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Recent advances in targeting autophagy in cancer

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Abstract

Autophagy is a cellular homeostasis mechanism that fuels the proliferation and survival of advanced cancers by degrading and recycling organelles and proteins. Preclinical studies have identified that within an established tumor, tumor cell autophagy and host cell autophagy conspire to support tumor growth. A growing body of evidence suggests that autophagy inhibition can augment the efficacy of chemotherapy, targeted therapy, or immunotherapy to enhance tumor shrinkage. First-generation autophagy inhibition trials in cancer using the lysosomal inhibitor hydroxychloroquine (HCQ) have produced mixed results but have guided the way for the development of more potent and specific autophagy inhibitors in clinical trials. In this review, we will discuss the role of autophagy in cancer, newly discovered molecular mechanisms of the autophagy pathway, the effects of autophagy modulation in cancer and host cells, and novel autophagy inhibitors that are entering clinical trials .

Keywords

Autophagy; Cancer; Immunotherapy; Chemotherapy; Hydroxychloroquine; Lysosome

Autophagy as a potential therapeutic target in cancer

The abnormal growth of cancer cells requires more nutrients than normal cells. Therefore, the pathways which control cellular growth and metabolism are a major focus of therapeutic drug development. Autophagy is an evolutionarily conserved intracellular degradation mechanism that recycles intracellular components to maintain homeostasis and to promote cell survival during stress [1]. The recycling capacity of autophagy arguably makes it a growth pathway and provides an internal source of nutrients to the growing cancer cell. In the harsh conditions of the **tumor microenvironment** (TME, see Glossary), which has disordered vasculature and hypoxia, this internal source of nutrients can play a critical role

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for tumor cell survival [2,3]. Autophagy was originally referred to as a tumor-suppressor mechanism, that prevents the accumulation of damaged organelles and subsequent genomic instability which is critical for tumorigenesis [4-6]. However, more recent evidence from mouse models detailed below suggests that autophagy does not play a strong tumor suppressor role in cells lacking multiple oncogenic mutations [7,8]. In fact, in the setting of advanced cancer, autophagy does not impair but promotes tumor cell survival [9-12]. Understanding the role of autophagy in different stages of tumorigenesis is important when trying to decide as a field whether to develop autophagy inhibitors or inducers as pharmacological agents to target autophagy in cancer.

Genetically engineered mouse models of cancer are the setting in which this controversy of whether autophagy should be promoted or inhibited in cancer came to light. For example, in a mouse model of pancreatic cancer (*Kras* mutant *Tp53*^{-/-}) with defective autophagy-related (*Atg*) gene 5 (*Atg5*) deleted, mice did not die of pancreatic cancer, but rather died of pancreatic polyps (benign tumors) overwhelming the pancreas [13]. In human cancer, very few cells destined to become tumors carry oncogenic mutations and non-cancerous cells do not; but in *Kras* mutant *Tp53*^{-/-} *Atg5*-null model all pancreatic cells had oncogenic mutations questioning the relevance of this model to human cancer. When autophagy deficiency was modeled in pancreatic cells without mutations in *Kras* or *Tp53*, no benign tumor growth was initiated [14]. This finding put to rest the concern that effective drugs that inhibit autophagy will induce tumorigenesis in patients. On the other hand, genetic or pharmacological autophagy inhibition has been shown to slow down tumor growth and prolong overall survival in numerous mouse models of advanced cancer [14-18].

Another major advance in understanding the role of autophagy in cancer is that autophagy in the non-mutated host cells in the TME also supports tumor growth [19-23]. A pharmacological autophagy inhibitor or inducer would modulate autophagy in all cells in the body, not just the tumor cells. Since autophagy inhibition in normal cells does not induce tumorigenesis, and autophagy inhibition in cancer cells promotes survival [14], these findings provide further rationale for focusing on autophagy inhibitors and not autophagy inducers for advanced cancer. Patients with advanced cancer often are willing to tolerate high rates of side effects in return for shrinkage of the tumor, but if targeting autophagy produces catastrophic toxicity, there is no chance for effective drug development. In a mouse model of lung cancer, in which autophagy was completely inhibited throughout the body, mice could tolerate a complete lack of autophagy for 2-3 months but fatal multisystem organ failure ensued eventually in these mice [15]. Although drug therapy can never achieve complete autophagy inhibition like genetic deletion can, clinical development of autophagy-modulating therapy needs to be done carefully.

Initial efforts in translating proof-of-principle *in vivo* studies, demonstrating that blocking autophagy enhances cancer therapy into clinical trials, focused on the lysosomotropic drug hydroxychloroquine (HCQ). The initial phase I trials of HCQ in combination with standard-of-care treatments demonstrated that autophagy inhibition can be achieved in patients without excess toxicity [24]. In some but not all clinical trials there is some evidence that the addition of HCQ to standard-of-care therapy can produce clinical antitumor activity [25-28]. However, both in pre-clinical models and in clinical trials, autophagy inhibition with HCQ

alone or in combination is not curative, suggesting there are resistance mechanisms to this approach [24,25,28-30].

Even though there is a large amount of evidence that autophagy is an important pathway in cancer, neurodegeneration, and infectious disease [1], to date no specific autophagy inhibitor or inducer has received regulatory approval in cancer or any other disease. These issues raise the need to find better pharmaceuticals for targeting autophagy in cancer. In this review, we will highlight newly discovered regulatory mechanisms of mammalian autophagy that could in the future serve as targets for drug discovery, different contexts in cancer where autophagy modulation may be safe and effective, and therapeutics that are emerging in preclinical studies and clinical trials to modulate autophagy in cancer.

Newly elucidated regulatory mechanisms of autophagy

The autophagy pathway has been studied in yeast and in eukaryotic cells for decades, but especially in mammalian cells, new regulatory elements are being discovered that may have implications for drug development. To understand the significance of the new discoveries we first will highlight a basic understanding of the canonical autophagy pathway, which consists of the creation of a circular doublemembrane vesicle called an autophagosome that sequesters and delivers cytoplasmic cargo for degradation in the lysosome (Figure 1).

The initiation of autophagy begins when either the nutrient sensor mammalian target of rapamycin complex 1 (mTORC1) is inhibited or the energy sensor AMP-activated protein kinase (AMPK) is activated. Metabolic or therapeutic stress that leads to either activation of AMPK or inhibition of mTORC1 results in an activated Unc51-like kinase 1 (ULK1) complex, the first complex in the autophagy pathway. When the ULK1 complex is activated, it phosphorylates and activates the vacuolar protein sorting 34 (VPS34) complex. The VPS34 complex has lipid kinase activity that prepares membranes for assembly of the ATG8/light chain 3 (LC3) conjugation machinery, which includes drug targets ATG4 and ATG7. This conjugation machinery acts like a ubiquitin ligase system but uniquely conjugates LC3 family members (such as LC3B) to lipid constituents on the forming autophagosome. Once LC3B is attached to the autophagosome membrane it not only serves as a marker of autophagy but a docking site for autophagy cargo receptors SQSTM1 (p62), NCOA4, and the neighbor of BRCA1 gene (NBR1) that sequester cargos like organelles and proteins that are marked by ubiquitination for their autophagic degradation. Autophagosome maturation is followed by fusion with the lysosome. The lysosome is an acidic organelle because of the presence of a proton pump, the vacuolar -ATPase (V-ATPase). Once an autophagosome fuses with the lysosome, acid-dependent enzymes such as cathepsins degrade autophagic cargo and nutrient transporters recycle macromolecules that fuel growth of the cell. For a comprehensive review of the molecular machinery of autophagy please see [1].

Although the basic mechanism of autophagy has been known for years, a number of recent studies related to each step provide potentially new autophagy regulators as targets for drug development (Figure 1). Related to autophagy initiation (Step 1), a recent study demonstrated that tank-binding kinase 1 (TBK1) phosphorylates Syntaxin17, which can

then assemble an ATG13-FIP200 protein complex independent of ULK1 activity to activate autophagy [31]. A novel regulator of autophagosome biogenesis (Step 2) is the endoplasmic reticulum resident transmembrane protein 41B (TMEM41B), which mobilizes lipids into the emerging autophagosomal membrane [32,33]. The elongation of emerging autophagosome (Step 2) is regulated by recently identified interaction between ATG2A and a lipid scramblase, ATG9. ATG2A delivers lipids to PI3P-WD repeat domain phosphoinositide-interacting (WIPI2) complexes, and ATG9 rearranges the lipids into a symmetrical bilayer that feeds into the autophagosome [34]. Maturation of the autophagosome (Step 5) is carried out by the recruitment of endosomal sorting complexes required for transport (ESCRT) proteins. ESCRT-1 recruits the filament-forming charged multivesicular body protein 2A (CHMP2A), with the help of FIP200. Filament polymerization brings together the open ends of the autophagosome followed by membrane fission. An AAA-ATPase VPS4 finally settles the fission process and closes the autophagosome [35]. Previously, SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) [36,37] were thought to be the key drivers of autophagosome-lysosome fusion (Step 6). However, a recent paper identified ATG14 as a bifunctional autophagy protein that was not only required for the initiating ULK1 complex but also for efficient autophagosome-lysosome fusion [38].

Finally, a recent study identified Lysosomal Enzyme Trafficking Factor (LYSET), a transmembrane protein in the Golgi that is required for lysosomal enzymes trafficking to the lysosome to enable lysosomal degradation (Step 7) [39]. One such lysosomal enzyme is palmitoyl-protein thioesterase 1 (PPT1), which was found to regulate lysosomal acidification by controlling the localization of palmitoylated subunits of the V-ATPase [40]. In addition, mTORC1 was recently found to regulate the assembly of V-ATPase [41]. Although not many new discoveries have been about the core autophagy conjugation machinery (Step 3-4), there is an emerging literature that there are autophagy-independent roles of certain ATG genes such as *ATG5* and *ATG7* including LC3-associated phagocytosis [42], and these roles may be very important in cancer progression [43]. Multiple autophagy genes, not just *ATG7* or *ATG5*, need to be tested in mechanism-focused laboratory research that is attempting to implicate autophagy in a biological process. Specifically, genetic or chemical modulation of one protein in the ULK1 complex, the VPS34 complex, the LC3 conjugation cascade, and the lysosome would be the most comprehensive way to imply that autophagy has a role in any given biological process.

Targeting tumor cell autophagy in combination with chemotherapy or targeted therapy

Patients with advanced cancer are treated with chemotherapy, targeted therapy, or immunotherapy, but in most cases, these treatments are not curative. Autophagy promotes the survival and resistance of cancer cells in the face of chemotherapy and targeted therapy. Its role in impairing anti-tumor immunity is only recently being appreciated. In each therapeutic context autophagy inhibition has been shown to enhance the efficacy of the anti-cancer modality mostly in preclinical models, but also in some clinical trials.

Autophagy inhibition augments chemotherapy

Multiple *in vitro* and *in vivo* studies suggest that traditional cytotoxic chemotherapy drugs induce autophagy in multiple cancer types and blocking autophagy with the lysosomal inhibitors chloroquine (CQ) or HCQ can augment chemotherapy efficacy [44]. The most compelling evidence that this can be translated into patient benefit comes in pancreatic ductal adenocarcinoma (PDAC). In a phase I/II trial of neoadjuvant HCQ and gemcitabine (i.e., given prior to surgery) was tolerable and produced a 61% reduction in the pancreatic cancer biomarker CA19-9 (cancer antigen 19-9) at the time of surgery [45]. This was followed by a randomized phase II clinical trial of neoadjuvant gemcitabine and nab-paclitaxel therapy, with or without HCQ in resectable PDAC [26]. Patients received standard adjuvant therapy but not HCQ after surgery. In this larger clinical trial, the HCQ + chemotherapy arm produced a significantly higher pathological response rate compared to the chemotherapy alone arm. However, there was no significant difference in relapse-free and overall survival indicating a possible development of drug resistance, or the mitigation of HCQ effects with time. A recent clinical update found that 31% of locally advanced PDAC patients treated with HCQ + gemcitabine survived > 5 years [25]. A correlative study looking at both of these neoadjuvant trials found that patients with SMAD4 transcription factor deficiency in their tumors had a higher rate of benefit with an HCQ based regimen compared to patients who did not have a SMAD4 deficient tumor [46]. Finally, a randomized phase II trial in Stage IV pancreatic cancer comparing gemcitabine/nab-paclitaxel + HCQ to gemcitabine/nab-paclitaxel also found a higher response rate but no difference in progression-free or overall survival by the addition of HCQ [28]. Taken together, these findings support that autophagy inhibition with chemotherapy could provide benefits for PDAC treatment, but perhaps a more potent and specific autophagy inhibitor is needed to produce superior durability of response compared to HCQ.

Autophagy inhibition augments targeted therapy

The most striking autophagy activation is seen in the setting of mitogen-activated protein kinases (MAPK) pathway inhibition [47]. MAPK signal transduction cascade progresses via sequential phosphorylation of RAS, RAF, MEK and ERK kinases. The first node of MAPK signaling link to autophagy was BRAF (v-raf murine sarcoma viral oncogene homolog B1), a serine/threonine protein kinase that plays a critical role in the MAPK signaling [48].

Although FDA-approved BRAF^{V600} inhibitors (BRAFi) combined with MEK inhibitors (MEKi) have revolutionized the treatment strategy of *BRAF*-mutated cancers, primary and acquired chemoresistance remains a major obstacle for patients [49]. Autophagy has been reported as a major druggable resistance mechanism to targeted therapy or in BRAF-mutant cancers by many groups [12,50-52]. This has led to the launch of the non-randomized BAMB (BRAF, Autophagy and MEK Inhibition in Melanoma) phase I/II clinical trial of dabrafenib (BRAFi), trametinib (MEKi) with HCQ [53]. The triple drug combination was tolerable and produced an 85% response rate in stage IV *BRAF* mutant melanoma. A high response rate was also observed in patients with elevated serum lactate dehydrogenase (LDH), which is an extremely poor prognostic marker in melanoma. Considering the safety and response rate achieved in BAMB trial, a double-blind randomized trial (BAMB2/EA6191) combining dabrafenib and trametinib with HCQ or placebo has been launched

([NCT04527549](#)) to discern whether the addition of HCQ to BRAF and MEKi is truly beneficial.

The other major MAPK genotype in which autophagy plays a role is *RAS* mutant cancers [47]. Several reports suggested that autophagy inhibition can synergize with the MEK or ERK inhibitors in *RAS* mutant cancers. A genetic screen demonstrated that combined knockdown of autophagy and MAPK components produced the maximum cytotoxicity in *RAS* mutant cells [54]. Kinsey *et al.* found that the MEKi trametinib induced autophagy in *RAS* mutant cancer lines. The combination of CQ and trametinib produced synergistic antitumor efficacy in *RAS* mutant PDX tumors [55]. Similarly, ERK inhibition in *KRAS*-driven human PDAC cells induces autophagy and targeting ERK with CQ produced additive anti-tumor activity *in vivo* [56]. Ongoing clinical trials combining MEKi or ERKi with HCQ are evaluating the safety and efficacy of this approach (e.g., [NCT04132505](#), [NCT04386057](#)).

The promising preclinical and clinical data from *BRAF* and *RAS* mutant cancer studies combining MAPK pathway and autophagy inhibitors suggests that this approach may produce benefits. However, a mechanistic connection between autophagy and MAPK pathway is still being investigated and may allow the development of more specific and efficient inhibitors for treating such cancers. In a recent study, a decrease in MYC following MAPK inhibition in PDAC cells enabled unrestricted access of MiT/TFE (the microphthalmia/transcription factor E) transcription factors to CLEAR (Coordinated Lysosomal Expression and Regulation) network transcriptional elements with a corresponding elevation in autophagy and lysosomal gene expression [57]. Two different studies showed that MEKi in PDAC cells upregulates ferritinophagy, an autophagy process that selectively degrades the iron storage protein ferritin in the lysosome to release iron in the cytosol [57-59]. This increase in iron promotes mitochondrial iron-sulfur cluster protein synthesis for enhanced mitochondrial oxidative phosphorylation in PDAC cells [57-59]. Targeting ferritinophagy could be an interesting new approach to autophagy inhibition but requires more research.

Autophagy inhibition in the TME augments anti-tumor immune responses

Immunotherapeutic strategies aim to boost anti-tumor immunity in cancer; however, the clinical outcomes have been less effective than anticipated. Recent findings suggest autophagy inhibition not only in tumor cells but in different cells present in the TME such as T cells, and tumor-associated macrophages (TAMs) could augment anti-tumor immunity and enhance immune checkpoint inhibitors therapies, such as anti-programmed cell death protein 1 (PD-1) antibody treatment [60,61]. These findings emphasize that autophagy in both the tumor cells and host cells supports tumor growth (Figure 2).

Autophagy inhibition impairs T regulatory cells (Tregs) survival and reduces cytotoxic T-cell exhaustion

Studies show that the extent of somatic DNA mutations in cancer cells dictates the mechanism of anti-tumor activity shown by host cells lacking autophagy, either enhancing antitumor immunity [62] or by reducing an oncometabolite production [63]. In one study when host animals with a whole-body deletion of *Atg7* gene were implanted with liver

cancer cells containing high tumor mutational burden (TMB); and therefore, a larger number of neoantigens for the immune system to recognize, a T cell-dependent anti-tumor immune response ensued that was characterized by reduced Tregs and intra-tumoral T-cell exhaustion [62]. Similar to these findings, in a breast cancer model, autophagy was found to impair anti-tumor effector functions of CD8⁺ T cells. CD8⁺ T cells lacking *Atg5* gene were more metabolically active, produced higher levels of interferon- γ (IFN- γ) and tumor necrosis factor α (TNF- α) and displayed an effector antitumor memory phenotype [64].

It is still unclear if autophagy inhibition only augments anti-tumor immunity in high TMB cancers. For instance, in one study, animals harboring low TMB liver cancer cells did not show an increased immune response with whole body *Atg7* knockout [63]. Tumors did shrink with *Atg7* deletion, but it was due to a metabolic compromise to the tumor cells. Loss of autophagy in normal hepatocytes resulted in reduced circulating levels of arginine which is an essential amino acid for tumor cells growth [63]. In contrast, the ability of autophagy to suppress an anti-tumor immune response was also uncovered by a genome wide CRISPR screening in commonly used mouse cancer models that are typically low TMB compared to human cells [65]. Taken together these findings suggest more work needs to be done in human tissues to understand the role of autophagy in regulating T cell immunity against cancer cells in high versus low TMB tumors.

Autophagy inhibition enhances antigen presentation by tumor cells

An adaptive immune response to cancer cells depends on antigenic peptide epitopes generated and presented by cancer cells via major histocompatibility complex (MHC) molecules to CD4⁺ and CD8⁺ T cells. Two recent studies have implicated autophagy in regulating antigen presentation.

Deng *et al.* demonstrated that autophagy specifically degrades components of the immunoproteasome in the cancer cell, which generate peptides presented by MHC. Inhibition of ULK1 blocked autophagic degradation of immunoproteasome components and enhanced the efficacy of PD-1 antibody in a liver kinase B1 (*LKB1*) mutant nonsmall cell lung cancer mouse model. This genotype is particularly immunotherapy resistant in humans and is especially sensitive to genetic autophagy inhibition [66].

One of the most potentially impactful ways that autophagy regulates tumor immunity is through its regulation of MHC class I. Yamamoto *et al.* demonstrated that MHC-I molecules are selectively targeted for lysosomal degradation by an autophagy-dependent mechanism that involves the autophagy cargo receptor NBR1 [67]. Blocking autophagy genetically or with CQ increased surface expression of MHC Class I improving T cell priming. Combining CQ with anti-PD-1 and anti-CTLA4 antibodies produced synergistic anti-tumor activity in an immune-cold mouse model of pancreas cancer [67]. Taken together, there appear to be multiple ways that autophagy can regulate antigen presentation, but confirmation in human tissues and in other cancer types needs to be done and is an area of active research.

Autophagy in TAMs

TAMs are a vital component of the TME, and sometimes make up to 40-50% of the tumor mass in certain tumors such as melanoma [68]. Depending on the phenotype, M1

or M2, TAMs display anti-tumor or pro-tumorigenic properties, respectively. Autophagy inhibition in TAMs promotes M1 polarization, resulting in increased proinflammatory cytokine secretion [20]. In melanoma models, HCQ treatment repolarizes macrophages from an M2 to M1 phenotype while also upregulating the stimulator of interferon gene (STING). Upregulated STING results in the phosphorylation of TBK1 and release of interferon β . TAM-induced interferon secretion enhances tumor-directed cytotoxic T cells, and HCQ treatment also enhanced the antitumor activity of anti-PD-1 antibody in two different low TMB melanoma mouse models [61]. Taken together, these findings suggest that targeting autophagy in TAMs may be a promising approach and may open up the possibility of different drug delivery modalities such as lipid nanoparticles that hone to macrophages, for autophagy drugs.

Autophagy in natural killer (NK) cells, dendritic cells (DCs) and cancer-associated fibroblasts (CAFs)

In addition to T cells and macrophages, there is evidence that inhibiting autophagy enhances the infiltration and cytotoxicity of NK cells in melanoma tumors [69]. Less is known about autophagy in DCs in a cancer context, but *Ppt1* deficient DCs were able to cross-present viral antigens more efficiently to T cells, suggesting autophagy inhibition could enhance DCs function in the TME [70]. CAFs present another important part of TME and produce structural components of extracellular matrix like collagen, and growth cytokines. Recent studies indicate that autophagy in CAFs promotes the secretion of interleukin-6 to support tumor growth [19]. In lung cancer, autophagy in CAFs were critical for enabling metastasis [22].

Autophagy modulators for preclinical and clinical trials

Given the evidence that autophagy supports tumor growth and promotes resistance to multiple types of cancer therapy [50-52,71,72], the identification of novel chemical inhibitors or inducers of autophagy is a major unmet need. Many of the proteins involved in autophagy have proven difficult to drug. In addition, despite the evidence provided above, the concern that an autophagy inhibitor will induce tumorigenesis weighs heavily on the field.

Since novel autophagy inhibitors or inducers could produce unknown side effects, they can only be ethically tested in patients with advanced cancer refractory to standard-of-care therapy. These patients are typically not as concerned about the development of polyps as they are about the known cancer that needs to be treated. If novel drugs are not safe or do not produce benefit in the advanced cancer setting, they are unlikely to be developed for other cancer contexts, such as a preventative medication in premalignant disease. The following section will detail efforts to develop novel autophagy inhibitors and their effects on models of advanced cancers.

ULK1/2 inhibitors

An ULK1/2 inhibitor (ULKi) blocks early autophagy and can be a promising therapeutic strategy for cancer. SBI-0206965 has been identified as a potential ULKi that arrests

tumor growth in multiple cancer types including non-small cell lung cancer (NSCLC), neuroblastoma, and renal carcinoma [73,74]. SBI-0206965 elevated the sensitivity of daunorubicin in acute myeloid lymphoma [75]. The specificity of SBI-0206965 to ULK1 is questionable, as it has also been found to inhibit focal adhesion kinase (FAK) and AMPK [73]. ULK-100 and ULK-101 are more potent and selective ULKi [76]. ULK-101 suppresses early autophagy induction and induced NSCLC cell death [76]. MRT67307 and MRT68921 are also novel ULKi. They showed *in vitro* performance and established tumor suppressive activity [61]. DCC-3116, another potent and selective ULKi showed preclinical antitumor activity in combination with MAPKi trametinib. A first-in-human phase 1 study of DCC-3116 monotherapy and with trametinib evaluating the safety, tolerability, clinical activity, pharmacokinetics, and pharmacodynamics in RAS or RAF mutant advanced or metastatic solid tumors patients is ongoing (NCT04892017). This clinical development positions ULK1 as the first autophagy-specific target that has a novel inhibitor in clinical trials.

VPS34 inhibitors

VPS34 is the major lipid kinase that prepares autophagic membranes for the conjugation machinery to be assembled. Targeting VPS34 kinase activity genetically or pharmacologically has a broad impact on the immune landscape of melanoma and colorectal cancer (CRC), by inducing the infiltration of NK cells, CD8⁺, and CD4⁺ T effector cells into the TME and turning an immune-cold to an immune-hot TME [60]. In addition, VPS34i (SAR405 or SB02024) treatment in animal models of melanoma or CRC improved the therapeutic efficacy of PD-1 and PD-L1 targeting antibodies [77]. Furthermore, the combination of anti-PD-1/PD-L1 antibodies and VPS34i augments the production of pro-inflammatory cytokines and chemokines such as CCL5, CXCL10, and IFN- γ , as well as the accumulation of CD4⁺, CD8⁺ T cells, and NK cells, DCs, and M1 macrophages into the tumor bed enhancing the efficacy of immunotherapy [72]. The major concern with VPS34 inhibitors is that VPS34 can play a role in non-autophagic endocytic trafficking [78], which could lead to off-target toxicity. So far, there is no VPS34 inhibitor that has entered in clinical trials.

V-ATPase inhibitors

V-ATPase is a multi-subunit transmembrane complex that acidifies the lysosomes. While not directly an autophagy target, targeting V-ATPase blocks autophagic flux [79]. 249C is a newly discovered class of dihydro-pyrazole-5-carboxamide compounds that targets the V-ATPase subunit ATP6V1H, inhibiting biochemical activity, lysosomal acidification, and micropinocytosis [80]. The excellent pharmacological properties and tolerability of 249C distinguishes it from other V-ATPase inhibitors such as bafilomycin, which can only be used as a tool compound. Cancer cells expressing *KRAS*^{G13D} and *KRAS*^{G12V} mutations are dependent on V-ATPase and are vulnerable to 249C treatment [80]. If a 249C derivative were to be developed for clinical trials, it would provide an alternative approach to targeting the lysosome in addition to PPT1 inhibitors.

PPT1 inhibitors

PPT1 is a lysosomal hydrolase that cleaves thioester linkages in palmitoylated proteins and facilitates their degradation. PPT1 regulates V-ATPase subcellular localization thereby regulating lysosomal acidification and autophagy [40,81]. Palmitoylation is an important post-transcriptional protein modification involved in protein-protein interactions and subcellular membrane localization of the substrates [82]. Advances in our understanding of CQ and HCQ action led to the identification of PPT1 as an autophagy target. It was previously shown that CQ and HCQ at high concentrations effectively block autophagy in cancer cells yet elicit little cytotoxicity as single agents [83]. However, the molecular target of these drugs was unknown limiting further development. CQ and its derivative HCQ accumulate preferentially in the lysosomes [84]. It was shown that Lys01, a dimeric form of CQ produced a 10-fold more potent autophagy blockade than CQ or HCQ [83]. Recently, leveraging functional knowledge gained by testing Lys01 in preclinical models, dimeric quinacrine [40] and long-linkered dimeric chloroquines [81,85] were generated that were 100-1000-fold more potent than CQ [40,81,85]. These molecules, along with Lys01 and CQ were used to identify PPT1 as their molecular target [40,81]. With this advance in the understanding of the molecular target of CQ, other novel inhibitors are now in development. For example, PPT1 inhibitor GNS561 has already entered clinical trials [86]. The completed GNS561 phase I trial established the safety of the molecule and a phase 2 combination drug trial of trametinib and GNS561 in KRAS mutant cholangiocarcinoma (NCT03316222) has recently been launched [86,87]. While excitement is growing about novel PPT1 inhibitors, more work needs to be done to identify which patients are most likely to benefit from PPT1 inhibition.

Autophagy inducers

While much effort has been placed on developing autophagy inhibitors, there are proponents of the concept of autophagy induction as a novel approach to cancer therapy [88]. ABTL0812 targets the peroxisome proliferator-activated receptor alpha and gamma (PPAR α and PPAR γ), Akt/mTOR axis and induces autophagic cell death in tumors [89,90]. ABTL0812 has completed its first-in-human clinical trial with a high safety and efficacy profile (NCT02201823). The recommended dose for the Phase II clinical trial has been determined. In this protocol, ABTL0812 has been administered as first-line therapy in combination with paclitaxel and carboplatin for the treatment of advanced NSCLC patients (NCT03366480)[91]. Although the development of ABTL0812 as an autophagy inducer is intriguing it is likely that the cell death induced by this drug is due to an autophagy-independent mechanism. Given the findings that host cell autophagy can enable tumor growth, there may be an added concern that in some cases autophagy induction could lead to more accelerated growth in advanced cancer.

Resistance mechanisms to autophagy inhibition in cancer

Multiple autophagy inhibitors have been tested in pre-clinical and clinical studies. Except for the pre-clinical data of potent PPT1 inhibitors such as GNS561 and DC661 [81,86,87], there is little evidence to suggest that autophagy inhibitors show single-agent anti-cancer activity. Genetic and pharmacological studies in animal models of cancer suggest that

cancer cells can induce complementary pathways to mitigate the effects of autophagy inhibition [92-94] (Figure 3). Targeting such adaptive pathways along with autophagy presents a different approach that can be tested in the clinic. In one study, knock out of some of the twelve essential autophagy genes in multiple autophagy-dependent cancer lines induced cell death in the majority of cells; however, a few clones survived and showed an adaptation to this loss of autophagy [93]. One common mechanism that enabled the survival of these clones was the upregulation of a nuclear factor erythroid 2-related factor 2 (*NRF2*)-dependent antioxidant response and protein homeostasis [93]. This *NRF2* dependency was also seen when autophagosome formation was completely lost during autophagy, by knocking out either upstream or downstream regulator genes of autophagosome formation, *RB1CC1/FIP200* and *ATG7*, respectively [95]. In another study, cancer cell clones which survived the selective pressure of autophagy inhibition were found to be defective in mitophagy [92]. These mitophagy defective clones increased their dependency on mitochondrial fusion, and an autophagy-independent mitochondrial-derived vesicles (MDVs) formation to degrade damaged mitochondria to maintain mitochondrial homeostasis [92]. This report suggested that co-inhibition of autophagy and MDVs trafficking could produce better outcome in cancer than targeting autophagy alone [92].

We have reported recently that following treatment with lysosomal autophagy inhibitors (LAI), HCQ or DC661, melanoma cells increased sphingolipid and cholesterol levels that were associated with increased GM1+ membrane microdomains (GMM) in plasma membranes and lysosomes. Targeting UDP-glucose ceramide glucosyltransferase (UGCG), a rate-limiting enzyme for glycosphingolipids synthesis, synergistically augmented LAI induced cytotoxicity *in-vitro*. Combining the FDA approved UGCG inhibitor eliglustat with LAI produced excellent anti-tumor activity *in vivo* [94].

Concluding remarks and future perspectives

The tumor cell enabling role of autophagy in cancer suggests that targeting autophagy in cancer is a reasonable approach to boost the efficacy of anti-cancer therapies in advanced cancer. The study of autophagy in cancer has expanded dramatically, but there remains an unmet need to clinically translate these findings. Even though there are a number of HCQ trials and a few novel autophagy inhibitors entering clinical trials, targeting autophagy in patients is still in its infancy as a field. There are multiple challenges and unanswered questions that have so far limited the development of novel autophagy inhibitors and inducers for cancer and other diseases (see outstanding questions).

To answer these questions there is a need to develop more potent and specific inhibitors of the pathway. The development of novel tool compounds to study autophagy could help us hone in on the best targets for drug development. Finally, identifying predictive and pharmacodynamic biomarkers that can be translated from the lab into the clinic would speed up clinical development of promising novel autophagy inhibitors for cancer.

Glossary

AMP-activated protein kinase (AMPK)

AMPK is a heterotrimeric protein complex which can sense intracellular ATP, ADP, and AMP concentrations to maintain cellular energy homeostasis.

Autophagy-related (ATG) genes/proteins

There are 40 ATG proteins that have been identified in total, of which at least 19 are directly involved in the biogenesis of the autophagosome, including nucleation, elongation, and closure of the phagophore membrane. ATG proteins also have autophagy-independent functions.

Cargo receptors

Autophagy cargos like organelles and proteins are marked by ubiquitination for their degradation. Ubiquitinated cargos are recognized by their specific cargo receptor proteins (selective autophagy), e.g., sequestosome-1 (SQSTM1/p62), nuclear receptor coactivator 4 (NCOA4), and the neighbor of BRCA1 (NBR1) gene (NBR1), and then recruited to the LC3-II in the autophagosome.

Major histocompatibility complex (MHC)

MHC is a group of genes that code for a variety of cell surface proteins on antigen presenting cells to display antigen peptides to specific T cell receptors. Major types of MHC molecules are MHC class I and class II that are presented on nucleated cells (almost all cells) and antigen presenting cells (e.g., macrophages, dendritic cells etc.), respectively.

Mammalian target of rapamycin complex 1 (mTORC-1)

mTORC-1 is a nutrient sensor and growth regulator. Under nutrient sufficient conditions activated mTORC-1 inhibits autophagy and promotes cellular growth.

Microtubule-associated protein (MAP) light chain 3 (LC3)

LC3 is a human homologue of yeast Atg8, is a crucial part of autophagy. The cytosolic form of LC3 is called LC3-I (18 kDa) and the phagophore membrane-bound form upon autophagy induction is LC3-II (16 kDa). An increase in the ratio of LC3-II/LC3-I serves as one of the markers for autophagosomes formation and can be detected using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Tumor microenvironment (TME)

The tumor microenvironment is the surrounding environment of a tumor that includes immune cells, fibroblasts, blood vessels and the extracellular matrix.

Unc51-like kinase 1 (ULK1) complex

ULK is the first complex of autophagy pathway and consists of ULK1, ATG13, family interacting protein of 200 kDa (FIP200), ATG13 and ATG101. AMPK inactivates mTORC-1 and activates ULK1 to initiate autophagy process.

Vacuolar protein sorting 34 (VPS34) complex (also known as Class III phosphatidylinositol 3-kinases (PI3K))

A family of lipid kinases that add a phosphate group to the 3' hydroxyl on the inositol ring of phosphoinositides and generates phosphatidylinositol 3-phosphate.

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Outstanding Questions Box

- Do macroautophagy levels vary in different cancer types and thus require different dosage of autophagy inhibitors?
- What is the best approach for autophagy modulation at different stages of cancer, either as a single agent or as a combined therapy?
- How are different types of selective autophagy programs regulated in specific cancers?
- Would it be more effective to target selective autophagy pathways like ferritinophagy compared to broadly targeting autophagy?
- Does autophagy inhibition enhance the efficacy of standard of care cancer therapies in the clinic?
- Is there a clinical context where an autophagy inhibitor can produce responses as a single agent?

Highlights:

Autophagy is a degradative and recycling process that is upregulated in cancer cells.

New advances in understanding the mechanism of autophagy have uncovered novel potential targets for drug development.

Clinical trials involving hydroxychloroquine have demonstrated the safety of targeting autophagy, but efficacy could be improved.

Autophagy regulates tumor immunity.

Novel autophagy inhibitors are entering clinical trials.

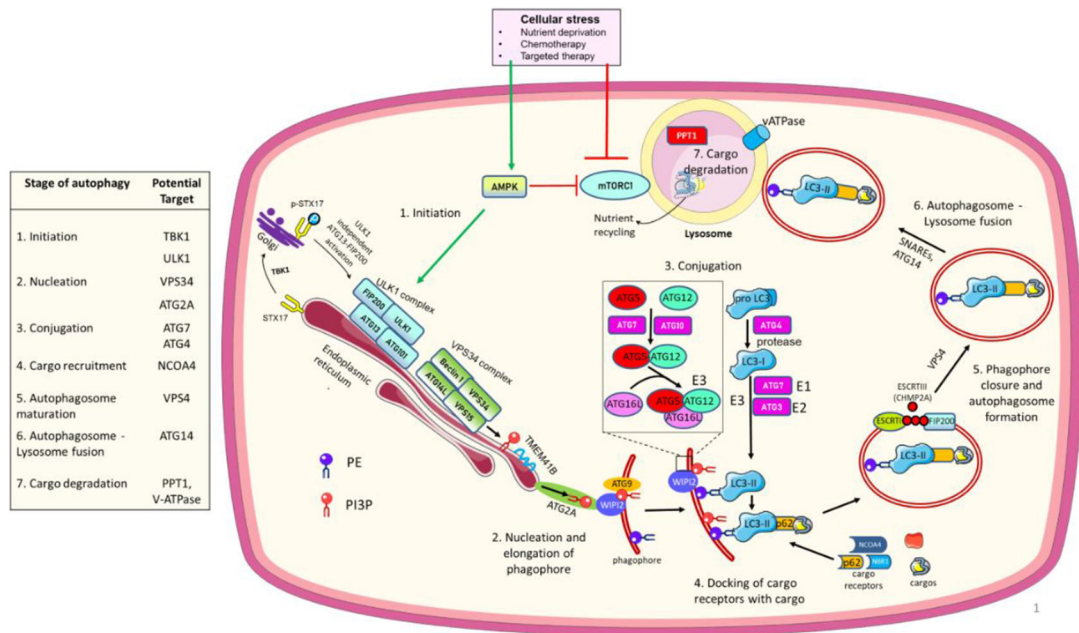


Figure 1. Autophagy regulatory pathway.

Cellular stress activates AMPK which directly and indirectly (through inhibition of mTORC1) activates the ULK1 complex and initiates autophagy. An ULK1 independent activation of autophagy can also occur via TBK1. ULK1 phosphorylates and activates the PI3KC3 lipid kinase complex which generates phosphatidylinositol 3-phosphate (PI3P) to commence phagophore membrane elongation with the help of TMEM41B and ATG2A, which deliver lipids to PI3P-WIPI2 complexes for the next conjugation steps. Two ubiquitin-like sequential conjugation reactions (ATG12 and ATG7 conjugate ATG8 (LC3) protein to phosphatidylethanolamine (PE). ATG7 and ATG3 act as E1 and E2-like enzymes, respectively, and the ATG12-ATG5-ATG16L complex as an E3-like enzyme to finally catalyze the binding of LC3I to PE by ATG3. Next, autophagy cargo receptors with their specific cargos dock onto LC3II in the phagophore/growing autophagosome. ESCRT-III protein polymerization brings together the open ends of the autophagosome which are closed by VPS4 to complete the autophagosome maturation. The autophagosome fuses with the lysosome to degrade and recycle autophagy cargo components. Proteins in the table present potential targets to inhibit different autophagy steps in cancer. For recent advances in each step, please see the main text.

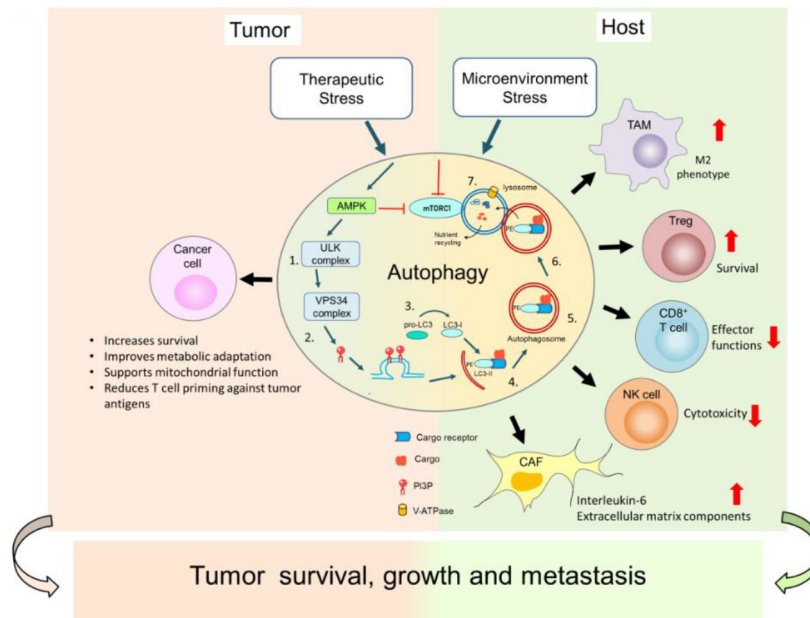


Figure 2. Autophagy supports tumor growth and survival by modulating cancer cells in the tumor, and host cells in the tumor microenvironment (TME). Stressors on cancer cells induce autophagy (please see Figure 1 for details) in both cancer cells as well as in non-cancerous host cells. In cancer cells, autophagy induction promotes their survival by altering their metabolism to provide nutrients and promoting their mitochondrial function under stress. Autophagy degrades immunoproteasome components and MHC-I molecules to escape antitumor immunity. In the TME in non-cancerous host cells, autophagy promotes an M2 phenotype of tumor associated macrophages (TAMs) that support tumor cell growth. Autophagy can also enhance T regulatory cells (Tregs) leading to immunosuppression in the TME. Autophagy limits infiltration and effector functions of CD8⁺ T cells and natural killer (NK) cells. Autophagy also enhances the activity of cancer associated fibroblasts (CAFs) in the extracellular matrix that supports tumor growth and metastasis. Together the functions of autophagy in cancer cells and host cells support tumor survival, growth, and metastasis.

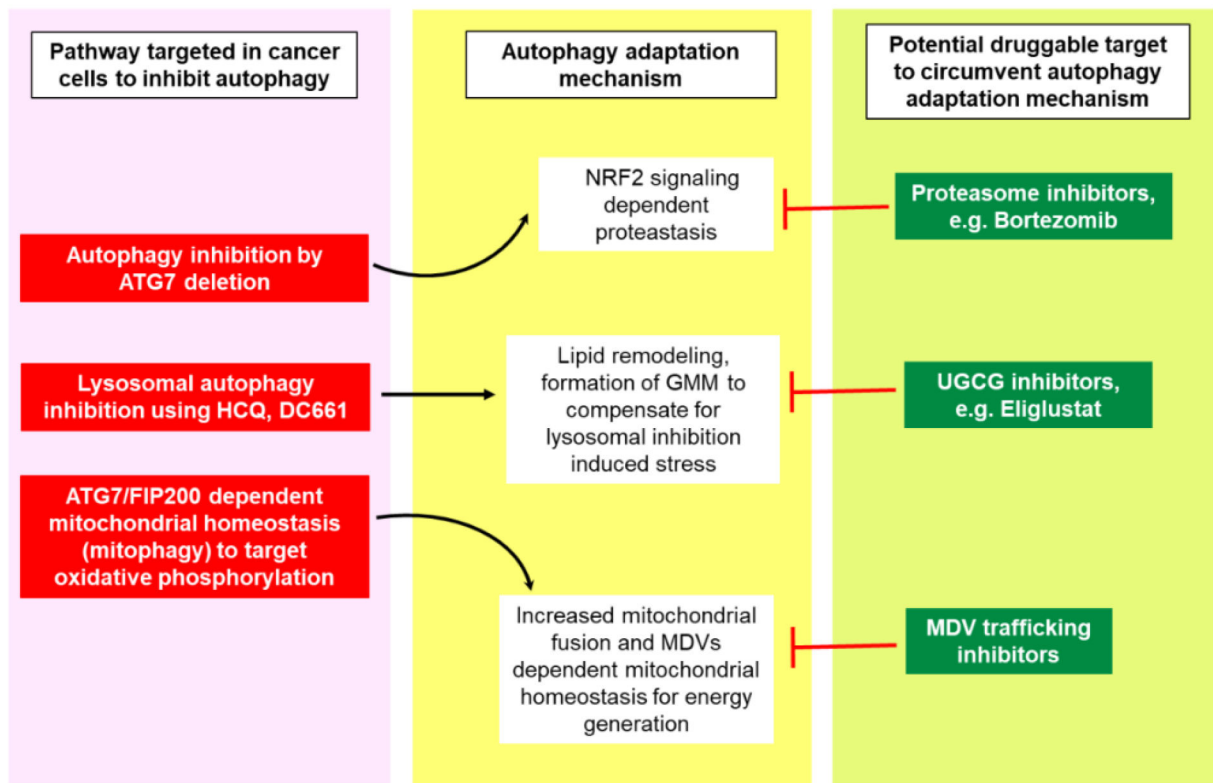


Figure 3. Autophagy adaptation mechanisms as novel potential targets to augment autophagy inhibitory effects on cancer.

Autophagy inhibition by different strategies can induce compensatory pathways which can mitigate the anti-cancer effects of targeting autophagy in cancer. Combining the inhibitors of autophagy resistance pathways with autophagy inhibiting agents in cancer is a new approach which could be used to augment anticancer effects of autophagy inhibitors as a single agent or in combination with standard of care drugs that induce cytoprotective autophagy. Mitochondrial-derived vesicles (MDVs); GM1 + membrane microdomains (GMM); NRF2, nuclear factor erythroid 2-related factor 2; UGCG, UDP-Glucose Ceramide Glucosyltransferase.