Application and interpretation of current autophagy inhibitors and activators

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Autophagy is the major intracellular degradation system, by which cytoplasmic materials are delivered to and degraded in the lysosome. As a quality control mechanism for cytoplasmic proteins and organelles, autophagy plays important roles in a variety of human diseases, including neurodegenerative diseases, cancer, cardiovascular disease, diabetes and infectious and inflammatory diseases. The discovery of ATG genes and the dissection of the signaling pathways involved in regulating autophagy have greatly enriched our knowledge on the occurrence and development of this lysosomal degradation pathway. In addition to its role in degradation, autophagy may also promote a type of programmed cell death that is different from apoptosis, termed type II programmed cell death. Owing to the dual roles of autophagy in cell death and the specificity of diseases, the exact mechanisms of autophagy in various diseases require more investigation. The application of autophagy inhibitors and activators will help us understand the regulation of autophagy in human diseases, and provide insight into the use of autophagy-targeted drugs. In this review, we summarize the latest research on autophagy inhibitors and activators and discuss the possibility of their application in human disease therapy.

Keywords: autophagy; cancer; neurodegenerative diseases; PI3K inhibitor; cycloheximide; lysosomal lumen alkalizer; Rack1 protein; ER stress inducer; rapamycin; LiCl

Introduction

Cell growth and homeostasis are governed by tightly regulated biosynthetic and catabolic processes. The major cellular pathways for protein and organelle turnover are autophagy and proteasome-mediated degradation. Autophagy is a universal, dynamic process that takes place in all eukaryotic cells. There are three primary forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy[1]. Of these classifications, macroautophagy, herein referred to as autophagy, is the major form of autophagy and has been well characterized in recent years.

Increasing studies have shown that autophagy is involved in many human diseases (such as neurodegenerative diseases and cancer) and plays roles in their pathogenesis[2,3]. Uncovering the role of autophagy in these diseases is essential because it may present a novel therapeutic target. Unfortunately, available methods to monitor autophagy are limited. Enhancing or blocking autophagy by chemical drugs or genetic means will help us to reveal autophagic mechanisms implicated in these disease processes, and autophagy inhibitors or activators may become possible therapeutic strategies. Various compounds or strategies that have been utilized to induce or suppress autophagy in mammalian systems are summarized and discussed in this review.

Mechanisms and pharmacologic targeting of autophagy

Although autophagy and autophagy-related processes are dynamic, they can be broken down into several discrete steps for the purpose of discussion: (1) induction, (2) autophagosome formation, (3) autophagolysosome formation, and (4) delivery and degradation of the autophagic body[1]. Autophagy is induced as a response to both extracellular stress conditions (nutrient deprivation, hypoxia, and oxidative stress) and intracellular stress conditions (endoplasmic reticulum stress, accumulation of damaged organelles, and aggregation of proteins). The large number of stimuli able to trigger autophagy implies the involvement of multiple signaling pathways in autophagosome formation.

The autophagy-related genes and their products are named as ATG and Atg, respectively[4]. Once the phagophore has
been formed, the membrane structure expands to sequester materials to form autophagosome; this process is mediated by two ubiquitin-like conjugation systems, the Atg12-Atg5 and Atg8 conjugation systems[9]. In fact, half of the ATG genes essential for autophagy are involved in these two conjugation systems, and they are well conserved among eukaryotes. In addition, Atg1 kinase and its regulators, the phosphoinositide 3-kinase (PI3K) complex, Atg9 and the Atg2–Atg18 complex, are all involved in autophagosome formation[6]. Of these Atgs, Beclin 1, the mammalian ortholog of yeast Atg6, interacts with class III PI3K (also named Vps34) and thus plays an important role in the initiation of autophagosome formation during autophagy[7]. Beclin 1 is the first autophagy-related tumor suppressor gene reported thus far, and it has been shown to stimulate autophagy and suppress tumorigenesis in cancer cells[8]. These specific Atgs may be potent autophagy-regulating targets for genetic intervention.

The central player in autophagic signaling complexes and pathways is the mammalian target of rapamycin (mTOR), which negatively regulates autophagy. mTOR forms two different multi-protein complexes, which are referred to as mTOR complex 1 (mTORC1) and mTORC2, which are largely defined by the presence of either raptor or rictor[9]. mTOR is highly conserved from yeast to mammals. As a central regulator of cell growth and metabolism, mTOR controls growth-related processes such as development, aging and the response to hypoxia[10]. Thus, inhibitors of mTOR may be useful for the treatment of human diseases such as neurodegenerative diseases and metabolic disorders. Apart from the classical mTOR pathway regulating mammalian autophagy, the Ca²⁺-calpain-G proteins and the cAMP-Epac-PLC-ε-IP3 pathways also play important roles in the regulation of autophagy in an mTOR-independent way[11]. Multiple unexplored drug targets may exist in these two mTOR-independent autophagic pathways.

With the development of intensive research on autophagy, it has been recognized that an increase in autophagosome number alone does not necessarily correlate with increased autophagic activity or flux. Instead, the striking accumulation of autophagic vacuoles (AV) in cells likely reflects an imbalance between the rates of autophagic sequestration and completion of the degradative process. In other words, these cells can be thought of as undergoing “autophagic stress”[12]. Maintaining the function of the lysosome and/or promoting its fusion with autophagosomes are critical for the completion of autophagic flux. Histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase, recruits misfolded proteins to dynein motors for transportation to aggresomes in an autophagy-dependent manner[13]. Mounting evidence suggests that although HDAC6 is not required for autophagy activation per se, it is critical for autophagosome-lysosome fusion[14, 15]. Our recent findings showed that HDAC6 and dynein participated in the degradation of MPP⁺-induced misfolded α-synuclein aggregates by regulating the aggresome-autophagy pathway[16, 17].

### Autophagy activators

Several recent articles address that autophagy upregulation may have therapeutic benefits in a range of diseases. New research related to autophagy activators has become a hot topic owing to their potential clinical value. The growing list of various compounds or strategies for inducing autophagy is shown below (Table 1).

### Starvation inducers

Physiologically, autophagy is induced by amino acid deprivation[18]. Electron microscopy studies have indicated that complete deprivation of serum and amino acids provides a useful model for the further study of cellular autophagy[19]. It is widely accepted that autophagy is induced in several

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**Table 1. Autophagy activators.**

| Name                                      | Mechanism                     | Target point                             | Solubility      | References       |
|-------------------------------------------|-------------------------------|------------------------------------------|-----------------|------------------|
| Earle’s balanced salt solution (EBSS)     | Starvation inducer            | Autophagy induction                      | Water-soluble   | [20, 21, 59]     |
| Brefeldin A                               | ER stressing inducer          | Autophagy induction                      | Water-insoluble | [28, 60, 61]     |
| Thapsigargin                              | ER stressing inducer          | Autophagy induction                      | Water-insoluble | [28, 29, 61]     |
| Tunicamycin                               | ER stressing inducer          | Autophagy induction                      | Water-insoluble | [28, 29, 60, 61] |
| Rapamycin                                 | mTOR inhibitor                | mTOR-dependent signaling pathway         | Water-insoluble | [32–34]          |
| CCI-779                                   | mTOR inhibitor                | mTOR-dependent signaling pathway         | Water-insoluble | [37, 39, 62, 63] |
| RAD001                                    | mTOR inhibitor                | mTOR-dependent signaling pathway         | Water-insoluble | [39, 64–66]     |
| AP23576                                   | mTOR inhibitor                | mTOR-dependent signaling pathway         | Water-insoluble | [39]             |
| Small molecule enhancers rapamycin (SMER) | mTOR-independent activator   | mTOR-independent signaling pathway       | Water-soluble   | [40–42]          |
| Trehalose                                  | mTOR-independent activator   | mTOR-independent signaling pathway       | Water-soluble   | [43–45]          |
| Lithium chloride                          | IMPase inhibitor              | mTOR-independent signaling pathway       | Water-soluble   | [47, 48]         |
| L-690,330                                 | IMPase inhibitor              | mTOR-independent signaling pathway       | Water-soluble   | [49]             |
| Carbamazepine                             | IMPase inhibitor              | mTOR-independent signaling pathway       | Water-soluble   | [50, 67, 68]     |
| Valproic acid sodium salt                 | IMPase inhibitor              | mTOR-independent signaling pathway       | Water-soluble   | [51, 67]         |
| N-Acetyl-D-sphingosine (C2-ceramide)      | Class I PI3K inhibitor        | mTOR-dependent signaling pathway         | Water-soluble   | [54, 69]         |
| Penitrem A                                | Ca²⁺ channel blocker         | mTOR-dependent signaling pathway         | Water-soluble   | [11, 70]         |
| Calpastatin                               | Calpain inhibitor             | mTOR-dependent signaling pathway         | Water-soluble   | [71]             |
| Xestospongin B                            | IP3R blocker                 | mTOR-dependent signaling pathway         | Water-insoluble | [58, 72–74]     |
cell types as a response to total nutrient and serum starvation by incubation in Earle's Balanced Salt Solution (EBSS) or DMEM without amino acids and serum[20, 21]. Drosophila Rack1, a receptor of activated protein kinase C1, increases 4.1- to 5.5-fold during nutrient deprivation in all three genotypes. Recently, it was reported that loss of Rack1 led to an attenuated autophagic response to starvation[22]. This starvation-induced protein potentially acts as a scaffold protein during autophagosome formation.

**Endoplasmic reticulum stress inducers**

Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) results in ER stress. Emerging data now indicate that ER stress is a potent inducer of autophagy, a process whereby eukaryotic cells recycle their macromolecules and organelles[23–25]. Sar1 and Rab1b are monomeric GTPases that control traffic from the ER to the Golgi, and there is evidence indicating that the activity of both proteins is also required for autophagosome formation[26]. ER stress enhances autophagy by negatively regulating the AKT/TSC/mTOR pathway[27]. ER stress inducers such as brefeldin A, thapsigargin and tunicamycin increase the formation of autophagic vesicles with the expression of Beclin and LC-3 (microtubule-associated protein1 light chain 3) II, two autophagic markers[28, 29]. However, conflicting data have also been reported. For example, Gordon et al showed that autophagy is inhibited by thapsigargin, which releases Ca²⁺ from ER stores and thus increases intracytosolic Ca²⁺ levels[30]. Another study demonstrated that thapsigargin did not affect autophagosome formation but did lead to accumulation of mature autophagosomes by blocking autophagosome fusion with the endocytic system[31]. The opposite effects shown by these ER stress inducers may be caused by crosstalk between regulatory pathways of ER stress and autophagy.

**Rapamycin and its derivatives**

Rapamycin, also called sirolimus, is a natural product with potent antifungal and immunosuppressive activities. It forms a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then stabilizes the raptor-mTOR association and inhibits the kinase activity of mTOR[32]. As an inhibitor of mTOR, rapamycin has been widely reported in the literature to induce autophagy both in vivo and in vitro[33, 34]. In organisms from yeast to humans, TOR proteins control several cellular processes other than autophagy, including the repression of ribosome biogenesis and protein translation and transcriptional induction of compensatory metabolic pathways[35, 36]. These effects may contribute to the complications observed with long-term rapamycin use, such as immunosuppression, which is not compatible with disease therapy. Temsirolimus (also known as CCI-779) is a water-soluble ester of rapamycin that decreased huntingtin aggregate formation in a mouse model of Huntington disease by inhibiting mTOR[37]. The results from a current clinical trial support the use of CCI-779 for mantle cell lymphoma intervention, likely in combination with other agents, such as antiangiogenic drugs or histone acetylase inhibitors[38]. Similar to CCI-779 (intravenous formulation), two other rapamycin analogs are RAD001 (or Everolimus, oral formulation) and AP23573 (intravenous formulation), both of which display a good safety profile with mild, dose-limiting toxicities compared with rapamycin[39]. These rapamycin derivatives are better therapeutic strategies against cancer.

**Small molecule enhancers of rapamycin**

Owing to the immunosuppressive side effects of rapamycin that preclude its use in therapy, a safer way of inducing autophagy urgently needs to be developed. Using a novel, two-step screening process, three small molecule enhancers of rapamycin (SMERs), SMERs 10, 18, and 28, were identified from 50729 compounds screened in 2007[40]. SMER10 is an aminopyrimidine, SMER18 is a vinylogous amide, and SMER28 is a bromo-substituted quinazoline. These SMERs have been confirmed to induce autophagy in mammalian cells, enhancing the clearance of autophagy substrates such as mutant huntingtin and A53T α-synuclein, which are associated with Huntington’s disease and familial Parkinson’s disease, respectively[41]. The SMERs did not affect the levels of various autophagic regulators, such as Beclin 1 (Atg6), Atg5, Atg7, and Atg12, nor did they enhance conjugation of Atg12 to Atg5, which is a critical step in autophagosome assembly preceding LC3 conjugation[41]. These SMERs induce mammalian autophagy in an mTOR-independent manner, appearing to act either independently or downstream of the target of rapamycin. However, a recent study shows that Atg5 plays an essential role in the degradation of Aβ and APP-CTF, which is induced by starvation or SMER28. In other words, SMER28 decreases Aβ and APP-CTF levels via an Atg5-dependent autophagic pathway[42].

**Trehalose**

Trehalose was first extracted from rye ergot by Wiggers in 1832. Trehalose protects the integrity of cells against various environmental stresses such as heat, cold, desiccation, dehydration, and oxidation by preventing protein denaturation. Recently, a new role for trehalose as an mTOR-independent activator of autophagy was discovered[43]. Trehalose-induced autophagy enhanced the clearance of autophagy substrates such as mutant huntingtin and A30P and A53T α-synuclein[43]. Furthermore, as a natural hemolymph sugar of invertebrates, trehalose may be a safe strategy for the treatment of two other neurodegenerative diseases, AD[44] and prion disease[45]. Notably, trehalose pre-treatment protected against pro-apoptotic insults by reducing mitochondrial load in addition to its autophagic induction role[43, 46]. It is difficult to conclude that prolonged upregulation of autophagy would be beneficial for treatment without any risk. However, the dual protective properties of trehalose and its less toxic characteristics make it a unique candidate for developing therapeutic strategies of autophagy-targeted drugs.
IMPase inhibitors
In 2005, Prof Rubinsztein found that the lithium chloride (LiCl) used to treat bipolar disorders induced autophagy in an mTOR-independent manner. It was revealed that LiCl induced autophagy via inhibition of inositol monophosphatase (IMPase), leading to free inositol depletion and reduced inositol-1,4,5-triphosphate (IP3) levels. This is the first report describing a pathway that regulates mammalian autophagy other than the canonical mTOR-dependent pathway. Autophagy induced by LiCl significantly promotes the degradation of mutant huntingtin and α-synuclein in neurodegenerative diseases.

Autophagy inhibitors
Autophagy could potentially be suppressed at any stage of autophagic flux. During the study of autophagy mechanisms, many chemical inhibitors have been identified and were used in various cell and animal models. These inhibitors target various steps of the autophagy process and can be used to investigate the role of autophagy in neurodegenerative diseases. The table below summarizes some of the autophagy inhibitors identified:

| Name               | Mechanism                              | Target point                      | Solubility   | References       |
|--------------------|----------------------------------------|-----------------------------------|--------------|-----------------|
| 3-Methyladenine    | PI3-kinase inhibitor                   | Autophagosome formation           | Water-soluble| [76, 78, 80]    |
| Wortmannin         | PI3-kinase inhibitor                   | Autophagosome formation           | Water-insoluble| [80, 102, 103] |
| LY294002           | PI3-kinase inhibitor                   | Autophagosome formation           | Water-insoluble| [80, 102, 103] |
| Cycloheximide      | protein synthesis inhibitor            | Autophagosome formation           | Water-insoluble| [85-87]         |
| Baflomycin A1      | Vacuolar-type H(+)-ATPase inhibitor    | Autophagolysosome formation        | Water-insoluble| [90, 91]        |
| Hydroxychloroquine | lysosomal lumen alkalizer              | Lysoseme                          | Water-soluble | [95, 104, 105] |
| Lys05              | lysosomal lumen alkalizer              | Lysoseme                          | Water-soluble | [97, 106]       |
| Leupeptin          | acid protease inhibitor                | Lysoseme                          | Water-soluble | [98, 107]       |
| E64d               | acid protease inhibitor                | Lysoseme                          | Water-insoluble| [100, 108]     |
| Pepstatin A        | acid protease inhibitor                | Lysoseme                          | Water-insoluble| [100]           |
Previously, it was thought that the overall effect of these inhibitors was typically to block autophagy because the class III enzymes that are required to activate autophagy act downstream of the negative regulatory class I enzymes. However, a recent study presents a surprising finding that 3-MA has a dual role in autophagic regulation[81]. 3-MA promoted autophagic flux when administered under nutrient-rich conditions with a prolonged period of treatment, although it was still capable of suppressing starvation-induced autophagy. The inhibitory effects of wortmannin are the opposite of those of 3-MA: it has persistent effects on class III PI3K and transient effects on class I PI3K[81]. Data from this study also suggest that wortmannin is a more suitable autophagy inhibitor than 3-MA due to its persistent inhibition of class III PI3K activity. However, it is notable that wortmannin induces the formation of vacuoles that appear similar to autophagosomes, although they are swollen late endocytic compartments[82].

In addition, studies also have shown that LY294002 activated autophagy by inhibiting the class I PI3K signaling pathway[83]. LY294002 increased intracellular calcium, at least in part, by mobilizing intracellular calcium stores and inhibiting calcium transients[84]. Therefore, experiments where calcium is relevant should avoid using LY294002. Understanding the complex role of PI3K inhibitors in autophagy may help in choosing the proper inhibitor for a particular situation.

Cycloheximide
Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms and is produced by the bacterium Streptomyces griseus. It is a widely used method in biomedical research to inhibit protein synthesis that is inexpensive and fast acting. Studies have demonstrated that cycloheximide suppresses cellular autophagy induced by hyperosmotic sucrose or cadmium chloride in mouse pancreatic acinar cells[85]. An in vitro study has shown regression of autophagic vacuoles in seminal vesicle cells following cycloheximide treatment[86]. Cycloheximide has proven to be a fast and effective inhibitor of autophagic segregation and may inhibit segregational steps occurring prior to the actual formation of autolysosomes[87]. Although it is still currently used to inhibit the autophagy-lysosome pathway[88], one should keep in mind that the inhibition of autophagic degradation and lysosomal enzyme delivery is rapidly reversed following the removal of cycloheximide[89] and that the mechanism of cycloheximide action in short-term experiments remains poorly understood.

Vacuolar-type H (+)-ATPase inhibitors
Vacuolar-type H (+)-ATPases (V-ATPases) are found within the membranes of many organelles including lysosomes, endosomes, and secretory vesicles, where they play a variety of roles crucial for organelle function. Bafilomycin A1 is a specific inhibitor of V-ATPase in cells, and it inhibits the acidification of lysosomes and endosomes. As early as 1998, bafilomycin A1 was reported to prevent maturation of autophagic vacuoles by inhibiting the fusion between autophagosomes and lysosomes in the rat hepatoma cell line H-4-II-E[90]. Inhibition of autophagy by bafilomycin A1 decreased proliferation and induced apoptosis in colon cancer cells[91]. However, Prof Daniel revealed an apparently contradictory result that bafilomycin A1 did not block the fusion of autophagosomes with lysosomes[92]. Data from the relatively recent literature also show that bafilomycin A1 and rapamycin potentiate ethanol-increased LC3 lipidation, whereas wortmannin and a BECN1-specific shRNA inhibit ethanol-promoted LC3 lipidation[93]. Furthermore, concanamycin A, another selective V-ATPase inhibitor, also increased the accumulation of autophagosomes[94].

Lysosomal lumen alkalinizers
Lysosomal lumen alkalinizers (chloroquine, hydroxychloroquine, NH4Cl, and neutral red) are used to block autophagic progress by impairing lysosomes. Of them, chloroquine and its analog hydroxychloroquine are the only clinically relevant autophagy inhibitors that are widely used as anti-malarial and anti-rheumatoid agents. It has been reported that chloroquine-mediated lysosomal dysfunction enhanced its anticancer effect[95]. A major concern with the use of hydroxychloroquine is that high micromolar concentrations, which are not consistently achieved in patients, are required to block autophagy in vitro.

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Acid protease inhibitors
The lysosome is the ultimate degradative autophagic compartment in the cell. Leupeptin is a naturally occurring protease inhibitor that inhibits cysteine, serine and threonine peptidases. It blocks autophagy at the step of degradation of the cytoplasm enclosed in lysosomes and causes the accumulation of autolysosomes and/or many cytoplasmic inclusions in the central vacuoles[98]. Cycloheximide administered simultaneously with leupeptin rapidly inhibited the formation of autophagic vacuoles and the sequestrations of both cytoplasmic and lysosomal enzymes[99].

Lysosomal cathepsins, which are enclosed in lysosomes, help maintain the homeostasis of the cell’s metabolism by participating in the degradation of autophagic bodies. Among the lysosomal hydrolases and proteases, cathepsins have an especially major role. E64d and pepstatin A are two autophagy inhibitors that function by suppressing lysosomal proteases. E64d is a membrane-permeable inhibitor of cathepsins B, H, and L, whereas pepstatin A is an inhibitor of cathepsins D and E. The lysosomal turnover of endogenous LC3-II could be investigated using E64d and pepstatin A. LC3-positive
puncta, i.e., autophagolysosomes, highly accumulated in the presence of E64d and pepstatin A under starvation conditions\[108\]. It was recently reported that E64d plus pepstatin A increased free GFP fragments resulting from the degradation of GFP-LC3 within the autolysosome\[109\]. This observation is confusing because the level of free GFP fragments is thought to reflect autophagic flux. One possible reason for this is that under certain conditions (non-saturating concentrations) some lysosomal inhibitors only partially suppress cathepsin activity and may not completely suppress lysosomal degradation (or cleavage) of GFP-LC3.

Genetic intervention
The existence of autophagy inhibitors and activators greatly facilitates the investigation of autophagy and its therapeutic potential in human diseases. However, most chemical inhibitors of autophagy are not entirely specific; thus, genetic intervention is suggested as a preferred approach to block autophagy. The ATG genes are essential for autophagosome information\[109\]. The use of ATG gene deletions/inacti-vations or functional knockdown (eg. RNAi against the ATG genes) methods may produce a more specific manipulation of autophagy. A growing number of studies indicate that Atg-deficient cells and animals provide available experimental models for monitoring autophagy in different organisms. Furthermore, microRNAs may also be used for autophagy-related studies.

The tumor-suppressive miRNA miR-101 has been identified as a potent inhibitor of basal and rapamycin-induced autophagy\[110\]. Recently, miR-30a has been shown to be a potent autophagic inhibitor by downregulating Beclin 1 and ATG5 expression. In contrast, knockdown of miR-30a by antagonim-30a increases the expression of Beclin 1 and ATG5\[111\]. Although previous reports have shown that down-regulation of ATG7, ATG5, or BECN1 by RNAi significantly decreases autophagy, it should be noted that autophagy may also occur in the absence of some of these key autophagic proteins. Recent evidence suggests that the mammalian autophagy may occur through an Atg5/Atg7-independent pathway\[112\]. Furthermore, Beclin 1-independent autophagy was also found in dying cortical neurons\[113\]. These interesting data emphasize the limitations of Atg5/Atg7 and Beclin 1 as autophagic markers in some situations.

Therapeutic implications for autophagy regulators
Malfunctioning autophagy is observed in many human diseases including cancer, neurodegenerative diseases, cardiac and muscular diseases, infectious and inflammatory diseases, diabetes and obesity\[114\].

However, the effect of autophagy on disease progression has not yet been discovered, and the identification and development of new drug targets is still a key focus. Further investigations are required to assess the clinical potential of autophagy activators and inhibitors in various diseases.

Autophagy is a universal, dynamic process that takes place in all eukaryotic cells and contributes to the turnover and rejuvenation of cellular components. It may also promote an autophagic death distinct from apoptosis, which is termed type II programmed cell death\[115\]. As a double-edged sword, autophagy plays a dual role in many diseases\[116\]. For example, autophagy acts both as a tumor suppressor and a protector of cancer cell survival in tumorigenesis\[117\]. A growing body of evidence demonstrates that cellular decisions toward autophagy depend on disease type, stage, microenvironment and drug treatment. Future work will be required to further investigate the mechanisms of autophagy underlying various diseases and to elucidate their exact roles in these diseases.

Many promising small molecules have been developed to regulate autophagy for therapeutic needs. Recently, a potent small molecule inhibitor of autophagy termed spautin-1 for specific and potent autophagy inhibitor-1 was discovered. Spautin-1 promotes the degradation of Vps34-PI3 kinase complexes by inhibiting two ubiquitin-specific peptidases, USP10 and USP13, that target the Beclin1 subunit of Vps34 complexes\[118\]. DEPTOR, an inhibitor of mTORC1 and mTORC2, accumulates upon glucose deprivation and mTOR inhibition and induces autophagy\[119\]. Similarly, the small-molecule inhibitor torin 1 was used to demonstrate that inhibition of mTOR kinase activity was a more potent inducer of autophagy than rapamycin\[120\]. These small molecule regulators of autophagy are more effective and likely to enhance the therapeutic arsenal against human diseases.

As mentioned above, many autophagy regulators are medicines that have been used in clinical therapy for years. Their new roles in autophagic regulation are surprising. FDA-approved antipsychotic drugs (fluspirilene, trifluoperazine, and pimozide) and FDA-approved drugs for cardiovascular indications (niguldipine, nicardipine and amiodarone) have been all described to promote autophagy\[121\]. The existence of autophagic regulators among FDA-approved drugs facilitates the investigation of the therapeutic potential of regulators of autophagy in vivo.

Novel regulators of autophagy with better therapeutic indexes are still needed. Because of their lower toxicity, traditional Chinese medicines should be considered for disease therapy by autophagic regulation. Recently, it was reported that the therapeutic effects of resveratrol\[122\] and oridonin\[122\] were both related to autophagy. Our studies also show that paeoniflorin, the principal bioactive component of Radix Paeoniae alba, potently protected PC12 cells against MPP+ or acidosis-induced injury by upregulating the autophagic pathway\[123\].

The regulation of autophagy is complex and involves many signaling pathways. Thus, the safety and effectiveness of autophagy activators or inhibitors must be taken into account before clinical therapy development. A combination of mTOR and PI3 kinase inhibitors showed a synergistic antitumor effect\[124\]. Another study showed that the combination of the mTOR inhibitor rapamycin and the IMPase inhibitor lithium ameliorates toxicity of polyglutamine-expanded huntingtin\[125\]. These lines of evidence shed some light on the advantage of combination therapy and suggest that the combination ther-
apy based on an mTOR inhibitor and an mTOR-independent activator deserves further investigation as a potential treatment.

Concluding remarks
Autophagy is a process that involves the sequestration of intracellular components and their subsequent degradation in secondary lysosomes that is highly conserved from yeast to mammals. In the past several decades, the molecular mechanisms of autophagy and its role in human diseases have been well elucidated. However, there are still many questions that remain to be intensively investigated. Although autophagy activators and inhibitors have been widely used to monitor autophagy (Figure 1), the exact therapeutic doses of these autophagic regulators need to be optimized according to individual experimental conditions such as experimental purpose, purpose, and specific experimental needs.

Figure 1. Pharmacological targeting of control points in the autophagic pathway. EBSS, Earle’s Balanced Salt Solution; ER, endoplasmic reticulum; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; SMER, small molecule enhancers of rapamycin; IMPase, inositol monophosphatase; IP3R, inositol-1,4,5-triphosphate receptor; HDAC6, histone deacetylase 6; 3-MA, 3-methyladenine; ATG, autophagy-specific gene.
cell type, culture characteristics, etc.

Discovering potential drug therapies that can be used to modulate autophagy is a major challenge that is likely to provide a huge therapeutic potential. Autophagy-targeted drugs should be selected based on the type and stage of the various diseases. Gene-targeting approaches may provide a novel therapeutic option for human diseases and deserve further exploration. Given the side effects caused by high drug dosage, the use of a combination therapy of autophagic regulators, rather than treatment with a single medicine, is strongly recommended whenever possible.

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