Potential therapeutic applications of autophagy

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Abstract | Autophagy is a dynamic process of subcellular degradation, which has recently sparked great interest as it is now recognized to be involved in various developmental processes and various diseases including cancer and neurodegeneration. Autophagy can function as a cytoprotective mechanism; however, it also has the capacity to cause cell death. A better understanding of autophagy is needed to allow its manipulation for therapeutic purposes, and new insights into the molecular mechanisms of autophagy are now leading to the discovery of exciting new potential drug targets.

Much attention in the field of cell biology has been focused on protein and organelle biogenesis; however, to maintain homeostasis, cells need to balance biosynthetic and degradative processes. Most subcellular degradation is carried out by two mechanisms: the proteasome and autophagy. The proteasome degrades proteins that have been tagged with ubiquitin and is sterically limited; that is, only unfolded proteins can fit within the degradative channel. By contrast, autophagy has the capacity to degrade folded proteins, protein complexes and entire organelles. Furthermore, autophagy has been implicated in a wide range of diseases including cancer, neurodegeneration, myopathies, major histocompatibility complex (MHC) class II antigen processing and the removal of certain bacterial and viral pathogens[1–15].

There are various processes that fall under the general term autophagy[16]. The one common element of autophagy-related pathways is the import of cytoplasmic components into the lysosome. In higher eukaryotes, autophagy functions solely as a degradative and remodelling pathway, whereas in yeasts it also plays a role in biosynthesis. In yeasts, at least two resident hydrolases are delivered to the vacuole, where they normally function, through the autophagy-related cytoplasm-to-vacuole targeting pathway[17]. The three main types of autophagy are chaperone-mediated autophagy, microautophagy and macroautophagy. Chaperone-mediated autophagy involves the direct translocation of cytosolic proteins that contain a particular pentapeptide motif across the lysosomal membrane and requires the action of cytosolic and lysosomal chaperones to unfold substrates. It is primarily involved as a secondary response to nutrient depletion and will not be discussed in this Review, although this process might be blocked in certain forms of Parkinson's disease[18]. The primary difference between microautophagy and macroautophagy is that microautophagy sequesters cytoplasm directly at the lysosomal surface by septation and/or invagination of the lysosomal membrane, whereas the sequestering membrane in macroautophagy is distinct from the lysosome (FIG. 1). Microautophagy has only been well characterized in yeasts and so will not be discussed further. Here, we will focus on macroautophagy (hereafter referred to as autophagy), with a brief review of what is currently known about the molecular machinery. By mobilizing nutrients that result from macromolecular degradation, autophagy can buffer against the effects of starvation in organisms from yeast to mammals. However, it also has roles in a number of disease processes. We will review these aspects and consider potential strategies to manipulate autophagy for therapeutic purposes.

The molecular machinery of autophagy

The morphological hallmark of autophagy is the formation of the sequestering vesicle, termed an autophagosome (FIG. 1), and most of the proteins required for autophagy appear to function at the step of vesicle formation[19]. The molecular components of the autophagy machinery were first identified in yeasts, but many of the corresponding proteins have homologues in higher eukaryotes[20,21]. TABLE 1 lists the autophagy genes that are known in mammals. Of the 11 autophagy-related genes (*ATG*) that have clear orthologues in mammals, 8 are involved in two separate but related conjugation reactions[22]. Atg8/LC3 and Atg12 are ubiquitin-like proteins that have some structural similarity, but do not have clear homology, with ubiquitin. Both proteins are activated by Atg7, which is a homologue of the ubiquitin activating enzyme, and are

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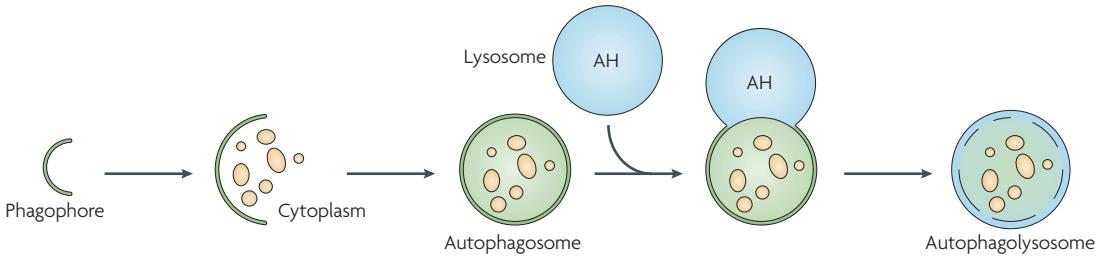

Figure 1 | **Schematic overview of macroautophagy.** A membrane of unknown origin forms the initial phagophore or isolation membrane. The phagophore expands, sequestering cytoplasm and on completion forms a double-membrane autophagosome. The autophagosome fuses with a lysosome, containing acid hydrolases (AH), which can now gain access to the inner vesicle, termed an autophagic body. The fused compartment where the autophagic body and its contents are degraded is called an autophagolysosome or autolysosome. Following breakdown, the resulting macromolecules are released back into the cytosol through permeases for reuse in metabolic processes (during starvation). Alternatively, the cargo might be inactivated or killed (when macroautophagy acts as part of the immune response to eliminate microbial pathogens), or the removal of cytoplasm might result in cell death. See main text for details.

subsequently conjugated to phosphatidylethanolamine or Atg5, respectively. The function of these proteins is not known, but Atg8/LC3 is the only Atg protein in higher eukaryotes that is known to be associated with the completed autophagosome.

The remaining mammalian Atg proteins represent three distinct complexes that include additional components in yeasts. Yeast Atg1 is a serine/threonine kinase that has limited similarity with human Ulk1. In yeasts, Atg1 is part of a multiprotein complex that includes several proteins that are specific for either autophagy or the biosynthetic cytoplasm-to-vacuole targeting pathway[23]. The presence of these proteins has led to the hypothesis that one function of the Atg1 complex is to regulate the conversion between these two types of autophagy; however, Atg1 also functions at the step of vesicle formation.

Beclin 1 (BECN1; also known as Atg6) is one component of a complex that includes the class III phosphotidylinositol (PtdIns)-3-kinase (PIK3C3; also known as Vps34) that is stimulatory for autophagy. Class I PtdIns-3-kinases control the activation of the kinases Akt1 and mammalian target of rapamycin (mTOR), and are inhibitory for autophagy[24–26] (FIG. 2). *BECN1* is haploinsufficient and is mutated in a number of cancer lines, particularly those that are involved in breast tumours[27,28].

The Atg9 protein is the only transmembrane protein in higher eukaryotes that is involved in autophagosome formation. Accordingly, Atg9 might mark the site(s) of the donor membrane that is used to allow expansion of the phagophore, the initial nucleating membrane that develops into an autophagosome[29] (FIG. 1). In yeasts, Atg9 does not appear to be present in the completed vesicle, which suggests that it is retrieved before or on completion of sequestration[30], whereas in mammals the situation is less clear.

Chemical regulation of autophagy

Autophagy occurs at basal rates in mammalian cells, but it can be regulated by a number of pathways (see TABLE 2 for a summary of small-molecule regulators of these pathways). Similar to yeasts, the classical pathway that regulates autophagy acts through mTOR, a protein kinase that is central to nutrient-sensing signal transduction, regulation of translation and cell-cycle progression[31–34]. Tumour suppressors such as phosphatase and tensin homologue (PTEN) and tuberous sclerosis 1 and 2 (TSC1 and TSC2) as well as the PtdIns-3-kinase p110α catalytic subunit oncogene (*PIK3CA*) are upstream of mTOR and control its activation in response to growth factors. mTOR activity negatively regulates autophagy, although the mTOR effectors that regulate mammalian autophagy have not been elucidated. mTOR is a pharmacologically tractable target, as it can be inhibited by rapamycins, which are lipophilic macrolide antibiotics that form a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then bind to and inactivate mTOR, leading to an upregulation of autophagy. Rapamycin is suitable for long-term human use (for example, to prevent renal-transplant rejection) and rapamycin analogues are in Phase III trials for gliomas and advanced renal carcinoma[35–37]. There are at least two distinct TOR-containing complexes in all eukaryotic cells, which are referred to as TORC1 (also known as CRTC1) and TORC2 (also known as CRTC2), and differ in their sensitivity to rapamycin (FIG. 2). TORC2 can be further subdivided into additional complexes based on alternative splicing of the mammalian stress-activated protein-kinase-interacting protein (SIN1)[38]. Interestingly, recent studies have illustrated that the rapamycin-insensitive mTOR complex, TORC2, directly regulates the activating phosphorylation of Ser473 on Akt1 (REF. 39). However, this can be destabilized by chronic exposure to rapamycin in certain cell types[40], which has triggered an interest in the development of mTOR-catalytic inhibitors for various cancer indications[41]. Whether an mTOR-catalytic inhibitor will differ from rapamycin in its ability to activate autophagy is unclear. Therefore, it will be important to evaluate any effects that these inhibitors might have on autophagy.

Autophagy can also be induced by an mTOR-independent route by lowering myo-inositol-1,4,5-triphosphate (IP_3) levels. This can be achieved with drugs such as lithium, carbamezapine and sodium valproate, which

Phosphotidylinositol-3-kinases
Class I phosphotidylinositol (PtdIns)-3-kinases are composed of catalytic p110 subunits and p85 adaptors. The main product of the class I enzymes is PtdIns(3,4,5)P_3. This PtdIns participates in inhibition of autophagy. The Class III PtdIns-3-kinase is Vps34, which is associated with a presumed regulatory protein kinase Vps15. The class III enzyme generates only PtdIns(3)-phosphate, which is stimulatory for autophagy.

Akt1
Akt1 (or protein kinase B) is a serine/threonine protein kinase that is activated by the phosphotidylinositol-3-kinase pathway that activates survival responses.

mTOR
(Mammalian target of rapamycin). A family of kinases that is conserved in all eukaryotes that are sensitive to rapamycin and function in nutrient-sensing signal transduction, regulate translation and promote cell-cycle progression.

Haploinsufficient
A gene that requires biallelic expression. Suppression of one allele would reduce the gene dosage below the critical level.

Table 1 | **Autophagy genes in mammals***

Gene	Protein function
ATG1, ULK1‡	Atg1 is a serine/threonine protein kinase; it may be involved in regulation and vesicle formation
ATG3	Atg3 functions as an ubiquitin-conjugating-like enzyme that covalently attaches Atg/LC3 to phosphatidylethanolamine
ATG4	Atg4 is a cysteine protease that cleaves the C-terminus of Atg8/LC3 to expose a glycine residue for subsequent conjugation
ATG5	Atg5 is covalently attached to Atg12, and binds Atg16 as part of a tetrameric complex of unknown function
ATG6, Beclin 1‡	Atg6 is a component of the class III phosphotidylinositol-3-kinase complex that is required for autophagy
ATG7	Atg7 is a homologue of the ubiquitin-activating enzyme; it activates both Atg8/LC3 and Atg12 before conjugation
ATG8, MAP1LC3‡	Atg8/LC3 has structural similarity to ubiquitin; it is conjugated to phosphatidylethanolamine, and is part of the autophagosome, but its function is not known
ATG9	Atg9 is a transmembrane protein that may be involved in delivering membrane to the forming autophagosome
ATG10	Atg10 functions as an ubiquitin-conjugating-like enzyme that covalently attaches Atg12 to Atg5
ATG12	Atg12 has some structural similarity to ubiquitin; it is conjugated to an internal lysine of Atg5 through its C-terminal glycine
ATG16	Atg16 binds Atg5 and homo-oligomerizes to form a tetrameric complex

*In this table we have only listed certain key genes in which the gene product has been confirmed to play a role in autophagy in higher eukaryotes. ‡Only the autophagy-related gene (*ATG*) name and number is listed except when that name is not used in higher eukaryotes. However, many of the autophagy genes are present as multiple isoforms, usually denoted as a, b and so on.

all act on inositol metabolism and results in decreased IP_3 (REF. 42). All of these drugs have been formulated for long-term use in humans. As far as we are aware, the mTOR and IP_3 pathways do not signal to each other, and the data suggest that autophagy can be enhanced to a greater extent by the inhibition of both pathways, compared to saturating doses of drugs that inhibit each pathway individually[42]. As IP_3 is a second messenger that mediates calcium release from the endoplasmic reticulum to the cytoplasm[43], it is possible that some of the IP_3 effects on autophagy might be modulated by calcium.

Diseases responsive to autophagy modulation

There are a number of diseases that could be treatable using therapies that induce autophagy. The strongest candidates are those for which the mutant proteins or causative agents have been demonstrated to be autophagy substrates, and include neurodegenerative conditions that are caused by intracytosolic aggregate-prone proteins and certain infectious diseases.

Proteinopathies. The formation of intracellular aggregates is a cardinal feature of many neurodegenerative diseases, including Parkinson's disease, polyglutamine expansion diseases — such as Huntington's disease and spinocerebellar ataxia type 3 (also known as Machado–Joseph disease) — and forms of dementia that are caused by mutations in the neuronal protein tau[44]. When these diseases are caused by dominant mutations, they mediate pathology primarily by gain-of-function mechanisms, whereby the mutation changes the protein into something toxic. Typically, these toxic proteins are aggregate-prone; for instance, the mutant proteins are much more likely to aggregate compared with their wild-type counterparts. However, it is still vigorously debated as to whether the large aggregates that are visible by light microscopy are the most toxic entities — some believe they are relatively protective or are neutral epiphenomena[44–46]. It is worth noting that no one has presented data showing that aggregates (also called inclusions) are protective relative to the wild-type protein. When the phrase 'protective roles for aggregates' is used, the data suggest that the cells with the visible inclusions show less toxicity, compared with cells in which the mutant protein appears to be diffusely expressed[45].

Aggregate-prone intracytosolic proteins, including mutant huntingtin fragments, expanded polyalanine tracts tagged to green-fluorescent protein and mutant forms of α-synuclein that cause autosomal-dominant Parkinson's disease, are highly dependent on autophagy for their clearance in cell models[47,48]. Their clearance is delayed by autophagy inhibitors — such as 3-methyladenine and bafilomycin A1 (REFS 47,48) — and knockdowns of autophagy genes[49], whereas inducers of mTOR-dependent and mTOR-independent autophagy enhance their clearance[47,48]. Subsequent studies have provided robust support for these assertions and suggest that autophagy is important for the clearance of larger mutant huntingtin fragments and the mutant full-length gene product[50,51]. Conversely, the wild-type full-length protein and wild-type fragments are far less dependent on autophagy for their clearance compared with mutant forms[50,51]. Rapamycin attenuates mutant huntingtin fragment toxicity in cells, and in transgenic *Drosophila melanogaster* and mouse models of Huntington's disease[52]. The main effect of rapamycin in this context is through autophagy[53].

3-methyladenine
3-methyladenine is an inhibitor of class III phosphotidylinositol-3-kinases and blocks autophagy. This compound acts as a competitive inhibitor of ATP.

Bafilomycin A1
Bafilomycin A1 binds integral membrane subunits in the vacuolar-type ATPase located in the lysosome membrane. The resulting inhibition of proton translocation, elevates lysosomal pH and blocks autophagy.

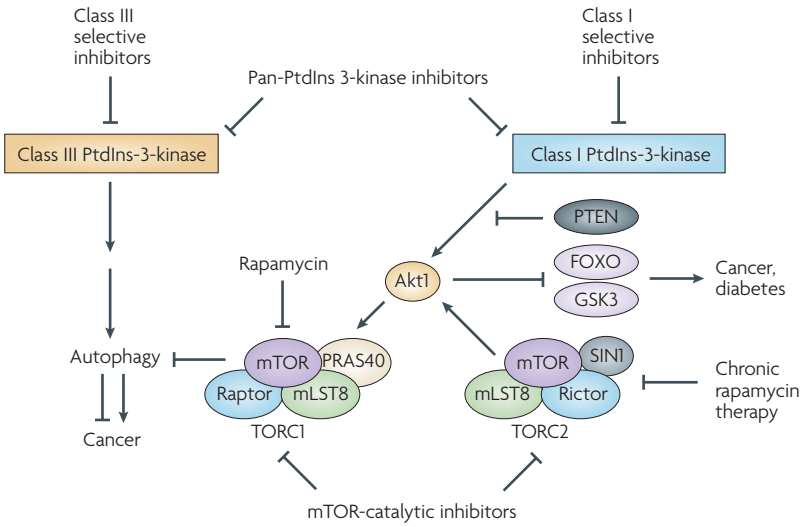

Figure 2 | **Considerations for pharmacological modulation of autophagy.** Autophagy is regulated by mammalian target of rapamycin (mTOR) through the action of phosphotidylinositol (PtdIns)-3-kinases. mTOR is a serine/threonine protein kinase that acts as a central regulator of cell growth and survival. The class I PtdIns-3-kinase product PtdIns(3)-phosphate indirectly activates Akt1 (also known as protein kinase B) and stimulates mTOR. Phosphatase and tensin homologue (PTEN) is a PtdIns(3,4,5)P_3 3-phosphatase, which lowers the activity of Akt1 and mTOR. Akt1 is another serine/threonine protein kinase that, in addition to activating mTOR, negatively regulates the activity of various downstream effectors including glycogen synthase kinase 3 (GSK3). GSK3 in turn plays a role in the regulation of several physiological processes and mediates apoptosis. Forkhead box O (FOXO) is a transcription factor that is phosphorylated by Akt1, which causes it to be retained as an inactive form in the cytosol. When FOXO enters the nucleus it blocks cell-cycle progression and cell growth. The mTOR complexes (TORC) control cell growth and cell-cycle progression in response to nutrient availability and certain growth factors; TOR acts in part as a global regulator of protein translation. TORC1 (also known as CRTC1) blocks autophagy and is sensitive to rapamycin, whereas TORC2 (also known as CRTC2) regulates Akt1 activation and is insensitive to rapamcyin. However, exposure of certain cell types to rapamycin for 24–48 hours can destabilize TORC2 and inhibit Akt1 activation. The class III PtdIns-3-kinase generates PtdIns(3)-phosphate and activates autophagy. Therefore, use of a pan-specific PtdIns-3-kinase inhibitor or a class III selective inhibitor would block autophagy, whereas a class-I-selective inhibitor would activate autophagy. mLST8, mammalian orthologue of mTOR-interacting protein from *Saccharomyces cerevisiae* Lst8; PRAS40, proline-rich AKT-substrate, 40 kDa; RAPTOR, regulatory-associated protein of mTOR; RICTOR, rapamycin-insensitive companion of mTOR; SIN1, stress-activated protein-kinase-interacting protein.

Recent data in cell and fly models suggest that rapamycin-mediated autophagy upregulation might be valuable for other intracellular proteinopathies, including spinocerebellar ataxia type 3, and for the management of both mutant and wild-type tau[53]. Tau was of particular interest as it is mutated in certain frontotemporal dementias, and wild-type tau is the main component of the neurofibrillary tangles that are believed to contribute to pathology in sporadic Alzheimer's disease. Other diseases caused by aggregate-prone toxic proteins that could also be amenable to autophagy upregulation include forms of motor neuron disease caused by mutations in superoxide dismutase 1 (*SOD1*)[54] and forms of peripheral neuropathy caused by mutations in peripheral myelin protein 22 (*PMP22*)[55].

Rapamycin treatment of intracytosolic proteinopathies reduces the levels of both soluble and aggregated species in cell and animal models in an autophagy-dependent manner[47–53]. Similar effects are seen when cell models are treated with drugs that activate autophagy in an mTOR-independent way[42]. However, it is not clear whether autophagy removes only soluble species and oligomers, or whether it can also clear larger aggregates directly. Our data with mutant forms of α-synuclein, which do not form inclusions in our cell lines, suggest that autophagy can efficiently target species that are not in aggregates visible by light microscopy[48]. Indeed, autophagy is likely to clear soluble species, as inclusions form in neurons of mice with neuronally restricted knockouts of autophagy genes[56,57]. As these mice were otherwise normal, these inclusions are presumably composed of wild-type cellular proteins that are not aggregate-prone under conditions that allow autophagy. This suggests that these aggregates arose from the failure to clear soluble proteins, which aggregated when their concentrations exceeded critical levels. Thus, the reduction of both soluble species and aggregates observed in Huntington's disease, cell and animal models after autophagy upregulation could be explained by a model in which autophagy does not directly clear aggregates themselves, but clears soluble monomeric and oligomeric species, as these are aggregate precursors. If these are removed, then the equilibrium is shifted away from aggregate formation, and even large aggregates can be degraded if the synthesis of the mutant protein is switched off (for example, as seen in an inducible mouse model after transgene expression is terminated[58]). The observation that induction of autophagy reduces the load of both soluble and aggregated forms of proteins, such as mutant huntingtin, suggests that it mediates its protective effect by lowering the overall toxin load. Thus, in the context of autophagy protection in these diseases, it becomes largely irrelevant whether aggregates are the most toxic species of these disease-causing proteins.

Although induction of autophagy reduces the load of the toxic proteins in these diseases, it is important to consider the theoretical possibility that autophagy might have some deleterious consequences, such as activation of autophagic cell death. We believe that this possibility is unlikely for the following reasons: first, induction of autophagy by either genetic or chemical means protects against subsequent pro-apoptotic insults in cell models and in *D. melanogaster*[59]. Second, rapamycin is designed for chronic use in humans. We are not aware that any of its side effects are due to autophagy induction — most can be explained by other consequences of inhibiting mTOR signalling (for example, immunosuppression that is due to cell-cycle inhibition). However, it is possible that other chemical inducers of autophagy might have toxic effects, especially if they have an impact on critical pathways that control cell viability (such as Bcl-2 inhibitors[60,61]). Thus, novel compounds and pathways will require appropriate safety testing.

An important question to consider is: why are aggregate-prone proteins logical autophagy substrates? Most of these proteins can be removed from the cytosol either

Bcl-2
(B-cell CLL/lymphoma 2). The founding member of a family of apoptosis-regulating proteins. Many Bcl-2-family members regulate mitochondria-dependent steps in cell-death pathways, with some suppressing and others promoting the release of apoptogenic proteins from these organelles.

Table 2 | **Known small molecules that influence autophagy**

Compound	Target	Effect	References
Rapamycin	Target of rapamycin	Induces autophagy	33,34
Lithium, sodium valproate, carbamezapine	Enzymes that ultimately affect myo-inositol-1,4,5-triphosphate levels, such as inositol monophosphatase	Lowers myo-inositol-1,4,5-triphosphate levels, induces autophagy	41
3-methyladenine	Class III phosphotidylinositol-3-kinase	Inhibits autophagy	92
Wortmannin	Class III phosphotidylinositol-3-kinase	Inhibits autophagy	93
Bafilomycin A1	Vacuolar-ATPase	Inhibits autophagy	81,82
Chloroquine	Lysosomal pH	Inhibits autophagy	71
Hydroxychloroquine	Lysosomal pH	Inhibits autophagy	83
Tamoxifen	Beclin 1	Increases Beclin 1, induces autophagy	70

by the ubiquitin-proteasome pathway or by autophagy. When these proteins acquire mutations that make them aggregate-prone, then the soluble oligomeric and higher-order soluble and insoluble aggregates cannot be cleared by the proteasome, which has a very narrow opening[62]. In these circumstances, autophagy becomes a default pathway. However, we cannot exclude the possibility that there might be some other, as yet uncharacterized, selective process whereby cells target aggregate-prone proteins preferentially through autophagy.

Pathogens. Another class of diseases that might be treatable by autophagy upregulation are certain bacterial and viral infections, in which the pathogens can be engulfed by autophagosomes and transferred to lysosomes for degradation. These include *Mycobacterium tuberculosis* (the bacterium that causes tuberculosis)[63], group A *Streptococcus* (the causative agent in strep throat, toxic-shock syndrome and necrotizing fasciitis)[64] and viruses such as herpes simplex virus type 1 (REF. 1). Conversely, some bacteria and viruses have also evolved to subvert the autophagic system and use autophagy for replication[1,8–15,65].

For example, *M. tuberculosis* infects over a billion people worldwide and follows a cycle of infection, latency, reactivation and transmission. One of the key points in the cycle occurs after the mycobacteria enter host macrophages, where they reside in phagosomes. The presence of these mycobacteria in phagosomes interferes with PtdIns(3)-phosphate generation and the maturation of phagosomes to phagolysosomes (so the mycobacteria do not get degraded). Induction of autophagy allows for degradation of the mycobacteria in the phagosomes, possibly by autophagosomes engulfing the phagosomes[63].

One of the difficulties with treating diseases with rapamycin is that this drug has immunosuppressive actions (as mTOR regulates the cell cycle, which gets partially arrested by this inhibitor). However, the discovery of mTOR-independent-autophagy enhancers[42] provides the possibility that these might be useful in such conditions.

Cancer. Another important group of diseases that might be susceptible to autophagy induction are cancers[7]. This possibility is supported by observations that many positive regulators of autophagy function as tumour suppressors (for example, Beclin 1). The recent identification of DRAM (damage-regulated autophagy modulator), a protein that is needed to induce p53-dependent autophagy, provides yet another link between autophagy and tumour suppression[66]. Conversely, certain negative regulators of autophagy promote tumorigenesis. However, it is not clear whether these pathways are acting directly through autophagy or because there are shared pathways that regulate both autophagy and tumorigenesis, such as the Akt1 pathway. Similarly, the autophagy effector Beclin 1 also interacts with the anti-apoptotic Bcl-2 protein. Our understanding of the role of autophagy in cancer is complicated by two additional factors. First, the actual mechanism of tumour suppression is not known. Autophagy might remove damaged organelles such as mitochondria that could otherwise generate reactive oxygen species that damage nuclear DNA, it might remove growth factors, and/or it might participate in programmed death of oncogenic cells[67]. Second, some cancer cells use autophagy for their own cytoprotective purposes. Thus, the role of autophagy varies depending on the particular type of tumour and the stage of disease progression[68,69]. Although the role of autophagy in tumorigenesis is not clear, the observation that certain autophagy-inducing drugs are antitumorigenic at least allows these to be considered for therapy. For instance, as mentioned previously, rapamycins are in Phase III trials for glioma therapy[35,36] and are being used in trials to treat hamartomas that are associated with tuberous sclerosis[70]. Similarly, the anticancer drug tamoxifen appears to function in part by upregulating the level of Beclin 1 and inducing autophagy[71].

Conversely, autophagy inhibition may also be beneficial in certain contexts. Recently, Thompson and colleagues showed that the inhibition of autophagy with either chloroquine or *ATG5* short-hairpin RNA enhanced the ability of either p53-activation or alkylating-drug therapy to induce tumour-cell death[72]. These data support the view that autophagy upregulation in certain cancer chemotherapeutic contexts might represent a survival response by the cancer cells, and that it might be possible to increase therapeutic efficacy by simultaneously blocking autophagy. Thus, either inhibition

p53
A tumour-suppressor protein that is involved in the regulation of cell-cycle events, including apoptosis.

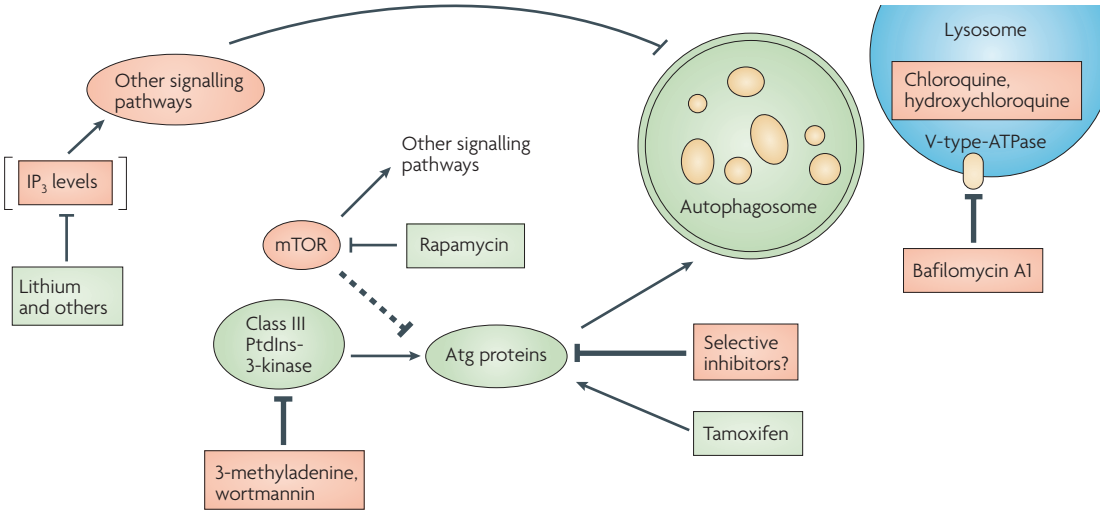

Figure 3 | **Small molecules that affect autophagy.** A schematic overview of the signalling pathways that are involved in autophagy modulation, and small molecules that interfere with these signalling pathways. Compounds and proteins that stimulate autophagy are shown in green and those that inhibit autophagy are shown in red. Rapamycin binds FK506-binding protein 12 (FKBP12) (not shown), and the complex inhibits mammalian target of rapamycin (mTOR) activity. mTOR is a negative regulator of autophagy, whereas the class III phosphotidylinositol (PtdIns)-3-kinase (PIK3C3) stimulates this process. Thus, compounds such as 3-methyladenine and wortmannin, which interfere with class III PtdIns-3-kinase function, block autophagy. The role of PtdIns(3)-phosphate in autophagy is not known, but some of the autophagic machinery, the Atg proteins, bind this phosphotidylinositol. Note that wortmannin inhibits both class I (inhibitory) and class III PtdIns-3-kinases, but the overall phenotypic effect is to inhibit autophagy. At present, there are no selective inhibitors that directly inactivate any of the Atg proteins. Tamoxifen is a drug that is widely used in the treatment of breast cancer. It competes with oestrogen for receptor binding and prevents the transcription of oestrogen-responsive genes, which results in reduced cell growth. There is some evidence that tamoxifen causes increased synthesis of Beclin 1 (BECN1; also known as Atg6), which may stimulate autophagy. Bafilomycin A1 is a selective inhibitor of vacuolar (V)-type ATPases, which results in elevated lysosomal pH. Chloroquine and hydroxychloroquine are chemicals that passively diffuse into the lysosome and become trapped upon protonation. These chemicals also cause an increase in lysosomal pH, which inhibits lysosome function and may block fusion of the autophagosome with the lysosome. Lithium is a nonspecific inhibitor of various enzymes including inositol monophosphatase, which results in a decrease in inositol(1,4,5)P_3 levels and an increase in autophagy.

or stimulation of autophagy may be indicated depending on the type of cancer, the progression of the disease and the nature of the treatment.

Autophagy in PtdIns-3-kinase cancer therapy

Class I PtdIns-3-kinase signalling is a central regulator of cellular survival and type I programmed cell death (apoptosis). Many cancer types have loss-of-function mutations in the PTEN phosphatase (PTEN downregulates class I PtdIns-3-kinase signalling), or activating mutations in *PIK3CA* (encoding a subunit of the class I PtdIns-3-kinase), which results in increased cell survival and tumorigenesis[73,74]. In addition to cancer, gene-knockout studies have revealed an important role for the class I PtdIns-3-kinase-γ and -δ isoforms in control of the immune system[75]. Not surprisingly, intensive efforts are in place to develop therapeutics that target the class I PtdIns-3-kinase family in cancer, inflammation and autoimmune indications[76].

A major question, however, is: what role does autophagy play during PtdIns-3-kinase therapy? Recent studies have suggested that cells with genetic inactivation of the type I programmed cell-death pathway can survive growth-factor and nutrient limitation owing to autophagy signalling, which suggests a cytoprotective role for autophagy[77,78]. It is predicted, therefore, that class I PtdIns-3-kinase inhibition would result in a similar phenotype. This suggests that the dual inhibition of class I PtdIns-3-kinase and the autophagy promoting PIK3C3 might be the optimal approach in targeting certain tumour types (FIG. 2). This might be achieved with a pan-PtdIns-3-kinase inhibitor or a combination of isoform-selective inhibitors. In fact, certain class I PtdIns-3-kinase inhibitors that are currently under development also inhibit the PIK3C3 (Vps34) albeit with low potency[79]. An example of such an inhibitor is the pyridinylfuranopyrimidine PI-103, which potently inhibits class I PtdIns-3-kinase and mTOR-catalytic activity and causes a weaker inhibition of PIK3C3 (hVps34)[80]. Interestingly, PI-103 demonstrated significant antiproliferative effects and cell-cycle arrest in cellular models of glioma irrespective of PTEN, p53 or epidermal growth factor receptor (EGFR) mutational status. This suggests that in addition to targeting class I PtdIns-3-kinase and mTOR signalling, the inhibition of a homeostatic pathway such as autophagy might contribute to the antitumour effects[80]. In the future it will be important to evaluate the activity of PtdIns-3-kinase inhibitors against PIK3C3 (Vps34)[79] in a functional cellular autophagy readout so that the optimal targeting strategy downstream of PtdIns-3-kinase can be chosen for each indication.

Future directions for chemical intervention

Our understanding of the relationships between autophagy and disease has benefited from the availability of chemical tools, such as rapamycin and bafilomycin A1 (FIG. 3). These compounds permit rapid, user-controlled activation or inhibition of autophagy, respectively, and, when combined with genetic methods, have proved to be powerful reagents for discerning the major players in the process. However, there is still much to be learned about the biology of autophagy, and, as such, there is a pressing need for additional small molecules. One of the limitations of known reagents is that their targets are either indirectly or non-exclusively involved in autophagy, and, therefore, their use also impinges on independent or parallel systems. For example, mTOR signalling has been identified as a nexus for integrating nutrient and growth signals, and inhibition of this pathway by rapamycin not only activates autophagy but also represses the translation of numerous proteins, causes immunosuppression, arrests the cell cycle and alters cell size[81]. These side effects may be unwelcome consequences if the study is designed to explore autophagy in isolation. Similarly, bafilomycin A1 is a vacuolar-type ATPase inhibitor with widespread effects on vacuolar integrity, which are apart from its influence on fusion of the autophagosome with the lysosome, or on lysosomal degradation of the autophagic body[82,83]. Thus, although the availability of these reagents has proved useful, there are significant disadvantages inherent in their mechanism. On the other hand, both chloroquine and hydroxychloroquine can also block autophagy[72,84]. These compounds might be more suitable autophagy blockers than bafilomycin A1, and both of these antimalarials are approved for other human clinical uses (for example, to treat rheumatoid arthritis).

One possible strategy that might have value for autophagy stimulation, particularly in the context of certain cancers, may be to disrupt the Bcl-2–Beclin 1 interaction. Indeed, Bcl-2 knockdown with RNA interference and drugs that target Bcl-2 result in autophagy induction[60,61]. This mechanism might be an important contributor to the regression of solid tumours previously reported with a Bcl-2 inhibitor[85].

At present, there are no known direct inhibitors of any of the Atg proteins. We propose that new insights could be made if each definable step of autophagy could be independently blocked with selective chemical probes that operate independently of off-target influences. Moreover, we posit that these research tools might provide the framework for the assembly of next-generation therapeutics. Last, we suggest that recent biological insights will provide a fertile foundation for launching this next round of small-molecule discovery. For example, Atg4 has recently been categorized as a cysteine protease that prepares the ubiquitin-like protein Atg8 for lipidation[22]. Based on its enzymatic activity, we suggest that rapid inactivation might be achieved using a selective protease inhibitor, and the design of such inhibitors might follow a standard path; in most cases, fluorinated ketones, vinyl sulphones or other 'warheads' are appended to short peptide sequences derived from the native substrate[86]. More than ten clinical trials are underway for various cysteine-protease inhibitors that are generated by similar approaches[86], and we propose that a specific inhibitor of Atg4 might be built on the basis of this strategy (possibly using the known structure of Atg4B as a starting point[87]). Similarly, the Atg1 kinase might represent another opportunity for future drug discovery. This cautious optimism is partly based on a recent surge in reports of selective and potent kinase inhibitors[88,89]. These discoveries are being driven by an abundance of structural information on the targets; thus, X-ray crystallography, nuclear magnetic resonance and computational-docking techniques will also be invaluable in the efforts to produce Atg1-kinase blockers.

Last, it has been suggested that autophagosome formation is driven by molecular machinery that includes several multiprotein complexes. Small molecules that control the formation or composition of these complexes might be powerful tools for studying autophagy. There is an ongoing emergence of sophisticated tools for the disruption of protein–protein interactions, and, with increasing alacrity, chemists are able to produce small molecules that inhibit or promote protein–protein contacts[90–92]. Again, we expect that a combination of structural and chemical methods will need to be focused on these goals. In turn, we expect that the insights made using these reagents will generate opportunities for drug discovery in the areas of neurodegenerative disease, infection and cancer.

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Competing interests statement

The authors declare competing financial interests: see web version for details.

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