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New Frontiers in Cancer Chemotherapy — Targeting Cell Death Pathways

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Abstract

Cell death plays an important role in tumorigenesis, growth, and progression and affects the efficiency of chemotherapy to a great extent. Apoptosis is usually regarded as the principal mechanism of chemotherapy-induced cell death. However, the dysregulation of apoptosis occurs commonly in many cancers, which lowers the effectiveness of therapy and allows cells to survive. The mechanisms by which cells acquire this resistance to chemotherapy are not fully understood. Several studies uncovered alternative cell death pathways that are mechanistically distinct from apoptosis. These pathways, including autophagy and necrosis, represent potential targets for novel cancer treatment. By modulating the key regulatory molecules involved in the different types of cell death, more effective and less toxic chemotherapy might be developed. In this chapter, we describe the signaling pathways and the molecular events that are involved in these three major forms of programmed cell death. Additionally, we also discuss the emerging therapies targeting these cell death pathways as new strategies against cancer.

Keywords: Cancer, apoptosis, autophagy, necrosis, targeted therapy

1. Introduction

According to the World Health Organization, cancers figure among the leading causes of death worldwide, accounting for 8.2 million deaths in 2012 [1]. The annual number of cancer cases are expected to rise from 14 million in 2012 to 22 million within the next two decades [1]. Cancer

can affect everyone - the young and the old, the rich and the poor, men and women - and poses a tremendous burden on patients, families, and societies. A substantial number of cancer patients experience a significant reduction in their quality of life due to physical pain, mental anguish, and economic hardship. Scientists and doctors are continuously making efforts to find better and more effective therapies against cancer. Currently, strategically targeted cancer therapies are emerging as treatments, which use drugs or other substances, such as tyrosine kinase inhibitors and apoptosis inducing agents, to interfere with specific molecules and processes involved in cancer cell growth and survival [2].

In multicellular organisms, the number of cells is tightly regulated to attain a balance between cell proliferation and death. Maintaining this balance is crucial for normal development and tissue size homeostasis [3]. Cell death is a fundamental process that not only plays a pivotal role in the regulation of normal physiological development and tissue balance but also acts as a defense mechanism against diseases such as cancer [4]. Over the past two decades, our knowledge of cell death and the mechanisms of its regulation have increased dramatically. Programmed cell death (PCD) is a principal mechanism of tumor suppression and is triggered in nonmalignant cells to eliminate unnecessary, aged, or damaged cells that may otherwise be harmful to the body [5]. Of note, apoptosis, autophagy, and programmed necrosis are the three main forms of PCD, easily distinguished by their morphological characteristics within the cell [6, 7]. Additionally, senescence and mitotic catastrophe (MC) are two other cell death mechanisms, often triggered in cancer cells and tissues in response to anticancer drugs [8]. Cell senescence, a state of permanent cell-cycle arrest characterized by specific changes in morphology and gene expression that differentiate it from reversible cell cycle arrest, is also considered as a type of cell death in the context of cancer therapy [9].

Accumulated data suggest that various chemotherapeutic agents can kill tumor cells through the induction of apoptosis [10]. Dysregulation of the apoptotic pathways can not only promote tumorigenesis [11, 12] but also render cancer cells resistant to chemotherapy. The ability of cancer cells to avoid apoptosis and continue to proliferate is one of the fundamental hallmarks of cancer and is a major target of cancer therapy development [12].Development of novel molecules that activate apoptosis by targeting both the intrinsic and extrinsic apoptotic pathways will advance our understanding of the mechanisms behind tumor cell proliferation, which may also lead to the development of effective cancer therapies. Autophagy is an evolutionarily conserved process that maintains cellular homeostasis by controlling protein and organelle turnover. It serves as critical adaptive response that recycles energy and nutrients during periods of starvation and stress to enable cell survival. Studies have shown that autophagy contributes to the adaptation of tumor cells to adverse microenvironments [13] and chemotherapy [13]. Autophagy may represent a major impediment to successful cancer therapy; therefore, targeting autophagy is considered a promising strategy in clinical cancer treatment. However, other studies have shown that deficiency in adequate autophagy results in various spontaneous tumors in mouse model [14], indicating a tumor suppressive role of autophagy in the process of tumorigenesis. It seems that autophagy plays dual roles as both promoter and suppressor in tumorigenesis. The dynamic role of autophagy in tumor development appears mainly dependent on tumor stage [15]. It is important to elucidate the

mechanisms by which autophagy influences tumorigenesis and treatment response. Analysis of autophagic signaling may identify novel therapeutic targets. Necrosis is generally considered a passive response to massive cellular damage. However, accumulating evidence supports the existence of programmed necrosis, which involves cell swelling, organelle dysfunction, and cell lysis [16, 17]. Given the fact that many cancers have defective apoptosis machinery, it is reasonable to consider the pros and cons of activating other cell death pathways, such as necrosis, senescence and MC, and assess their therapeutic potential.

In this chapter, we discuss three major forms of PCD at molecular, cellular, and physiological levels. We also discuss the regulation mechanisms of these cell death pathways. Finally, the emerging therapies and strategies targeting these cell death pathways in the treatment of cancers are examined.
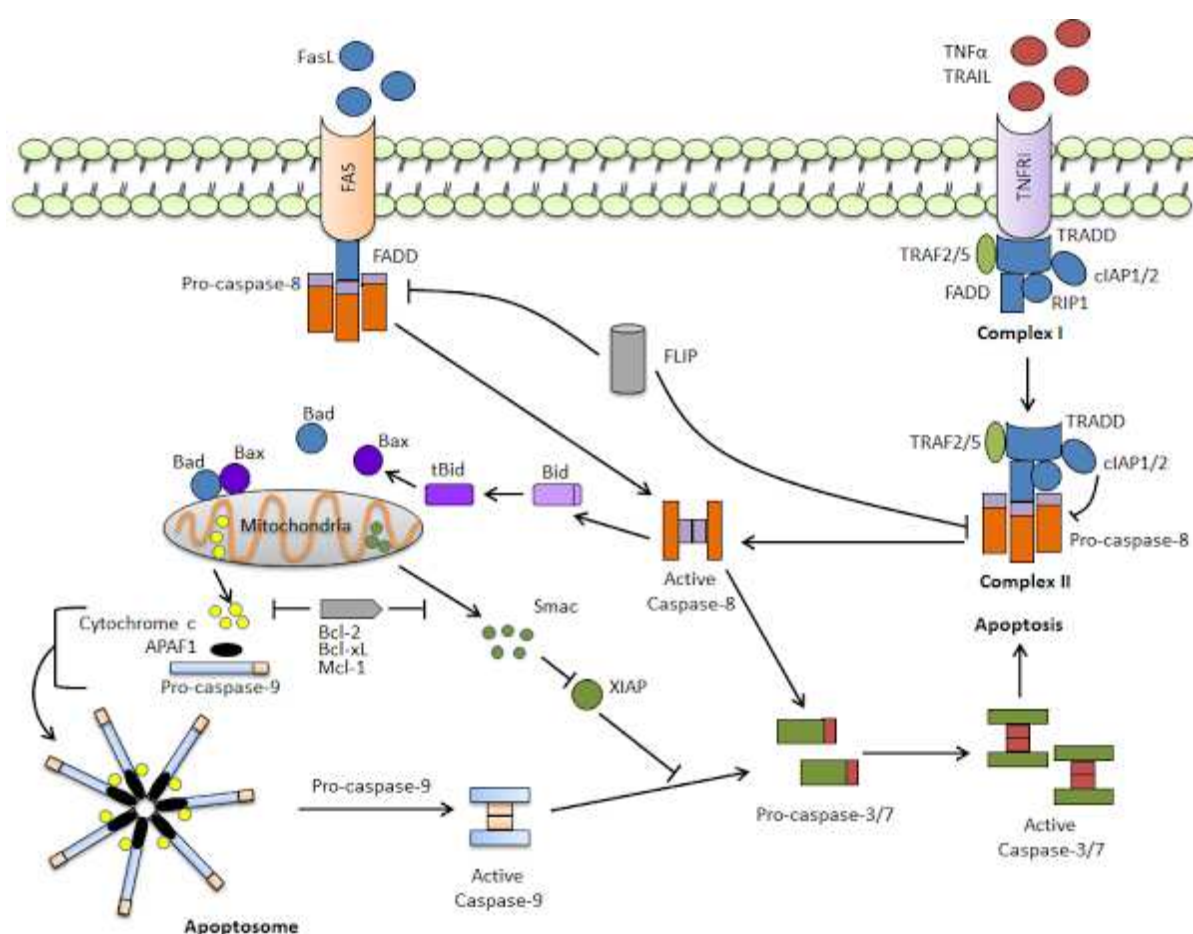
2. Apoptosis

The term "apoptosis" originates from Greek words *apo*, which means "since, " and *ptosis*, which means "dropping off, " and it refers to leaves falling off trees or petals dropping off flowers. It was first coined by Kerr *et al.* in 1972 and used to describe a regulated form of cell death with specific morphological features, which is different from the necrotic cell death resulting from acute tissue injury [18]. Since then, apoptosis has become one of the most extensively studied forms of PCD that plays a critical role in normal biological processes, such as embryonic development, immune response, tissue homeostasis, and cell turnover [19], as well as in a variety of pathological conditions including cancer [20]. Studies have shown that a cell undergoing apoptosis can be described by a series of characteristic morphological changes, including cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation [21]. In addition to the morphological changes, biochemical changes happening during apoptosis have also been revealed, and the three main ones are (1) the activation of caspases, (2) the breakdown of DNA and protein, and (3) the modifications of cell surface markers tagging the apoptotic cells for recognition by phagocytic cells [22].

Tissue homeostasis is maintained by an elaborate balance between cell growth by proliferation and/or survival on one side and cell death via apoptosis and other pathways on the other side. Any changes in the contribution of cell growth versus cell death can seriously affect the tissue homeostasis leading to human diseases. Accumulated evidence indicates that defect in apoptosis can contribute to cancer or onset of autoimmune responses, while excessive cell death can cause acute or chronic degenerative diseases, immunodeficiency, and infertility [23]. Under normal conditions, apoptosis represents a safeguard mechanism to prevent tumorigenesis, which indicates that evasion or resistance to apoptosis is a pivotal feature of cancer [24]. Alterations in cancer cells, which lead to impaired apoptotic signaling, not only promote tumor formation, progression, and metastasis but also contribute to treatment resistance [6-8, 24]. Thus, a better understanding of the molecular events that are involved in the regulation of apoptosis and their dysregulation in human cancers is expected to provide novel strategies for cancer therapy.

2.1. Two main signaling pathways involved in drug-induced apoptosis

Apoptosis can be triggered by various stimuli from outside or inside of the cells, for example, by ligation of cell surface receptors, by DNA damage as a results of treatment with cytotoxic drugs or irradiation, by a lack of survival signals or by developmental death signals. These death signals of diverse origins eventually converge to activate a series of cysteine aspartyl-specific proteases (caspases) through two main pathways, namely, extrinsic (death receptor) and intrinsic (mitochondrial) pathways [25]. Caspases are central to the mechanisms of apoptosis, which cleave key cellular proteins and dismantle the cells (Figure 1). Given the death-causing effects of caspase activation, these two pathways are strictly and closely regulated at each step. Apart from the two pathways mentioned, endoplasmic reticulum (ER)-mediated apoptosis is a lesser known third pathway [26].

Figure 1. The extrinsic and intrinsic apoptosis signaling pathways. The extrinsic pathway primarily involves the activation of procaspase 8 by death receptors (e.g., TNFR1 and Fas/Apo 1), whereas the intrinsic pathway involves the release of factors from mitochondria, such as cytochrome *c*, that forms a complex with APAF1 and procaspase 9, resulting in the cleavage and activation of procaspase 9. In mammals, either active caspase 8 or caspase 9 is capable of activating effector caspases such as caspase 3 or caspase 7, which then cleave apoptotic substrates leading to apoptosis. A link between the extrinsic and intrinsic pathways is observed in certain cells. This involves the cleavage of the Bcl-2 family member Bid by caspase 8, leading to the release of cytochrome *c* from the mitochondria and activation of caspase 9. For detailed signaling pathways, please see Sections 2.1.1-2.1.3.

Apoptosis in response to cancer therapy proceeds through the activation of the core apoptotic machinery, including the receptor and the mitochondrial signaling pathway [10]. In many tumor cell types, the main signaling pathway leading from drug-induced damage to cell death involves the mitochondrial release of proapoptotic molecules under the control of the B-cell lymphoma 2 (Bcl-2) family of proteins. However, death receptors of the tumor necrosis factor receptor (TNFR) superfamily, mainly CD95 (APO-1/Fas), have also been shown to play a role in linking drug-induced damage to the apoptotic machinery and modulating drug response.

2.1.1. Extrinsic (death receptor) pathway

The extrinsic apoptotic pathway is activated by the binding of death ligands to cell surface death receptors, which transmit extracellular death signals to the intracellular apoptotic machinery to elicit cell death [27]. Although several death receptors have been identified, the best known death receptors belong to the TNFR superfamily, including TNFRs, CD95 (Fas/Apo 1), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors [28]. These receptors become activated once bound by their cognate ligands such as TNF, CD95 (Fas), and TRAIL, which in turn results in death receptor aggregation, and recruitment of various adaptor proteins to the intracellular death domains (DD) of the death receptors and formation of death-inducing signaling complex (DISC). In this complex, Fas-associated death domain (FADD) recruits other DD- and/or death effector domain (DED)-containing proteins, such as procaspase 8 and procaspase 10, via homotypic death domain interactions (Figure 1) [29]. In contrast, TNF receptor-associated death domain (TRADD) recruits proteins leading to the formation of two complexes [30]. For example, TNFR1 binds to and forms complex I with TRADD, TNF receptor-associated factors 2 and 5 (TRAF2/5), receptor interacting protein 1 (RIP1), and the cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2) (Figure 1). This complex is important for the TNF-induced activation of NF-κB and MAPKs and the subsequent transcription of antiapoptotic genes. In certain circumstance, RIP1 is deubiquitinated by cylindromatosis and leads to the dissociation of RIP1 and TRADD from complex I. RIP1 and TRADD then form complex II with FADD, caspase 8, and/or caspase 10, which is analogous to the DISC induced by FasL and TRAIL (Figure 1) [4, 31, 32]. The activation of caspases 8 and 10 leads to the activation of the downstream caspase cascade to mediate apoptosis. In some cells, named type I cells, the activation of effector caspases, such as caspases 3, 6, and 7, by caspase 8/10 alone can induce apoptosis [33]. However, in type II cells, activated caspase 8/10 triggers the activation of intrinsic apoptotic pathway by the cleavage of the Bcl-2-homology 3 (BH3)-only protein Bid. Cleaved Bid is myristoylated to form tBid and translocates to the mitochondria membrane, which promotes the oligomerization of Bax and Bak and causes the release of apoptotic mediators from the mitochondria (Figure 1) [34, 35].

2.1.2. Regulation of the extrinsic pathway

Caspase 8 is the predominant initiator caspase in the extrinsic pathway, which plays a pivotal role in determining the cell fate following the death receptor activation. Therefore, the major signals that affect the recruitment of caspase 8 and its activation can modulate this signaling pathway. For example, cellular FADD-like interleukin-1β-converting enzyme inhibitory

protein (cFLIP) shares significant structural similarities with caspases 8 and 10, which allows it to compete for binding sites and thus displace caspase 8/10 in the DISC complex. cFLIP lacks a functional caspase domain, suggesting it to be a dominant-negative inhibitor [36]. Besides caspase 8/10, cFLIP can also bind to FADD and TRAIL receptor 5 (DR5), and this interaction in turn prevents the formation of the DISC complex and the subsequent activation of caspase cascade [37]. Similarly, A20-binding inhibitor of NF-κB 1 (ABIN1) exerts its antiapoptotic effect by interfering with the interaction of RIP1 and FADD with caspase 8 [38].

cIAP1/2 contain a signature baculovirus IAP repeat (BIR), a caspase-recruitment domain (CARD), and a really interesting new gene (RING) domain at their C-terminal that exhibits E3 ubiquitin ligase activity, which help to recruit TRAF1/2 and inhibit TNFα-apoptotic signaling. Although cIAP1/2 are not efficient caspase 8 inhibitors, they can play a regulatory role in extrinsic pathway through the activation of prosurvival signals, such as NF-κB pathway. This effect was shown to result from the cIAP1/2 induction of RIP1 ubiquitination and the recruitment of TAK1, TAB2/3, and the IKK complex [39]. The NF-κB signaling pathway has been linked to death receptor signaling because RIP, which serves as an adaptor molecule for TNFR1 in the NF-κB pathway, can be cleaved by caspases. Upon TNF receptor signaling, this modulates the balance between proapoptotic and antiapoptotic signals and may even stimulate an autocrine "death loop" [10, 40]

Ubiquitination has been shown to regulate the activity of caspase 8. A clear example is that the polyubiquitination of the p10 subunit of caspase 8 by a cullin3-based E3 ligase can enhance its enzymatic activity [41]. This modification occurs after the recruitment of caspase 8 to DISC complex and allows for the binding of active caspase 8 to the polyubiquitination-binding protein, p62, which is thought to increase the stability of cleaved caspase 8 [41]. The deubiquitinating (DUB) enzyme A20 was reportedly involved in reversing this modification [41].

2.1.3. Intrinsic (mitochondrial) pathway

The intrinsic pathway, as implied by its name, is activated by internal stimuli such as DNA damaging agents, growth factor deprivation, oxidants, hypoxia, overload of calcium, and microtubule targeting drugs [42]. Upon the detection of the internal stimuli, two proapoptotic Bcl-2 family members, Bax and Bak, undergo structural changes and subsequent oligomerization at the outer membrane of the mitochondria, leading to the induction of mitochondrial outer membrane permeabilization (MOMP) and the release of mitochondrial cytochrome *c* (Cyt-c) into the cytosol [43-46]. The released Cyt-c assembles a multiprotein caspase-activating complex, known as the "apoptosome" [47]. The central component of the apoptosome is Apaf1 that is transiently bound by released Cyt-c in the presence of ATP or dATP, which leads to the oligomerization of Apaf1 and then the exposure of its CARD [48]. Subsequently, Apaf1 binds to procaspase 9 via interaction between their CARDs. In this complex, procaspase 9 dimerizes and autoactivates. Activated caspase 9 then cleaves and activates the downstream executioner caspases 3 and 7 to perpetrate cell death rapidly (Figure 1) [49]. Besides Cyt-c, other apoptotic factors are also released from the mitochondrial intermembrane space into the cytoplasm such as apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis (IAP)-binding protein with low pI (Smac/DIABLO), and Omi/high temperature requirement protein A2 (Omi/HtrA2) [50]. Smac/DIABLO and Omi/HtrA2

promote caspase activation by neutralizing the inhibitory effects on IAPs, while AIF causes DNA condensation [51-53]

2.1.4. Regulation of the intrinsic pathway

The intrinsic pathway is tightly regulated by the intricate interactions between pro- and antiapoptotic members of the Bcl-2 family, which are categorized according to the organization of their Bcl-2 homology (BH) domains: (1) antiapoptotic members such as Bcl-2, Bcl-xL, Bcl-w, A1, and Mcl-1, which all possess the four BH1-BH4 domains and inhibit proapoptotic counterparts; (2) effector proapoptotic members such as Bax, Bak, and Bok, which all possess the three domains BH1-BH3; and (3) BH3-only proteins, including Bid, Bad, Bim, Bik, Bmf, Hrk, Noxa, Puma, Blk, BNIP3, and Spike, which only have the short BH3 motif and promote MOMP, either by inhibiting antiapoptotic proteins or by activating Bax and Bak [54-56]. Antiapoptotic Bcl-2 members block the oligomerization of Bax and Bak or their association with BH3-only proteins, thus preventing MOMP and Cyt-c release. However, upon a cytotoxic stimulus, the effects of antiapoptotic members are counteracted by BH3-only proteins, such as Bim and Noxa. BH3-only proteins release Bax-Bak from inhibition and allow them to promote MOMP and apoptosis.

The inhibitor of apoptosis protein (IAP) family represents another negative regulator of the intrinsic apoptotic pathway. So far, eight members have been identified, including cIAP1, cIAP2, X-linked IAP (XIAP), neuronal apoptosis inhibitory protein (NAIP), melanoma IAP (ML-IAP), survivin, Apollon, and IAP like protein 2 (ILP2) [57]. All IAPs contain BIR domains and 70 amino acid motifs, which are essential for antiapoptotic properties of IAPs because BIR domains bind the active sites of caspases and inhibit proteolytic function. Indeed, XIAP, survivin, and cIAP1/2 have been found to directly inhibit caspases 3, 7, and 9 [58]. In case of XIAP, its BIR3 domain directly binds to the small subunit of caspase 9, while its BIR2 domain interacts with the active-site substrate-binding pocket of caspases 3 and 7 [59, 60]. Some IAPs such as cIAP1/2 and XIAP contain a highly conserved RING domain that possesses E3 ubiquitin ligase activity and may target effector caspases for ubiquitination and subsequent proteasomal degradation [61, 62].

Other apoptotic factors, for example, Smac/DIABLO, when released from the mitochondrial intermembrane space during mitochondrial apoptotic events, are able to bind to various IAPs, mainly XIAP in a manner that displaces caspases from XIAP and enables their activation. In addition, the binding of Smac/DIABLO to IAPs facilitates the latter to be degraded by proteasome [63]. However, unlike Cyt-c, the ablation of Smac/DIABLO, Omi/HtrA2, or both proteins does not lead to the inability to activate caspases or undergo apoptosis [64-66]. This suggests that there may be considerable redundancy in XIAP inhibition, and in fact other proteins have also been demonstrated to inhibit XIAP [67].

3. Autophagy

The term autophagy (from the Greek *auto*, meaning "oneself, " and *phagy*, meaning "eating") was first introduced by Christian de Duve based on the observation that cells were able to

digest their own components [68]. Nowadays, autophagy is defined as a self-digestive cellular process by which eukaryotes degrade and recycle long-lived proteins, cellular aggregates, and damaged cellular organelles to maintain cellular homeostasis. Three types of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), have been identified to date, which could be distinguished from one another via different modes of delivery of the cargo to the lysosome and their function. Microautophagy is a direct engulfment of cytoplasmic components into the lysosomal lumen for degradation [69], while in CMA, a subset of soluble cytosolic proteins containing a KFERQ motif are recognized by molecular chaperons, including the HSPA8/HSC70 (heat shock 70 kDa protein 8), and directly translocated into lysosomes through a receptor (LAMP-2A) on the lysosomal membrane [70]. Macroautophagy is a process that is responsible for the delivery and degradation of macromolecules and organelles by generating specialized cytosolic vesicles (hereafter referred to as autophagy) [71].

Autophagy is activated under physiological and pathological conditions, such as nutrient starvation, hypoxia, hyperthermia, and oxidative stress, and in response to drugs and radiation. This dynamic process generates cellular energy resources that allow a cell to adapt its metabolism to energy demand. Defects in the autophagy process lead to the accumulation of damaged proteins and/or genomic damage and can cause diseases such as neurodegeneration, infectious diseases, heart diseases, and cancers [72, 73]. Although autophagy can suppress tumor growth, it clearly plays a role in promoting tumor cells to survive under stress [74]. The suppression of autophagy can sensitize cancer cells to anticancer therapy [75, 76], but under apoptosis deficiency condition, autophagy can also cause cell death through a process termed "autophagic cell death" [72, 77].

3.1. Pathway of autophagy

After induction by a stress signal such as starvation, the process of autophagy begins with the formation of autophagosomes, which assemble around and encapsulate the targeted proteins or organelles, and then fuse with lysosomes to form autolysosomes for degradation. This complex process can be divided into five major steps, namely, nucleation, elongation, maturation, fusion, and degradation, which are tightly controlled by a subset of molecules encoded by autophagy-related genes (ATGs) (Figure 2A). The first step of autophagy is the nucleation of the phagophore, an isolation membrane that most likely derives from the ER [78, 79]. Besides the ER, studies have also shown that the plasma membrane and membranes of mitochondria and Golgi are also involved in the formation of the phagophore [80, 81]. The phagophore then extends and sequesters the substrates destined for degradation and finally forms the characteristic double membrane vesicle, known as autophagosome. The outer membrane of the mature autophagosome then fuses with the lysosome or inner body to generate a structure named autolysosome, where the inner membrane of autophagosome and its contents are degraded by the activity of acidic hydrolases provided by the lysosome [82, 83]. The catabolic products are then either recycled into different metabolic pathways or undergo further degradation to yield energy (Figure 2A).
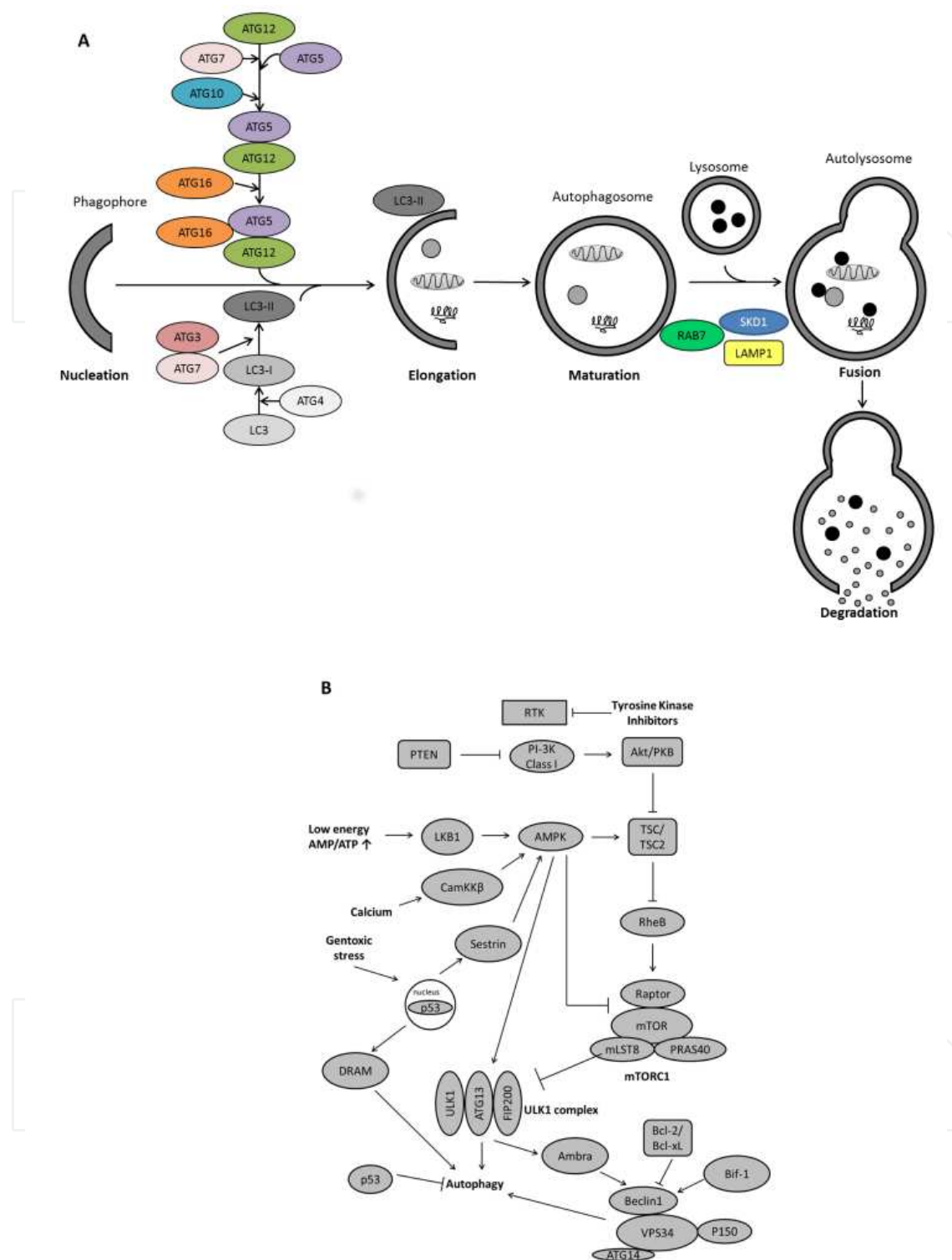
Figure 2. Schematic representations of the autophagy pathway and its regulation. (A) The five major steps of autophagy, namely, nucleation, elongation, maturation, fusion, and degradation, are illustrated. Phagophore membrane elongation and subsequent sealing of the autophagosome require two ubiquitin-like conjugation systems that mediate the formation of ATG5-ATG12 complex and LC3-II. (B) The signaling molecules and pathways involved in autophagy regulation (see Sections 3.1-3.3 for details).

Autophagy is a highly regulated process by ~30 ATGs discovered hitherto in mammals. Several signaling pathways that initiate autophagy converge at a serine/threonine protein kinase mammalian target of rapamycin (mTOR), a key regulator of the autophagic pathway, which inhibits autophagy in the presence of nutrients and growth factors [84]. In mammals, the initiation of phagophore formation is regulated by a great deal of macromolecular complexes or groups of proteins, including the ULK1 kinase and its regulators, the autophagy-specific phosphatidylinositol 3-kinase (PI3K) complex, and the multi-spanning transmembrane protein ATG9 [85-88]. The PI3K complex, which consists of the active enzyme VPS34, a class III PI3K, together with p150 and Beclin 1, the counterparts of yeast Vps15 and Vps30/Atg6, and ATG14, catalyze the production of phosphatidylinositol-3-phosphate, thereby triggering the recruitment of effectors proteins, such as double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting (WIPI) family proteins [89-93]. The elongation of the isolation membrane and the subsequent closure of the autophagosome require two ubiquitin-like conjugation systems. First is the ATG12-ATG5-ATG16L system: ATG12 is conjugated to ATG5 by the ATG7 (E1-like enzyme) and ATG10 (E2-like enzyme). The resulting ATG5-ATG12 complex interacts with ATG16L and then oligomerizes to form a large ATG16L complex, which localizes on the outer surface of the extending autophagosomal membrane, but it dissociates from the membrane before autophagosome formation is completed (Figure 2A) [94]. A recent study demonstrated that under certain stress conditions, autophagy can occur independently of ATG5/ATG7, suggesting the existence of an alternative pathway for autophagosome formation [95]. Second is the phosphatidylethanolamine (PE)-light chain 3 (LC3) system: LC3 (the mammalian homologue of yeast Atg8) is cleaved by the cysteine protease ATG4 and then conjugated to the lipid PE by the activity of ATG7 and ATG3 (E2-like enzyme) [94, 96]. The lipidated form of LC3 (LC3II) specifically accumulates on nascent autophagosomes and recruits cargo adaptor proteins (also known as autophagy receptors), such as p62, Nbr1, or NIX. These proteins, in turn, recruit cargo from the cytoplasm, for example, ubiquitinated protein aggregates and damaged organelles, to promote the closure of the autophagosome [97-99]. Once autophagosome formation is complete, it fuses with lysosomes through mechanisms that remain largely unknown in mammalian cells. Some regulators have been found to be involved in the autophagosome-lysosome fusion process, including LC3, the lysosomal proteins LAMP-1 and LAMP-2, the small GTP-binding protein RAB7, and the AAA-type ATPase SKD1 [100-102]. Autophagosome-lysosome fusion then leads to the activation of the hydrolases and the degradation of the sequestered cargo (Figure 2A).

3.2. Signaling pathways involved in the regulation of autophagy

3.2.1. Mammalian target of rapamycin

The mTOR, a PI3K-related serine/threonine protein kinase, plays a key role in maintaining the balance between cell growth and proliferation. It has also been found to regulate the autophagy in response to nutrient status, growth factor signals, and cell stress [103]. In higher eukaryotes, mTOR exists in at least two distinct protein complexes, known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [104, 105]. The mTORC1 consists of mTOR, mLST8 (mam-

malian lethal with SEC13 protein 8), RAPTOR (regulatory-associated protein of mTOR), PRAS40 (proline-rich Akt substrate of 40 kDa), and DEPTOR (DEP domain containing mTOR-interacting protein) and is considered to be the principal regulator of autophagy [106]. mTORC1 is downstream of PI3K and is activated in response to mitogenic stimuli or nutrient availability. When nutrients and growth factors are available, mTORC1 inhibits autophagy by phosphorylating ULK1-ATG13-FIP200 complex, which is required for the phagophore formation [107, 108]. In addition, activated mTORC1 promotes mRNA translation via activating S6K and inhibiting 4EBP1. The inhibition of mTORC1 strongly induces autophagy, for example, rapamycin (an mTORC1 inhibitor), and potently induces autophagy even in the presence of rich nutrients. As indicated (see Figure 2B), mTOR activity is activated by different signaling pathways, which converge on the tuberous sclerosis complex (TSC) and the ras homolog enriched in brain (Rheb), a small GTPase that activates mTORC1 when in its GTP-bound state [109]. TSC, which is comprised of TSC1 (harmartin) and TSC2 (tuberin), acts as a GTPase-activating protein (GAP) for Rheb, promoting hydrolysis of its bound GTP and thus inhibiting Rheb and mTORC1 activity [110]. The inhibition of TSC1/2 by Akt phosphorylation allows Rheb-GTP to accumulate and activates mTOR [111]. Phosphatase and tensin homology (PTEN), a phosphoinositide-3 phosphatase, is a negative regulator of the PI3K/Akt pathway and thus an inducer of autophagy [112].

3.2.2. AMP-activated protein kinase

AMP-activated protein kinase (AMPK), a serine/threonine protein kinase, is another sensor of cellular energy status and regulates the metabolism of glucose and lipids in response to changes in nutrient and intracellular ATP concentration. In conditions of nutrient deprivation, reduced ATP production causes an elevated AMP/ATP ratios that activates the energy-sensing serine/threonine kinase 1(LKB1)-AMPK signaling axis [113]. Elevated AMP/ATP ratio activates the LKB1 and subsequently phosphorylates and activates AMPK. The activation of AMPK mediates the phosphorylation of TSC, which results in the inactivation of mTORC1 and induction of autophagy [114, 115]. Moreover, AMPK has also been found to directly phosphorylate RAPTOR, an activating component of mTORC1, thereby inhibits mTORC1 in a TSC-independent manner (Figure 2B) [116].

AMPK also directly regulates autophagy through the phosphorylation and activation of ULK1. Studies have shown that AMPK interacts with the N-terminal proline/serine (PS)-rich domain of ULK1, and this interaction is required for the ULK1-mediated autophagy (Figure 2B) [117]. Furthermore, AMPK has been shown to associate with and directly phosphorylate ULK1 on several amino acid sites, and this modification is required for ULK1 activation in response to nutrient deprivation [118]. It was reported that AMPK can interact with and phosphorylate ULK1 at Ser555, Ser637, and Thr659 and that AMPK-dependent phosphorylation of ULK1 is involved in the localization of ATG9 and increases autophagy efficiency [119]. Similarly, Kim *et al.* found that under the conditions of glucose starvation, AMPK activates ULK1 through direct phosphorylation of ULK1 on Ser317 and Ser777, thereby activating ULK1 and promoting autophagy [120]. By contrast, Shang *et al.* found that ULK1 undergoes dramatic dephosphor-

ylation on Ser638 and Ser758 upon starvation, and the dephosphorylation of ULK1 leads to its dissociation from AMPK and becoming more active in autophagy induction [113, 121].

3.3. Regulation of autophagy by oncogenes and tumor suppressor

3.3.1. Bcl-2 family members

Bcl-2 family members were initially identified and characterized as regulators of apoptosis; however, more and more evidence has revealed that the members of this family also regulate the autophagy process. The antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1, have been found to interact with Beclin 1 and inhibit autophagy [122-124] (see Figure 2B). Because the overexpression of these antiapoptotic genes is commonly seen in cancer cells, the inhibition of autophagy may promote the oncogenic properties of these Bcl-2 family proteins. Indeed, the small interfering RNA (siRNA)-mediated knockdown of Bcl-2 can trigger autophagy and apoptosis in tumor cells [125, 126]. The antiapoptotic proteins were found to interact with Beclin 1 through their BH3 receptor domain and the BH3 domain of Belcin 1, thereby inhibiting Beclin-1-dependent autophagy [122]. The disruption of such interaction by ABT737, a BH3-mimetic agent, or the expression of other proteins with Bcl-2 homology 3 (BH3) domain that competitively disrupt the interaction can induce autophagy [123]. Additionally, the death-associated protein kinase, DAPK, a protein that phosphorylates Beclin 1 thereby disrupting Beclin-1-Bcl-2 interaction and inducing autophagy, is another inducer of autophagy that is commonly silenced by methylation in different types of human cancers.

In contrast to antiapoptotic proteins, the proapoptotic BH3-only proteins, such as BNIP3L, Bad, Noxa, Puma, BimEL22, and Bik, can promote autophagy [127]. For example, autophagy induced by hypoxia occurs through a hypoxia inducible factor-1 (HIF-1)-dependent transcriptional activation of BNIP3L that disrupts the interaction between Bcl-2 and Beclin 1 [128]. Furthermore, Puma, another "BH3-only" protein, induces mitochondrial autophagy in response to mitochondrial perturbations in a Bax/Bak-dependent manner [129].

3.3.2. p53 and p53-related regulators

The p53 tumor suppressor protein is well known for its role as a transcription factor that regulates the expression of a series of genes, contributing to cell cycle arrest, DNA damage and repair, apoptosis, and senescence [130, 131]. Underscoring its importance in the regulation of proliferative homeostasis, it is the most commonly mutated tumor suppressor in human cancer [132]. In mammalian cells, p53 shuttles between the nucleus and the cytoplasm; activated p53 translocates to the nucleus and induces the expression of target genes. Although p53 is best known as a nuclear transcription factor, studies also demonstrate that cytoplasmic p53 mediates mitochondrial outer membrane permeabilization and transcription-independent apoptosis [133, 134].

Similarly, p53 has been shown to modulate autophagy depending on its subcellular localization. Nuclear p53 stimulates autophagy in a transcription-dependent fashion by modulating the expression of a number of regulators that inhibit the mTOR pathway. For example, p53

activates the genes encoding AMPKβ1 (a component of AMPK), TSC1/2 and PTEN, which are all known negative regulators of mTORC1, leading to the activation of autophagy [135]. In addition, sestrin 1 and sestrin 2, two p53 target genes, have been identified as a critical link between p53 activation and mTORC1 activity [136]. Sestrin is induced in response to DNA damage and oxidative stress in a p53-dependent manner and inhibits mTORC1 activity via AMPK-mediated TSC activation (Figure 2B) [136]. Furthermore, sestrin 2 was shown to be required for autophagy induction in response to various cellular stress conditions, including nutrient starvation and rapamycin exposure [137]. In addition, p53 can promote autophagy in an mTOR-independent manner via the upregulation of damage regulated autophagy modulator (DRAM), a lysosomal protein mediating autophagic cell death [138]. However, p53 can also suppress autophagy [139], an effect attributable to cytoplasmic rather than nuclear p53 (Figure 2B). A recent study indicates that cytoplasmic p53 can regulate autophagy through direct interaction with FIP200 [140]. In addition, several autophagy inducers, such as nutrient starvation, rapamycin, and ER stress, stimulate proteasome-mediated degradation of p53; hence, the inhibition of p53 degradation can suppress autophagy induced by these cellular stress signals.

4. Necroptosis/programmed necrosis

For a long time, necrosis has been considered as a form of cell death that is uncontrolled and lacks underlying signaling events resulting in dramatic irreversible alterations in essential cell parameters of metabolism and cell structure [141]. This might be true for cell death in response to severe physical or chemical damage or adverse conditions; however, accumulating evidence supports the notion that necrosis is a regulated process involving multiple developmental, physiological, and pathological scenarios [142, 143]. For such a reason, it is called necroptosis or programmed necrosis. Necroptosis is characterized by cytoplasmic and organelle swelling, followed by the disruption of the cell membrane integrity, leading to the release of the cellular contents into the extracellular milieu, which may result in an inflammatory response. Unlike apoptosis, the nuclei of necrotic cells remain largely intact [144]. Necroptosis can be induced by inhibition of cellular energy production, generation of ROS, imbalance of intracellular calcium flux, or extracellular cell death signals, which are also able to induce apoptosis, suggesting that different types of cell death may share, at least in part, common mechanisms. In this sense, time and intensity of stimulus may determine the type of cell death. Indeed, one study showed that depending on glutathione depletion and oxidative stress level, apoptosis can switch to necroptosis [145].

4.1. Molecular mechanisms of necroptosis

Necroptosis can be triggered by ligands through numerous death receptors, including TNFR1 and TNFR2, TRAILR1 and TRAILR2, CD95 (Fas), and toll-like receptors (TLRs) [146-148], as well as by different kinds of physical-chemical stress stimuli, such as anticancer drugs, ionizing radiation, and calcium overload [149].

The death receptors are activated by their ligands followed by the recruitment and activation of caspase 8 that trigger the apoptosis in the absence of NF-κB survival pathway. However, under conditions that fail to trigger apoptosis, necroptosis may be an alternative cell death pathway. As shown in Figure 3, in the context of TNFR1 signaling, TNFα activates TNFR1, which in turn induces the recruitment of RIP1 kinase and other proteins, including TRADD, TRAF2, and cIAP1/2, to form a transient molecular complex referred to as complex I [144]. In complex I, RIP1 is rapidly modified by k63-linked polyubiquitination mediated by E3 ligases, cIAP1, and cIAP2. The ubiquitination of RIP1 serves as a platform to dock additional signaling molecules, IKK complex, key mediators that lead to the activation of the canonical NF-κB signaling, or inflammatory pathways [150-152]. RIP1 can be subsequently deubiquitinated by the enzyme cylindromatosis (CYLD) [153] and, together with proteins involved in cell death signaling, form complex II, which comprises as key components RIP1, TRADD, FADD, and caspase 8 [154]. The formation of complex II initiates the cell death signals, and the cell death via apoptosis or necroptosis is determined at this step. In the absence of cIAP1 or FLIP, RIP1, FADD, and caspase 8 form complex IIa to activate the caspase cascade and to induce apoptosis [31]. However, when caspase 8 activation is inhibited due to genetic or pharmacological inhibition, RIP1 together with RIP3 forms a complex that leads to the necroptotic signal transduction pathway [155]. This RIP1/RIP3-containing cytoplasmic necroptotic protein complex is called complex IIb (also known as necrosome), which constitutes a key molecular platform of necroptosis. RIP1 and RIP3 can phosphorylate reciprocally in an autocrine/paracrine manner, leading to the activation of their kinase activity [32, 156].

The mixed lineage kinase domain-like protein (MLKL), also detected in complex IIb, is the most downstream effector of necroptosis so far identified [157, 158]. The N-terminal domain of MLKL is required for assembly of higher order structure and recruitment of MLKL to the plasma membrane, followed by permeabilization of the plasma membrane [159-161]. The C-terminal pseudokinase domain of MLKL interacts with RIP3 and is phosphorylated by the latter at the threonine 357 and serine 358 residues, and these phosphorylation events are critical for necroptosis [161]. In fact, blocking MLKL activity leads to necroptosis inhibition. Besides the plasma membrane, activated MLKL may translocate to intracellular membranes, possibly leading to the permeabilization of the ER, mitochondria and lysosome [160]. In addition, RIP3 phosphorylates MLKL, which in turn activates the mitochondrial phosphatase phosphogly-cerate mutase 5 (PGAM5), a central downstream effector of the necrosomal complex. PGAM5 in turn initiates the dephosphorylation of GTPase dynamin-related protein 1 (DRP1), a mitochondrial fission regulator, which leads to the mitochondrial fission and mitochondrial fragmentation. This process is essential for the necroptotic pathways, as necrosis cannot occur without mitochondrial fission [162].

In addition to necrosome, another necroptosis-inducing complex referred to as "ripoptosome" has also been identified [163, 164]. Under normal conditions, the core components of this complex, namely, RIP1, FADD, and caspase 8, are ubiquitinated by IAPs, which leads to the degradation of these core components and thereby suppresses ripoptosome formation (Figure 3). However, when exposed to Smac mimetics or genotoxic stress, IAPs are downregulated, resulting in the spontaneous formation of the ripoptosome and triggering of caspase-8-
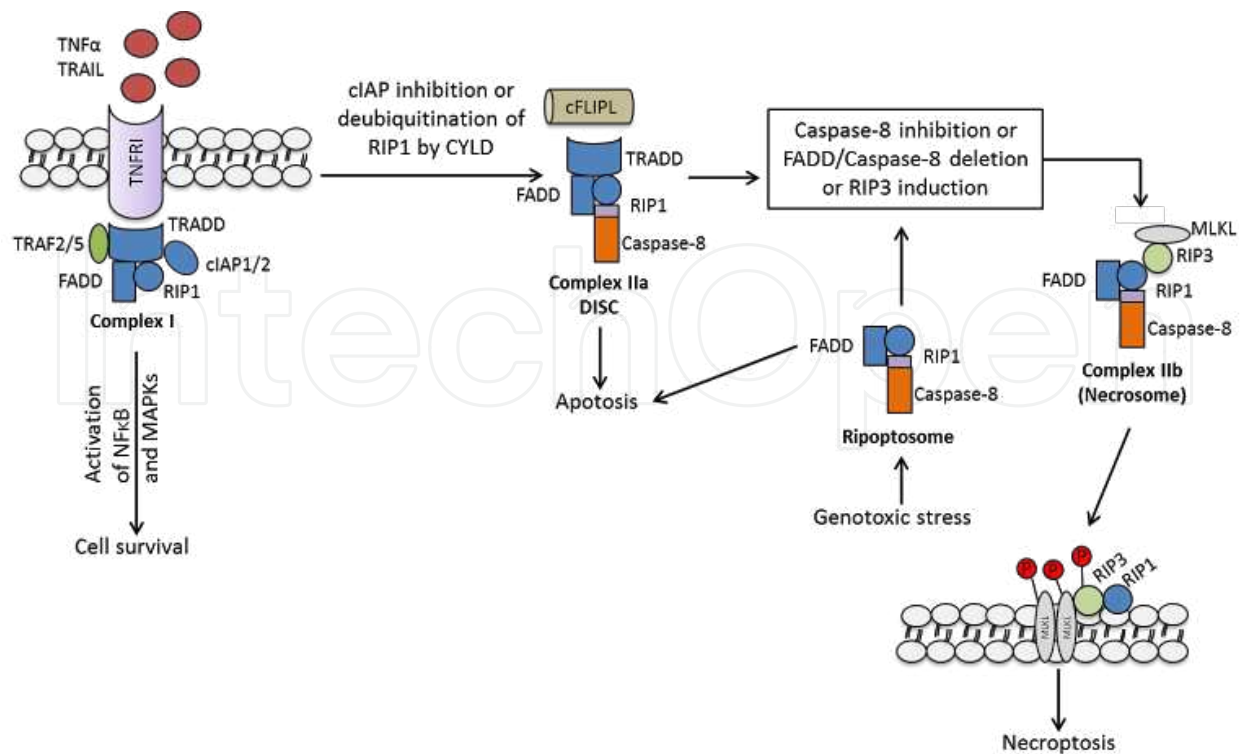
Figure 3. TNFR1 engagement- or genotoxic stress-induced formations of divergent signaling complexes, leading to the activation of NF-κB, apoptosis and necroptosis. Binding of ligands to receptors leads to the intracellular formation of complex I. Lys63-linked polyubiquitination of RIP1 (in complex I) by cIAP ligases results in cell survival through the activation of NF-κB and MAPKs. Deubiquitination of RIP1 by CYLD or inhibition of cIAP proteins leads to the conversion of complex I to complex IIa, activating the caspase cascade for apoptosis induction. Under conditions where caspase 8 activity is inhibited or RIP3 is highly expressed, RIP1 interacts with RIP3 to form complex IIb (necrosome), which mediates necroptosis. The formation of complex IIb requires the kinase activity of RIP1. RIP3 and MLKL are phosphorylated in complex IIb and translocated to the plasma membrane, where the complex mediates membrane permeabilization. In addition, the downregulation of IAPs by Smac mimetics or genotoxic stress results in the spontaneous formation of the ripoptosome, which can trigger caspase-8-mediated apoptosis or caspase-independent necroptosis.

mediated apoptosis or caspase-independent necroptosis. There are three signaling pathways initiated following ripoptosome formation. (1) The formation of caspase 8 homodimers within the complex results in full catalytic activity and thus apoptosis. (2) The formation of caspase-8-cFLIPL (long splice form of FLIP) heterodimers instead results in limited catalytic activity, which is able to cleave RIP1 but is not sufficient to trigger apoptosis, leading to ripoptosome disassembly and cell survival. (3) The formation of caspase-8-cFLIPS (short splice form of FLIP) heterodimers predominates within the complex, and caspase 8 activation and RIP1 cleavage are prevented, thereby promoting ripoptosome formation. This in turn leads to the mode of cell death instead of being switched to necroptosis [163, 164].

A schematic overview of major signal transduction pathways induced by various stimuli and ultimately leading to necroptosis can be found in the review article (Figure 1) by Kaczmarek *et al.* [165].

4.2. Regulation of necroptosis

4.2.1. FLIP and necroptosis

cFLIP molecule has been shown to be able to modulate the activation of procaspase 8 and thereby prevents the apoptosis mediated by death receptors [166]. In the cytoplasm, RIP1 can form complex with FADD, caspase 8, and TRADD (further referred to as ripoptosome) following stimulation of T-cell receptor, TLR3, or TRL4. Remarkably, in response to genotoxic stress (DNA damage), the spontaneous formation of the ripoptosome occurs independent of death receptor activation [4, 144, 156, 163]. The ripoptosome can induce caspase-dependent or caspase-independent cell death, depending on the cellular context or differential regulation of caspase 8 by cFLIP. The cleavage of RIP1 in the ripoptosome complex by caspase 8 homodimers triggers the downstream activation of effector caspases, leading to the induction of apoptosis. Further, ripoptosome-mediated cell death or necroptosis also depends on the type of FLIP isoform. The caspase 8/FLIPL heterodimers may induce RIP cleavage, thus leading to ripoptosome disassembly and necroptosis inhibition, whereas caspase 8-/cFLIPS lacks proteolytic activity necessary for RIP1 degradation, thus leading to necroptosis induction via RIP1 and RIP3 [164]. Therefore, it is possible to divert cells to undergo apoptosis via the inhibition of necroptosis through the modulation of ratio of FLIPL to FLIPS. Indeed, previous studies have demonstrated that cFLIP protects cIAP antagonist-treated cells from Fas-induced cell death, which involves both apoptosis and necroptosis [167]. cFLIPL inhibits the formation of the cell death-inducing "ripoptosome," which functions in TLR3-induced apoptosis and necroptosis [164]. Furthermore, siRNA-mediated silencing of cFLIPL sensitizes cells to TNF-induced RIP1/RIP3-dependent necroptosis [168]. Therefore, FLIP plays a pivotal role not only in the regulation of apoptosis but also in necroptosis via the formation of ripoptosome and by switching between apoptotic and necroptotic mechanism.

4.2.2. IAPs and necroptosis

The members of the IAP protein family exhibit E3 ubiquitin ligase activity and are characterized by BIR domains that bind the active sites of caspases and inhibit proteolytic function [169]. During the intrinsic pathway of apoptosis, Smac/DIABLO is released from mitochondria to cytosol thereby releases the caspases from the trap of IAP, leading to the activation of caspases followed by apoptotic cell death. Smac protein was shown to induce the autodegradation of cytosolic IAP1 and IAP2, allowing the formation of a caspase-8-activating complex consisting of RIP1, FADD, and caspase 8 [170]. Several mammalian IAPs may utilize ubiquitination to regulate their own stability. It has been recently found that Chal-24-induced autophagy activation can result in the degradation of c-IAP1 and c-IAP2 and the formation of ripoptosome, thus contributing to necroptosis induction [171]. Remarkably, in the absence of IAPs and under conditions where caspases are blocked, necroptosis can be stimulated via RIP1 and its downstream kinase [172]. It has been demonstrated that loss of cIAPs promotes the spontaneous formation of ripoptosome induced by genotoxic stress or TLR3 stimulation through poly (I:C), a synthetic homologue of virus-derived double stranded DNA. Such event occurs independently of death receptor stimulation and is suppressed by the cIAP1 or cIAP2

that cause RIP1 ubiquitination and degradation [164]. FLIPL knockdown is able to enhance ripoptosome aggregation, thus sensitizing cells to etoposide or TLR3-mediated cell death. The role played by ripoptosome is complex since it can stimulate caspase-8-mediated apoptosis or caspase-independent necroptosis depending on the cell types [163].

4.2.3. Regulation of necroptosis by caspase 8 activity

The concept of apoptosis blocking necrosis by caspase activity was firstly proved in 1998 by the finding that the pharmacological inhibition of caspase activity sensitizes TNF-mediated necrotic cell death in L929 cells [173]. Since then, this concept has been accepted as an established theory. Indeed, zVAD-fmk, a pan-caspase inhibitor, has been widely used to induce necroptosis in a variety of cell lines as well as in mice models [173, 174]. Among the caspases, caspase 8 is responsible for the switching between apoptosis and necroptosis [174]. Necroptosis but not apoptosis was observed in caspase-8-deficient Jurkat cell lines in response to Fas and TNFR stimulation. *In vivo* studies have also shown that caspase-8-deficient mice have significant necroptotic death, leading to embryonic lethality, which support the roles of caspase 8 in blocking necroptosis. T-cell- or intestinal epithelial cell-specific deletion of caspase 8 in mice also exhibited severe necroptotic features, inducing immunodeficiency or terminal ileitis, respectively [175, 176].

The critical roles of caspase 8 on necroptosis are known to induce the cleavage of RIP1 and RIP3 [31, 177]. Therefore, cells treated with caspase inhibitor or deficient in caspase 8 increase the RIP1-RIP3 complex formation as well as necroptosis [156, 172, 178]. In addition, CYLD was recently identified as a target of caspase 8 and a critical mediator. Caspase 8-mediated CYLD cleavage at Asp215 prevents necroptosis, whereas the expression of mutant CYLD (D215A), which is resistant to caspase 8-mediated cleavage, enhances necrosome formation and necroptosis [178].

4.2.4. RIP1/RIP3 and necroptosis

RIP1 and RIP3 are key signaling molecules in inducing necrosis and are regulated by caspases and ubiquitination. The activity of RIP1 is specifically associated with necrosis and not with apoptosis, which is demonstrated by use of Necrostatin-1 (Nec-1) that specifically blocks the kinase activity of RIP1 [179]. Nec-1 inhibits TNF-induced necrosis in L929 cells and FasL-induced necrosis in Jurkat cells that were pretreated with caspase inhibitor zVADfmk or deficient in FADD [180]. In addition to RIP1, RIP3 kinase activity is also involved in caspase-independent cell death [177], and it has become clear that RIP3 determines cells to undergo necrosis in response to TNF treatment [32, 156, 172]. In contrast to RIP1, RIP3 is not required for TNF-induced NF-κB activation [156, 181]. RIP1 activity is essential for necrosome formation and its C-terminal RHIM domain (RIP homotypic interaction motif), allowing homotypic interaction with RIP3 to from a TNF-induced complex, which is important for stabilizing the necrosome [182]. Under necrotic cell death conditions, RIP3 also binds to other metabolic enzymes, such as the cytosolic glycogen phosphorylase (PYGL), the cytosolic glutamate-ammonia ligase (GLUL), and the glutaminol-ysis-initiating enzyme GLUD1, which positively modulates RIP3 enzymatic activity [32,

182]. These interactions result in glutamine production and regulate glycogenolysis. The knockdown of PYGL, GLUL, or GLUD1 partially reduced the degree of TNF- and zVAD-fmk-mediated ROS production and necrosis. It seems that both RIP1 and RIP3 are responsible for an increased cellular metabolism of carbohydrate and glutamine, leading to higher ROS formation and subsequent necrotic cell death [182, 183]. The activity of caspase 8 inhibits the necrotic cell death, likely by the cleavage of RIP1 and RIP3 [184], and downstream, through caspases 3 and 7 activation and poly-ADP-ribose polymerase (PARP)-1 [185]. Again, this demonstrates the importance of RIP1/3 and the enhanced ROS formation during the inhibition of caspases for the subsequent induction of necrosis [185-187].

5. Targeting cell death pathways in cancer treatment

5.1. Activating apoptosis in cancer treatment

5.1.1. TNF-Related Apoptosis-Inducing Ligand (TRAIL)

The TRAIL has been considered as a promising anticancer drug since it was found that TRAIL preferentially triggers cell death in cancer cells compared to normal cells. Furthermore, unlike TNF and FasL, TRAIL and antibodies against the TRAIL receptors were confirmed to be well tolerated and safe in nonhuman primates even at relatively high concentrations [28, 188]. Recombinant human TRAIL has been shown to have the capacity to induce apoptosis in a variety of cancer cells *in vitro* and in tumor xenografts [11]. TRAIL receptor agonists, including recombinant TRAIL, as well as humanized antibodies against TRAIL receptors have been evaluated in clinical trials [189-191]. However, several clinical trials using such drugs as single agents to induce cancer cell death did not recapitulate the promising results obtained in animal studies, which might be due to insufficient cross-linking of TRAIL receptors by the available TRAIL agonists. This has led to the investigation of TRAIL-based combination therapies to maximize antitumor activity. It has been shown that both conventional chemotherapy with DNA damaging agents and radiotherapy induce the expression of TRAIL receptors in response to DNA damage, thus suggesting a potential synergistic effect when combining these therapies with TRAIL-targeted treatment [188, 192]. For example, histone deacetylase (HDAC) inhibitors can induce the expression of TRAIL, thereby leading to the apoptosis in acute myelogenous leukemia (AML) [193]. HDAC inhibitors enhance the synthesis of several proteins involved in TRAIL signaling, such as DR5, and are able to sensitize the TRAIL-resistant cancer cells when combined with TRAIL treatment [194, 195]. In addition, enhanced assembly of the TRAIL DISC has been proposed to confer increased sensitivity in TRAIL-based combination therapies [196]. Many cytotoxic chemotherapeutic agents have been shown to induce the stabilization of p53 tumor suppressor protein in response to DNA damage and other cellular stresses, which transcriptionally activates DR5 and other proapoptotic proteins that synergizes with TRAIL. Therefore, TRAIL combination with such agents could be a useful therapeutic strategy for cancer.

However, the efficiency of TRAIL-based therapy in human cancers is not satisfactory due to the existence of both agonistic receptors (TRAIL-R1 and TRAIL-R2) and antagonistic decoy receptors (TRAIL-R3 and TRAIL-R4) in human cells. This implies that recombinant TRAIL ligand is capable of eliciting proapoptotic or antiapoptotic signals depending on the availability of these different receptors on the cell surface. Moreover, in some cancer cells, TRAIL can induce the activation of NF-κB, thus promoting cancer cell survival rather than apoptosis [188, 197]. Thus, the context-based effect of TRAIL signaling may explain the lack of efficacy seen in recent studies of TRAIL-targeted anticancer therapy. It was reported that TRADD is a key component that activates NF-κB in TRAIL signaling, and siRNA-mediated knockdown of TRADD in cancer cells sensitizes them to TRAIL-induced apoptosis. Therefore, TRADD may serve as a target for sensitizing cancer cells to TRAIL cytotoxicity [198].

5.1.2. Bcl-2 family

Antiapoptotic proteins of the Bcl-2 family, such as Bcl-2, Bcl-xL, and Mcl-1, are promising targets for anticancer drug development because they play a crucial role in regulating apoptosis, and the overexpression of these proteins is frequently observed in a variety of tumor types. Currently, three main strategies targeting this pathway are under investigation: (1) small molecules that affect gene or protein expression, (2) silencing of the upregulated antiapoptotic proteins with antisense oligonucleotides, and (3) BH3-only peptides or synthetic small molecule inhibitors interfering with Bcl-2 like protein function. With regard to transcription silencing, studies have shown that, depending on the tissue origin of the malignancy, the expression of Bcl-2 or Bcl-xL can be downregulated in specific types of cancer and leukemia cells by small molecule drugs that modulate the activity of retinoic acid receptors (RAR), retinoid X receptors (RXR), peroxisome proliferator-activated receptors (PPAR), vitamin D receptors (VDR), and certain other members of the steroid/retinoid superfamily of ligand-activated transcription factors (SRTFs). Consequently, RAR and RXR ligands as well as PPAR modulators have been developed and evaluated for the treatment of some types of leukemia, lymphoma, and solid tumors, such as breast and prostate cancers [23, 199]. HDAC inhibitors, which function as transcriptional repressors via interaction with retinoid receptors and other transcription factors, can also favorably modulate the expression of Bcl-2 or Bcl-xL in some tumor lines [23]. These findings provide the basis for developing novel strategies for cancer treatment by suppressing the expression of antiapoptotic Bcl-2-family genes in cancer.

Besides the chemical compounds, antisense oligonucleotides have also been studied to knockdown the Bcl-2 family of antiapoptotic proteins. One agent that is currently most advanced in clinical trials is Genasense (also known as oblimersen or G3139), which is a synthetic, 18-base, single-stranded phosphorothioate oligonucleotide targeting Bcl-2 mRNA that was developed by Genta Inc. (Berkeley Heights, NJ). More precisely, Genasense is in phase II and phase III clinical trials treating a wide variety of adult and childhood tumors [200]. In addition, treatment with Genasense markedly improved the antitumor activity of many chemotherapeutic agents, such as taxanes, anthracyclines, alkylators, doxorubicin, or dacarbazine [201-203]. In a phase III clinical trial, Genasense in combination with dacarbazine was reported to significantly improve multiple clinical outcomes and increase overall

survival in patients with advanced melanoma [203]. Furthermore, a bispecific antisense oligonucleotide selectively targeting Bcl-2 and Bcl-xL has been reported to simultaneously downregulate the expression of both Bcl-2 and Bcl-xL and enhance chemosensitivity in various cancer cells [204-206].

Intracellular stress signals can activate BH-3 only proteins to antagonize antiapoptotic Bcl-2 family members. An attempt to mimic the BH3-only action was the development of BH3 mimetic compounds containing exposed BH3 domain that occupy the BH3-binding site on Bcl-2 or Bcl-xL, abrogating their antiapoptotic functions. ABT-737 and its oral derivative, ABT-263 (also called navitoclax), are among the first promising BH3 mimetics in cancer therapy [207, 208]. Both drugs avidly bind and inhibit Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1 or A1. ATB737 has been shown to be effective as a single agent against certain lymphomas and small cell lung cancer *in vitro* and *in vivo* [207] and against non-small cell cancer [209]. ATB737 has also been reported to have synergistic cytotoxicity with conventional chemotherapeutic agents, and other targeted agents, including tyrosine kinase inhibitor, EGFR inhibitor, MEK inhibitor, and BRAF inhibitor, to reverse drug resistance and kill tumor cells [210, 211]. Initial clinical trials have demonstrated a significant antitumor activity of ATB263 as a single agent in the treatment of B-cell malignancies, especially CLL [212]. Several preclinical studies have shown promising effects of combinatorial use of ATB263 with conventional cytotoxic agents or targeted therapy in both solid tumor and hematologic malignancy models [210, 213]. However, the practical use of ATB263 is limited due to its propensity to induce acute throm-bocytopenia. ABT-199, a newer BH3 mimetic that specifically targets Bcl-2, has been shown to suppress the growth of Bcl-2-dependent tumors *in vitro* and *in vivo* without causing thrombo-cytopenia since it does not antagonize Bcl-xL, which is critical for platelet survival [214-216]. Also, ATB263 has been demonstrated to enhance the antitumor activity when administered in combination with other chemotherapeutic agents [217, 218]. Other BH3 mimetics, such as WEHI-539, BXI-61, BXI-69, Obatoclax, S1, JY-1-106, Gossypol, and its derivatives (apogossypol, apogossypolone, and TW-37) as well as selective Mcl-1 inhibitors, have been developed, and their antitumor effects have also been investigated or are under investigation (reviewed by Vogler [211]).

5.1.3. IAP inhibitors

IAPs play a critical role in the control of cell survival and death by regulating key signaling events such as caspase activation and NF-κB signaling that makes them become attractive molecular targets. XIAP has been reported to be the most potent inhibitor of apoptosis among all IAPs, consequently targeting XIAP using antisense oligonucleotides, or siRNA molecules have been developed in the treatment of cancer. Indeed, targeting XIAP by antisense oligo-nucleotides or siRNA has been demonstrated to be able to induce apoptosis and sensitize cancer cells to death receptor- and chemotherapeutic agents-induced cell death in a variety of cancer *in vitro* and *in vivo* [219-223]. Similarly, the siRNA-mediated downregulation of other IAPs, such as cFLIP and survivin, has also been shown to enhance chemotherapy activity in a range of cancers [224-226]. In addition, some chemical compounds, for example, mTOR inhibitors and HDAC inhibitors, can suppress the cFLIP expression via blocking its translation and transcription, respectively [226, 227].

Another approach for targeting IAPs is to disrupt IAP binding to caspases by small molecule IAP antagonists. IAP antagonists bind to the BIR2 or BIR3 domain of XIAP, cIAP1, and cIAP2, leading to the activation of caspase and induction of apoptosis [228]. Most of IAP antagonists are Smac mimics, and in addition to monovalent compounds that contain one Smac-mimicking unit, bivalent or dimeric IAPs have also been developed, which consists of two Smac-mimicking units that are connected via a chemical linker [229]. When used as a single agent, IAP antagonists can only effectively trigger cell death in a small subset of human malignancies, suggesting that IAP antagonist-based combination therapies might be required for the effective treatment of a majority of tumors. A variety of chemotherapeutic agents (including doxorubicin, etoposide, gemcitabine, paclitaxel, cisplatin, vinorelbine, SN38, 5-fluorouracil (5-FU), cytarabine, and HADC inhibitor vorinostat), death receptor agonists, and signal transduction modulators (including proteasome inhibitors, various kind of kinase inhibitors and monoclonal antibodies targeting growth factor receptor) have been shown to act cooperatively with IAP antagonists to enhance antitumor activity *in vitro* and in preclinical models of cancers [230-236]. For instance, beneficial synergistic effects were observed when IAP antagonists were used in combination with other compounds, such as bortezomib, TRAIL, or DNA damaging agents, such as melphalan, to reduce tumor burden in multiple myeloma models [237]. Along these lines, LBW242 was also highly beneficial in an FLT3-mutated AML xenograft mouse model when administered along with the protein kinase inhibitor PKC412 [238]. A recent study depicted the combinatorial effect of Pak1, a downstream Rac effector, inhibition on IAP antagonist treatment in NSCLC cell lines, rendering these cells hypersensitive to apoptotic cell death [239]. The development of combination therapy is warranted as it promotes better patient survival, as shown in a metastatic breast cancer Phase III clinical trial [240]. Combination therapy might promote synergistic effects leading to low drug dosage, as well as suppressing resistance to therapy if multiple cell survival pathways are targeted at once, although the probability of toxicity is also increased [241]. Fortunately, clinical trials with IAP antagonists have not showed any dose-limiting toxicity [242].

5.2. Targeting autophagy in cancer treatment

Therapeutic targeting of the autophagy pathway as a new anticancer strategy has been under extensive investigation. Since autophagy can play roles in tumor growth depending on the context, such as tumor type or stage, both of the autophagy-enhancing and autophagy-inhibiting agents may elicit beneficial effects in the treatment of cancer.

5.2.1. Inhibition of autophagy

High levels of autophagy are commonly observed in tumor cells following anticancer therapy. For example, chemotherapeutic agents (e.g., doxorubicin, temozolomide, camptothecin, and tamoxifen), HDAC inhibitors (e.g., SAHA), tyrosine kinase inhibitor (e.g., imatinib, sorafenib), and monoclonal antibody (e.g., trastuzumab) have all been demonstrated to induce autophagy in a variety of tumor cells [243-248]. Furthermore, a number of studies have shown that genetic knockdown of ATGs or pharmacological inhibition of autophagy can effectively promote cell death induced by various anticancer agents in many cancer lines and in multiple tumor models [249-252]. These findings suggest that the activation of autophagy is a protective strategy for

tumor cells to avoid being entirely killed by anticancer agents. The prosurvival ability of autophagy renders tumor cells resistant to anticancer agents, which greatly compromises curative efficacy of chemotherapy. In these contexts, the inhibition of autophagy can be a promising strategy to reestablish or increase the sensitivity of tumor cells to therapeutic agents.

The common inhibitors of autophagy can be categorized into three types according to their action mechanisms: (1) inhibit the formation of autophagosome via restraining the recruitment of Class III PI3K to the membrane, such as 3-methyladenine (3-MA) and Wortmannin; (2) prevent the degradation of proteins within autophagosome by disrupting lysosomal function, such as chloroquine (CQ) and its analog hydroxychloroquine (HCQ); and (3) intervene in the fusion of autophagosome with lysosome, such as bafilomycin A1 (BafA), a direct inhibitor of vacuolar ATPase [253].

CQ and its derivative HCQ are the most common autophagy inhibitors used in clinical trials. Preclinical studies have shown that CQ and HCQ are equipotent at autophagy inhibition and potentiate the anticancer effects of different drugs both *in vitro* and *in vivo*. For example, Amaravadi *et al*. reported that in a Myc-induced lymphoma mouse model, QC and HCQ significantly enhance the cytotoxic effects of p53 expression and alkylating agents and substantially impair the recurrence of tumor after chemotherapy [254, 255]. In chronic myelocytic leukemia (CML) cell lines, the inhibition of autophagy by CQ markedly augments the cell death induced by imatinib, a tyrosine kinase inhibitor that is a first-line therapeutic agent for BCR/ABL-positive CML [256]. CQ has also been shown to promote the cytotoxic effects of SAHA, an HDAC inhibitor, to overcome imatinib-resistant CML cells [246]. In a colon cancer xenograft model, CQ in combination with vorinostat was shown to significantly reduce tumor burden and increase apoptosis [257]. Similarly, CQ enhances the anticancer effect of the saracatinib, an src inhibitor, in a xenograft mouse model of prostate cancer [258]. Currently, phase I/II clinical trials are ongoing to evaluate the potential benefits of CQ and HCQ in combination with standard cancer therapies for a variety of cancers [259], and these clinical trials are listed at http://www.clinicaltrial.gov/.

In addition to CQ and its derivatives, other potential autophagy inhibitors have also been studied for their anticancer efficacy *in vivo* and *in vitro*, including 3-MA, BafA, monensin, and pepstatin A [260-262]. For example, the inhibition of autophagy by 3-MA increases cell death induced by 5-fluorouracil (5-FU) in colon cancer xenograft model [263] as well as enhances cytotoxicity induced by imatinib in glioma cell lines [264]. BafA in combination with tyrosine kinase inhibitors, such as imatinib, nilotinib, or dasatinib, significantly increase cell death in CML cells [256]. However, it must be remembered that the cytotoxic effects of these different agents might not be solely due to the inhibition of autophagy; targeting key autophagy proteins would be a more potent and specific approach. These include ULK1, Beclin 1, or ATG proteins.

5.2.2. Induction of autophagy

Although the concept of "autophagic cell death" in mammalian cells remains largely contro-versial, studies do show that autophagy is required for the efficient killing of tumor cells in certain circumstances [252]. Certain tumor cells become highly resistant to apoptosis and chemotherapy by overexpression of Bcl-2 or Bcl-xL, lack of Bax and Bak, loss of Beclin 1, or

exposure to pan-caspase inhibitors [76]. Most anticancer drugs exhibit limited effect on this subset of tumor cells. Fortunately, studies have demonstrated that the induction of autophagy may be an alternative way for cancer treatment when apoptosis is blocked [265, 266]; however, the conditions under which autophagy can function as a primary cell-death mechanism remain to be defined.

Among the potential targets in autophagy, Akt-mTOR pathway is the most investigated one. mTOR inhibitors, including rapamycin and its analogs everolimus (RAD-001), temsirolimus (CCI-779), and deforolimus (AP-23573), have been developed and studied for their ability to induce autophagy and cell death. Everolimus and temsirolimus have been approved for the treatment of renal cell carcinoma and mantle cell lymphoma [267]. Rapamycin has been shown to inhibit cell growth and initiate cell death in mantle cell lymphoma cell lines and various primary tumor cells, such as malignant gliomas, breast cancers, renal cell carcinomas, non-small cell lung cancers, and cervical and uterine cancers [13, 253]. Everolimus induces massive autophagy in leukemia [268], in advanced pancreatic cancer [269], and in many other cancers [270], accompanied by reduced tumor burden. In addition, everolimus in combination with etoposide, cisplatin, or doxorubicin display synergistic effects without significant increase in toxicity [271]. However, rapamycin and its analogs would inevitably activate Akt kinases, which associate with the induction of insulin receptor substrate-1, jeopardizing the antitumor effects of these mTOR inhibitors [272]. Other inhibitors, including ATP-competitive inhibitors of both mTORC1 and mTORC2 as well as the dual PI3K-mTOR inhibitor NVP-BEZ235 [273, 274], have exhibited more potent capacity to induce autophagy in cancer cells [275, 276].

Antiapoptotic Bcl-2 family members are frequently overexpressed in many human tumor types, rendering tumor cells resistant to apoptosis. Bcl-2 family members are important regulators involved in both apoptosis and autophagy. As a result, the modulation of Bcl-2 family proteins leads to not only apoptotic but also autophagic cell death. The underlying mechanism of this effect reflects the fact that Bcl-2/ Bcl-xL proteins can bind and disrupt the autophagic function of Beclin 1, which contains a BH3 domain [277]. This is notably the case for BH3 mimetics (ABT737, ABT236, gossypol, obatoclax) that targets Bcl-2/Bcl-xL, thus allowing Beclin 1 to be released to trigger autophagy [277, 278]. Obatoclax has been shown not only to induce cell death on its own but also to potentiate the effects of other anticancer agents such as the dual EGFR/HER2 inhibitor lapatinib, or HDAC inhibitors [279]. Although the inhibitory effect affects both apoptosis and autophagy, the tumor cells preferentially undergo autophagic cell death in apoptosis-defective cells. For example, doxorubicin primarily induces autophagy at low doses while apoptosis at high doses, and the combination of Bcl-2 siRNA treatment with a low dose of doxorubicin enhances the autophagic response, tumor growth inhibition, and cell death [126].

In pursuit of new drugs to selectively kill renal cell carcinoma (RCC), Giaccia and colleagues identified a compound, STF-62247, that strongly induced autophagy and massive vacuolization in VHL (a tumor suppressor gene lost in 75% of RCCs)-deficient RCC cells with no apparent apoptosis induction. Blocking autophagy using ATG5 or ATG7 siRNA or 3-MA prevents STF-62247-induced cell death, indicating that this compound induces cell death by

autophagy in VHL-deficient RCC cells [280]. In addition, other autophagic cell death-inducing anticancer agents have been developed and studied [279].

5.3. Modulation of necrosis for cancer treatment

The accumulating data have indicated that necrotic cell death can be activated to induce the damage of tumor tissue. DNA-damaging agents are the most widely used and effective chemotherapeutic approach for cancer treatment, which has been shown to be able to stimulate a regulated form of necrosis [281]. The PARP is activated in response to DNA damage, which facilitates the access of DNA repair enzymes to damaged DNA. The hyperactivation of PARP depletes cytosolic nicotinamide adenine dinucleotide (NAD) and induce necrosis [282], which may lead to selectively killing tumor cells because highly proliferating tumor cells depend on cellular NAD to generate energy through aerobic glycolysis. PARP-mediated necrosis may explain the phenomenon that neither Bax/Bak nor p53 deficiency impedes cell death in response to DNA-damaging agents. Jouan-Lanhouet *et al*. found that the death receptor ligand TRAIL induces necroptosis in human HT29 colon and HepG2 liver cancer cells via RIP1/RIP3-dependent PARP1 activation and depletion of cellular ATP levels, suggesting PARP1 activation as an effector mechanism downstream of RIP1/RIP3 [283].

There are more and more compounds and anticancer drugs that have been demonstrated to induce cancer cell death through necrosis, such as shikonin, FTY720, staurosporine, derivatives of amiloride (5-benzylglycinyl-amiloride and glycinyl-amiloride), and BI2536 (a small molecule inhibitor of the mitotic kinase Plk1). Most of them were not necessarily designed in a mechanism-based fashion but were only later found to induce necrotic features in the dying cells [284-289]. Shikonin, a naturally occurring naphthoquinone, was reported to induce necroptotic cell death in cancer cells that can be prevented by RIP1 inhibitor necrostatin-1, a specific inhibitor of necroptosis. Moreover, shikonin-induced necroptosis can overcome drug- and apoptosis-resistant cancer cell lines overexpressing P-glycoprotein, MRP1, BCRP, Bcl-2, or Bcl-xL [284, 285]. FTY720, a sphingolipid analogue drug that mimics ceramide, was shown to target the I2PP2A/SET oncoprotein, which results in the activation of tumor suppressor PP2A and subsequently induces RIP1-mediated necroptotic cell death and tumor growth inhibition [286]. Staurosporine, an inhibitor of a broad spectrum of protein kinases, has been shown to induce necroptosis in leukemia cells when caspase activation is inhibited. The induction of necroptosis was blocked by several pharmacological inhibitors, including necrostatin-1, HSP90 inhibitor geldanamycin, MLKL inhibitor necrosulfonamide, and a cathepsin inhibitor CA-074-OMe, which has been demonstrated to rescue the caspase-independent necrotic cell death of leukemia cells treated by staurosporine [289]. Other anticancer agents have also been shown to induce necroptosis via different mechanisms.

Bonapace *et al*. reported that obatoclax (GX15-070), a putative antagonist of Bcl-2 family members, could overcome glucocorticoid resistance in childhood acute lymphoblastic leukemia (ALL) through the induction of autophagy-dependent necroptosis, which bypassed the block in mitochondrial apoptosis [290]. Obatoclax was also shown to promote the assembly of the necrosome on autophagosomal membranes, thereby connecting obatoclax-induced autophagy to necroptosis signaling pathways [291]. Coimmunoprecipitation assays demon-

strated that obatoclax promoted the physical interaction of ATG5, a constituent of autopha-gosomal membranes, with FADD, RIP1, and RIP3 as key components of the necrosome [291].

Small molecule inhibitors targeting IAPs such as Smac mimetics, which have been developed to induce apoptotic cancer cell death, have been found to also engage necroptotic cell death. He *et al.* first reported that, upon the inhibition of caspase activity, Smac mimetics in combi-nation with TNFα provoke a strong necroptotic response in Smac mimetic-resistant cancer cells [172]. Similarly, Smac mimetic BV6 promotes TNFα-induced necroptosis not only in leukemia cell lines deficient in caspase 8 or FADD but also in primary, patient-derived ALL cells [292]. This Smac mimetic/TNFα-triggered necroptosis occurred in an RIP1-dependent but caspase-independent manner in these leukemia cells lacking caspase 8 or FADD; however, in FADD- or caspase-8-proficient leukemia cells, the same cotreatment of Smac mimetic and TNFα induced apoptotic cell death [292]. This illustrates that Smac mimetic can prime leukemia cells to TNFα-mediated cell death via either necroptosis or apoptosis depending on the cellular context. Mechanistically, by promoting the degradation of cIAP proteins, Smac mimetics stimulate the necrosome formation and promote necroptosis [293]. Furthermore, a recent study demonstrated that Smac mimetic cooperates with demethylating agents to synergistically induce cell death and can circumvent apoptosis resistance of AML cells by switching to necroptosis [294].

Targeted toxins are fusion proteins that combine a targeting protein, such as a ligand for a specific receptor, and a toxic peptide derived from a bacterial pathogen [295]. Diphtheria toxin GM-CSF (DT-GMCSF) was shown to kill AML cells by simultaneously activating both caspase-dependent apoptosis and caspase-independent necroptosis [296]. Interestingly, DT-GMCSF-induced necroptotic cell death even occurred in apoptosis-resistant AML cells, indicating that necroptosis may open new perspectives for cancer drug development in AML [296].

6. Conclusions and future perspectives

The dysregulation of the cell death process is closely related to cancer progression and resistance to chemotherapy. The concept to therapeutically target apoptotic signal transduc-tion pathways has significant implications for cancer therapy since intact apoptosis programs are critically required for the antitumor activity of most current cancer therapies that are used in clinical oncology. The reactivation of apoptosis not only directly induces cell death in tumor cells but also sensitizes tumor cells to other chemotherapeutic or targeted therapy agents. However, given the wide variety of genetic and epigenetic defects that lead to apoptosis resistance in most cancers, understanding the mechanism and regulation of other cell death pathways in response to antitumor agents is important. Autophagy and necrosis are two nonapoptotic cell death models that can be triggered as a consequence of cancer therapy and may have overlapping but separable regulatory networks. Unlike apoptosis or necrosis, autophagy has been shown to exert dual functions in cancers. On one hand, autophagy can function as a survival pathway in response to anticancer agents; hence, autophagy inhibitors may be used as adjuvants to standard cancer therapies. On the other hand, autophagy can lead

to cell death in certain circumstances; thus, autophagy inducers may help to eradicate cancer cells. As the effect of autophagy on cancer therapy varies depending on cell context such as cell type, phases, and microenvironment, personalized pharmacotherapeutic strategies should be adopted alone or most likely in combination with standard chemotherapeutic agents.

The success of compounds like the BH3 mimetics, Smac mimetics, TRAIL, and mTOR inhibitors in preclinical studies is proof that the reactivation of defective cell death pathways in cancer cells is possible and can effectively eradicate tumor cells. Therefore, efforts should be put to further delineate and identify regulatory components of the different cell death pathways that can be targeted and manipulated to execute death. Understanding the crosstalk between the different cell death pathways as well as between cell death and non-cell death pathways in cancer is crucial to spot prospective convergence points between pathways. Targeting the convergence points will allow the switching between pathways and improvisation of alternate means of inducing cancer cell death. For example, TNF signaling to the NF-κB prosurvival and inflammatory pathway can be rerouted to the necroptotic pathway to promote cell death just by modulating cIAP levels [163].

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