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Leaving the lysosome behind: novel developments in autophagy inhibition

The search for a single silver bullet for the treatment of cancer has now been overshadowed by the identification of multiple therapeutic targets unique to each malignancy and even to each patient. In recent years, autophagy has emerged as one such therapeutic target. In response to both therapeutic and oncogenic stress, cancer cells upregulate and demonstrate an increased dependence upon this intracellular recycling process. Particularly in malignancies that currently lack targeted therapeutic options, autophagy inhibitors are the next hopeful prospects for the treatment of this disease. In this review, we discuss the rapid evolution of autophagy inhibitors from early lysosomotropic agents to next-generation lysosome-targeted drugs and beyond.

The rise of autophagy as a therapeutic target

Cancer is the second leading cause of death in the USA by a minute margin expected to close within the next decade [1]. In 2015, the Surveillance, Epidemiology, and End Results program sponsored by the National Cancer Institute projects 1,658,370 new cancer cases and 589,430 cancer-associated deaths in this country alone [2]. Such statistics are sobering and continue to fuel the work of translational medicine. Although the silver bullets of imatinib in BCR-ABL-expressing leukemia and trastuzumab in HER2-overexpressing breast cancer are encouraging, the vast majority of cancer patients still receive a generic therapeutic regimen consisting of cytotoxic chemotherapy and radiation [3]. As biomedical research has progressed, it has become clear that cancer is not a single disease: each malignancy is as unique as the individual hosting it. This unfortunate fact has presented the biomedical research community with the immense challenge of treating each patient uniquely, which is a concept coined 'precision medicine'.

In theory, precision medicine is simple: for example, if a patient's tumor harbors an activating mutation in the *EGFR* gene and shows dependency upon EGFR signaling,

the patient would be treated with an EGFR inhibitor. In reality, several caveats complicate the precision medicine theory and have slowed the development of a corresponding pharmacological toolkit [4]. First, malignancies are often driven by more than one mutation. The genomic landscape of cancer is incredible, with individual tumors acquiring an average of 50, and as many as 200, somatic mutations [5]. Although the majority of these mutations do not support tumorigenesis, it is estimated that as many as eight or more mutations will play leading roles in this process [5]. As a result, combination therapy approaches are required to treat this disease. However, within current clinical use, combination strategies often result in toxicities that limit their use in human patients. Second, target-matched therapeutic options are extremely limited. In fact, it is estimated that only 5% of the cancer genome has been successfully drugged [6]. In the case of most tumor suppressors and the prominent oncogene *RAS*, small molecule inhibitors have been unsuccessful thus far, labeling these genomic drivers of disease undruggable. With such a limited therapeutic toolbox, the overall impact of precision medicine has slowed. Last, patients receiving some form of precision therapy often still experience thera-

Abigail R Solitro*¹
& Jeffrey P MacKeigan**¹

¹Center for Cancer & Cell Biology, Van Andel Research Institute, 333 Bostwick Avenue, Grand Rapids, MI 49503, USA

*Author for correspondence:

Tel.: +1 616 234 5681

Fax: +1 616 234 5733

abigail.solitro@vai.org

Author for correspondence:

jeff.mackeigan@vai.org

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Key terms

Autophagosome: A double-membrane vesicle capable of sequestering cytosolic material and delivering it to the lysosome for degradation.

Lysosomotropic: The characteristic of being drawn to and trapped within the acidic lysosomal compartment.

peutic resistance as tumors employ bypass mechanisms for survival [7]. Although a malignancy may exhibit a dependency upon EGFR signaling, the tumor has the ability to switch signaling dependencies when exposed to a therapeutic insult. This phenomenon has been observed to contribute to the clonal evolution that permits tumor relapse following initial treatment [8,9].

Despite the challenges currently facing precision medicine, biomedical researchers have discovered their own bypass mechanisms in an attempt to outsmart human tumors. One strategy is to target a signaling node common to several pathways in the cancer cell. Since it has been established that tumors display multiple drivers of disease and potential back-up drivers, targeting a single node would seemingly limit toxicity to the patient while still inhibiting several of the tumor's dependencies. The roadblock that has limited the development of this strategy is the lack of a therapeutic margin. Ideally, precision medicine would minimize the negative effects to nontransformed cells that are often observed with cytotoxic chemotherapy and radiation. Unfortunately, the top prospects for targetable signaling nodes play essential roles in the survival of both transformed and nontransformed cells. A second strategy seeks to target a downstream effector pathway of a currently undruggable target. This approach is best exemplified in the work

of the National Cancer Institute's RAS Initiative. Since the RAS isoforms lack small molecule inhibitors, extensive research has identified targetable effector pathways that are preferentially activated by oncogenic RAS mutations [10]. Among other exciting discoveries, autophagy has been implicated as one such effector pathway.

Autophagy is defined as an intracellular recycling process in which cells degrade cytosolic material for reuse. As illustrated in Figure 1, the process is initiated with the engulfment of cytosolic material such as damaged mitochondria into a double membrane organelle called the **autophagosome**. The process is complete after the fusion of a lysosome with the autophagosome allows the degradation of the engulfed material. Although all cells are thought to undergo a basal level of autophagy to maintain cellular homeostasis, the oncogenic mutations harbored by cancer cells often upregulate this process [11,12]. As in KRAS-mutated non-small-cell lung cancer, the upregulation of autophagy has been synonymous with an increased dependence upon this process, theoretically providing a therapeutic window where a patient's malignancy could be preferentially targeted by autophagy inhibitors. These recent findings coupled with the existence of FDA-approved autophagy inhibitors has allowed for an expedited preclinical and clinical investigation of autophagy's role in tumorigenesis. In this review, we pay tribute to the lessons learned from the first autophagy inhibitors and discuss the field's rapid evolution toward clinical relevance.

Antimalarial drugs as autophagy inhibitors

The first compounds termed autophagy inhibitors were not designed as such, but were rather reper-

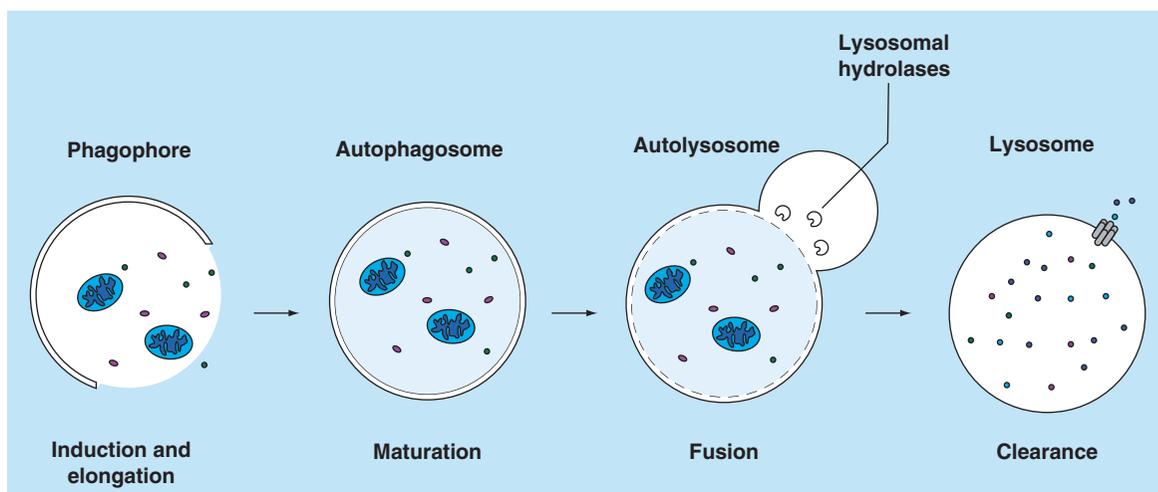


Figure 1. The stages of autophagy. Autophagy is categorized into stages defined by the primary organelle present in the process. The induction and elongation stages describe the formation of a double membrane structure called the phagophore, and its subsequent elongation. Maturation occurs when the phagophore closes to form a cytosolic material-filled autophagosome. The fusion stage defines the formation of the autolysosome, a product of fusion between a mature autophagosome and a functional lysosome. Clearance describes the active degradation and recycling of engulfed material by lysosomal hydrolases.

posed from their initial use as antimalarial agents. The development of these autophagy inhibitors has a long, rich history that began with the Peruvian people's use of cinchona tree bark to ameliorate fever and other malaria-associated symptoms in the early 1600s (major events are reviewed in Figure 2). When Jesuit priest missionaries visited Peru, they observed the natives' practices and, recalling the deadly effects of malaria in Europe, transported the bark across the Atlantic Ocean [13]. In the 1800s, French chemists successfully extracted pure quinine from the cinchona bark and showed its curative effects on malaria patients. This achievement marked the beginning of the race for antimalarial compounds. Extracted quinine was used extensively throughout the 19th century; in fact, over 25,000 kg was used by Union troops alone in the American Civil War [13]. Its use was limited, however, by access to cinchona bark. During World War I, these limitations resulted in casualties experienced by both sides; many sources have even claimed that malaria posed a greater threat to human life than the war itself. As synthetic chemistry advanced, German scientists at the Bayer pharmaceutical company introduced their first line of quinine-related compounds during World War II [14]. Included in the line-up was chloroquine, now considered the founder of lysosome-targeted autophagy inhibitors. Interestingly, due to toxicity issues, chloroquine was dismissed for human use by both Germany and the USA in early clinical studies; it took an extensive clinical trial comparing all synthetic antimalarial compounds to show that chloroquine was in fact superior in human patients [13]. If not for the persistence of scientists, autophagy inhibitors

like chloroquine might have never been developed [14].

Although chloroquine was predominantly used to treat malaria and inflammatory-related diseases in the early and mid-1900s, the rise of biomedical research and the identification of autophagy sparked several key observations related to cancer. In the 1960s, chloroquine was coined as a fibroblast-inhibiting agent following observations of slowed proliferation and migration *in vitro* [15]. In addition, the efficacy of radiation was improved by chloroquine treatment *in vivo* and lysosomal dysfunction was observed in treated animals' livers [16,17]. During the following decade, several instances of lysosomal damage were reported in animals receiving chloroquine treatment, officially labeling the compound as **lysosomotropic** [18–21]. The primary discovery at this point was chloroquine's mechanism of action: the compound readily crossed the lysosomal membrane and became protonated, causing its accumulation within the lysosome. Chloroquine's continued sequestration caused a significant increase in the lysosome's pH, inactivating acid hydrolase enzymes and rendering the lysosome nonfunctional [22–24]. In the case of malaria, in which parasites hijack the lysosomal system within red blood cells to provide a continuous nutrient supply, the past successes of chloroquine in malaria patients were elucidated.

As the biomedical research field's understanding of autophagy expanded in the 1980s, chloroquine's known effects on the lysosome suggested a connection to the intracellular recycling process. The first studies of chloroquine's effects on autophagy illustrated an accumulation of autophagosomes following

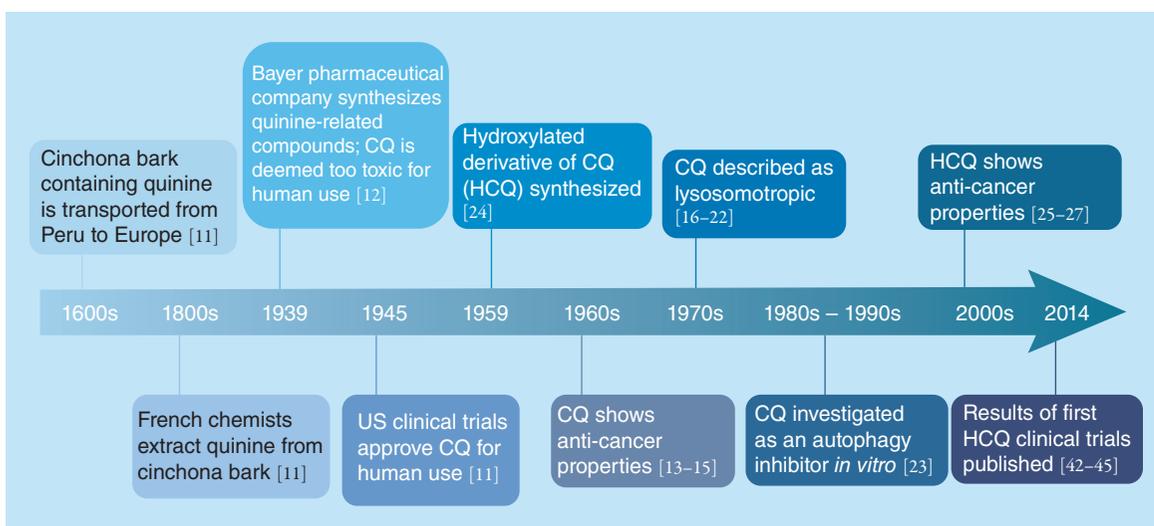


Figure 2. Key events in the history of hydroxychloroquine. The serendipitous identification of the first autophagy inhibitors, CQ and HCQ, has a long, rich history. Events critical to the clinical investigation of HCQ in autophagy research are outlined.

CQ: Chloroquine; HCQ: Hydroxychloroquine.

treatment, which led researchers to incorrectly conclude that chloroquine was inducing autophagy [25]. In the 1990s and early 2000s, it was discovered that chloroquine affected autophagy by inactivating the lysosome, just as had been established in malaria research. It was at this point that a more complete picture of autophagy came into view: the accumulation of autophagosomes observed across multiple malignancies both *in vitro* and *in vivo* occurred as a result of the process being blocked in the final stages. Although the field had serendipitously uncovered an autophagy inhibitor, chloroquine was not the perfect compound. Although it was FDA approved and well characterized, chloroquine was known for severe side effects in human patients, especially after prolonged use. In 1959, a hydroxylated version of chloroquine was synthesized to reduce the retinopathy, indigestion and tinnitus effects of treatment, while maintaining the benefits of oral bioavailability and fast gastrointestinal absorption [26]. Like chloroquine, hydroxychloroquine was primarily investigated in malaria and inflammatory disease research until the 2000s. At the turn of the century, the autophagy field was primed to investigate the next lysosomotropic agent as a potential autophagy inhibitor. *In vitro* studies were intriguing, showing apoptosis after hydroxychloroquine treatment across numerous cancer cell lines as well as the stalling of growth and proliferation in breast cancer cells [27–29]. These initial studies paved the way for further investigation of hydroxychloroquine in the context of cancer.

Hydroxychloroquine in cancer clinical trials

The biomedical research field was slow to translate the observations of lysosomotropic agents out of the malaria research field. However, the importance of autophagy in the process of tumorigenesis had been well established. The first records of neoplastic autophagy occurred in the 1970s in lung tumors, but the phenomenon quickly expanded to both breast and liver tumors as well [30–32]. These early observations were all made using biochemical techniques, such as acid phosphatase staining of the lysosome and transmission electron microscopy to visualize autophagosomes [33,34]. From the initial observations of basal autophagy in cancer cells, the important discovery that standard chemotherapeutic agents induced autophagy was made. In the 1980s, the popular cytotoxic therapies vincristine and vinblastine were shown to cause autophagosome accumulation [35,36]. Around the same time, radiation was found to do the same across multiple malignancies [37]. These findings led to the widely accepted hypothesis that tumors use autophagy as a chemoresistance mechanism.

The establishment of basal and therapeutic stress-induced autophagy in several cancer contexts supported the investigation of hydroxychloroquine in clinical trials. Unfortunately, progress on this front was stalled by controversies in the field regarding the true role of autophagy in cancer. A seminal publication revealed that haploinsufficiency of the essential autophagy gene *BECN1* accelerated tumor formation in a mouse model of breast cancer [38]. This work suggested that autophagy was tumor suppressive in nature and therefore, argued against the use of autophagy inhibitors in cancer patients. In addition, several accounts of the so-called autophagic cell death process caused the field to question autophagy's role as a survival mechanism [28]. As the field expanded, several studies demonstrated autophagy's tumor-promoting role in cancer [39–41]. The first supporting evidence was born from the observation that autophagy could be exploited as a survival mechanism. Upon growth factor withdrawal, bone marrow cells activated autophagy to recycle essential nutrients and maintain cellular homeostasis and viability [42]. Building upon these findings, *in vitro* and *in vivo* analyses showed that loss of autophagy sensitized apoptosis-defective, tumorigenic epithelial cells to necrotic cell death [43]. In addition, due to the advancement of RNA interference technology, comprehensive analyses of mammalian homologues of the 30 autophagy-related (ATG) genes in yeast were performed [44]. These *in vitro* findings coupled with the most recent *in vivo* studies of cancer mouse models have led the field to accept a binary role for autophagy in cancer: autophagy acts as a tumor suppressor in early tumorigenesis and promotes tumor progression in later stages of the process. Perhaps the best examples of the tumor-promoting capabilities of autophagy come from recent mouse models of oncogenic *KRAS*- and *BRAF*-driven non-small-cell lung cancer, which have shown the necessity of functional autophagy for the development of invasive disease [12,45]. Lymphoma mouse models have also been informative, as chloroquine derivatives have been shown to synergize with chemotherapy through autophagy inhibition to improve disease outcomes [46,47].

Once the preclinical evidence for the role of autophagy in tumorigenesis was deemed sufficient, clinical trials investigating hydroxychloroquine across multiple malignancies ensued. The ClinicalTrials website reports over 50 studies of hydroxychloroquine in cancer that are either completed or currently active. The majority of these studies have been at the Phase I or II level, providing limited information on tumor burden and patient survival benefits. However, eight clinical trials have been successfully completed with published results that are summarized in Table 1. First,

hydroxychloroquine was administered as a single agent twice daily for 2 months to metastatic pancreatic ductal adenocarcinoma (PDAC) patients who had previously received standard-of-care chemotherapy [48]. Data were collected from 20 patients and revealed that both 400 and 600 mg doses of hydroxychloroquine were safe and tolerable. In addition, the *in vitro* autophagy marker LC3 proved to be a reliable indicator of autophagy inhibition in peripheral lymphocytes *in vivo*. The major challenge encountered during this study was the variability of autophagy inhibition observed between patients receiving the same dose of hydroxychloroquine. In August of 2014, results from six clinical trials were published simultaneously. One study, performed in dogs with spontaneous lymphoma, indicated cooperation between hydroxychloroquine and doxorubicin to achieve a 93% overall response rate [49]. Along with determining a maximum tolerated dose (MTD) of 12.5 mg/kg/day for hydroxychloroquine, drug accumulation was found to be enriched in the tumor tissue when compared with plasma. An additional trial evaluated the safety and tolerability of hydroxychloroquine in combination with bortezomib in relapsed or refractory myeloma patients [50]. Efficacy of hydroxychloroquine was demonstrated by the accumulation of autophagic vesicles in bone marrow cells, and nearly half of all patients demonstrated some length of stable disease during treatment. Hydroxychloroquine-attributed toxicities were not observed and a 600 mg dose was recommended for future studies. A third study investigated a combination treatment strategy of the mTOR inhibitor temsirolimus and hydroxychloroquine [51]. In 27 patients harboring a variety of solid tumor malignancies, escalating doses of hydroxychloroquine were administered twice daily in the presence of a fixed dose of temsirolimus. Results from this study showed the safety and tolerability of hydroxychloroquine in this drug combination, as well as a stable disease state in over two-thirds of the patient cohort. Although a MTD of hydroxychloroquine was not reached, the study sponsors recommended a 600 mg dose for future trials. A separate trial also investigated patients with various solid tumor malignancies, but employed a combination therapy approach of temozolamide and hydroxychloroquine [52]. In total, 40 patients were dose-escalated from 200 to 1200 mg of hydroxychloroquine daily with a fixed dose of temozolamide. Confirming the results from the previous study, a MTD was not achieved and 600 mg was recommended for future studies. Additionally, stable disease was observed in a fraction of patients and was enriched in those with melanoma. Another study published in this set was conducted to investigate hydroxychloroquine in combination with vorinostat in

patients with advanced solid tumors [53]. Similar to the previous study, patients were treated with escalating doses of hydroxychloroquine with a fixed dose of vorinostat. A MTD of 600 mg for hydroxychloroquine was achieved and recommended for future studies. In a study of hydroxychloroquine in addition to temozolamide and radiation in patients with glioblastoma multiforme, escalating doses of hydroxychloroquine were administered and 600 mg was determined to be the MTD due to toxicities such as neutropenia and sepsis [54]. Autophagy inhibition as assessed by LC3 protein levels and autophagosome number in peripheral blood mononuclear cells correlated with dose escalations. The latest clinical trial with published results investigated hydroxychloroquine in combination with gemcitabine in PDAC patients [55]. With no dose-limiting toxicities, this treatment strategy caused significant decreases in CA 19–9, the most common disease biomarker for PDAC. In addition, the mean overall survival was extended to nearly 3 years, and the results of these preliminary trials appear promising as *in vitro* markers of autophagy have been corroborated and stable disease has been achieved in some instances. However, with only eight completed trials thus far, it is difficult to make concrete statements about the efficacy and potential success of hydroxychloroquine in the treatment of cancer. Clearly, although generally safe and tolerable, the MTD of hydroxychloroquine is expected to vary between malignancies and within combination strategies. It will be crucial for those clinical trials currently underway to report similar data as soon as results become available.

Improving autophagy inhibition at the lysosome

As both preclinical and clinical trials of hydroxychloroquine continue, a parallel movement has begun to investigate and develop more potent lysosomotropic agents. Although hydroxychloroquine offers the advantages of being FDA approved, well characterized and orally available, there is room for improvement. One negative aspect of hydroxychloroquine is the requirement of micromolar levels to obtain sufficient autophagy inhibition in patients. The pioneering clinical trials for hydroxychloroquine in cancer have shown significant interpatient variability of autophagy inhibition, even with 1200 mg doses taken twice daily. In addition, the compound's half-life registers at 50 days, and inflammatory disease (e.g., rheumatoid arthritis) patients have been shown to retain hydroxychloroquine in their system up to 5 years after receiving therapy [56]. This characteristic of hydroxychloroquine likely accounts for the chronic side effects observed in malaria and inflammatory disease patients, includ-

Table 1. Hydroxychloroquine success in clinical trials.				
Study identifier	Disease context	Treatment regimen	Major findings	Ref.
NCT01273805	PDAC	HCQ	<ul style="list-style-type: none"> • HCQ was safe and tolerable at 600 mg • LC3 protein levels were a reliable marker of drug efficacy <i>in vivo</i> • Degree of autophagy inhibition varied between patients 	[48]
n/a	Lymphoma	HCQ and doxorubicin	<ul style="list-style-type: none"> • HCQ was safe and tolerable at 12.5 mg/kg in dogs • HCQ was enriched in tumor tissue • 93% overall response rate was achieved 	[49]
NCT00568880	Myeloma	HCQ and bortezomib	<ul style="list-style-type: none"> • HCQ was safe and tolerable at 600 mg • Stable disease was achieved in 45% of patients • Accumulation of autophagic vesicles was observed in bone marrow cells 	[50]
NCT00909831	Various solid tumors	HCQ and temsirolimus	<ul style="list-style-type: none"> • HCQ was safe and tolerable at 600 mg • Stable disease was achieved in 67% of patients 	[51]
NCT00714181	Various solid tumors	HCQ and temozolamide	<ul style="list-style-type: none"> • HCQ was safe and tolerable up to 1200 mg • Stable disease was achieved in a small fraction of patients • Melanoma patients responded better than those with other malignancies 	[52]
NCT01023737	Various solid tumors	HCQ and vorinostat	<ul style="list-style-type: none"> • HCQ was safe and tolerable at 600 mg 	[53]
NCT00486603	GBM	HCQ, temozolamide and radiation	<ul style="list-style-type: none"> • HCQ was safe and tolerable at 600 mg • Neutropenia and sepsis were observed in this treatment regimen • LC3 protein levels and autophagosome number in PBMCs correlated with autophagy inhibition 	[54]
NCT01128296	PDAC	HCQ and gemcitabine	<ul style="list-style-type: none"> • HCQ was safe and tolerable up to 1200 mg • PDAC biomarker CA 19–9 levels were decreased during treatment • Mean overall survival was extended to 34.8 months 	[55]

Currently, eight clinical trials investigating hydroxychloroquine in cancer have published results. The defining characteristics and major findings are summarized. GBM: Glioblastoma multiforme; HCQ; Hydroxychloroquine; PBMC: Peripheral blood mononuclear cell; PDAC: Pancreatic ductal adenocarcinoma.

ing retinopathy and indigestion. It would be naive to assume that these same side effects would not occur when taken long-term in cancer patients. Coupled with the high doses of hydroxychloroquine required *in vivo*, the compound's long half-life could eliminate any therapeutic margin gained in patients. As mentioned previously, there is evidence that upregulated autophagy and **autophagy dependence** are unique to cancer cells, theoretically providing a therapeutic window to preferentially affect a tumor with autophagy inhibitors. However, the maintenance of high levels of hydroxychloroquine in a cancer patient will inevitably affect nontransformed cells as well. Lastly, the limited acute toxicities observed in malaria and inflammatory disease patients have not been maintained in a cancer context. As discussed previously, the clinical trials performed thus far have shown dose-limiting toxicities such as sepsis when hydroxychloroquine is used in combination treatment strategies. Increased potency, or the same level of autophagy inhibition at a lower dose, coupled with a shorter

half-life compound, will be important improvements if lysosomotropic agents are to be successfully paired with other anticancer agents. Significant progress has already been made both in the identification of existing compounds that display lysosomotropic characteristics and in the development of novel compounds. In many cases, compounds designed for other uses have been observed to show lysosomal accumulation. For example, the S2R agonist siramesine, developed for the treatment of anxiety, was serendipitously found to induce lysosomal dysfunction *in vitro*. Upon further investigation, the compound's mechanism of action was found to be identical to that of chloroquine and its derivatives; the passive accumulation of siramesine in the lysosome led to an increased pH and inactivation of acid hydrolase enzymes [57]. Within a breast cancer context, this repurposed lysosomotropic agent illustrated autophagosome accumulation, indicative of autophagy inhibition [57]. Similarly, ML-9, an inhibitor of AKT and other kinases, exhibited lysosomotropic properties resulting in an inactivated lysosome

and autophagy inhibition. In prostate cancer cell lines, ML-9's autophagy inhibitory effects sensitized cells to chemotherapeutic treatment [58]. In addition to small molecule agents, antimalarial compounds that possess structural similarity to chloroquine and its derivatives have been investigated for their lysosomotropic properties. Mefloquine, which is currently FDA approved as an antimalarial compound, not only shows lysosome-inactivating capabilities in breast cancer cells, but illustrates improved potency over chloroquine *in vitro* [59,60]. Structurally related to mefloquine is quinacrine, a compound that has been extensively evaluated across numerous malignancies. By the same mechanism elucidated in all previous lysosomotropic agents, quinacrine has exhibited autophagy inhibition in brain cancer, osteosarcoma and melanoma contexts [60,61]. Although quinacrine has shown as much as a 60-fold increase in potency over chloroquine, its prior use in sterilization techniques has tainted its name and raised questions about the potential long-term side effects of use [62]. The successes observed in repurposing compounds as lysosomotropic agents have largely been *in vitro*. Although suggestive of clinical relevance, there remains a significant amount of work to be done in validating the safety and efficacy of these compounds in preclinical models. In addition, the majority of *in vitro* studies have compared the efficacy and potency of novel agents to chloroquine. As mentioned previously, chloroquine is regarded as the founder of lysosomotropic autophagy inhibitors, but is no longer relevant in human patients due to the long-term toxicity profiles observed across numerous diseases [26]. Hydroxychloroquine, which shows improved toxicity, should be

Key term

Autophagy dependence: The state of relying on autophagy for cell survival, as in oncogene addiction.

the benchmark that all compounds are compared with, especially in research expected to translate to clinical trials.

The identification of existing lysosome-targeted autophagy inhibitors has occurred concurrently with the development of novel lysosomotropic agents. The idea for the first novel compound, developed from Amaravadi and colleagues, was born out of extensive research in both the preclinical and clinical space. Upon observing the success of hydroxychloroquine in patients, a chemical synthesis program was launched to construct a bivalent aminoquinoline compound based on the structure of two chloroquine molecules fused together. Lys05 was the product of this program, and has since shown tenfold improved potency over hydroxychloroquine both *in vitro* and *in vivo*. Additionally, Lys05 has shown antitumor activity in mouse models of multiple malignancies, albeit with acute toxicities including pseudo-obstruction of the gastrointestinal tract [63]. Nevertheless, Lys05 represents a promising example of a novel drug development effort. In an extensive attempt to synthesize novel lysosomotropic agents, a recent study used chloroquine as a backbone to rationally synthesize over 60 new compounds [24,60]. Each compound was screened for their autophagy inhibitory effects and as a result, two compounds were identified as superior to chloroquine in both efficacy and potency. Coined VATG-027 and VATG-032, the drugs showed cytotoxic and cytostatic proper-

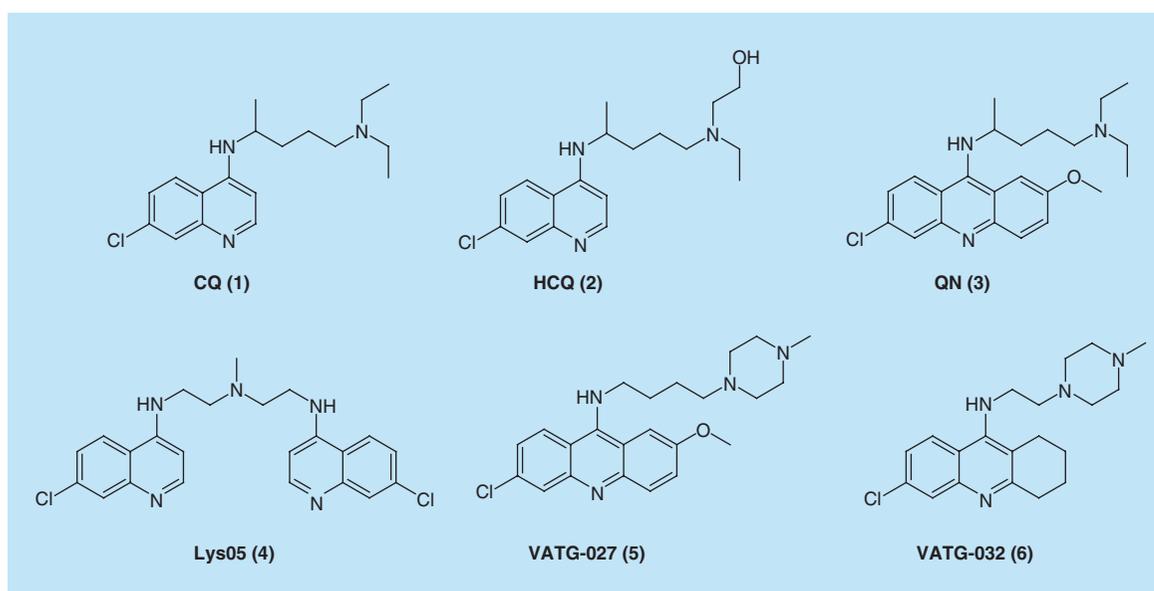


Figure 3. Structures of prominent lysosomotropic agents. Chloroquine (CQ, 1), hydroxychloroquine (HCQ, 2), quinacrine (QN, 3), Lys05 (4), VATG-027 (5) and VATG-032 (6).

ties, respectively, providing opportunities to investigate their potential synergy in combination treatment strategies. The structures of these newly synthesized compounds are compared with the parent antimalarial compounds in Figure 3 (chloroquine – 1, hydroxychloroquine – 2, quinacrine – 3, Lys05 – 4, VATG-027 – 5, VATG-032 – 6).

The identification and development of potent lysosomotropic agents that supersede hydroxychloroquine are early-stage research endeavors. There is a necessity for *in vivo* validation of lead compounds in established tumor models, in order to establish the characterization of lysosome-targeted compounds as a worthy endeavor. However, the benefits of lysosomotropic autophagy inhibitors are significant. By blocking autophagy in the final stage of the process, full inhibition is ensured since the lysosome is the only degradative organelle capable

of recycling autophagosomal material. In addition, it has been suggested that the accumulation of autophagosomes is, in itself, toxic to cells; only a blockade in the final stages of autophagy would achieve this effect. Researchers have recently identified a tendency for oncogenic *RAS*-driven cancer cells, among others, to display a dependence upon **macropinocytosis** [64]. A process used to engulf extracellular material, macropinocytosis utilizes endosomes to transport engulfed material to the lysosome for degradation and recycling. Autophagy inhibitors acting at the level of the lysosome would undoubtedly inhibit other pathways that cancer cells rely on, such as macropinocytosis. There are likely processes not yet discovered that converge at the lysosome and support cancer cell survival; by inhibiting autophagy at this point, all pathways would be blocked simultaneously [65]. Unfortunately, the negative side

Table 2. Therapeutic potential of essential autophagy machinery.

Autophagy-associated proteins	Role in process	Potential for therapeutic targeting	Ref.
ATG1/ULK1	Protein kinase	§	[67–69]
ATG3	E2-like enzyme	‡	[70]
ATG4	Cysteine protease	‡	[71]
ATG5	E3-like enzyme	‡	[72]
ATG6/BECLIN1	VPS34-associated protein	†	–
ATG7	E1-like enzyme	‡	–
ATG8/LC3	Membrane-associated protein	†	[70]
ATG9	Transmembrane protein	†	–
ATG10	E2-like enzyme	‡	–
ATG12	Scaffolding protein	†	–
ATG13	ATG1-associated protein	†	–
ATG14	VPS34-associated protein	†	–
ATG16	ATG5/12-associated protein	†	[72]
ATG17/FIP200	ATG1-associated protein	†	–
ATG18/WIP1	ATG9-associated protein	†	–
ATG29	ATG1-associated protein	†	–
ATG31	ATG1-associated protein	†	–
ATG101	ATG1-associated protein	†	–
DFCP1	Membrane-associated protein	†	–
RUBICON	VPS34-associated protein	†	–
UVRAG	VPS34-associated protein	†	–
VPS15	VPS34-associated protein	†	–
VPS34	Lipid kinase	§	[73–77]

All autophagy-related (ATG) and autophagy-associated proteins currently known were scored for their potential to be therapeutically targeted.

†Unlikely to be directly targeted.

‡Low therapeutic potential.

§High therapeutic potential and/or drug development is underway.

effects associated with these compounds most likely stem from the full inhibition of an essential organelle; in this way, the compounds' top strength can also be viewed as their biggest weakness. Also, the lysosomotropic nature of this class of drugs results in longer half-lives and requires higher doses to be effective. It is likely that such compounds will always be inferior to small molecule inhibitors in this regard. As a result of these opposing variables, it is essential for lysosomotropic agents, rediscovered or newly developed, to be fully evaluated *in vivo*.

Novel therapeutic targets in the autophagy pathway

Potential therapeutic targets of autophagy inhibitors can seemingly be divided into two categories: key molecules associated with autophagy and major steps within the process. As displayed in Figure 1, the autophagy pathway is organized into distinct phases: induction of the membrane source forming the phagophore, elongation of the membrane and engulfment of intracellular material, maturation into the cargo-filled autophagosome, fusion with the lysosome and degradation of engulfed material. Lysosomotropic agents, as discussed in the previous section, inhibit the steps of autophagy involving the lysosome. The additional phases could be therapeutically targeted as well; however, since these earlier phases involve organelles lacking distinct characteristics such as a nonphysiological pH, it would be difficult to design compounds capable of inhibiting these structures. The only step that has been targeted, in addition to the lysosome's degradation of intracellular material, is the fusion of the autophagosome with the lysosome. Bafilomycin A1 is characterized as a vacuolar-type proton pump inhibitor that is capable of increasing the lysosome's pH by preventing the influx of hydrogen ions. Studies have also shown that this compound inhibits autophagy at the fusion stage, however the mechanism remains to be elucidated [66].

The majority of the field has turned its focus to the development of inhibitors targeting key molecules associated with autophagy. As mentioned previously, there exist at least 30 *ATG* genes in yeast, all of which have homologues in mammalian systems. These do not include the non-*ATG* genes known to encode proteins involved in this recycling process. The proteins that have been established as associated with, and in many cases essential for, the autophagy process include protein and lipid kinases (i.e., ATG1, VPS34), ubiquitin-like enzymes (i.e., ATG3, ATG7) and a cysteine protease (i.e., ATG4), all of which show therapeutic potential. Table 2 includes a list of proteins essential for autophagy and their suspected therapeutic potential.

Key terms

Macropinocytosis: The process of engulfing extracellular material for digestion and recycling by the lysosome.

Structure–activity relationship: A description of the common characteristics linking a molecule's physical state to its function.

Although efforts are only just beginning to identify and develop small molecule inhibitors of autophagy, several preliminary studies are worth reviewing. ULK1, the mammalian homologue of the yeast ATG1 protein, is the only known protein kinase essential for autophagy and consequently, is an ideal therapeutic target [67]. Only two studies investigating ULK1 inhibitors have been published thus far, however, several pharmaceutical companies are currently collaborating with academic laboratories to expand the biomedical research field's knowledge of autophagy and the subsequent development of small-molecule autophagy inhibitors. One approach currently employed is to utilize high-throughput screening methods to evaluate existing kinase inhibitors for selectivity toward ULK1. The single published study in this realm illustrated nanomolar half maximal effective concentration (EC_{50}) values of TBK1 inhibitors against ULK1 [68]. Although effects on autophagy as a whole were obvious and easily measured by LC3 protein levels, challenges arose in verifying ULK1 inhibition. Since the field's current knowledge of direct substrates of ULK1 is limited, and as antibodies for known substrates are few and far between, validating a bona-fide ULK1 marker will prove to be the biggest hurdle for those entering the ULK1 inhibitor race. Another approach is to develop novel kinase inhibitors selective for ULK1. In the only study completed thus far, a small molecule inhibitor of ULK1 was developed using a highly cross-reactive FAK inhibitor as a chemical backbone [69]. Pyrimidine analogs were then screened for ULK1 inhibition, and **structure–activity relationship** analyses were then used to expand upon the pyrimidine scaffold. In addition, novel substrates of ULK1 were elucidated using phosphorylation site consensus mapping and were used to verify ULK1 selectivity with newly developed antibodies. Preliminary analyses *in vitro* showed low micromolar EC_{50} values for the ULK1 inhibitor, as well as improved inhibition compared with chloroquine. Not only has this study established a benchmark for novel ULK1 inhibitors, but Shaw and colleagues have provided a working toolbox for the future validation of small molecule inhibitors of autophagy.

As the only lipid kinase currently known in the autophagy pathway, VPS34 shows potential for therapeutic targeting equivalent to that of ULK1. The few studies of VPS34 inhibitors have all employed

The advancement of lysosome-targeted autophagy inhibitors, though necessary and relevant, will likely be overshadowed by the novelty of small molecule inhibitors. The newness of the autophagy field in general has spawned emotions of awe and wonder as key players within the pathway are discovered and further elucidated every month. The list of targetable molecules in Table 2 will continue to grow and be fine tuned. While the field develops, top prospects like ULK1 and VPS34 are expected to make headlines, assuming their continued success in the preclinical space. Especially in the case of ULK1, as numerous collaborations are being born, the time to clinical relevance will be streamlined. Over the next decade, it is possible that an ULK1 inhibitor will make its way to a clinical trial.

This is indeed an exciting time for the autophagy field. From the observation of the lysosome in the 1950s by Christian de Duve, the biomedical research community's knowledge of autophagy and its involvement in human disease has grown exponentially. The serendipitous mechanism of action of antimalarial compounds accelerated investigations of autophagy inhibition in cancer patients. With hydroxychloroquine leading the way, the field now has the privilege

of watching more potent lysosomotropic agents and small molecule inhibitors hit the stage. As research and development continues over the next several years, autophagy inhibitors are expected to be the topic of numerous publications, scientific meetings and hallway conversations. No matter the area of cancer research, autophagy inhibitors will make a significant impact.

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Executive summary

The rise of autophagy as a therapeutic target

- Autophagy has emerged as a valid therapeutic target across multiple malignancies. Not only is autophagy employed as a chemoresistance mechanism, but numerous cancer types display a natural dependence upon this intracellular recycling process.

Antimalarial drugs as autophagy inhibitors

- The first autophagy inhibitors were born out of antimalarial research. When the lysosome-targeted mechanism of action for chloroquine and its derivative hydroxychloroquine was elucidated, the drugs became natural prospects for the inhibition of autophagy.

Hydroxychloroquine in cancer clinical trials

- The path to clinical trials for hydroxychloroquine was expedited due to its prior FDA approval and extensive characterization in the treatment of both malaria and inflammatory diseases. Published results have thus far shown the safety, efficacy and tolerability of hydroxychloroquine in cancer patients, both as a single agent and in combination treatment strategies. In some cases, stable disease has even been achieved.

Improving autophagy inhibition at the lysosome

- Despite the success of hydroxychloroquine in early clinical trials, more potent lysosomotropic agents are under development to address potential toxicity issues. Although studies are limited to *in vitro* characterization and mouse models, several lysosome-targeted compounds have shown improved potency over hydroxychloroquine.

Novel therapeutic targets in the autophagy pathway

- As the autophagy field continues to expand in both breadth and depth of knowledge within cancer, small molecule inhibitors of key autophagy enzymes are under investigation. Top prospects currently include ULK1 and VPS34, two kinases that are essential for the induction of autophagy.

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