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Expert Review

Programmed Cell Death Pathways and Current Antitumor Targets

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Abstract. Apoptosis and autophagic cell deaths are programmed cell deaths and they play essential roles in cell survival, growth and development and tumorigenesis. The huge increase of publications in both apoptosis and autophagic signaling pathways has contributed to the wealth of knowledge in facilitating the understanding of cancer pathogenesis. Deciphering the molecular pathways and molecules involved in these pathways has helped scientists devise and develop targeted strategies against cancer. Various drugs targeting the apoptotic TRAIL, Bcl-2 and proteasome pathways are already in Phase II/III clinical trials. The first mTOR inhibitor, temsirolimus has already been approved by the FDA, USA for the treatment of advanced renal cell carcinoma and more mTOR inhibitors are expected to be in the market in a few years time. Strategizing against aberrant autophagy activities in various cancers by using either pro-autophagics or autophagy inhibitors are currently been investigated. This review aims to discuss the most recent antitumor strategies targeting the apoptosis and autophagy signaling pathways and the latest outcome of clinical trials of the above drugs.

KEY WORDS: apoptosis; autophagy; autophagic cell death; cancer; programmed cell death.

INTRODUCTION

Cell death plays an important role in the development and homeostasis of normal tissues (1,2) and has profound effects on cancer growth and progression (3–5). An imbalance between cell proliferation and cell death is known to link to human diseases including cancer, autoimmune disease, neurodegenerative disorders, viral infections and AIDS (6–10). Current cancer therapies are based on the removal of solid tumor masses and are usually followed by a series of chemical or physical treatments such as chemotherapy and radiotherapy. The ability of these therapies to induce death of these rapidly growing cells forms the very basis of cancer therapy. Most chemotherapeutic agents as well as ionizing radiation utilize the endogenous mechanisms to induce programmed cell deaths (11,12). Further deciphering of the mechanisms and signaling pathways of these cell deaths have brought forward a new paradigm in which cancer may be efficiently targeted. Novel and specific cancer therapeutics and techniques directed at members of the cell deaths signaling pathways are being developed and currently being tested in clinical trials. The two major types of cell deaths, namely apoptosis and autophagic cell death, the most recent antitumor strategies targeting these cell death pathways and some of the novel anticancer drugs will be discussed.

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APOPTOSIS, CANCER AND THERAPEUTIC TARGETS

Historically, the term programmed cell death (PCD) was first mentioned by Lockshin and Williams in 1965 (13). The phenomenon was used to describe the coordinated deaths of certain larval muscles during transformation into adult moths. Kerr and co-workers later described a series of similar morphological characteristics which accompanied the deaths of a variety of tissue sources, which was then coined as "apoptosis" (14). The phenomenon of apoptosis was in fact discovered earlier by Carl Vogt in 1842 (15). However, it was decades later before the term apoptosis was adopted and generated a continuous interest in this field of science. Apoptosis or Type 1 cell death is a distinct mode of cell death that is fundamentally different from other forms of cell death based on its morphology, biochemistry and incidence (12). Apoptosis and mitosis share some commonality and are thought to be equal and opposite in a kinetic sense, envisaged as a push–pull relationship, which in normal conditions, exists in dynamically equilibrium state (16). Apoptotic processes are known to have widespread biological significance as they play important roles in cell development, proliferation/homeostasis, differentiation, regulation of the immune system and in the removal of defect and harmful cells.

The earliest recognized morphological changes in apoptosis involve compaction and segregation of nuclear chromatin and condensation of the cytoplasm (12,14). The plasma membrane convolutes or blebs in a florid manner, producing

fragments of cells (apoptotic bodies). These fragments are membrane-bounded and contain nuclear components (12,14,17). The apoptotic bodies are quickly taken up by nearby cells and degrade within their lysosomes, usually with no associated inflammation (12,14). Biochemically, apoptosis is characterized by the double stranded cleavage at the linker regions between nucleosomes, resulting in the formation of multiple DNA fragments (17), phosphatidylserine externalization (18) and is accompanied by a series of genes and protein expressions. Currently, there are two signaling pathways mediating apoptosis. In the extrinsic pathway, apoptosis is mediated by death receptors on the cell surface; while in the intrinsic pathway, mitochondria play an important role. In both pathways, activated caspases (cysteine aspartic acid specific proteases) cleave and activate other downstream cellular substrates. Caspases are synthesized as inactive zymogens (or pro-enzymes) and are usually cleaved to form active enzymes or undergo auto-proteolysis in a cascade manner.

In the extrinsic pathway, apoptosis is mediated by death receptors, which belong to TNF (tumor-necrosis factor) receptor superfamily and are characterized by extracellular cysteine-rich domains (CRDs) and intracellular death domain (DD). Ligands such as TNF ligand, TNF ligand superfamily member 10 (TNFSF10), Fas ligand and TRAIL (TNF-related apoptosis-inducing ligand) interact with their respective death receptors, recruit Fas-associated DD adapter protein (FADD) and form the death inducing signaling complex (DISC). This complex further recruits pro-caspase-8 and pro-caspase-10, leading to the activation of the executioner caspase-3, caspase-6, and caspase-7 (19,20). Active caspase-3 or caspase-7 proteolytically cleaves DFF45 (DNA fragmentation factor-45), which subsequently releases active DFF40; the inhibitor's associated endonuclease. It is responsible for the degradation of chromosomes into nucleosomal fragments, the characteristic hallmark of apoptosis (21,22).

On the other hand, the intrinsic pathway is mediated by the mitochondria. In response to apoptotic stimuli, such as DNA damage, γ-irradiation, and serum deprivation, cytochrome *c* (Cyt *c*) (20,23,24), SMAC/DIABLO (direct inhibitor of apoptosis-binding protein) (25–27), AIF (apoptosis-inducing factor, promotes chromatin condensation) (28), and EndoG (endonuclease G, facilitates chromatin degradation) are released from the mitochondria (29,30). Cyt *c* binds to and activates Apaf-1 (apoptotic protease activating factor-1) protein in the cytoplasm. This induces the formation of apoptosome which subsequently recruits the initiator pro-caspase-9, yielding activated caspase-9 and finally mediates the activation of caspase-3 and caspase-7.

Bcl-2 (B-cell lymphoma 2) family of proteins are found to play an important role in the regulation of mitochondrial-linked apoptosis (31). Bcl-2 subfamilies such as Bax, Bak, and BH (Bcl-2 homolog) 3-only subfamily (e.g. Bid) are pro-apoptotic while Bcl-2 and Bcl-X_L are functionally anti-apoptotic. Activated Bax and Bak form homo-oligomer which creates pores on the mitochondrial membrane and releases Cyt *c* from the mitochondria. Bcl-2 and Bcl-X_L inhibit the action by blocking the activation of Bax and Bak and prevent the release of the pro-apoptotic protein (32). Nevertheless, the activation of Bax and Bak can be restored with the presence of pro-apoptotic BH3-only proteins. BH3-only proteins function as antagonists of specific subsets of their pro-survival relatives (33). These death ligands induce apoptosis by unleashing Bak or Bax from control by the pro-survival proteins (34). In certain cell types, where the apoptotic extrinsic pathway are triggered but lower levels of DISC complex are formed, and thus lower levels of active caspase-8; amplification of the death signal is possible through the cleavage of Bid by caspase-8, which directly mediates Bax/Bax oligomerization and triggers the release of Cyt *c* (35,36). The release of Cyt *c* from mitochondria results in the formation of apoptosome, followed by the recruitment of procaspase-9, and subsequently activation of downstream effector caspases (36).

In addition, inhibitors of apoptosis (IAPs) also play an important role in the regulation of apoptosis. Currently, eight human IAPs have been identified such as X-linked IAP (XIAP), IAP-like protein-2 (IAP-2), c-IAP-1, c-IAP-2, ML-IAP, neuronal apoptosis inhibitory protein (NAIP), survivin, and apollon (37). Human IAP family members such as XIAP, cIAP1 and cIAP2 are potent caspase inhibitors (38,39). XIAP, c-IAP1 and c-IAP2 block Cyt *c*-induced activation of caspase-9, thus preventing the activation of caspases-3, -6 and -7. Furthermore, these IAPs bound to and inhibit the enzymatic activity of caspase-3 following its activation by caspase-8, thereby arresting the proteolytic cascade initiated by the initiator caspase (40). XIAP primarily inhibits caspase by disrupting the conformation of the active caspase and masks the substrate binding active site (37).

Apoptosis is an essential developmental process which maintains tissue homeostasis and serves as a barrier to oncogenic transformation (41). Defective apoptosis regulation is known to cause neoplastic cells to gain extended lifespan, develop genetic mutations, capable of growth under stress conditions, and tumor angiogenesis (7). Tumor resistance to apoptotic cell death is often a hallmark of cancer and contributes to chemo-resistance (7). Several key pathways controlling apoptosis are commonly altered in cancer (42). For example, overexpression of certain anti-apoptotic proteins, such as Bcl-2, Bcl-X_L, Akt, nuclear factor-κB (NF-κB) and IAP protein family, are found in many types of human tumors (43). Since cancer cells are highly dependent on these genetic changes (specifically in the apoptotic pathways) for survival, designing novel anticancer drugs that selectively kill cancers cells while sparing normal cells are considered logical and appropriate (41).

THE APOPTOSIS SIGNALING PATHWAYS AS ANTI-TUMOR TARGETS

TRAIL (TRAIL Ligands, Monoclonal Antibodies Against TRAIL-R1 and TRAIL-R2)

TRAIL (Apo2 ligand) is a death protein and it induces cell death via the extrinsic pathway by recruiting and activating caspase-8 and caspase-10 to its R1 and R2 receptors (44). TRAIL also activates the intrinsic pathway via the TRAIL-caspase-8-tBid-Bax cascade, through the cleavage of Bid, which promotes Bax and Bak oligomerization, leading to Cyt *c* release and activation of caspase-9 (45). These actions collectively amplify the activities of the related executioner caspases. TRAIL is set to be a promising cancer therapeutic agent as it induces apoptosis in a wide variety of

tumor cells, sparing normal cells (46,47). TRAIL activity is also known to be independent of p53 status, making it potentially effective against chemotherapy-resistant tumors (48). Early clinical trials have been initiated in cancer patients, using soluble recombinant TRAIL (rhAPO2L, co-developed by Genentech and Amgen) (49,50), monoclonal antibodies (agonists) targeting TRAIL-R1, such as mapatumumab (HGS-ERT1 developed by Human Genome Sciences), and anti-TRAIL-R2, such as lexatumumab (HGS-ETR2 developed by HGS), AmG 655 (developed by Amgen) and apomab (developed by Genentech) (51).

In a phase I safety and pharmacokinetic trial of rhAPO2L used as a single agent in patients with advanced solid tumors and non-Hodgkin lymphoma (NHL), of 36 patients with available data, 32 of them had at least one post-baseline tumor assessment with 17 (53%) having stable disease, and 13 (41%) with disease progression. Only a single patient was reported to have a partial response to the drug (49). Although there were no reported cases of toxicities, patient's response towards rhAPO2Lwas clearly unremarkable. In another phase IB study of rhAPO2L in combination with rituximab in six patients with low-grade NHL, two patients showed complete response, one with partial response and two with stable disease (52). According to the authors, the combination of rhApo2L/TRAIL and rituximab appeared safe and showed evidence of activity in these subjects with low grade NHL. However, the low number of subjects may render the results inconclusive. In another phase IB trial of rhTRAIL in combination with paclitaxel, carboplatin and bevacizumab in patients with advanced non-small cell lung cancer (NSCLC), the overall response rate reported was 56% (53).

Mapatumumab either used alone or in combination with other chemotherapy drugs in phase I or phase II trials involving either solid tumors, NHL or NSCLC have yet to produce impressive trial outcomes, as in most cases, few patients were observed with partial response or stable disease (54–57). Similarly for lexatumumab, AMG 655 and apomab, the percentage of patients developed partial response or stable disease in several phase I trials was low (51,58–60). However, these drugs were quite well tolerated by patients. There was generally a lack of data on patient's TRAIL status and response to therapy in these early trials. Preferential TRAIL sensitivity and presence of TRAIL-R1 and TRAIL-R2 expression in certain cancers are considered factors in patient's response. Therefore, rTRAIL and agonistic anti-TRAIL-R therapies may be limited to patients with TRAIL-sensitive tumors. It was suggested that the efficacy of TRAIL targeting therapies may be improved when diagnostic methods determining TRAIL sensitivity of clinically detectable human cancers are available (51). Trials are still ongoing, especially involving combination of these agents with current chemotherapy drugs.

BCL2 Family Proteins (BH3 Mimetics and Bcl-2 Antisense)

The Bcl-2 family is characterized by specific regions of homology termed Bcl-2 homology (BH1, BH2, BH3, and BH4) domains. In human, the Bcl-2 family is divided into anti-apoptotic and pro-apoptotic proteins. Anti-apoptotic proteins have BH1–BH4 domains (e.g. Bcl-2 and Bcl-X_L). Pro-apoptotic proteins have either BH1–BH3 domains (e.g. Bax and Bak) or BH3-only domains (e.g. Bid, Bim, Puma, Bad, Noxa, Hrk, Bik) (34,61,62). These domains are critical to the function of these proteins, especially their impact on cell survival, cell death and their ability to interact with other family members and regulatory proteins. The molecular surface of the multidomain anti-apoptotic Bcl-2 protein contains a BH3 binding groove, which accommodates BH3 domain from pro-apoptotic Bcl-2 proteins family members. The BH3-only proteins are known to function as antagonists of anti-apoptotic Bcl-2-family proteins and act as tumor suppressors (34). This has provided the platform for subsequent drug discovery strategies based on mimicking BH3 peptides with chemical compounds that bind in the same groove (63).

Apoptosis deregulation in cancer cells appears to primarily affect the signaling pathways upstream of Bax/Bak and mitochondria, leaving the downstream core apoptotic machinery mostly intact (43,64). Therefore, this has become a very appealing strategy for restoring apoptosis in cancer cells by manipulating the equilibrium between the pro- and anti-apoptotic Bcl-2 family members (41). Since pro-apoptotic BH3 domains bind directly to the hydrophobic grooves of pro-survival proteins with high affinity, and are necessary and sufficient for initiation of apoptosis (65), agents mimicking the BH3 domains may provide some degree of selectivity against cancer cells. This is mainly because cancer cells are postulated to be more sensitive to inhibition of pro-survival proteins compared with their normal counter parts (7). Cancer cells often express high levels of Bcl-2-like anti-apoptotic proteins to evade the apoptotic fate imposed by unscheduled cell proliferation, activation of oncogenes, or DNA damage (66,67). Therefore, it is possible to design BH3 mimetics to target specific anti-apoptotic proteins that are over-expressed in a particular type of cancer for improved specificity (41). Several chemicals mimicking BH3 peptides exclusively targeting the Bcl-2 anti-apoptotic proteins have since been described (63,68–70). Another antitumor strategy is direct inhibition of Bcl-2 mRNA, in the form of antisense.

One of the earliest small-molecule BH3 mimetics or more accurately Bcl-2 and Bcl-X_L inhibitors that went through several Phase I/II clinical trials is Gossypol, an orally-available compound derived from cottonseed extracts (71). It binds to the BH3-binding grooves of Bcl-2, Bcl-X_L and Mcl-1 (72). Several past clinical trials have failed to indicate this compound as a meaningful anticancer agent. For example, in a previous clinical study carried out in 23 patients with advanced refractory solid tumors, none of the patients showed evidence of tumor regression during treatment (73). A phase II trial of gossypol (10 mg orally twice daily) performed in 27 patients with pathologically confirmed recurrent glial tumors showed low treatment response and their plasma levels did not correlate well with response or toxicity (74). In another phase I/II study of 20 women with metastatic breast cancer refractory to doxorubicin and paclitaxel, the drug produced no therapeutic responses (75). This may explain the lack of clinical trials involving gossypol ever since. Recently, AT-101, a derivative of R-(−)-gossypol, was found to be well tolerated in an ongoing phase I trial involving chronic lymphocytic leukemia (CLL) patients (76). Currently, a semi-synthetic analog of gossypol with improved pharmacologic properties, such as apogossypolone (ApoG2)

was found to inhibit the growth of diffuse large cell lymphoma cells *in vitro* and *in vivo* (77).

GX015-070 (obatoclax mesylate) is an indole-derivative and a broad-spectrum inhibitor of pro-survival Bcl-2 family proteins and has been evaluated in clinical trials. A phase I clinical trial of obatoclax mesylate in 44 patients with refractory leukemia and myelodysplasia has demonstrated that the drug was well tolerated up to the highest dose. A single patient with acute myeloid leukemia (AML) with mixed lineage leukemia t(9;11) rearrangement achieved a complete remission, which lasted 8 months. Three of 14 patients with myelodysplasia showed hematologic improvement (78). In another phase I trial, where obatoclax was administered to patients with advanced CLL, activation of Bax and Bak was demonstrated in peripheral blood mononuclear cells and induction of apoptosis was related to overall obatoclax exposure, as monitored by the plasma concentration of oligonucleosomal DNA/histone complexes. Obatoclax mesylate was noted to have biological activity and modest single agent activity in heavily pretreated patients with advanced CLL (79).

Currently, there is a nuclease-resistant phosphorothioate antisense oligonucleotide targeting Bcl-2 mRNA (oblimersen sodium), which has shown promising activity for CLL and malignant melanoma in randomized phase III trials. It is an 18-mer phosphorothioate antisense oligonucleotide designed to bind to the first six codons of the human bcl-2 mRNA (80). The use of oblimersen in combination with chemotherapy in a variety of cancers has shown diverse response rates with good tolerability. In the Oblimersen Melanoma Study Group, the addition of oblimersen to dacarbazine has significantly improved multiple clinical outcomes in patients with advanced melanoma and there was an increased in overall patient's survival (81). In another phase III trial, the addition of oblimersen to fludarabine and cyclophosphamide has significantly increased the complete response/nodular partial response rate in patients with relapsed or refractory CLL (82). However, not all combination therapies produce desirable outcomes. In the Cancer and Leukemia group B study 10107 (CALGB), although the combination of oblimersen and imatinib was safe and feasible, there were no observed clinical benefits in the imatinib-resistant chronic myeloid leukemia (CML) patients (83). In the randomized phase II study of carboplatin and etoposide with or without oblimersen for extensive-stage small-cell lung cancer (CALGB 30103), the addition of oblimersen to a standard regimen for this disease did not improve any clinical outcome measure (84).

Proteasome Inhibition

The proteasome is a multicatalytic enzyme complex that degrades intracellular proteins by a targeted and controlled mechanism (85). Degradation of protein via the ubiquitin/proteasome pathway involves two steps, i.e. conjugation of multiple ubiquitin moieties to the substrate and degradation of the tagged protein by the downstream 26S proteasome complex into oligopeptides (85,86). The proteasome degrades a wide range of protein substrates involved in cell cycle regulation, apoptosis, and other cellular functions (87). Some of the important proteins that are degraded by proteasomes include key signaling molecules involved in tumor suppression and pro-apoptotic proteins. Therefore, proteasome inhibition has emerged as an attractive target for cancer therapy. Preclinical studies have shown that the proteasome inhibitor, bortezomib decreases proliferation, induces apoptosis, enhances the activity of chemotherapy and radiation, and reverses chemoresistance in a variety of hematologic and solid malignancy models *in vitro* and *in vivo* (87).

Bortezomib is the first proteasome inhibitor that has undergone clinical trials after a series of successful preclinical studies. So far, phase I and II trial results produced encouraging prospects. In a current retrospective study (based on data from Phase II (SUMMIT or CREST) or Phase III (APEX) registration studies) to clarify the utility of bortezomib as a repeat therapy, bortezomib retreatment appears to be safe and effective in patients with relapsed multiple myeloma (88). In a separate phase I/II trial, weekly bortezomib plus oral cyclophosphamide and prednisone produced more than 50% complete response rate and an encouraging 1-year survival in relapsed/refractory patients with multiple myeloma (89). Some phase III trials in patients with multiple myeloma are currently being initiated (90). However, clinical trials involving other cancers such Hodgkin's lymphoma (91), advanced solid tumors such as breast, ovarian and prostate (92) and metastatic gastroesophageal cancer (93) lacked favorable outcomes.

Other Potential Target in Preclinical Stages

Smac/DIABLO (Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low p*I*) is one of the many proteins released from the mitochondria into the cytosol in response to apoptotic stimuli. Smac/DIABLO promotes apoptosis by antagonizing the IAPs, such as XIAP, cIAP-1, and cIAP-2, which are often up-regulated in many cancer cells (94). This activity has prompted the synthesis of peptides that mimic Smac/DIABLO functions and potentially act as therapeutic agents capable of inducing death or to increase the apoptotic effect of chemotherapeutic agents (25,94). In a recent study, the synthesized Smac/DIABLO-N7 peptide was found to increase the apoptosis-inducing potential of chemotherapeutic drugs (paclitaxel, doxorubicin and tamoxifen) and irradiation, and sensitized TRAIL-resistant cells to undergo apoptosis (95). However, none of the drugs in this category have yet progressed to the human clinical trials. The summary of the apoptosis pathways and antitumor targets are as shown in Fig. 1. Table I summarizes the various drugs targeting the apoptosis pathways and clinical trial stages based on published reports.

AUTOPHAGY, AUTOPHAGIC CELL DEATHS, CANCER AND THERAPEUTIC TARGETS

Autophagy literally means self-digestion in Greek (96). It is responsible for the turnover of unnecessary or dysfunctional organelles and proteins, such as damaged mitochondria (97). These processes are important to maintain a well-controlled balance between anabolism and catabolism to facilitate normal cell growth and development. It is also a survival pathway, required during starvation or growth factor deprivation as it provides an alternative energy source (98,99). Autophagy plays an essential role during starvation,

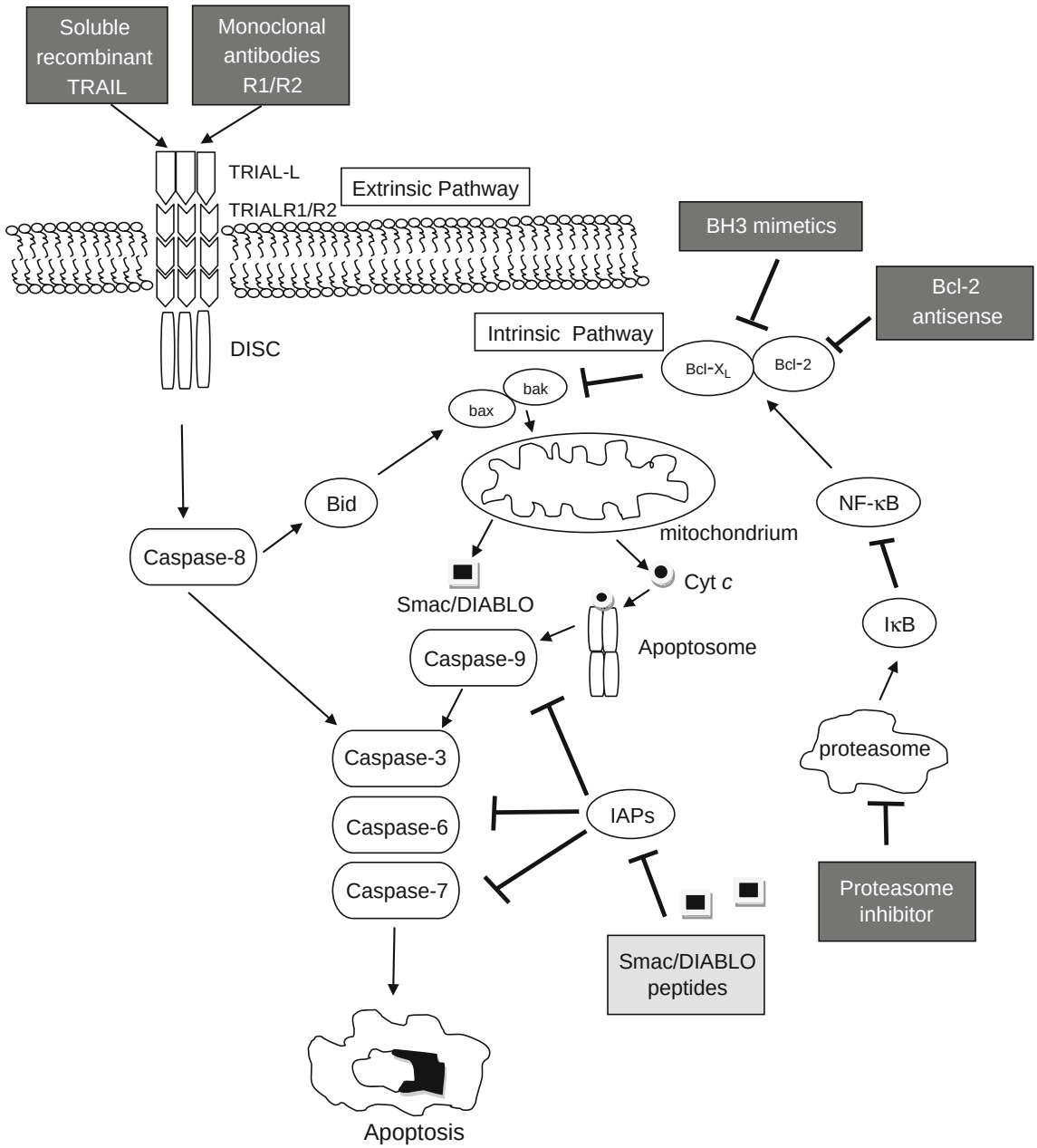

Fig. 1. Apoptosis signaling pathways (extrinsic and intrinsic pathways) and current antitumor targets. Drugs targeting TRAIL (rhTRAIL, agonists TRAIL-R1 and TRAIL-R2), BH3 mimetics, Bcl-2 antisense and proteasome inhibitor are currently in various phases of clinical trials.

cellular differentiation, cell death, cell survival, aging and tumor prevention (97,100–103).

Briefly, the autophagy process begins with the formation of a pre-autophagosomal structure known as isolation membrane or phagophore (104). The isolation membrane engulfs cytosolic components and further elongates to form the autophagosome, surrounding the components destined to be recycled. The autophagosome, which is a double membrane-bounded structure undergoes maturation and fuses with both endosomal and lysosomal vesicles to form autolysosomes (104–106). The sequestered contents are subsequently degraded by lysosomal hydrolases and are recycled. In mammalian autophagy, LC3 protein is used as an index of autophagosome formation. Following the synthesis as Pro-LC3, C-terminal fragment is cleaved off by hAtg4Bp to yield a cytosolic precursor form, LC3-I (soluble, unlipidated). LC3-I subsequently conjugates with a phospholipid via a ubiquitylation-like system to form the active LC3-II (lipidated, membrane-bounded), which is then targeted to the early autophagosome membrane (107). LC3-II is localized to preautophagosomes and autophagosomes, making this protein an autophagosomal marker (108). Following the fusion of autophagosomes with lysosomes, intra-autophagosomal LC3-II is rapidly degraded by lysosomal hydrolytic enzymes (109).

In general, autophagy promotes survival to stress. However, there are increasing evidences that when autophagy is over-stimulated, it can progress to autophagic cell death (110–112). It has been also documented that malignant cell

Table I. Current Therapeutic Targets and Clinical Trial Stages

Type of cell death	Therapeutic targets	Current drugs	Type of cancer	Clinical trial stages (published reports)
Apoptosis				
TRAIL	TRAIL	rhApo2L	Advanced solid tumors and non-Hodgkin lymphoma (NHL) (with and without metastasis)	Phase I (2008)
			Low grade NHL, NSCLC	
	TRAIL-R1 receptor	Mapatumumab	Advanced solid tumors or NHL	Phase I/II (2008)
			Advanced NSCLC	
	TRAIL-R2 receptor	Lexatumumab	Advanced solid tumors	Phase I (2008)
		AMG 655	Advanced solid tumors	Phase I (2007)
		Apomab	Advanced solid tumors	Phase I (2007)
BCL2 family proteins	Bcl2 and Bcl-XL inhibitors	Gossypol	Advanced solid tumors, adult malignant gliomas, metastatic breast cancer	Phase I (2001)
		AT-101 (derivative of gossypol)	CLL	Phase I (2006)
		Obatoclax mesylate	Advanced hematologic malignancies	Phase I (2008)
	Bcl2 mRNA	Oblimersen sodium	Advanced melanoma and hematologic malignancies	Phase II/III (2008)
Proteasome	Proteasome inhibitor	Bortezomib	Multiple myeloma	Phase II/III (2008)
			Hodgkin's lymphoma	Phase II (2008)
			Advanced solid tumors	Phase I/II (2008)
Autophagy and autophagic cell death				
mTOR signaling pathway	mTOR inhibitors	Everolimus	Advanced renal cell carcinoma	Phase III (2008)
		Deforolimus	Advanced hematologic malignancies	Phase I/II (2008)
	PI3K/mTOR inhibitor	NVP-BEZ235	Advanced solid tumors	Phase I, ongoing
Autophagy	Pro-autophagic	Temozolomide	Advanced NSCLC	Phase II (2009)

types undergo autophagic cell death when responding to anticancer agents and traditional herbs, indicating the potential utility of autophagic cell death induction in cancer therapy (101,106,113). Morphologically, autophagic cell deaths are characterized by an increase in the number of autophagic vacuoles in the cytoplasm, followed by cell demise. This has been observed in various diseases such as Alzheimer's disease (114) Huntington's disease (115–118), Parkinson's disease (119). However, details on how the physiological autophagy process can progress to autophagic cell death remain to be investigated.

One of most studied and important pathways involved in the regulation of autophagy is the PI3K-Akt-mTOR signaling pathway. The mammalian target of rapamycin, commonly known as mTOR, is a serine/threonine kinase which belongs to the family of phosphatidylinositol kinase-related kinase. It regulates translation and cell growth by its ability to phosphorylate both 4E-BP1 and p70s6k. Upstream PI3K (phosphoinositide 3-kinase) and Akt activation by growth factors leads to the activation of mTOR and subsequently phosphorylation of these substrates. Phosphorylation of p70s6k promotes ribosome biogenesis and increases the capacity of the translational machinery for protein synthesis (120). Phosphorylation of 4E-BP1 initiates the transcription of a subset of mRNAs important for cell growth and proliferation (120–123). The mTOR kinase is also a key regulatory component that controls the induction of autophagy (106,124,125). Inhibition of mTOR (by nutrient-depletion, starvation or rapamycin) leads to cell cycle arrest, inhibition of cell proliferation, immunosuppression and induction of autophagy. Increased levels of the mTOR kinase are found to inhibit the autophagy process resulting in an increased in cell growth and tumor development (106). Rapamycin is a specific mTOR inhibitor. It complexes with the cytosolic receptor FK506-binding protein (FKBP-12), and subsequently binds to a distinct region of mTOR upstream of the catalytic domain (126). It induces autophagy and inhibits the proliferation of a variety of cells (127).

Beclin-1 is a 60KDa tumor suppressor protein and is identified from a yeast two-hybrid screen as interacting with Bcl-2 (128). Beclin-1 is found to be mono-allelically deleted in a high percentage of ovarian, breast and prostate cancers (based on the 17q21 and gene mapping studies). It has been demonstrated to have a direct link between tumorigenesis and the disruption of autophagy (129). Beclin-1 binds to Class III P13K, which promotes the trafficking of lysosomal enzymes to the lysosomes, which Beclin-1 may be involved in the process (106). 3-methyladenine (3MA) is a known inhibitor of Class III PI3K, inhibits autophagy. Reduced expression of Beclin-1 is associated with a reduced autophagic vacuole formation (130,131). Overexpression of Beclin-1 in MCF-7 human breast cancer cells is found to facilitate autophagy induced by serum and amino-acid deprivation, which indicates that Beclin-1 is a necessary regulator for autophagy (129).

It is widely postulated that the autophagic pathway is deregulated in tumour cells. It has been reported that several proteins and pathways related to autophagy signaling are deregulated during cancer development (129,132). Cell lines derived from hepatic, pancreatic and breast carcinoma exhibit low autophagic activity, as compared with normal cells from the same origin (129,133). Autophagic capacity is known to

increase during premalignant stages of pancreatic carcinogenesis, and then decreases during the transition of pancreatic adenoma into adenocarcinoma, suggesting that decreased autophagic activity possibly contributes to the malignancy of pancreatic cancer (113,134,135). A decrease in autophagic capacity is also observed during animal experimental carcinogenesis, where cells from preneoplastic liver nodules, or primary hepatocellular carcinomas induced by chemical carcinogens showed a decreased autophagic capacity as compared to normal cells from the liver (113,135–137). Therefore, there are good reasons to suggest that manipulation of autophagy may provide useful ways to increase the efficacy of cancer treatments, prevent cancer development and limit tumor progression.

However, autophagy is divergent in nature in both tumor suppression and tumor progression (138). Although the argument supports that if cells cannot activate autophagy, protein synthesis will predominate over protein degradation and cellular growth continues (typical characteristic of tumor cells). There were some exceptional cases. For example, studies in human epidermoid lung carcinoma cells revealed that the autophagic pathway in response to nutrient deprivation is not down-regulated when compared to their normal counterparts (139). Human colon cancer cells which were able to survive for long periods of time in the absence of nutrients, have a high rate of autophagy activity (140). Studies in colorectal cancer cells revealed that these cancerous cells harbor functional autophagic machinery functions to prolong cell survival during shortages of nutrients (141). A recent study by Fujii and co-workers has also showed that strong LC3 expression in the peripheral area of pancreatic cancer tissue is correlated with poor outcome and short disease-free period (142). The authors have even concluded that activated autophagy associated with pancreatic cancer cells may be a response to factors in the cancer microenvironment, such as hypoxia and poor nutrient supply (142).

Autophagy could be activated in more advanced stages of cancer to guarantee survival of cancer cells under extreme conditions, such as the restricted access of cells located in the inner areas of solid tumors to nutrients (143). It is believed that during the early stages of development of solid tumors, blockage of autophagy maintains continuous growth. As the tumor mass grows, internal cells will probably switch to a catabolic state to guarantee their survival, although cells in the periphery close to blood vessels can maintain the anabolic state. Although autophagy is suppressed during the early stages of tumor progression, it will be up-regulated during later stages as a protective mechanism against stress conditions such as low oxygen and/or nutrient levels (144). Both oxygen and glucose supply is usually insufficient for the aggressively proliferating cancer cells in locally advanced cancers (145–147). Therefore, an increase in the autophagic activity in some cancer cells may contribute to survival in harsh microenvironments in which cancers are known to progress (142). Autophagy is known to promote the survival of normal cells during nutrient starvation. Similarly, it may enhance the survival of rapidly growing cancer cells that have outgrown their oxygen and nutrient supply. Autophagy may also promote the survival of cancer cells by targeting damaged mitochondria and other organelles for lysosomal degradation, thereby buffering oxidative stress that can be triggered by activated cancer-causing genes or by cancer treatments (148).

Suppression of autophagy may contribute to the initial rapid growth of tumors, however, in more advanced stages of cancer, autophagy may be required to provide essential nutrients to the cells in the inner part of a solid tumor that do not have direct access to the circulation (149). Therefore, some of the recent strategies for cancer treatment suggested include inducing autophagy in early developed cancers while inhibiting autophagy in advanced tumor cells with intact autophagy response to sensitize the cells to a variety of anticancer agents (150).

THE AUTOPHAGY SIGNALING PATHWAYS AS ANTI-TUMOR TARGETS

The mTOR Signaling Pathways (mTOR Inhibitors)

Rapamycin, as the first prototype of an mTOR inhibitor has poor aqueous solubility and strong immunosuppressive properties. Therefore its utilization at doses susceptible to produce an effect as an anticancer agent is limited (151). Various rapamycin analogues have since been developed (152). temsirolimus (CCI-779) is the first mTOR inhibitor approved by the U. S. Food and Drug Administration for cancer treatment and is considered a first-line treatment for patients with advanced RCC (renal cell carcinoma) with poor prognostic features (152). The other rapamycin analogs currently in clinical development as anticancer agents include everolimus (RAD-001) and deforolimus (AP23573) (152). These agents have demonstrated significant anticancer activities in preclinical studies.

Everolimus, a derivative of rapamycin, and is structurally similar to temsirolimus, binds to an intracellular protein, FKBP-12, forming a complex that inhibits the mTOR kinase. A recent phase III, randomised, double-blind, placebo-controlled trial of everolimus in patients with metastatic renal cell carcinoma, revealed that everolimus as a single therapy was associated with a reduction in the risk of progression or death compared with placebo in patients with metastatic renal cell carcinoma whose disease had progressed after treatment with VEGF-targeted therapies (153). Deforolimus (AP23573) has been tested in phase I and II clinical trials and has showed promising results in several tumor types including sarcoma (151,154). However, in a recent phase II trial study on efficacy and safety of single-agent deforolimus in patients with relapsed or refractory hematologic malignancies, the results were unremarkable. Of the 52 patients evaluated, partial responses were noted in five subjects while hematologic improvement/stable disease was only observed in 21 patients (155).

Pro-Autophagics

The first proautophagic chemotherapy to overcome apoptosis resistance in cancer cells comes from the use of temozolomide, a proautophagic cytotoxic drug, which has demonstrated therapeutic benefits in glioblastoma patients and is currently in clinical trials for several types of apoptosis-resistant cancers (144). Temozolomide is a prodrug, a monofunctional alkylating agent and is chemically related to dacarbazine. It is the 3-methyl derivative of the experimental

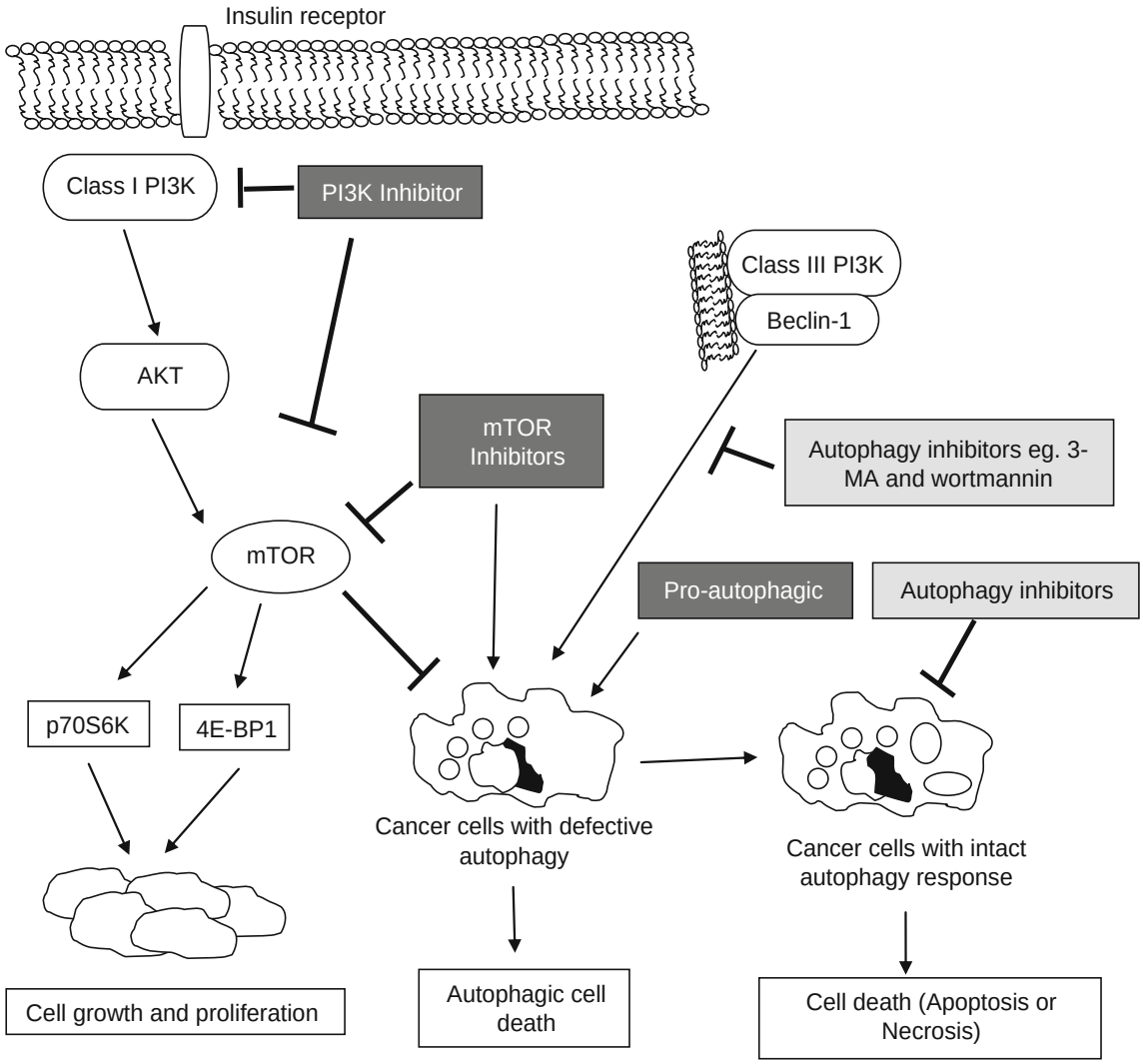

Fig. 2. Autophagy signaling pathways and current antitumor targets. PI3K inhibitor (NVP-BEZ235), mTOR inhibitors (everolimus and deforolimus) and pro-autophagic (temozolomide) are currently in various phases of clinical trials.

anticancer drug, mitozolomide. The ability of temozolomide in inducing autophagic cell death is reported in various preclinical studies (156–159). In addition, temozolomide has also demonstrated pro-apoptotic activities in malignant melanoma cells (160).

In a systematic assessment of three randomized controlled trials whether temozolomide holds any advantage over conventional therapy for high grade gliomas, it was concluded that temozolomide was an effective therapy for glioblastoma multiforme (GBM). The drug has helped prolonged survival and delayed progression as part of primary therapy and has a low incidence of early adverse events (161). Similar outcomes were observed in a recent prospective, single-arm, phase II study involving erlotinib in combination with radiation therapy and temozolomide to treat GBM and gliosarcoma. The median survival of patients was 19.3 months in the treatment group as compared with 14.1 months in the historical control studies. Patients treated with the combination of erlotinib and temozolomide during and following radiotherapy had better survival than historical controls (162). However, good therapeutic effects were not observed in patients with NSCLC. In a current efficacy and safety study of temozolomide in pretreated patients with NSCLC, the results revealed that only two patients achieved a partial response and three had stable disease of a total of 31 patients evaluated (163). The researchers have also pointed out that prolonged low daily doses of temozolomide have demonstrated minimal activity in patients with advanced NSCLC. Perhaps more phase II and III studies to characterize the efficacy of this drug in various cancers are warranted.

PI3K Inhibitors

NVP-BEZ235 is an imidazo[4,5-c]quinoline derivative that inhibits PI3K and mTOR kinase activity by binding to the ATP-binding cleft of these enzymes and induces G1 arrest (164). Preclinical studies have suggested that NVP-BEZ235 is a potent dual PI3K/mTOR modulator with favorable pharmaceutical properties. For example, it has been found to potently inhibit VEGF-induced HUVEC cell proliferation and survival *in vitro* and VEGF-induced angiogenesis *in vivo* (165). The compound has also shown to inhibit microvessel permeability in BN472 mammary carcinoma grown orthotopically in syngeneic rats, suggesting that this compound is

potentially anti-angiogenic (165). Deregulated angiogenesis and high tumor vasculature permeability are known VEGF-mediated characteristics of human tumors. In addition, NVP-BEZ235 is found to produce significant tumor growth inhibition in xenograft models of pancreatic cancers and breast cancer cells (166,167). NVP-BEZ235 is currently in phase I clinical trials (164). Reports on the safety and efficacy of this drug in humans have yet to be published.

Autophagy Inhibitors

Since autophagy activities are known to differ according to stages of cancer, modulation of autophagy is postulated to enhance efficacy of anticancer therapy. In one preclinical study, effects of imatinib with or without different types of autophagy inhibitors on human malignant glioma cells was carried out (168). It was demonstrated that suppression of imatinib-induced autophagy by 3-methyladenine (3-MA) or small interfering RNA against Atg5 (which inhibit autophagy at an early stage), attenuated the imatinib-induced cytotoxicity. On the other hand, inhibition of autophagy at a late stage by bafilomycin A1 or RTA 203 enhanced imatinib-induced cytotoxicity through the induction of apoptosis (168). Thus, the authors have even suggested that therapeutic efficiency of imatinib for malignant glioma may be augmented by inhibition of autophagy at a late stage, which could help sensitize tumor cells to anticancer therapy (168). Drugs in this category have yet progress to the human clinical trials. The summary of the autophagic pathways and antitumor targets are as shown in Fig. 2. Table I summarizes the various drugs targeting the autophagic pathways and clinical trial stages based on published reports.

FUTURE DIRECTIONS

Fundamental knowledge in programmed cell deaths has generated a great deal of information and insights on the pathogenesis and development of targets against cancer. Both apoptosis and autophagy signaling pathways have been frequently found to be impaired in many human tumors, which served as an important consideration in devising cancer pharmacotherapy strategies. Thus, modulating apoptosis and autophagy by various means may be an important strategy to fight against the disease. Cancers, which are resistant to the apoptotic effects of certain chemotherapy drugs may be sensitive to drugs that evoke autophagy or autophagic cell deaths and vice versa. Devising personalized pharmacotherapeutic strategies based on the autophagy activities of the tumors may be another option. This is in view that autophagy may have different effects in different stages of cancer progression. For example, one author suggested that if autophagy response and activities are normal in tumors, combining standard chemotherapy drugs with autophagy inhibitors may sensitize the tumor cells to anticancer agents i.e. by reactivation of apoptosis (150). Cancer cells which present defects in the autophagic pathway may be countered by replacement of autophagy-inducing signals, e.g. up-regulation tumor suppressor proteins such as Beclin-1 or PTEN or by inhibiting mTOR kinase (106). In some other cases, utilizing both autophagy and apoptosis inducers may present a deadly strategy against highly resistant tumors.

Latest findings have pointed that both apoptosis and autophagic cell death pathways may be intertwined. Studies have demonstrated that certain compounds have to ability to trigger both apoptosis and autophagy cell deaths simultaneously in cancer cells (169,170). Studies have also showed that blocking one of the pathways will trigger the activation of another. Researchers have even hypothesized that there are factors (either external or internal) that may affect the preferential shunting into either biochemical cascades that will ultimately result in either apoptosis or autophagic deaths (171). One of the questions remained to be answered includes whether the concept of cell death switches exist and if they do, how do they turn on and off? Understanding the complex relationships between these two cell death mechanisms may bring about a new paradigm towards the fight against cancer.

In conclusion, as more knowledge is uncovered on both apoptosis and autophagic pathways, strategies to arrest the growth and inducing death in tumor cells may be further improvised. So far, targeted drugs like obatoclax, oblimersen, bortezomib and mTOR inhibitors such as everolimus and deforolimus have showed promising activities in clinical trials. These newer classes of drugs appear to work synergistically in combination with other chemotherapeutics and have also showed specific activities against certain cancers. Since these drugs are specifically targeted against certain molecules or receptors in the pathway, further unveiling of the tumor's characteristics such as receptor or protein status may be critical in assessing patient's response and clinical trial success. More studies to characterize the efficacy of these drugs in various cancers are certainly warranted. This newer generation of targeted molecules may one day replace the standard chemotherapy drugs as first line drugs in treating cancer patients.

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