Key autophagic targets and relevant small-molecule compounds in cancer therapy

X.-P. Tong^{*,§a}, Y. Chen^{*,a}, S.-Y. Zhang^{*,a}, T. Xie^{*}, M. Tian^{*}, M.-R. Guo^{*}, R. Kasimu[†], L. Ouyang^{*} and J.-H. Wang^{†,‡}

^{*}State Key Laboratory of Biotherapy & Collaborative Innovation Center of Biotherapy, West China Hospital, Sichuan University, Chengdu, 610041, China, [†]School of Pharmacy, Xinjiang Medical University, Urumqi, 830054, China, [‡]School of Traditional Chinese Materia Medica & Key Laboratory of Structure-Based Drug Design and Discovery of Ministry of Education, Shenyang Pharmaceutical University, Shenyang, 110016, China and [§]School of Pharmacy, China Pharmaceutical University, Nanjing 211198, China

Received 1 July 2014; revision accepted 31 August 2014

Abstract

Autophagy is a highly conserved lysosomal degradation process which can recycle unnecessary or dysfunctional cell organelles and proteins, thereby playing a crucial regulatory role in cell survival and maintenance. It has been widely accepted that autophagy regulates various pathological processes, among which cancer attracts much attention. Autophagy may either promote cancer cell survival by providing energy during unfavourable metabolic circumstance or can induce individual cancer cell death by preventing necrosis and increasing genetic instability. Thus, dual roles of autophagy may determine the destiny of cancer cells and make it an attractive target for small-molecule drug discovery. Collectively, key autophagy-related elements as potential targets, oncogenes *mTORC1*, class I *PI3K* and *AKT*, as well as tumour suppressor class III *PI3K*, *Beclin-1* and *p53*, have been discussed. In addition, some small molecule drugs, such as rapamycin and its derivatives, rottlerin, PP242 and AZD8055 (targeting *PI3K/AKT/mTORC1*), spautin-1, and tamoxifen, as well as oridonin and metformin (targeting *p53*), can modulate autophagic pathways in different types of cancer. All these data will shed new light on targeting the autophagic

process for cancer therapy, using small-molecule compounds, to fight cancer in the near future.

Introduction

Autophagy is an evolutionarily conserved, multi-step lysosomal process, in which cellular materials are delivered to lysosomes for degradation and recycling (1). At least three forms of autophagy have been identified: macroautophagy, chaperone-mediated autophagy and microautophagy, which are different in physiological functions and modes of cargo delivery to the lysosome (2). Among them, macroautophagy (hereafter referred to as autophagy) is the major regulated catabolic mechanism by which an autophagosome sequesters cytoplasm then fuses with a lysosome for degradation (3). It has been demonstrated that autophagy is modulated by a limited number of AuTophagy-related genes (*ATGs*) (4).

Cancer is a multi-step process caused by genetic alterations involving mutations of oncogenes or tumour suppressors which drives progressive transformation of normal cells to malignancy (5). On the one hand, autophagy can help in cell maintenance by specifically degrading damaged or superfluous organelles; in this context it can be regarded as a powerful promoter of metabolic homeostasis (6). On the other hand, an opposing role of autophagy has been discovered, since it can promote cell death in cancer, which helps classify autophagy as type II programmed cell death (PCD) (7). Different oncogenic pathways, including Bcl-2, mTOR, class I phosphatidylinositol 3-kinase (*PI3K*), *AKT* and *MAPKs*, or tumour suppressive pathways, such as *PI3K*, *Beclin-1*, *Bif-1*, *UVRAG*, *DAPKs*, *PTEN* and *p53*, have distinct influence on various types of cancer (8). Thus, autophagy may either promote cancer cell survival or induce cancer cell death under different conditions.

Correspondence: L. Ouyang, State Key Laboratory of Biotherapy & Collaborative Innovation Center of Biotherapy, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu 610041, China. Tel/Fax: +86 28 85164063; E-mail: ouyangliang@scu.edu.cn; and J.-H. Wang, School of Pharmacy, Xinjiang Medical University, Urumqi 830054, China. Tel/Fax: +86 28 85164063; E-mail: profwjh@126.com

^aThese authors contributed equally to this work.

Since a number of small molecule compounds have been found to target key regulatory autophagy-related components, and regarding their great potential in affecting malignancy, they have received considerable attention. For example, drugs, including rapamycin and its derivatives, rottlerin, PP242 and AZD8055, target the PI3K/AKT/mTOR signalling pathway to induce autophagy, while other agents such as spautin-1 and tamoxifen effect their function by regulating Beclin-1 activity in autophagy. In addition, drugs such as oridonin and metformin may induce cell death *via* p53-mediated autophagy. Thus, small molecule compounds can target oncogenic or tumour suppressive autophagic pathways in different types of cancer, which may shed new light on finding novel and potential drugs for future cancer therapeutics.

The machinery of autophagy

To the best of our knowledge, the autophagic process can be dissected into five different steps: induction, nucleation, elongation & completion, docking & fusion and degradation & recycling (9).

Induction of autophagosome formation is modulated by the complex comprised of Unc51-like kinase 1/2 (ULK1/2), the mammalian homolog of ATG13 (mATG13) and RB1-inducible coiled-coil 1/focal adhesion kinase family interacting protein of 200 kD (RB1CC1/FIP200) (10,11). Directly binding to ATG13, C12orf44/ATG101 is also essential for the induction process (12). The mammalian ortholog of the yeast protein kinase TOR (mTORC1) is influenced by different nutrient levels (13). When mTORC1 is associated with the complex, it phosphorylates and subsequently inactivates ULK1/2 and mATG13; however, when mTORC1 dissociates from the complex, it results in induction of autophagy (13).

Vesicle nucleation proceeds, which requires ATG14-containing PI3KCIII complex, containing Beclin-1, PI3KCIII/VPS34, and PIK3R4/p150. Anti-apoptotic protein Bcl-2 may bind to Beclin-1 and prevent its interaction with PI3KCIII (14). RUBICON, a Beclin-1-binding protein, inhibits PI3KCIII activity in the UVRAG-associated Beclin-1 complex (15). A further two positive regulators of the complex are AMBRA1 which directly binds to Beclin-1, and Bif-1 which can interact with Beclin-1 through UVRAG (16,17).

The process of elongation & completion calls for two ubiquitin-like pathways. First is the ATG12-ATG5-ATG16 complex, of which ATG12 is catalyzed by ATG7 and ATG10, and is then fused with ATG5 (18). In conjunction with ATG12-ATG5, ATG16L leads the complex to the autophagic isolation membrane (19). The second is the ATG8/LC3 system, in which the ATG4-

processed form of LC3 is regarded as LC3-I and PE-conjugated form is called LC3-II (22). Transmembrane protein ATG9 also functions in elongation, whose mechanism is not yet clearly understood (23). Once autophagosome expansion is completed, LC3 detaches from LC3-II, and then is released into the cytosol (24).

Fusion of autophagosomes with endosomes may involve protein components from SNARE machinery, such as VAM7 and VAM9, while UVRAG can also activate the GTPase RAB7, thus promoting fusion with lysosomes (25,26). A further component of SNARE, syntaxin 17, interacts with SNAP29 and endosomal/lysosomal SNARE VAMP8 (27).

Degradation & recycling proceed. In this way, autophagy helps cells respond to a wide range of extracellular and intracellular stresses (Fig. 1).

Roles of autophagy with relevant compounds in cancer

Autophagy has been demonstrated to be able to act as either cell guardian or executioner in cancer. Self-recycling properties of autophagy can function as a tumour-promoting mechanism, while its cell death characteristics may help autophagy work as type II PCD (28). Both oncogenesis and tumour suppression are influenced by perturbations of the molecular machinery that control autophagy. Numerous oncogenic proteins, including Bcl-2, Ras, mTOR, PI3KCI, AKT and MAPKs may suppress autophagy, while several tumour suppressor proteins, such as Beclin-1 interactome, DAPK1, LKB1/STK11 and PTEN promote it (29). However, it is surprising to find that p53, one of the most important tumour suppressor proteins, regulates autophagy based on its different subcellular localization (30). Small molecule compounds that regulate protein function and affect biological processes have been extensively employed to dissect biological pathways and to study different diseases. With deepening research into cancer treatment *via* different autophagic signalling pathways, several kinds of small molecule compounds have been revealed to promote or suppress autophagy, by targeting corresponding autophagy-related signalling pathways. Here, we have provided examples of specific mechanisms of some widely accepted autophagic modulators such as PI3KCI/AKT/mTORC1 signalling pathway, Beclin-1 interactome and p53, as mentioned above.

PI3KCI/AKT/mTORC1 pathway with relevant small molecule compounds

mTOR-dependent signalling pathway, induced by insulin or amino acid depletion, is the major mechanism which

Figure 1. The machinery of autophagy.

controls autophagic activities. As a principal regulator of cell population growth, mTORC1 is deregulated in most human cancers, and can be promoted by activation of class I phosphatidylinositol 3-kinase (PI3KCI) and its downstream components, such as AKT kinase (31). Enhanced activity of PI3KCI is often found *via* activating kinase mutations or gene amplification in cancers, thus inhibiting autophagy (32). Further, AKT mutational activation may suppress autophagy in response to metabolic stress, whereas AKT kinase inhibition potently induces autophagy by inactivating mTORC1 (33). After activation of PI3KCI, AKT is phosphorylated by PDK1, a pleckstrin homology domain (PH-domain)-containing protein. Subsequently, AKT regulates a number of targets – kinases, transcription factors and other regulatory molecules (36). Over-expression of two tumour suppressors, PTEN and ARHI, induce autophagy by negatively regulating the PI3KCI/AKT/mTORC1 pathway (34). A further well-known major homeostatic regulator, AMPK suppresses activation of mTORC1 by direct phosphorylation of mTOR; thus, targeting AMPK can be an important therapeutic strategy to overcome cancer. By mediating protein translation, mTORC1 regulates autophagy, and the mTORC1 sub-network may occupy a central position in autophagic pathways. Thus, autophagy is controlled by a complicated network of signalling routes mostly dependent on PI3KCI/AKT/mTORC1, an important oncogenic signalling mechanism involved in many human cancers.

Our understanding of the interplay between autophagy and cancer first benefited from availability of rapamycin, a natural product that inhibits mTORC1 by dissociating raptor from mTOR, thus limiting access of mTOR to some substrates (35). With its exquisite selectivity, rapamycin is used as an indispensable pharmacological probe for elucidating biological functions of mTOR serine/threonine kinase, in governing cell population growth and proliferation (36). Since rapamycin has significant therapeutic effects, its synthetic analogues including temsirolimus (CCI-779), everolimus (RAD001) and ridaforolimus (AP23573), have also been developed to improve pharmacokinetic properties and produce advantageous intellectual property positions (37). Temsirolimus is a pro-drug of sirolimus, and contains a dihydroxymethyl propionic acid ester moiety at the C-40-O position. This is quickly hydrolyzed after intravenous administration, aiding treatment of cancers such as acute myeloid leukaemia, non-small-cell lung cancer (NSCLC) and renal cell carcinoma (38). In addition, everolimus enhances anti-tumour effects of oncolytic adenovirus Delta-24-RGD, by inducing autophagy in gliomas (36). Its effect in acute lymphoblastic leukaemia, prostate cancer and NSCLC causes increased autophagy and reduced tumour size (36). With a dimethyl phosphate group at C-40-O position, ridaforolimus has been aimed to treat various cancers, such as advanced malignancies and relapsed haematological ones (38). Four additional mTORC1 inhibitors, rottlerin,

niclosamide, perhexiline and torin1, promote autophagy by directly inhibiting mTORC1 function or inhibiting proteins upstream of the mTOR pathway in cancer cells, under nutrient-rich conditions (39). Interestingly, rottlerin also targets TSC2, a negative regulator of mTORC1, thereby suppressing mTORC1 signalling (39). In addition, metformin, primarily known as an activator of AMPK, represses expression of mTORC1 by its interaction with AMPK. After activation of AMPK by treating metformin, activated AMPK directly phosphorylates mTOR and suppresses its activation, resulting in activating substrates of mTORC1, including the ULK1 complex, which is essential for autophagosome formation.

ATP-competitive inhibitors directly prevent mTOR function and suppress AKT phosphorylation in primary leukaemic cells and stromal cells cultured alone or in combination with leukaemic cells (40). As ATP-competitive inhibitors, PP242 and AZD8055 block phosphorylation of mTORC1 substrates and AKT, while AZD8055 also shows excellent selectivity against all PI3K isoforms and other members of PI3K-like kinase family (40,41). Both these inhibitors greatly prevent cell proliferation and suppresses tumour growth by inducing autophagy in malignancy, such as cancer of the head and neck, squamous cell carcinoma and acute myeloid leukaemia.

Phenethyl isothiocyanate, a promising chemo-preventive agent of human prostate cancer, induces autophagic cell death by suppressing phosphorylation of both AKT and mTORC1 (42). 2-deoxyglucose and glucose 6-phosphate also reduce mTORC1 and AKT phosphorylation, which have been used in the treatment of acute lymphoblastic leukaemia and other lymphoid malignancies (43). As a main active component of marijuana, THC induces autophagic cell death and enhances endoplasmic reticulum (ER) stress by inhibiting AKT and mTORC1 in cancer cells (44). Resveratrol, a polyphenol present in grapes, peanuts and other plants, is known to display anti-tumour activities, promoting cell death by triggering autophagy and thereby activating AKT and mTORC1 in ovarian cancer cells (45). The standard first-line systemic drug sorafenib is used to treat advanced hepatocellular carcinoma (HCC); it can activate AKT through an mTOR feedback loop, switching protective autophagy into autophagic cell death (46).

A class of PI3KCI inhibitors with multiple-target abilities has also emerged. The 1H-imidazo [4,5-c]quinoline derivative NVP-BE235 inhibits activities of PI3KCI/AKT/mTOR cascade by binding to the ATP-binding cleft of both PI3KCI and mTOR, in glioma cells (47). PI103 is a potent inhibitor of both PI3KCI and mTOR, thereby preventing AKT phosphorylation and resulting in enhancement of autophagy. This compound

may also inhibit proliferation and invasion of cancer cells, as it suppresses tumour growth in a variety of human cancers with genetic abnormalities of PI3KCI activation (48). With a morpholino pyridinopyrimidine core structure, KU0063794 (AstraZeneca) has, presumably, been developed using PI103 as lead compound, and has high potential and selectivity as an mTOR inhibitor (49). In addition, the synthetic glucocorticoid dexamethasone potently inhibits the PI3KCI/AKT/mTOR signalling pathway in acute lymphoblastic leukaemia cells, and is used clinically as a chemotherapeutic agent against many haematological malignancies (15). Also, PTEN, a tumour suppressor frequently mutated in human tumours, induces autophagy by inhibiting the PI3KCI/AKT/mTOR signalling cascade. It has been found that magnolol blocks PI3K/PTEN/AKT and induces H460 autophagic cell death, underlining potential utility of its induction as a new cancer treatment (50) (Fig. 2).

Beclin-1 interactome with relevant small molecule compounds

Beclin-1, mammalian homologue of ATG6, enhances autophagy by combining with PI3KIII/Vps34 in autophagic induction, as the evolutionarily conserved domain of Beclin-1 interacts with PI3KIII/Vps34 (51). This Beclin-1-Vps34 complex is primarily regulated by post-translational modification and several Beclin-1 binding partners (52). Beclin-1 is also a direct substrate of caspases-3/7/8 in apoptosis, whose caspase cleavage is sufficient to suppress autophagy in cancer (53). Beclin-1 is mediated by positive regulators such as UVRAG, Bif-1, AMBRA1, high mobility group box 1 (HMGB1), survivin, and PTEN-induced putative kinase 1 (PINK1), as well as having negative regulators including Bcl-2, Bcl-X_L, Mcl-1 and RUBICON (54). UVRAG interacts with Beclin-1 and PI3KIII/Vps34, while Bif-1 fuses with Beclin-1 through UVRAG, thereby enhancing autophagy (55). AMBRA1 promotes Beclin-1 interaction with its target PI3KIII/Vps34 and mediates autophagosome nucleation (56). As an extracellular damage-associated molecular pattern molecule, HMGB1 disrupts interaction between Beclin-1 and its negative regulator Bcl-2, by competitively binding to Beclin-1 (57).

A further positive modulator is the anti-apoptotic protein, survivin, which presents a possible mechanism in crosstalk between autophagy and apoptosis by Beclin-1-mediated degradation (58). PINK1, a serine/threonine protein kinase in mitochondria, also interacts with Beclin-1 thus inducing autophagy (17). Beclin-1 contains a BH-3 motif necessary for binding to Bcl-2, Bcl-X_L and Mcl-1. Bcl-2 blocks Beclin-1 interaction

Figure 2. PI3K/Akt-mTORC1 pathway and its autophagy-modulated compounds in cancer.

with PI3KIII/Vps34 and reduces PI3KIII activity, while Bcl-X_L and Mcl-1 inhibit Beclin-1 activity by stabilizing Beclin-1 homo-dimerization (59,60).

Contrary to UVRAG and Bif-1, RUBICON prevents kinase activity of PI3KIII/Vps34 and blocks autophagosome maturation; it is also involved in endocytic pathways by inducing aberrant endosomes and blocking EGFR degradation (50). Interaction of Bcl-2/Bcl-X_L with the Beclin-1/Vps34 complex reduces PI3KIII/Vps34 activity, and this interaction can be competitively disrupted by BH3-only proteins, such as Bad. Thus, in cancers, Beclin-1 enhances autophagy and inhibits tumorigenesis, by mediation of positive and negative regulators.

Although currently no Beclin-1-related drugs have undergone successful trials for clinical use, a number of small molecule compounds can target Beclin-1 in different cancer cells. As a possible lead compound for development of anti-cancer drugs, spautin-1 is also a potent inhibitor of autophagy. It promotes degradation of PI3K-III/Vps34 complexes by inhibiting two ubiquitin-specific peptidases, USP10 and USP13, which target the Beclin-1 subunit of Vps34 complexes (61). Xestospon-

ging B disrupts Beclin-1 through an indirect link established by Bcl-2, and then interferes with the molecular complex formed by PI3KIII and Beclin-1 (62). BH3 mimetic ABT-737 specifically reduces interaction between Bcl-2 and Bcl-X_L with the BH3 motif of Beclin-1, which stimulates Beclin-1-dependent activation of PI3KIII (63). A chemotherapeutic vitamin D analogue, EB1089, promotes massive autophagy by disrupting inhibitory interaction between two BH3 domains of Beclin-1 and Bcl-2 (64) and a further small molecule inhibitor, gossypol, interrupts interaction between Beclin-1 and Bcl-2/Bcl-X_L at ER and induces autophagy in a Beclin-1-/Atg5-dependent manner (65). In addition, tamoxifen, a well-recognized anti-tumour drug for breast cancer treatment, is able to increase levels of Beclin-1 to stimulate autophagy (66). Generally regarded as an mTORC1 inhibitor, RAD001 (everolimus) has also been found to increase Beclin-1 expression and induce autophagy in leukaemia (67,68).

Some small molecule compounds directly inhibit PI3KIII, thereby influencing expression of Beclin-1. 3-Methyladenine is also used as a specific inhibitor of autophagic sequestration, controlling PI3KIII and thus

Figure 3. Beclin-1, PI3KCIII and their autophagy-regulating compounds in cancer.

providing a target for its action (69). Furthermore, anti-proteolytic effects of wortmannin ($IC_{50} = 30$ nM) and LY294002 ($IC_{50} = 10$ μ M) have been found to be accompanied, not by an increase in lysosomal pH or a reduction in intracellular ATP, but by inhibition of autophagic sequestration (70). Thus, PI3KCIII inhibitors wortmannin and LY294002 inhibit autophagy in isolated hepatocytes (Fig. 3).

p53 with relevant small molecule compounds

Embedded within a highly interconnected signalling pathway, p53 regulates key cell processes such as DNA repair, metabolism, development, inflammation, endocytosis and cell death (71). It has been demonstrated that p53 can positively or negatively control autophagy, but its role in autophagy is paradoxical as it depends on its subcellular location; it occurs in two forms, cytoplasmic p53 and nuclear p53 (72). During responses of p53 to DNA damage, a number of cell death genes are transcriptionally activated, some of which play important roles in autophagy. When exposed to stress, nuclear p53 induces autophagy by acting at multiple levels on the AMPK-mTOR axis. It may down-regulate mTOR by transcriptional regulation of main activators of AMPK-Sestrin1/2, and target it to phosphorylate tuberous sclerosis 2 (TSC2) (73). Damage-regulated autophagy

modulator (DRAM) can also be regarded as a transcriptional target of nuclear p53 in autophagy modulation, explaining the complexity of mutual regulation in apoptosis and autophagy (74). Regarding this crosstalk, the pro-autophagic role of p53 can influence expression of Bcl-2 protein family members, including Bcl-2, Bcl-X_L, Mcl-1, and Bad, while reduction of p53 induces release of Beclin-1 from sequestration (75). p53-inducible BH3-only protein PUMA induces mitochondrial autophagy, but Bax alone can induce mitochondria-selective autophagy in the absence of PUMA activation (76). Following activation, cytoplasmic p53 translocates to the nucleus and regulates expression of a number of target genes (77). In this condition, PUMA enhances cell death by targeting mitochondria to autophagy, and in response to mitochondrial dysfunction, p53 also induces DRAM1-dependent autophagy (78). Cytoplasmic p53 also inhibits autophagy through inhibition of AMPK and activation of mTOR, resulting in hyper-phosphorylation of AMPK, TSC2, acetyl CoA carboxylase (ACC α) and p70^{S6K} (79). As a target of cytoplasmic p53, TP53-induced glycolysis and apoptosis regulator (TIGAR) inhibits autophagy by negatively modulating glycolysis and suppressing reactive oxygen species (80). However, divergent effects of nuclear and cytoplasmic p53 are still controversial. p53 variants in the cytoplasm significantly inhibit autophagy, whereas those in the nucleus may fail

Figure 4. p53 and its autophagy-regulating compounds in cancer.

to suppress it. Thus, p53 plays a role in controlling the basal level of autophagy, as nuclear p53 boost it and cytoplasmic p53 reduces it, independent of its transcriptional activity.

There are numbers of small molecule compounds identified to target p53 in different cancer cells. Tetracycline diterpenoid natural product oridonin activates p53 and ERK in murine fibrosarcoma L929 cells. Nitric oxide has been demonstrated to play a pivotal role in oridonin-induced autophagy, whose production may lead to oridonin-induced p53 and ERK activation, oridonin inducing autophagy in L929 cells *via* the nitric oxide-ERK-p53 positive-feedback loop (79,80). As a selective Met tyrosine kinase inhibitor, SU11274 activation leads to autophagic cell death in NSCLC A549 cells. It has been demonstrated that p53 can be activated after SU11274 treatment, while interruption of p53 activity reduces SU11274-induced autophagy (81). Treatment with polyphenolic compound, galangin, inhibits cell proliferation and induces autophagy, particularly leading to accumulation of autophagosomes and increase in p53 expression. This mediates autophagy through a p53-dependent pathway in HCC HepG2 cells (82). Additionally, metformin increases expression of p53, but reduces

mRNA level of anti-apoptotic Bcl-2. It has been demonstrated that anti-melanoma effects of metformin are mediated through autophagy associated with p53/Bcl-2 modulation, mitochondrial damage and oxidative stress (83). Fangchinoline is a highly specific anti-tumour agent which induces autophagic cell death *via* p53/Sestrin2/AMPK signalling in HCC HepG2 and PLC/PRF/5 cells (84). Previously used to treat high blood pressure and anxiety, prazosin has been found to induce patterns of autophagy by a p53-mediated mechanism in HPC2 cells, since cells exposed to prazosin have higher levels of phospho-p53 and phospho-AMPK (85). What is more, dihydrotychacol A, a macrocyclic bisbibenzyl derivative, increases p53 expression, induces p53 phosphorylation and up-regulates p21 (Waf1/Cip1), thus mediating autophagy associated with p53, in human osteosarcoma U2OS cells (86) (Fig. 4).

Conclusions

As an evolutionarily conserved lysosomal degradation process, autophagy is Janus-faced in cancer; it primarily acts as a survival mechanism, but can lead to cell death under certain circumstances. Autophagy can be modu-

lated by some key oncogenes (for example, *PI3KCI*, *AKT*, and *mTORC1*) and tumour suppressors (Beclin-1 and p53), which are implicated in autophagic signalling pathways. This seals the fate of the cancer cells. In addition, several small molecule drugs have also been demonstrated to target or regulate corresponding autophagic signalling pathways in cancer. To a certain extent, our understanding of the links between autophagy and cancer has benefited from the availability of rapamycin, an mTOR inhibitor, proven to be powerful at discerning major players in cancers associated with autophagy.

Autophagy interacts with cell death pathways via multiple mechanisms in different contexts, although its effect on cell death decisions is not always predictable. However, there is promising pre-clinical and clinical evidence, suggesting autophagy modulators may enhance certain types of anti-cancer treatment and reduce resistance to therapies, in many cases. Better understanding of how autophagy determines cell death decisions in specific contexts and development of new drugs, which will allow fine tuning of the autophagic response, have great potential to lead to novel anti-cancer treatment.

Although many small molecule compounds that regulate autophagy are presently utilized in autophagy research, some of these molecules have multiple functions and targets. As a result, more selective inducers and inhibitors of autophagy need to be developed to unravel the network of processes. The best hope for cancer therapeutics may lie in discovering candidate small molecule drugs that target core oncogenic or tumour-suppressive autophagic pathways or even the autophagic network itself, rather than individual genes or proteins. Application of combinatorial treatment may overcome weaknesses and limitations of traditional single-target drugs by employing a network-level perspective, and inhibiting multiple pathways. Thus, further elucidation of the complicated mechanisms involved in autophagy will provide promising information to promote discovery of novel small molecule drugs targeting oncogenic and tumour suppressive autophagic networks, in the near future.

Acknowledgments

This work was supported by grants from Key Projects of the National Science and Technology Pillar Program (no. 2012BAI30B02) and National Natural Science Foundation of China (nos 81402496, 81473091, U1170302, 81160543, 81260628, 81303270 and 81172374).

Conflict of interest

We declare that none of the authors has a financial interest related to this work.

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