. Reggiori, and P. Codogno, *Emerging regulation and functions of autophagy.* Nat Cell Biol, 2013. **15**(7): p. 713-720.
6. Wullschleger, S., R. Loewith, and M.N. Hall, *TOR Signaling in Growth and Metabolism.* Cell, 2006. **124**(3): p. 471-484.
7. Mizushima, N., *The role of the Atg1/ULK1 complex in autophagy regulation.* Current Opinion in Cell Biology, 2010. **22**(2): p. 132-139.
8. Itakura, E. and N. Mizushima, *Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins.* Autophagy, 2010. **6**(6): p. 764-776.
9. Mari, M., S.A. Tooze, and F. Reggiori, *The puzzling origin of the autophagosomal membrane.* F1000 Biology Reports, 2011. **3**: p. 25.
10. Lamb, C.A., T. Yoshimori, and S.A. Tooze, *The autophagosome: origins unknown, biogenesis complex.* Nat Rev Mol Cell Biol, 2013. **14**(12): p. 759-774.
11. Hamasaki, M., et al., *Autophagosomes form at ER-mitochondria contact sites.* Nature, 2013. **495**(7441): p. 389-393.
12. Ravikumar, B., et al., *Plasma membrane contributes to the formation of pre-autophagosomal structures.* Nat Cell Biol, 2010. **12**(8): p. 747-757.

13. Jaber, N. and W.-X. Zong, *Class III PI3K Vps34: essential roles in autophagy, endocytosis, and heart and liver function.* Annals of the New York Academy of Sciences, 2013. **1280**(1): p. 48-51.
14. Burman, C. and N.T. Ktistakis, *Regulation of autophagy by phosphatidylinositol 3-phosphate.* FEBS Letters, 2010. **584**(7): p. 1302-1312.
15. Pattingre, S., et al., *Bcl-2 Antiapoptotic Proteins Inhibit Beclin 1-Dependent Autophagy.* Cell, 2005. **122**(6): p. 927-939.
16. Russell, R.C., et al., *ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase.* Nat Cell Biol, 2013. **15**(7): p. 741-750.
17. Tanida, I., T. Ueno, and E. Kominami, *LC3 conjugation system in mammalian autophagy.* The International Journal of Biochemistry & Cell Biology, 2004. **36**(12): p. 2503-2518.
18. Glick, D., S. Barth, and K.F. Macleod, *Autophagy: cellular and molecular mechanisms.* The Journal of pathology, 2010. **221**(1): p. 3-12.
19. Eskelinen, E.-L., *Maturation of Autophagic Vacuoles in Mammalian Cells.* Autophagy, 2004. **1**(1): p. 1-10.
20. Gutierrez, M.G., et al., *Rab7 is required for the normal progression of the autophagic pathway in mammalian cells.* Journal of Cell Science, 2004. **117**(13): p. 2687-2697.
21. Jiang, P., et al., *The HOPS complex mediates autophagosome–lysosome fusion through interaction with syntaxin 17.* Molecular Biology of the Cell, 2014. **25**(8): p. 1327-1337.
22. Nixon, R.A., *The role of autophagy in neurodegenerative disease.* Nat Med, 2013. **19**(8): p. 983-997.
23. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nat Cell Biol, 2011. **13**(2): p. 132-141.
24. Russell, R.C., H.-X. Yuan, and K.-L. Guan, *Autophagy regulation by nutrient signaling.* Cell Res, 2014. **24**(1): p. 42-57.
25. Jung, C.H., et al., *mTOR regulation of autophagy.* FEBS Letters, 2010. **584**(7): p. 1287-1295.

26. Wang, J., et al., *A Non-canonical MEK/ERK Signaling Pathway Regulates Autophagy via Regulating Beclin 1.* Journal of Biological Chemistry, 2009. **284**(32): p. 21412-21424.
27. Hardie, D.G., F.A. Ross, and S.A. Hawley, *AMPK: a nutrient and energy sensor that maintains energy homeostasis.* Nat Rev Mol Cell Biol, 2012. **13**(4): p. 251-262.
28. Park, K.-J., et al., *Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death.* Biochemical and Biophysical Research Communications, 2009. **382**(4): p. 726-729.
29. Bellot, G., et al., *Hypoxia-Induced Autophagy Is Mediated through Hypoxia-Inducible Factor Induction of BNIP3 and BNIP3L via Their BH3 Domains.* Molecular and Cellular Biology, 2009. **29**(10): p. 2570-2581.
30. Mizushima, N., T. Yoshimori, and B. Levine, *Methods in Mammalian Autophagy Research.* Cell. **140**(3): p. 313-326.
31. Kimmelman, A.C., *The dynamic nature of autophagy in cancer.* Genes & Development, 2011. **25**(19): p. 1999-2010.
32. Qu, X., et al., *Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene.* Journal of Clinical Investigation, 2003. **112**(12): p. 1809-1820.
33. Gozuacik, D. and A. Kimchi, *Autophagy as a cell death and tumor suppressor mechanism.* Oncogene, 0000. **23**(16): p. 2891-2906.
34. Liang, X.H., et al., *Induction of autophagy and inhibition of tumorigenesis by beclin 1.* Nature, 1999. **402**(6762): p. 672-676.
35. Mathew, R., V. Karantza-Wadsworth, and E. White, *Role of autophagy in cancer.* Nature reviews. Cancer, 2007. **7**(12): p. 961-967.
36. Kung, C.-P., et al., *Autophagy in tumor Suppression and cancer therapy.* Critical reviews in eukaryotic gene expression, 2011. **21**(1): p. 71-100.
37. Mathew, R., et al., *Autophagy Suppresses Tumorigenesis through Elimination of p62.* Cell. **145**(2): p. 322.
38. Yang, Z.J., et al., *The Role of Autophagy in Cancer: Therapeutic Implications.* Molecular Cancer Therapeutics, 2011. **10**(9): p. 1533-1541.

39. Paoli, P., E. Giannoni, and P. Chiarugi, *Anoikis molecular pathways and its role in cancer progression.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2013. **1833**(12): p. 3481-3498.
40. Liu, E.Y. and K.M. Ryan, *Autophagy and cancer – issues we need to digest.* Journal of Cell Science, 2012. **125**(10): p. 2349-2358.
41. Wei, H., et al., *Suppression of autophagy by FIP200 deletion inhibits mammary tumorigenesis.* Genes & Development, 2011. **25**(14): p. 1510-1527.
42. Kim, M.-J., et al., *Involvement of Autophagy in Oncogenic K-Ras-induced Malignant Cell Transformation.* Journal of Biological Chemistry, 2011. **286**(15): p. 12924-12932.
43. Son, J.H., et al., *Neuronal autophagy and neurodegenerative diseases.* Exp Mol Med, 2012. **44**: p. 89-98.
44. Lynch-Day, M.A., et al., *The Role of Autophagy in Parkinson's Disease.* Cold Spring Harbor Perspectives in Medicine, 2012. **2**(4): p. a009357.
45. Winslow, A.R., et al., *α-Synuclein impairs macroautophagy: implications for Parkinson's disease.* The Journal of Cell Biology, 2010. **190**(6): p. 1023-1037.
46. Martinez-Vicente, M., et al., *Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease.* Nat Neurosci, 2010. **13**(5): p. 567-576.
47. Martin, D.D.O., et al., *Autophagy in Huntington disease and huntingtin in autophagy.* Trends in Neurosciences, 2015. **38**(1): p. 26-35.
48. Wolfe, D.M., et al., *Autophagy failure in Alzheimer's disease and the role of defective lysosomal acidification.* The European journal of neuroscience, 2013. **37**(12): p. 1949-1961.
49. Nixon, R.A. and D.-S. Yang, *Autophagy failure in Alzheimer's disease—locating the primary defect.* Neurobiology of Disease, 2011. **43**(1): p. 38-45.
50. Choy, A. and C.R. Roy, *Autophagy and bacterial infection: an evolving arms race.* Trends in Microbiology, 2013. **21**(9): p. 451-456.
51. Levine, B., *Eating Oneself and Uninvited Guests: Autophagy-Related Pathways in Cellular Defense.* Cell, 2005. **120**(2): p. 159-162.
52. Kuballa, P., et al., *Autophagy and the Immune System.* Annual Review of Immunology, 2012. **30**(1): p. 611-646.

53. Deretic, V., *Multiple Regulatory and Effector Roles of Autophagy in Immunity.* Current opinion in immunology, 2009. **21**(1): p. 53-62.
54. Shi, C.-S. and J.H. Kehrl, *MyD88 and Trif Target Beclin 1 to Trigger Autophagy in Macrophages.* The Journal of Biological Chemistry, 2008. **283**(48): p. 33175-33182.
55. Hou, W., et al., *Strange attractors: DAMPs and autophagy link tumor cell death and immunity.* Cell Death Dis, 2013. **4**: p. e966.
56. Matsuzawa, T., et al., *IFN-γ Elicits Macrophage Autophagy via the p38 MAPK Signaling Pathway.* The Journal of Immunology, 2012. **189**(2): p. 813-818.
57. Ma, Y., et al., *Autophagy and Cellular Immune Responses.* Immunity. **39**(2): p. 211-227.
58. Kimura, S., T. Noda, and T. Yoshimori, *Dissection of the Autophagosome Maturation Process by a Novel Reporter Protein, Tandem Fluorescent-Tagged LC3.* Autophagy, 2007. **3**(5): p. 452-460.
59. Johansen, T. and T. Lamark, *Selective autophagy mediated by autophagic adapter proteins.* Autophagy, 2011. **7**(3): p. 279-296.
60. Zhang, X.-j., et al., *Why should autophagic flux be assessed?* Acta Pharmacol Sin, 2013. **34**(5): p. 595-599.
61. Rubinsztein, D.C., P. Codogno, and B. Levine, *Autophagy modulation as a potential therapeutic target for diverse diseases.* Nat Rev Drug Discov, 2012. **11**(9): p. 709-730.
62. Yang, S., et al., *Pancreatic cancers require autophagy for tumor growth.* Genes & Development, 2011. **25**(7): p. 717-729.
63. Ballou, L.M. and R.Z. Lin, *Rapamycin and mTOR kinase inhibitors.* Journal of Chemical Biology, 2008. **1**(1-4): p. 27-36.
64. Stallone, G., et al., *Sirolimus for Kaposi's Sarcoma in Renal-Transplant Recipients.* New England Journal of Medicine, 2005. **352**(13): p. 1317-1323.
65. Bové, J., M. Martínez-Vicente, and M. Vila, *Fighting neurodegeneration with rapamycin: mechanistic insights.* Nat Rev Neurosci, 2011. **12**(8): p. 437-452.
66. Bursch, W., et al., *Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy.* Carcinogenesis, 1996. **17**(8): p. 1595-1607.

67. Amaravadi, R.K., et al., *Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma.* Journal of Clinical Investigation, 2007. **117**(2): p. 326-336.
68. White, E. and R.S. DiPaola, *The Double-Edged Sword of Autophagy Modulation in Cancer.* Clinical Cancer Research, 2009. **15**(17): p. 5308-5316.
69. Amaravadi, R.K., et al., *Principles and Current Strategies for Targeting Autophagy for Cancer Treatment.* Clinical Cancer Research, 2011. **17**(4): p. 654-666.
70. Kim, T., Y. Kim, and J. Woo, *The Adenosine A3 Receptor Agonist Cl-IB-MECA Induces Cell Death Through Ca2+/ROS-Dependent Down Regulation of ERK and Akt in A172 Human Glioma Cells.* Neurochemical Research, 2012. **37**(12): p. 2667-2677.
71. Morello, S., et al., *Cl-IB-MECA inhibits human thyroid cancer cell proliferation independently of A3 adenosine receptor activation.* Cancer Biology & Therapy, 2008. **7**(2): p. 278-284.
72. Kim, S.-J., et al., *Inhibition of cell proliferation through cell cycle arrest and apoptosis by thio-Cl-IB-MECA, a novel A3 adenosine receptor agonist, in human lung cancer cells.* Cancer Letters, 2008. **264**(2): p. 309-315.
73. Panjehpour, M. and F. Karami-Tehrani, *An adenosine analog (IB-MECA) inhibits anchorage-dependent cell growth of various human breast cancer cell lines.* The International Journal of Biochemistry & Cell Biology, 2004. **36**(8): p. 1502-1509.