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Targeted Protein Degradation via Lysosomes

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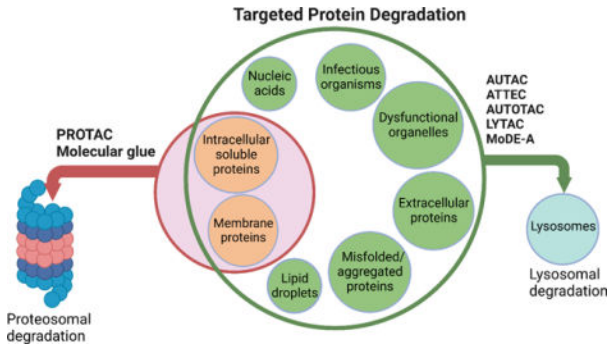
Abstract

Among the scope of targeted protein degradation (TPD), Proteolysis Targeting Chimeras (PROTACs), leveraging the ubiquitin-proteasome system, have been extensively studied. However, they are limited to degrading soluble and membrane proteins, excluding the aggregated and extracellular proteins and dysfunctional organelles. As an alternative protein degradation pathway, lysosomes serve as a feasible tool to access these untouched proteins/organelles by proteosomes. Here, we focus on reviewing the emerging lysosome mediated TPD, such as AUTAC, ATTEC, AUTOTAC, LYTAC, and MoDE-A. Intracellular targets, such as soluble and aggregated proteins and organelles, can be degraded via the autophagy-lysosome pathway. Extracellular targets, such as membrane proteins, and secreted extracellular proteins can be degraded via the endosome-lysosome pathway. In addition, we summarize the mechanism and regulation of autophagy, available methods/assays monitoring the autophagy process, and the recently developed chemical probes for autophagy pathways.

Graphical Abstract



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Author Contributions

R.R.P., D.L., S.R.C. and E.Y.M. wrote the manuscript. J.W. edited and proofread the manuscript.

Conflicts of interest

J.W. is the co-founder of CoActigon Inc. and Chemical Biology Probes LLC.

INTRODUCTION

Protein homeostasis is a coordinated and complex web of building blocks that maintains the cellular concentrations, folding, interactions, and localization of proteins essential for cellular functions. One vital role protein homeostasis plays is the clearance of unwanted misfolded proteins or proteins that fail to fold due to mutations.[1] It has been demonstrated that the Ubiquitin-proteosome system (UPS) and lysosomal system are the two principal and complementary approaches for protein degradation.[2,3] The UPS pathway degrades intracellular, soluble, and short-lived proteins.[4,5] On the other hand, the lysosomal system can degrade many fully folded, long-lived, aggregated proteins, extracellular proteins, nucleic acids, lipids, damaged organelles and infectious organisms such as bacteria and viruses.[6] In the past decade, proteolysis-targeting chimeras (PROTACs), leveraging the proteasome pathway, have made a significant progress since they emerged in 2001.[7–9] Recently, several PROTACs have entered clinical trials, which demonstrated their potential in medicinal chemistry and chemical biology.[10] However, the UPS related small molecule-based PROTACs approach has some limitations since it can only degrade intracellular, soluble, and short-lived proteins, limiting their applications in many diseases caused by extracellular proteins and protein aggregates, such as in Huntington's and Alzheimer's disease. Therefore, lysosomal degradation of biomolecules can greatly enrich the toolbox of targeted protein degradation (TPD) and expand their applications in human diseases. Lysosomes are ubiquitous acidic organelles that can degrade proteins, nucleic acids and other biomaterials. One of their cellular functions is to degrade and recycle the intracellular and extracellular materials using acidic hydrolases.[11,12] There are two major lysosomal-based degradation pathways: the degradation of cytoplasmic proteins and damaged organelles by lysosome through autophagy called autophagy-lysosomal pathway and the degradation of extracellular proteins by lysosome through endocytosis called endosome-lysosomal pathway.

Autophagy-lysosomal pathway

Autophagy is a conserved catabolic process for the turnover and recycling of cytoplasmic components, such as proteins and organelles, which are then trafficked into lysosomes.[13–15] Autophagy has a crucial role in maintaining cells' homeostasis and energy balance. Any disruption in the autophagy process causes various diseases such as cancers, neurodegenerative disorders (NDDs), immune disorders, and metabolic diseases.[16] Autophagy has multifaceted roles in cancers, depending on the tumor genotypes and therapeutic agents. On one hand, it controls tumor growth by removing cancer-causing cells and organelles. On the other hand, it protects tumor cells from therapy-induced death and helps to promote tumor growth.[17,18] Additionally, the autophagic removal of aggregated proteins and organelles from the heart is beneficial since autophagic death of unwanted cardiac cells may lead to heart failure.[19] Autophagy can also be used as a defense mechanism against intracellular pathogens.[20]

Autophagy is divided into three types, namely microautophagy,[21,22] chaperon-mediated autophagy (CMA)[23,24], and macroautophagy[25]. Microautophagy is the direct engulfment of cytoplasmic components by lysosomes, whereas chaperone-mediated autophagy degrades

specific proteins containing a KFERQ-like motif recognized by the molecular chaperon HSPA8/HSC70, which directs the protein to the lysosomal surface protein LAMP2A for lysosomal engulfment. Macroautophagy (hereafter autophagy) involves the formation of a double-membrane vesicle called an autophagosome. The targeted, degraded material is trapped inside the autophagosome, which is later delivered into the lysosome through membrane fusion between the autophagosome and lysosome.[26] The mechanism of the autophagic process is highly regulated by autophagy-related proteins. More than 40 autophagy-related genes and their encoding proteins have been identified in yeast, and most of them are also present in mammals, which indicates that autophagy is an evolutionarily conserved process.[27]

Mechanism and regulation of autophagy

The regulation of autophagy is a complex mechanism involving several autophagy-related (Atg) proteins. The autophagy mechanism consists of two ubiquitin-like conjugation systems resulting in the modified complexes of autophagy regulators LC3-II and Atg12-Atg5-Atg16L, respectively.[28] The autophagy machinery can be divided into three stages: a) induction of autophagy b) nucleation, elongation, and maturation of autophagosome; and c) fusion with lysosome and degradation (Figure 1).

Autophagy occurs at a low level under basal conditions and is triggered by cellular stress such as nutrient starvation, oxidative stress, and mTORC1 inhibition.[29–31] The initiation of autophagosome formation occurs after the activation of ULK1/2 (unc-51 like kinase) proteins which can form ULK1/2 complexes with other proteins such as Atg13, Atg101, and FIP200.[32] Autophagy nucleation is facilitated by forming the class lll phosphatidylinositol 3-kinase (PI3K) complex containing VPS34, VPS15, Beclin 1, and Atg14L.[33,34] The PI3K complex also helps to phosphorylate phosphatidylinositol (PI) to phosphatidylinositol-3-phosphate (PI3P). PI3P is needed for the correct localization of Atg proteins Atg18 (WIPI1/2) and Atg2, which help to recruit Atg9 protein to the autophagosome during its nucleation step.[27,35] Atg9 vesicles are the source of autophagosome membranes in nucleation step by coalescence with the ULK1 complex.[36] The elongation or maturation step involves two ubiquitin-like conjugated systems, LC3-II (Atg 8 in yeast) and Atg12-Atg5-Atg16L.[37] ATG4B cleaves proLC3 isoforms to form LC3-I, where the glycine residue will be the C-terminus.[38] LC3-I is activated by Atg 7 (E1-like) and conjugated with Atg 3 (E2-like), followed by covalent binding to phosphatidylethanolamine (PE). This results in the lipidated LC3 (i.e., LC-3-I-PE or LC3-II). LC3-II is covalently bound to the membrane of the autophagosome.

GABARAP is another Atg 8 homologue in mammals, which is less explored than LC3.[37] Atg4B also plays a role to cleave LC3-II to LC3-I referred as delipidation or deconjugation, which helps to recycle LC3.[39,40] Similarly, Atg12 is activated and conjugated by Atg7 (E1-like) and Atg10 (E2-like) proteins, respectively, followed by association with Atg5 and Atg16L, resulting in an E3-like complex Atg12-Atg5-Atg16L. The Atg12-Atg5-Atg16L complex, which helps the PE-conjugation, dissociates after autophagosome formation.[38,41] Atg conjugation systems are not essential for the autophagosome formation, although they facilitate and normally occur during autophagosome formation. Most importantly, Atg3

from the conjugation system is required for the opening and efficient degradation of the autophagosomal inner membrane after fusing with lysosomes.[42] LC3-II acts as a binding platform for autophagy receptors (for example, p62, NBR1).[43] The receptor proteins help traffic the double-membrane autophagosome to the lysosome. With the help of Rab-SNARE proteins, the fusion of lysosome and autophagosome results in autolysosomes[44,45] which release their inner components as well as inner membrane into the lysosome hydrolase for their degradation.[26,27,35,46,47]

Selectivity in autophagy

Although autophagy was considered non-selective initially, recent studies have revealed that certain types of macroautophagy are selective.[48] Most studied selective autophagy is controlled by the cargo receptor proteins, which can recognize cargos and bind to the LC3 proteins located on the isolation membrane/phagophore.[49] Selective autophagy of cell organelles has been classified based on the type of organelles that act as cargos, such as mitochondria (mitophagy), protein aggregates (aggrephagy), pathogens (xenophagy), ribosomes (ribophagy), lysosome (lysophagy), liposome (lipophagy), endoplasmic reticulum (ER-phagy or reticulophagy), and ferritin (ferritinophagy).[50–57] The general mechanistic pathway for selective autophagy in mammals are regulated by autophagy receptors such as p62, NBR1, NDP52, TAX1BP1, OPTN[58,59] and CCT2.[60] During the selective autophagy processes, autophagy receptors undergo post-translational and structural modifications, such as ubiquitylation, phosphorylation, acetylation, and oligomerization.[43] In the ubiquitin-dependent cargo selection, misfolded proteins are tagged with a polyubiquitin chain which is recognized and bound to receptors through their ubiquitin-binding domain (UBD) and ultimately delivered into the autophagosome. In contrast, the ubiquitin-independent pathway involves the recognition of the specific cargos such as proteins, lipids, or sugar-based signals by the specialized autophagy receptors.[61,62] For example, Dowdle et al. discovered that the selective autophagy of ferritin (i.e., ferritinophagy) is mediated by the ubiquitin-independent receptor NCOA4.[57,63]

p62/SQSTM1 as a receptor protein for selective autophagy

p62/SQSTM1 is a specific autophagy receptor protein found in the metazoans.[64] p62 plays a crucial role in the selective autophagic degradation of ubiquitinated protein aggregates and contains several conserved domains to bind various substrates.[65,66] The p62 domains facilitate the degradation of ubiquitinated cargo by binding to the UBD, undergo self-oligomerization through the PB1 domain, and then delivering the protein aggregates to the autophagosome by interacting with the autophagosome membrane protein LC3 via LC3 interacting region (LIR) (Figure 2).[67–70]

Some proteolytic systems are based on recognizing N-terminal residues (N-recognins) as essential components for their degradation (N-degrons), which is called the N-end rule pathway.[71,72] Cha-Molstad et al. studied the mechanism of the p62 binding with ER-residing proteins BiP for the autophagy of misfolded cytosolic proteins tagged with ubiquitin (Figure 3), which is evident that p62 is a critical molecule in the crosstalk between UPS and autophagy.[73] In this study, they observed that p62 follows the N-end rule pathway to the autophagosome biogenesis.[66]

The ZZ domain of p62 also plays a role in inducing autophagy by selectively modulating the N-recognin site and binding to N-terminal degrons, including N-terminal arginine (Nt-R) (Figure 3). The binding of the arginylated substrates (Nt-R) to the ZZ domain of p62 facilitates disulfide bond-linked p62 self-aggregation and its interaction with LC3, which ultimately leads to the delivery of p62 and its cargo into the autophagosome.[66,67] Cha-Molstad et al. developed two small molecule ligands (XIE62–1004 and XIE2008) binding to the ZZ domain of p62, which were also able to induce autophagy through p62 self-aggregation and LC3 interaction (Figure 4).[74]

Autophagy assays

Autophagy has been extensively studied in the biomedical field. However, one of the significant challenges in the field is the limited number of methods to accurately measure autophagic activity in cells. Historically, an increase in the number of autophagosomes measured by electron microscopy has been one of the methods to measure autophagy, but autophagy is a highly dynamic process involving several steps.[75] Measuring only the increase in the number of autophagosomes can be misleading since it can either mean the induction of autophagy by starvation or other stress factors or the reduction of lysosomal degradation of the autophagosomes due to lysosomal dysfunction. Measuring the autophagic flux has become a suitable alternative for autophagic activity in cells.[76] Autophagy flux refers to the amount of degradation of cytoplasmic materials per unit time. In the last few years, many assays have been developed which can measure autophagic flux with excellent reliability. These assays are based on cellular expressions of specific proteins involved in autophagy, such as microtubule associated protein LC3 and autophagy receptor protein p62.[76–78]

1) Monitoring autophagy flux using LC3—The following three LC3-based methods have been widely used to measure the autophagic flux.

(i) LC3 turnover assay: LC3, a mammalian homologue of yeast Atg8, has been used as a marker of autophagosome formation. As discussed before, ATG4 processes LC3 to become LC3-I which is subsequently conjugated to phosphatidylethanolamine (PE) to become LC3-II. LC3-II is found in autophagosomes and can indicate the formation of the same. LC3 turnover has been used to measure autophagic flux. Western blot has been used to visualize both LC3-I and LC3-II. The experiments are done in the presence and absence of lysosome inhibitors, and the comparison of LC3-II amounts in samples is semi-quantitatively used to determine the autophagic flux. When flux is high, the difference in LC-II between the samples will be high and vice-versa.[25] Commonly used lysosomal inhibitors are lysosomal protease inhibitors, such as E64d and pepstatin A, and bafilomycin A1, and lysosomotropic reagents, such as chloroquine.[79] The main advantage of this method is that it measures endogenous autophagic flux without transfection. However, the method has many drawbacks. Firstly, lysosomal inhibition can interfere with mTOR activity, resulting in further acceleration of autophagic activity. Secondly, care must be taken in selecting the type and concentration of the lysosomal inhibitor. For example, 100 nM of bafilomycin A1 blocks the fusion of autophagosomes with lysosomes affecting the measurement of autophagic flux.[80]

(ii) RFP-GFP-LC3 (tfLC3) reporter assay: In 2007, Kimura et al. reported an autophagy probe that analyzed the dynamics of autophagosomes using fluorescence.[81] Prior to discovering an autophagy probe, scientists used GFP-LC3 as a marker probe to demonstrate the formation of autophagosomes. However, it was evident that autophagosome formation did not correlate directly to autophagy flux.[82] The new probe was a novel marker protein, mRFP-GFP-LC3 tandem-tagged fluorescent protein (tfLC3), which emits both green and red fluorescence so that autophagosomes appear yellow. Once it is trafficked to autolysosome, the GFP loses fluorescence quickly due to low pH, but RFP maintains its fluorescence. The appearance of yellow or red fluorescence indicates the presence of more autophagosomes or autolysosomes. The tfLC3 probe offers many advantages over the earlier systems. For example, it can measure autophagic flux without using lysosomal inhibitors, and it can be used for selective substrates. The disadvantage to using the probe is that the system depends on transfection, and there is high background due to RFP accumulation in lysosomes.

(iii) GFP-LC3-RFP(LC3ΔG) reporter assay: In 2018, Kaizuk et al. developed GFP-LC3-RFP(LC3ΔG), a second-generation tandem-tagged fluorescent assay single molecular probe.[83] In this probe, the GFP-LC3 is conjugated to the N-terminal of RFP-LC3ΔG (ΔG means the RFP-LC3 lacks the C-terminal glycine). In cells, the probe is hydrolyzed by ATG4 family proteases generating equimolar amounts of GFP-LC3 and RFP-LC3ΔG. GFP-LC3 is conjugated to PE, localizes in autophagosome, and is subsequently degraded. Whereas the RFP-LC3ΔG is not degraded due to the lack of the C-terminal glycine. As a result, the RFP-LC3ΔG acts as an internal control and stays in the cytosol. The GFP/RFP signal ratio inversely correlates to autophagic activity. The authors applied this probe to measure autophagic flux in cells, in addition to mice and zebrafish. The probe was also used to do a high throughput screening of 1054 approved drugs to find novel autophagy inducers and inhibitors. Another advantage of this probe is that it can measure the basal autophagic activity among different tissues since the basal autophagic activity is generally too low to be measured by other probes. A significant limitation of this probe is that during transfection, a substantial proportion of clones express GFP-LC3ΔG. Thus, the authors recommend isolating clones expressing GFP-LC3-RFP-LC3ΔG after transfection.

(2) Monitoring autophagy flux using p62—p62, also called sequestosome 1 in humans, binds directly to LC3 and polyubiquitinated substrates. It becomes incorporated into the autophagosome and is itself degraded into autolysosome, thus acts as a marker of autophagic flux.[76,78] The subcellular localization and level of endogenous p62 can be measured by western blotting and immunostaining.[78] p62 is a multifunctional scaffold protein so it is important to validate autophagy measurement of p62 level with other available assays.

Luciferase assay-based method is also developed for the measurement of both LC3 and p62 quantification. Farakas et al[84] have developed the luciferase assay-based method for LC3 measurement in autophagic flux which was later extended to p62 measurement by Min et al.[77] In this method, the ratio of luciferase activity with p62 and its UBA domain deletion mutant has been used to determine the autophagic flux. Similarly, Bresciani et al.[85] have developed TR-FRET assays for LC3B and p62. These assays can be used as

high-throughput screening tools to identify the autophagy regulators. In this method, they have used Tb labeled LC3-II donor and D2 labelled LC3-II acceptor antibodies or Tb labelled donor p62 and Alexa-647 labeled acceptor p62 antibodies. Autophagy up-regulator induces the close proximity of LC3-II antibodies resulting in the signal for accumulation of the autophagosome vesicle.[85]

There are some other assay methods monitoring the autophagy markers such as ULK1, PtdIns3K, Atg9, Atg12-Atg5, Atg14, Atg16L1, WIPI family, BECN1, and STX17 (SNARE protein). Autophagic components other than LC3-family can be monitored to define specific steps of the process.[76]

Chemical probes for autophagy pathways:

Since tumor cells can activate autophagy to compensate for the energy shortage during cellular stress, several autophagy inhibitors targeting the Atg-related proteins have been developed to control this tumor growth mechanism by autophagy.[86,87] There are some studies on the repurposed drugs for autophagy regulation, such as chloroquine and hydroxychloroquine (clinically approved anti-malarial drugs) as autophagy inhibitors.[88] Some of the recently discovered chemical probes for autophagy are listed below (Table 1).

Endosome-lysosomal Pathway

Endocytosis is a cellular process of internalization of various components such as transmembrane proteins, receptors, receptor ligands, extracellular proteins and other biomolecules by the invagination of the plasma membranes and the formation of vesicles and vacuoles. Several steps are involved in the formation of endosomes and are classified as early endosomes, recycling endosomes, and late endosomes.[109,110] The detail mechanisms of the endosome formation have been described in some other reviews.[109,111] Briefly, endocytosis starts with the invagination of cargo proteins by the plasma membranes mediated by cytosolic proteins and multi-subunit complexes such as Rab proteins to form early endosome.[110] Early endosomes serve as major sorting stations to send back the recycling materials into recycle endosomes or degradation materials to lysosomes by converting into late endosomes. The fusion of late endosomes with lysosomes allows the degradation of the enclosed components by the lysosomal hydrolases.[109,110] Since, the UPS and autophagy based targeted degradation are only capable for degrading intercellular proteins, whereas the endosome-lysosomal pathway, described later in this review, has been applied for the degradation of extracellular proteins.[112–115]

Progress in the lysosome-based targeted protein degradation

Targeted protein degradation has been widely explored through PROTACs as therapeutics or chemical probes. However, this technology still has some limitations. Some other emerging technologies based on autophagy mechanisms such as autophagy-targeting chimera (AUTAC), autophagosome-tethering compounds (ATTEC), lysosome targeting chimera (LYTAC), and AUTOphagy-TArgeting Chimera (AUTOTAC) are under development. These recently acquired chemical biology platforms may overcome the PROTACs limitations.

In this contribution, we summarize current lysosome-based degraders for different target proteins (Figure 5, Table 2).

AUTACs

The Arimoto group developed autophagy-targeting chimera (AUTAC) molecules to remove targeted cytosolic proteins or mitochondria in xenophagy.[76,116–118] The S-guanylation of group A *Streptococcus* (GAS) bacteria by 8-nitro-cGMP promotes the K63-linked polyubiquitination, which ultimately signals the selective transportation to autophagosome followed by degradation.[119] The Arimoto group took advantage of GAS bacteria's selective process to develop a chimeric molecule capable of targeted protein degradation. AUTAC molecule contains a *p*-fluorobenzylguanylation tag (FBnG unit) and a target-specific binder linked to polyethylene glycol (PEG). Arimoto et al. developed AUTAC1 containing a methionine aminopeptidase 2 (MetAP2) protein binder fumagillin that successfully degraded the MetAP2 at 1 μM. Similarly, AUTAC2 was designed to degrade FK506-binding protein (FKBP12), whose non-covalent synthetic ligand of FKBP (SLF) has been used to degrade with 10 μM concentration through the AUTAC system. AUTAC3 was also designed to target nuclear protein Brd4 with its binder JQ1, but degradation was not as effective as cytosolic proteins MetAP2 and FKBP12. Most importantly, AUTAC4 was designed for selective degradation of mitochondria. AUTAC4 contains a phenylindole moiety that binds to mitochondrial translocator proteins (TSPO) located on the outer mitochondrial membrane (OMM) and can selectively remove the dysfunctional mitochondria. In summary, one end of AUTAC molecule can selectively bind to the protein of interest (POIs), and the other end, which contains S-guanine moiety, helps to induce K63 polyubiquitination. The autophagy receptors such as p62 recognize K63 polyubiquitinated protein cargoes and traffic them to the autophagosomes for subsequent degradation. The major limitation for AUTAC is that the mechanism K63 polyubiquitination induced by *S*-guanylation is still unknown.

ATTECs

Lu and coworkers developed a new approach for targeted protein degradation called autophagosome-tethering compounds (ATTEC).[120–123] ATTECs are bifunctional chimeric compounds that tether the POI to a specific protein degradation machinery (PDM) component, such as LC3. These compounds are found to be allele selective for a specific protein. They also designed a small molecule microarray screening for compounds that interact with LC3 and disease-causing protein mutant Huntington (HTT) with an expanded polyglutamine (PolyQ) stretch that is found in Huntington's disease.[122] From the microarray screening, the group identified four allele selective compounds (10O5, 8F20 or Ispinesib, AN1, and AN2) that can interact only with mHTT but not with wild-type (WT) HTT. These compounds also reduced some other polyQ expansion proteins such as ATXN3. Once the ATTEC molecule tether expanded polyQ proteins to autophagosome through LC3 domain, it is directed for subsequent degradation.

Recently, Lu group has applied their ATTEC strategy to degrade non-protein biomolecules, such as lipid droplets (LDs), and developed a new class of molecules called LD-ATTEC.[123] Since lipid droplets are composed of lipids, PROTACs and AUTACs cannot target or degrade the LDs. LD-ATTECs were designed as bifunctional molecules that link

the selective LD detecting probe (i.e., Sudan dyes) and LC3-binding molecules. These molecules constitute a ternary complex between triacylglycerol and LC3, which induces the proximity of LDs and autophagosomes in cells leading to autophagic degradation. During this mechanism, only the LDs induced by oleic acid in fibroblasts and endogenous LDs in differentiated adipocytes were degraded, leaving other lipid-containing membranes unaffected. Also, the global autophagy was not influenced by this selective LD degradation. The successful degradation of stored fats in the cell with LD-ATTEC opens a novel approach against the diseases caused by the accumulation of lipid droplets, such as obesity, cardiovascular diseases, or fatty liver disease. Although ATTEC technology is potentially effective in many cell types, the binding site in LC3 is not yet known.

Pei and coworkers have demonstrated an LC3 targeting autophagy chimeric molecule that can successfully degrade the BRD4 protein through the autophagy pathway. They have used a reversible BET bromodomain inhibitor JQ1 as a warhead for BRD4 and GW5074 for LC3 and linker to get the potent AUTAC molecule that downregulates the level of BRD4.[124] Recently, Sheng group have applied the ATTEC principle to develop the first generation of autophagic degrader of nicotinamide phosphoribosyl transferase (NAMPT). The NAMPT ATTEC were synthesized by connecting NAMPT inhibitor and LC3 binding warhead Ispinesib through a flexible linker.[125] Mechanistic studies confirmed that the NAMPT degradation occurs via autophagy-lysosomal pathway.

AUTOTACs

Ji et al. developed another autophagy based chemical tools called AUTOphagy-TArgeting Chimera (AUTOTAC).[126] It is a bifunctional molecule which contains an autophagy targeting ligand (ATL) or p62 binding ligand and a target binding ligand (TBL). Autophagy targeting ligands bind to the ZZ domain of the autophagy cargo receptor p62, which can activate them for self-oligomerization for the autophagosome biogenesis through LC3 interactions. p62 binding ligands follows the N-end rule pathways. PHTPP-1304, Vinclozolin M2 and Fumagillin have been used as target binding ligands (TBLs) for estrogen receptor beta (ERβ), androgen receptors (AR) and methionine aminopeptidase-2 (MetAP2), respectively. The resulting AUTOTACs were able to degrade the respective target proteins in nanomolar range, which was not possible with ATL or TBL alone. AUTOTAC is applicable for the autophagic clearance of a wide range of intracellular target proteins. It can target not only monomeric proteins but also aggregated oligomeric proteins with sustained efficacy. Another feature of AUTOTAC is that unlike PROTACs, its potency is not critically dependent on the linker length, rendering straightforward AUTOTAC design.

LYTACs and MoDE-As

The Bertozzi group developed lysosomal targeting chimeras (LYTACs) for the degradation of the extracellular secreted proteins and plasma membrane-associated proteins.[112,113] LYTACs consist of a target binding moiety (small molecule or antibody) linked to a glycan (polypeptide) ligand that can bind to the lysosome targeting receptor such as the cation-independent mannose-6-phosphate receptor (CI-M6PR) or asialoglycoprotein receptor (ASGPR) for liver-specific lysosomal degradation. LYTAC contains a glycan tag to help recognize the extracellular and membrane bound protein of interest (i.e., EGFR, CD71,

PD-L1) for lysosomal degradation. They used neutravidin (NA), a fluorescently labeled protein that is stable under endosome and lysosome condition, to measure its uptake when combined with the LYTAC molecule. In parallel, the Tang group also developed a trivalent-*N*-acetylgalactosamine (tri-GalNAc) to target LYTACs to ASGPR on hepatocytes.[115] They conjugated the ASGPR ligand (tri-GalNAc) to biotin and antibodies generating a new class of degraders, which were able to internalize and degrade neutravidin and EGFR. Capitalizing a similar concept, the Spiegel group developed MoDE-As (molecular degraders of extracellular proteins through the asialoglycoprotein receptor (ASGPR)), a small molecule version of ASGPR targeted LYTACs.[114] MoDE-As molecules were able to recruit and induce the degradation α-DNP antibody and cytokine MIF protein.

The degradation of ASGPR was dependent on ASGPR internalization through the clathrin-mediated endocytosis-lysosomal system. The capability of lysosomes to maintain homeostasis stability was not affected in cells treated with LYTACs, suggesting that this modality may be safe at the cellular level. The success of LYTAC degraders results from endogenous kinetics of protein trafficking and turnover for the targeted protein. However, LYTACs cannot be applied to intracellular targets due to the nature of the degraders. As small molecules, MoDE-As might achieve deeper tissue penetration compared with antibody-based LYTACs.

Tissue Selectivity of Lysosome-Mediated Targeted Protein Degradation

For different lysosome-mediated TPD technologies, different key partner proteins in the lysosomal pathway are involved. Based the quantitative proteomics study in human tissues[128], we examined the tissue distribution levels of p62 (gene: SQSTM1), LC3 (genes: MAP1LC3A and MAP1LC3B), ASGPR (genes: ASGR1 and ASGR2), and CI-M6PR (gene: IGF2R) based on their tissue specificity (TS) scores (Figure 6). It is interesting to note that p62 and ASGPR are highly enriched in skeletal muscle and liver, respectively. In brain, while LC3 is enriched, CI-M6PR is deficient compared to other tissues. Future work can take advantage of the differential expression of the lysosome pathway related proteins to achieve tissue selectivity.

Conclusion and Future Perspectives

The regulatory cycle of proteins has a crucial role in the fate of a living cell. Unwanted proteins need to be recycled through degradation into their constituent amino acids. Targeted protein degradation is one of the recently developed therapeutic approaches which is used to degrade disease-causing proteins. PROTACs are the most studied TPD technologies based on the UPS pathway, but these technologies are limited only to soluble and membrane proteins. There are some PROTAC molecules reported for the degradation of protein aggregates, for examples; tau,[129] huntingtin,[130] and α-synuclein proteins.[131] Ubiquitination-dependent PROTAC cannot be applied to degrade insoluble and complex protein aggregates and defective cell organelles such as mitochondria. Therefore, advancements lysosomal-based degradation are inevitable. When studying autophagy-mediated lysosomal degradation, multiple assays are available. However, it is highly recommended not to use any single assay to measure autophagy and to validate the results using various assays depending on the experimental design.

Some newly explored TPD approaches such as AUTAC, ATTEC, AUTOTAC, and LYTAC are based on the lysosomal pathways. These novel approaches can selectively recognize and traffic the proteins/organelles to lysosomes for degradation. The lysosome-based degradation of the target proteins can overcome the limitations of proteasome-mediated degradation. However, complete understanding and broad application of these approaches are still in its infancy. AUTAC and ATTEC are used to degrade intracellular proteins or biomolecules, but their mechanism is not completely understood. The critical unanswered questions with these techniques are how S-guanylation of AUTAC induces K63 -polyubiquitination. ATTEC molecules are more selective for the targets, but their binding mechanism to LC3 is still unknown. The Kim group discovered that AUTOTAC can activate the autophagy cargo receptor (i.e., p62), which is self-polymerized, sequestered, and delivered into phagophore for autophagic degradation.[126] To further improve our understanding of these Autophagy Lysosome System (ALS) mediated degraders, assays commonly used in PROTAC studies should be adopted, such as proteomics profiling to evaluate the degradation specificity, target engagement in cells, protein degradation kinetics and in vivo PKPD modeling.[126] Comparing PROTACs with the ALS mediated degraders, we observed that the ALS mediated degraders tend to have μM or sub-μM potencies, while PROTACs can usually achieve single digit nM or sub-nM potencies. This may be due to the difference of degradation kinetics between the UPS and the ALS. To differentiate from PROTACs, the development of ALS-based degraders should primarily focus on targets that cannot be degraded by PROTACs, such as misfolded proteins or aggregates and dysfunctional organelles. Similarly, LYTACs and MoDE-As are primarily designed for extracellular protein degradation. These modalities need to find their niche applications that can differentiate from neutralizing antibodies, which are proven clinical modalities. Nonetheless, the lysosomal-based degradation approach will greatly expand the TPD toolkit, not only as chemical biology tools but also as therapeutic modalities. Lysosomal-based degradation techniques might not be able to degrade nuclear proteins efficiently, as the lysosomal system mostly operate in the cytoplasm. Moreover, the degradation efficiency of these methods may vary among different cell and tissue types (Figure 6), which could be taken advantage of to achieve tissue selectivity. In summary, as an alternative platform, lysosome-mediated targeted protein degradation will further expand the TPD field and provide exciting opportunities for therapeutic development.

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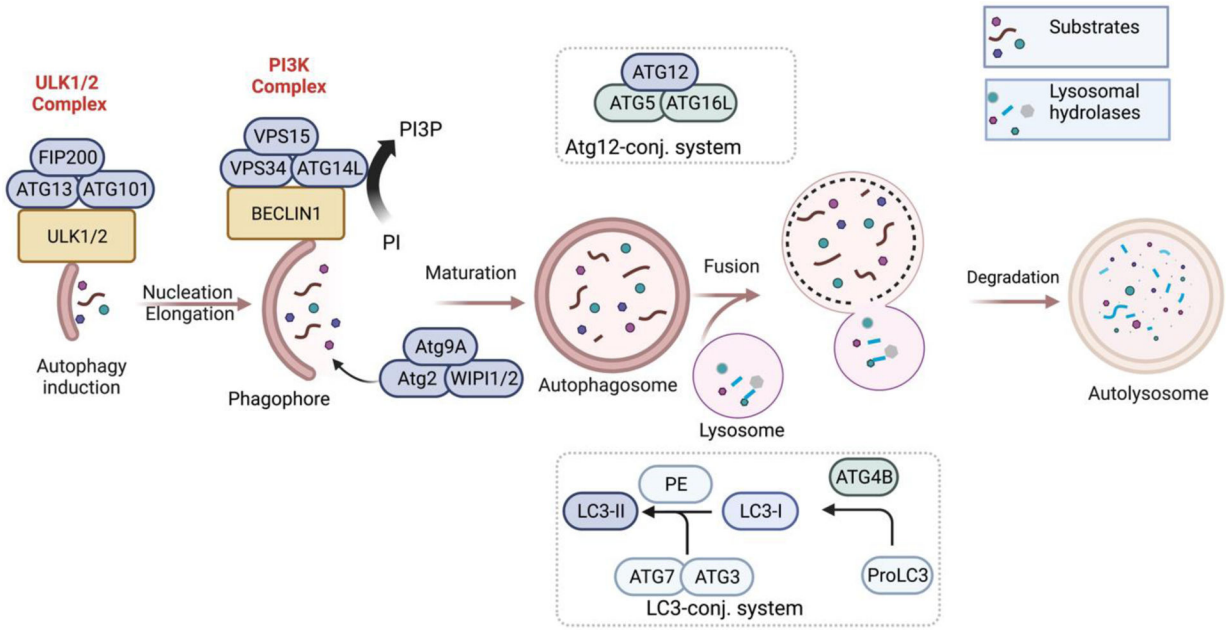


Figure 1:
Schematic representation of autophagy.

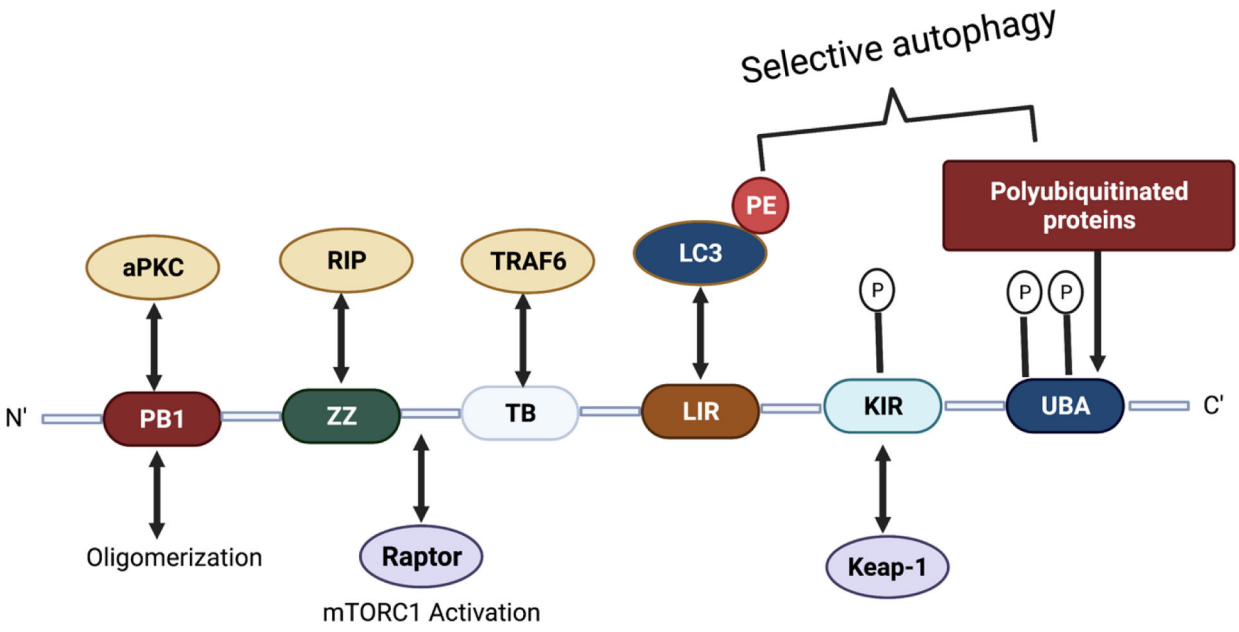


Figure 2:
Schematic representation of p62 structure and functional domains.

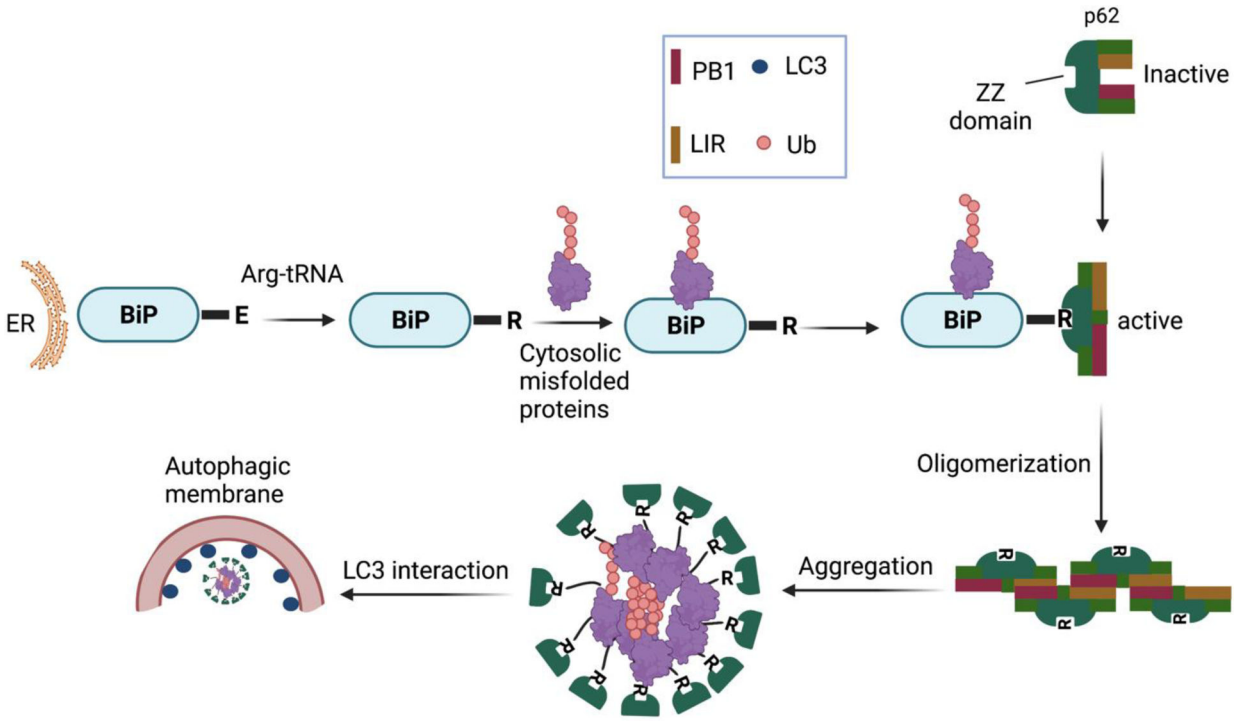
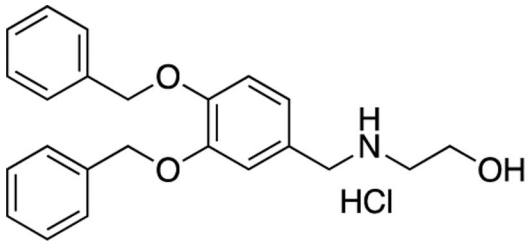
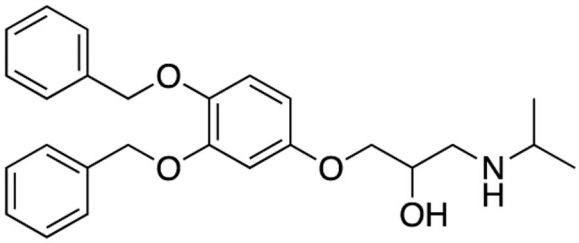


Figure 3:

A model illustrating the role of the N-end rule pathway in N-terminal arginylation of ER-residing proteins for the regulation of autophagy through p62 binding.[73,74]



XIE62-1004



XIE62-2008

Figure 4:
Structures of XIE62–1004 and XIE2008.

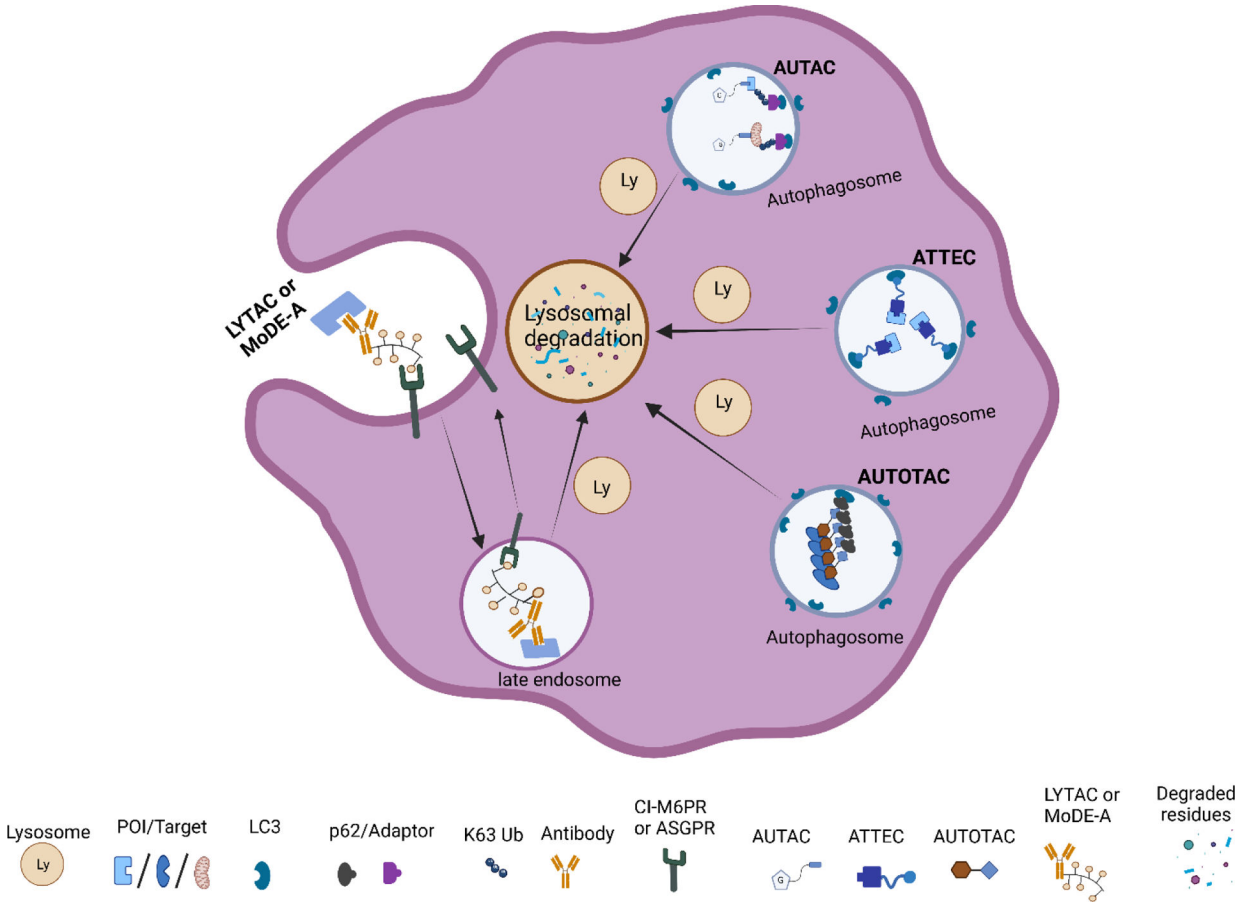


Figure 5:
Graphical illustrations of lysosomal-based degradation technologies.

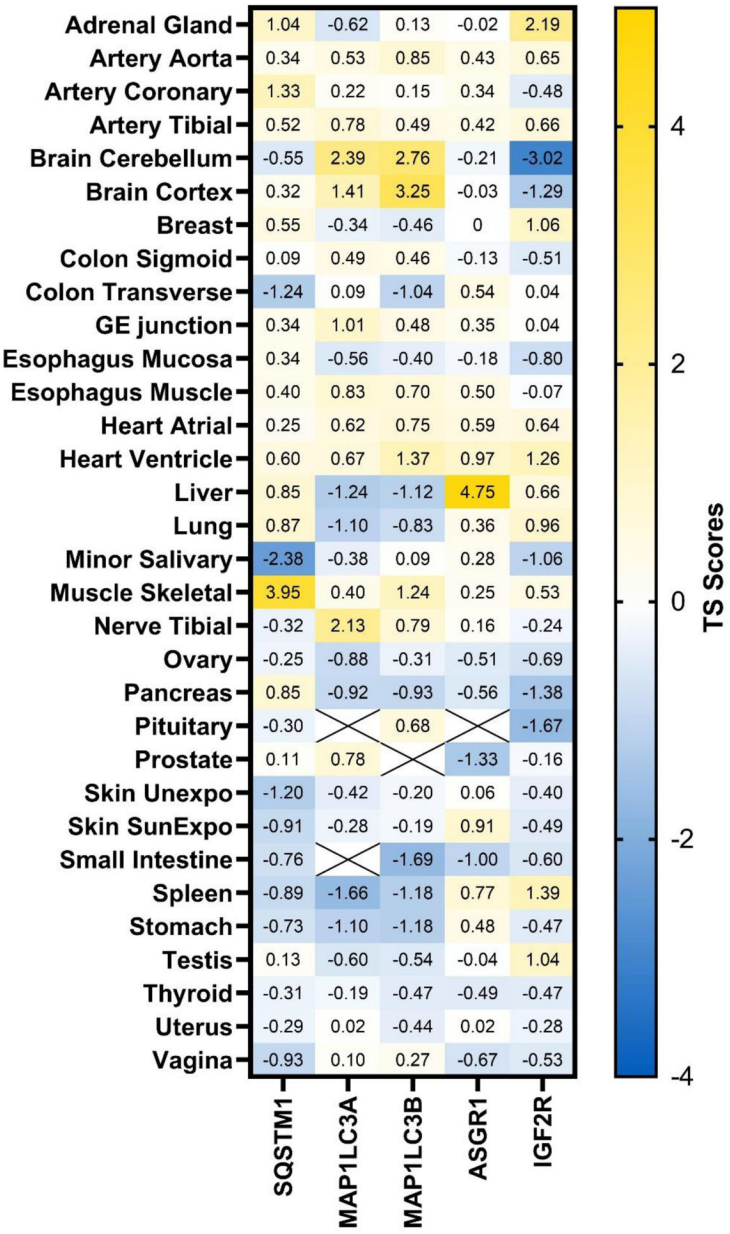
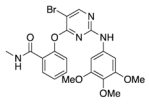
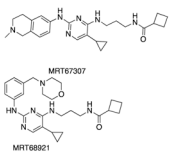
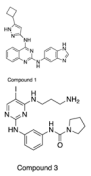
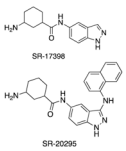
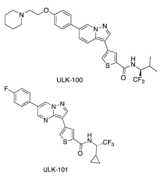
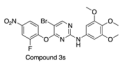
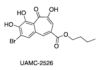
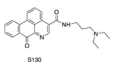


Figure 6:

Tissue distribution of lysosome pathway related proteins. Protein abundance was measured using quantitative proteomics.[128] Tissue selectivity (TS) scores were calculated and plotted. TS score >2.5 is considered highly enriched in certain tissues. SQSTM1: sequestosome 1 or p62; MAP1LC3A: microtubule associated protein 1 light chain 3 α; MAP1LC3B: microtubule associated protein 1 light chain 3 β; ASGR1: asialoglycoprotein receptor 1; ASGR2: asialoglycoprotein receptor 2; IGF2R: insulin like growth factor 2 receptor or CI-M6PR. The TS score for ASGR2 is unavailable.

Table 1:

Some of the recently discovered chemical probes for the autophagy pathways.

Compound(s)	Chemical structure(s)	target	Activity (IC_{50} otherwise mentioned)	References
SBI-0206965	 SBI-0206965	ULK1/2 Inhibitor	108 nM (ULK1)	89,90
MRT67307 MRT68921	 MRT67307 MRT68921	ULK1/2 Inhibitors	45 nM (ULK1) 38nM (ULK2) 2.9nM (ULK1) 1.1 nM (ULK2)	91
Compound 1 Compound 3	 Compound 1 Compound 3	ULK1/2 Inhibitors	5.3 nM (ULK1) 13 nM (ULK2) 120 nM (ULK1) 360 nM (ULK2)	92,93
SR-17398 SR-20295	 SR-17398 SR-20295	ULK1 inhibitors	22.4 μM 45 nM	94
DCC-3116	N/A	ULK1 inhibitor (Preclinical Phage I)	4.7 nM (ULK 1), 36 nM (ULK2)	95
ULK100 ULK101	 ULK-100 ULK-101	ULK1 inhibitors	1.6 nM 8.3 nM	96
Compound 3s	 Compound 3s	ULK1 inhibitor	<20 μM	97
NSC185058	 NSC185058	Atg4B inhibitor	51 μM	98
UAMC-2526	 UAMC-2526	Atg4B inhibitor	Observed both *in vivo* and *in vitro*	99
S130	 S130	Atg4B inhibitor	3.24 μM	100

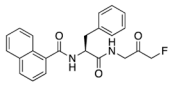
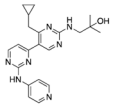
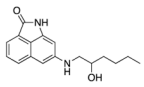
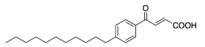
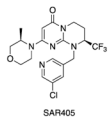
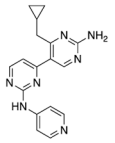
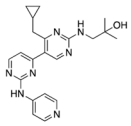
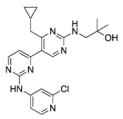
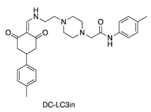
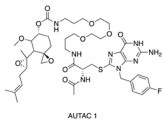
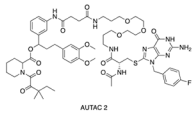
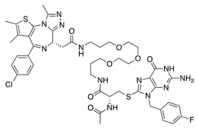
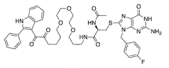
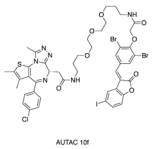
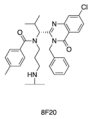
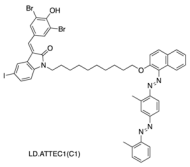
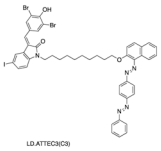
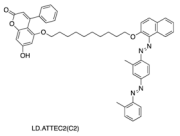
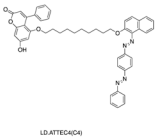
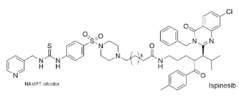
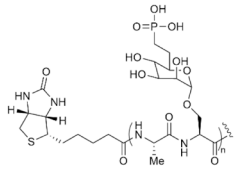
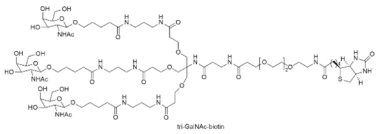
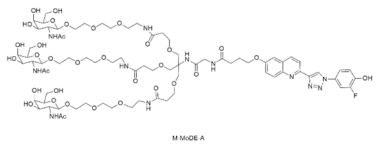
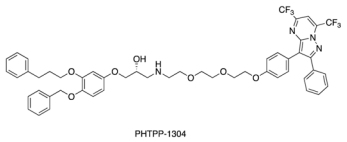
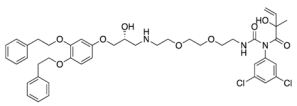
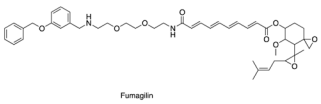
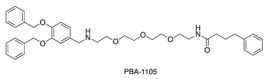
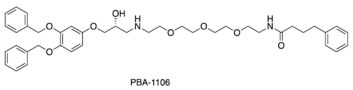
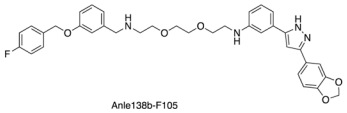
FMK-9a	 FMK-9a	Atg4B inhibitor	260 nM	101
LV-320	 Compound 19	Atg4B inhibitor	24.5μM	102
Compound 33	 Compound 33	Atg4B inhibitor	12 μM	103
Compound 21f	 *(E)*-4-Oxo-4-(4-undecylphenyl)but-2-enoic acid (**21f**)	Atg4B inhibitor	11 μM, Ki = 3.1 μM	104
SAR405	 SAR405	VPS34 inhibitor	1.2 nM Kd = 1.5 nM	105
PIK-III	 PIK-III	VPS34 inhibitor	18 nM	57
Compound 19	 Compound 19	VPS34 inhibitor	15 nM	106
VPS34-IN1	 VPS34-IN1	VPS34 inhibitor	25 nM	107
DC-LC3in	 DC-LC3in	LC3 inhibitor	3.06 μM	108

Table 2:

Summary of recently discovered lysosome-based degraders.

Compound(s)	Chemical structure(s)	Binding protein(s)	Target of interest	Activity (DC_{50}, otherwise mentioned)	References
AUTAC1	 AUTAC 1	p62 LC3 K63 Ub	MetAP2	1 μM	[118]
AUTAC2 (MTXSLF)	 AUTAC 2	p62 LC3 K63 Ub	FKBP12	10 μM in HeLa cells 25 μM in MES-SA cells	[117,118,127]
AUTAC3	 AUTAC 3	p62 LC3 K63 Ub	BRD4	-	[117,118]

AUTAC4	 AUTAC 4	p62 LC3 K63 Ub	TSPO	-	116–118
AUTAC 10f	 AUTAC 10f	LC3	BRD4	0.5 μM in MDA-MB-231 and MDA-MB-468 cells	124
10O5, 8F20, AN1 & AN2	 10O5  8F20  AN1  AN2	LC3	mHTT	Kd 7.7 μM (10O5) 29.2 nM (8F20) 9.8 μM (AN1) 7.0 μM (AN2)	120,122
C1, C2, C3, C4	 LD.ATTEC1(C1)  LD.ATTEC3(C3)  LD.ATTEC2(C2)  LD.ATTEC4(C4)	LC3	LC3B	Kd 4.2 μM (C1), 1.9 μM (C3), 4.9 μM (C2), 1.3 μM (C4)	123
Compound A3	 NAMPT inhibitor Ispinesib Compound A3	LC3	NAMPT	-	125
Poly(M6Pn)-biotin (LYTAC)	 poly(M6Pn)-Biotin	Cl-M6PR	NA-647	-	112,113

Tri-GalNAc-biotin		ASGPR	NA-650	-	115
Tri-GalNAc-Ctx antibody (Ctx-GN)	-	ASGPR	EGFR	40% downregulation	113, 115
M-MoDE-A		ASGPR	MIF	-	114
PHTPP-1304		p62 LC3	ERβ	1.48 nM	126
VinclozolinM2-2204		p62 LC3	AR	211.08 nM	126
Fumagilin-105		p62 LC3	MetAP2	0.701 μM	126
PBA-1105		p62 LC3	tauP301L	1-10 nM	126
PBA-1106		p62 LC3	tauP301L	1-10 nM	126
Anle138b-F105		p62 LC3	tauP301L	~3.0 nM	126