Pharmacologic agents targeting autophagy

Helin Vakifahmetoglu-Norberg,1 Hong-guang Xia,2 and Junying Yuan2

1Division of Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden. 2Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, USA.

Introduction

Autophagy is a major intracellular catabolic mechanism that directs selected cytoplasmic components and organelles to the lysosome for degradation. Macroautophagy (hereafter referred to as “autophagy”) is responsible for the bulk turnover of cytoplasmic material and is a tightly regulated process. Initiation of autophagy involves the formation of an isolation membrane (phagophore), a critical step in the formation of the autophagosome, and is regulated by multiple signaling mechanisms. The phagophore, which sequesters a region of cytoplasm or selected substrates, elongates and eventually matures into an autophagosome, a double-membrane vesicle that is subsequently trafficked to fuse with a lysosome.

In most cells, autophagy occurs at low basal levels but is often induced to confer stress resistance and sustain cellular survival under adverse conditions, as an essential cytoprotective response (1). Mutations in the autophagic machinery components are associated with a number of human disorders (2). Malfunction of autophagy is implicated in pathophysiology such as cardiomyopathies, infectious diseases, Crohn’s disease, and neurodegenerative disorders including Alzheimer’s, Huntington’s, and Parkinson’s diseases (3). Overactivation of autophagy has also been suggested to play an important role in promoting cancer cell survival in the tumor microenvironment in vivo and contributing to resistance to chemotherapies and metabolic changes to sustain tumor cell survival under stress and promote metastasis and dormancy (4–6).

Small molecules are important tools for dissecting molecular mechanisms of biologic pathways and for investigating the potential therapeutic strategies in human diseases. The approach of using small-molecule modulators provides pharmacologic methods similar to those of genetic manipulations to selectively inhibit or activate specific pathways and has therefore been termed “chemical genetics” (7). Compared with conventional genetic approaches, selective small-molecule modulators provide the possibility to conditionally regulate the activities of gene products in different settings, including cultured cell models and animal models. In contrast to permanent genetic modification, small-molecule tools provide a means to regulate target activity in a temporal and often reversible manner. During the past several years, a variety of small-molecule modulators that either activate or inhibit autophagy pathways have been developed that will be the subject of this Review.

Due to its pathophysiologic significance, autophagy has been the subject of intensive study, in the effort to gain a better understanding of the process at the molecular level and to discover potential new therapeutic targets. Small-molecule activators of autophagy have been shown to reduce the amounts of toxic protein aggregates and to promote cell survival under stress, which may be of therapeutic benefit in certain neurodegenerative diseases. In addition, the induction of autophagy has been shown to increase the longevity of certain experimental organisms (8), suggesting that the activation of autophagy may help to maintain normal homeostasis during aging and promote longevity. On the other hand, there are also circumstances in which autophagic activation may permit pathogenesis. For example, the inhibition of autophagy as a therapeutic approach might eliminate stress-tolerant cancer cells that escape chemotherapeutic treatment and which currently pose a fundamental barrier to successful anticancer treatments. Accordingly, both pharmacologic activators and inhibitors of autophagy may be considered as potential new drug candidates for the treatment of autophagy-relevant human diseases.

Herein we review the current landscape in the development of screening technology and the discovery of molecules and pharmacologic agents that target various regulatory mechanisms of the autophagic machinery. We highlight recent advances in the discovery of small-molecule regulators of autophagy and evaluate their utility in dissecting autophagic pathways and the potential therapeutic application of these compounds in human pathogenesis.

Screening strategies for isolating autophagy regulators

The hallmark for activation of autophagy is the formation of double-membrane autophagosomes that can only be clearly discerned at the EM level, which is incompatible with the demand for...
a high-throughput screening approach. Fortunately, the identification of GFP-tagged light chain 3 (GFP-LC3), one of the mammalian homologs of Atg8 in yeast that targets the isolation membrane upon the formation of autophagosomes as a fluorescent marker for autophagy (9), and the recent development of high-throughput microscopy technology made it possible to conduct such image-based screens using light microscopy. In fact, image-based screens for small-molecule modulators of autophagy represent a prominent example of the successful use of image-based, high-throughput screens by multiple labs.

**GFP-LC3-II-based methods.** GFP-LC3 was developed as an image-based method to quantitate autophagosomes as ring-shaped structures or small dots in the cytoplasm upon induction of autophagy (9). Zhang et al. adapted this method into a high-throughput imaging-based screening system using human glioblastoma H4 cells stably expressing human GFP-LC3 (10). The level of autophagy was analyzed by measuring the number, size, and intensity of GFP-LC3 spots using high-throughput fluorescence microscopy. Treatment with rapamycin, a known autophagy inducer, leads to significant increases in the fluorescence levels of GFP-LC3 compared with DMSO-treated cells. However, since the induction of autophagy is commonly associated with activation of cellular stress and cell death, which can be very common when screening a small-molecule library, the hit rate of autophagy inducers can be very high. To eliminate those compounds that induce autophagy as a consequence of cell death, it is important to have a high-throughput screening approach.
and demonstrated the feasibility of identifying small molecules that can both induce autophagy and block inflammation.

Figure 1. Small-molecule modulators of autophagy that target different steps of the autophagic machinery. Autophagy can be activated by inhibitors against class I PI3K (LY294002), mTOR (rapamycin), AKT (perifosine), and IMPase (Li+) as well as activation of AMPK by metformin. While Ca++ channel inhibitors (fluspirilene, verapamil) can stimulate autophagy, thapsigargin, an endoplasmic reticulum Ca++ ATPase inhibitor, elevates Ca++ and suppress autophagy. The initiation of autophagy is regulated by the activity of the class III PI3K complex, which can be inhibited by 3-MA and PT21 as well as by spautin-1 that can promote the degradation of the class III PI3K complexes. Once the phagophore formation is initiated, compounds such as verteporfin can interfere with LC3-interacting region motifs and potentially block the selective recruitment of cargos such as mitochondria. The trafficking of autophagosomes to the lysosome is facilitated by the cytoskeleton. Thus, microtubule destabilization by vinca alkaloids can block the maturation of autophagosomes, whereas stabilization by taxol may increase the fusion between autophagic vacuoles and lysosomes. On the other hand, paclitaxel was shown to inhibit autophagy by blocking the activation of the VPS34 complex by inducing inhibitory phosphorylation of VPS34 as a substrate of CDK1. Lysosomotropic agents that increase the lysosomal pH, such as CQ, NH4Cl, and monensin, interfere with lysosomal function and block autophagy at a late stage. The final step of the autophagy pathway can also be blocked by inhibitors of lysosomal enzymes, such as E64d and pepstatin A, and by bafilomycin A1, a specific inhibitor of v-ATPase.

to include secondary criteria in the screens, e.g., by determining the effects of the compounds on cell viability at the same time. To test whether the activation of autophagy might accelerate the degradation of misfolded proteins, Zhang et al. used the accumulation of mutant polyglutamine as another marker, which can be degraded upon the activation of autophagy. With this screening method, both autophagy activators and inhibitors were isolated successfully (10, 11).

Shaw et al. used a dual fluorescent reporter mCherry–GFP-LC3, which can potentially distinguish between increases in GFP punctae as a result of increased autophagic flux and impaired lysosomal function due to the ability of acidic lysosomal pH to quench the GFP signal but not the mCherry signal (12). Since defects in key autophagy genes such as Atg16l1 have been shown to lead to increased intestinal inflammation (13), Shaw et al. tested the possibility of isolating autophagy inducers that can also block the production of the inflammatory mediator IL-1β. This screen was able to identify compounds that can induce autophagy as well as block IL-1β production and demonstrated the feasibility of identifying small molecules that can both induce autophagy and block inflammation.

p62/SQSTM1-based methods. Another imaging-based screening method for autophagy regulators involves the measurements of p62, an autophagy receptor that functions by linking autophagic cargo to ATG8/LC3 family members located on the autophagic membranes. Larsen et al. set up a cell system based on tetracycline-induced expression of GFP-p62 using Flp-In T-REx HEK293 cells, with GFP-p62 recombined into a single integrated FRT site in the genome (14). This system allows for the monitoring of inducible autophagic flux by following the degradation of the fusion proteins after promoter shut-off. This inducible GFP-p62 system does not require the quantification of autophagosome size or intensity and thus may be more straightforward for adoption by other laboratories.

Mutant protein-based methods. Williams et al. developed a method based on the detection of removal of hemagglutinin-tagged mutant α-synuclein or GFP-fused mutant huntingtin pro-
teins, both of which are aggregate prone and are known autophagy substrates (15). The expression levels of these mutant proteins in cells treated with small molecules are measured using antibodies against hemagglutinin or GFP as a surrogate marker for autophagy. Their expression levels are subsequently measured using anti-LC3 antibodies and normalized against the expression of actin.

Pharmacological agents that target autophagic machinery

Here we provide a discussion of small-molecule activators and inhibitors of autophagy that have been used in research and their potential application in developing new treatments for human diseases (Figure 1 and Tables 1 and 2). We caution the reader that potential application in developing new treatments for human diseases (Figure 1 and Tables 1 and 2) and their use in clinical trials should be undertaken with caution. Although autophagy is a physiological process that is involved in the clearance of damaged organelles and the degradation of misfolded proteins, it is also a major contributor to the survival and proliferation of cancer cells. By promoting intracellular recycling, autophagy can be used to treat and prevent malaria.

Table 2. Compounds that inhibit autophagy

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mechanism and use</th>
<th>Reference</th>
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<tbody>
<tr>
<td>3-MA</td>
<td>Inhibits class III PI3K</td>
<td>38</td>
</tr>
<tr>
<td>Wortmannin/LY294002</td>
<td>Inhibits class III PI3K</td>
<td>41</td>
</tr>
<tr>
<td>PT21</td>
<td>Inhibits VPS34 kinase</td>
<td>38</td>
</tr>
<tr>
<td>Spautin-1</td>
<td>Inhibits USP10/13 to promote the degradation of VPS34 complexes</td>
<td>11, 45</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>A non-competitive inhibitor of SERCA that blocks the fusion of autophagosomes with lysosomes</td>
<td>56</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Microtubule stabilizer that inhibits autophagy by inducing inhibitory phosphorylation of VPS34 at T159 and blocking autophagosome–lysosome fusion</td>
<td>68, 69</td>
</tr>
<tr>
<td>SAHA</td>
<td>Inhibits HDACs and blocks the fusion of autophagosomes and lysosomes</td>
<td>75</td>
</tr>
<tr>
<td>CQ</td>
<td>Neutralizes the acidic pH of intracellular vesicles and inhibits autophagy by blocking lysosomal degradation; used to treat and prevent malaria</td>
<td>77</td>
</tr>
<tr>
<td>HCQ</td>
<td>A CQ derivative that blocks lysosomal degradation</td>
<td>78</td>
</tr>
<tr>
<td>Lys05</td>
<td>A CQ derivative with improved lysosomal accumulation</td>
<td>79</td>
</tr>
<tr>
<td>Monensin</td>
<td>Interferes with autophagosome–lysosome fusion; used as an antibiotic</td>
<td>81</td>
</tr>
<tr>
<td>Lucanthone</td>
<td>Interferes with lysosomal degradation; used as a chemotherapeutic agent and DNA intercalator</td>
<td>82</td>
</tr>
<tr>
<td>Matrine</td>
<td>Blocks autophagic degradation by elevating intraluminal pH of lysosomes</td>
<td>83</td>
</tr>
<tr>
<td>Xanthohumol</td>
<td>Prenylated chalconoid that inhibits p53 and blocks autophagosome maturation</td>
<td>85</td>
</tr>
<tr>
<td>DBeQ</td>
<td>Reversible ATP-competitive inhibitor of p57 that inhibits autophagosome maturation</td>
<td>86</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>Inhibits the activity of v-ATPases and blocks autophagosome–lysosome fusion; used as an antibiotic</td>
<td>89</td>
</tr>
<tr>
<td>E64d/Pepstatin A</td>
<td>Inhibitors of cysteine and aspartic proteases that inhibit autophagy by blocking lysosomal degradation</td>
<td>90</td>
</tr>
<tr>
<td>Bafilomycin A1</td>
<td>Inhibitor of v-ATPase that blocks the lysosomal proton transport, leading to the inhibition of lysosomal hydrolases</td>
<td>87</td>
</tr>
<tr>
<td>Concanamycin A</td>
<td>Inhibitor of v-ATPase that blocks the lysosomal proton transport and leads to the inhibition of lysosomal hydrolases</td>
<td>88</td>
</tr>
<tr>
<td>Verteporfin</td>
<td>Used in combination with photodynamic therapy to eliminate abnormal blood vessels</td>
<td>91</td>
</tr>
<tr>
<td>DCMI</td>
<td>Inhibits autophagosome–lysosome fusion; used as an antidepressant</td>
<td>92</td>
</tr>
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mTOR is the catalytic subunit of two functionally distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 inhibitors, including rapamycin or rapalogs, can affect the activity of Unc-51 like autophagy activating kinase 1 (ULK1), a key Ser/Thr kinase that acts both as a downstream effector and negative regulator of mTORC1 to regulate autophagy (17–21). Activators of autophagy by rapamycin and its analogs has been shown to be protective in different animal models of neurodegenerative disorders (22–25). These data suggest that autophagy prevents the accumulation of misfolded proteins. A new generation of ATP-competitive inhibitors of mTOR display potent activation of autophagy. Unlike that of rapamycin, which only inhibits mTORC1, this new generation of inhibitors, such as PP242 and Torin 1, inhibits both mTORC1 and mTORC2 (26–29).

Although rapamycin and other inhibitors of mTOR are strong activators of autophagy and have been tested as anticancer therapeutics, caution should be taken in equating the activation of autophagy with cancer suppression, as accumulating evidence suggests that activation of autophagy can promote the survival of cancer cells. By promoting intracellular recycling, autophagy can confer stress resistance and sustain cell survival under adverse conditions, which may promote metastasis (30). Thus, the efficacy of rapamycin and other inhibitors of mTOR as an anticancer therapy may have to do with their inhibitory effects on protein translation and metabolism, rather than autophagy activation.

Activators of AMPK activate autophagy. AMPK, the main sensor of intracellular energy, is an important regulator of both ULK1 and mTOR. The activation of AMPK by increases in the intracellular ratio of AMPK/ATP leads to the activation of autophagy via both inhibition of mTOR and activation of ULK1.
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(31). Hence the administration of AMPK activators such as metformin stimulates autophagy. The mechanism of metformin is still being debated: metformin has been reported to target the LKB1/AMPK complexes (32) and/or inhibit the respiratory chain complex I, which leads to a reduction in intracellular ATP, thereby indirectly activating AMPK (33).

**Inhibitors of VPS34 kinase complexes block autophagy.** Recent evidence suggests that modulation of the activity of class III PI3Ks, including VPS34, PIK3R4, ATG14L/Barkor, UVRAG, BIF-1, Rubicon, and Ambra1 (34–36), regulates autophagy. Class III PI3K complexes mediate the production of phosphatidylinositol-3-phosphate (PI3P), a key lipid-signaling molecule that serves as an important checkpoint involved in regulating the mTOR-independent induction of autophagy under normal nutritional conditions (37). PI3P is required for the formation of the phagophore and, subsequently, the autophagosome.

Autophagy is positively regulated by PI3P, the product of class III PI3K (VPS34 complexes), and is negatively regulated by phosphatidylinositol triphosphate, the production of class I PI3K. 3-Methyladenine (3-MA) has been widely used as an inhibitor of class III PI3K to block autophagosome formation; however, 10 mM 3-MA is required to inhibit autophagy. A structural study suggested that 3-MA preferentially inhibits VPS34 in vitro (38). In vivo, 10 mM 3-MA can also inhibit class I PI3K, which may explain why it can promote autophagy flux under nutrient-rich conditions (39, 40). The PI3K inhibitors wortmannin and LY294002, which inhibit both class I and III PI3K complexes, have also been used to inhibit autophagy (39, 41, 42).

An x-ray crystallographic study of the class III PI3K VPS34 demonstrated that it exhibits distinguishing structural features that differentiate it from class I PI3Ks. The P loop of VPS34, which binds the phosphates of ATP, curls inward toward the ATP-binding pocket and is more constricted than that of class I PI3K. The hinge between the N and C lobes of VPS34 is one residue shorter than in class I PI3Ks. Therefore, VPS34 lacks the bulged-out space at the adenine-binding pocket hinge that serves as an important checkpoint involved in regulating the mTOR-independent induction of autophagy under normal nutritional conditions (37). PI3P is required for the formation of the phagophore and, subsequently, the autophagosome.

The stability of VPS34 complex components, including VPS34, beclin 1, VPS15/p150, ATG14L, and UVRAG, are coordinately regulated so that the reduction of one component may lead to the reduction in the levels of other components (43). Thus, it is possible to regulate the levels of entire complexes by targeting one component. Spautin-1, isolated from an imaged-based screen for small-molecule modulators of autophagy (11), promotes the degradation of VPS34 complexes by targeting the ubiquitin-specific peptidases, USP10 and USP13, two deubiquitinating enzymes that regulate beclin 1 and VPS34 stability. Spautin-1 promotes ubiquitination and proteasomal degradation of beclin 1 and VPS34 complexes under glucose-free conditions (44), consequently inhibiting autophagy.

Spautin-1 treatment can promote the death of cancer cells in the setting of nutrient deprivation when autophagy is activated to promote survival. Moreover, inhibition of autophagy by spautin-1 under nutritional deprivation conditions leads to the activation of chaperone-mediated autophagy, a lysosome-dependent pathway that mediates degradation of missense mutant p53 protein (45). Increasing evidence suggests that certain p53 mutants may promote oncogenesis through a dominant gain-of-function mechanism; thus, the reduction of accumulated mutant p53 may be an important therapeutic goal in cancer treatment. Development of improved spautin-1 analogs with good safety and in vivo stability profiles may represent a novel anticancer therapeutic strategy.

**Inhibitors of class I PI3K signaling activate autophagy.** In contrast to the positive role of the class III PI3K products in promoting autophagy, increases in the levels of class I PI3K products (phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate) negatively regulate autophagy (46). Targeting this class I PI3K with inhibitors such as CH512799, GDC-0980, and GDC-0941, leads to the activation of autophagy (47–49).

Protein kinase AKT is a major downstream mediator of class I PI3K signaling. Pharmacologic targeting of AKT by inhibitors such as perifosine leads to autophagy activation (50). Moreover, treatment with inhibitors of receptor tyrosine kinases that leads to the inhibition of AKT can activate autophagy, possibly by inhibiting mTORC1 or by regulating beclin 1. The activation of the EGFR tyrosine kinase-mediated signaling pathway may inhibit autophagy through multiple mechanisms that target beclin 1 (51, 52).

**Modulators of intracellular Ca²⁺ affect autophagy.** Ca²⁺ is an important intracellular second messenger involved in the regulation of many cellular processes, including autophagy, which can be inhibited by increasing intracytosolic Ca²⁺ levels (53). L-type Ca²⁺ channels are voltage-dependent channels that are involved in regulating excitation-contraction coupling of muscle and hormone secretion in endocrine cells. Several L-type Ca²⁺ channel antagonists were isolated from multiple screens for small-molecule modulators of autophagy. Zhang et al. (10) identified multiple antagonists of L-type Ca²⁺ channels, including fluspirilene, verapamil, loperamide, and amiodarone, as autophagy activators that promote the degradation of long-lived proteins and misfolded polyglutamine but do not cause cell death. In another small-molecule screen for autophagy modulators using the clearance of the autophagy substrate A30P α-synuclein as a biomarker, Williams et al. (15) also uncovered multiple antagonists of L-type Ca²⁺ channels including verapamil, loperamide, amiodarone, nimodipine, and nitrendipine as autophagy inducers that do not exhibit cytotoxicity.

The ability of these L-type Ca²⁺ channel antagonists to induce autophagy suggests the possibility that the levels of intracellular Ca²⁺ play an important role in regulating autophagy. Elevation of intracellular Ca²⁺ leads to the activation of calpains, which are Ca²⁺-dependent, non-lysosomal cysteine proteases that are expressed ubiquitously in mammals and other organisms. The treatment of fluspirilene, an antipsychotic drug used for the treatment of schizophrenia, was found to reduce intracellular Ca²⁺ and prevent the calpain-mediated cleavage of ATG5, which is activated by elevated intracellular Ca²⁺ (54). In addition, calpain 1 can cleave the Gus subunit of heterotrimeric G proteins, which increases its activity after calpain cleavage. Gus enhances adenyl cyclase activity, which in turn inhibits autophagy (55). Thus, a reduction in the intracellular Ca²⁺ concentrations and calpain activity can lead...
to the activation of autophagy. These studies suggest that calpain 1 may have multiple mechanisms to control the levels of autophagy in cells under normal nutritional conditions by regulating the levels of key proteins involved in autophagy.

Thapsigargin, a noncompetitive sarcoplasmic reticulum/ER Ca\(^{2+}\) ATPase inhibitor, increases cytosolic Ca\(^{2+}\) concentrations by inhibiting Ca\(^{2+}\) pumps in the sarcoplasmic reticulum and ER. Depletion of Ca\(^{2+}\) in the ER may in turn cause secondary activation of plasma membrane Ca\(^{2+}\) channels, allowing further Ca\(^{2+}\) influx into the cytosol. Thapsigargin does not affect autophagosome formation but leads to accumulation of mature autophagosomes by specifically inhibiting the fusion of autophagosomes with lysosomes (56). Thapsigargin blocks autophagosomal recruitment of the small GTPase Rab7, which is required for complete autophagosome formation but leads to accumulation of mature autophagosomes with lysosomes (56). Thapsigargin blocks autophagosomal recruitment of the small GTPase Rab7, which is required for complete autophagosome formation but leads to accumulation of mature autophagosomes with lysosomes (56).

**ER stress can induce autophagy, disruption of intracellular Ca\(^{2+}\) within the endocytic system (56). Thus, while the activation of ER stress can induce autophagy, disruption of intracellular Ca\(^{2+}\) homeostasis can inhibit autophagy.**

**Inhibitors of inositol monophosphatase activate autophagy.** Lithium, used widely as a mood-stabilizing drug for the treatment of bipolar disorder, was shown to induce autophagy and reduce mutant huntingtin aggregates and cell death (57). Sarkar et al. found that lithium inhibits inositol monophosphatase (IMPase), leading to depletion of free inositol and a reduction in the levels of myo-inositol-1,4,5-triphosphate (IP3), which may represent an mTOR-independent pathway for regulating mammalian autophagy. Similar effects were observed with other IMPase inhibitors and mood-stabilizing drugs such as L-690330 and carbamazepine, which also decrease inositol levels (58). Since IP3 receptor (IP3R) is a potential binding partner of beclin 1, the IP3R antagonist Xestospongin B can stimulate autophagy by releasing beclin 1 from IP3R to assemble into the VPS34 complex (59, 60). In addition, rilmenidine, which is an imidazoline receptor 1 agonist and an FDA-approved drug for hypertension, has been shown to induce autophagy, enhance mutant huntingtin clearance and reduce toxicity in a mouse model of Huntington’s disease. Rilmenidine is currently being tested in a safety trial in patients with Huntington’s disease (61). Any success of such clinical trials would encourage the development of additional autophagy modulators for the treatment of neurodegenerative diseases.

**Induction of cell death by Tat–beclin 1 peptide.** Tat–beclin 1 peptide, a fusion peptide of 18 amino acids derived from beclin 1 and the HIV Tat protein transduction domain, was shown to induce autophagy and decrease the accumulation of polyglutamine expansion protein aggregates by binding to Golgi-associated plant pathogenesis-related protein 1 (GAPR-1, also known as GLIPR2), a protein that associates with lipid rafts at the cytosolic leaflet of the Golgi membrane. In addition, Tat–beclin 1 inhibits the replication of several pathogens (including HIV-1) in vitro and reduces mortality in mice infected with chikungunya or West Nile virus (62). On the other hand, treatment with Tat–beclin 1 can induce cell death (63), and knockdown of components of the autophagy machinery, such as beclin 1, Atg4L, and Atg5, can block cell death induced by Tat–beclin 1, which suggests that Tat–beclin 1 may activate autophagic cell death.

**Targeting cytoskeletal components to regulate autophagy.** The destabilization of microtubules by either vinblastine (64), nocodazole, or cytochalasin B and D blocks the maturation of autophagosomes (65); conversely, taxol-mediated microtubule stabilization increases the fusion between autophagic vacuoles and lysosomes (66, 67). Paclitaxel, which also stabilizes microtubules, was shown to inhibit autophagy through two distinct mechanisms dependent on the cell cycle stage. In mitotic cells, paclitaxel blocks the activation of the VPS34 complex by inducing inhibitory phosphorylation of VPS34 at T159, which is mediated by mitotic kinases such as CDK1 (68). In non-mitotic cells, paclitaxel inhibits autophagosome trafficking to block maturation of autophagic vesicles (69). The ability of microtubule targeting agents to negatively regulate autophagy suggests that their efficacy as anticancer therapeutics may be partially attributable to their ability to inhibit autophagy. Additionally, other autophagy-inhibiting agents may not enhance the therapeutic effects of paclitaxel, nocodazole, or vinblastine, as these agents already inhibit autophagy.

**Histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase, has been shown to function as a central component of basal autophagy that targets protein aggregates to the dynein motor for transport (70, 71). HDAC6 was shown to promote autophagy by recruiting cortactin-dependent, actin-remodeling machinery, which assembles an F-actin network that stimulates autophagosome-lysosome fusion and substrate degradation (72). HDAC6 deficiency impairs autophagosome maturation; accordingly, inhibitors of HDAC6 and other HDACs have been shown to block autophagy (73) through acetylation and suppression of autophagy-associated proteins such as ATG7. However, the effect of HDAC inhibitors on autophagy has been a controversial issue, and other studies propose HDAC inhibitors as autophagy activators (74–76).**

**Lysosomal alkalizers block autophagic flux and degradation.** The lysosomal lumen alkalizers inhibit autophagy by neutralizing the acidic pH in the lumen of lysosomal vesicles, which is required for the activities of lysosomal hydrolases involved in autophagic degradation. Thus, alklylation of lysosomal vesicles leads to the accumulation of autophagosomes by blocking lysosomal degradation (77). Chloroquine (CQ) and its derivative hydroxycloroquine (HCQ) have been investigated in preclinical studies and clinical trials as anticancer drug candidates (78). Some efforts have been made to generate more potent inhibitors of lysosomal functions. For example, Lys05, a dimeric CQ with an improved ability to accumulate in the lysosomes, may have more potent antitumor activity than HCQ (79, 80). Other compounds with CQ-like activities in alkalizing lysosomal compartments have also been identified. Monensin, a polyether antibiotic, is also a lysosomotropic drug that prevents the acidification of lysosomes and interferes with the fusion of autophagosomes and lysosomes (81). In addition, lucanthone, an anti-schistosome agent, inhibits autophagy via a similar mechanism to CQ (82). Finally, matrine, a quinolizidine alkaloid, can also block autophagic degradation by elevating the luminal pH of lysosomes (83).
starvation, suggesting that p97 might be selectively required for autophagic degradation of ubiquitinated substrates. Xanthohumol, a prenylated chalcone that can bind directly to the N domain of p97, was identified as a p97 ATPase inhibitor that can modulate autophagy (85). DBeQ, a selective, reversible and ATP-competitive p97 inhibitor, also impairs autophagic degradation of LC3-II mediated by DBeQ negatively regulates both autophagy and proteasome-mediated degradation, the two key catabolic mechanisms of cells, by DBeQ effectively induces caspase activation and apoptosis.

Vacular H+-ATPases block autophagic flux. Vacular H+-ATPases (v-ATPases) on the membrane of intracellular organelles with acidic lumen, such as endosomes and lysosomes, couple proton transport with ATP hydrolysis to maintain the proton gradient and the acidic pH environment. Treatment of inhibitors of v-ATPases, such as bafilomycin A1 or concanamycin A (87, 88), block lysosomal proton transport and lead to the inhibition of lysosomal hydrolases, which are only activated at a low pH. As a consequence, inhibition of v-ATPase blocks autophagic flux.

Azithromycin, a macrolide antibiotic drug commonly used to treat certain bacterial infections, has been identified as an autophagy inhibitor by preventing lysosomal acidification (89). Inhibition of autophagy by azithromycin has been suggested to lead to inhibition of intracellular killing of mycobacteria within macrophages and result in chronic infection with non-tuberculous mycobacteria in patients with chronic inflammatory lung diseases such as cystic fibrosis, who may require long-term use of antibiotics such as azithromycin.

Other inhibitors of autophagic degradation. Inhibitors of lysosomal hydrolases and proteases, such as E64d and pepstatin A, are commonly used to target cysteine, serine, and threonine peptidases such as leupeptin or cystatin B, or cathepsins that are localized in the lysosomal lumens to block autophagic flux (90).

Verteporfin, a benzoporphyrin derivative and an FDA-approved drug used clinically in photodynamic therapy, was identified in an image-based screen for chemicals that can prevent autophagosome formation in the presence of the lysosomal inhibitor CQ (91). Since the cytoplasm of cells treated with verteporfin exhibits the accumulation of single-membrane vesicles that are empty by EM analysis, it was hypothesized that verteporfin might inhibit the membrane expansion of phagophores, leading to defects in capturing cytoplasmic cargo.

Clomipramine, an FDA-approved drug used for the treatment of psychiatric disorders, and its active metabolite, desmethylclomipramine (DCMI), have been shown to interfere with autophagic flux by blocking autophagosome-lysosome fusion (92). DCMI-dependent blockage of autophagy enhances the efficacy of doxorubicin, a chemotherapeutic agent (92), but further studies are needed to clarify its anti-autophagic mechanism.

Concluding perspectives and future directions

Since defects in autophagic pathways have been implicated in many human disorders from inflammatory diseases to cancers and neurodegeneration, augmentation or inhibition of autophagy could potentially be used to treat multiple human diseases. While we are still in the early days in terms of developing pharmacologic modulators of autophagy, the identification of small-molecule modulators has already provided important insights into the mechanisms that regulate autophagy. High-throughput, image-based screens have been developed by multiple labs for isolating small-molecule modulators of autophagy. This was made possible by the recent technology development on fluorescence microscopy and related software platforms that allow quantitative analysis of autophagic vesicles in fluorescent imagery. The combination of high-throughput, image-based screens and additional criteria such as cell viability and production of inflammatory cytokines has made it possible to identify small-molecule modulators of autophagy with certain specificities. With the advancement in our understanding of autophagy pathways, we expect that there will be more target-specific design of autophagy modulators, such as inhibitors of VPS34 kinase activity. Small molecules that interfere with the various steps of the autophagic machinery that act as inducers or inhibitors will provide important tools for understanding the mechanism of autophagy and provide potential lead candidates for developing novel therapeutics for human diseases.

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Address correspondence to: Junying Yuan, Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115, USA. Phone: 617.432.4170; E-mail: jyuan@hms.harvard.edu.


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