Autophagy modulators to enhance host control against bacterial infections

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By

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Veena

DECLARATION

I hereby declare that the work described in this thesis entitled **'Autophagy modulators to enhance host control against bacterial infections'**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.

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CERTIFICATE

This is to certify that the work described in this thesis entitled **'Autophagy modulators to enhance host control against bacterial infections'**is the result of investigations carried out by Ms. Veena A at Autophagy laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, under my supervision.

31st March, 2016 Dr. Ravi Manjithaya

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SYNOPSIS

Macroautophagy (herein autophagy) is a cellular catabolic pathway in which cytoplasmic components are captured by vesicles (autophagosomes) that eventually fuse with lysosomes to degrade the cargo. Autophagy occurs in basal levels in all eukaryotic cells to maintain cellular homeostasis and at conditions of stress, superfluous organelles and proteins not essential for survival are degraded. The degraded products provide building blocks for cellular sustenance especially during starvation periods. Apart from these functions, cargos like aggregated proteins, damaged organelles and intracellular pathogens which are otherwise harmful to cells are also selectively captured by autophagy and destined for degradation. As expected, dysfunctional autophagy is linked to several human diseases such as Alzheimer's disease, Parkinsons's disease, Huntingtons's disease where inability of autophagy machinery to clear aggregated proteins is one of the major causative agent for such neurodegenerative diseases. Apart from aggregate clearance, autophagy is also important in the context of infectious diseases where intracellular pathogens are cleared by a form of autophagy known as xenophagy. The process of xenophagy provides a broad spectrum of defense mechanism to capture bacterial, viral and protozoan pathogens. Many of the pathogens have evolved ways to subvert xenophagy and establish their intracellular niche for replication. In addition, mutation(s) in some of the autophagy genes are shown to cause pre-disposition towards intracellular infections. **Chapter I introduces these concepts.**

Given the wide application of autophagy, its modulation by genetic or pharmacological means using small molecules could be a potential therapeutic approach. Although many small molecule autophagy inducers and inhibitors are known, there is still a need for more potent modulators that are functional *in vivo* and with better specificity rather than promiscuously disturbing many signaling pathways within cells.

Yeast based high throughput screening done previously in lab have identified some potential autophagy inducers and inhibitors. I have tested the ability of these autophagy inducers to clear intracellular *Salmonella* population. The details of experimental assays that were carried out for this work have been explained in **Chapter II.** In this study we have identified a potential xenophagy inducer which shows intracellular pathogen clearance in different cell types and against candidate gram positive and gram negative bacterial pathogens. The results pertaining to

screening and validation are summarized in **Chapter III.** The results also indicate that the potential xenophagy modulator could be helpful in targeting larger number of infectious pathogens in both epithelial and macrophage cell line. Further experiments were done to understand the mechanism of action of the compound whose results and interpretations are explained in **Chapter IV**. The results collectively suggest the involvement of autophagy and recruitment of xenophagy proteins to pathogens. Future studies will involve finding the exact mechanism through which compound work and finding its intracellular target.

Chapter 1: Introduction

1.1 Cellular homeostasis

Cells undergo a continuous process of synthesis and degradation of their constituent proteins and organelles which help them in maintaining homeostasis and also for rapid modulation of specific protein/organelle levels to respondchanging extracellular environment.Although much information about organelle turnover is not available, cells are known to identify and degrade damaged or surplus organelles such as mitochondria and peroxisomes. In thecase of proteins, theturnoverrate varies between proteins with half-lives ranging from minutes to years across different species(*1*).An important function of protein turnover is toeliminate non-functional and damaged proteins, thus preventing the accumulation of toxic protein aggregates. Cells have the ability to distinguish aberrant proteins and selectively degrade them and thus preserve proteostasis(*2*).Major intracellular degradation systems include ubiquitin proteosome system (UPS), autophagy and related pathways, endoplasmic reticulum associated protein degradation (ERAD), multivesicular body pathway (MVB)(*3*).

UPS is a two step degradation process occurring in eukaryotes. It involves tagging of a substrate protein with multiple ubiquitin molecules through covalent attachment and subsequent degradation of the tagged protein by 26S proteosome. UPS involves concerted action of three ATP dependentenzymes to conjugate ubiquitin to lysine residues of the substrate- E1 (Ubiquitin activating enzyme), E2 (Ubiquitin conjugating enzyme) and E3 (Ubiquitin ligase). Only poly-ubiquitination on specific lysine (K) residues like K48 and K29 on target proteins isdestined for degradation through proteosome. Proteosome is a tunnel shaped protein complex consisting of a 20S core particle and two 19S cap subunits. The poly-ubiquitinated proteins are recognized by the 19S regulatory caps and proteolysis occurs in the 20S core particle finally releasing short peptides of 7-9 residues long. Ubiquitin is recycled back after targeting the ubiquitinated protein to proteosome by the action of deubiquitinating enzymes(*4*).

Most of the secreted and transmembrane proteins in eukaryotic cells are folded in the endoplasmic reticulum before they get transported to their final destination. Any misfolded protein or trapped protein intermediates due to mutations and cellular stress have to be rectified using chaperons and if not possible to be repaired, have to be degraded. A specific cellular

process geared towards this function called Endoplasm Reticulum Associated Degradation (ERAD) recruits machinery to recognize misfolded and mutated proteins and retro-translocates them to cytosol for degradation. Once in the cytosol, UPS mediated degradation of ERAD cargo occurs.ER stress leads to accumulation of proteins like Inositol Requiring Protein (IRE1), Activating Transcription Factor 6 (ATF6) which can translocate to thenucleus and upregulate expression of gene involved in UPS (*5*).

Macroautophagy (herein autophagy) is a cellular process in which a part of the cytoplasm is sequestered in a double membrane vesicle called autophagosomes and fuses with lysosomes resulting in degradation of its contents. The breakdown constituents such as amino acids are recycled back to the cytoplasm(*6*) (Figure 1). This fuels anabolic pathways and helps in cell sustainability during conditions of stress. The cargo for degradation could be cytoplasmic long lived proteins, aggregated or misfolded proteins, damaged or superfluous organelles, or intracellular pathogens. This process occurs at basal level in normal cells whereas gets induced under certain conditions like nutrient starvation, hypoxia, infection.Autophagy is a conserved process across yeast, plant and animal cells.

1.2 Autophagy

Christian de Duveintroduced the concept of lysosomes based on his observation of hydrolytic enzymes being concentrated in small fractions of isolated cytoplasm particles(*7*). Subsequent electron microscopy studies by Essner and Novikoff showed dense bodies that are also positive for acid phosphatase(*8*).Later Porter and his group reported presence of higher number of dense bodies than usual in electron micrograph of rat liver cells induced with glucogen(*9*). Since the dense bodies contained degenerated mitochondria it was termed as autophagic vacuoles and the process was coined as autophagy, meaning “self eating”by de Duve in 1963 (*10*). Subsequent studies revealed that cytoplasmic contents were sequestered in double membrane vesicles called autophagosomes which eventually fused with lysosomes to generate autolysosomes (also sometimes referred to as autophagolysosomes) and results in cargo degradation and recycling back of building blocks such as amino acids into the cytoplasm. Bioinformatic and cell biology assays revealed autophagy genes in several lower and higher eukaryotic organisms including yeasts, fungi, hydra, flies, dictyostelium, worms, plants and mammalian model systems suggesting that autophagy is a evolutionarily conserved process.

In the 1990s, the molecular machinery of autophagy was mainly identified through yeast genetic screen. So far 41 genes named as autophagy related genes (ATG) have been identified, many of whose orthologues in higher eukaryotes are also now known. In addition, several proteins involved in vesicular trafficking pathways including those involved in fusion with lysosomes such RABs, tethers, SNAREs and cytoskeleton associated proteins are also associated in autophagy process.

Large numbers of proteins involved in the process were mapped usingbioinformatic, genetic and biochemical toolsat basal and starved conditions. The mass spectrometry analysis of human cellsrevealed autophagy interaction network containing 409 candidate proteins with 751 interactions(*11*). Characterization of some of the proteins shed light on the autophagosome biogenesis and flux that comprises of initiation, elongation, maturation and fusion of the double membrane vesicles called autophagosomes with lysosomes.

Following section describes the vesicular and molecular events that take place during macroautophagy.

1.3 Autophagy Machinery:

1.3.1 Induction of Autophagy

Pre-autophagosomal structure or Phagophore assembly site (PAS) is the site of autophagosome biogenesis in yeastand is found close to vacuolar membrane. Depending on the stage of autophagosome formation, different proteins of autophagy machinery is present atPAS. In mammalian cells, the exact location of initiation is not well defined. Studies show that autophagosomes arise fromendoplasmic reticulum (ER), mitochondria and other cytosolic membrane structures like trans-Golgiand late endosomes including plasma membrane and recycling endosomes(*12*).

In yeast, Atg1 is a serine/threonine kinase which associates with other Atg proteins- Atg13, Atg17, Atg29, Atg31 to form the initiation complex. Atg17-Atg29-Atg31 is a stable complex found in both nutrient rich and starvation conditions. Interaction of Atg1 and Atg13 with Atg17-Atg29-Atg31 is enhanced during starvation due to dephosphorylation of Atg1 and Atg13.The mammalian homologues of Atg1 are Unc-51- like kinases1 and 2 (ULK1/2) and that of Atg17 is

FIP200. ULK1/2 interacts with Atg13, FIP200 and Atg101to form a complex. Atg101 is known as Atg13 binding protein and does not have a yeast homolog.

Atg6/Vacuolar protein sorting (Vps30) whose mammalian homologue is Beclin1is important for localization of autophagy proteins to thesite of autophagosome formation. It binds to class III type phosphoinositide 3-kinase(Vps34) to form Beclin1-Vps34-Vps15 core complexas a result of ser14 phosphorylation of Beclin1by ULK1. This complex can bind to several protein partners which can enhance (Atg14L, UVRAG, Bif1) or inhibit (Bcl2, Bclxl, Rubicon) autophagy(*13*).

The regulatory signaling component that affects induction of autophagy process is Target of rapamycin (Tor) complex1 (*14*).This is a key nutrient sensing pathway within cells and controls the phosphorylation status of Atg13 that determines its interaction with Atg1/Ulk1/2 for generation of the initiation complex. Under nutrient rich conditions, Tor causes hyperphosphorylation of Atg13 and this formhas lower affinity for Atg1. This prevents the formation of the initiation complex and as a result, autophagy is inhibited.Starvation conditions or treatment of cells with the TOR inhibitor rapamycin leads to dephosphorylation of Atg13 allowing induction of autophagy. Tor is also involved regulating phosphorylation status of several other effectors like Tap42, Sit4, Ure2 and Gln3 transcription factors, some of which arerequired for autophagy. The mammalian/yeast TOR consisttwo complexes, TORC1and TORC2. Though induction of autophagy is mainly controlled by TORC1, studies have also showed that mTORC2 is a negative regulator of autophagy(*15*).

1.3.2 Vesicle expansion and nucleation:

The isolation membrane formed during initiation grows by sequential addition of membranes and is completed when the vesicle is completely sealed separating the cargo from rest of the cytoplasm. The growth of the double membrane autophagosome requires membrane addition. Various membrane sources such as ER, plasma membrane, trans-Golgi and late endosomes provide membrane to the developing autophagosome. Atg9 is a transmembrane protein implicated in this transport of membrane formsfrom peripheral source to the PAS in yeast or the phagophore initiation site in mammals.

Thereare twoubiquitin like proteins, Atg12 and Atg8 functioning in the membrane expansion process.Atg12is covalently attached to lysine residue of Atg5 through the action of Atg7 and

Atg10 which acts like E1 and E2 like enzymes respectively.The tetrameric complex of Atg12-Atg5 is formed with the help of Atg16. This multimeric complex of Atg12-Atg5-Atg6 is required for the formation of the autophagosome vesicles. The second ubiquitin conjugation system is involved in lipidation of Atg8. Atg8 in yeast is a single protein whose homolog in mammalian cells, microtubule associated protein 1A/1B-light chain 3(LC3) has 6 isoforms. The C-terminal arginine is cleaved by Atg4 protease revealing the glycine residue. The glycine residue is activated by E1 like enzyme, Atg7 and transferred to E2 like enzyme and finally addition of phosphatidylethanolamine (PE) by the action of Atg5-Atg12-Atg16 complex which acts like E3 ligase enzymes.This conjugation converts Atg8 from a soluble protein (LC3-I in mammals) to membrane associated protein (LC3-II in mammals).The growing phagophore membrane gets transformed into matured autophagosomesduring which Atg5 leaves the membrane and only Atg8-PE/LC3II is present(*16*).

1.3.3 Vesicle fusion:

The completely formed autophagosomes (300-900nm in diameter) can move on microtubules on both directions, but is generally biased towards microtubule organizing centre where the lysosomes are enriched. Proteins specifically needed for fusion gets enriched on the membrane site destined for fusion. These proteins are similar to those needed for homotypic vacuole fusion, like Rab7, ESCRT, SNARES and class C Vps/HOPS complex proteins.

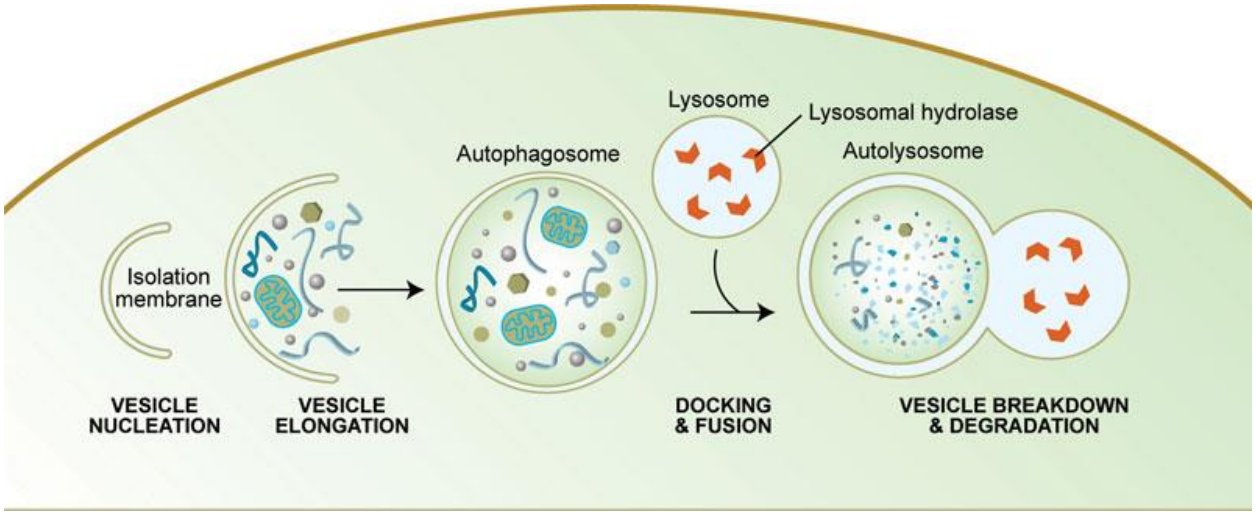


Figure 1: **Steps in autophagy process**–Cytoplasmic material gets sequestered by an expanding double membrane sac called phagophore. The completed autophagosome fuses with the lysosomes to form autolysosomeand the inner single membrane contents gets degraded by the action of lysosomal hydrolases (www.wormbook.org).

1.4 Types of Autophagy:

Autophagy can be primarily classified under three types: macroautophagy, microautophagy and chaperone mediated autophagy (CMA) (the later one has been reported only in mammalian cells). Macroautophagy is a bulk sequestration of cytoplasmic constituents into double membrane vesicles, called autophagosomes leading to fusion with lysosomes. On the other hand,microautophagy involves invagination of the lysosomal membrane that results in capturing of local cytosolic constituents. The cargo for microautophagy is majorly cytoplasmic components but specific cases of cargo capture are seen in case of micropexophagy (microautophagy of peroxisomes), micromitophagy(microautophagy of mitochondria)and piecemeal autophagy of nucleus (PMN/nucleophagy)(*17*).

CMA differs from the other two in its cargo selection. The three events that occur during CMA include recognition of substrate for degradation, unfolding the recognized protein and lastly, translocate them into lysosomes. The substrates for CMA are mainly cytosolic proteins which carry KFERQ amino acid motif. This CMA targeting motif is recognized by cytosolic chaperone

heat shock cognate protein of 70KDa (Hsc70) which then targets them to thelysosomal surface(*18*). Lysosome-associated membrane protein type 2A (LAMP2A), a receptor on lysosomal membrane binds the substrate-chaperone complex. Unfolding occurs most likely through the action of Hsc70 along with its co-chaperones- Bag1, Hip, Hop and Hsp40. Binding of the complex triggers the monomeric LAMP2A to form multimeric LAMP2A components which act as unfolded protein translocation unit. During periods of prolonged stress (more than 10 hours), CMA is hyperactivated and can remain at high activity for until 3 days. The physiological importance of the process includes degrading proteins specifically not needed during starvation so that it can be used as building blocks for producing essential proteins (*19*). CMA gets upregulated even during oxidative stress and exposure to toxic chemicals. CMA occurs only in mammalian cells and no equivalent pathway of CMA is known in yeast or other organisms.

These processes could be selective or non-selective depending on the specificity of the cargo sequestered for degradation. It is non-selective when bulk cytoplasmic contents are captured for turnover whereas selective when cargos are specifically targeted using adaptor proteins that bridge the cargo with the core autophagymachinery as seen in the case of damaged/superfluous organelles and invading microbes.

1.5 Autophagic adaptor hypothesis:

Recent studies have identified a number of proteins involved in recognition of cargodestined forautophagic capture showing that the process is more selective than originally anticipated. These proteins are called as adaptor proteins as they facilitate recruiting autophagic machinery to the cargo. In most cases, there is direct interaction of the adaptor protein with the cargo and the autophagosome membrane marker LC3 through motifs known as Atg8-InteractingMotif (AIM) or LC3 Interacting Region(LIR) (Figure 2). Proteins containing LIRs include cargo receptors (p62, NDP52), some members of basal autophagy process (Atg1, Atg3), proteins involved in vesicle transport (RabGTPase-activating proteins) and signaling molecules (Starch binding domain containing protein, Stdb1) that gets degraded by autophagy. Analysis of almost 40 LIRs revealed the core consensus sequence [W/F/Y]xx[L/I/V], where x is any amino acid(*20*).

Adaptor proteins can be classified according to the cargo they bind. The cargo binding domains of adaptors could be either post translationally modified (PTM) domain or a transmembrane domain of the cargo. One the commonly targeted PTM specific binding domain is ubiquitin, that can be targeted bya number of adaptor proteins. Protein specific transmembrane domain is seen as in case of yeast vacuole enzymes, aminopeptidase 1 (Ape1p) and α-mannosidase (Ams1p)(*21*).

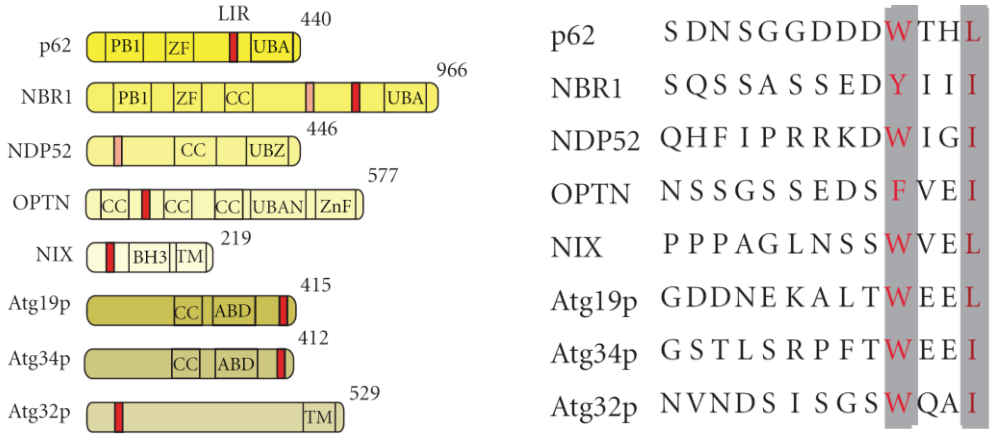


Figure 2: Domain architecture and sequence alignment of LIR motifs of adaptor proteins(*21*).

Table: Types of selective autophagy and their adaptor proteins

Selective Autophagy	Adaptors involved	Cargo binding domain
Mitophagy	Atg32, Atg33, Nix, Bnip3	Mitochondrial outer membrane
Nucleophagy (PMN)	NVJ1	-
Pexophagy	Atg30, Atg36	Pex3
Xenophagy	p62, NDP52,NBR1,OPTN	Ubiquitin, Galectin, DAG
Aggrephagy	p62, NBR1	Ubiquitin
Cytoplasm to vacuole targeting (Cvt)	Atg19p, Atg34p	Protein specific binding e.g.:aminopeptidaseI and α-mannosidase

1.6 XENOPHAGY

Several intracellular pathogens such as bacteria, viruses and protozoa invade specific human cells such as epithelial and macrophages. Once inside the mammalian cells, they encounter the innate immunity defense system of the host cells. Almost all of the pathogens have diverse strategies to counter or evade the host approach to capture and destroy. Working in concert with the innate immune response, macroautophagy is also recruited by the cell to capture the pathogen. Thisprocess of capturing and elimination of intracellular pathogen by autophagy is termed as xenophagy. Studies on xenophagy have been done in*Dictyosteliumdiscoideum, Caenorhabditiselegans, Drosophila*, plants and mammalian cells.

Xenophagy remained unknown for two decades post the discovery of general autophagy in 1963. The first evidence of xenophagy came from the study by Rikihisa in 1984, where guinea pig polymorphonuclear leukocytes (PMNs) incubated with *Rickettsiae* (gram negative pleomorphic bacteria) showed autophagosome like structures containing bacteria. These structures were also positive for acid phosphatase, a component of lysosomes showing that it is a degradative compartment for the entrappedbacteria (*22*).

The process of xenophagy is now proven by many groups as a cellular innate defense mechanism against huge number of intracellular pathogens in both phagocyte and non-phagocyte cells.

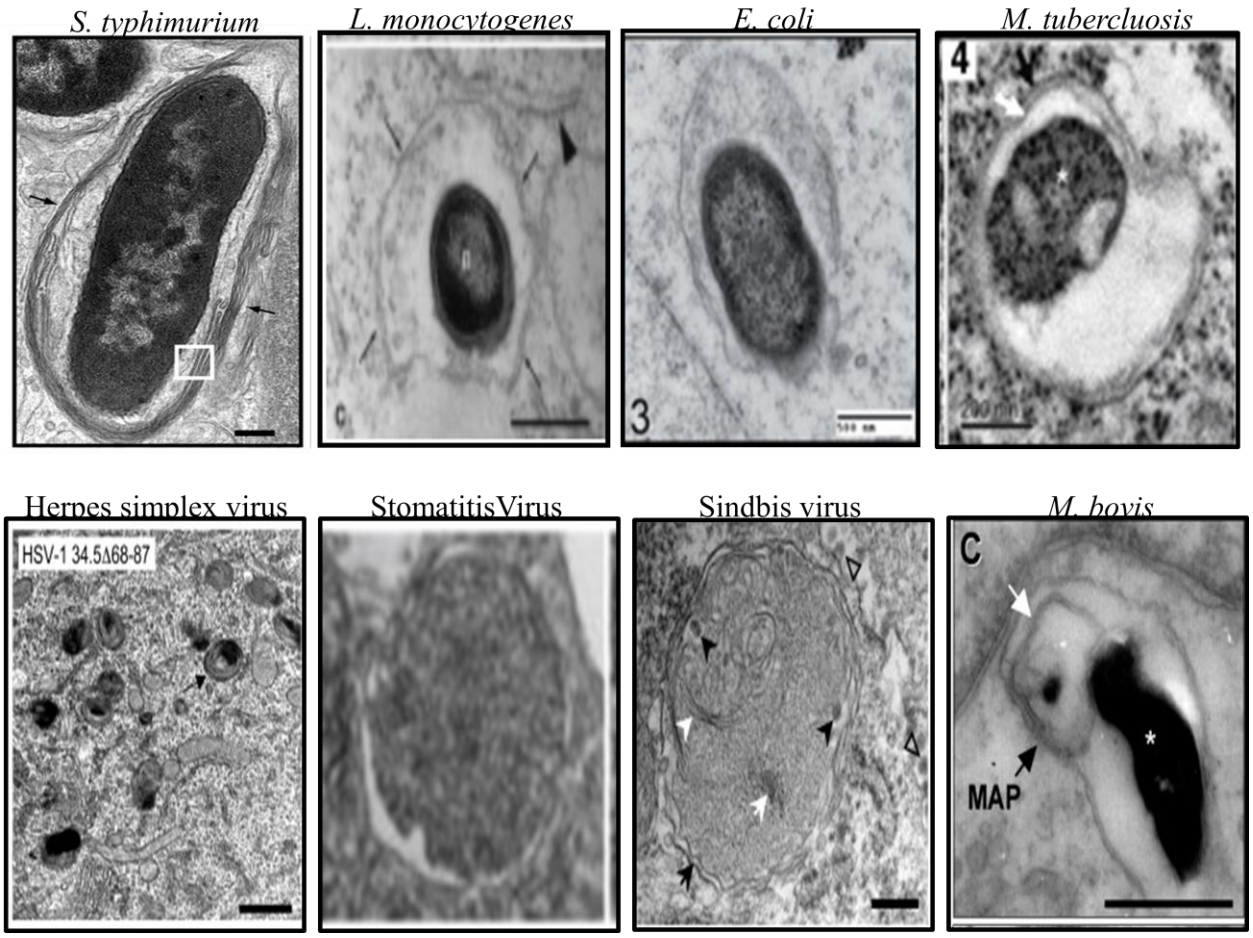


Figure 3: Electron micrograph images of bacterial and viral pathogens captured by double membrane autophagosomes(*23*)(*24-26*).

1.6.1 Importance of xenophagy

Several studies including the pioneeringwork by Yoshimori's group, showed thatintracellularGroup A *Streptococcal*load was increased in atg5 knockout MEF cell line suggesting that functional autophagy is required for restricting and eliminating intracellular pathogens (*27*). An interesting work by Okawa et al. showed that *Shigella* produce protein that bind autophagy proteins and inactivate xenophagy mechanism (*28*).

Inhibition of autophagy using chemical modulators like 3-Methyladenine has shown increased intracellular survival of *Helicobacter pyroli* in AGS cells (*29*). Similarly, pathogens like

Porphyromonasgingivalis and *Brucellaabortus* which have evolved ways to survive and replicate inside autophagosomes, is also shown to be inhibited when treated with 3-Methyladenine which prevents the autophagosome formation (*30*).

Mutation of autophagy gene, ATG16L1 leads to genetic predisposition to bacterial infections:

Crohn's disease is an inflammatory bowel disease affecting the entire digestive tract due to massive infiltration of leukocytes into intestinal mucosa leading to chronic inflammation. Genome wide association studies (GWAS) have provided evidence for thecontribution of two autophagy genes, ATG16L1 and IRGM in the disease pathogenesis. A pioneering GWAS publication revealed that single nucleotide polymorphisms occurring at ATG16L1 (T300A) and several risk polymorphisms of IRGM(*31*).

Several subsequent studies addressing the importance of ATG16 and IRGM have revealed that ATG16 null mutant mice die one daypost delivery and mice deficient for IRGM develop normally but are extremely susceptible to bacterial infections. Similarly, over expression of ATG16 T300A does not impair the general autophagy process but show deficits in intracellular bacterial clearance. Thus, impairment of xenophagy causes chronic infection that plays amajor role in Crohn'sdisease(*32*).

1.6.2 Mechanistic insights into xenophagy

Pathogens enter cells by phagocytosis (in case of phagocyte cells) or by endocytosis (in case of non-phagocyte cells).The conventional pathway is for the phagosome/endosome to fuse with lysosomes for degradation. But, pathogens have developed several ways by which they can subvert this pathway and ensure intracellular replication.

One of the common strategies of the pathogens to evade fusion with lysosomes is to block the pathway preventing maturation to next stage or escape into cytosol. This leads to existence of many subsets of pathogenic population after enteringthe hostcell(Figure 4).The fate of the first

subset of population leads to fusion of phagosome/endosome with lysosomes for degradation. Secondly, pathogens can modify the endosome compartment into their replicative niche by preventing the maturation of endosome such that it cannot fuse with lysosomes. Thereis also a third subset of population that damages the endosomes and escape into the cytosol. This subset where they replicate faster is believed to be the primary target forxenophagy(*33*). A proportion of phagosomes that are positive for LC3 are called as LC3 associated phagosomes and the process is called LC3 Associated Phagocytosis.

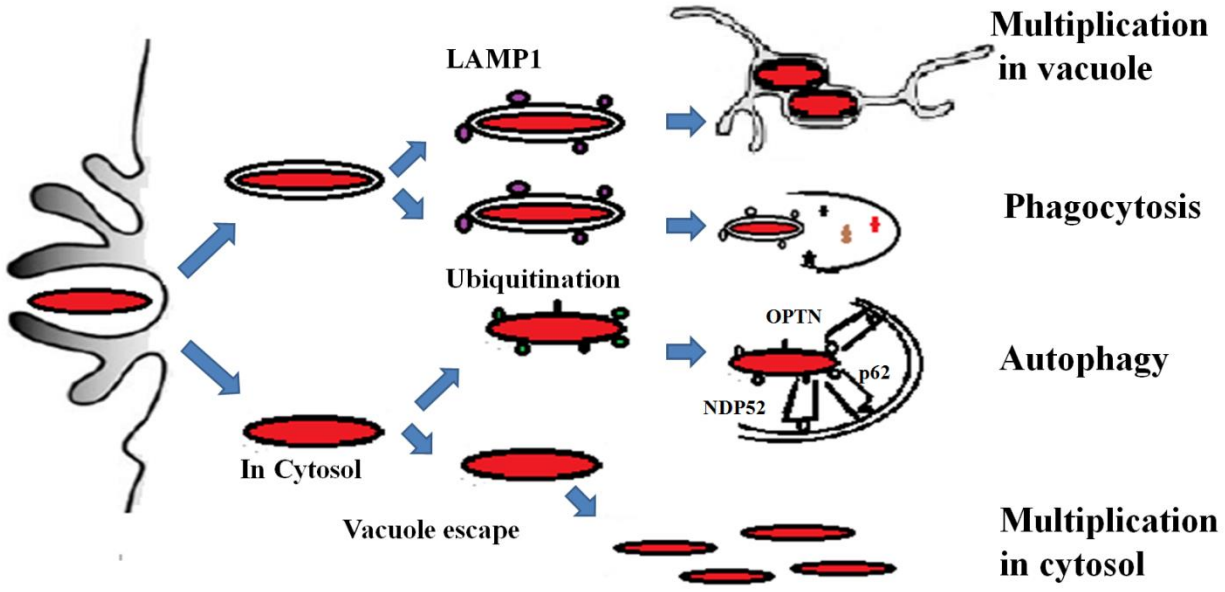


Figure 4: Fate of different intracellular *Salmonella* populations – upon entry into cells, the pathogen can exist in any of the one subset namely a- in *Salmonella* containing vacuoles where slow replication occurs, b- phagosomes targeted for degradation, c- cytosolic bacteria captured by autophagy proteins for fusion with lysosomes, d- cytosolic population where rapid replication is possible. (Image modified from (*33*)).

LC3 Associated Phagocytosis

LC3 Associated Phagocytosis (LAP) is a cellular defense process against pathogens where LC3 gets recruited to a single membrane phagosome as against the conventional autophagy where LC3 gets recruited to double membrane autophagosomes (Figure 6). Though the exact

mechanism of LAP initiation is not known, few studies have shown NOD1and NOD2 recruit Atg16L to the bacterial entry site on the plasma membrane, facilitating LC3II recruitment to phagosome membrane and the LC3 coated phagosomes are shown to rapidly get matured(*24*). This process requires the class III PI3K activity and involves autophagy proteins like LC3, Rubicon, Beclin-1 and Vps34 but is independent of ULK1(*34*).

There is no known distinct marker present only on LAP membranes but they can be distinguished from autophagic bodies using electron microscopy (Figure 5).

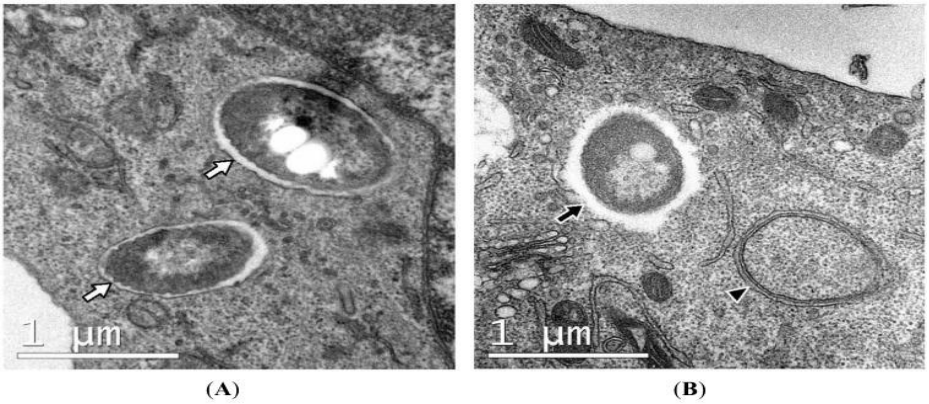


Figure 5: Transmissionelectron micrograph of RAW macrophages expressing GFP-LC3 infected with *Burkholderiapseudomallei*. The single membrane LAP structures (panel A) can be differentiated from double membrane autophagosomes (panel B, arrow head) and pathogens in cytosol (panel B, black arrow)(*24*).

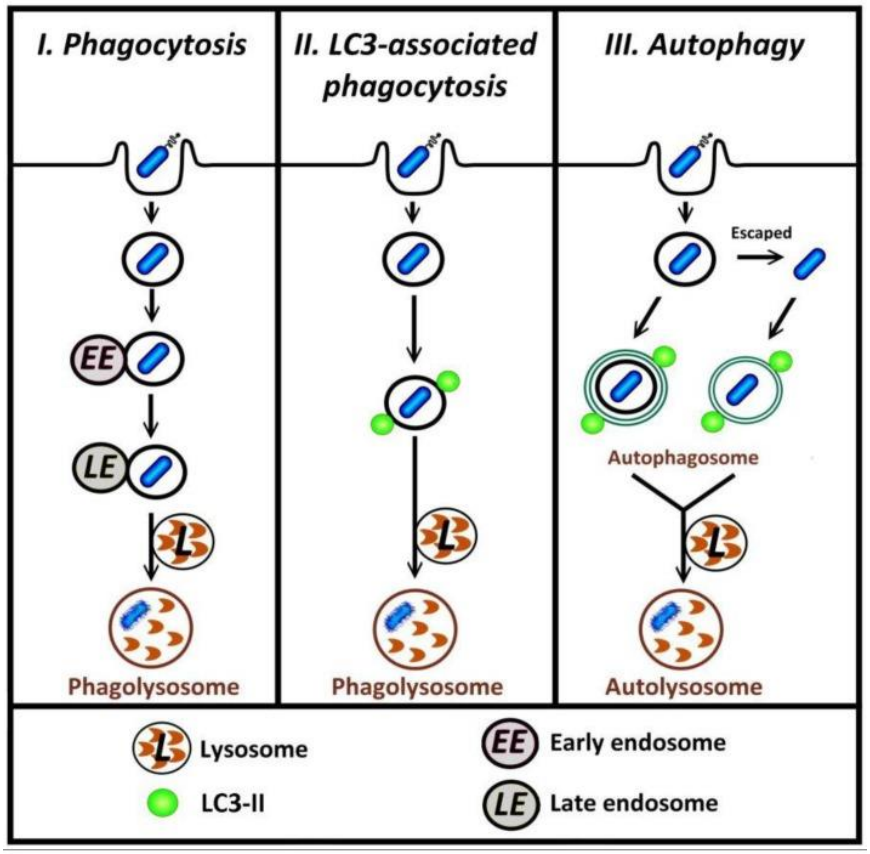


Figure 6: **Different pathways for bacterial degradation**. I- Phagocytosis is the process by which the the pathogen entering through phagosome is trafficked to fuse with lysosome for degradation. II- LAP functions by recruiting autophagic protein, LC3 to phagosomes and enhances fusion with the lysosome. III- Autophagy captures the pathogens that escape to cytosol and the damaged pathogen containingphagosomes(*24*).

1.6.3 Targets for xenophagy

Pathogens like GAS, *Listeria monocytogenes* produce pore forming cytolysins like streptolysin and listeriolysin respectively enabling them to escape into cytosol and hence avoid fusion with lysosomes. This would also provide pathogens with sufficient nutrients from cytosol to replicate faster. (*35*).Xenophagyexist as a major defense mechanism of host to capture the cytoplasmic pathogens using its machinery proteins explained below.

The xenophagy can also capture phagosomes that are either intact or in most cases damaged phagosomes and remnants of rupturedphagosomal membrane that is captured by the double membrane autophagosomal structure(*36*).

Pathogen recognition by Autophagy Machinery

Host cellshave receptors to bind bacterial cell wall proteins and some of the receptors studied are pattern recognition receptors (PRRs), such as Toll-like Receptors (TLRs) or nucleotide-binding oligomerization domains (NOD)-like receptors (NLRs), which can recognize the pathogen patterns like lipopolysaccharides or peptidoglycans. TLR signaling is known to increase the recruitment of LC3 to facilitate theformation of autophagosomes. Even the cytosolic receptors, NOD1and NOD2 are shown to recruit ATG16L to the site of *Shigella* entry(*37*).

Both gram positive and gram negative organisms are implied in autophagic clearance. Autophagy machinery gets employed by recognizing the ubiquitinated cargos. Adaptor proteins that recruit the autophagy machinery by recognizing ubiquinated proteins include p62, NDP52 (CALCOC2), Optineurin (OPTN) (Figure 7). These proteins also have LC3 interacting region (LIR) through which they deliver the cargo into autophagosomes. LC3, commonly studied autophagosome membrane protein, belongs to two subfamilies of proteins namely, LC3 and GABARAP/GATE16. LC3 protein family isoforms includes LC3A, LC3B, LC3C whereas GABARAP/GATE16 family consists of GABARAP, GABARAPL1, GABARAPL3 and GATE16 (also called as GABARAPL2) all are known to be involved in xenophagy process(*20*).

In *Salmonella typhimurium* infection, a secondary pathway that is independent of ubiquitin bound cargos is through diacylglycerol (DAG) a lipid messenger, also bind to *Salmonella* and improves theautophagic capture(*38*). Galectin-8, a cytosolic lectin binds to glycans during bacterial invasion and recruits NDP52 to activate autophagy. It is postulated to recognize damaged salmonella containing vacuoles and target them to autophagosomes(*36*).

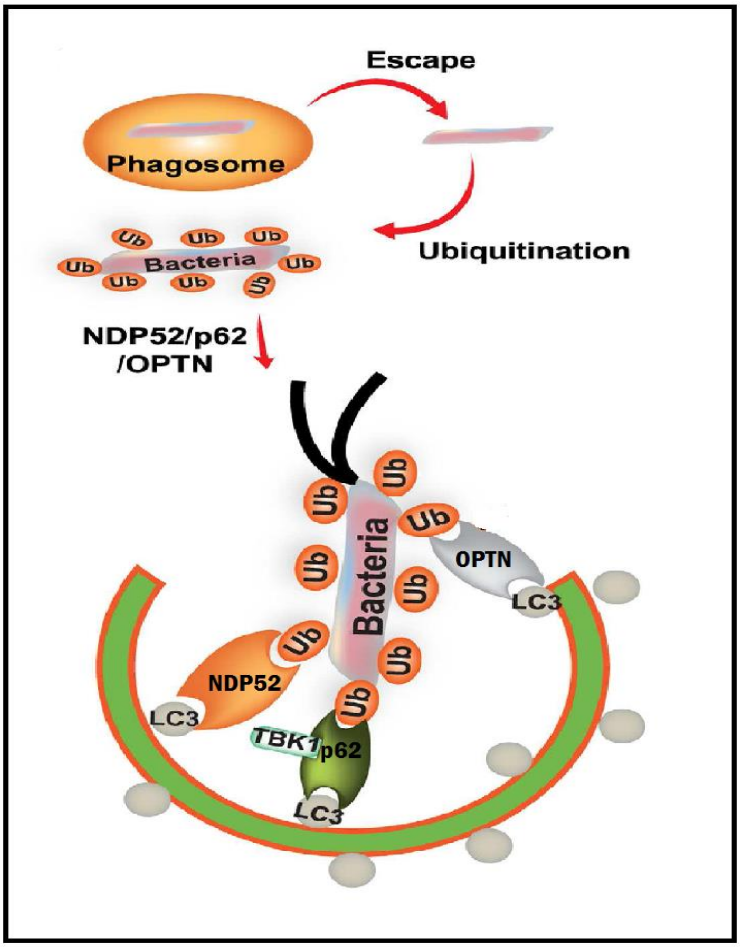


Figure 7: Molecules involved in autophagic pathogen recognition(*39*).

1.6.4 Microbial model systems for studyingxenophagy:

Bacterial pathogens have their own way of survival inside cells that would help defend themselves against host defense mechanism. Some pathogens come into cytosol whereas some remain in the endosomes and prevents fusion to lysosomes. The mechanism of autophagy capture also varies between different pathogens.

1.6.4.1 Pathogens that are restricted by xenophagy

Bacterial infections

Mycobacterium tuberculosis

Mycobacterium tuberculosis is an intracellular pathogen that can persist within phagosomes and prevents the fusion with lysosomes. *Mycobacterium* interferes with the delivery of V_0 H^+ ATPase to the phagosomal membrane and thus reduces the acidification of pathogen-containing phagosomes. Induction of autophagy by starvation orrapamycin, treatment with interferonγ, vitamin D has shown to acidify the *Mycobacterium* containingphagosomes, followed by increased co-localization with late endocytic markers and LC3, which indicate theformation of phagolysosomes, hence removing the block in phagosome maturation pathway and concomitant reduction in intracellular *Mycobacterium*titre(*40*).

Group A Streptococcus (GAS)

Streptococcus pyrogenes are gram positive bacteria also known as Group A Streptococcus (GAS) due to the presence of antigen A on their cell wall. Virulent GAS leads to hemolysis characterized by destruction of blood cells. Their primary means to avoid host defense is to produce streptolysin, a pore formingcytolysin that damages the endosome and leads to GAS entry into thecytosol. Studies have shown colocalization of LC3 with GAS one hour post infection. This colocalization is lost in Atg5 knockout MEFs and in 3-methyladenine, an autophagy inhibitor treated samples, proving the involvement of autophagy(*27*).

Salmonella typhimurium

One of the commonly studied model system for xenophagy is *Salmonella typhimurium.* It is a rod shaped flagellated, gram negative bacterium that causes gastroenteritis referred to as salmonellosis in humans and typhoid likedisease in mice. *S. typhimurium* is a member of genus *Salmonella enterica*, with more than 2500 serotypes reported.

Salmonella enters through contaminated food or water and their targets for infection are macrophages and intestinal epithelial cells. After reaching theintestine, it invades the epithelial cells using type three secretion system (T3SS). T3SS is composed of almost 30 proteins, and its structure is similar to bacterial flagella. This complex of proteins is involved in virulence of *Salmonella* species. The specialty of T3SS is its needle structure and it is involved in translocation of bacterial proteins called effectors to host cytoplasm. *Salmonella* encodes for two virulence related secretion systems namely, T3SS1 is involved in breaching the epithelial cell wall and T3SS2 is involved in intracellular replication(*41*).

After entry into cells, *Salmonella* converts its niche into a vacuolar compartment called as *Salmonella* Containing Vacuole (SCV). SCVs avoid fusion with lysosomes as it can prevent the delivery of NADPH oxidase and induction of nitric oxide. At later time points like 6-8 hour post infection, SCVs mature into a replicative compartment and develop long membranous structures called *Salmonella* induced filaments (Sifs). SCVs are positive for some late endosome markers like vATPases and LAMP1 but absent for few like mannose-6-phosphate receptor(*42*).

A proportion of *S. typhimurium* loses their SCV and enters cytosol as early as one hour post infection and they are the primary target for ubiquitin coating. This ubiquitinated population is shown to co-localize to autophagy marker, LC3 by means of autophagy adaptor proteins(*33*). In the presence of autophagy inhibitor or in autophagy deficient cell line this co-localization is lost going to show that it is indeed an autophagy dependent association.

The mechanism and kinetics of *Salmonella*xenophagyis well studied and reported. Studies have shown around 20-30% of intracellular *Salmonella* being captured by autophagy machinery at early time points (1 hour) post infection(*43*). In spite of therecognition of *Salmonella* by ubiquitin, DAG and galectin 8 by the host cell which leads to autophagic clearance, the pathogen can overcome the process. Subversion happens because autophagic capture is seen highest soon after infection but *Salmonella* beginshyper replication4 to 6 hour post infection and the reason behind autophagy not being able to capture at later time points is not well known. One reason speculated is the translocation of *Salmonella* virulence effectors of SPI-2genes into host cell cytosol through T3SS mechanism. One of the effectors translocated is sseL which has deubiquitinase activity(*41*). This could essentially prevent the ubiquitination of the pathogen and hence affect the pathogen recognition by autophagy proteins. Studies on mutant sseL*Salmonella* strain has shown enhanced clearance by autophagy (*44*). Thus using *Salmonella* as a model system to study the effect of autophagy induction which would enable the host cell to overcome the block in capturing imposed by the pathogen would be insightful.

Viral infections

The anti-viral role of xenophagy targets the viral components or virions for degradation to lysosomes. The first study of xenophagy on viruses came from CNS infection with sindbis virus(*45*). Overexpression of beclin-1 in mice brains reduced the viral titres and the number of

sindbispositive cells and thereby reducing the Sindbis virus encephalitis(*46*). The mechanism of this effect is yet to be clearly understood, but it is postulated that it could either be due to enhanced clearance of the virus or neuro-protection against apoptosis due to upregulation of autophagy.

Both DNA (*Herpesviridae)* and RNA (*Alphaviridae, Tobamoviruses, Rhabdoviridae)* viruses are shown to be manipulated by autophagy. In these studies, LC3 protein family members are used as amajor marker protein to label virus containing autophagosomes(*46*).

Binding of viruses like herpes virus and adenovirus to the target cell (CD46 receptor) in some cases are shown to stimulate autophagy through includes down regulation of mTOR pathway and involvement of Toll7 receptor as in thecase of vesicular stomatitis virus(*47*).

Other autophagy genes that are linked to anti-viral autophagy are Atg3, Atg7, Atg8, Atg12. Deletion of these genes has shown an increase in viral replication in case of Tobacco mosaic virus and Vesicular stomatitis virus(*46*).

Human Immunodeficiency Virus (HIV)

During chronic and advanced infection with HIV, called as Acquired Immunodeficiency Syndrome (AIDS) the major trouble is not due to HIV viremia but the inability of the host cells to defend itself from infectious diseases. This occurs due to depletion of large amounts of CD4+ T-cells and massive release of inflammatory cytokines like IL-1β, IL-6, and TNF-α(*48*).

Studies on autophagy during HIV infection has revealed dual role for the processwherein there is down regulation of autophagy process due to activation of mTOR in the infected Dentritic cells which could contribute to lesser clearance rate and on the other hand, autophagy is seen involved in establishing viral reservoir that is maintained for years inside host cells.HIV employs autophagosomes for its virion assembly and replicationand also prevents the last stage of fusion with lysosomes.

1.6.4.2 Pathogens that evade autophagy

Both bacteria and virus have evolved mechanisms to resist against the host cell defenses. There are many examples of intracellular pathogens where they avoid or counteract autophagy.

Francisellatularensis

Francisellatularensis, is a gram negativecoccobacillus bacterium, which causes tularemia, infection of blood monocytes, down regulates the expression of many autophagy genes and hence recognition of the bacterium by autophagy machinery is very less. Similar down regulation of autophagy genes is also reported recently in *Mycobacterium* infection. This inhibition is non-specific and is mediated by *Mycobacterial*protein,Eis(*49*).

Shigellaflexneri

*Shigella*is a human pathogen that causes shigellosis, a disease manifested by bacillary dysentery. It enters through fecal-oral route and infects the epithelium of colon and rectum and the resident macrophages beneath the M cells. *Shigella* can escape from the phagosome/endosome and move within the host cells by directing actin polymerization using its *vir*G gene. VirG is an outer membrane protein that accumulates on one end of the bacterium and mediates bacteria's polar movement. It is also known to be the target of autophagy machinery via interaction with Atg5. Recent studies have shown that an effector protein of *Shigella*, IcsB acts as anti-Atg5 binding protein, by having a strong affinity for the same binding region on VirG as that of Atg5. Hence, mutants of IcsB are captured by autophagosomes more rapidly(*28*).

Listeria monocytogenes

Listeria monocytogenes is a virulent food-borne pathogen causing listeriosis. The bacterium can escape into cytosol by producing a pore forming lysin called listeriolysin that raptures the phagosome. In the cytosol, Listeria can move through the host cell by actin polymerization occurring at one end of the bacterium mediated by actA protein. Though recruitment of LC3 to *Listeria* at early time points post infection is seen, they escape into thecytosol and due to their high cellular motility, capture by autophagy is much reduced and the mutants of ActA*Listeria*gets captured by autophagy machinery rapidly (*50*).

1.6.4.3 Pathogens that exploit autophagy for survival

Staphylococcus aureus

S. aureus can convert the $LC3^+$ double membrane autophagosomes into a replicative niche and prevents fusion with lysosomes. At later time points post infection (10-12 hours), bacteria can come into cytosol and cause apoptosis of the host cell. Similarly, Atg5 deficient MEFs show reduced replication of *S. aureus* confirming the need for induction of autophagy for the bacteria's replication(*51*).

Manipulation of autophagy by viruses

Large numbers of viruses are also shown to manipulate autophagy and can make use of the process for their own survival and replication. Kaposi's sarcoma herpesvirus (KSHV) and Herpes simplex virus-1 target beclin-1and inhibits autophagosome formation(*52*). KSHV encodes a viral protein that is similar mammalian bcl2, which binds with higher affinity to beclin and thus prevents forming complex with vps34(*13*).

1.7 Adaptive immunity and Autophagy:

Cytokines of innate and adaptive immunity are known to be involved in autophagy process against pathogens. Secretion of IFN-γ, TNF-α stimulates autophagy whereas, IL-4 and IL-13 inhibits autophagy(*53*). Other roles played by autophagy include mitigating inflammation reactions and removal of apoptotic debris by which prevents acute tissue inflammation.

Autophagy connection to immunity is also related to genes like ATG5, ATG16L and IRGM (autophagy-stimulatory immunity-related GTPase). Mutation/disruption of them is linked to Crohn's disease, systemic lupus erythematosus (SLE)(*54*).

1.7.1 Role of Autophagy in Antigen presentation:

Recent studies have shown that autophagy process which is used to deliver cytoplasmic pathogens or pathogen molecules to lysosomes, can also contribute to MHC class II presentation in dendritic cells and B cells. Also,DC specific deletion of atg5 has impaired T cell priming and in thepresentation of phagocytosed antigens. MHC class II molecules represent peptides from exogenous proteins to $CD4^+$ T cells. Studies have shown that when exogenous nuclear or cytosolic proteins from bacteria and virus are over expressed and treated with autophagy inhibitors like 3-MA or wortmanin, there was decrease in the MHC-II presentation (*55*).

1.8 Autophagy as therapeutic agents:

Autophagy in health and disease is commonly referred to as a double edged sword. Malfunctioning of autophagy, either upregulation or downregulation has been implicated in various pathophysiology like neurodegenerative diseases, infectious diseases, cancer, Crohn's disease(*56*).

Cancer is a well known example where inhibition of autophagy proved beneficial. Autophagy gives tumor cells the ability to adjust for the metabolic stress in their tumor micro-environment and thus increases the tumor cell survival whereas, in caseofneurodegeneration and infectious disease, enhancing autophagy aids in clearing toxic protein aggregation and pathogens respectively.

The different pathways that converge in regulating autophagy are being studied extensively and could be potential therapeutic targets to control the process. Small molecule modulators of autophagy are the most commonly used tools to control the target pathway temporarily and in a reversible manner.

Though vast number molecules are already known to modulate autophagy, its efficacy in-vivo is yet to be proven. Recent studies on animal models where autophagy gene is tagged or deleted have made possible tissue specific and time specific manner examination of theeffect of modulating autophagy. Excessive modulation of autophagy could be deleterious and hence, there is aneed for autophagy modulators that would precisely target the diseased cell type.

Few of the autophagy modulators that are currently in clinical trials are mentioned in the table below(*57*).

Drug	Autophagy target	Disease	Phase
Chloroquine	Lysosomal inhibitor	Stage IV small cell lung cancer	1
Chloroquine	Lysosomal inhibitor	Relapsed and refractory multiple myeloma	2
Hydrochloroquine	Lysosomal inhibitor	Breast cancer	2

Carbamazepine	Autophagy inducer	Alpha1- antitrypsin deficiency liver cirrhosis	2
Lithium carbonate	Autophagy inducer	Amyotrophic lateral sclerosis	2
Trehalose	Autophagy inducer	Vascular aging	N/A

1.8.1 Xenophagy modulators as therapeutic agents

In regard to therapeutic effect of chemical modulatorstowards xenophagy, preliminary studies have shown positive effects.

Studies from Kim et al has shown that antimycobacterial effect of two commonly used drugs, isoniazid and pyrazinamide is mainly due to activation of autophagy in host cells though it exhibits in-vitro antibacterial effect and the drug mediated effect was perturbed in atg7 mutant *Drosophilia*and the flies exhibited decreased survival rates(*58*).

Tat-Beclin1, an antimicrobial peptide was synthesized by Beth Levine's group by analyzing the binding region of Beclin1 protein with HIV's virulence factor, Nef. This peptide has shown to have an effect on number of viruses like Sindibis virus, HIV, West Nile virus, chikungunya virus and intracellular bacterium, *Listeria monocytogenes*. The mechanism of function of the peptide is shown to be because of its interaction with the autophagy inhibitor, Golgi-associated plant pathogenesis Related Protein1 (GAPR1). In a cellular context GAPR1 is Beclin1 binding protein that negatively regulates autophagy(*59*).

BRD5631, a synthetic molecule has shown to decrease in protein aggregates of over expression of poly Q repeats in cells. The compound also increased co-localization of LC3 and NDP52 with Salmonella typhimurium and also decreased secretion of IL-1β(*60*).

Chapter 2: Materials and Methods

2.1 Cell Culture:

HeLa, U1752, RAW 264.7 cell lines were maintained in growth medium comprising of Dulbecco's modified Eagle's medium (DMEM)(Sigma-Aldrich, D5648) supplemented with3.7 g/L sodium bicarbonate, 10% fetal bovine serum (PAN, 3302-P121508) and 100 units/ml of penicillin and streptomycin (Sigma-Aldrich, P4333) at 5% CO_2 and 37ºC.

2.2 Bacterial Strains:

The strains used in the study are WT *Salmonella typhimurium* SL1344 (kind gift from Prof. C V Srikanth, RCB, India) and WT *Salmonella typhimurium* 14028s (kind gift from Prof. Linda Kenney, MBI, Singapore). They were grown in Luria Bertani media at 37ºC. To study the expression of virulence genes of SPI-2, *S. typhimurium* 14028s strain containing sifA-LacZ transcriptional fusion was used (kind gift from Prof. Linda Kenney, MBI, Singapore).

2.3 Antibodies and reagents:

The antibodies used in the study are as follows: LC3B (L7543, Sigma-Aldrich), p62 (PM045, MBL), β-tubulin (E7-c, Biogenuix), Anti-rabbit IgG, HRP linked antibody (7074P2, CST), Atto 663 (18620, Sigma), Atto 488 (62197, Sigma).

Reagents used are: O-NitrophenolGalactoside (N1127, Sigma-Aldrich)

2.4 Colony forming assay

Single colony of *Salmonella typhimurium*SL1344 was grown for six hours in 37˚C shaking incubator. Secondary culture (0.2% inoculum) was grown overnight in micro-aerophilic conditions (tube kept stationary in 37˚C incubator). U1752, HeLa (WT andAtg5KO), RAW264.7 cell lines were infected at a multiplicity of infection (MOI) of 250 for one hour. The cells were treated with media containing gentamycin at the concentration of 100µg/ml for 2 hours to kill the extracellular bacteria. The cells were then treated with the compound and incubated for 3 hours (6 hours in case of RAW264.7). At the end, the cells were lysed using

lysisbuffer (0.1% SDS, 1% Triton X-100, 1X PBS) and the intracellular *Salmonella* was plated and the CFU was counted.

Colony forming assay for Methicillin Resistant *Staphylococcus aures* (MRSA)

RAW 264.7 macrophage cells (2X 10^5) were grown in 90% DMEM and 10% FBS containing media. MRSA cells (10^7 CFU/mL) were suspended in 90% DMEM and 10% FBS for 1h for opsonization and were added to macrophages for 1 hour. Macrophages were washed with PBS twice and treated with Gentamycin (50 µg/ml) for 1 hour to remove any extracellular bacteria. Macrophages were washed with PBS twice and incubated with compound G for 6 hours. Cells were suspended in 0.5 ml of ice cold water for 1 h after washing twice with PBS. Bacteria were harvested from the lysates and plated.

* Experiment was done in collaboration with Dr. JayantaHaldar, NCU, JNCASR.

2.5 CellTitreGlo assay

U1752 and HeLa cells were counted and equal numbers (9000/well) in growth medium were plated on a 96 well plate. Dose response of compound G (.001µM- 100µM) was done for 3 hours. Also, toxicity of the chosen concentration of 50µM was checked at different time points ranging from 3 hours to 10 hours and incubated in 5% CO_2 incubator at 37˚C. After the incubation time point, CellTitreGlo reagent (G7570, Promega) was added to each well and luminescence was measured using Varioskan Flash (Thermo Scientific).

2.6 Traffic light assay

HeLa cells were grown on coverslips until 70-80% confluence. Lipid mediated transfection of RFP-EGFP-LC3 plasmid (Addgene #21074) was done using lipofectamine 3000 (E7510, Sigma). A mix of 2.5µg of DNA and 5µl of p3000 reagent in 100µl OPTI-MEM was prepared and mixed with 5µl of lipofectamine 3000 diluted in 100µl of OPTI-MEM (31985-070, Invitrogen). The lipid-DNA complex was allowed to form by incubating for 5 minutes and was added to cells. After 6hours the lipofectamine containing media was changed to fresh media. After 24 hours, the cells were used for different treatments. Drug treatment was done by adding fresh media containing 50µM of compound G and starvation was induced by maintaining the cells in Earle's balanced salt solution (E7510, Sigma) for 2 hours. After treatment, cells were

fixed using 4% paraformaldehyde and permeabilized using 0.25% triton X-100. The coverslips were mounted on glass slides using vectashieldantifade reagent (H-1000, Vector laboratories) and were sealed with nail polish. Imaging was done using Delta vision microscope (GE Healthcare). On an average 15 stacks were taken and a projected image was obtained after deconvolution using enhanced ratio algorithm using the Softworx software (GE).

2.7 Western blotting

HeLa cells were treated with fresh growth medium or growth medium containg compound G for two hours. After incubation, cells were washed with ice cold 1X PBS and lysed using lysis buffer (10% w/v SDS, 10mM DTT, 20% v/v glycerol, 0.2M Tris-HCL pH 6.8, 0.5% w/v bromophenol blue) and collected using cell scrapper. The lysates were boiled at 99˚C for 15 minutes and stored at -80˚C. Western blotting was performed using standard methods.

2.8Growth Curve of *Salmonella typhimurium*

A single colony of *Salmonella typhimurium*SL1344 grown overnight at 37˚C was dilutedin Luria Broth media to get an Abs_{600} of 0.2. The diluted culture was used for treatments with compound G (50µM) and compound G with gentamycin (100µg/ml). The growth curve of 80 µl culture taken in a 384 well plate was obtained by measuring the absorbance at 600nm using Varioskan Flash Plate reader Spectrophotometer (Thermo Scientific) at 300 rpm at every 30 minutes interval for 10 hours and was plotted using GraphPad Prism.

2.9 βgalactosidase activity assay

Single colony of WT *Salmonella typhimurium* 14028s and sifA-lacZ fused 14028s was grown overnight in LB media and was transferred to secondary LB culture (1% inoculum) and was left to grow until 1 O.D in the presence of compound G. βgalactosidase activity assay was performed by mixing cell culture (500µl) with 900µl of Z lysis buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH7.0), two drops of chloroform and 0.1% SDS followed by vigorous vortexing to lyse the bacteria. O- NitrophenolGalactoside (4mg/ml), made in sodium phosphate buffer of pH7.4 was added (200µl) and transferred to 96 well plate after centrifugation (4000g, 10 minutes), 150µl per well and absorbance values at 420nm and 590nm was noted at fixed intervals. The βgalactosidase activity was calculated using the Miller formula [$(A_{420}*1000)$ / (time*volume*OD_{590})] considering the absorbance values at 30 minutes.

Intracellularβgalactosidase activity assay

Single colony of WT *Salmonella typhimurium* 14028s and sifA-lacZ fused 14028s was grown overnight in LB media and was transferred to secondary LB culture (1% inoculum) and was left to grow until 1 A_{600}. The HeLa cells were infected with $1*10^9$ *Salmonella* per well of a 6 well plate. After infection for an hour, gentamycin treatment for two hours followed by three hours of compound G treatment the HeLa cells were scrapped and collected and centrifuged for 10 minutes at 4000g. The pellet was dissolved in 100µl of 1X PBS and 10µl aliquot was removed for plating on LB plates. βgalactosidase activity assay was performed by mixing cell culture (500µl) with 900µl of Z lysis buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH7.0), two drops of chloroform and 0.1% SDS followed by vigorous vortexing to lyse the bacteria. O- NitrophenolGalactoside (4mg/ml), made in sodium phosphate buffer of pH7.4 was added (200µl) and transferred to 96 well plate after centrifugation (4000g, 10 minutes), 150µl per well and absorbance values at 420nm and 590nm was noted at fixed intervals. The βgalactosidase activity was calculated using the miller formula [(OD_{420}*1000) / (time*volume*OD_{590})] considering the absorbance values at 120 minutes.

2.10 Immunofluroscence:

HeLa cells were infected with *Salmonella typhimurium* WT strain SL1344 with MOI of 400 for 15 minutes followed by gentamycin treatment at the concentration of 100µg/ml for 10 minutes to kill the extracellular bacteria. The cells were treated with or without compound G and incubated for different time points at 37˚C, fixed and subjected to immune fluorescence using anti-p62 antibody (1:500 dilution)and anti-LC3 antibody (1:500 dilution) followed by secondary antibodies (Atto 633, Sigma 1:200 dilution). The images were obtained using deconvolution microscopy (Delta Vision, GE). The p62 and LC3 co-localization with *Salmonella typhimurium* SL1344 was quantified using ImageJ-Cell counter plugin (NIH).

2.11 Live Cell Microscopy

GFP-LC3transfected HeLa cells was infected with mcherry- *Salmonella typhimurium* SL1344for 15 minutes (MOI=400) and was treated with gentamycin for 10 minutes. The cells were then washed with 1X PBS and changed to either only media or media containing compound G and

imaged by FV10i- olympus confocal live cell imaging microscope, using 60x water immersion lens, with confocality aperture set to 1.0. Images were taken at an interval of 15 minutes (Five Z sections of 1 micron each was taken). The intensity of the Red channel was measured using image J – Stacks T function- Intensity vs time plot plugin.

* I acknowledge Dr. Deepak Saini, MRDG, IIsc for providing access to live cell microscope facility and Mr. Vignesh for his kind help during the experiment

Chapter 3

Screening and validation of compounds for xenophagy

Hits from yeast based high throughput screening done in lab previously for autophagy modulators were further verified using secondary assays in mammalian cells and for their ability to clear intracellular pathogens.

3.1 Results and Discussion

3.1.1 Screening for xenophagy enhancers

The screening for xenophagy involves monitoring the intracellular clearance of *Salmonella typhimurium*in the presence of the compounds over a period of time. The assay protocol involves infection of *Salmonella typhimurium* on host cells (HeLa/U1752/RAW 264.7) for an hour followed by gentamycin treatment for two hours to remove extracellular bacteria. Compound treatment for three hours was followed by lysing the host cells and plating the intracellular bacteria. The screening was done in U1752 and the effect of the compound on clearance of the pathogen was quantified by counting the colony forming units (Figure 1A). The concentrations of the drugs used for screening were based on the cell viability assay done for the compounds.

Though all the compounds screened for xenophagy are autophagy inducers, not all the compounds could mediate clearance of intracellular *Salmonella typhimurium*except for the compound G at 50µM which showed two fold and more reduction (Figure 1B).

3.1.2 Validation of effect of compound G in three cell lines

Compound G showed consistent clearance of intracellular *Salmonella* in the screening (Figure2). As most pathogens target epithelial cells and macrophages, the effect of the drug was further verified in HeLa epithelial and RAW 264.7 macrophage cell lines. Consistent results were obtained in all the three cell lines used to check the efficiency of compound G. This shows that the effect of the compound in clearing intracellular *Salmonella* is not cell type specific.

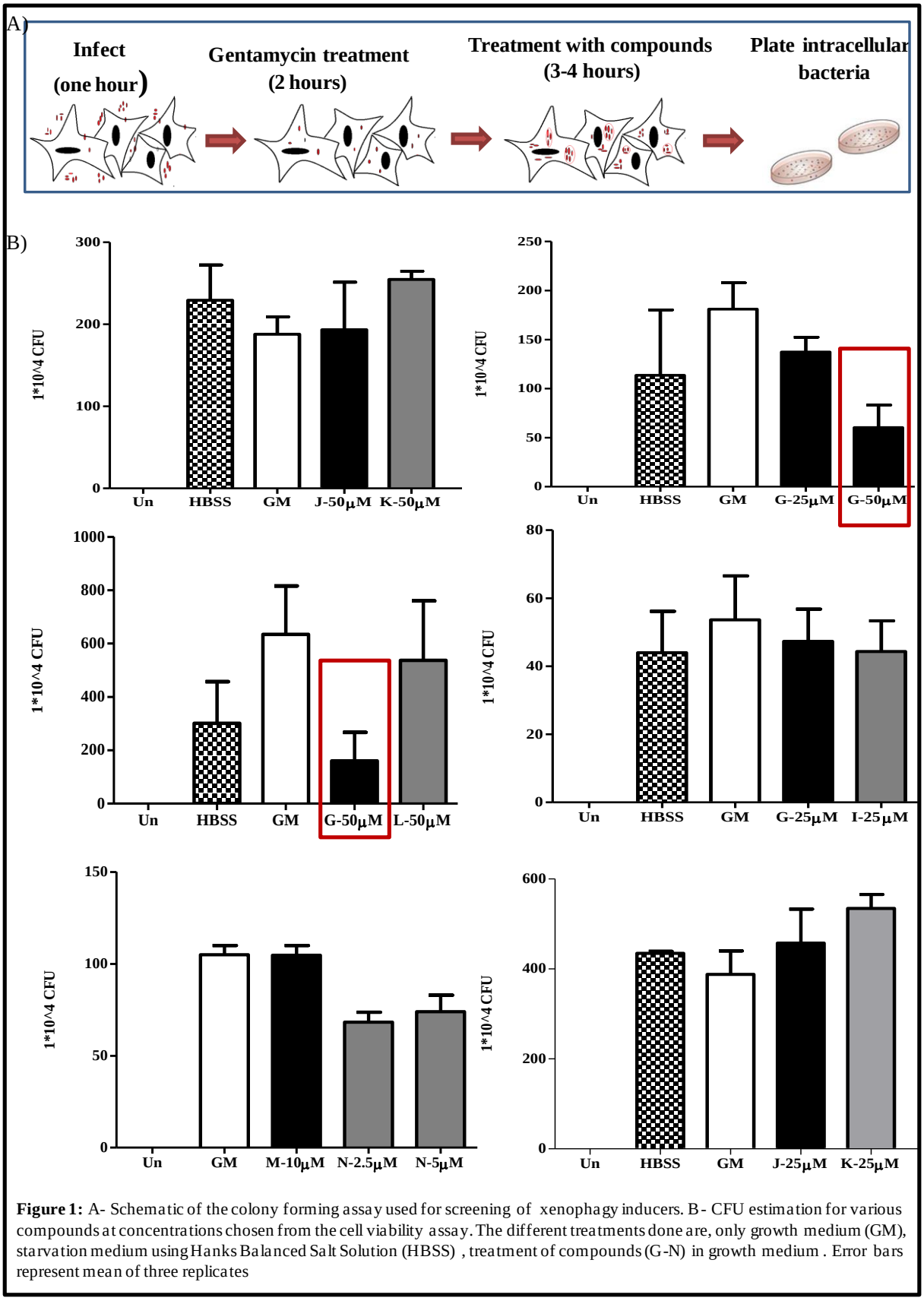
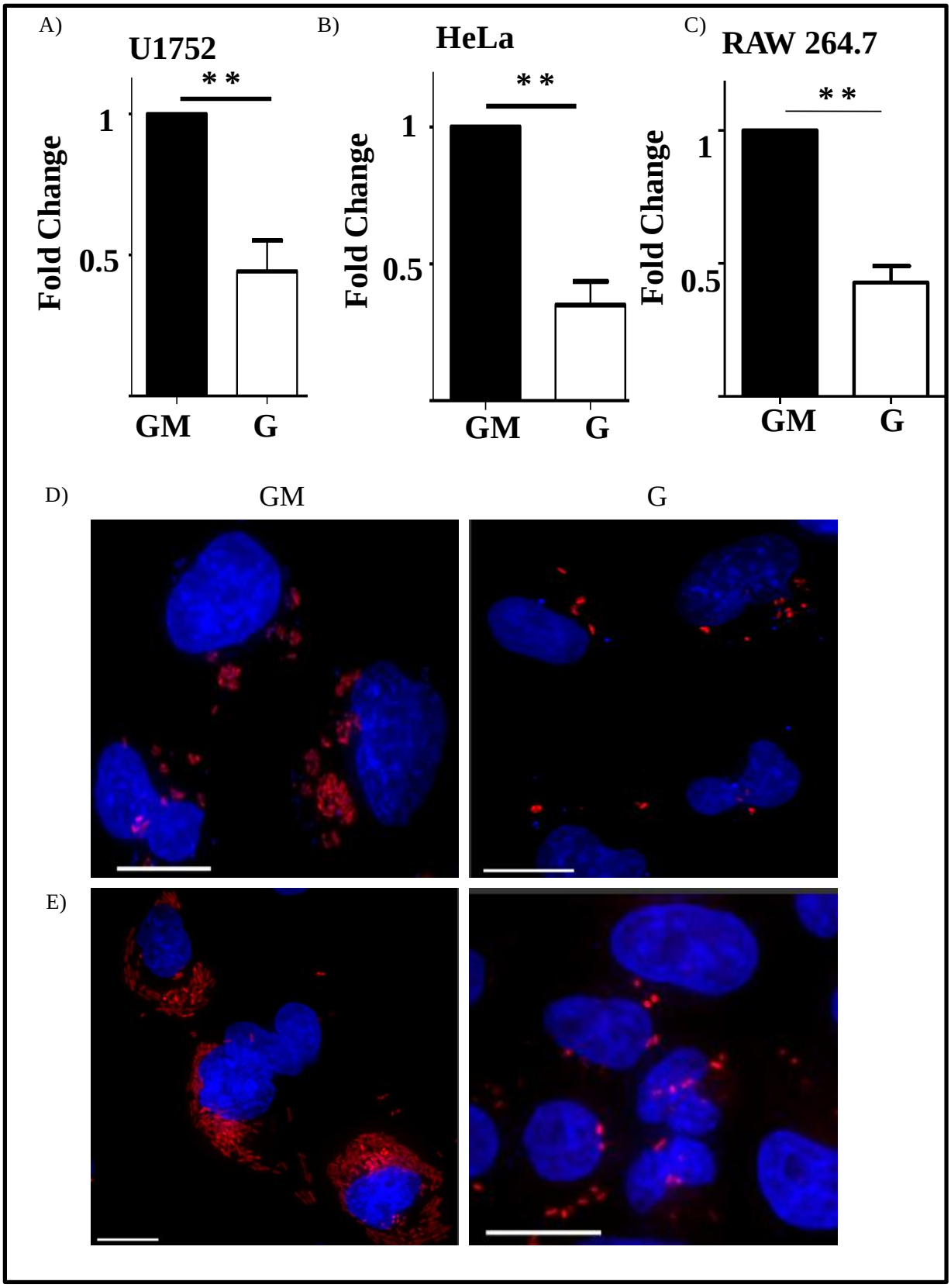


Figure 1: A- Schematic of the colony forming assay used for screening of xenophagy inducers. B- CFU estimation for various compounds at concentrations chosen from the cell viability assay. The different treatments done are, only growth medium (GM), starvation medium using Hanks Balanced Salt Solution (HBSS) , treatment of compounds (G-N) in growth medium . Error bars represent mean of three replicates


A)
U1752
* *
Fold Change
1
0.5
GM
G
B)
HeLa
* *
Fold Change
1
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GM
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C)
RAW 264.7
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Fold Change
1
0.5
GM
G
D)
GM
G
E)

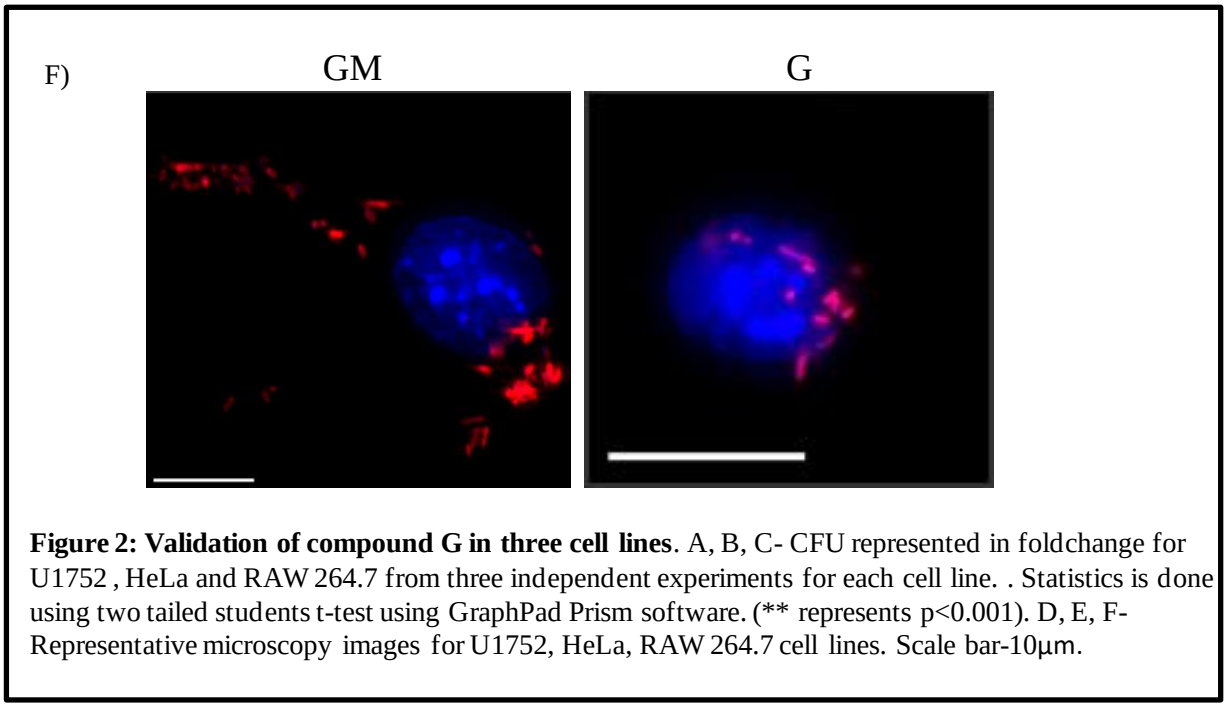


Figure 2: Validation of compound G in three cell lines. A, B, C- CFU represented in foldchange for U1752 , HeLa and RAW 264.7 from three independent experiments for each cell line. . Statistics is done using two tailed students t-test using GraphPad Prism software. (** represents p<0.001). D, E, F- Representative microscopy images for U1752, HeLa, RAW 264.7 cell lines. Scale bar-10µm.

3.1.3 Cell viability assay

CellTitre-Glo cell viability assay is a luminescence based method to determine the number of viable cells in a culture by measuring the amount of ATP present which is generated by the metabolically active cells. CellTitre-Glo reagent is added to cells, which lyses the cells and the ATP released fuels the reaction ofluciferin in the reagent to form oxyluciferin. This product generates the luminescent signal that is directly propotional to the amount of ATP which indicates the number of viable cells.

This assay was used to test the effect of compound G on the viability of mammalian cells. HeLa and U1752 cells were treated with compound G at 50µM concentration for different time points. At the end of each time point the cells were lysed using CellTitre-Glo reagent and the luciferase reading was measured. The compound at 50µM concentration did not affect the viability of cells till 10 hours (Figure 2). So, for the further experiments the treatment of compound G at 50µM was used at time points of 6 hours and below.

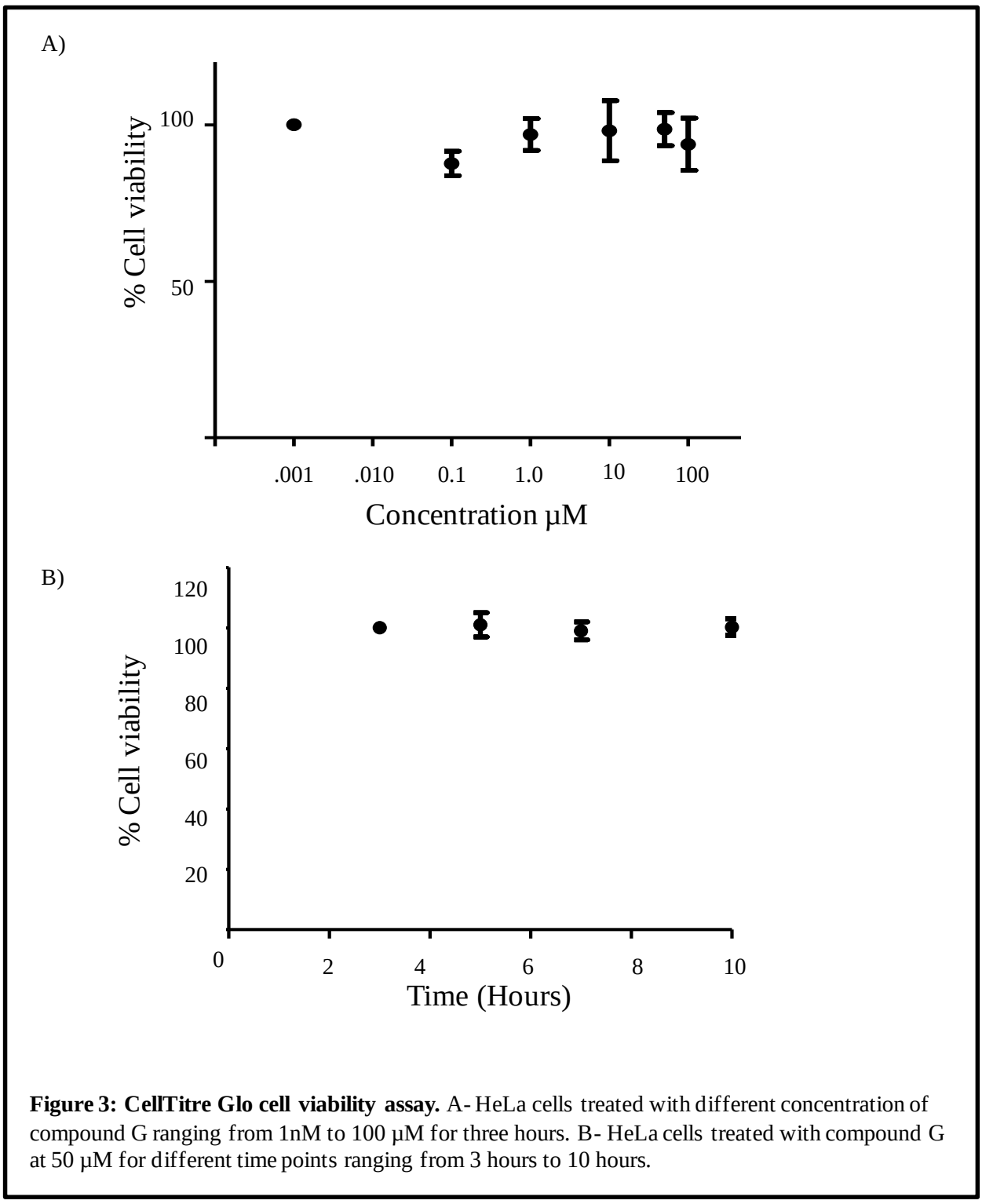


Figure 3: CellTitre Glo cell viability assay. A- HeLa cells treated with different concentration of compound G ranging from 1nM to 100 µM for three hours. B- HeLa cells treated with compound G at 50 µM for different time points ranging from 3 hours to 10 hours.

3.1.4 Induction of autophagic flux by compound G

Traffic light assay

Traffic light assay is useful to study the autophagic flux in which LC3, a member marker for autophagy process is tandemly tagged to mRFP and GFP fluorescent proteins. This construct when expressed in mammalian cells can be used to differentiate between the autophagosome and autolysosome populations. This assay depends on the quenching of GFP signal in acidic pH of the autolysosomes. Hence LC3 found on autophagosomes appear yellow due to double tagging whereas LC3 found on autolysosomes appear red. The assay can be used to easily identify autophagy enhancement or inhibition caused due to chemical modulators or stress conditions by measuring the yellow and red puncta within cells as given below.

	AUTOPHAGOSOMES	AUTOLYSOSOMES
Autophagy induction	↑	↑
Autophagy inhibition at early steps	↓	↓
Autophagy inhibition at late steps	↑	↓

Traffic light assay was done for compound G to investigate whether the induction of autophagy as shown in yeast high throughput assay was due to increase in autophagosome formation or due to increase in fusion of autophagosomes with lysosomes to form autolysosomes. Compound G at 50µM concentration in growth medium was treated on HeLa cells transfected with ptfLC3 for two hours. Increase in number of both autophagosomes and autolysomes was observed in comparison with only growth medium treated cells. The increase was more pronounced in case of autolysosomes going to show that compound G treatment has lead to increase in the fusion step (Figure 4B).

3.1.5 Western blot analysis of autophagy induction

LC3 is a protein found in cytoplasm in LC3-I form whereas becomes membrane associated LC3-II form when activated by autophagy due to phosphoethylamine (PE) attachment. The conversion from LC3-I to LC3-II form can thus be used to identify autophagy induction and

inhibition. Compound G when treated on HeLa cells for hours at 50µM concentration lead to accumulation of LC3II form going to show the induction of autophagy process (Figure 4C).

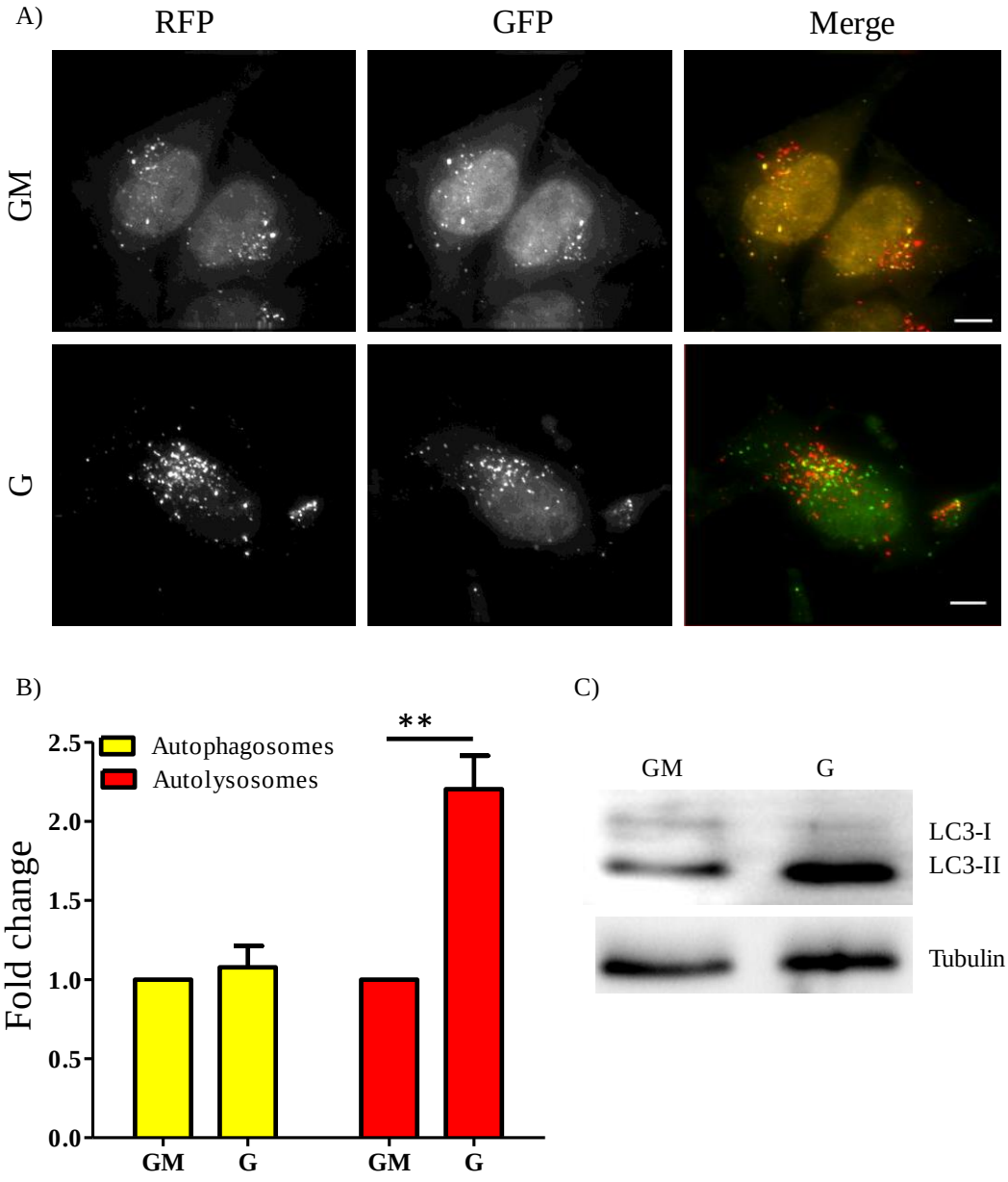


Figure 4: **Traffic light assay**. A- Treatment of compound G at 50µM for two hours in ptfLC3 expressing HeLa cells. Representative image from n=50 from three independent experiments shown. B- Quantitation of number of puncta per cell is expressed in fold change. Statistics is done using two tailed students t-test using GraphPad Prism software. (** represents $p<0.001$). Scale bar represents 5 µm. C- Western blot analysis showing autophagy induction and increased LC3-II form conversion.
Note- Traffic light assay was done along with Mr. Piyush Mishra, JNCASR.

Effect of compound G on intracellular Methicillin resistant *Staphylococcus aureus* (MRSA)

In order to check the effect of compound G on wider range of pathogens, gram positive MRSA was used in the colony forming assay in RAW 264.7 macrophages. Initial studies have shown more than two fold decrease (Figure 5) as seen in the case Salmonella going to show that the compound is not specific for a particular pathogen type.

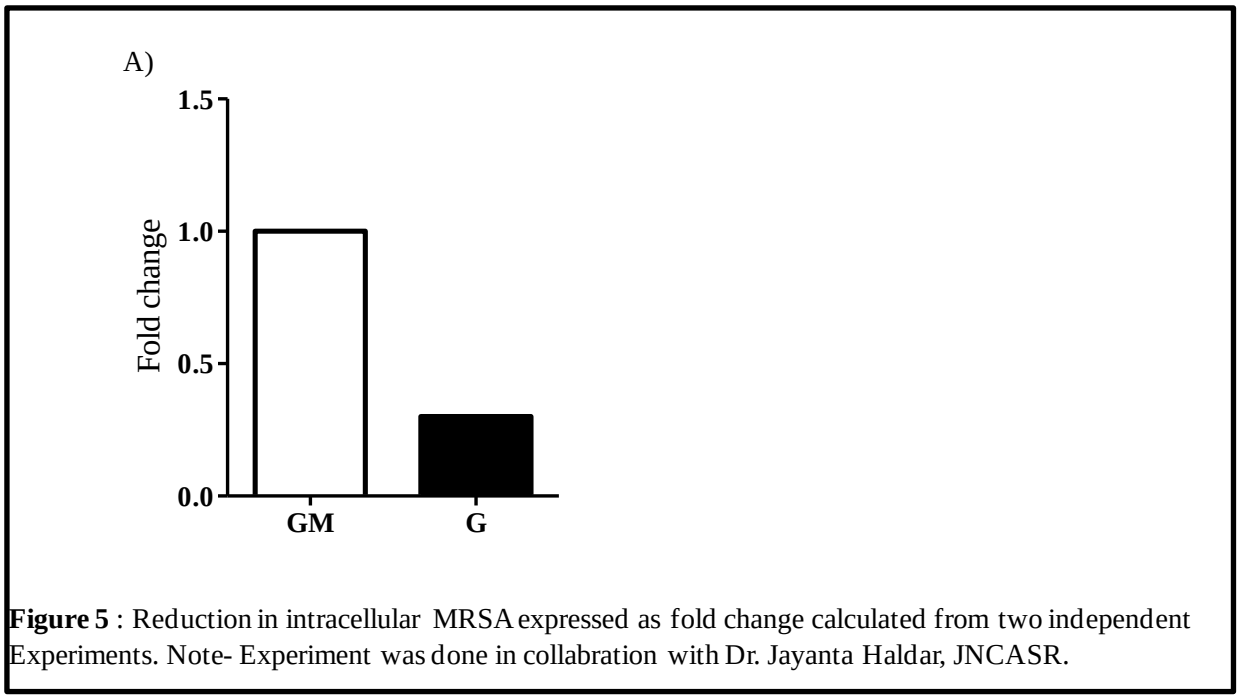


Figure 5 : Reduction in intracellular MRSA expressed as fold change calculated from two independent Experiments. Note- Experiment was done in collabration with Dr. Jayanta Haldar, JNCASR.

The experiments so far has shown that compound G has the ability to clear intracellular *Salmonella*. It is also evident that the effect of the compound is not due to a particular cell type but is also applicable in other cell types like macrophage cell line that behave differently to pathogen insult. General autophagy assays has shown that compound is an autophagy inducer in mammalian cells even in the absence of infection. Hence the compound could be a potential xenophagy inducer having a broad spectrum of action against wide number of pathogens as seen in gram negative *Salmonella typhimurium* and gram positive MRSA.

Chapter 4

Insights into mechanism of action of compound G

Compound G as shown in the previous chapter is a potential xenophagy inducer that has the ability to clear intracellular pathogens. This chapter addresses the potential mechanism of action especially the role of autophagy.

Two major directions taken to understand the mechanism of compound action are

- Effect of the compound on pathogen directly
- Ability of the compound to induce host cellular defense mechanisms.

4.1 Results and Discussions

4.1.1 Effect of the compound on pathogen directly

As the compound is able to inhibit the growth of intracellular pathogens, there is a possibility that the compound could have a bacteriocidal or bacteriostatic effect directly on the pathogen. Also, replication of pathogens depends on the expression of virulence genes. In case of *Salmonella typhimurium,* the virulence genes are clustered in the form of *Salmonella* pathogenicity islands (SPI). Any inhibition to the expression of virulence genes will make the pathogen weak and incapable of replication. In order to check these possibilities, growth curve of *Salmonella typhimurium* and the expression of SPI-2 gene were checked.

4.1.1.1 Growth Curve

The effect of compound G on *Salmonella* growth *in vitro* in Luria Broth was studied by monitoring the growth curve. A single colony of *Salmonella typhimurium* SL1344 grown overnight was diluted to get an O.D of 0.1. The diluted culture was used for treatments with compound G and gentamycin (100µg/ml) and transferred to 384 well plate containing 80µl culture per well. The growth of the culture was obtained by measuring the A_{600}every 30 minutes for 10 hoursusing varioskan Flash PlatereaderSpectrophotometer at 300 rpm and the values were

plotted using GraphPad Prism.It was seen that there was no lag in the growth of cultures treated with compound G showing that the compound does not have any direct anti-microbial activity on *Salmonella*(Figure 1).

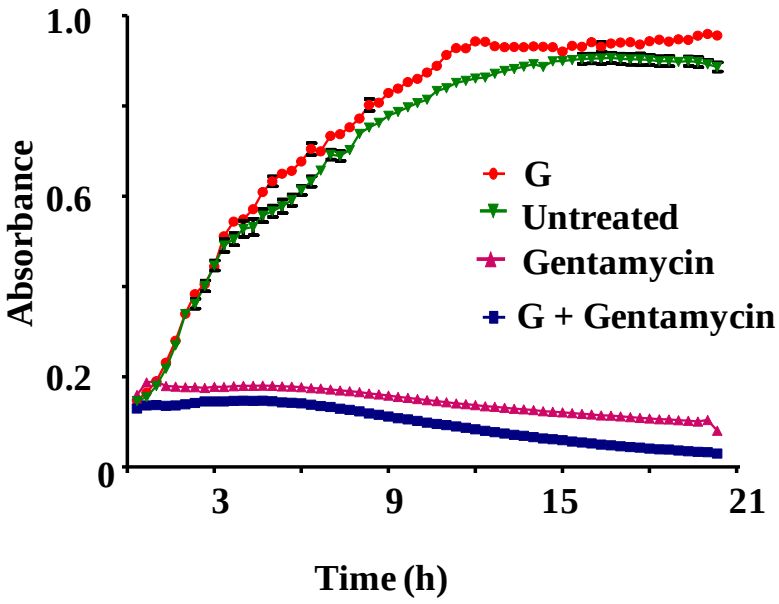


Figure 1: Growth curve of *Salmonella typhimurium*. Growth curve of *Salmonella typhimurium* culture (untreated) and culture treated with compound G or gentamycin was obtained by measuring the A_{600} in a 384 well plate using Varioskan Flash plate spectrophotometer.

4.1.1.2 Expression of SPI-2 virulence gene of *Salmonella typhimurium*

β Galactosidase assay

Using lacZ as a reporter gene, the effect of the compound on sifA gene expression was studied. sifA is a member of SPI-2 virulence genes required to form *Salmonella* induced filaments (Sifs) on the *Salmonella* containing vacuoles (SCVs), which are the replicating niches of *Salmonella* within cells. Sifs gives stability to SCVs preventing fusion with lysosomes and thus establishing infection. The expression was not affected when lacZ-sifA*Salmonella typhimurium* 12048s was grown in the presence of compound G (Figure 2B).

To check if the compound acts on expression of virulence genes post infection, intracellular *Salmonella* after treatment with compound G was isolated and tested. The expression was not affected even in the intracellular pathogens treated with compound (Figure 2C).

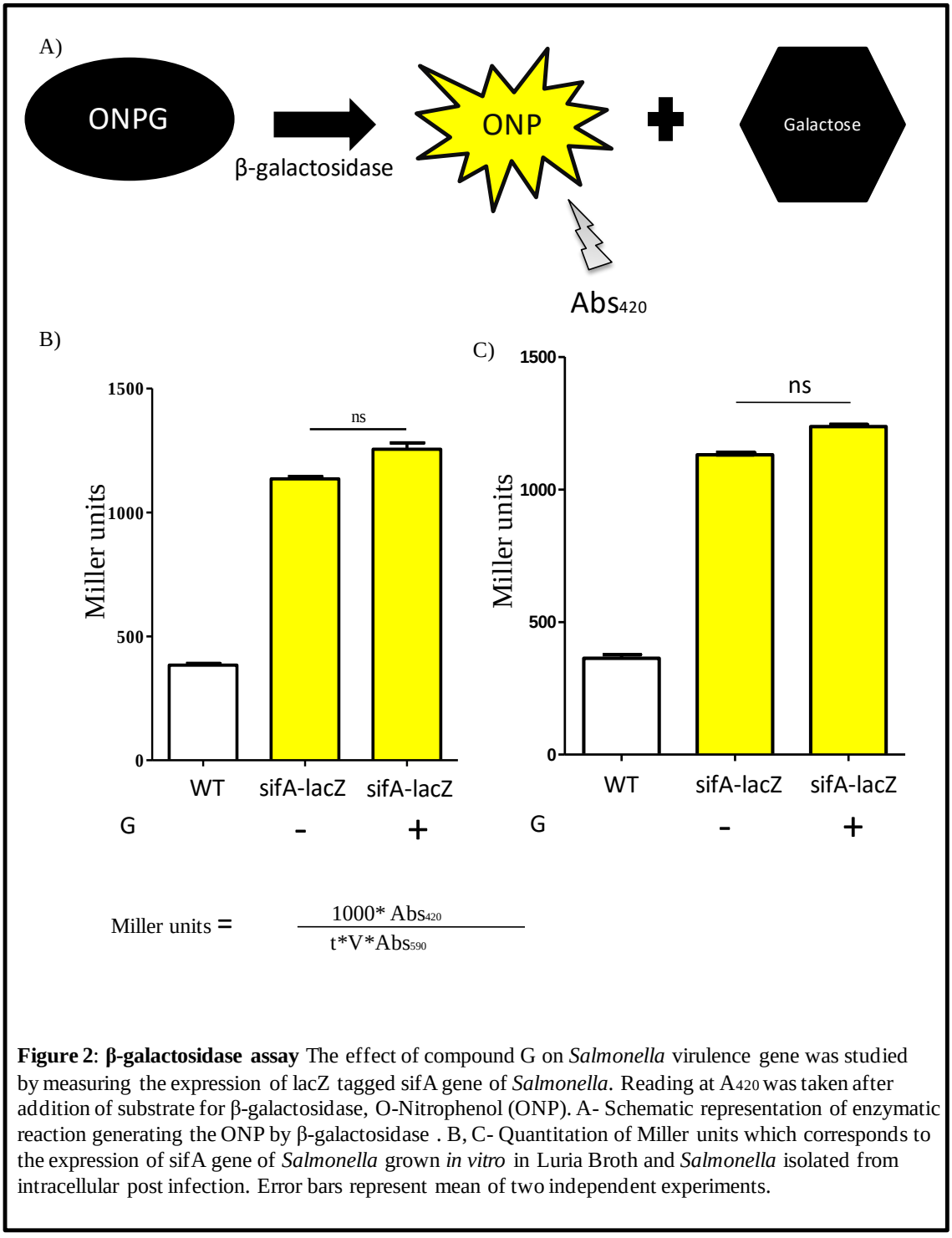


Figure 2: **β-galactosidase assay** The effect of compound G on *Salmonella* virulence gene was studied by measuring the expression of lacZ tagged sifA gene of *Salmonella*. Reading at A_{420} was taken after addition of substrate for β-galactosidase, O-Nitrophenol (ONP). A- Schematic representation of enzymatic reaction generating the ONP by β-galactosidase . B, C- Quantitation of Miller units which corresponds to the expression of sifA gene of *Salmonella* grown *in vitro* in Luria Broth and *Salmonella* isolated from intracellular post infection. Error bars represent mean of two independent experiments.

4.1.2 Ability of the compound to induce host defense mechanisms

As the compound did not have any apparent effect on growth and expression of virulence genes of *Salmonella typhimurium*, next the effect of the compound on inducing host defense mechanisms were checked. As the compound is already shown as an autophagy inducer, the compound could enhance the xenophagy process to capture the intracellular pathogens. In order to check if the process of autophagy is indispensible for compound G's effect, the clearance of intracellular pathogens by compound G was tested in autophagy null conditions created by both genetic and pharmacological ways. Also, induction of xenophagy was checked by monitoring the recruitment of xenophagy machinery.

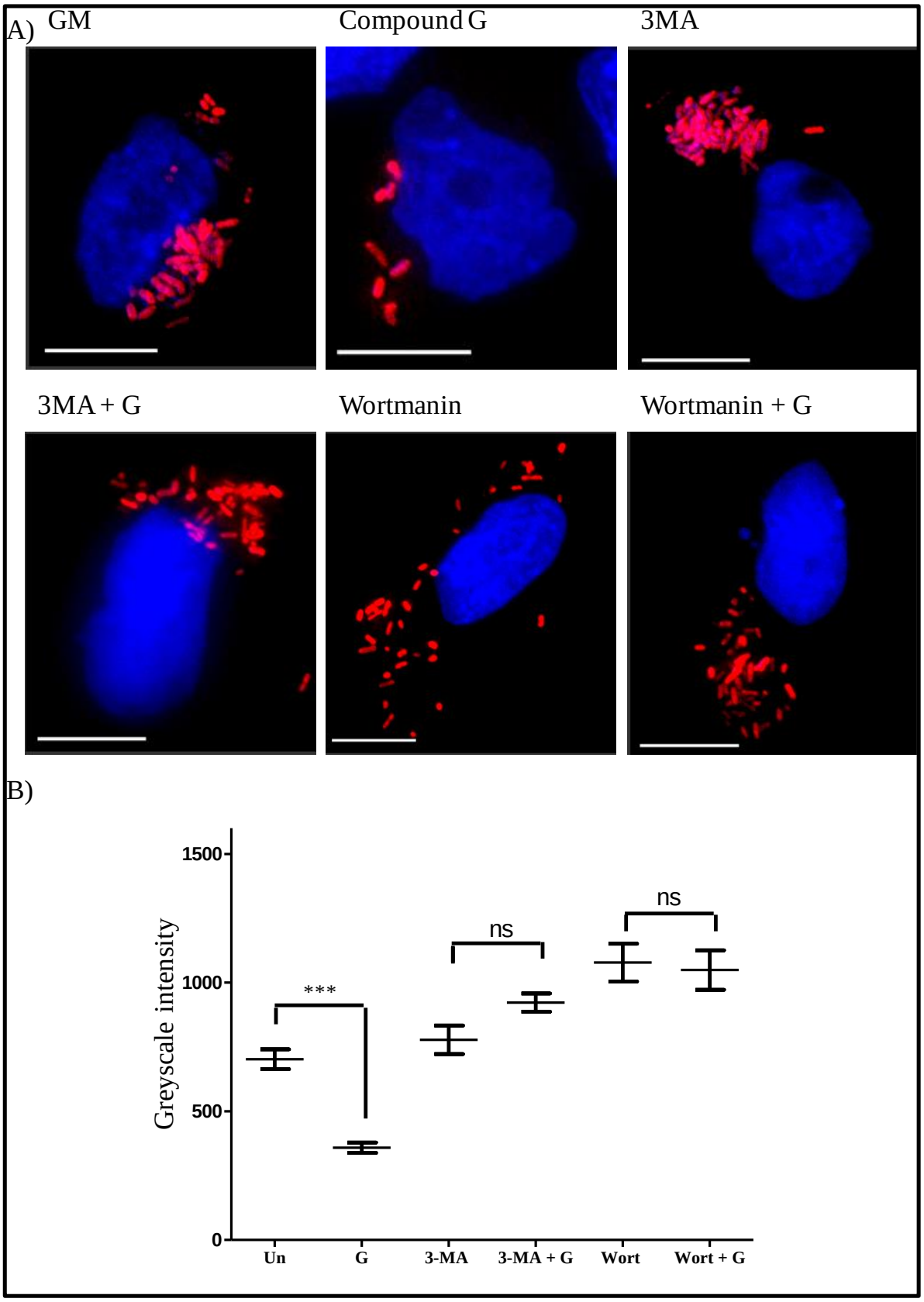
4.1.2.1 Dependence of compound G on autophagy

Using Atg5 KO HeLa cell line

In order to check if the compound G's effect is dependent on autophagy process, the colony forming assay as described in chapter IIwas performed using Atg5 KO HeLa cell line. It was observed that the intracellular clearance of *Salmonella* by the compound was ineffective in the knockout cell line going to show that the mechanism of drug action is dependent on autophagy process (Figure 3C).

Using chemical inhibitors

Another way to inhibit autophagy in cells is by using chemical molecules that act on autophagy pathway and inhibit the process. Two of the commonly used inhibitors are 3-methyladenine (3-MA) and wortmanin, both of which are PI3K inhibitors. Treatment of 3-MA or wortmanin along with compound G prevented the clearance of intracellular *Salmonella* thus confirming the involvement of autophagy process in compound G's mode of action (Figure 3B).


A)
GM
Compound G
3MA
3MA + G
Wortmanin
Wortmanin + G
B)
Greyscale intensity
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G
3-MA
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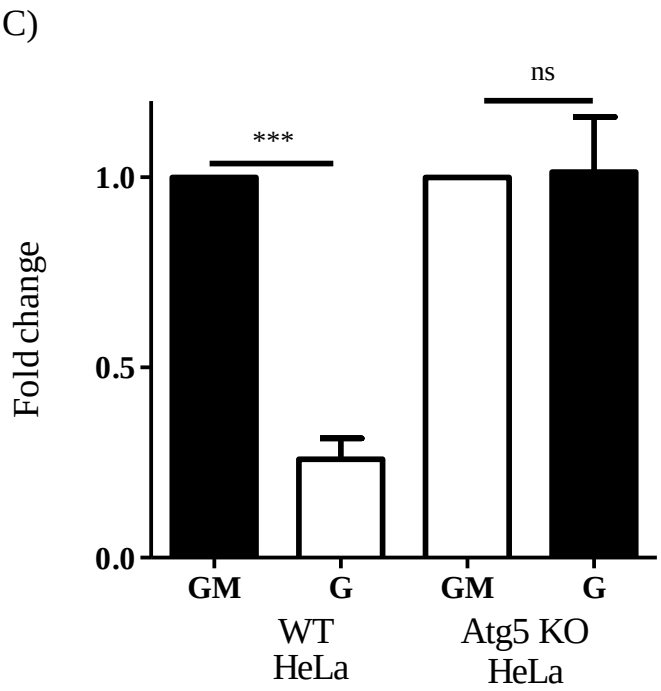


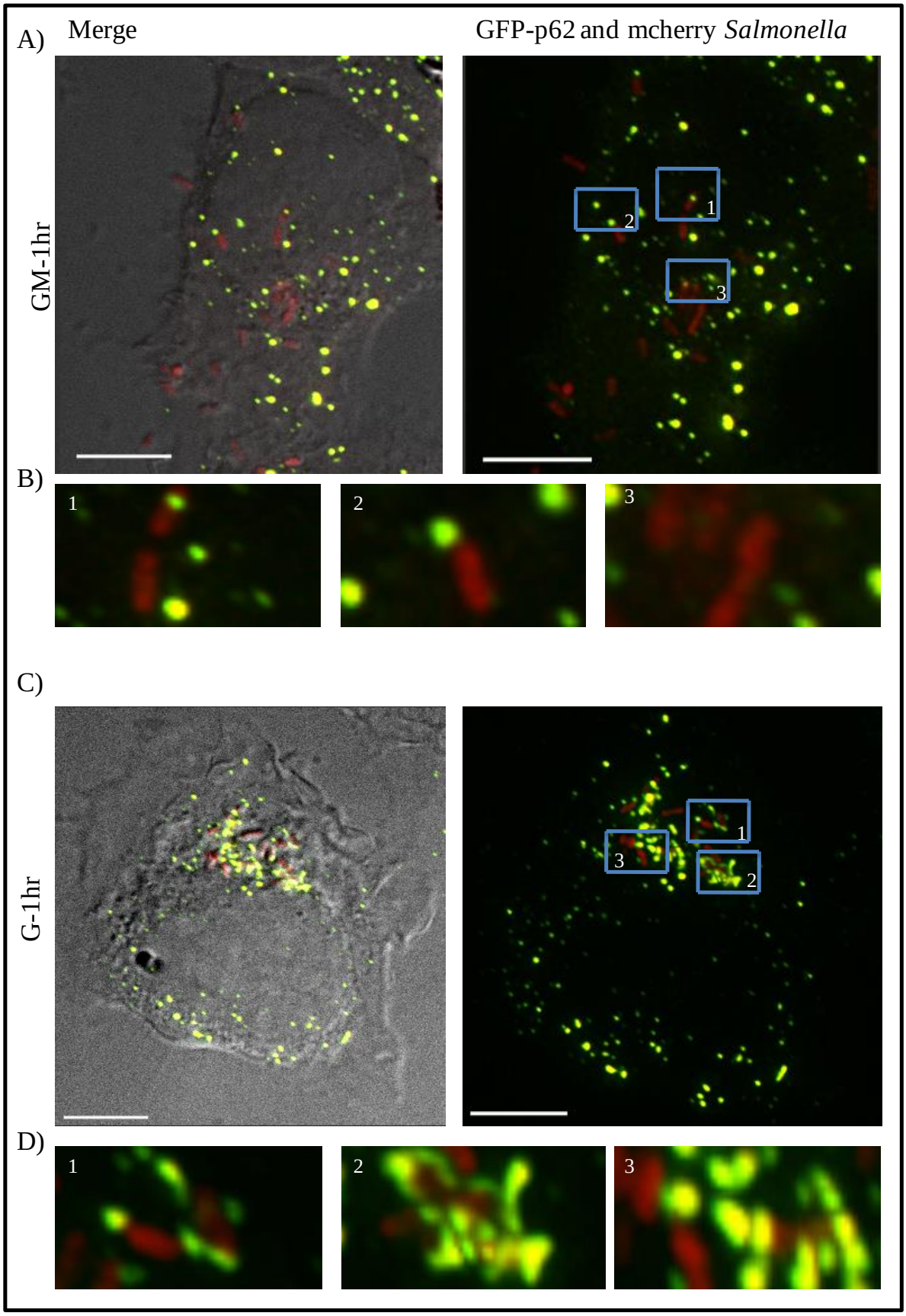
Figure 3: A- Representative microscopic images for HeLa cells treated with chemical inhibitors, wortmanin or 3MA along with the compound G taken from n=45 from three independent experiments . B- Quantitation for the effect of chemical inhibitors along with compound G. Statistics is done using two tailed students t-test using GraphPad Prism software. (*** represents p<0.0001). C- Quantitation represented as fold change for effect of compound G on Atg5 KO cell line and control WT HeLa cells. Statistics is done based on three independent experiments using two tailed students t-test using GraphPad Prism software.

4.1.2.2 Recruitment of xenophagy machinery to *Salmonella*

Xenophagy machinery recruits adaptor proteins (p62, NDP52, OPTN, NBR1) for capture of ubiquitinated bacterial cargos. The adaptor proteins are in turn recognized by autophagy membrane proteins. The membrane protein families include LC3 (its isoforms-LC3A, LC3B, LC3C) and GABARAP family (GABARAP, GABARAPL1, GABARAPL2).

Immunofluorescence was done against one of the commonly studied adaptor and membrane proteins, p62 and LC3B respectively in order to check for its recruitment to intracellular *Salmonella* population. HeLa cells were infected with mcherry tagged *Salmonella typhimurium* SL1344 and were immunostained for p62 and LC3B at different time points after treatment with compound G. Recruitment of p62 and LC3 increased with prolonged times of drug treatment unlike the only growth medium treated samples where recruitment was highest only in the initial

time point say one hour and dropped at later time points. Recruitment was distinct in compound G treated samples as there was clustering of p62 and LC3 protein seen in the regions of *Salmonella*. This clustering recruitment is quantitated by taking the ratio of red (mcherry-Salmonella) and green channel (p62) within a Region of Interest (ROI) (Figure 4I). This shows that the compound G is able to keep the cells in autophagy induced state and recruit xenophagy machinery proteins.


A)
Merge
GFP-p62 and mcherry *Salmonella*
GM-1hr
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C)
G-1hr
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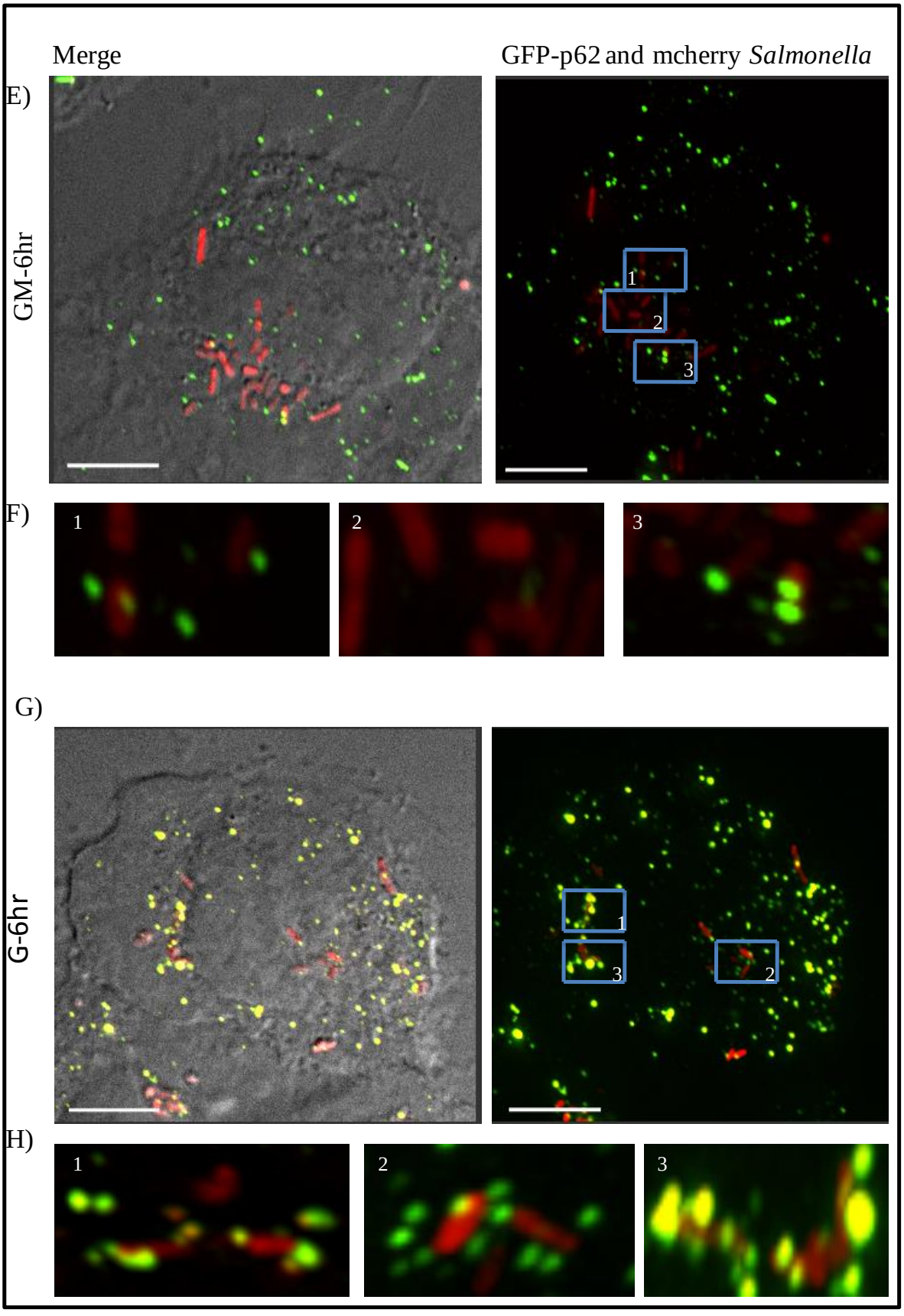
Merge
GFP-p62 and mcherry *Salmonella*
E)
GM-6hr
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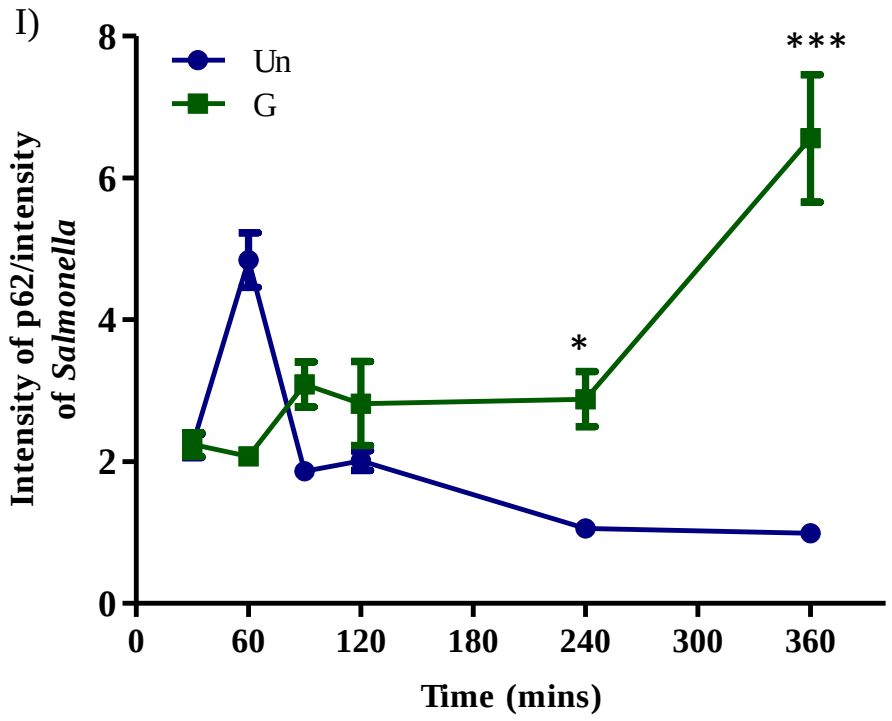


Figure 4: Recruitment of p62 to *Salmonella*. HeLa cells infected with mcherry- *Salmonella* and treated with compound G were immunostained for p62. A,C- Representative image for samples collected post one hour drug treatment and zoomed in images of the same are shown below (B,D). E,G- Representative image for samples collected post six hour drug treatment and zoomed in images of the same are shown below (F,H). I- Quantitation of p62 recruitment to *Salmonella* plotted as ratio of red channel (mcherry-*Salmonella*) and green channel (p62) within a region of interest (ROI) that is kept constant across all cells. Over 100 ROIs was measured from three independent experiments for each time point and the ratio is plotted for untreated (growth medium) and G treated using GraphPad Prism. Statistics is done using two tailed students t-test using GraphPad Prism software. (* represents $p<0.01$*** represents $p<0.0001$). Scale bar-5 µm.

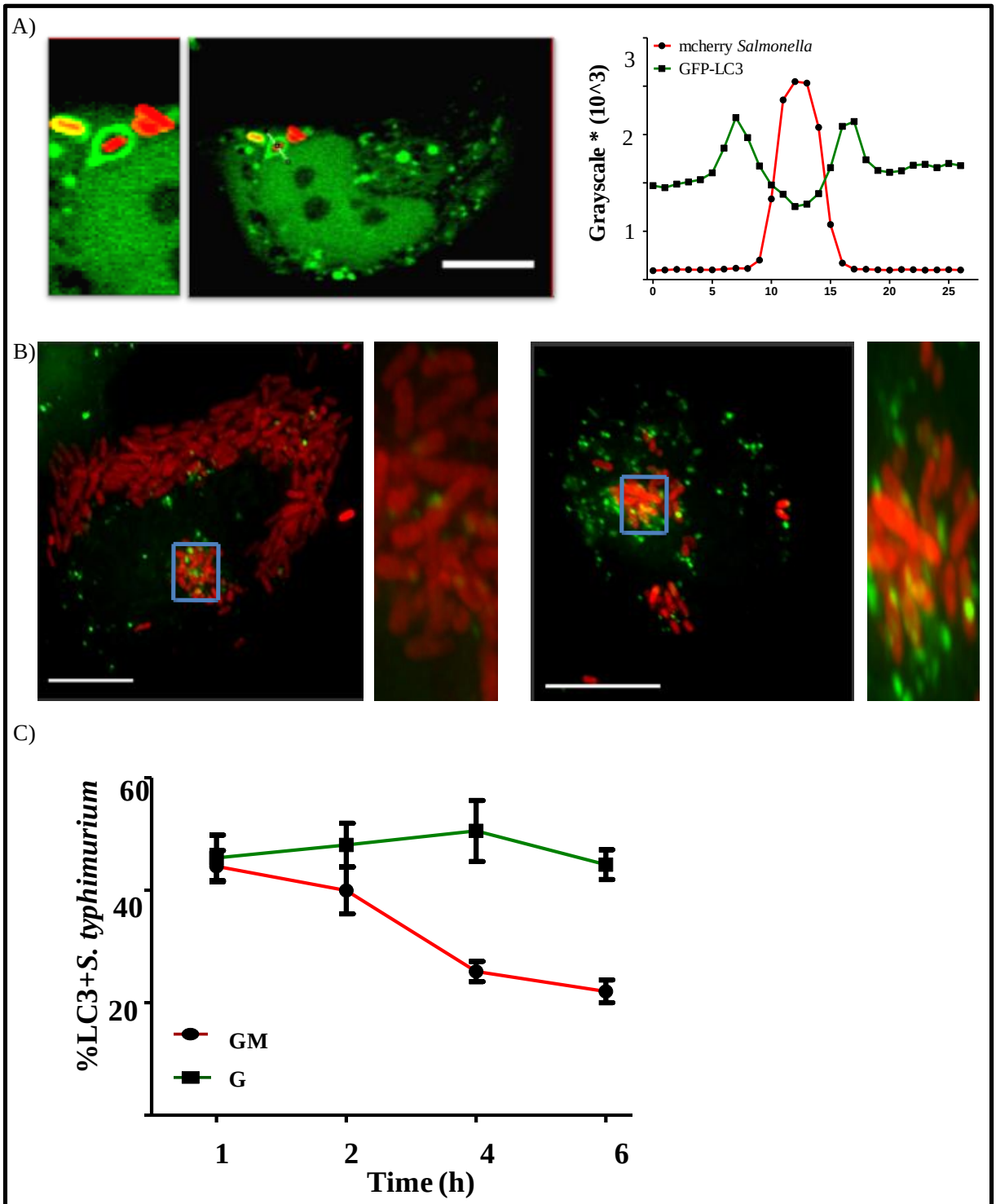
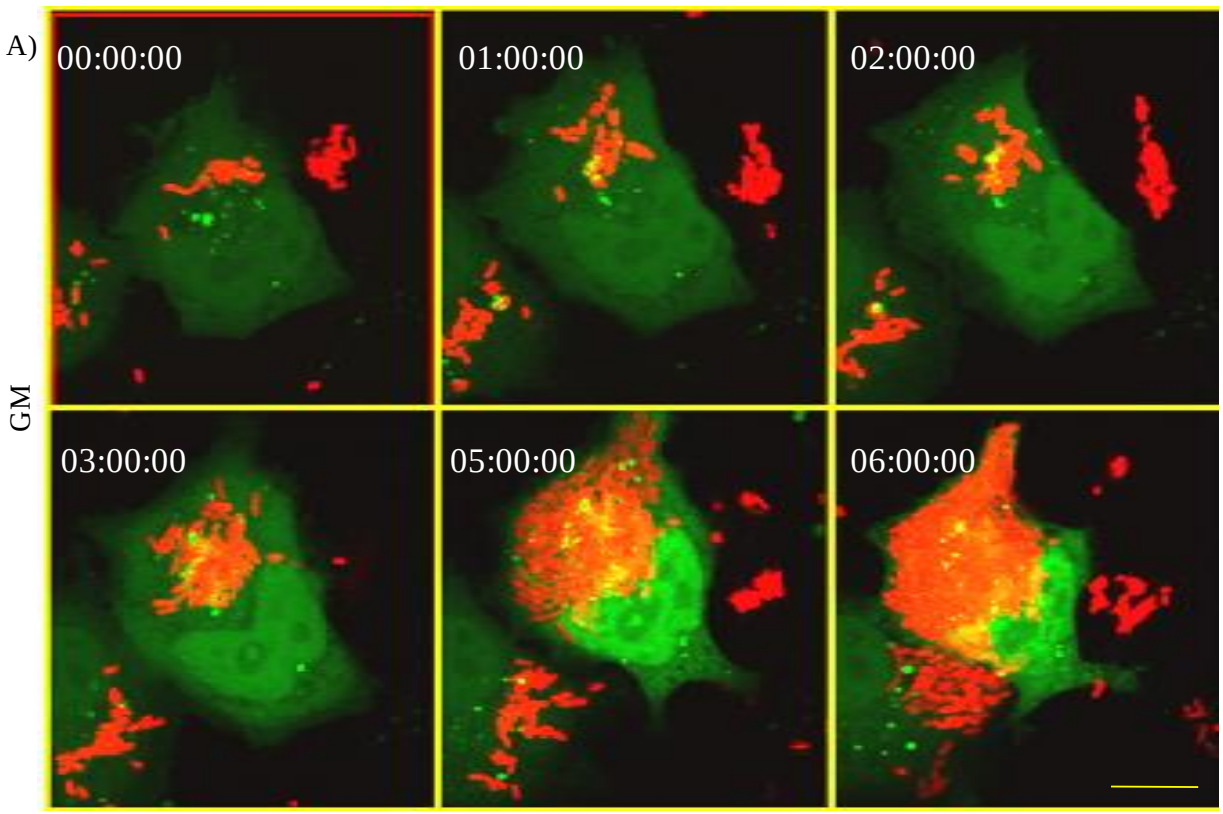


Figure 5: LC3 recruitment to mcherry *Salmonella*. A- line profile for a *Salmonella* captured by GFP-LC3. B- Representative microscopic images for HeLa cells infected with mcherry *Salmonella* treated with growth medium (GM) and compound G for 6 hours. C- Quantitation of LC3 recruitment to *Salmonella* measured by measuring the percentage of *Salmonella* positive for LC3 puncta is plotted for GM and G treated using GraphPad Prism. Scale bar-5 µm.

4.1.2.3 Live cell analysis of mechanism of compound G action

In order to study the events of capturing and degradation by the compound, live cell imaging was done on mcherry - *S.typhimurium* infected HeLa cells that is transfected with GFP-LC3 and followed every 15 minutes for 6 hours. Observation of live cell videos indicated that the compound G halts the replication of *Salmonella* and is arrested mostly in LC3 positive vesicles. There was no evident degradation observed in the videos atleast at the end of six hours (Figure 6C).

*I acknowledge Dr. Deepak Saini, MRDG, IIsc for providing access to live cell microscope facility and Mr. Vignesh for his kind help during the experiment.


A)
GM
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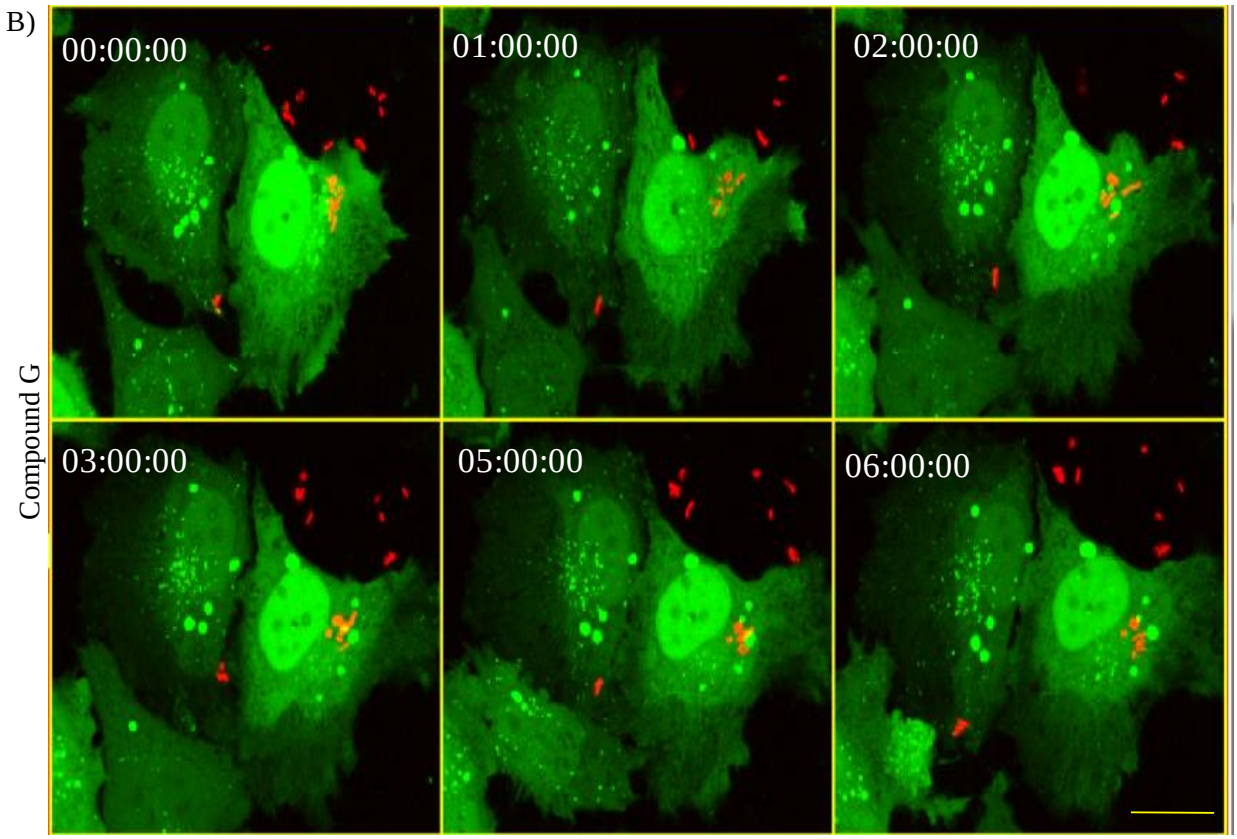
B)
Compound G
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01:00:00
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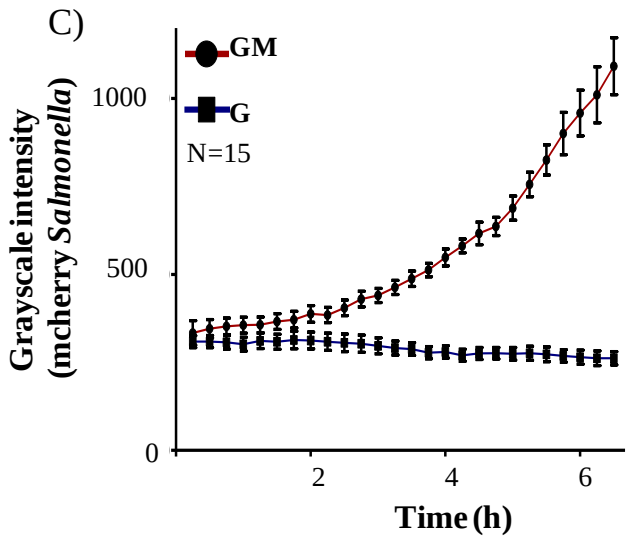

Figure 6: Live cell microscopy A, B- HeLa cells expressing GFP-LC3 was infected with mcherry *Salmonella* supplemented with only growth media (GM) or growth medium with compound G and imaged using confocal live cell microscope. Scale bar- 5µm. C- Replication of intracellular Salmonella was quantitated by measuring the intensity of red channel (mcherry – *Salmonella*) over time.
Live cell microscopy was done in Prof. Deepak Saini's lab, MRDG, IISc

The conventional xenophagy process involves capturing of cargo leading to fusion with lysosomes degrading the captured bacteria. It was seen through microscopic techniques that compound G mediated capturing of bacterial cargo, prevented the replication of *Salmonella* but not its degradation till six hours. It is possible that the compound has the ability to restrict intracellular bacteria. As xenophagy is a downstream effect, it is possible that the compound enhances signaling upstream especially those related immune response that makes compound G a potent xenophagy inducer among other autophagy inducers.

Chapter 5: Discussions and Future plans

Xenophagy targets the intracellular pathogen populations for an autophagy-mediated lysosomal degradation fate. In addition, this process also helps in antigen presentation, initiating the secondary immune response inside host cells. Not surprisingly, pathogens have evolved strategies to block or manipulate xenophagy as it is a common host defense mechanism against wide range of microbes. Hence modulation of xenophagy as a new paradigm for clearance of intracellular pathogens is being explored in addition to research on new generations of antibiotics.

Antibiotics which directly target microbes are currently the most effective tool to destroy pathogens, although their*invivo* efficacy depends on various factors mentioned below.

- Ability of the antibiotic to cross the hydrophobic membrane of host cells. For e.g. Gentamycin and streptomycin do not cross the cell membrane and are active on pathogens only in the tissue fluids.
- Pathogens get trapped into intracellular vesicles like phagosomes and are protected from action of antibiotics that do not enter such vesicles thus establishing pathogen reservoirs leading to chronic infection. Hence antibiotics that that has ability for intraphagosomal accumulation is more effective(*61*).
- Ability of the antibiotic to be active inside cells. This depends on the intracellular environment of the host cell. Aminoglycoside and macrolide antibiotics for example are less active in acidic pH of lysosomes(*62*).
- Interaction of the antibiotic with host defense factors. As an example, although clindamycin gets accumulated inside cells, it is ineffective because it inhibits the action of antimicrobial peptides and superoxide production in phagocytic cells(*63*).

Another major hurdle in dealingwith infectious diseases is antibiotic resistance. A potent antibiotic over time becomes ineffective due to spread of resistant strains. This forces a situation to avoid repeated use of antibiotics, commonly referred to as “antibiotic overuse” and there is a constant need for finding new generation of antibiotics.

Also, xenophagy is a mechanism that is active against a broader spectrum of microbes like bacteria, virus, fungi and protozoans unlike antibiotics which is specific against a particular subset of pathogens depending upon the mechanism of action of the drug. Hence new strategies to target the pathogens would be a boon to the field and will make it difficult for the pathogens to gain resistance against a range of defense mechanisms.

The objective of this project is to find novel compounds that would enhance innate immunity through xenophagy and would thus be an alternative approach to antibiotics to combat against infectious diseases. Compound G identified previously in lab as an autophagy inducer in a high throughput screen, was further confirmed using secondary assays. This compound showed potential among other autophagy inducers when screened for selective autophagy pathway such as xenophagy, especially against *Salmonella*. Further detailed microscopic experiments like immunofluorescence and live cell imaging showed that compound G halted replication of intracellular pathogens with an increased recruitment of autophagy machinery proteins such as p62 and LC3 to bacterial surface. Next, the clearance effect of the drug was abrogated when used on Atg5 KO HeLa cell line going to show that effect of drug is autophagy dependant. However, interestingly, this compound did not affect *in vitroSalmonella* growth *per se* in Luria Broth. The expression of virulence genes of *Salmonella*, specifically of Pathogenicity Island-2 (SPI-2) was also not affected by the drug.

These results thus indicate that an inducer of xenophagy can be used as a potential drug that could reduce the intracellular population of bacteria.

Future plans

Future studies with compound G will deal with unfolding the mechanism of drug action. The different directions in which I would like to test the compound's mode of action are as follows:

To understand the upstream signaling activation that could possibly converge on xenophagy pathway activation.

Curiously, not all the compounds identified by high throughput screen as autophagy inducers functioned in clearing intracellular pathogens. What makes compound G so unique as a a potent xenophagy inducer? Hence the additional effect of compound G could be because it activates

some immune signaling pathways that ultimately lead to activation of autophagy mediated clearance. It is already shown in literature that NODs, MAPK, Nrf immune signaling pathways converge on autophagy activation. Hence overexpression and knockdown studies of immune pathway components will likely provide epistatic understanding of xenophagy induction by compound G.

To check for morphology of the *Salmonella* entrapped vesicles in compound treated samples.

The growth kinetics of bacteria in different niches within host cells varies. Electron microscopy will be done to see the morphological appearance of the bacteria containing vesicles, which would reveal if the drug treated samples entrap the bacteria in a population of vesicles say autolysosomes which is not permissible for replication. EM studies will also show if the increased recruitment xenophagy machinery proteins to *Salmonella* by is due to the ability of the compound G to mediate large cargo capture as the size of bacteria containing autophagosomes is 25-100 folds higher than starvation induced autophagosomes.

To check if the pathogen virulence is affected by the compound

Pathogens like *Salmonella* express and also translocate the virulence effectors into cytosol of host cells to modify the intracellular environment to suit their survival. Perturbation in this process would make the *Salmonella* incapable of active replication.

Translocation of host proteins to pathogen surface

Apart from the xenophagy machinery proteins that recognize pathogens, compound treatment could lead to translocation of some additional host proteins that prevents pathogen's replication. This could be studied by doing mass spectrometry analysis of intracellular pathogens treated with compound G.

Global gene expression of host and pathogen

Genes that get globally up regulated/down regulated in the presence of drug will be checked using microarray or RNA sequencing of host and pathogen. This would reveal all the candidate

genes involved in regulation of xenophagy and would also possibly reflect on the novel pathways involved xenophagy mediated immune response.

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