CHAPTER

12

Autophagy Inhibition and Chemosensitization in Cancer Therapy

Emmanuel K. Cudjoe, Jr.[*,**], S. Lauren Kyte*[*,**], Tareq Saleh*[*,**], Joseph W. Landry*[*,**], David A. Gewirtz*[*,**]

*Virginia Commonwealth University, Richmond, VA, United States
**Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, United States

Abstract

Autophagy is a cellular degradation mechanism involving protein turnover as well as the recycling of excessive and dysfunctional organelles. Autophagy is generally considered to occur at basal levels in cells, but may be induced to higher levels under conditions of stress, such as starvation, hypoxia, ionizing radiation treatment, or chemotherapy. Aberrations in the autophagic machinery may represent either cause or effect in various diseases. In cancer, autophagy has been shown to be one of the mechanisms affecting the response to chemotherapy and/or radiation treatment. In this chapter, the different types and functional forms of autophagy, the autophagy signaling pathway, as well as the role of autophagy in transformation and tumor suppression are discussed. Modulation of autophagy for improving tumor cell chemosensitivity and radiosensitivity is currently being explored in clinical trials; the potential impact and limitations of these studies are discussed. We also briefly review the intersection between autophagy and the immune system with regard to the effectiveness of chemotherapy. The chapter concludes with future directions in cancer treatment in relation to novel therapies and potential drug targets for autophagy modulation.

ABBREVIATIONS

AMBRA1	Autophagy/Beclin-1 regulator 1
AMPK	AMP kinase
Atg	Autophagy-related
AV	autophagic vacuole
BMPCs	Bone marrow plasma cells
CTCs	Circulating tumor cells
CMA	Chaperone-mediated autophagy

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CQ	Chloroquine
DAMP	Danger associated molecular pattern
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
HCQ	Hydroxychloroquine
HIF-1a	Hypoxia-induced factor 1a
HMGB1	High-mobility group box 1
ICD	Immunogenic cell death
LC3	Microtubule-associated protein light chain 3
mTOR	Mammalian target of rapamycin
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PtdIns3K	Phosphatidylinositol 3-kinase
ROS	Reactive oxygen species
SMERs	Small-molecule enhancers of rapamycin
SR-2	Sigma-2 receptor
STAT3	Signal transducer and activator of transcription
Ubl	Ubiquitin-like
ULK	Unc-51-like kinase
UVRAG	Ultraviolet radiation resistance-associated gene
Vps34	Vacuolar sorting protein 34

12.1 INTRODUCTION

Cancer currently constitutes one of the highest disease burdens in the United States and the world at large. In addition to surgery and radiation therapy, chemotherapy remains one of the primary therapeutic options for this disease. Unfortunately, there are various strategies whereby cancer cells are able to evade chemotherapy-induced cell death and continue to proliferate. These include, but are not limited to, the upregulation of multidrug efflux pumps, enhanced DNA repair capability, evasion of apoptosis, and autophagy, which is the focus of this chapter [1]. This chapter will also address how autophagy may, in theory and in practice, be manipulated to improve the response to conventional or targeted cancer therapy.

Over the last few decades, autophagy has become a widely studied cellular phenomenon, the discovery of which earned Dr. Yoshinori Ohsumi the 2016 Nobel Prize in Physiology or Medicine. Autophagy is a conserved homeostatic cellular process that is present primarily in eukaryotic cells [2]. Autophagy has been defined as a cytoplasmic protein and organelle degradation mechanism involving the lysosomal machinery in which dysfunctional, damaged and/or excess cellular components are recycled for homeostasis, survival, and the production of energy and metabolic precursors [1–5]. Autophagy is also induced above basal levels in various cells in response to stress such as starvation, radiation, and chemotherapy.

12.2 AUTOPHAGY FUNCTION AND ROLE IN CANCER

12.2.1 The Autophagic Machinery

Autophagy has been classified into three main classes, specifically microautophagy, macro-autophagy, and chaperone-mediated autophagy (CMA) [4,6]. This classification is based

largely on the method of delivery of the substrates to the lysosomes, where the substrates are degraded. For example, in CMA, the substrate protein is first selectively identified by a chaperone molecule in the cytosol, then delivered to the surface of the lysosome where it unfolds and crosses the lysosomal membrane [7]. Unlike macro- and microautophagy, CMA has only been identified in mammalian cells, is always targeted to selective cargo, and is used to degrade soluble proteins [4,7,8]. It is known that the proteins targeted for CMA have a motif of amino acids in their sequence that makes them recognizable to their chaperones [9]. In contrast, macro- and microautophagy may be selective or nonselective. The terminology "macroautophagy" is used interchangeably with autophagy in many publications and represents the large-scale autophagy that involves the formation of a double-membrane phagophore which matures into an autophagosome. The autophagosome then fuses with a lysosome to form an autolysosome in which the contents are degraded by hydrolases. Finally, in microautophagy, there is no direct involvement of a delivery machinery and the substrates are engulfed directly by the lysosome where they are degraded by hydrolases [4,10]. Microautophagy, among other processes, is stimulated primarily through cellular nitrogen depletion [11].

The molecular mechanisms of autophagy have been elucidated primarily utilizing yeast models. These studies have led to the discovery of about 36 Atg (autophagy-related) genes, 34 of which are listed by Klionsky et al. [12,13]. The majority of the Atg family proteins discovered in yeast have been found to have mammalian homologs which have essentially similar roles in yeast and in human cells. The initial phase of the (macro)autophagy process has four stages, specifically the induction, nucleation, elongation, and completion steps [8]. When cells are deprived of nutrients via starvation, a classical approach for inducing autophagy, the induction step involves the mammalian target of rapamycin (mTOR—a protein that inhibits autophagy via the inhibition of the class III PI3 kinase/Beclin-1 complex formation), which is inhibited by AMP-kinase, a protein that responds to energy changes within the cell [14–16]. Inactivation of mTOR in turn leads to its dissociation from and hence activation of the Unc-51-like kinases ULK1 and ULK2. The activated kinases then phosphorylate Atg13 and FIP200 following which Atg101 binds and stabilizes Atg13 in the ULK-Atg13-FIP200 complex [17] in the nucleation step. The phosphatidylinositol 3-kinase (PtdIns3K) complex which comprises vacuolar sorting protein 34 (Vps34—a class III PI3 kinase), p150, Atg14, and Beclin-1 is required for the initiation of vesicle nucleation [15,17]. The class III PI3 kinase Vps34, the only one in mammalian cells, is known to associate with Beclin-1, which facilitates its phosphorylation of phosphatidylinositol to phosphatidylinositol-3-kinase (PtdIns3K) [15,18]. Beclin-1 has several binding partner proteins which may serve to promote the process of autophagy, as is the case with Atg14L and the UV radiation resistance-associated gene (UVRAG); conversely, binding of Beclin-1 to Bcl-2 and Bcl-XL results in inhibition of autophagy [15]. After the nucleation phase, elongation is mediated by conjugation of two ubiquitin-like (Ubl) protein complexes—Atg5–Atg12–Atg16 and Atg8–phosphatidylethanolamine—which then leads to the completion step that involves the autophagy-related (Atg) proteins 3, 5, 7, 10, 12, and 16L and microtubule-associated protein light chain 3 (LC3) [8,17,19]. The formation of the Atg5–Atg12–Atg16 complex plays a central role in this process and leads to the conversion of cytosolic LC3-I to the lipidated membrane-bound isoform, LC3-II [19]. Completion of autophagy involves the fusion of the autophagosome with the lysosome to form the autolysosome where the cargo (contents of

the autophagosome) are broken down by hydrolases to generate energy, amino acids for protein synthesis, or other precursors of metabolism.

It should be noted that the mTOR signaling pathway is not the exclusive pathway for the induction of autophagy. As demonstrated by Sarkar et al. [15,20], there are a number of small molecules that may induce autophagy independent of rapamycin and mTOR. These molecules were shown to enhance the effects of rapamycin-induced growth arrest in yeast and were thus termed small-molecule enhancers of rapamycin (SMERs) by Sarkar et al. [20]. It was also previously believed that Atg5 and Atg7 are necessary for autophagy in mammalian cells but an alternative pathway independent of Atg5/Atg7 has been identified [21].

The importance of the ULK1 kinase complex—Vsp34 axis—has been recognized over the last few years. The Beclin1–Atg14L–Vps34–Vps15 complex that has been shown to be important in autophagosome formation has been found to be regulated by ULK1. In recent studies of the relationship between ULK1 and Vps34, it was found that amino acid or glucose starvation resulted in increased activity of Vps34 lipid kinases associated with Atg14L, suggestive of a direct role of ULK1 in the regulation of autophagy through the phosphorylation of Beclin-1 [18,22].

12.2.2 The Functions of Autophagy

Autophagy is primarily considered to be a protective mechanism owing to its recycling and homeostatic functions at basal levels. It is largely believed that cells undergo autophagy as a defense mechanism against death by starvation and other sources of stress. However, there is substantial evidence to indicate that autophagy is not always protective. Other forms of autophagy that have been reported include one that is toxic (also referred to as autophagic cell death), one that results in cell stasis, and one that is nonprotective [23–29]. Briefly, autophagy is cytoprotective when its induction directly results in survival of the cell (i.e., resistance to treatment), in which case its inhibition, either by genetic or pharmacologic means, results in increased sensitivity to treatment and hence cell death. Conversely, cytotoxic autophagy is determined to occur when the promotion of autophagy leads to cell death. This form of autophagy is not obligatorily associated with apoptosis. Confirmation of cytotoxic autophagy is based on the observation that inhibition or suppression of the autophagy promotes cell survival [23]. Autophagy is termed cytostatic when its induction leads to cell growth arrest without pronounced cell death. The fourth and most controversial or least understood functional form of autophagy is the nonprotective form. It is unclear what benefit this form of autophagy might be to the cell in terms of the response to chemotherapy or radiation treatment as sensitivity of cells to treatment is not enhanced with suppression of nonprotective autophagy either through pharmacological or genetic means (i.e., the autophagy by definition does not provide a protective advantage) [26,30,31].

Autophagy has traditionally been considered as only a catabolic process but recent studies have provided evidence to indicate that it has additional functions including cellular secretion (via both the conventional pathway involving the ER/Golgi apparatus and nonconventional pathways involving alternate pathways independent of the ER/Golgi machinery). In cancer, autophagy has been found to have both anti- and protumorigenic roles. It is widely believed that moderate induction of autophagy is protective in most cancer cells, whereas the excessive induction of autophagy in some cancer cells following chemotherapy and/or radiation treatment promotes autophagy-mediated cell death [19].

12.2.3 Autophagy in Transformation and Tumor Suppression

As mentioned previously, autophagy occurs in cells at normal or basal levels and is believed to be part of the cell homeostatic machinery that ensures that excess, damaged, or dysfunctional macromolecules that may induce cellular transformation are removed. Cellular transformation involves the transition of normal cells into the tumorigenic state and is accompanied by alterations in cell morphology as well as cell function, particularly the acquisition of the capacity for uninhibited growth. Autophagy therefore represents one of the checks and balances in place against processes that may lead to uncontrolled proliferation and tumorigenesis. In mice with heterozygous knockdown of the essential autophagy gene Beclin-1, tumorigenesis was significantly higher than in mice with wild-type Beclin-1; similar results have been seen with Atg5, Atg7, and autophagy/Beclin-1 regulator 1 [32]. Also, in many tumor cells, the autophagy pathway is downregulated due to the upregulation of autophagy inhibitors such as the class I PI3 kinase pathway proteins [33]. This lends credence to the assertion that autophagy is a tumor suppression mechanism that limits cellular transformation.

Various tumor cells differ in the extent of their dependence on autophagy. In some, such as pancreatic tumor cells, there is a high level of basal autophagy necessary to maintain the high metabolic demand of proliferating cells [34,35]. Autophagy protects some tumors from cell death by eliminating damaged proteins and preventing the generation of reactive oxygen species (ROS) that lead to DNA damage in developing tumors [31]. In such cases, defects in autophagy have been found to be associated with increased sensitivity to metabolic stress and DNA damage [19,31]. This is evident in studies of pancreatic and breast cancer tumors where inhibition of autophagy or defective autophagy (in association with defective apoptosis) was observed to promote ROS production, DNA damage, and tumorigenesis [35–37]. The antitumorigenic effects, however, stem from its ability to protect cells from the DNA damage (that leads to transformation into cancer cells) via damaged protein clearance and the blockage of ROS generation [31].

The fundamental aim of cancer treatment strategies is to eliminate the tumor cells by the promotion of apoptosis or alternative cell death pathways. Where this is not feasible, interfering with tumor cell proliferation is the less desirable alternative. In efforts to achieve these ends, the logical approach has been to identify and/or develop agents that will cause irrevocable injury to the tumor cells and to abrogate cellular integrity. Protective autophagy, one of the processes that serves to maintain tumor cell viability and tumor progression, may be inhibited to facilitate tumor cell death. In tumor cells that are unable to undergo autophagy, promoting autophagy would be counterintuitive since it would prevent metabolic stress, ultimately resulting in survival [38]. It is for this reason that clinical trials have been initiated to evaluate the influence of pharmacological autophagy manipulation on sensitivity to chemotherapy.

12.3 CURRENT APPROACHES IN AUTOPHAGY MODULATION

During the course of cancer therapy, it is highly uncertain as to the potential consequences of autophagy inhibition. Dysfunctional autophagy can lead to defective organelle degradation and turnover, ROS production, mutated or damaged DNA, and inflammation [39].

In addition, impairing autophagy has been reported to prevent the release of ATP and the subsequent recruitment of dendritic cells and T lymphocytes to the tumor microenvironment, potentially resulting in a diminished immune response and attenuation of tumor cell killing [40]. Conversely, inhibiting autophagy *that is cytoprotective* should sensitize tumor cells to cancer chemotherapy, such as DNA-damaging compounds (doxorubicin, temozolomide, etoposide), as well as to radiation therapy [41,42]. If tumor cells survive treatment, their ability to undergo autophagy could allow for tumor dormancy and recurrence [43]. Therefore, it may be beneficial to inhibit autophagy both during and after cancer therapy.

The outcome of autophagy inhibition during cancer treatment is currently under investigation in a multitude of clinical trials. All of these studies utilize either chloroquine (CQ), a clinically used antimalarial drug, or hydroxychloroquine (HCQ), an FDA-approved analog of CQ, for autophagy inhibition. The mechanism by which HCQ and CQ inhibit autophagy is not fully understood; however, it is known that these compounds are weak bases that undergo ion trapping when located in acidic environments, such as lysosomes [44]. Because HCQ and CQ are unable to cross plasma membranes once ionized, they will accumulate and subsequently increase the lysosomal pH. A basic pH can inhibit the fusion of autophagosomes with lysosomes, thereby preventing the late-stage autophagy wherein the cellular cargo is degraded.

Clinical trials have been completed with HCQ alone or in combination with chemotherapy and/or radiotherapy to determine the clinical outcome of autophagy inhibition in cancer patients. Phase I trials, those primarily concerned with the safety of investigative new drugs, have revealed that HCQ can be safely administered chronically at doses up to 1200 mg per day [45–48]. Determining the highest tolerated dose regimen is critical since steady-state concentrations of HCQ can only be achieved after weeks of administration, rather than days [49]. The Phase I trials were conducted in patients with advanced solid tumors, melanoma, relapsed/refractory myeloma, or metastatic colorectal cancer [45–48]. The secondary outcome, overall survival, was also assessed in some of the Phase I studies. For example, it was found that HCQ in combination with temsirolimus allows for a median progression-free survival of 3.5 months in patients with advanced solid tumors [45]. Phase I/II and II trials have also been performed to determine the efficacy of HCQ in prolonging the survival of cancer patients. In patients with glioblastoma, HCQ in combination with both radiation and temozolomide resulted in a median survival of approximately 16 months [50]. However, when given alone, HCQ presented negligible efficacy in metastatic pancreatic adenocarcinoma patients [51]. CQ has performed similarly in clinical trials with high tolerance in a wide range of cancer patients, including those with colorectal, breast, non-small cell lung, ovarian, and renal cancers, and has been shown to increase median survival in glioblastoma patients by 13 months [52,53].

Overall, HCQ is capable of promoting partial responses to therapy and prolonging a stable disease state. When comparing the outcomes of the various cancer patient populations, it appears that a subset of cancer types may be more sensitive to HCQ or CQ. It has also been noted that cancer cells are more susceptible to HCQ-induced autophagy inhibition than normal, nontransformed cells and peripheral blood mononuclear cells (PBMCs); yet, the mechanism behind this selectivity remains to be determined [54].

Despite these promising findings, the toxicity of HCQ and CQ must be considered. Fortunately, HCQ is less toxic than CQ, with most clinical trials observing a lack of dose-limiting side

effects with the administration of 1200 mg/day for up to 1 year [55]. However, HCQ has been found to cause retinopathy, an irreversible disease of the retina that leads to impairment or loss of vision, in as much as 7.5% of patients treated with the drug [56]. Those with a high risk for developing retinopathy, such as patients with renal disease or who are receiving tamoxifen, can be given HCQ in low doses (200–400 mg/day) for up to 10 years. However, the efficacy of HCQ may be compromised by reducing the dose. In addition, fatigue, gastrointestinal side effects, and myelosuppression have also been observed [50]. Yet, the main concern for HCQ use is the variability in toxicity when combined with different cancer therapy regimens. Therefore, it is doubtful that a safe, standard dose of HCQ can be determined; individual clinical trials may be necessary for each chemotherapy drug and/or radiotherapy regimen administered in combination with HCQ. In the meantime, increasing the frequency of screening for changes in vision and more accurate calculations of HCQ for real patient weight, rather than ideal weight, can lower the risk for retinopathy.

Despite the progress being made in active clinical trials for autophagy inhibitors, the interpretation of outcomes will remain a challenge for a number of reasons. First, the promotion of autophagy is being assessed by the extent of autophagic vacuole (AV) formation; yet, the presence of AVs in patient tissues does not explicitly identify the functional state of autophagy; that is whether autophagy progression has been compromised in the presence of HCQ or if autophagy is simply being induced by chemotherapy and/or radiation. Greater clarity can be obtained by determining the ratio of LC3-I to LC3-II expression. Secondly, there are currently no unequivocally accurate clinical biomarkers of autophagy. The most common indication of autophagy inhibition in the tumor is actually determined by evaluating the level of autophagy in PBMCs, which frequently exhibit less autophagy inhibition than tumor cells [52]. Other cell types, such as bone marrow plasma cells (BMPCs), have been found to possess AVs following HCQ administration when none is detected in PBMCs [47]. Another option for detecting autophagy is by comparing the formation of AVs in circulating tumor cells (CTCs) vs in the solid tumor to determine if CTCs are the best surrogate tissue, especially for metastatic cancers [49]. Lastly, autophagy-associated secreted proteins in the blood plasma can be measured as an indicator of intratumoral autophagy [57]. The establishment of an accurate clinical biomarker is crucial in order to determine if the use of a specific cancer therapy to treat a particular cancer is inducing cytoprotective autophagy, which can then be modulated by autophagy inhibition.

In some ways, testing autophagy inhibitors in a clinical setting may actually be premature in the absence of accurate information relating to the roles of autophagy in patient tumors. It would be beneficial to identify which cancers are sensitive to autophagy inhibition to better determine the appropriate patient population and therapeutic approach. On the other hand, moving forward clinically may be the best option in that by progressing to phase III trials the effect of the autophagy inhibitor on tumor sensitivity to chemotherapy and/or radiation can be evaluated. These clinical trials would also provide an opportunity to explore whether intermittent high levels or continuous low levels of autophagy inhibition are more appropriate in modulating the efficacy of chemotherapy and/or radiation. There is also a need to assess not only the toxicity of the autophagy-inhibiting drugs, but also the consequences of chronic autophagy inhibition. It has been proposed that modulating autophagy can initiate the development of tumors [54]. However, the current use of autophagy inhibitors is being investigated in patients with advanced cancers; therefore, hindering the progression

of the life-threatening cancer is a greater priority than the possible development of secondary, and potentially, benign tumors.

12.4 AUTOPHAGY AND THERAPY-INDUCED IMMUNE RESPONSE

While the importance of the antitumor immune response to the effectiveness of cancer therapeutics has been recognized for over 100 years [58], it has only recently been appreciated as a reliable means to suppress tumor growth [59]. Therefore, any discussion of the consequences of cancer therapeutics must include consideration of the contribution of the antitumor immune response. In the context of antitumor immunity, autophagy has been reported to have contradictory functions, both stimulatory and inhibitory. Hypoxia-induced autophagy, a common occurrence in large tumors, has immune protective functions [60]. As a consequence of hypoxia-induced autophagy, the signal transducer and activator of transcription 3 (STAT3) is activated through a hypoxia-induced factor 1a (HIF-1a)-regulated pathway [61,62]. Once activated, STAT3 can stimulate tumor cell secretion of the immune suppressive cytokines IL-10, IL-23, and TGF-beta [63]. Inhibition of antitumor immunity through hypoxia-induced autophagy has been confirmed using the B16-F10 TRP2 vaccine tumor model where tumors exposed to the pharmacological autophagy inhibitor, HCQ, exhibited a significant decrease in tumor growth in nonvaccinated and vaccinated mice [62]. This model shows that autophagy suppresses CD8 T-cell antitumor immunity. These effects could be relevant to the observation that CQ or HCQ increased long-term survival and enhanced immune cell proliferation and tumor infiltration using MC38 and B16-F10 tumor models [62,64].

In addition to activating STAT3, hypoxia-induced autophagy inhibits NK cell-mediated antitumor immunity. This occurs in two ways. First, via autophagosome fusion with endocytosed cytolytic perforin and granzymes released from NK cells [65], limiting their activities. Second, hypoxia-induced autophagy can disrupt the NK cell–tumor cell immune synapse through hypoxic stress inducing the endocytosis and degradation of connexin 43, a major component of gap junctions [66]. Because perforin and granzymes and the immune synapse are also relevant to CD8 cytotoxic lymphocytes, these inhibitory activities would presumably be relevant to suppression of T-cell function, as well.

In contrast to these immune inhibitory activities, there is evidence that autophagy can promote antitumor immunity [40]. A limited subset of chemotherapeutic agents has been shown to stimulate the antitumor immune response through a process called immunogenic cell death (ICD). Although it is not understood why some therapies induce ICD, this appears to be mediated through an autophagy and apoptosis-dependent process [67]. Several reports have identified danger-associated molecular pattern (DAMP) molecules as having critical roles in stimulating therapy-induced ICD [68]. These DAMPs are not normally accessible to the immune system but become accessible during ICD. The most well-studied DAMPs include the nuclear high-mobility group box 1 (HMGB1), cellular ATP, and endoplasmic reticulum-localized chaperone calreticulin [69]. While calreticulin is exposed on the plasma membrane in the early stages of therapy-induced ICD, later stages of ICD promote the secretion of HMGB1 and ATP through compromised plasma membranes [70]. DAMPs stimulate immune cell migration to the tumor [71], tumor cell phagocytosis by antigen presenting cells, and the antitumor activity of T-cells and NK cells [72] largely

through binding receptors on immune cells [73]. The consequence of DAMP secretion and ICD are an enhanced adaptive and innate antitumor immune response, with adaptive immune cell memory.

The positive correlations between antitumor immunity and autophagy are not reserved to animal models only and are also observed in the clinic. As a group, patients with solid or hematologic malignancies with elevated autophagy have lived longer [74–76] and their tumors have been shown to retain elevated levels of CD8 T-cells [77]. These observations provide some evidence that induction of autophagy could prolong survival and improve antitumor immunity. In addition, fasting and other less aggressive caloric restriction regimens induce autophagy organism-wide in animal tumor models with therapeutic benefits to ICD-inducing therapies [78]. While effective in animal models, a caloric restriction strategy is not likely to be plausible in humans because it could exacerbate life-threatening cancer-induced cachexia [79].

Like many of its functions in cancer biology, autophagy's roles in regulating the antitumor immune response are complex, with both pro- and antitumor activities. Roles for hypoxia-induced autophagy generally appear to promote tumor survival and therefore might benefit from a strategy to inhibit autophagy. Conversely, therapy-induced autophagy, which would likely be experienced by many cancer patients, has antitumor action and therefore autophagy inhibition might be detrimental to patients. This raises serious reservations relating to current clinical trials combining CQ or hydroxylchloroquine with chemotherapy (or radiation). These trials are largely based on data from cell culture and tumor xenograft experiments where inhibition of the cytoprotective form of autophagy sensitizes the tumor cells to therapy. However, in the clinical situation where the immune system is presumably functional, interference with autophagy could be counterproductive if, in fact, this results in immunosuppression.

12.5 FUTURE IMPLICATIONS, NOVEL THERAPIES, AND DRUG TARGETS

As discussed above, the finding that conventional cytotoxic cancer therapy can promote autophagy has provided the basis for autophagy modulation in therapy. Many cancer therapeutics as well as radiation can induce autophagy by the activation of multiple pathways including the PI3K/AKT/mTOR pathway as well as the DNA damage response [80]. Most efforts to modulate autophagy in cancer cells are focused on inhibiting autophagy as a protective mechanism that facilitates survival. For this purpose, the use of classical autophagy inhibitors, such as HCQ, has been widely investigated. However, a number of limitations to the current approach have been described. These include multiple off-target effects of the lysosomotropic agents and the uncertainty of sufficient autophagy inhibition in humans, which is complicated by the absence of a widely accepted approach for evaluating autophagy inhibition in patients' tumors. Furthermore, the antitumor effects of CQ or HCQ can be attributed to mechanisms other than autophagy (reflecting the nonspecificity of their antitumor effects) in addition to unfavorably interfering with autophagy in nontransformed cells [81].

Accordingly, there is a compelling need for developing novel autophagy modulators that can affect autophagy more specifically in patients' tumors. Current efforts are directed

toward developing more specific and potent lysosomotropic agents. Despite the fact that HCQ, which has a safer therapeutic profile than CQ, has FDA approval and is used in many clinical trials, high-dose regimens must be administered in order to achieve sufficient autophagy inhibition in patients, presumably due to the drug's lack of lysosomal specificity. Other antimalarial drugs, such as mefloquine and quinacrine, have been shown to be more potent as lysosomal inhibitors, possibly resulting in more effective or sustained sensitization to cancer therapy [82]. Several other compounds have been shown to affect lysosomal function. For example, siramesine, an S2R (sigma-2 receptor) agonist and an anxiolytic compound, was found to induce lysosomal dysfunction in a manner similar to CQ where it passively accumulates in the lysosome, resulting in an increased pH [83]. In addition, the active metabolite of clomipramine, a tricyclic antidepressant, has been shown to block the autophagosome–lysosome fusion, while lucanthone, an antischistosomal drug with potential antineoplastic activity, can inhibit autophagy by increasing lysosomal membrane permeability, leading to a significant increase in cathepsin D cytosolic levels, and ultimately, apoptosis [84].

Derivatives of CQ, such as the analog Lys05, have also been developed, which can accumulate to extremely high levels within the lysosome, causing a large increase in lysosomal pH. The resulting inhibition of the autophagic flux was found to sensitize several cancer cell lines to therapy more effectively than HCQ. In addition, Lys05 showed a significant capability to interfere with autophagy *in vivo*, allowing for the development of a potent lysosomotropic agent that can be used at lower concentrations than HCQ in patients [85]. ARN5187 is a dual inhibitor of REV-ERBβ and autophagolysosome formation that exerts improved cytotoxic activity, which is yet to be evaluated *in vivo* [86]. VATG-027 was identified via high-throughput screening of antimalarial compounds as another autophagy inhibitor which had antitumor effects in melanoma cells [87].

A better understanding of the different pathways that regulate autophagy has allowed for the identification of novel, promising molecular targets. The ability to modify these signaling pathways should provide more specific means to modulate autophagy, as opposed to lysosomotropic agents which can sensitize tumor cells to therapy independent of autophagy. Potential autophagy targets include Atg7, Atg4, BECLIN1, and ULK-1. ULK-1 is the initial upstream kinase in the autophagic pathway [54,88]. Genetic knockdown of ULK-1 results in dramatic dysfunction in the autophagic response [18]. MRT68921 is a kinase inhibitor that was found to significantly interfere with autophagy primarily due to its effect on ULK-1 [89]. SBI-0206965 was also presented as an additional catalytic inhibitor of ULK-1 that might possibly serve as a new small molecule to modulate autophagy [90]. These agents are still in the initial phases of development and their effect on therapy-induced autophagy in tumor cells has not been elucidated. Furthermore, there have not been any clinical trials for any of the ULK-1 inhibitors.

Vsp34 is emerging as an important target for novel anticancer therapy. Vsp34 is downstream to ULK-1 and is involved in many vesicular trafficking pathways including autophagy [18]. Genetic deletion of Vsp34 results in the failure of autophagosome formation. Spautin-1 inhibits two ubiquitin-specific proteases USP10 and USP13, resulting in the degradation of Vps34 and consequently, the inhibition of autophagy [91]. Initial studies showed a synergistic effect of Spautin-1 with imatinib, suggesting a potential role of autophagy inhibition in the treatment of chronic myeloid leukemia [92]. A novel small

molecule, SAR405, was found to inhibit autophagy by preventing the catalytic activity of the two Vps34 complexes, Atg14L and UVRAG-containing Vps34 complexes, providing an additional means of selective autophagy inhibition [93]. Lastly, PIK-III, a kinase inhibitor of the bisaminopyrimidine family, inhibits the degradation of autophagy adapters, such as p62, following catalytic inhibition of mTOR. PIK-III can also interfere with mitophagy, the autophagic degradation of mitochondria [94].

Both ULK-1 and Vsp34 are not specific to the autophagic pathway; however, they provide more selective drug targets that should replace the highly nonspecific pan-PI3K inhibitors, such as 3-methyladenine. Continuous efforts to improve these novel small molecules or to identify other targets in the autophagic machinery is under way. However, another challenge for inhibiting autophagy in tumor cells arises from the identification of multiple roles or function of autophagy induced by cancer therapy [95]. Consequently, even with a more specific target, inhibition of cytotoxic autophagy might have deleterious therapeutic outcomes. The strategy of autophagy inhibition would greatly benefit from the identification of autophagy-related biomarkers suggestive of a cytoprotective role of autophagy or by restricting these approaches to tumors with an established dependence on autophagy sometimes referred to as "addiction to autophagy".

12.6 CONCLUSION

The roles of autophagy in cancer and cancer treatment are diverse and remain to be completely elucidated. Thus far, it has been found that autophagy can play a tumor-suppressive role by preventing oncogenic mutations, as well as dysfunctional mitochondria and the subsequent release of genotoxic ROS. On the other hand, autophagy can adopt a tumor-promoting role following tumor formation by allowing tumor cells to adapt to the hypoxic and nutrient-deprived tumor microenvironment. It has been proposed that these various functions of autophagy are dependent upon multiple factors including the specific oncogenic mutation(s) (e.g., p53), the functional and/or impaired signaling pathways, such as the mTOR and PI3K/Akt pathways, and the level of cellular stress. With regard to the response to chemotherapy and/or radiation, autophagy can also manifest and play various roles, each of which contribute in a unique way to either cell survival or death. The current FDA-approved modulators of autophagy, HCQ and CQ, could prove to be effective in prolonging the survival of cancer patients; however, their limited efficacy and potential toxicities have hindered their progression toward becoming a common component of cancer therapy. The search continues for an accurate method for detecting autophagy in the tumors of patients, for identifying the nature of that autophagy as a basis for therapeutic intervention, and for a specific and potent autophagy inhibitor that can successfully sensitize tumor cells to the established cancer chemotherapy and radiation regimens.

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