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Targeting Autophagy in Cancer

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Preface

Autophagy is a mechanism by which cellular material is delivered to lysosomes for degradation allowing basal turnover of cell components and providing energy and macromolecular precursors. Autophagy has opposing, context-dependent roles in cancer and interventions to both stimulate and inhibit autophagy have been proposed as cancer therapies. This has caused therapeutic targeting of autophagy in cancer to be sometimes viewed as controversial. Here we suggest a way forward for effective targeting of autophagy by understanding the context-dependent roles of autophagy and capitalizing on modern approaches to clinical trial design.

Introduction

Advancements in the understanding of autophagy and how we can harness this pathway to improve clinical outcomes have come a long way since the introduction of the term by Christian de Duve in 1963[1] (Fig 1). The importance of autophagy in health and disease was recently highlighted when Yoshinori Ohsumi was awarded the Nobel Prize for Physiology or Medicine for his work elucidating the mechanism of autophagy[2]. Of particular importance is the role of autophagy in cancer. It is thought that autophagy prevents cancer development. Conversely, once cancer is established, increased **autophagic flux** often enables tumor cell survival and growth[3,4]. Thus an important question for cancer therapy is should we try to enhance autophagy or inhibit it? In premalignant lesions much evidence suggests that enhancers of autophagy might prevent cancer development[5]. Conversely, in advanced

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cancers, both enhancing autophagy and inhibiting it have been suggested as therapeutic strategies[3,6,7].

Despite this potential for confusion, clinical interventions to deliberately manipulate autophagy in cancer therapy are already underway[7] with the vast majority focused on inhibiting autophagy. Indeed, a search of the ClinicalTrials.gov website in February 2017 using the search term “autophagy and cancer” returned 51 studies focused on inhibiting and evaluating autophagy to improve patient outcomes. As with other areas of cancer biology, such as the potential for the immune system to both promote and inhibit tumor formation and progression, the key to successful autophagy-focused therapeutic intervention comes from understanding the biology of how autophagy effects tumor initiation and progression. Here in this Review we discuss recent studies that clarify and support this concept. By considering past clinical trial results, current clinical trial design, the development of biomarkers of autophagy dependence and response, and the role of autophagy in chemoresistance we will explore how cancer therapy can be maximized by autophagy manipulation. Review of these topics is especially timely now with the continued convergence of a better mechanistic understanding of how autophagy influences therapeutic response both at the tumor cell intrinsic level and within the host, with increasing information from autophagy focused clinical studies. This convergence will allow us to better target autophagy to improve clinical outcomes in oncology patients.

Autophagy

Macroautophagy (referred to hereafter as autophagy) is an evolutionarily ancient and highly conserved catabolic process involving the formation of double membraned vesicles called autophagosomes that engulf cellular proteins and organelles for delivery to the lysosome[8,9] (Fig 2). Autophagy is controlled by a highly regulated set of signaling events, occurs at a basal level in all cells, and is induced by diverse signals and cellular stresses[7]. There may be important differences between stimulus-induced autophagy and basal autophagy but our understanding of such differences is poor. Formation and turnover of the autophagosome involves evolutionarily-conserved genes called autophagy related (*ATG*) genes[9] and is typically divided into distinct stages: initiation, nucleation of the autophagosome, expansion and elongation of the autophagosome membrane, closure and fusion with the lysosome, and concluding with degradation of intravesicular products (Fig 2). Initiation begins with activation of the ULK1 (also known as ATG1) complex (involving ULK1, ULK2, ATG13, FIP200 (also known as RB1CC1) and ATG101), which activates a class III PI3K complex (VPS15, VPS34 (also known as PIK3C3), ATG14, Beclin 1, UV radiation resistance-associated gene protein (UVRAG; also known as p63), and activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1), all of which are scaffolded by a putative tumor suppressor Beclin 1[10]. The ATG5-ATG12 complex conjugates with ATG16 to expand the autophagosome membrane and members of the LC3 and GABARAP families of proteins are conjugated to the lipid phosphatidylethanolamine (PE) and recruited to the membrane. ATG4B, in conjunction with ATG7, conjugates LC3-I and PE to form LC3-II (also known as MAP1LC3B). This lipid-conjugated form of LC3 commonly serves as an autophagosome marker[11]. Ultimately, the autophagosome fuses with the lysosome, the contents are degraded and macromolecular precursors are recycled or used to fuel metabolic pathways. The

adaptor protein sequestosome 1 (also known as p62) that targets specific substrates to autophagosomes and LC3II are degraded along with other cargo proteins and can be used as a measure of autophagic flux[11].

Many of these steps in the autophagy pathway represent potentially druggable targets providing ways to both positively and negatively influence autophagy (Fig 2). Although current efforts in the clinic to inhibit autophagy are focused on inhibiting the lysosome using chloroquine (CQ) or the related hydroxychloroquine (HCQ), inhibitors against other autophagy regulators such as VPS34[12–14], ULK1[15,16] and ATG4B[17] have been reported and shown to inhibit tumor cell growth or induce tumor cell death *in vitro*[15–17] and in preclinical mouse models[17]. Next generation lysosomal inhibitors are also in development including Lys05, a bisaminoquinoline that inhibits autophagy and impairs melanoma and colorectal adenocarcinoma growth as a single agent in preclinical mouse models[18]. Lys05 is a more potent autophagy inhibitor than HCQ due to a greater deacidification of the lysosome[18]. Other potent lysosomal inhibitors such as quinacrine and VATG-027 and VATG-032 (novel acridine and 1,2,3,4-tetrahydroacridine derivatives of quinacrine) have also been shown to be effective in patient derived BRAF mutant melanoma cell lines[19]. Conversely, induction of autophagy is feasible using existing drugs (e.g. BH3 mimetics[20] and mTOR inhibitors[21]) but also **nutraceuticals** such as trehalose[22] and caloric restriction mimetics[23] or exercise[24].

Other, less studied forms of autophagy include microautophagy and chaperone-mediated autophagy (CMA). Non-selective microautophagy is mediated by direct engulfment of cytoplasm and its components by tubular membrane invaginations into lysosomes. Selective microautophagy involves direct targeting of specific organelles into lysosomes such as peroxisomes (micropexophagy), nonessential components of the nucleus (piecemeal microautophagy of the nucleus), and mitochondria (micromitophagy). While microautophagy has been associated with the development of neurodegenerative disorders such as Alzheimer disease and Huntington's disease as well as lysosomal glycogen storage diseases such as Pompe disease, it has not been implicated in cancer[25]. CMA is a form of selective autophagy in which cytosolic proteins with motifs related to the pentapeptide KFERQ are recognized by Heat Shock cognate 70 kDa Protein (HSC70; also known as HSPA8) forming a chaperone complex[26,27] that translocates into the lysosome via the lysosomal-associated membrane protein 2A, LAMP2A. CMA has been implicated in cancer[28] and drugs targeting the lysosome could affect all types of autophagy.

Substrates that are degraded by autophagy may differ depending on the autophagic stimulus. One example is the role of autophagy in iron homeostasis[29]. Degradation of ferritin by autophagy is initiated when cells sense that they are deficient in iron and is mediated by nuclear receptor co-activator 4 (NCOA4), allowing release of iron into the cell. Selective autophagy of specific substrates can also occur due to oncogenic stress. For example, degradation of the nuclear lamina occurs in human primary fibroblast cells transformed with oncogenic HRASV12 and genotoxic insults, but not during starvation stress[30]. It is often implicitly assumed that a measured increase in autophagy must have the same consequence irrespective of the stimulus. These studies suggest that this assumption is wrong, and there may be a high degree of selection with regards to the cargo being degraded, depending on the autophagy stimulus. This could perhaps explain the context-dependent consequences of

autophagy on cellular processes and better understanding of such mechanisms in cancer could provide a way to more selectively target autophagy for therapeutic purposes.

Cancer Clinical Trials

Extensive pre-clinical evidence exists to support the idea of inhibiting autophagy to improve clinical outcomes in cancer patients. Animal tumor models driven by specific oncogenes have been shown to cause tumors that regress upon subsequent genetic or pharmacological inhibition of autophagy (see below for further discussion). Similarly, following an initial finding in 2007 by Amaravadi and colleagues[31] (Fig 1), a large number of *in vitro* studies, genetically engineered mouse models (GEMMs) and patient-derived xenograft (PDX) mouse models have demonstrated improved anti-tumor effects when various different types of anti-cancer drug are combined with either genetic or pharmacological autophagy inhibition[3,6,32].

CQ and HCQ are currently the only clinically available drugs used to inhibit autophagy. These drugs deacidify the lysosome and block fusion of autophagosomes with lysosomes preventing cargo degradation (Fig 2)[33]. CQ is also able to sensitize cancer cells to chemotherapeutic agents through autophagy-independent mechanisms[34] and has other anti-cancer effects independent of its effect on autophagy[35,36]. Some of the first clinical evidence of improving outcomes using autophagy inhibition came from a small trial involving 18 patients with glioblastoma. Those treated with CQ in conjunction with radiation and the alkylating agent temozolomide experienced a statistically significant prolonged median survival compared to controls (33 months compared with 11 months)[37]. Follow up clinical trials, and retrospective data from Briceno *et al.* supported the findings of the initial study (Table 1)[38,39]. Additional early studies combining CQ with radiation for brain metastasis also found improved intracranial tumor control[40,41].

The next major series of clinical trials utilized HCQ and had the additional benefit of attempting to correlate **pharmacokinetic (PK)-pharmacodynamic (PD) parameters** with autophagy inhiibition[42–48]. These early phase clinical trials were performed in patients with a wide variety of malignancies and tested multiple drug combinations (Table 1). Notably, these trials provided important lessons on the implementation of autophagy-targeted therapy. A canine lymphoma study of HCQ combined with the chemotherapy doxorubicin modeling a dose escalation phase I human study provided the initial proof of principle that combining HCQ with chemotherapy was safe[42]. Importantly, it also provided preliminary evidence of the clinical activity of HCQ with an observed objective response rate of 93%[42]. Additional human studies included a broad range of tumors including advanced solid tumors and melanoma[43–45], glioblastoma[46], and refractory myeloma[47]. As predicted, the **maximum tolerated dose** (MTD) of HCQ varied in relation to the concurrent therapy utilized. A phase I study of vorinostat with HCQ in refractory solid tumors defined the MTD of HCQ to be 600 mg daily when combined with vorinostat at 400 mg daily[43]. Similar findings related to safety were observed when combining HCQ with concurrent radiation therapy and temozolomide in patients with glioblastoma[46]. In contrast, combining HCQ with 25 mg daily of the mTOR inhibitor temsirolimus in another solid tumor patient population found the combination to be safe with HCQ used at 600mg twice daily[44]. Common dose limiting

toxicities in these trials included gastrointestinal toxicity and fatigue[43–45]. Importantly, HCQ-induced neurotoxicity was not observed as might have been predicted from *Atg7* gene knockout mouse models wherein mice developed significant neurodegeneration upon complete deficiency of autophagy[49]. The MTD of HCQ as a single agent has not been measured and 600 mg twice daily of HCQ is the highest dose tested so far when administered in combination with standard chemotherapy agents[44]. Additional studies of potentially higher HCQ doses or more potent lysosomal autophagy inhibitors such as Lys05, quinacrine, and VATG-032 [18,19,50,51] might maximize autophagy inhibition and anti-tumor activity.

Clinical response to autophagy inhibition has varied widely (Table 1). While initial glioblastoma studies utilizing CQ in combination with chemotherapy and radiation found more than a doubling of median survival compared to controls[37–39], a phase I/II trial utilizing HCQ in combination with chemotherapy and radiation found no significant improvement in survival of patients with glioblastoma[46]. Of note, in this particular study with HCQ there was inconsistent inhibition of autophagy between patients and dose-limiting toxicities including **myelosuppression** that prevented intensification of HCQ therapy, which may explain the different responses in these trials. A phase II trial of HCQ monotherapy in patients with previously treated metastatic pancreatic cancer demonstrated no clinical benefit and inconsistent evidence of autophagy inhibition[48]. However, this study was performed in patients with advanced disease with limited potential for single agent HCQ to improve end-stage disease outcomes. Pre-clinical data from PDX studies of pancreatic cancer had demonstrated a response to single agent HCQ[52]. Furthermore, pre-operative treatment with HCQ in combination with gemcitabine resulted in a decrease in the serum tumor marker cancer antigen (CA) 19-9 in 60% of patients with pancreatic adenocarcinoma[53]. Interestingly, in this same cohort, those patients with a greater than 51% increase in LC3-II puncta labeling in peripheral blood mononuclear cells (PBMC) (suggesting effective autophagy inhibition) experienced both an increase in progression free survival (PFS) (15.03 months compared with 6.9 months) and overall survival (OS) (34.83 months compared with 10.83 months)[53].

Biomarkers

A major limitation in all of the clinical studies has been identifying appropriate pharmacodynamic biomarkers evaluating changes in autophagy. Barnard *et al.*[42] showed that increased intra-tumoral HCQ was associated with an expected increase in LC3II puncta formation (a measure of autophagosome turnover) and accumulation of sequestosome 1 compared to treatment naive tumors. This provided evidence that the clinical use of HCQ could inhibit autophagic flux within tumors, and supports the use of LC3II and sequestosome 1 immunohistochemistry as potential biomarkers for future trials. Several human trials have also utilized transmission electron microscopy (TEM) to evaluate the number of double membraned vesicles (presumed to be autophagosomes) in PBMC. However, this was found to be an unreliable method to monitor autophagy inhibition due to a lack of correlation with levels of autophagy inhibition in tumor samples as measured by changes in the lysosomal protease CTSD cathepsin D as well as sequestosome 1 and LC3II by immunohistochemistry[43].

There can be up to a 100-fold difference in HCQ uptake in tumors compared to plasma, suggesting that plasma analysis is a poor surrogate for tumor specimen analysis[42]. Additionally, CQ uptake into tumor tissue is affected by tumor pH, presenting a difficulty in blocking autophagy in more acidic tumors[50]. Such pH variations could explain some of the differences in accumulation of the drug between tumours. Finally, higher doses of HCQ (1200 mg/daily) may be better at causing an accumulation of autophagic vesicles in both PBMCs and tumor biopsies[47], although this cannot always be achieved due to dose limiting toxicities. This highlights the potential benefit of newer autophagy inhibitors. For example, Lys05 (and its parent compound Lys01) more potently accumulate within and deacidify the lysosome, allowing for greater autophagy inhibition at lower doses. These effects can be seen using standard biomarkers including accumulation of LC3II by western blot analysis and the accumulation of autophagosomes as measured by TEM[18]. Due to the limitations of current autophagy inhibitors, and as we continue to evaluate upcoming new inhibitors, better biomarkers of autophagy manipulation are needed.

Ongoing clinical trials are attempting to define additional biomarkers (Table 2). Functional imaging techniques are being used to correlate intra-tumor hypoxia with autophagy via positron emission tomography (PET)/computed tomography (CT) scans using hypoxia tracers 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide (EF5) labeled with 18F-fluorine isotope (18F-EF5) and [18F]-HX4 [18F-flortanidazole] (NCT01881451[54] and NCT02233387[55]). Similarly, the relationship between cancer metabolism and autophagy is being evaluated in a clinical trial combining HCQ with chemotherapy in patients with advanced colorectal cancer (NCT01206530[56]). Other studies plan to correlate the effects of combined proteasome and vorinostat mediated histone deacetylase (HDAC) inhibition on autophagy and serum metabolic profiles (NCT02042989[57]). HDAC family members have been shown to increase autophagy through several mechanisms including the regulation of gene transcription of essential genes[58]. Increased activity of autophagy after treatment with HDAC inhibitors has been shown to significantly blunt HDAC anticancer activity[58]. Induction of autophagy has also been shown to occur in response to proteasome inhibitors and is believed to play a role in resistance[59]. This is the basis for early phase and ongoing clinical trials inhibiting autophagy in combination with HDAC[43] and proteasome[47] inhibitors.

Pre-clinical studies have also identified the use of the transcriptional regulators belonging to the microphthalmia /transcription factor E (MiT/TFE) family as a potential biomarker of autophagy regulation. Microphthalmia-associated transcription factor (MITF) or TFE3 overexpression was associated with an increase in autophagy and MiT/TFE-dependent autophagy and lysosome gene expression in established pancreatic ductal adenocarcinoma (PDAC) cell lines, primary PDAC tumors, and primary patient-derived PDAC cell lines[60]. Therefore, evaluating the expression levels of MiT/TFE family members, as well as their associated proteins within tumor samples, has the potential to identify patients with autophagy activation under the control of MiT/TFE proteins. Another interesting study by Follo *et al.* found that quantification of autophagy initiation by ATG13 puncta was correlated between patient tumor derived *ex vivo* spheroids and formalin fixed clinical tumor samples, and that differences between ATG13 levels correlated with clinical outcomes in mesothelioma[61]. This is especially important as current measures of autophagic flux require

the use of inhibitors of lysosomal proteases to detect the accumulation of LC3II, which is not possible in formalin-fixed samples[11]. In contrast, ATG13 is a static marker making it potentially much more of a clinically relevant biomarker of autophagy.

Surrogate markers from peripheral blood could provide another method to assess autophagy inhibition. Autophagy regulates cellular secretion of cytokines and other signaling molecules[62]. The autophagy-regulated secretome[63], e.g. secretion of the cytokine interleukin-6 (IL-6)[64], has been suggested as a potential biomarker of autophagic activity. Modern clinical procedures such as endoscopic retrograde cholangiopancreatography (ERCP) allow for sampling of such factors from organ associated ducts[65] or the peripheral blood so such an approach is technically feasible. Better understanding of how autophagy regulates secretion, and the molecules secreted, may allow us to incorporate such methods into a biomarker strategy. Together, these studies suggest a potential multi-dimensional biomarker strategy that would incorporate the direct molecular evaluation of autophagy in biopsies, monitoring of autophagy-regulated soluble factors, and functional imaging techniques. While somewhat involved, all these assays are clinically feasible and could be incorporated into clinical trial protocols.

Targeting Autophagy: a good idea?

The collective results of published clinical trials (Table 1) present evidence for the safe use of CQ and HCQ as a cancer therapy. The reported positive clinical outcomes are encouraging for the role of autophagy inhibition in cancer therapy, but care needs to be taken to understand the underlying contexts where autophagy inhibition will be beneficial and where it could potentially be detrimental.

Autophagy is a known survival mechanism conserved from yeast to mammals[66]. It has also been identified as a survival mechanism across several tumor types[67–70]. The association between tumor cell survival and autophagy can be explained, in part, by the role of autophagy in protecting cells from undergoing programmed cell death[71]. This provides a logical rationale for why the inhibition of autophagy could improve the response to other agents and forms the basis for the completed (Table 1) and ongoing (Table 3) clinical trials. However, the effect of autophagy on the ability of tumor cells to undergo apoptosis is not always protective. For example, within the same tumor cell population, autophagy can promote or inhibit apoptosis under different cellular contexts in response to similar death stimuli such as CD95 ligand (CD95L) or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), which both act as death receptor agonists[72]. The mechanisms underlying these opposing effects are due to the degradation of different pro- or anti-apoptotic regulators by autophagy[72,73]. A take home message from this work is that a much better understanding of how autophagy regulates apoptosis sensitivity (i.e. what substrates it degrades) is needed, if the aim is to predict whether a tumor cell is more or less likely to be killed in response to a particular death signal when autophagy is blocked. Moreover, increases in cell death can depend on the stage of the autophagy pathway that is inhibited. For example, prevention of autophagosome maturation can decrease necroptosis while inhibition of autophagosome turnover potentiates necroptosis in the same prostate cancer cells[74]. These observations exemplify the underlying problem of autophagy manipulation in

cancer therapy– autophagy has context dependent and even opposing effects on tumor cell behavior. Such context-dependent effects are poorly understood, emphasizing the importance of a better understanding of the molecular mechanisms that determine how autophagy affects cancer cell behaviors.

Arguments against inhibiting autophagy in cancer therapy

Several studies, especially from Kroemer and colleagues, have suggested that autophagy inhibition is a bad idea in cancer treatment because it would reduce anti-tumor T cell responses[75–77]. The rationale is that autophagy in dying tumor cells is required for immunogenic cell death leading to efficient recognition by the immune system and activation of an effective anti-tumor immune response[78,79]. One caveat to these studies is that they focused on highly immunogenic tumor models, including the CT26 colon cancer mouse model[80], which may have influenced some of the responses seen. In opposition to this idea, a recent study using less immunogenic B16 mouse melanoma and 4T1 human mammary carcinoma cell mouse models, found equivalent T cell responses between autophagy-competent tumor-bearing mice and tumor-bearing mice wherein autophagy was blocked by either genetic deletion of autophagy genes or pharmacologically through treatment with CQ[81]. Another study from the Kroemer lab took the idea of autophagy being required for immunogenic cell killing one step further by concluding that enhanced autophagy (using caloric restriction mimetics) could boost anti-tumor immune responses[82]. This led to the suggestion that not only should autophagy not be inhibited but that interventions aimed at increasing autophagy during cancer therapy should be considered.

Autophagy can stimulate tumor antigen cross- presentation[83] providing another potential mechanism by which autophagy inhibition could interfere with a robust anti-tumor immune response. Correlative evidence suggests that these mechanisms may be associated with better outcomes[83]. Higher LC3II puncta combined with the presence of nuclear high mobility group protein B1 (HMGB1), a non-histone chromatin-binding protein known to stimulate anti-cancer immune responses, in resected breast cancer specimens was associated with improved metastatic free survival and breast cancer specific survival[84] and increased immune infiltration of the tumor[85]. A caveat for these studies is that the available markers (e.g. autophagosome vesicles in PBMCs) are, as noted above, poor measures of the actual level of autophagic flux that is taking place in the tumor tissue. Countering these ideas, other studies report that some anti-tumor immune responses are enhanced by autophagy inhibition[86,87]. Thus, there are arguments both for and against autophagy inhibition even when just considering the effects on anti-tumor immune responses.

Another potential use of autophagy to influence an immune response has been demonstrated in an ongoing study led by the Second Affiliated Hospital, School of Medicine at Zhejiang University that has proposed the use of the combination of autophagy and proteasome inhibition in *ex vivo* tumor cells in the development of a tumor vaccine (NCT03057340[88]). Pre-clinical data has shown that inhibition of autophagy in tumor cells treated with a proteasome inhibitor results in enrichment of short-lived proteins (SLiPs) and misfolded proteins known as defective ribosomal products (DRiPs) in autophagosomes, named DRibble corpuscles[89]. DRiPs and SLiPs are highly expressed in tumors and have the ability

to support an anti-tumor immune response, but are inherently unstable and under normal conditions are degraded by proteasomes. Inhibiting proteasome degradation stabilizes these proteins that are then concentrated in autophagosomes (DRibble corpuscles). Inhibition of autophagy at this stage prevents breakdown of DRiPs and SLiPs that have concentrated in the autophagosome, and allows for fractionation and collection of the DRibble corpuscles to provide the protein needed to create effective DRibble vaccines[89]. DRibble tumor vaccines developed from these proteins have been shown to induce cross-reactive T-cell responses and tumor antigen cross- protection[90]. Preliminary analysis of a Phase II trial evaluating the use of DRibble vaccines in patients with non-small cell lung cancer (NSCLC) demonstrated that at 12 weeks, PBMCs from treated patients had multiple induced and increased antibody responses[91]. Other studies are also attempting to exploit autophagy to improve the efficacy of cancer immunotherapies. For example, a Phase I trial evaluating DNX2401, an oncolytic adenovirus, in glioblastoma patients (NCT01956734[92]) hypothesizes that autophagy stimulated in response to temozolomide therapy could help viral replication in the tumor cells. This is an example where autophagy inhibition would be counterproductive to the intended purpose of the primary therapy.

In patients, autophagy inhibition is not specifically targeted to tumor cells, thus potential toxicity from global autophagy inhibition represents another reason for pause when considering the value of targeting autophagy. This is exemplified in a study where knockout of an essential autophagy gene (*Atg7*) in all tissues was achieved in adult mice[49]. *Atg7* deletion led the eventual death of all mice due to severe neuronal toxicity, disruption of glucose homeostasis, and increased susceptibility to infection. However, it is important to remember that while the removal of an essential component of the canonical autophagy pathway in every cell in the body might mimic the effect of a “perfect” autophagy inhibitor, such a strategy is markedly different than that of the clinical application of an autophagy inhibitor, which is unlikely to be as effective at inhibiting autophagy as the complete deletion of an essential autophagy regulator. In support of this idea, chronic use of HCQ for treatment of rheumatological disorders and treatment of some cancer patients with CQ as an autophagy inhibitor for extended time periods without adverse toxicity[93] demonstrates that long term treatment with lysosomal autophagy inhibitors is feasible. Most importantly, as long as cancer cells are more dependent on autophagy than normal tissues, even a drug that causes some normal tissue toxicity can have a useful therapeutic window allowing it to be an effective cancer treatment. Indeed, in the inducible *Atg7* knockout mouse, the growth of KRAS-driven lung tumors were profoundly inhibited by *Atg7* deletion before any signs of neurotoxicity[49], indicating that just such a window for autophagy inhibition exists to treat some cancers.

Possible mechanisms and markers of autophagy dependence

Although autophagy may be functional in many cancer cells and needed to respond to stresses like amino acid deprivation, some cancer cells may be especially dependent on autophagy even in the absence of added stress[94]. This idea has been called autophagy addiction or autophagy-dependence and is important because in some studies it was recognized that only autophagy-dependent tumors responded to pharmacological autophagy inhibition *in vivo*[94]. Moreover, drug synergy between autophagy inhibitors and other anti-

cancer drugs can occur in autophagy-dependent tumor cells, while the same drug combination was sometimes even antagonistic in autophagy-independent tumor cells[93,94]. This implies that if autophagy inhibitors are combined with other drugs in autophagy-independent tumors in the clinic, the effects could be counterproductive. A reliable way to identify autophagy-dependent cancers is now needed to incorporate this concept into clinical decisions. Multiple mechanisms of autophagy addiction are beginning to be uncovered (Fig 3) that may help identify the most autophagy-dependent tumors, and many of these mechanisms are amenable to the development of biomarkers that could potentially be used to select patients whose tumors are most likely to respond to autophagy inhibition therapy.

Mutations in the RAS pathway are often associated with high levels of autophagy needed to maintain tumor cell metabolism[95–97]. For example, pancreatic cancer has very high rates of *KRAS* mutation and, together with increased activity of transcription factors that promote autophagy[60] and pancreatic stellate cells in the tumor microenvironment that use autophagy to fuel tumor cell metabolism[98], is thought to cause pancreatic tumors to be especially dependent on autophagy[97]. Similarly, tumors in mouse models of lung cancer and melanoma driven by the $Braf^{V600E}$ mutation are highly sensitive to *Atg7* gene deletion[99,100] while autophagy inhibition is sufficient to kill $BRAF^{V600E}$ –expressing, but not wildtype BRAF-expressing brain tumor cell lines[93].

These data might lead us to conclude that RAS and BRAF mutant tumors define autophagy-dependency and would be good markers to select patients in whom we should try to inhibit autophagy therapeutically[101,102]. However, even here there are context-dependent effects that we should keep in mind. Nuclear p53 has been shown to facilitate autophagy while cytoplasmic p53 is associated with inhibition autophagy[103,104] indicating that overall the role of p53 in autophagy is complex. Whilst p53 has both autophagy promoting and autophagy inhibiting activities, it is not known if these activities determine whether or not tumor cell growth is increased, or decreased through cell death, upon autophagy inhibition.

In one KRAS mutant mouse pancreatic cancer model, homozygous deletion of *Trp53* in the pancreas switched loss of autophagy from being an inhibitor of tumor growth to a promoter[105]. Based on this study, it was suggested that patients whose tumors had both KRAS and p53 mutations might experience tumor growth following autophagy inhibition[106]. However, this concern may be unfounded because human pancreatic tumors do not present with homozygous deletion of *TP53* occurring simultaneously with activation of KRAS, instead these tumours typically presents as p53 loss of heterozygosity (LOH)[52]. Subsequent studies performed in mouse models using conditional pancreatic *Trp53* LOH that more closely resembles the human disease indicate that p53 status does not affect response to autophagy inhibition in pancreatic cancer[52]. Huo *et al.* were able to show that impaired autophagy following monoallelic loss of *Becn1* in mice resulted in a reduction of partner and localizer of BRCA2 (*Palb2*)-associated mammary tumorigenesis (a model of hereditary breast cancer) in the presence of wild type *Trp53*, but not in a p53 null background[107]. A similar conclusion was reached using immortalized, HRAS mutant-expressing primary human ovarian surface epithelial cells, skeletal muscle myoblasts and embryonic kidney cells; some displayed growth inhibition when autophagy was blocked, others showed growth promotion[108]. Moreover, analysis of a large number of human cancer

cell lines with KRAS mutations did not find them to be more sensitive to knockdown of ATG genes than tumor cell lines without KRAS mutations[35]. Taken together these data suggest that while studying RAS and p53 may provide further important insights into the biological mechanism by which autophagy can both promote and inhibit tumor growth, the status of these two genes alone may not identify tumors where autophagy inhibition would be most valuable.

In a panel of breast cancer cell lines selection for or against a library of shRNAs that targeted over 100 autophagy regulators was used to identify those tumor cell lines that could survive and/or proliferate following global genetic interference of the autophagy pathway[94]. This study revealed that some breast cancer cells grow perfectly well when autophagy is globally inhibited, whilst others are dependent on autophagy for survival. These effects were associated with autophagy regulation of signal transducer and activator of transcription 3 (STAT3) activity and autophagy-dependent secretion of interleukins, especially IL-6[64]. In colon cancer, functional JUN N-terminal kinase 1 (JNK1) was required for hypoxia-induced autophagy[109] and ongoing clinical studies are underway to test the use of JNK1 as a marker of autophagy dependence (NCT01206530[56]). Epidermal growth factor receptor (EGFR) mutated or amplified tumors are another potential target for inhibitors of autophagy. Activation of EGFR leads to the downstream regulation of several pathways that influence autophagy, including PI3K-AKT-mTOR, STAT3, and RAS family signaling and Beclin1-associated signaling pathways[110]. Specifically, tumors expressing EGFR variant III (EGFRvIII), a common mutation in the extracellular domain of EGFR are shown to require upregulation of metabolism[111] and are autophagy dependent[112].

Importantly, clinical trials are already utilizing these markers of dependence, or gathering further data for biomarker validation (Table 2). For example, the BRAF, autophagy and MEK inhibition in metastatic melanoma (BAMM) trial (NCT02257424[113]) is specifically assessing HCQ autophagy inhibition for BRAF V600E or BRAF V600K-expressing metastatic melanoma. An additional trial in glioblastoma will evaluate the role of using EGFRvIII to identify patients who will respond to CQ autophagy inhibition in combination with chemotherapy and radiation (NCT02378532[114]).

Autophagy in Cancer Escape Mechanisms

There is mounting evidence of the potential role of autophagy in the ability of cancers to develop resistance to chemotherapy. Patients with melanoma whose tumors become resistant to the BRAF inhibitor, vemurafenib, via an ER stress response display higher levels of autophagy[115]. Moreover, inhibition of autophagy could reverse acquired resistance to vemurafenib that resulted from continued culture of melanoma cell lines in the presence of the drug[115]. Similarly in the clinical setting, a patient with BRAF mutant brain cancer, who had initially responded to vemurafenib treatment, but then acquired resistance to the drug was successfully treated by a combination of CQ and vemurafenib[93]. Thus, in this patient, a tumor could be re-sensitized by treatment with the autophagy inhibitor. Importantly though, only the combination therapy of kinase inhibitor with autophagy inhibitor and not autophagy inhibition as a single agent was effective for long-term control of the tumor growth,

indicating that the clinical benefit is due to overcoming resistance rather than the acquisition of new sensitivity to autophagy inhibition alone[93].

Further laboratory and clinical studies found that genetic and pharmacological autophagy inhibition could overcome multiple molecularly-distinct mechanisms of resistance to BRAF inhibition and was effective in both low and high-grade BRAF mutant brain tumors[116]. Although only a few patients with clinically-acquired resistance to the BRAF inhibitor have been treated with combinations of CQ and the BRAF inhibitor vemurafenib, it is encouraging that each person obtained clinical benefit suggesting that the autophagy inhibitor is consistently able to overcome resistance to the kinase inhibitor in patients[93,116]. Additional pre-clinical studies have shown the ability of autophagy inhibition to overcome resistance to tyrosine kinase inhibition in bladder cancer[117], thyroid cancer[118], NSCLC[119,120], and ALK-positive lung cancer[121]. Because current attempts to circumvent resistance to kinase inhibitors tend to focus on either targeting the same pathway (often the same kinase) in a different way, or targeting a parallel signaling pathway, this strategy of inhibiting an entirely independent process (i.e. autophagy) may represent a fundamentally different way to tackle acquired drug resistance.

Autophagy has also been implicated in resistance to multiple standard chemotherapeutics, often in some of the most difficult to treat tumors. Recent studies have found autophagy induction to be a cause of resistance to the cytotoxic drug paclitaxel in ovarian cancer[122]. Resistance to the chemotherapy cisplatin has been shown to be due to autophagy induction in ovarian and esophageal cancer[123,124], and via hypoxia induced autophagy in lung cancer[125]. Like in melanoma[115], autophagy induction due to an ER stress response results in resistance in primary patient chronic lymphocytic leukemia cells to cyclin dependent kinase (CDK) inhibitors[126] and in resistance in glioblastoma cell lines to HDAC inhibitors such as Tubastatin A[127]. As the link between autophagy and resistance to chemotherapy is strengthened, autophagy will undoubtedly continue to develop as a promising target in cancer therapy[128–132].

Autophagy has also been implicated in supporting the survival of dormant tumor cells and, more importantly, may be critical for such tumor cells to start growing again. In pancreatic cancer mouse models where tumor regression was induced by silencing oncogenic KRAS, rare surviving tumor cells that persist after complete inhibition of the oncogenic driver rely, in part, on autophagy[133]. A recent study using a *Drosophila* tumor model found that dormant tumors from autophagy-deficient animals reactivate tumor growth when transplanted into autophagy-proficient animals. This suggests that non-tumor cell autonomous autophagy in the surrounding cells of the microenvironment is critical for re-growth of dormant tumors[134]. If similar effects occur in mammals, this study would suggest that efforts to enhance autophagy after apparently successful treatment of cancer might have the unintended side effect of promoting recurrence from residual dormant tumor cells.

Conclusion

Within the world of oncology, autophagy has competing and context-dependent effects thus a “one size fits all” approach with interventions designed to inhibit or enhance autophagy in

cancer therapy will not be successful. Given this situation one might presume the best strategy would be to simply avoid trying to manipulate autophagy at all in cancer therapy. However, altered autophagy is unavoidable. Many of our current treatments (e.g. those that affect the mTOR pathway) themselves affect autophagy. In addition physiological stimuli, especially those that often affect tumors differently compared with normal tissues like nutrient deprivation or hypoxia, will also alter autophagy in the tumor. This means that we need to understand what effect these changes have and try to tailor interventions to the particular situation. Initially at least, such interventions are most likely to revolve around inhibiting autophagy. This means that the key decision is in which patients to offer autophagy inhibition therapy.

Clinical trials utilizing CQ or HCQ as autophagy inhibitors have demonstrated the safety of targeting autophagy for cancer therapy. No devastating neurological toxicities have been observed in patients receiving these agents, suggesting that the neurodegeneration seen in mouse models after complete and irreversible inhibition of autophagy is not necessarily informative of the extent of toxicity that will occur after pharmacological treatment with autophagy inhibitors. The survival benefit associated with combining CQ with the BRAF inhibitor, vemurafenib, in brain tumor[93,116] patients provides clinical evidence that autophagy targeted therapy is a feasible clinical strategy in appropriately selected patient populations. To date, the focus of clinical trials has been on the use of lysosomal inhibition with CQ and its derivatives. More potent and autophagy specific inhibitors are in development including better lysosomal inhibitors such as Lys05[18] and drugs targeting earlier steps in the autophagy pathway including ULK1[15,16], VPS34[12–14] and ATG4B[17] While preliminary data is encouraging, these compounds are still in early pre-clinical studies. Issues with selectivity as well as the need for the use of higher drug concentrations may limit clinical utility and optimization of the lead drugs through chemical modifications of the structures will be needed before moving to clinical trials[135].

An important unanswered question that is raised with inhibitors that target early steps in the autophagy pathway is whether it is better to stop the formation of autophagosomes, or to block the degradation of autophagosomes with lysosomal inhibitors. Autophagosomal structures can serve as scaffolds to induce apoptosis[136] and necroptosis[74,136]. Thus, accumulation of autophagosomes might promote such signaling under some circumstances. If this idea is correct, it might be better to block autophagosome degradation with a lysosomal inhibitor rather than inhibit autophagosome formation which might prevent tumor cell killing. And finally, there remains the question of the use of autophagy inducers to prevent oncogenesis. Arguments have been made that increasing autophagy suppresses the development of cancer by limiting genomic mutations, promoting oncogene-induced senescence, and reducing tumor initiating inflammation[137]. This remains a complex question due to the interaction of autophagy with different genetic backgrounds such as with p53 mutations in pancreatic[105] and breast cancer[107] where p53 status may influence response to autophagy stimulation, making it either pro- or anti-tumorigenic.

We have begun to combine anti-cancer drugs of many different classes with autophagy inhibitors and inducers, but with little rationale for deciding which combinations to test or a serious attempt to select patients who are most likely to benefit from these therapies.

Fortunately, modern clinical trial design often allows collection of samples from tumors and blood before and after treatment. This may aid the development of better biomarkers to serve as pharmacodynamic markers of the efficacy of autophagy inhibitors and better identify which patients we should or should not be treating. If we combine improved clinical studies with detailed molecular and cellular analysis to understand the mechanisms underlying the context-dependent effects of autophagy on cancer it should be possible to develop a more rational basis for deciding when and in which direction we should try to manipulate autophagy during cancer therapy. Since we cannot avoid autophagy being altered in tumors and we know that such alterations will change tumor behavior, ignoring the problem is not a good option; a better answer is to understand the biology and then apply that knowledge in well-designed clinical trials.

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Glossary terms

Autophagic flux
a measure of the amount of cellular cargo and the rate at which it is degraded through the autophagy pathway.

Nutraceuticals
a food with medicinal benefit

Pharmacokinetic (PK)–pharmacodynamic (PD) parameters
the study of the time course of metabolism (PK) and the biochemical and physiological effects (PD) of a drug.

Maximum tolerated dose (MTD)
the highest dose of a treatment that is effective whilst not causing unacceptable side effects.

Myelosuppression
a decrease in bone marrow activity resulting in fewer red blood cells, white blood cells, and platelets.

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Biographies

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Christina G. Towers is a post-doctoral fellow in the Department of Pharmacology at the University of Colorado School of Medicine with Andrew Thorburn. Her research is focused on the role of autophagy in cell death, and the development of novel CRISPR *in vitro* and *in vivo* models to better understand the process of autophagy.

Andrew Thorburn is a Professor and Chair of the Pharmacology Department at the University of Colorado School of Medicine. His laboratory focuses on understanding the role of autophagy in cell death, especially as it relates to cancer therapy.

Key Points

- Macroautophagy (autophagy) is a highly regulated multi-step process involved in the bulk degradation of cellular proteins and organelles to provide macromolecular precursors that are recycled or used to fuel metabolic pathways.
- Autophagy can be targeted for both stimulation and inhibition. Stimulation can be achieved through cellular stress (nutrient deprivation) and mTOR inhibition, while inhibition can be achieved through multiple targets both upstream (ULK1, Beclin 1 and VPS34 inhibitors) as well as downstream at the site of lysosomal fusion with the autophagosome.
- Early clinical trials have demonstrated the feasibility and potential benefit of clinically inhibiting autophagy in multiple cancer models including glioblastoma, pancreatic cancer, melanoma, sarcoma, and multiple myeloma.
- Ongoing studies are developing novel clinical biomarkers that can be used to monitor autophagy in patients including electron microscopy evaluation of autophagosome number in peripheral blood mononuclear cells and tumor samples, LC3II and ATG13 puncta by immunohistochemistry and novel imaging techniques utilizing positron emission tomography and metabolomics profiles.
- The role of autophagy in regulating tumor immune responses is unclear, with arguments both for and against autophagy inhibition. Further research is needed to define the safety and utility of autophagy inhibition while also maximizing tumor immune responses for improved clinical outcomes.
- Markers of autophagy dependence have the potential to identify patients who will best respond to autophagy inhibition therapy. Such markers include altered RAS signaling, BRAF mutations, STAT3 activation, autophagy-dependent secretion of interleukins and p53 status.
- Autophagy can be an effective cancer escape mechanism and is implicated in the development of resistance is multiple cancers including BRAF mutated central nervous system (CNS) tumors and melanoma, non-small cell lung cancer (NSCLC), bladder cancer, and thyroid cancer. Combination therapy with autophagy inhibition in these cancers has the potential to reduce and reverse resistance to therapy.

Table of Contents Summary

Autophagy is a process that delivers cytoplasmic components to lysosomes for degradation. This Review discusses clinical interventions to target autophagy in cancer and explains how understanding the context-dependent role of autophagy in cancer should dictate future clinical trial design.

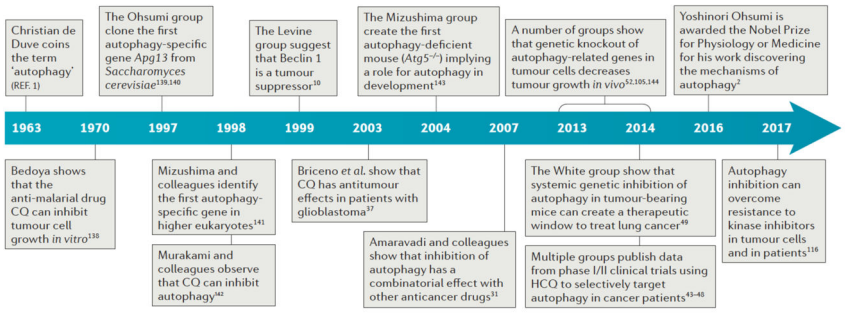

Figure 1. Timeline of the Major Discoveries Leading to the Successful Targeting of Autophagy in Cancer

De Duve first coined the term ‘autophagy’ during a lysosomal conference in 1963. Since then key discoveries have been made elucidating the mechanisms of the process from yeast to cultured cell lines, into mice, and finally culminating in successful clinical trials and case studies in patient tumors. The timeline concludes with the Nobel Prize awarded to Yoshinori Ohsumi for Physiology or Medicine in 2016, emphasizing the impact of his work as well as that of many others along the way. CQ, chloroquine; HCQ, hydroxychloroquine

1963 - Christian de Duve coins the term “autophagy”[1]

1970 - Bedoya shows that the anti-malarial drug, CQ can inhibit tumour cell growth *in vitro*[138]

1997 - The Ohsumi group clone the first autophagy specific gene, *ATG1*, from *Saccharomyces cerevisiae*[139,140]

1998 - Mizushima and colleagues identify the first autophagy specific gene in higher eukaryotes[141]

1998 - Murakami and colleagues observe that CQ can inhibit autophagy[142]

1999 - The Levine group suggest that Beclin 1 is a tumor suppressor gene[10]

2003 - Briceno *et al.* show that CQ has anti-tumour affects in patients with glioblastoma[37]

2004 - The Mizushima group create the first autophagy deficient mouse ($Atg5^{-/-}$), implicating autophagy in development[143]

2007 - Amaravadi and colleagues show that inhibition of autophagy has a combinatory effect with other anti-cancer drugs[31]

2013 - A number of groups show that genetic knock out of autophagy-related genes in tumour cells decreases tumour growth *in vivo*, e.g. [52,105,144]

2014 - The White group show that systemic genetic inhibition of autophagy in tumour-bearing mice can create a therapeutic window to treat lung cancer[49]

2014 - Multiple groups publish data from Phase I/II clinical trials using HCQ to selectively target autophagy in cancer patients[43–48]

2016 - Yoshinori Ohsumi is awarded the Nobel Prize for Physiology or Medicine for his work discovering the mechanisms of autophagy[2]

2017 - Autophagy inhibition can overcome resistance to kinase inhibitors in tumour cells and in patients[116]

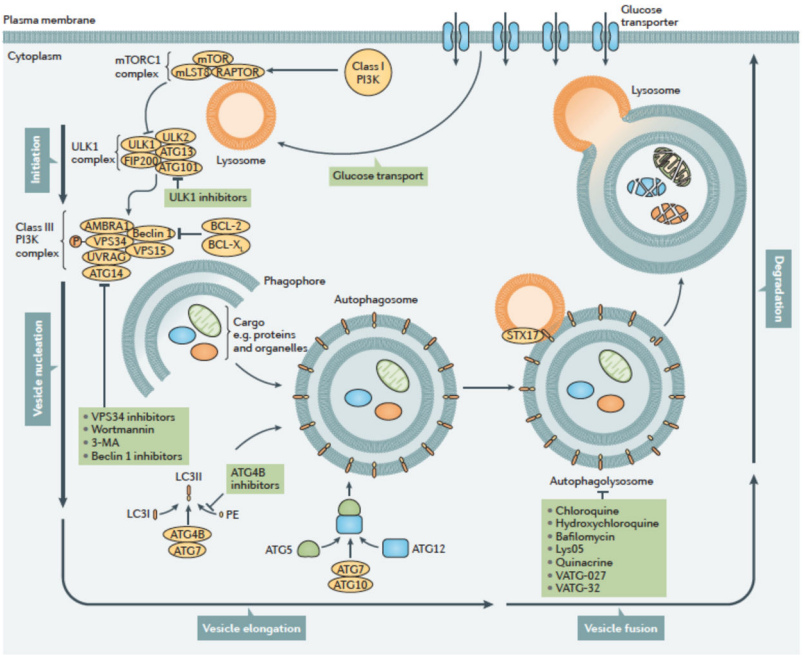

Figure 2. Autophagy can be inhibited at multiple stages

The process of autophagy is divided into five distinct stages: initiation, vesicle nucleation, vesicle elongation, vesicle fusion and cargo degradation. Nonspecific macroautophagy is initiated by upstream activation through either nutrient starvation or growth factors. Under starvation conditions, a drop in glucose transport results in a release of mTOR inhibition of the ULK1 complex, allowing for the progression of autophagy. The ULK1 complex (comprising ULK1, ULK2, FIP200, ATG101 and ATG13) induces vesicle nucleation which is then mediated by a class III PI3K complex consisting of multiple proteins. Beclin 1, a BCL-2 homology (BH)-3 domain only protein, is phosphorylated by ULK1 and acts as an overall scaffold for the PI3K complex, facilitating localization of autophagic proteins to the phagophore. BCL-2 and BCL-XL interact with Beclin 1 at the BH3 domain to decrease the pro-autophagic activity of Beclin 1 by interrupting the Beclin 1–VPS34 complex formation and decreasing the interaction of Beclin 1 with UVRAG. Additional negative regulation of this process occurs with the phosphorylation of VPS34 (also known as PIK3C3), which decreases its interaction with Beclin 1. In contrast, AMBRA binds Beclin 1 and stabilizes the PI3K complex. ATG14 and UVRAG also bind Beclin 1 to promote interactions between Beclin 1 and VPS34 and phagophore formation. VPS15 is required for optimal VPS34 function by enhancing VPS34 interaction with Beclin 1. The growing double membrane undergoes vesicle elongation to eventually form an autophagasome: a process mediated by two ubiquitin-like conjugation systems. The first system involves the conjugation of phosphatidylethanolamine (PE) to cytoplasmic LC3-I to generate the lipidated form, LC3-II which is facilitated by the protease, ATG4B, and the E1-like enzyme, ATG7, whereby LC3-II is incorporated into the growing membrane. The second conjugation system is mediated again by ATG7 as well as the E2-like enzyme, ATG10, resulting in an ATG5-ATG12 conjugate. Subsequently, the SNARE protein, syntaxin 17 (STX17) facilitates autophagosome fusion with the lysosome, resulting in an autophagolysosome. The low pH of the lysosome results in degradation of the autophagosome contents. This process can be

targeted pharmacologically upstream by means of direct ULK1, VPS34, or Beclin 1 inhibition. It can also be targeted by wortmannin and 3-methyladenine (3-MA) which act as PI3K inhibitors. Downstream targets include direct ATG4B inhibitors as well as chloroquine or hydroxychloroquine and bafilomycin, which act to prevent autophagosome fusion with the lysosome. PE, phosphatidylethanolamine.

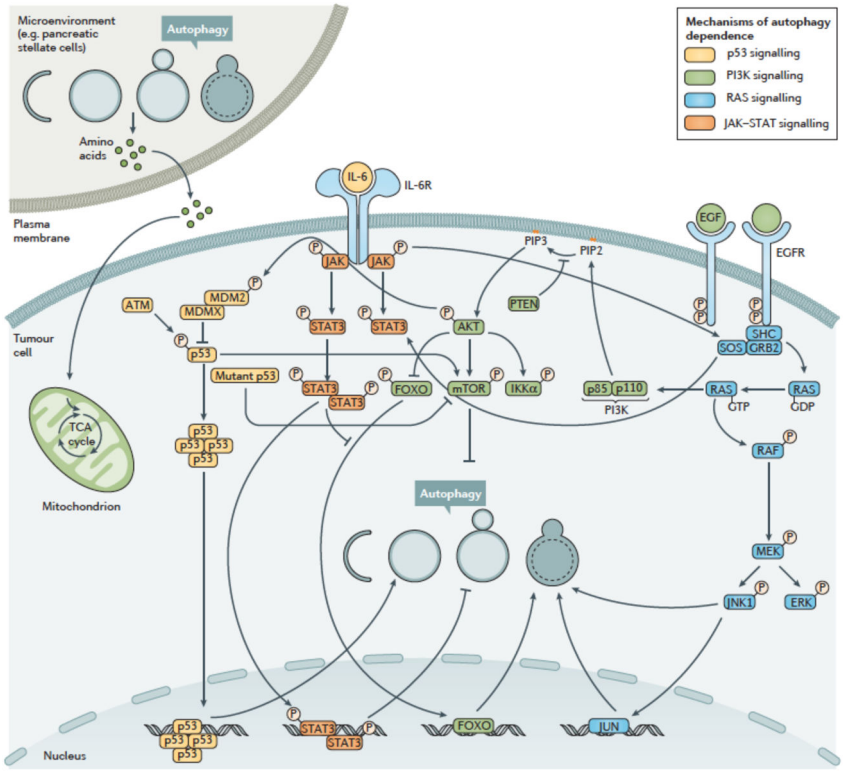

Figure 3. Molecular Mechanism of Autophagy Dependence

Pre-clinical and clinical models have indicated that the tumor microenvironment, for example pancreatic stellate cells in the case of pancreatic cancer, p53 status, RAS family status, activation of JAK–STAT and PI3K signaling may all play roles in the determination of autophagy dependence within cancer cells, both *in vitro* and in patients. These pathways have all been shown to affect autophagy either positively or negatively and many participate in cross-pathway signaling. Signaling through p53 can both promote and inhibit autophagy and may interact with other proteins activated by mutations to enhance autophagy dependence, especially in pancreatic cancer. Activation of EGFR via amplification or mutation leads to the downstream up-regulation of the PI3K-AKT-mTOR pathway as well as activation of STAT3 and the RAS pathway. Although autophagy inhibition can occur through mTOR activation, these downstream effects collectively result in stimulation of autophagy and an increase in autophagy dependence. Mutations or alterations in the RAS family (specifically KRAS) have been shown to promote autophagy, enhancing tumor growth and therapy resistance. Specific mutations in *RAF* such as $BRAF^{V600E}$ promote autophagy dependence in multiple tumors including central nervous system (CNS) tumors and melanoma. Finally, autophagy regulation of JAK-STAT signaling through IL-6 has been identified as a mechanism of autophagy dependence in breast cancer. All of these pathways are complex and interact on multiple levels. Identification of tumors with these pathways and as of yet to be identified pathways will provide methods of detection of autophagy dependent tumors.

Table 1

Published Autophagy Trials in Cancer

Tumour Type	Autophagy Inhibitor	Clinical trial Phase	Additional treatment	Clinical Response	Grading of Side effects	Biomarker measures	Ref
Non-Hodgkin lymphoma	HCQ	I (in Dogs)	Doxorubicin	PFS- 5 months ORR-93.3%	Grade 1 or 2: Mild lethargy GI upset Grade 3 or 4: None	Plasma concentrations of HCQ LC3 positive cells by flow cytometry EM of PBMC for AVs	42
Solid tumors	HCQ	I	Vorinostat	1 patient (renal cell carcinoma) durable PR 2 patients (colorectal cancer) prolonged SD	Grade 1 or 2: nausea, diarrhea, fatigue, weight loss, anemia, elevated creatinine Grade 3: fatigue and/or myelosuppression in a minority of patients	EM of PBMC for AVs IHC for LC3II	43
Solid tumors and melanoma	HCQ	I	Temsirolimus	67% SD (solid tumors) 74% SD (melanoma)	Grade 1 or 2: fatigue, anorexia, nausea, stomatitis, rash, weight loss Grade 3 or 4: Anorexia, fatigue, nausea	EM of PBMC for AVs	44
Solid tumors and melanoma	HCQ	I	TMZ	Solid tumor patients: 10% PR 27% SD Metastatic melanoma patients: 14% PR 27% SD	Grade 2: fatigue, anorexia, nausea, constipation, diarrhea	EM of PBMC for AVs	45
Solid tumors	HCQ	I	Rapamycin with Metronomic cyproterone and docetaxel	40% PR 44% SD	Grade 1 and 2: fatigue, diarrhea, mucositis Grade 3: Fatigue, myelosuppression, diarrhea, nausea, vomiting, cardiotoxicity, hepatic toxicity	Not evaluated	145
Sarcoma	HCQ	Case series (10 patients)	Rapamycin	6 PR 3 SD 1 PD	Grade 1: rash, nausea, diarrhea, constipation	Evaluation of 18FDG-PET as measure of tumor response after two weeks	146
Glioblastoma	CQ	III	TMZ and radiation	Median survival 24 months (controls 11 months)	Grade 1: Myelosuppression	Not evaluated	39
Relapsed Glioblastoma	CQ	Case series (5 patients)	Radiation	2 month response: 2 PR 1 SD	None	Not evaluated	147
Glioblastoma	HCQ	I/II	TMZ and radiation	Median survival 15.6 months	Grade 2 or 3: myelosuppression, nausea, fatigue, constipation, diarrhea	Plasma concentrations of HCQ EM of PBMC for mean AVs PBMC LC3-II:Actin ratio	46

Tumour Type	Autophagy Inhibitor	Clinical trial Phase	Additional treatment	Clinical Response	Grading of Side effects	Biomarker measures	Ref
					Grade 4: myelosuppression, constipation		
Glioblastoma	CQ	III	TMZ and radiation	Median survival: 33 months (controls 11 months)	Increased seizure frequency	Not evaluated	37
Brain metastases: Non-small cell lung cancer, small cell lung, breast, ovarian	CQ	Pilot	Radiation	Median OS: 5.7 months PFS of brain metastasis at one year 55%	Grade 1: radiation dermatitis Grade 2: alopecia	Not evaluated	40
Brain metastases: Non-small cell lung cancer, breast cancer	CQ	II	Radiation	ORR 54% (control 55%) PFS of brain metastasis at one year 83.9% (control 55.1%)	Grade 1 or 2: headache, dizziness, nausea, vomiting, anorexia, myelosuppression Grade 3: nausea, constipation, headache, drowsiness	Not evaluated	41
Refractory myeloma	HCQ	I	Bortezomib	14% very good PR 14% minor response 45% period of SD	Grade 1 or 2: myelosuppression fatigue, peripheral neuropathy, nausea, vomiting, diarrhea, constipation Grade 3 or 4: nausea, constipation, diarrhea, anorexia, myelosuppression fatigue	Plasma concentrations of HCQ EM of PBMC and bone marrow plasma cells for mean AVs PBMC LC3-II:Actin ratio	47
Metastatic PDAC	HCQ	II	None	2 months PFS 10% Median PFS 46.5 days (1.5 months) OS: 69 days (2.3 months)	Grade 3 or 4: lymphopenia, elevated alanine aminotransferase	PBMC LC3-II:β-Actin ratio	48
PDAC	HCQ	I/II	Gemcitabine	61% with decrease in CA19-9 If >51% increase in LC3-II, improved disease-free survival to 15.03 months vs 6.9 months, OS 34.83 months v 10.83 months	Grade 3: Myelosuppression, hyponatremia, elevated AST, hypoalbuminemia, hyperbilirubinemia rash hyperglycemia ileus	CA19-9 as measure of tumor response LC3-II in PBMC	53
NSCLC	HCQ	I	Erlotinib	1 PR 4 SD ORR 5%	Grade 1 or 2: Nausea, fatigue, vomiting, anemia, anorexia Grade 3 or 4: Rash, nausea, nail and skin changes, myelosuppression Grade 5: Pneumonitis	Plasma concentrations of HCQ	148

AVs: Autophagic vacuoles
CA 19-9: cancer antigen 19-9
CQ: chloroquine

CR: Complete response
EM: Electron microscopy
GI: gastrointestinal tract
HCQ: hydroxychloroquine
IHC: Immunohistochemistry
NSCLC: non-small cell lung cancer
ORR: Overall response Rate
OS: Overall Survival
PBMC: Peripheral blood mononuclear cell
PDAC: pancreatic adenocarcinoma
PD: Progressive disease
FDG-PET: fluorodeoxyglucose-positron emission tomography
PFS: Progression free survival
PR: Partial response
SD: Stable disease
TMZ: temozolomide

Side effects are graded according to the Common Terminology Criteria for Adverse Events using a scale of 0–5 with 0 representing no adverse side effects and 5 representing death as a result of an adverse side effect.

Table 2

Autophagy Biomarker Identification Trials

Autophagy biomarker	Methods of measurement	Tumor type	Clinical trial ID
Tumor hypoxia	18F-EF5 PET Tissue LC3II staining Autophagy gene expression	Clear cell ovarian	NCT01881451[54]
Tumor hypoxia	18F-HX4 PET Autophagy gene expression	Cervical	NCT02233387[55]
Autophagosomes	Autophagic vesicles in PBMC	Myeloma	NCT01594242[149]
Metabolic alterations	18FDG PET Autophagic vesicles in PBMC	Colorectal	NCT01206530[56]
Metabolic alterations	Serum metabolic studies	Advanced p53 malignancies	NCT02042989[57]
Metabolic alterations	MRI including magnetic resonance spectroscopy and diffusion weight imaging	Cervical	NCT01874548[150]

18F-EF5, fluorine 18 (18F)-2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide (EF5); 18 FDG, 18F-fluorodeoxyglucose; HX4, flortanidazole; MRI, magnetic resonance imaging; PBMC, peripheral blood mononuclear cells; PET, positron emission tomography

Table 3

Open Trials Targeting Autophagy Dependent Cancers

Tumor type	Autophagy-dependence marker	Phase	Autophagy Inhibitor	Additional treatment	Clinical Trial ID
Colorectal	JNK1	I/II	HCQ	FOLFOX+ Bevacizumab	NCT01206530[56]
Glioblastoma	EGFRvIII	I/II	CQ	Temozolomide + radiation	NCT02378532[114]
Pancreatic	Mutant RAS	I/II	HCQ, nab-Paclitaxel	Gemcitabine, nab-Paclitaxel	NCT01506973[151]
Pancreatic	Mutant RAS	II	HCQ, nab-Paclitaxel	Gemcitabine, nab-Paclitaxel	NCT01978184[152]
Pancreatic	Mutant RAS	I/II	HCQ	Gemcitabine	NCT01128296[153]
BRAF Mutant Melanoma	Mutant BRAF	I	HCQ	Vemurafenib	NCT01897116[154]
BRAF Mutant Melanoma	Mutant BRAF	I/II	HCQ	Dabrafenib, Trametinib	NCT02257424[113]

CQ, chloroquine; EGFRvIII, epidermal growth factor variant III; HCQ, hydroxychloroquine, JNK1, JUN N-terminal kinase 1; nab-paclitaxel, nanoparticle albumin-bound-paclitaxel