Targeting autophagic pathways for cancer drug discovery

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Abstract

Autophagy, an evolutionarily conserved lysosomal degradation process, has drawn an increasing amount of attention in recent years for its role in a variety of human diseases, such as cancer. Notably, autophagy plays an important role in regulating several survival and death signaling pathways that determine cell fate in cancer. To date, substantial evidence has demonstrated that some key autophagic mediators, such as autophagy-related genes (ATGs), PI3K, mTOR, p53, and Beclin-1, may play crucial roles in modulating autophagic activity in cancer initiation and progression. Because autophagy-modulating agents such as rapamycin and chloroquine have already been used clinically to treat cancer, it is conceivable that targeting autophagic pathways may provide a new opportunity for discovery and development of more novel cancer therapeutics. With a deeper understanding of the regulatory mechanisms governing autophagy, we will have a better opportunity to facilitate the exploitation of autophagy as a target for therapeutic intervention in cancer. This review discusses the current status of targeting autophagic pathways as a potential cancer therapy.

Key words Autophagy, cancer, cell death, survival, drug discovery

Autophagy, a term derived from the Greek words “auto” (self) and “phagy” (to eat), refers to an evolutionarily conserved, multi-step, lysosomal degradation process in which a cell degrades long-lived proteins and damaged organelles[1,2]. Three forms of autophagy have been identified based upon the mode of delivery to the lysosome, namely macroautophagy, microautophagy, and chaperone-mediated autophagy[3,4]. Macroautophagy (hereafter referred to as autophagy) is a major regulated catabolic process that involves the delivery of cytoplasmic cargo sequestered inside double-membrane vesicles to the lysosome. Autophagy is strictly regulated by a number of autophagy-related genes (ATGs) that were originally discovered in yeast (Figure 1)[5]. To date, over 35 distinct ATGs have been identified in yeast, and even more ATGs are probably expressed in mammals[6,7].

Under most conditions, autophagy promotes cell survival by allowing cells to adapt to stressful conditions; thus, cells are provided with the energy required for minimal cellular functions even when nutrients are scarce[8]. Alternatively, many studies have demonstrated that autophagy plays a pro-death role in type II programmed cell death (type II PCD) but not in apoptosis (type I PCD)[9]. Therefore, depending on the cell type and context, autophagy appears to play opposite roles in determining cell fate. Autophagic cell fate is regulated by several key mediators [e.g., Beclin-1, phosphatidylinositol 3 kinase (PI3K), mammalian target of rapamycin (mTOR), Bcl-2, and p53], which have an astonishing number of links to many human diseases, most notably cancer[10,11]. In cancer cells, autophagy can either serve as a temporary survival mechanism, which may provide a means of recycling macromolecules, or lead to cell death if autophagy is excessively induced by cellular stresses[12,13]. Therefore, autophagy may play a two-faced role, acting either as a guardian or as an executioner in cancer, depending on the stage of cancer initiation and progression or on the surrounding cellular environment[14].

Molecular Pathways of Autophagy Regulation in Cancer

Autophagy is a complicated process that involves...
Chin J Cancer; 2013; Vol. 32 Issue 3

Figure 1 Multiple stages of autophagy and the involved molecular regulators. Autophagy is stimulated by nutrient deprivation, hypoxia, cytokines, hormones, and DNA damage. The early stages of activation require ATG1 and ATG13, which in turn can be inhibited by mTOR. Vesicle nucleation depends on Beclin-1-class III PI3K-Vps15 core complexes and other proteins. Vesicle elongation and completion are mediated by the Atg16L complex and LC3. Docking and fusion refer to the maturation of autolysosomes and are promoted by Rab7, LAMP1, LAMP2, SKD1, Vti1b, and the ESCRT complex. In the last stage, autophagosomal cargoes are digested and then nutrients and energy are recycled.
input from many upstream regulatory signaling pathways\textsuperscript{[15,16]}. Although the regulatory mechanisms of autophagy are partially known, the exact function of autophagy in cancer is still controversial. When analyzing the intricate relationship between autophagy and cancer, a common challenge is to determine whether autophagy protects cell survival or contributes to cell death\textsuperscript{[16,17]}. To resolve the role of autophagy in cancer cell fate, several hypotheses have been put forward. One hypothesis proposes that the role of autophagy varies depending on the stage of tumor development. For instance, autophagy limits tumor formation in early stages but favors tumor cell survival, invasion, and metastasis after tumors have formed\textsuperscript{[16,18]}. Another hypothesis suggests that autophagy can affect tumorigenesis in a cell- or tissue-specific manner\textsuperscript{[16,17]}. Therefore, at the molecular level, autophagy plays either a pro-survival or a pro-death role by regulating tumor suppressor genes or oncogenes. These autophagic pro-survival or pro-death genes, and the corresponding proteins, can integrate into cancer cell signaling networks and ultimately regulate cell survival or death\textsuperscript{[16,18]}.

ATGs play a key role in the formation of autophagosomes and regulation of autophagic activity; furthermore, they are closely linked to cancer initiation and progression. Silencing some essential ATGs, such as ATG3, ATG4, Beclin-1/ATG6, ATG10, and ATG12, can sensitize cancer cells to a wide spectrum of stressful conditions\textsuperscript{[19]}. Additionally, targeting selected protein kinases involved in autophagy regulation with small molecule kinase inhibitors may be another feasible approach in cancer treatment. A number of protein kinases regulate the induction of autophagy following nutrient deprivation or other cellular stresses. The following protein kinases have been reported to activate protective autophagy in cancer cells as a response to cytotoxic agents, including AMP-activated protein kinase (AMPK), glycogen synthase kinase 3 (GSK3) beta, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and eukaryotic elongation factor-2 kinase (eEF-2K)\textsuperscript{[20–22]}.

mTOR, an evolutionarily conserved serine/threonine kinase, serves as the main negative regulator of autophagy in cancer cells. mTOR forms two complexes in mammalian cells. Only mammalian target of rapamycin complex 1 (mTORC1) is sensitive to inhibition by rapamycin; therefore, we focus on the role of mTORC1 in autophagy\textsuperscript{[22]}. Three major mTORC1-inducing pathways have been elucidated, including the PI3K-Akt pathway and the MAPK/ERK pathway, consisting of Ras-proto-oncogene serine/threonine-protein kinase (Raf-1), mitogen-activated protein kinase 1/2 (MEK1/2), and extracellular signal-regulated kinase 1/2 (ERK1/2). The LKB1-AMPK pathway, consisting of liver kinase B1 and AMPK, can inhibit mTORC1\textsuperscript{[24]}. The TSC2/TSC1 complex, which has a tumor suppressor function in various cancers, is a key point upstream of mTORC1 since TSC2/TSC1 can suppress mTORC1 by inactivating the mTORC1-interacting protein, Rheb\textsuperscript{[25,26]}. Upon PI3K activation, Akt phosphorylation of TSC2 destabilizes TSC2 and disrupts its interaction with TSC1 to abolish the negative regulatory effect of the TSC2/TSC1 complex on mTORC1\textsuperscript{[27]}. Phosphorylation of TSC2 by AMPK can increase the GAP activity of TSC2, stabilize the TSC2/TSC1 complex, and inactivate Rheb, resulting in the inactivation of mTORC1 and the initiation of autophagy\textsuperscript{[26]}. In mammals, two homologs of ATG1, namely uncoordinated 51-like kinase 1 (ULK1) and ULK2, mammalian autophagy-related protein 13 (mATG13), and scaffold protein FIP200 have been identified. Under nutrient starvation conditions, mTORC1 disrupts the binding of ATG13 with ULK and destabilizes ULK, thereby inhibiting the ULK-dependent phosphorylation of FIP200 and autophagy induction by phosphorylation of ULK and ATG13\textsuperscript{[26]}. Moreover, mTORC1 regulates autophagy by mediating protein translation and cell growth through phosphorylation of 4E-binding protein 1 (4E-BP1) and p70S6K. Phosphorylation of 4E-BP1 leads to its dissociation from eukaryotic translation initiation factor 4E (eIF4E) and up-regulates cap-dependent translation. Phosphorylation of p70S6K by mTORC1 enhances p70S6K activity and allows it to phosphorylate downstream targets\textsuperscript{[23]}. Once activated, p70S6K phosphorylates eukaryotic elongation factor 2 kinase (eEF2K) to relieve elongation factor 2 (eEF2) from inhibition by eEF2K in addition to promoting autophagy\textsuperscript{[29]}. DEPTOR, an inhibitor of mTORC1 and mTORC2, inhibits mTORC1 and mTORC2 by directly binding to them both. DEPTOR is subjected to proteasome-dependent degradation upon serum stimulation to ensure mTOR activation.

p53, a well characterized human tumor suppressor gene involved in genotoxic stress response and DNA damage repair, also participates in autophagy regulation\textsuperscript{[30]}. Intriguingly, the role of p53 in autophagy seems to be paradoxical depending on its subcellular localization, which may dictate whether p53 contributes to cancer cell survival or death\textsuperscript{[31]}. In the nucleus, p53 can activate AMPK to inhibit mTOR and induce the autophagic process. Also, p53 can promote autophagy through targeting multiple genes that code for pro-autophagic modulators, including DAPK-1, damage-regulated autophagy modulator (DRAM), pro-apoptotic Bcl-2 proteins (e.g., Bad, Bax, BNI3, and PUMA), Sestrin1/2, and TSC2\textsuperscript{[18]}. Notably, Sestrin1 and Sestrin2, which are usually expressed under conditions of DNA damage and oxidative stresses, are negative regulators of mTORC1 and execute their function through activation of AMPK and TSC2; thus, Sestrin1/2 establish a connection between p53 and autophagy through mTORC1\textsuperscript{[22]}. Opposite to nuclear p53, cytoplasmic p53 inhibits autophagy by mTORC1 activation. Tasdemir et al.\textsuperscript{[23]} have shown that depletion of p53 in mice induces autophagy. The autophagy induced by loss of p53 promotes the survival of p53-deficient cells to sustain...
high ATP levels under conditions of hypoxia and nutrient depletion[34]. Therefore, p53 signaling controls autophagy in an ambiguous fashion that depends on its subcellular localization and plays a two-sided role in cancer.

Beclin-1, the mammalian homolog of ATG6 and a Bcl-2 interacting coiled-coil protein, is essential for the formation of double-membrane autophagosomes, which are required in the initial step of autophagy[35]. Beclin-1 can promote interaction of Bcl-2 with other autophagy regulators, such as Vps34 (PI3K), p150, UVRAG, Bif1, ATG14L, and Rubicon, to form huge protein complexes[40]. Additionally, Beclin-1 is a haploinsufficient tumor suppressor gene. Beclin-1 can enhance autophagy by combining with PI3KIII in the initiating stage of autophagy. UVRAG, a major positive mediator of Beclin-1, can directly and markedly enhance PI3KIII lipid kinase activity, thus, facilitating autophagy. Through mediating the Beclin-1/PI3KIII complex, UVRAG can promote autophagy and inhibit tumorigenesis. Bif1, another positive mediator of Beclin-1, can interact with Beclin-1 through UVRAG to regulate autophagy and suppress tumorigenesis[37]. Following dissociation of Beclin-1 from Bcl-2, autophagy may be activated depending on whether Bcl-2 has been phosphorylated by the starvation-activated c-JUN N-terminal kinase (JNK)[38]. The tumor suppressor function of Beclin-1 is supported by the identification of its mediators in tumorigenesis[35]. Bcl-2 inhibits autophagy through interacting with Beclin-1 as Beclin-1 contains a BH3 domain that facilitates the interaction of Beclin-1 with Bcl-2[40]. By interacting with Beclin-1, Bcl-2 blocks the interaction of Beclin-1 with PI3KIII, decreases PI3KIII activity, and down-regulates autophagy. Overall, Beclin-1 may enhance autophagy and inhibit tumorigenesis by forming signaling complexes mediated by positive and negative regulators, which suggests a crucial role for Beclin-1 in cancer.

Mounting evidence has demonstrated that mitochondria, the main source of reactive oxygen species (ROS) in cells, may orchestrate the autophagic process in cancer initiation and progression. For instance, ROS play multifaceted roles as a “molecular switch” in the regulation of several core autophagic pathways (e.g., ATG4-ATG8/LC3, Beclin-1, PTEN, p53, PI3K-Akt-mTOR, and MAPK signaling) that may jointly seal the fate of cancer cells. MAPKs and p21-activated kinases (PAK), two classes of downstream signaling molecules regulated by ROS, are thought to be the major signaling pathways for driving cancer cell metastasis[41,42].

In summary, all of the aforementioned survival/death signaling pathways involved in ATGs, the mTOR subnetwork, the Beclin-1 interactome, p53 signaling, and ROS may play crucial roles in autophagy-related cancer signaling networks. This suggests that autophagic pathways could be promising new targets in cancer drug development, which we will discuss in the following section.

### Autophagy-modulating Agents for Cancer Treatment

Evidence suggests that induction of autophagy may help transform tumor phenotypes during cancer therapy. At this time, several novel strategies are being used to target autophagy-inducing pathways for drug discovery in cancer treatment because a number of autophagy-inducing drugs have been identified as potential cancer therapeutic agents[43-46]. Several autophagy-inducing agents are already being used to treat different human cancers and should be further explored both at the bench and in the clinic.

Tumor cells can use autophagy to supply nutrients and energy, and promote tumor survival when nutrients are limited. Therefore, some drugs have been developed to block autophagic processes so as to suppress tumor progression. Autophagy process can be divided into several phases, including the initiation period, docking and fusion of autophagosomes with lysosomes, and catalytic degradation of cytoplasmic materials inside autolysosomes. PI3K inhibitors, including 3-methyladenine (3-MA), wortmannin, and LY294002, can interfere with or block autolysosome formation and result in the inhibition of autophagy[47]. Also, it has been observed that cancer cells undergo increased autophagy and are more sensitive to lysosomotropic agents. Bafilomycin A1, vinblastine, and Nuokaoadzuo can inhibit the fusion process to interrupt autophagy[47]. Bafilomycin A1, a type of macrolide antibiotic derived from *Streptomyces griseus*, has been reported to block the fusion of autophagosomes with lysosomes in tumor cells. Two anti-malarial drugs, hydroxychloroquine (HCQ) and chloroquine (CQ), can inhibit lysosomal acidification and prevent the degradation of autophagosomes, thereby suppressing autophagy in Myc-driven lymphoma and increasing the antitumor effects of cyclophosphamide[48-50]. In imatinib-resistant BCR-ABL-positive chronic myeloid leukemia (CML) cell lines, CQ enhanced cell death by inhibiting autophagy and strengthened the activity of vorinostat, an histone deacetylase (HDAC) inhibitor[51,52]. In combination with the anti-malarial drug quinacrine, CQ remarkably sensitized gastrointestinal stromal tumor cells to imatinib, both in vitro and in vivo, and reinforced the efficiency of quinacrine[53]. CQ has recently been reported to be able to inhibit therapy-induced autophagy and to increase cell death in established tumors, leading to tumor regression[54].

Nevertheless, autophagy is not only a survival response that opposes growth factor and nutrient deprivation but also an important mechanism for tumor
cell suicide. Recently, an increasing amount of data have suggested that autophagy, as a mechanism of type II PCD, may present new opportunities for developing alternative anti-cancer therapies. Tamoxifen, an antagonist of estrogen receptor (ER), has a high binding affinity for the microsomal antiestrogen binding site (AEBS), a hetero-oligomeric complex involved in cholesterol metabolism. Tamoxifen and other AEBS ligands induce breast cancer cell autophagy through inducing sterol accumulation[59,60]. These data indicate a therapeutic implication for selective AEBS ligands in breast cancer management and reveal a mechanism that may explain the induction of autophagy in MCF-7 cells by tamoxifen and other selective ER modulators[67,68].

Imatinib (Gleevec), an inhibitor of tyrosine kinases, can induce autophagy in multidrug-resistant Kaposi’s sarcoma cells[59,60]. HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), have been reported to be able to induce autophagy and cell death in HaLa cells independent of caspase-dependent apoptosis[64,65]; thus, initiation of autophagic cell death by SAHA has clear therapeutic implications for apoptosis-defective tumors. It is well known that mTOR is a major regulator of cell growth that has also been implicated in tumorigenesis[64,65]. The tumor suppressing action of rapamycin, an inhibitor of mTOR, is linked to induction of autophagic cell death. In addition to these agents, there are also other interesting examples of autophagy-inducing agents from traditional Chinese medicine. One such traditional Chinese compound, arsenic trioxide (As$_{2}$O$_{3}$), has been reported to be able to induce apoptosis through cytochrome c release and caspase activation[66,67]. Interestingly, recent studies showed that treatment of human T-lymphocytic leukemia cells with As$_{2}$O$_{3}$ led to cytotoxicity through inducing autophagy. A Bcl-2 family member, Bcl-2–adenovirus E1B 19-kDa-interacting protein 3 (BNIP3), was reported to play a pivotal role in As$_{2}$O$_{3}$-induced autophagic cell death in malignant glioma cells[68,69]. Additionally, Polygonatum cyronema lectin (PCL) was shown to be able to induce autophagic cell death via a mitochondria-mediated ROS-p38-p53 pathway in human melanoma A375 cells[70,71]. Based on the aforementioned examples, autophagy may play an important role in the cytotoxic effects of these compounds that could spark new autophagy-targeted cancer therapeutic strategies[72,73].

Additionally, DNA damage agents have been found to be able to induce autophagy in tumor cells. For example, temozolomide (TMZ), an alkylating agent, is widely used to treat primary and recurrent high-grade gliomas. The cytotoxicity of TMZ is thought to result from the formation of 0-6-methylguanine in DNA, which mispairs with thymine during DNA replication and triggers futile cycles of the mismatch repair system and subsequent DNA damage. It was shown that TMZ induces autophagy and that pharmacologic inhibition of autophagy could influence cellular outcome. Much work is needed to determine how modulators of autophagy impact cancer initiation, progression, and therapeutic response, and to determine exactly why targeting autophagic signaling pathways may be a valuable strategy for cancer drug development.

**Concluding Remarks and Future Directions**

Autophagy plays a dual role in the regulation of pro-survival and pro-death signaling pathways in a variety of diseases, including cancer. Several key autophagic mediators, including ATGs, PI3K, mTOR, p53, Beclin-1 interacome, and ROS, have been demonstrated to play pivotal roles in the complex autophagic network in cancer cells. However, much work is needed to determine the intricate molecular mechanisms of autophagy in cancer, to define how crucial modulators of autophagy in cancer impacts cancer initiation and progression, and to elucidate why targeting autophagic signaling pathways is promising for cancer therapeutics. Furthermore, recent biological insights can provide a fertile foundation for launching this next round of small-molecule drug discovery. These discoveries are being driven by an abundance of structural information on the potential targets; therefore, X-ray crystallography, nuclear magnetic resonance (NMR), and structural bioinformatics-docking techniques will be invaluable in the efforts to target autophagic pathways for drug discovery. More importantly, there is an increasing emergence of sophisticated mathematical models, such as the Naive Bayesian framework and support vector machine (SVM), for the disruption of protein-protein interactions (PPIs). The best hope for targeting autophagy as a therapeutic intervention may lie in the discovery of agents that are able to target the altered autophagy-regulating signaling pathways, or even the autophagic network, rather than targeting the individual genes or proteins. A better understanding of the autophagic PPI network will provide useful insights into how these hub proteins and autophagy-related signaling pathways can be exploited as potential therapeutic targets for treatment of human diseases (Figure 2). Due to the complex, two-sided nature of autophagy, establishing the dual role of autophagy in tumor survival vs. death may assist in determining therapeutic potential. Inhibiting autophagy may enhance the efficacy of currently used anti-cancer drugs and radiotherapy. In addition, promoting autophagy may induce cancer cell death with a high threshold to apoptosis. Therefore, both strategies have significant potential to be translated into ongoing clinical trials that may provide more valuable
information regarding whether targeting autophagic pathways in tumor cells would be a promising avenue for cancer therapeutics.

Received: 2012-01-11; revised: 2012-04-08; accepted: 2012-04-08.
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