Autophagy: A Druggable Process

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Abstract

Macroautophagy (hereafter called autophagy) is a vacuolar, lysosomal pathway for catabolism of intracellular material that is conserved among eukaryotic cells. Autophagy plays a crucial role in tissue homeostasis, adaptation to stress situations, immune responses, and the regulation of the inflammatory response. Blockade or uncontrolled activation of autophagy is associated with cancer, diabetes, obesity, cardiovascular disease, neurodegenerative disease, autoimmune disease, infection, and chronic inflammatory disease. During the past decade, researchers have made major progress in understanding the three levels of regulation of autophagy in mammalian cells: signaling, autophagosome formation, and autophagosome maturation and lysosomal degradation. As we discuss in this review, each of these levels is potentially druggable, and, depending on the indication, may be able to stimulate or inhibit autophagy. We also summarize the different modulators of autophagy and their potential and limitations in the treatment of life-threatening diseases.

INTRODUCTION

Autophagy is a stress-responsive catabolic process by which lysosomal enzymes degrade intracellular components (1). It is now widely accepted that autophagy plays crucial roles in cellular and tissue homeostasis as well as metabolism, development, and, through pathogen clearance, immunity. Several degradation pathways feed into lysosomes (**Figure 1**): microautophagy, by which cytosolic material is degraded after direct internalization into the lysosome; chaperone-mediated autophagy, through which a subset of cytosolic proteins are degraded selectively; and macroautophagy (hereafter termed simply autophagy), which handles cytoplasmic material isolation and degradation (2).

Autophagy is induced by many different physiological stimuli or pathophysiological situations. It also plays a role in the quality control of the cytoplasm, as it removes protein aggregates, damaged organelles (e.g., defective mitochondria), and intracellular pathogens (bacteria and viruses). Autophagy participates in regulation of levels and recycling of intracellular components, such as amino acids, lipids (lipid droplets), and carbohydrates, and thereby contributes directly to cell metabolism and energy regulation (3). Finally, autophagy protects against cell modifications related to aging (4). Thus, this complex membranous pathway, regulated by many factors, is involved in cellular strategies to protect organisms from cancers; infectious diseases; and metabolic, muscle, inflammatory, and neurodegenerative disorders. Conversely, complete or partial dysregulation of the autophagic pathway can exacerbate or underlie these pathologies. Here we summarize our current understanding of the molecular pathway of autophagy and provide an overview of pharmacological and chemical regulators of various steps in the pathway.

AUTOPHAGY: A ONE-WAY TICKET TO THE LYSOSOME

Signaling, Biogenesis, and Maturation of the Autophagosome

Autophagy is initiated by the formation of a special, double-membrane organelle called the autophagosome. The biogenesis of the autophagosome is orchestrated by multiple signaling pathways and dynamic membrane complexes containing autophagy-related (ATG) proteins that induce the biogenesis of a preautophagosomal structure, termed the phagophore, from the omegasome, a site on the endoplasmic reticulum (ER) positive for phosphatidylinositol 3-phosphate (PI3P) and the PI3P-binding protein double FYVE-domain-containing protein 1 (5). The phagophore elongates and then closes up to form a mature autophagosome that will later fuse with lysosome (**Figure 1**).

Initiation of autophagosome biogenesis is multifactorial (**Figure 1**); it can be induced by energy status, nutrient amounts, and various stresses. The mediators frequently include signaling kinases from the mammalian target of rapamycin (mTOR) pathway, notably mTOR complex 1 (mTORC1), which is inhibited by nutrient starvation as well as by rapamycin (6). mTORC1 is indeed at a crossroads of stress and energy sensing and autophagy regulation and is responsible for integration of signaling from the class I phosphatidylinositol 3-kinase (class I PI3K) pathway and AMP-activated protein kinase (AMPK). mTORC1 blocks the autophagic pathway by phosphorylation of UNC51-like kinase 1 (ULK1, called ATG1 in yeast) or ULK2 in the ULK complex, which also contains ATG13, FIP200, and ATG101 (7).

Inhibition of mTORC1 leads to activation of the ULK complex, which in turn activates the class III PI3K or Vps34 complex, inducing synthesis of a specific pool of PI3P (8) that is necessary for autophagosome biogenesis (**Figure 1**). The complex formed by association of Vps34 (bearing kinase activity), Vps15 (or p150), Beclin1, ATG14, and AMBRA1 is recruited by the ER-resident protein VMP1 (9). Subsequent steps in autophagy require PI3P-binding proteins, such as members of the WIPI family (10). For example, WIPI2 participates in the recruitment of ATG16L1 and

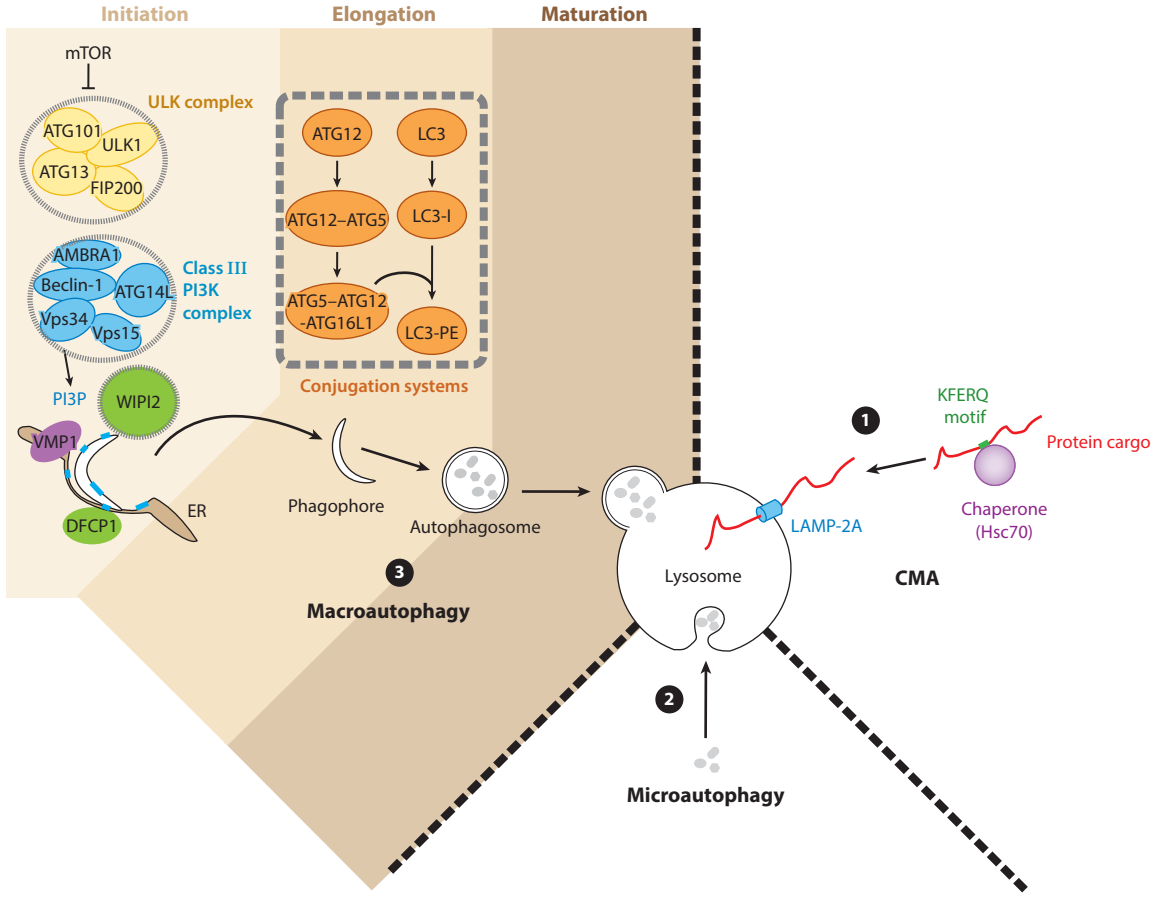

Figure 1

The autophagic pathway in mammalian cells. Three autophagic pathways target cargoes for lysosomal degradation in mammalian cells: ❶ CMA translocates substrates to the lysosome through interaction with LAMP-2A, a protein located on the lysosome surface. CMA requires a targeting motif (KFERQ) on the substrate; this motif is recognized by the chaperone Hsc70. ❷ Microautophagy sequesters cargoes by direct invagination into the lysosome. ❸ Macroautophagy involves the formation of double-membrane organelles called autophagosomes in which cargoes are sequestered (initiation and elongation steps). Autophagosomes fuse with lysosomes, where the cargo is degraded (maturation step). There are three important stages in the macroautophagy pathway: the initiation of autophagosome formation (involving formation of the phagophore), the elongation/closure step (which results in formation of a sealed autophagosome), and autophagosome maturation (which includes the fusion with the lysosome). The different modules that constitute the ATG protein core machinery are delimited by the dotted lines. Energy depletion and amino acid and serum starvation activate the ULK complex via different signaling pathways, including the mTOR pathway. The phagophore emanates from a domain of the ER called the omegasome. The omegasome is characterized by the presence of the PI3P-binding protein DFCP1. PI3P is represented by blue lines. Also shown is the ER-located transmembrane protein VMP1, which interacts with the class III PI3K complex. Stimulation of the ULK complex activates this complex, which consists of the core structure (Beclin1, Vps15, and Vps34) and two regulators (ATG14L and AMBRA1). This induction allows the production of PI3P to promote the recruitment of the WIPI proteins upstream of the two ubiquitin-like conjugation systems that elongate and seal the autophagosomal membrane. The first conjugation system results in the formation of the ATG12–ATG5-ATG16L1 complex that acts as an E3-like enzyme in the second conjugation system, which generates the lipidated PE-conjugated form of LC3 (LC3-II) downstream of the unconjugated form LC3-I. Abbreviations: AMBRA, activating molecule in Beclin 1-regulated autophagy; ATG, autophagy-related; CMA, chaperone-mediated autophagy; DFCP1, double FYVE-domain-containing protein 1; ER, endoplasmic reticulum; LC3, light chain 3; mTOR, mammalian target of rapamycin; PE, phosphatidylethanolamine; PI3P, phosphatidylinositol 3-phosphate; ULK, UNC51-like kinase 1; VMP1, vacuole membrane protein 1; Vps, vacuolar protein sorting; WIPI, WD repeat domain phosphoinositide-interacting protein.

consequently the ATG12–ATG5 conjugation system (**Figure 1**) to allow the targeting of the LC3 protein (the yeast ATG8 homolog) to the omegasome membranous structure by lipidation of the protein. This elongation step marks the transition from omegasome to phagophore organelle (**Figure 1**). The phagophore is a transient, double-membrane, cup-shaped structure; its elongation and closure results in the autophagosome. Importantly, other membrane sources, such as the Golgi apparatus, the plasma membrane, and endosomes also may participate, directly or partially, in autophagosome biogenesis from phagophore generation to growth of the organelle (8).

The last step of autophagy is the maturation of the autophagosome (**Figure 1**). Once an autophagosome has formed, it fuses with a lysosome to form an autolysosome in which engulfed cytosolic components are digested by acidic hydrolases and recycled. The autophagosome can fuse with endosomal compartments to generate an intermediate vesicle called an amphisome, which can fuse with a lysosome (11). Regulators of the fusion of autophagosomes with lysosomes include Ras-related proteins in brain small GTPases (such as Rab7 and Rab11), soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARES, such as syntaxin-17, SNAP-29, and VAMP8), homotypic fusion and vacuole protein sorting (HOPS) complex components (such as Vps16, Vps33A, and Vps39), endosomal sorting complexes required for transport (ESCRT) components (such as SKD1, CHMP2B, and CHMP4B), and FYVE and coiled-coil domain-containing protein (FYCO1) (12). The multivalent adaptor protein PLEKHM1, which interacts with Rab7, LC3, and the HOPS complex, is a central hub that influences both the progression of autophagy and endocytosis (13). Despite this growing list of factors involved in autophagic maturation, their individual roles and how they act sequentially are still not fully documented.

Selective Autophagy

Originally, autophagy was considered to be a nonselective degradation process. It has become evident, however, that autophagy can be highly selective. Selective autophagic responses are named according to the type of cellular material targeted: aggregated proteins (aggrephagy), mitochondria (mitophagy), peroxisomes (pexophagy), lipid droplets (lipophagy), ribosomes (ribophagy), ER (reticulophagy), pathogens (xenophagy), glycogen (glycophagy), zymogen (zymophagy), nucleus (nucleophagy), chromatin (chromatophagy), myelin (myelinophagy), ferritin (ferritinophagy), lysosomes (lysophagy), stress granules (granulophagy), and proteasome (proteaphagy). Despite the growing lists of substrates that are processed selectively by the autophagy machinery, the exact mechanisms of substrate recognition by the autophagic pathway are still under investigation.

Clearly, two different cellular pathways—ubiquitin-independent and ubiquitin-dependent—exist to recognize signals on cargoes to deliver them into the autophagosome. The ubiquitin-independent pathway involves receptors (such as NIX for mitophagy and FAM134B for reticulophagy) that bind directly to certain substrates and then interact with autophagosomes via LC3-interacting regions (LIRs). The recognition of ubiquitinated intracellular cargo is dependent on sensing of ubiquitin by a receptor with a ubiquitin-binding domain and an LIR motif. Examples of such receptors are p62 (for aggrephagy), optineurin, and NDP52 (for mitophagy). For more details, see previous reviews (14, 15).

POTENTIAL DRUG TARGETS IN THE AUTOPHAGIC PATHWAY

Rationale for Targeting Autophagy

Dysfunction in autophagy underlies many human diseases. This is to be expected, given the fundamental role of autophagy in protein and organelle quality control, metabolic homeostasis, and stress response (16, 17). Autophagic failures that occur in systemic disorders and organ-specific

Table 1 Examples of human diseases associated with defective autophagy[a]

	Primary autophagy defects	Secondary autophagy defects	Both
Organ-specific	**Lung** Asthma (*ATG5*) **Heart** Ischemia/reperfusion **Intestine** Crohn's disease (*ATG16L1*, *NOD2*, *IRGM*) Ulcerative colitis (*SMURF1*) **Bone** Paget's disease of bone (*SQSTM1*) **Central nervous system** Static encephalopathy of childhood with neurodegeneration in adulthood (SENDA) (*WDR45*)	**Central nervous system** Huntington's disease Tauopathies Stroke **Heart** Cardiomyopathy **Skeletal muscle** Muscle atrophy Autophagic vacuolar myopathies Collagen VI–related myopathies Inclusion body myositis **Liver** α1-Antitrypsin deficiency Nonalcoholic fatty liver disease (NAFLD)	**Central nervous system** Alzheimer's disease (*PSEN1*) Parkinson's disease (*PINK1*, *PARK2*) Amyotrophic lateral sclerosis (*SQSTM1*)
Multisystemic	**Cancer** Breast (*BECN1*) Ovarian (*BECN1*) Prostate (*BECN1*) Brain (*PARK2*) Lung (*PARK2*) Gastric (*UVRAG*, *PARK2*) **Immune disease** Vici syndrome (*EPG5*) *Mycobacterium tuberculosis* infection (*IRGM*) *Mycobacterium leprae* infection (*NOD2*) **Lysosomal disease** Danon disease (*LAMP2*)	**Cancer** Carcinoma Sarcoma **Immune disease** Infection **Metabolic dysfunction** Type II diabetes Metabolic syndrome Obesity **Vascular disease** Ischemia/reperfusion **Lysosomal disease** Pompe disease Gaucher disease	**Immune disease** Lupus erythematosus (*ATG5*)

[a]Primary and secondary autophagy alterations linked to organ-specific or multisystemic human diseases. Genes with mutations, polymorphisms, and haplo-insufficiencies that affect molecular components of the autophagic pathways are indicated in red, brown, and blue, respectively. The proteins encoded by *ATG5* (autophagy protein 5), *ATG16L1* (autophagy-related 16 like 1), *BECN1* (Beclin 1), and *WDR45* (WD repeat domain 45) participate in autophagosome formation, whereas those encoded by *UVRAG* (UV radiation resistance associated), *EPG5* (ectopic P-granules autophagy protein 5 homolog), *PSEN1* (presenilin 1), and *LAMP2* (lysosomal-associated membrane protein 2) participate in autophagosome maturation and degradation in bulk autophagy. LAMP2 (isoform a) also contributes to chaperone-mediated autophagy. *SMURF1* (SMAD specific E3 ubiquitin protein ligase 1), *SQSTM1* (sequestosome 1), *PARK2* (Parkin RBR E3 ubiquitin protein ligase), and *PINK1* (PTEN induced putative kinase 1) encode proteins involved in selective autophagy. *IRGM* (immunity-related GTPase family, M) and *NOD2* (nucleotide binding oligomerization domain containing 2) encode autophagy regulators.

pathologies are summarized in **Table 1**. Different stages in the process, including autophagy signaling, autophagosome formation, and autophagosome maturation, contain targets for drug development.

Signaling and Autophagosome Formation

In the life span of autophagosome formation, the first complex that can be targeted by drugs is the signaling complex that interacts directly with the mTOR pathway. mTORC1 is targeted by

rapamycin, a natural product, and rapamycin analogs such as AP23573, CCI-779, and RAD001 (18). Torin-1 and PP242, which act via ATP competition, inhibit mTORC1 and mTORC2 (19). The AKT/class I PI3K pathway also regulates autophagy initiation (20). Specific targeting of this pathway is of interest because inhibition of mTORC1 or mTORC2 could be compensated for by AKT stimulation. One drug candidate, PI-103, inhibits class I PI3K directly and thus induces autophagy robustly (21). Finally, metformin, used classically as an antidiabetic molecule, inhibits the kinase activity of AMPK, which is central to autophagy because it regulates both the mTOR response and ULK1 phosphorylation, acting thus as a positive regulator of autophagy (22).

The mTORC1 pathway negatively controls the activity of the ULK complex, which promotes Beclin1 activation and, ultimately, initiation of autophagosome formation. Thus, ULK1 kinase is an attractive candidate for drug screening, and numerous promising drug candidates, including compound 6 (23), MRT67307 (24), and MRT68921 (25), inhibit the catalytic activity of ULK1 (and potentially ULK2). Further investigations are necessary to confirm that autophagy initiation is inhibited specifically.

The class III PI3K complex is responsible for the formation of the PI3P pool, which is essential for autophagosome biogenesis (see above and Reference 26). Formation of this complex is blocked efficiently by well-characterized drugs such as wortmannin and 3-methyladenine (3-MA) (27), which block autophagy by inhibiting PI3P synthesis. These drugs also alter endosomal functions because Vps34 is important during either multivesicular body formation or retrograde transport, two trafficking pathways that require local PI3P synthesis (28). In addition, depending on the concentration, wortmannin and 3-MA also target class I and class II PI3K (29).

Researchers have identified other compounds that block class III PI3K complex activity by specific inhibition of the Vps34 complex. These include Spautin-1 (which acts indirectly by inhibition of Beclin1 activity), LY294002 (a Pan-PI3K inhibitor that has effects similar to those of 3-MA), SAR405, Vps34-IN1, PIK-III, and compound 31 (Vps34 inhibitors) (all reviewed in 27). Most of these compounds have been shown to or are suspected to negatively modulate autophagy. Finally, another indirect regulator of class III PI3K complex activity is a cell-permeable Tat-Beclin1—composed of the HIV-1 Tat protein transduction domain and of 18 amino acids of Beclin1—that is a potent autophagy inducer (30).

Posttranslational Modifiers of Autophagic Proteins

Posttranslational modifications influence the structures of proteins, their stabilities, and their biological functions. These modifications may influence localization and interactions with other proteins or with ligands. Most of the core autophagy proteins are posttranslationally modified by phosphorylation or β-linked *N*-acetylglucosamine on Ser or Thr residues or by acetylation or ubiquitination on Lys residues (31, 32). Some inhibitors of these posttranslational modification processes have been identified that affect autophagy in vitro, but they are poorly selective, limiting their potential use in clinical applications.

Phosphorylation regulates both bulk and selective autophagy pathways. Inhibitors of tyrosine kinases act by increasing levels and the stability of parkin (a ubiquitin E3 ligase) and by impeding its interaction with Beclin1. This facilitates the formation of ubiquitinated targets and promotes the clearance of aggregates in animal models of Alzheimer's, Huntington's, and Parkinson's diseases (33). Among these inhibitors, nilotinib (Tasigna), which was approved by the US Food and Drug Administration (FDA) for use in treatment of myeloid and lymphoblastic leukemias in 2007, has potential in the treatment of neurodegenerative diseases (34, 35).

Another posttranslational modification involves the addition of an acetyl group to lysine; the acetyl group is transferred from acetyl CoA to the lysine by K(lys) acetyl transferase or N-terminal

acetyl transferase. Acetylation regulates autophagy both indirectly and directly. Indirectly, modification of nuclear proteins such as histones and cytoplasmic and nuclear pools of ATG or transcriptional factors such as forkhead box O (FOXO) influence the expression of ATG genes (36, 37). Activities of autophagic regulators or executors themselves are also influenced by acetylation. Changes in the acetylation status induced by spermidine (a competitor of p300 acetyltransferase) and resveratrol (sirtuin deacetylase activator) treatments have been demonstrated to activate autophagy (38). Other pharmaceutical agents that influence acetyl-CoA metabolism such as sirtuin1 activators or other histone deacetylase (HDAC) inhibitors (39) also have potential for use in the treatment of diseases related to autophagy.

Ubiquitination is essential for bulk autophagy and for cargo recognition in many forms of selective autophagy (14, 15). Ubiquitination depends on three types of enzymes (ubiquitination-activating E1, ubiquitin-conjugating E2, and ubiquitin-ligating E3). Ubiquitin ligases are responsible for the substrate specificity of the selective pathway. The isopeptide bond between ubiquitin and its targets can be cleaved by deubiquitinating enzymes (DUBs).

ATG proteins such as ULK1, Beclin1, and class III PI3K are targets of ubiquitination. TRAF6 mediates the ubiquitination of ULK1; ubiquitination stabilizes ULK1, promoting its activity during autophagy induction. The complex of TRAF6 and AMBRA1 also induces a posttranslational modification of Beclin1 to favor its association with the class III PI3K complex to trigger autophagy. Beclin1 is also a substrate for the E3 ubiquitin ligase NEDD4; ubiquitination of Beclin1 promotes its degradation through the proteasome pathway. Deubiquitination of Beclin1 by the DUBs ubiquitin-specific peptidases 10 and 13 triggers autophagy. Spautin-1, a small-molecule inhibitor of these DUBs, is presently in preclinical evaluation, and development of improved Spautin-1 analogs with good safety and in vivo stability profiles may be clinically useful (35).

Enhancing autophagy could have positive effects in patients with neurodegenerative proteinopathies such as Huntington's and Parkinson's diseases and in patients with certain infectious diseases that result in xenophagy substrates. Targeting the receptors that mediate selective autophagy would allow modulation of autophagy à la carte without interfering with the bulk process (40).

Transcription Factors

Emerging studies suggest that transcriptional regulation modulates and adapts the autophagic process to different stress conditions. The transcriptional network controlling autophagy is complex and involves numerous transcription factors, most of which are under the control of mTOR. A list of transcription factors known to be involved in the control of autophagy has been published recently (41). Below, we summarize briefly the major transcriptional contributors to autophagy regulation for which efforts are under way to develop transcription-modulating strategies.

Forkhead box O proteins. The evolutionarily conserved FOXO family proteins are major regulators of cell proliferation, survival, and stress responses. FOXOs activate protein catabolism by inducing two major protein degradation systems: the ubiquitin/proteasome pathway and the autophagic/lysosomal pathway (42). FOXO1 and FOXO3 function as essential activators of autophagy in response to stress, such as nutrient deprivation (43, 44), by regulating the expression of genes encoding ATG proteins and by modulating intracellular glutamine levels (45). Autophagy-promoting nontranscriptional cytoplasmic functions have been described for FOXO proteins as well (46, 47).

Researchers have investigated FOXO function in various disease models. Clinical studies suggest that FOXO proteins play significant roles in diabetes mellitus, oxidative stress, immune system

function, and cancer (48). Patients with some diseases, such as diabetes or diabetic complications, would benefit from inhibition of FOXO activity, but in situations such as wound healing, cardiovascular disease, oxidative stress associated with aging, ovarian follicle depletion, and cancer, FOXOs agonists may be beneficial.

Transcription factor EB and Zinc-finger protein with KRAB and SCAN domain 3. The lysosome is a sensor of cellular metabolic cues that can influence whole-body metabolism. A major player in this pathway is the transcription factor EB (TFEB) (49, 50), a master regulator of lysosomal biogenesis and autophagy that is induced by starvation and links the autophagic pathway to cellular energy metabolism by controlling lipid catabolism via Ppargc1α and Ppar1α (51). Thus, TFEB represents an exceptional tool to induce lysosomal function and autophagy globally in vitro and in vivo, and indeed its overexpression has already been shown to reduce the amount of accumulated substrates successfully in a rapidly growing number of mouse disease models. These include lysosomal storage disorders; α1-antitrypsin deficiency; spinal bulbar muscular atrophy; and neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's diseases (52). Moreover, TFEB overexpression in the liver prevents weight gain and metabolic syndrome in both diet-induced and genetic mouse models of obesity (51). In addition, TFEB and TFE3, a transcription factor highly homologous to TFEB that promotes cellular clearance by regulating similar genes (53), have been shown recently to regulate the integrated stress response (ISR) by activating activating transcription factor 4 (ATF4) (54).

Conversely, another transcription factor, ZKSCAN3 (a zinc-finger protein with KRAB and SCAN domains) has been identified recently as the master transcriptional repressor of autophagy and autophagosome biogenesis (55). Its functions are essentially opposite those of TFEB, as it represses expression of many of the same genes that TFEB activates. This finding could have broad implications for the development of therapeutic strategies to positively modulate autophagy.

Activating transcription factor 4 and C/EBP homologous protein. ATF4 is a prosurvival factor that regulates the ISR gene expression program involved in amino acid metabolism, differentiation, metastasis, angiogenesis, resistance to oxidative stress (56), and drug resistance (57). ATF4 contributes to the fine-tuning of autophagy (58). Under prolonged stress conditions, ATF4 leads to stress-induced apoptosis by promoting expression of C/EBP homologous protein (CHOP), a transcription factor that inhibits expression of Bcl-2 and promotes expression of GADD34 (59). Inhibition of ATF4 may enhance the efficacy of current chemotherapeutic agents without causing toxicity, and therefore it represents an attractive therapeutic target in cancer drug development.

AUTOPHAGOSOME MATURATION AND LYSOSOMAL DEGRADATION

Microtubules are critical at different steps of autophagy, from autophagosome formation to autophagosome transport (60). The destabilization of microtubules by vinblastine or nocodazole blocks the maturation of autophagosomes (61, 62). In contrast, Taxol and paclitaxel stabilize microtubules and increase the efficiency of fusion between autophagosomes and lysosomes (63). Whether the antitumor action of these agents is at least partially attributable to their effectiveness as modulators of autophagy remains to be determined (60).

HDAC6, a microtubule-associated deacetylase, plays a central role in the targeting and retrograde transport of aggregate-containing inclusion bodies to the lysosomal compartment (64). HDAC6 is also required for autophagosome maturation, as it controls the assembly of the filamentous actin network that stimulates the fusion of autophagosomes with lysosomes (65). Inhibitors of HDAC6 and other HDACs also block autophagy through the inhibition of the deacetylation of autophagy-associated proteins, as described above.

LYSOSOME-TARGETING AGENTS

Lysosomes are the final destinations of macromolecules targeted for degradation through endocytosis, phagocytosis, and autophagy. Lysosome dysfunction underlies lysosomal storage disorders, neurodegenerative disorders, cancer, and cardiovascular diseases. Thus, the lysosome is a validated target of therapeutic intervention.

Lysosomotropic agents accumulate inside the lysosome because of their protonation and result in an increase in lysosomal pH. Among these compounds, the antimalarials chloroquine (CQ) and hydroxychloroquine (HCQ) also have potential in cancer therapy, particularly in cases of chemotherapy resistance (66). In fact, CQ and HCQ have synergistic effects with certain anticancer drugs used to treat breast, melanoma, lung, multiple myeloma, glioma, kidney, colorectal, and advanced/refractory solid tumors without resulting in additional side effects (67). Although the antitumor effect of CQ is primarily due to inhibition of autophagy, it affects the tumor endothelial architecture to promote vessel normalization, leading to the reduction of hypoxia in the tumor environment, the reduction of invasive potential, and an increase in the drug delivery response (68). CQ also targets pancreatic cancer stem cells via inhibition of CXCR4 and hedgehog signaling (69).

Pellegrini et al. (70) showed that CQ does not enter cancer cells efficiently because of protonation in the acidic extracellular milieu that characterizes the microenvironment of many tumors. Researchers have developed novel derivatives of CQ or CQ-based hybrid molecules in attempts to improve anticancer activity. For example, Lys-05 accumulates within and deacidifies the lysosome with 10-fold higher efficacy than CQ, resulting in impaired autophagy and reduced tumor growth in nude mice xenographed with HT-29 colon cancer cells (71). Quinacrine, another antimalarial drug, is more active than CQ, and its analogs have been described recently as potent lysosome-targeted autophagy inhibitors (72) that inhibit autophagy in colon cancer cells (73).

Bafilomycin-A1 and concanamycin A are another type of autolysosomal formation inhibitor. These agents block vacuolar-type H^+-ATPases (V-ATPases), which are proton pumps responsible for creating the acidic environment of lysosomes (20, 74, 75). Recently, the ER-calcium ATPase Ca-P60A/SERCA was identified as a novel target of bafilomycin A1. Thus, independently of its action on V-ATPase-dependent acidification, bafilomycin A1 also disrupts autophagic flux by inhibiting Ca-P60A/SERCA-dependent autophagosome-lysosome fusion (76).

Among the lysosomal hydrolases, the best known are the cathepsin family of proteases. Inhibitors of lysosomal cathepsins include pepstatin A, E64d, leupeptin, and cystatin B (77). Interestingly, in a transgenic mouse model of Alzheimer's disease, systemic administration of the small-molecule Z-Phe-Ala-diazomethylketone, used at a low concentration, enhances the expression and activity of cathepsin B, resulting in clearance of intracellular amyloid-β and reduced extracellular deposits (78). These data support the idea that the positive modulation of the lysosomal system by small molecules will facilitate removal of toxic deposits to limit, or possibly reverse, the progression of neurodegenerative diseases (79).

Proton pump inhibitors such as omeprazole modulate autophagy and lysosomal transport pathways, leading to cell death in pancreatic cancer cells (80). Thapsigargin A, an ER stressor, causes accumulation of mature autophagosomes by blocking fusion with lysosomes, resulting in the inhibition of autophagosomal recruitment of the small GTPase Rab7 (81).

PHARMACOLOGICAL MODULATORS OF AUTOPHAGY IN CLINICAL TRIALS

Here we provide a brief discussion of pharmacological modulators of autophagy that are being evaluated in clinical trials (**Tables 2** and **3**). Readers interested in a more detailed discussion of

Table 2 Autophagy inducers currently in clinical testing

Class	Therapeutic regimen	Disease	Target	http://clinicaltrials.gov identifier	Clinical trial phase
mTOR inhibitors	Sirolimus (rapamycin)	Angiofibromas tuberous sclerosis	mTORC1	NCT01526356	II
		Autosomal dominant polycystic kidney disease		NCT00920309	II/III
	Rapamycin alone or in combination with mycophenolate or prednisone	Kidney transplantation		NCT01014234	II
	Sirolimus plus trastuzumab	Breast cancer		NCT00411788	II
	Sirolimus plus pemetrexed	Non-small-cell lung cancer		NCT00923273	I/II
	Everolimus (RAD001)	Chronic myeloid leukemia		NCT01188889	I/II
		Advanced gastrointestinal neuroendocrine tumors		NCT01648465	II
		Prostate cancer		NCT00657982	II
		Renal cell cancer		NCT00830895	II
		Small-cell lung cancer		NCT00374140	II
	Everolimus plus alemtuzumab	Kidney transplant failure and rejection		NCT00166712	IV
		Chronic lymphocytic leukemia		NCT00935792	I/II
	Everolimus alone or in combination with irinotecan and cetuximab	Colorectal cancer		NCT00522665	I/II
	Everolimus plus OSI-906			NCT01154335	I/II
	Everolimus alone or in combination with temozolomide and radiation	Glioblastoma		NCT01062399	I/II
	Everolimus in combination with paclitaxel and carboplatin	Melanoma		NCT01014351	II
	Everolimus plus panobinostat	Multiple myeloma		NCT00918333	I/II
	Everolimus plus sorafenib	Multiple myeloma, lymphoma		NCT00474929	I/II
	Everolimus plus bevacizumab	Ovarian, peritoneal, and fallopian tube cancer		NCT01031381	II

(*Continued*)

Table 2 (*Continued*)

Class	Therapeutic regimen	Disease	Target	http://clinicaltrials.gov identifier	Clinical trial phase
	Everolimus plus docetaxel	Metastatic head and neck cancer		NCT01313390	I/II
	Everolimus plus pasireotide	Prostate cancer		NCT01313559	II
	Everolimus in combination with docetaxel and bevacizumab			NCT00574769	I/II
	Everolimus plus paclitaxel	Advanced prostate cancer		NCT00574769	I/II
	Temsirolimus	Relapsed follicular or mantle cell lymphoma		NCT01078142	I
	AZD8055	Advanced solid malignancies	ATP-competitive inhibitor of mTOR kinase activity	NCT00973076	I/II
		Liver cancer		NCT00999882	I
		Recurrent gliomas		NCT01316809	I
PI3K inhibitors	NVP BEZ235	Advanced breast tumor	Dual ATP-competitive PI3K class I and mTOR inhibitor	NCT00620594	I/II
		Pancreatic neuroendocrine cancers		NCT01628913	II
		Renal cell cancer		NCT01453595	I/II
	PF-04691502	Breast cancer		NCT01430585	II
	CAL-101	Chronic lymphocytic leukemia	PI3K inhibitor	NCT01539512	III
	LY294002	Neuroblastoma		NCT02337309	I
	GDC-0941	Glioblastoma/ gliosarcoma	Inhibitor of PI3K α/δ	NCT02430363	I/II
	GDC-0068		Pan-AKT inhibitor of AKT1/2/3		
	MK-2206	Advanced biliary cancers	Selective inhibitor of AKT1/2/3	NCT01859182	II
		Advanced liver cancer		NCT01239355	
		Melanoma		NCT01519427	III/IV
		Colorectal cancer		NCT01186705	II
	Perifosine	Waldenström's macroglobulinemia		NCT00422656	II
		Chronic lymphocytic leukemia, small lymphocytic lymphoma		NCT00873457	II

(*Continued*)

Table 2 (*Continued*)

Class		Therapeutic regimen	Disease	Target	http://clinicaltrials.gov identifier	Clinical trial phase
		Triciribine	Breast cancer		NCT01697293	II
			Advanced hematological malignancies		NCT00642031	I
AMPK		Metformin	Diabetes	AMPK activator	Approved by the FDA	
			Prostate cancer		NCT01433913	II
			Esophageal cancer in patients with Barrett's esophagus		NCT01447927	II
		Metformin plus 5-fluorouracil	Advanced colorectal cancer		NCT01941953	II
		Metformin plus chemotherapy	Pancreatic cancer		NCT01210911	II
		Resveratrol	Friedreich's ataxia	Activation of AMPK → NAD^+ → SIRT pathway via PDE1, PDE3, and PDE4 inhibition	NCT01339884	I/II
			Metabolic syndrome X		NCT02114892	II
			Cardiovascular disease		NCT01449110	II
			Small-cell lung cancer		NCT01079481	II
		Cannabinoids	Cancer-related side effects		Approved by the FDA	
			Primary gliomas		NCT00314808	I/II
		Enzalutamide	Prostate cancer	Activation of AMPK	NCT01547299	III
mTOR-independent inhibitors	IMPase	Lithium	Bipolar disorder	Depletion of free inositol, reduction of IP3 levels, and increase in levels of Beclin1	NCT00194129	III
			Depression		NCT01880593	II
			Acute monoblastic leukemia		NCT01820624	I
			Prostate cancer		NCT02198859	I
		Carbamazepine	Bipolar disorder	Reduces intracellular inositol levels	NCT02623504	IV
			α1-antitrypsin deficiency, liver cirrhosis		NCT01379469	II

(*Continued*)

Table 2 (*Continued*)

Class		Therapeutic regimen	Disease	Target	http://clinicaltrials.gov identifier	Clinical trial phase
	Imidazoline receptor 1 agonist	Rilmenidine	Chronic kidney disease, hypertension		NCT00892892	IV
			Resistant hypertension and atherosclerotic renal artery stenosis		NCT02539810	IV
			Huntington's disease			I
Ca^{2+} channel inhibitors		Fluspirilene	Psychotic disorders	Ca^{2+} channel antagonist	NCT02374567	III
		Verapamil	Kidney disease in diabetic patients		NCT00235014	III
			Atrial fibrillation		NCT00589303	III
			Hypertension, coronary artery disease		NCT00133692	IV
		Loperamide	Fecal incontinence		NCT00727649	III
			Diarrhea, *Clostridium difficile* infection		NCT01570634	II
		Amiodarone	Atrial fibrillation		NCT00127712	IV
		Nicardipine	Aortic aneurysm, thoracic surgery		NCT00508118	II
			Cerebral vasospasm		NCT01810302	II
			Ischemic stroke, high blood pressure		NCT01422616	III
			Brain tumors		NCT01951950	I
		Nimodipine	Dementia		NCT00814658	IV
		Nitrendipine	Isolated systolic hypertension		NCT02088450	II

Abbreviations: AKT, Ak murine thymoma; AMPK, AMP-activated protein kinase; FDA, US Food and Drug Administration; IMPase, inositol monophosphatase; IP3, inositol 3-phosphate; mTOR, mammalian target of rapamycin; NAD, nicotinamide adenine dinucleotide; PDE, phosphodiesterase; PI3K, phosphatidylinositol 3-kinase; SIRT, sirtuin.

how compounds that modulate autophagy are used in research and their potential applications in new treatments for human diseases should see several recent reviews (27, 34, 35, 82).

Because we are still in the early phase of development of small-molecule modulators of autophagy, no small molecules are in clinical testing that target the machinery of autophagy directly; most of the described compounds affect the regulation of autophagy.

Pharmacological Inducers of Autophagy

In this section, we discuss compounds that stimulate autophagy and that are in clinical trials (**Table 2**).

Table 3 Autophagy inhibitors currently in clinical testing

Class		Therapeutic regimen	Disease	Target	Identifier	Phase
Blocking the recruitment of cargoes		Verteporfin alone or in combination with photodynamic therapy	Macular degeneration, choroidal neo-vascularization	Interferes with LC3-interacting region motifs	NCT00436553	III
			Basal cell carcinoma, nevoid basal cell carcinoma syndrome, Gorlin syndrome		NCT00049959	III
Autophagosome maturation	Inhibitor of autophago-some and lysosome fusion	CQ or HCQ	Malaria, rheumatoid arthritis, lupus erythematosus	Prevents endosomal acidification	Approved by the FDA	
		CQ	HCV		NCT02058173	IV
			Glioblastoma		NCT00224978	III
			Breast cancer		NCT01023477	I/II
		CQ alone or in combination with methotrexate and sulfasalazine	Early aggressive rheumatoid arthritis		NCT00259610	IV
		HCQ	Alopecia areata	Prevents endosomal acidification	NCT00176982	IV
			Cutaneous and systemic lupus erythematosus		NCT01551069	III
			Hashimoto's thyroiditis		NCT01760421	II
			Oral lichen planus		NCT00102557	II
			Breast cancer		NCT01292408	II
			Chronic lymphocytic leukemia		NCT00771056	II
			Pancreatic cancer		NCT01273805	II
			Prostate cancer		NCT00726596	II
			Small-cell lung cancer		NCT00969306	I/II
		HCQ plus erlotinib	Non-small-cell lung cancer		NCT01026844	I

(*Continued*)

Table 3 (*Continued*)

Class		Therapeutic regimen	Disease	Target	Identifier	Phase
		HCQ in combination with AKT inhibitor MK2206	Advanced solid tumor		NCT01480154	I
		HCQ in combination with RTK inhibitor sunitinib			NCT00813423	I
		HCQ in combination with alkylating agent temozolomide			NCT00714181	I
		HCQ plus temsirolimus			NCT00909831	I
		HCQ in combination with sirolimus or vorinostat			NCT01266057	I
		HCQ in combination with microtubule stabilizer ixabepilone	Breast cancer		NCT00765765	I/II
		HCQ in combination with RTK inhibitor imatinib	Chronic myeloid leukemia		NCT01227135	II
		HCQ in combination with FOLFOX and angiogenesis inhibitor bevacizumab[a]	Colorectal cancer		NCT01206530	I/II
		HCQ with XELOX and bevacizumab[b]			NCT01006369	I/II
		HCQ in conjunction with radiation therapy and concurrent and adjuvant temozolomide	Glioblastoma		NCT00486603	I/II
		HCQ in combination with proteasome inhibitor bortezomib	Multiple myeloma		NCT00568880	I/II
		HCQ with cyclophosphamide, dexamethasone, and rapamycin			NCT01689987	I

(*Continued*)

Table 3 (*Continued*)

Class		Therapeutic regimen	Disease	Target	Identifier	Phase
		HCQ in combination with EGFR inhibitor erlotinib			NCT00568880	I/II
		HCQ plus bortezomib			NCT01689987	I
		HCQ in combination with EGFR inhibitor erlotinib	Non-small-cell lung cancer		NCT00977470	II
		HCQ in combination with EGFR inhibitor gefitinib			NCT00809237	I/II
		HCQ in combination with bevacizumab, carboplatin, and paclitaxel			NCT00933803	I/II
		HCQ in combination with nucleoside analog gemcitabine	Pancreatic cancer		NCT01128296	I/II
		HCQ in combination with docetaxel	Prostate cancer		NCT00786682	II
	Targeting cytoskeletal components	Vinblastine	Melanoma, skin cancer	Disrupts microtubules	NCT00885534	II
			Prostate cancer		NCT00003084	II
			Metastatic Ewing's sarcoma		NCT00061893	II
		Standard APO versus regimen including vinblastine[c]	Lymphoma		NCT00059839	III
Inhibition of the lysosomal degradation		Lucanthone	Schistosomiasis	Permeabilizes lysosomal membrane	Approved by the FDA	
		Lucanthone in combination with temozolomide and radiation	Glioblastoma multiforme	Inhibits topoisomerase II and interferes with DNA repair	NCT01587144	II

(*Continued*)

Table 3 (*Continued*)

Class	Therapeutic regimen	Disease	Target	Identifier	Phase
	Azithromycin	Gastroparesis	Prevents lysosomal acidification	NCT01323582	II
		Acne vulgaris		NCT00392223	III
		Vaginitis bacterial cervicitis		NCT01072136	III
		Respiratory tract infections		NCT00599053	II
	Azithromycin plus CQ	Falciparum malaria infection in pregnant women		NCT01103063	III
	Clomipramine	Obsessive-compulsive disorder	Inhibits autophagosome-lysosome autophagic flux	NCT00074815	III
		Premature ejaculation		NCT01439984	III

Abbreviations: CQ, chloroquine; EGFR, epidermal growth factor receptor; FDA, US Food and Drug Administration; HCQ, hydroxychloroquine; HCV, hepatitis C virus; RTK, receptor tyrosine kinase.
[a]FOLFOX is a chemotherapy regimen for treatment of colorectal cancer, made up of folinic acid (leucovorin), 5-fluorouracil, and oxaliplatin.
[b]XELOX consists of capecitabine plus oxaliplatin.
[c]Standard APO is doxorubicin, prednisone, and vincristine.

Inhibitors of mammalian target of rapamycin signaling activate autophagy. Rapamycin and its analogs are strong inducers of autophagy that may eventually find utility in the treatment of neurodegenerative disease (83). These drugs are in Phase I and II testing for efficacy in patients with several types of cancer. Caution must be taken in equating the activation of autophagy with suppression of cancer, because evidence suggests that autophagy activation can promote the survival of cancer cells. By promoting intracellular recycling, autophagy can confer resistance to stress and enhance cell survival under unfavorable conditions, which may promote metastasis. Thus, the effectiveness of rapamycin and other analogs as anticancer therapies may have to do with their inhibitory effects on protein translation and metabolism rather than their activation of autophagy.

Activators of adenosine monophosphate–activated protein kinase activate autophagy. AMPK is the main sensor of intracellular energy and is an important regulator of both mTOR and ULK1. One example of an AMPK activator with therapeutic potential in neurodegenerative disease is metformin (83). The biguanide metformin is an indirect AMPK activator that targets the complex I of electron transport in the mitochondria (84, 85). Metformin is a commonly used drug for treating type II diabetes that is also endowed with antineoplastic properties (86). Nilotinib is an antileukemia drug that is protective in multiple mouse models of neurodegeneration. It induces autophagy via AMPK activation (87).

Inhibitors of class I phosphatidylinositol 3-kinase signaling activate autophagy. Protein kinase AKT is a major downstream mediator of class I PI3K signaling. The inhibitors of AKT perifosine, triciribine, and GDC-0068 lead to autophagy activation. However, researchers have

raised concerns that AKT inhibition may increase susceptibility to cell death, rendering class I PI3K/mTORC1 inhibitors unsuitable as therapies for neurodegenerative disease. Moreover, treatment with inhibitors of receptor tyrosine kinases, which results in the inhibition of AKT, can activate autophagy, possibly by inhibiting mTORC1 or by regulating Beclin1. Dual class I PI3K/mTOR inhibitors such as NVP BEZ235 are potent mTOR-dependent autophagy inducers. Energetic metabolism inhibitors such as cannabinoids are also potent inducers of AMPK-dependent autophagy in pancreatic cancer cells (88, 89).

Although these small molecules affect the regulation of autophagy, caution should be taken in equating inhibition of regulatory mechanisms with direct inhibition of the machinery of autophagy. For this reason, we have not reported clinical trials currently under way to evaluate inhibitors of receptor tyrosine kinases or activators of epidermal growth factor tyrosine kinase.

Inhibitors of inositol monophosphatase activate autophagy. Autophagy can be induced by lowering intracellular inositol or myo-inositol-1,4,5-trisphosphate (IP3) levels independently of mTOR (90). Lithium and other mood-stabilizing agents used for the treatment of bipolar disorder, such as carbamazepine, enhance the clearance of autophagy substrates by inhibiting inositol monophosphatase, leading to depletion of free inositol and a reduction in the levels of IP3. The IP3 receptor, which is an IP3-activated calcium channel at the ER, interacts with Beclin1 indirectly via Bcl-2. Upon antagonist binding and a subsequent cellular reduction in IP3 levels, this interaction is disrupted (91). This mechanism may explain how drugs that cause a decrease in IP3 levels induce autophagy in an mTOR-independent manner (92).

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 for safety in patients with Huntington's disease (83). Any success of such clinical trials would encourage the development of additional autophagy modulators for the treatment of neurodegenerative diseases.

Inhibitors of Ca^{2+} channels activate autophagy. Autophagy can be induced by lowering intracytosolic Ca^{2+} levels. Several antagonists of L-type Ca^{2+} channels, including fluspirilene, verapamil, loperamide, and amiodarone, induce autophagy and promote the degradation of long-lived proteins and misfolded polyglutamine without causing cell death. In addition, fluspirilene, which is an antipsychotic drug, reduces intracellular Ca^{2+} and prevents the calpain-mediated cleavage of ATG5, which is activated by elevated intracellular Ca^{2+} (93).

Autophagosome biogenesis inducers. Gemcitabine is a nucleoside analog used as a cancer chemotherapeutic. Gemcitabine-induced cytotoxicity is dependent on autophagy mediated by vacuole membrane protein 1 (VMP1) (94), an ER protein required for organelle biogenesis, protein secretion, and development.

Pharmacological Inhibitors of Autophagy

In this section, we discuss compounds that inhibit autophagy and that are in clinical trials (**Table 3**).

Inhibitors of class III phosphatidylinositol 3-kinase block autophagy. Class III PI3K mediates the production of PI3P, a key lipid-signaling molecule that serves as an important checkpoint for the recruitment of the autophagy machinery at the phagophore (**Figure 1**). Miller et al. (95) exploited structural characteristics of class III PI3K and designed several promising inhibitors

such as PT21. Using small-molecule screen for catalytic inhibitors of Vps34, two groups have identified inhibitors that belong to the pyrimidinone and bisaminopyrimidine families (96, 97). To our knowledge, none of the Vps34 inhibitors are being evaluated clinically.

Lysosomal alkalizers block autophagic flux and cargo degradation. Among the autophagy-altering regimens currently being tested in clinical trials for efficacy in cancer patients, 83% are treatments based on HCQ and 15% include CQ. As none of the putative targets of CQ or its derivatives are involved exclusively in autophagy, there is a risk of side effects.

Other compounds that block autophagic flux have also been identified. For example, lucanthone, an antischistosomal agent, inhibits autophagy via a mechanism similar to that of CQ. Azithromycin, a macrolide used to treat certain bacterial infections, prevents lysosomal acidification (98). Inhibition of autophagy by azithromycin may inhibit intracellular killing of mycobacteria within macrophages, resulting in chronic infections with nontuberculous mycobacteria in patients with chronic inflammatory lung diseases such as cystic fibrosis. Clomipramine, an FDA-approved drug long used for the treatment of psychiatric disorders, and its active metabolite desmethylclomipramine interfere with autophagic flux by blocking the fusion of autophagosome with lysosome (99).

CONCLUSION AND PERSPECTIVES

The more we learn about autophagy, the more we discover the complexity of the regulation of this process and its importance in human physiology and disease. Novel modulators of autophagy that target the formation or maturation of the autophagosome have emerged from our increasing knowledge of the autophagy machinery. The deciphering of the molecular selectivity of autophagy has also been a source of novel modulators that act specifically on selective forms of autophagy. Tremendous recent progress has opened new possibilities for modulating autophagy in complex diseases, including cancer and neurodegeneration.

FUTURE ISSUES

Several key questions must be answered before we can effectively block disease progression by targeting autophagy:

1. Whether or not strategies for systemic or tissue-targeted modulation of autophagy are necessary will depend on the disease. To develop tissue-targeted modulators, we must understand how the interplay between tissues (or between different cell populations within a tissue) regulates autophagy.
2. We must delineate when the roles of ATG proteins are independent of autophagy to avoid potential adverse side effects.
3. There is a need for techniques that allow accurate measurement of the autophagic flux in vivo. Such progress should avoid using stimulators of autophagy initiation, as these agents could worsen the phenotype when the accumulation of autophagosomes is the consequence of the blockade of autophagosome consumption by the lysosomal compartment.
4. Validated methods are needed for measurement of activity at each step in the autophagic pathway in vivo. These techniques are necessary before we can determine when and how to modulate autophagy in disease. Autophagic parameters, including an autophagy-dependent secretome signature, would be also useful as predictive markers or biomarkers of pathologies.

5. Although questions remain, the targeting of autophagy is an attractive new path for the treatment of many complex diseases. The goal of intervention is to restore the homeostatic capacity of autophagy with activators or to use inhibitors when addiction to autophagy exacerbates pathological situations.

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