Chapter 8

# Drug Resistance and Molecular Cancer Therapy: Apoptosis Versus Autophagy

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Additional information is available at the end of the chapter

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## 1. Introduction

The majority of chemo/radiotherapies inhibit cancer cell growth by activating cell death pathways, such as apoptosis, necrosis, and autophagy-associated cell death. However, as the disease progresses, cancer cells can acquire a variety of genetic and epigenetic alterations, which leads to dysregulation of cell death-associated signaling pathways and chemo/radioresistance. Designing novel drugs and enhancing therapeutic strategies to improve survival and quality of life for cancer patients must specifically target pathways responsible for drug resistance. Two cellular mechanisms can contribute to chemo/radioresistance: inhibition of apoptotic cell death pathways and induction of autophagy, a cell survival response. The development of novel drugs and extensive research studies has provided significant insight into the aberrant regulation of apoptosis and key apoptosis inhibitor proteins during tumorigenesis. However, the extensive dysregulation of cell growth pathways in cancer cells makes it necessary to target multiple pathways in order to elicit a lasting death response. Autophagy, classically designated as a cell "survival" mechanism, appears to play a greater role in cell death than previously conceived. This contradiction between autophagy-associated cell survival versus cell death has intensified the interest in this field of research in cancer therapeutics. Understanding how autophagic cells cross the threshold from cell survival to cell death during drug treatments is imperative for identifying more potent therapies. Utilizing novel treatments that will re-activate apoptotic cell death pathways, while driving autophagy-associated cell death will lead to more effective chemotherapies, thereby enhancing overall patient survival.

**Keywords:** apoptosis; autophagy; programmed cell death; molecular therapy; personalized medicine; signaling pathways

## 2. Apoptosis pathways

Cancer cells can acquire apoptosis-resistance during treatment by up-regulating multiple pro-survival factors, such as inhibitors of apoptosis proteins (IAPs), nuclear factor-κB (NF-κB), and the B cell CLL/lymphoma-2 (BCL-2) family proteins. There are two major apoptosis signaling pathways, the extrinsic and intrinsic apoptosis signaling pathways (Figure 1). The extrinsic (death receptor) apoptosis pathway is induced by the binding of cell death ligands, such as FAS ligand or TNF to cell death receptors, FAS receptor or tumor necrosis factor receptor,TNFR1, respectively. Activation of these death receptors results in caspase 8 activated cell death [1]. The intrinsic (mitochondrial or BCL-2 regulated) apoptosis pathway can be activated by cellular stresses or chemo/radiotherapies that lead to functional activation of the pro-apoptotic BCL-2 family proteins, which induce mitochondrial outer membrane permeabilitization (MOMP) and cytochrome *c* release into the cytosol. Once in the cytosol, cytochrome *c* induces formation of the apoptosome complex, which contains cytochrome *c*, caspase 9 and apoptotic protease-activating factor-1 (APAF-1), followed by activation of downstream caspases 3, and 7[2]. While the intrinsic apoptosis pathway is considered to be regulated by BCL-2, the extrinsic pathway can also be regulated by BCL-2 family members via crosstalk with the intrinsic pathway. This crosstalk occurs through caspase 8 cleavage and activation of the BH3-interacting domain death agonist (BID). The cleavage product, truncated BID (tBID), is required for death receptor-induced apoptosis in some cell types. During tumorigenesis, both the extrinsic and intrinsic apoptosis signaling pathways become dramatically dysregulated thereby leading to increased cell survival upon chemo/radiotherapy. This chapter will discuss exploitation of factors regulating apoptosis, such as second mitochondria-derived activator of caspases (SMAC) and BH3-only proteins, as molecular targets utilized to overcome apoptosis resistance in cancer cells.

## 3. IAP family proteins promote apoptosis-resistance

IAPs are a pivotal class of pro-survival factors that suppress apoptosis against a large variety of apoptotic stimuli, including chemotherapeutic agents, radiation, and immunotherapy in cancer cells[3-5]. Elevated expression of IAPs is a common occurrence in multiple cancer types, while eliciting a wide range of biological responses that promote cancer cell survival and proliferation[6]. Therefore, IAPs are attractive molecular targets for anti-cancer therapies in order to decrease apoptosis-resistance, thereby enhancing cancer therapeutics and increasing patient survival.

IAPs are characterized by baculoviral IAP repeat (BIR) domains, which are required for the majority of IAP-mediated protein-protein interactions and inhibition of apoptosis[7]. Eight IAPs have currently been identified in humans, but the most studied IAP members include the X chromosome-linked IAP protein (XIAP), cellular IAP1 (cIAP-1), and cellular IAP2 (cIAP-2)[8]. IAPs inhibit both the intrinsic and extrinsic apoptotic pathways (Figure 1). XIAP binds to and inhibit caspases 3, 7, and 9, while cIAPs negatively regulate caspase 8 activation through TNFR1 signaling[9]. IAPs also contain a carboxyl-terminal RING domain, which enables them to function as E3 ubiquitin ligases[6]. XIAP and cIAPs can promote

cancer cell survival and proliferation by inhibiting caspase activation, IAP-antagonist binding, or by acting as critical mediators of the NF-κB pathway.

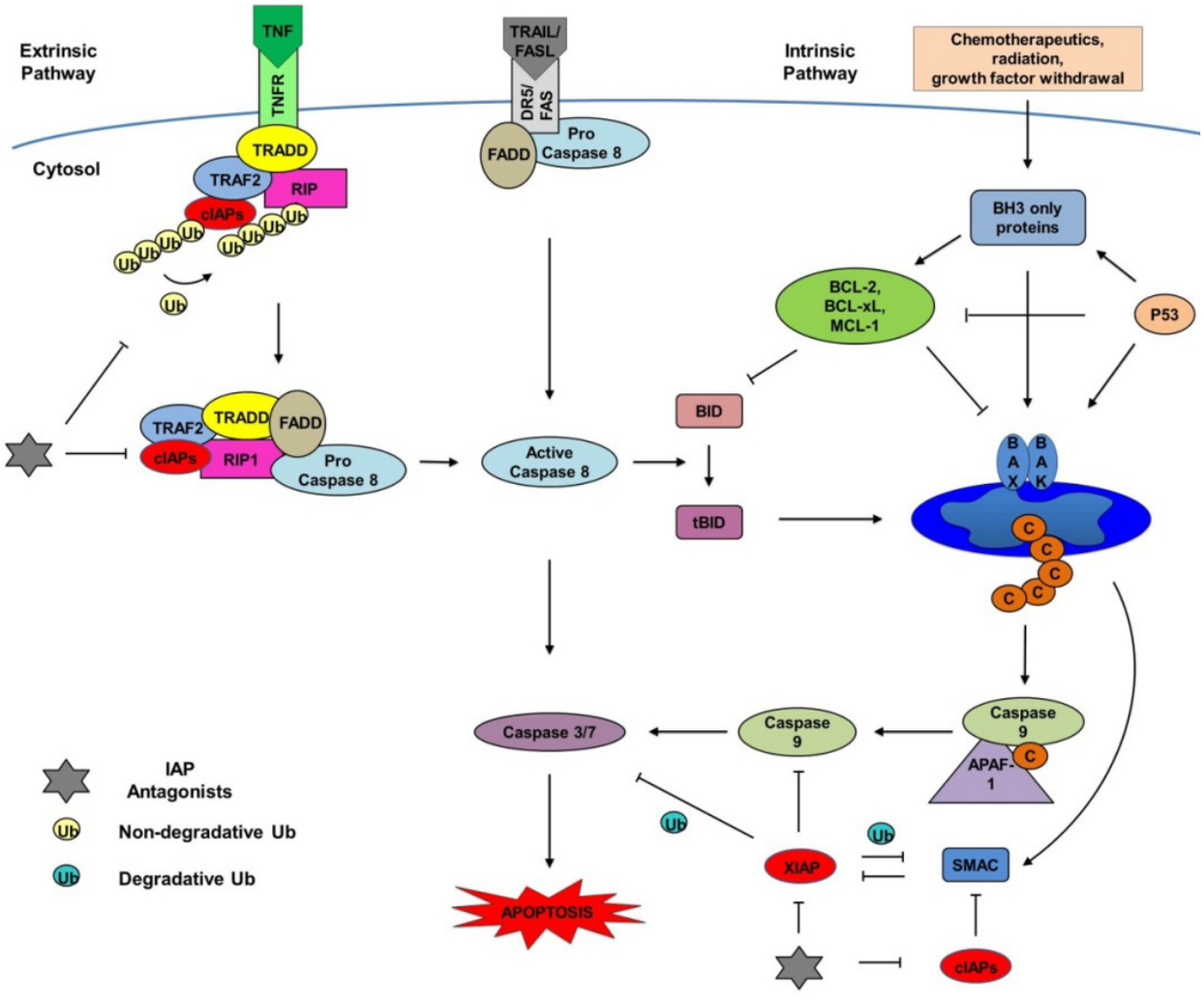


**Figure 1. Extrinsic and intrinsic apoptosis signaling pathways.** The extrinsic (death receptor) apoptosis pathway is induced by the binding of cell death ligands, TNF, FASL or TRAIL, to cell death receptors TNFR, FAS, or DR5, respectively. Activation of the death receptors results in caspase 8 activated cell death. The intrinsic (mitochondrial or BCL-2 regulated) apoptosis pathway can be activated by cellular stresses or chemo/radiotherapies. This leads to functional activation of the pro-apoptotic BCL-2 family proteins which induces cytochrome *c* or SMAC release into the cytosol. Cytochrome *c* induces formation of the apoptosome complex, which contains cytochrome *c*, caspase 9, and APAF-1, followed by activation of downstream caspase 3 and 7. SMAC can promote apoptosis by binding to XIAP, which results in the subsequent release of caspase 9 and downstream activation of apoptosis. cIAPs are capable of inhibiting SMAC by blocking this interaction. The crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8 cleavage and activation of the BID. The cleavage product, tBID, is required for death receptor-induced apoptosis in some cell types. During tumorigenesis, both the extrinsic and intrinsic apoptosis signaling pathways become dramatically dysregulated thereby leading to increased cell survival during chemo/radiotherapy. IAP antagonists can inhibit the anti-apoptotic actions of XIAP and cIAPs in both the intrinsic and extrinsic apoptosis pathways.

## 4. XIAP is a potent caspase inhibitor

XIAP protein is the first well-characterized IAP family member[4, 10, 11]. XIAP is overexpressed in approximately 25% of the 60 NCI human cancer cell lines and can predict response to chemotherapy[12-16]. Although it was initially believed that all IAP proteins blocked apoptosis by directly binding caspases, it was later found that only XIAP directly binds to and inhibits caspases 3, 7, and 9 (Figure 1)[10, 17, 18]. As caspase 9 is an initiator caspase, it is considered the most critical target for XIAP's anti-apoptotic function[19]. Structural studies have outlined the protein interactions utilized by XIAP to inhibit caspase function. The BIR3 domain of XIAP binds to the catalytic domain of caspase 9 while the linker region between XIAP BIR1 and BIR2 binds to caspase 3 or 7[20, 21],[22]. In addition to binding and blocking caspase catalytic sites, XIAP also utilizes its E3 ubiquitin ligase function for targeting and ubiquitylating caspase 3 for proteasome degradation[23]. Therefore, due to XIAP's ability to inhibit multiple caspases, either directly or via ubiquitylation, XIAP has become a premiere molecular target for current chemotherapies (Figure 1).

## 5. cIAP regulation of the NF-κB signaling pathway

Although IAPs are typically known to bind and inhibit caspases, cIAPs also modulate ubiquitin-dependent signaling events of the extrinsic apoptosis pathway and regulate activation of NF-κB[24]. cIAPs are required for stimulus-dependent activation of the canonical pathway and for constitutive suppression of the non-canonical NF-κB pathway (Figure 2)[8]. NF-κB is a transcription factor involved in angiogenesis, metastasis, and cell proliferation[8]. Upon activation, NF-κB regulates transcription of pro-survival genes such as TNF$\alpha$, cIAPs, BCL-2 and other apoptosis-related proteins. Furthermore, blocking NF-κB pathway can sensitize cancer cells to chemotherapeutic agents and radiation[25-27].

In the canonical NF-κB pathway, the inhibitor of NF-κB (IκB$\alpha$) binds to NF-κB, thereby preventing NF-κB nuclear translocation from the cytoplasm into the nucleus in unstimulated cells[28]. TNF-mediated activation of NF-κB requires the assembly of an ubiquitin-dependent signaling complex[29]. TNF ligand binding to TNFR1 induces the formation of a signaling complex by initially recruiting TNFR1-associated death domain protein (TRADD) and TNFR-associated factor 2 (TRAF2), followed by recruitment of receptor-interacting protein 1 (RIP1) and c-IAP proteins (Figure 2)[30, 31]. Within this complex, cIAPs promote nondegradative polyubiquitylation of RIP1, in addition to themselves, to generate a binding platform for assembly of the IκB kinase (IKK) complex[32-34]. This leads to the activation of IKKβ, which results in phosphorylation of IκB$\alpha$, prompting IκB$\alpha$ polyubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and activate target genes[28]. Therefore, cIAPs positively regulate the canonical NF-κB pathway.

Alternatively, in the non-canonical pathway, cIAPs negatively regulate NF-κB transcription by ubiquitylating and targeting NF-κB-inducing kinase (NIK) for proteasomal degradation[35]. In unstimulated cells, a cytoplasmic complex composed of cIAPs, TRAF2, TRAF3 and NIK, maintains constitutive ubiquitin-dependent proteasomal degradation of

NIK (Figure 2) [35-41]. Accumulation of NIK is acquired by dissociation of this cytoplasmic complex. Upon ligand binding, receptors of the TNFR family, such as CD40, recruit TRAF2, TRAF3 and the cIAP proteins into their respective signaling complexes. This results in cIAP ubiquitylation and degradation of the cIAPs, TRAF2, and TRAF3, which leads to stabilization and accumulation of NIK and downstream activation of NF-κB anti-apoptotic target genes[9, 42]. The conflicting roles that cIAPs play in inducing or inhibiting NF-κB signaling pathway display an additional layer of complexity when developing therapeutic drugs targeting cIAPS.

## 6. SMAC: IAP-antagonist

SMAC is a regulator of the intrinsic apoptosis pathway and becomes released from the mitochondria upon mitochondrial outer membrane permeabilitization (MOMP) (Figure 1). Structural studies show that SMAC induces apoptosis by binding to and sequestering IAPs from binding to caspases[43-45]. As previously mentioned, the BIR3 domain of XIAP binds to the N-terminus of small subunit p12 of processed caspase 9. SMAC protein contains a region homologous to the caspase 9 p12 subunit, therefore, it can also bind to XIAP BIR3 domain[20]. SMAC binding of XIAP allows the subsequent release of caspase 9 and activation of downstream signaling leading to apoptosis[46]. While cIAPs are not potent inhibitors of caspases, cIAPs are able to bind to SMAC with high affinity, thereby preventing SMAC from disrupting XIAP-mediated inhibition of caspases[6].

### 6.1. The role of IAP and SMAC and clinical outcome

Due to the importance of apoptosis resistance during chemo/radiotherapy, the expression of IAP proteins and IAP inhibiting proteins, such as SMAC, have demonstrated significant correlation with clinicopathological data[6, 47]. Altered expression of cIAPs in cancer cells is typically due to chromosomal aberrations, such as genomic ampifications, translocations and deletions. Genomic amplification at the 11q21-q23 genomic loci of both cIAP1 and cIAP2 has been detected in many cancers, including esophageal squamous cell carcinomas, liver cancer, lung cancer, and cervical cancer[48-51]. Furthermore, immunohistochemical analysis of cervical cancers from patients treated only with radiotherapy had high levels of nuclear cIAP1 staining and demonstrated that both overall survival and local recurrence-free survival was significantly poorer compared to patients with little or no nuclear cIAP1[50]. Genomic translocations, such as t(11;18)(q21;q21), results in the fusion of the BIR domains of cIAP2 with paracaspase mucosa-associated lymphoid tissue lymphoma translocation protein 1(MALT1) and occurs frequently in mucosa-associated lymphoid tissues[52-54]. The resulting cIAP2-MALT1 fusion protein constitutively activates the NF-κB signaling pathway[53, 55].

As previously discussed, cIAPs act as oncogenes in most cancers, however, cIAPs in multiple myeloma has demonstrated tumor suppressive properties. In multiple myeloma, chromosomal deletions of cIAP-1/2 resulted in stabilization of NIK, which induced constitutive aberrant activation of the non-canonical survival NF-κB pathway[37, 39]. This

further delineates the important balancing act of cIAPs in regulating the NF- κB pathways and cell survival.

XIAP expression is also dysregulated in many cancers and correlates with clinical outcome[6]. XIAP is upregulated in clear-cell renal cell carcinoma and correlates with increasing tumor stage, dedifferentiation, and aggressive growth[56]. XIAP was also shown to be an independent prognostic marker for non-muscular invasive bladder cancer, colon cancer, and liver cancer[57-59]. In invasive breast ductal carcinoma, nuclear expression of XIAP correlated with shortened overall survival[60]. Interestingly, a prostate cancer study showed patients with high XIAP levels had a much lower probability of tumor recurrence than those with lower XIAP expression. Furthermore, patients with high-grade prostate tumors who had high XIAP levels had a lower risk of recurrence compared with patients whose tumors express low XIAP[61]. This demonstrates that while many cancers have a correlation with high XIAP expression levels and poor prognosis, some cancers have additional altered mechanisms associated with poor clinical outcome. This further supports the need for tumor expression profiling in order to determine whether an individual's tumor is apoptosis-resistant. Pre-treatment screening will allow physicians to identify the proper treatment regimen in order to avoid unnecessary toxicity and relapse.

The down-regulation of IAP inhibitor, SMAC, has also been shown to play a significant role in inhibiting IAPs in cancer and correlates with clinical outcome[6]. In rectal cancer, high expression levels of SMAC correlated with 5-year recurrence free survival rate and 5-year local relapse-free survival rate[62]. Down-regulation of SMAC has been shown to be associated with disease progression in many cancer types, such as lung, hepatocellular carcinoma, testicular cancer[63-65]. In renal cell carcinoma, low levels of SMAC correlated with advanced tumor stage, poor prognosis, and a reduced probability of recurrence-free survival[56, 66]. Furthermore, XIAP expression increased with stage and grade, while mRNA and protein expression levels of SMAC did not significantly change. This results in a relative increase of anti-apoptotic XIAP over pro-apoptotic SMAC, thereby contributing to apoptosis resistance in renal cell carcinoma[66].

## 6.2. IAP antagonists as therapy to overcome apoptosis-resistance

Due to the dysregulation and contribution of IAPs towards chemo/radioresistance, researchers have developed several targeting strategies, such as small-molecule IAP antagonists, including SMAC mimetics, and antisense oligonucleotides. Table 1 shows a subset of IAP antagonists currently used in clinical trials.

## 6.3. SMAC-mimetics

Several studies have shown that overexpression of SMAC sensitizes neoplastic cells to apoptotic cell death[67, 68]. Therefore, SMAC mimetics have been developed in order to sensitize cancer cells to apoptotic stimuli, such as chemo/radiotherapy. Synthetic SMAC N-terminal peptides fused to cell-permeabilizing peptides were initially used as SMAC

mimetics for treating cancer cells. These peptides were found to bypass mitochondrial regulation and sensitize both human cancer cells in culture and tumor xenographs in mice to apoptosis when combined with TNF-related apoptosis-inducing ligand (TRAIL) or chemotherapeutic drug treatments[69, 70]. While appearing effective, SMAC peptides did not possess good pharmacological properties and, therefore, could not be used as therapeutic agents. Researchers then utilized 3D structure analysis of SMAC bound to XIAP BIR3 domain to design and synthesize small molecule SMAC mimetics[71-73]. These compounds show at least 20-fold enhanced binding to XIAP BIR3 domain over the natural SMAC peptide in a cell-free system[72-74]. Small molecule SMAC mimetics also bind and inhibit cIAP-1 and cIAP-2 activities and promote apoptosis synergistically with proapoptotic stimuli, such as TRAIL or TNF$\alpha$, in cancer cells that were previously determined to be resistant to TRAIL or TNF$\alpha$[71].

| Drug | **Cancer type(s)** | **Clinical Trial** | **Co-therapy** | **Outcome** |
|---|---|---|---|---|
| AT-406 | Solid tumors, lymphoma | Phase 1 | None | Ongoing.[225] |
| | AML | Phase 1 | Daunorubicin and Cytarabine | Ongoing.[225] |
| AEG35156 | AML | Phase 1/2 | High-dose Cytarabine and Idarubicin | AEG35156 treatment led to dose-dependent decreases of XIAP mRNA and protein levels. Apoptosis induction was detected.[195] |
| | AML | Phase 1/2 | Cytarabine and Idarubicin | Very effective when combined with chemotherapy in patients with AML refractory to a single induction regimen.[87] |
| YM155 | Advanced refractory solid tumors | Phase 1 | None | The safety profile, plasma concentrations achieved, and antitumor activity.[209] |
| | NSCLC | Phase 2 | None | Modest single-agent activity in patients with refractory, advanced NSCLC. A favorable safety/tolerability profile was reported.[210] |

AML– Acute myeloid leukemia; Non-small cell lung cancer – NSCLC

**Table 1.** Selective list of IAP antagonists undergoing clinical trials with and without combination therapy.

Pre-clinical and clinical data has demonstrated that SMAC mimetics may show more therapeutic promise in combination with conventional chemotherapeutic drugs, death receptor agonist or radiation therapy (Table 1). Research from our lab demonstrated that the

SMAC mimetic, SH130, disrupts the binding between XIAP/cIAP and SMAC. Upon combination treatment, SH130 enhances ionizing radiation-induced apoptosis *in vitro* and induces 80% tumor regression in hormone-refractory prostate cancer models[75]. We also demonstrated that SMAC mimetic, SH122, can induce cell death via both the extrinsic and intrinsic apoptosis pathways. In combined treatment with death receptor ligand, TRAIL, SH122 induces TRAIL-mediated cell death in prostate cancer cell lines by blocking IAPs and NF-κB[76].

SMAC mimetics have proven tremendous efficacy when used in combination with treatments to induce apoptosis in apoptosis-resistant cells[77]. Interestingly, it was also shown that SMAC mimetic treatment alone could induce apoptosis in a subset of non-small-cell lung cancer cell lines[78]. It was later determined that autocrine-secreted TNF$\alpha$-mediated apoptosis signals that were inhibited by IAP proteins. Treatment with the SMAC mimetic promoted formation of RIP1-dependent caspase 8-activating complex leading to apoptosis in these cells[78]. It has also been demonstrated that SMAC mimetic binding of cIAPs leads to rapid ubiquitination and proteasomal degradation of cIAPs[35]. Therefore, in addition to targeting XIAP to relieve caspase 9 inhibition in the intrinsic cell death pathway, SMAC mimetics can induce cIAPs auto-ubiquitination and degradation, which leads to NF-κB activation and TNF$\alpha$ secretion. The autocrine TNF$\alpha$ signaling in turn induces caspase 8 activation and cancer cell death (Figure 2).

### 6.4. cIAP- and XIAP-selective antagonists

SMAC mimetics have broad specificity by inhibiting both XIAP and cIAPs. Currently, the individual roles of IAPs in apoptosis resistance, as well as BIR domain structure, are unexplored. Therefore, more selective antagonists are designed in order to provide greater specificity for the diverse IAPs. CS3 is a cIAP1/2 selective antagonist and has been shown to induce degradation of cIAP1/2, activate canonical, non-canonical NF-κB signaling pathways, and induce cell death[79]. Although CS3 is capable of inducing cell death, cIAP-selective antagonists are significantly less potent in promoting apoptosis than pan-selective compounds[79].

Embelin, the active ingredient of traditional herbal medicine, is a potent IAP antagonist that binds to the XIAP BIR3 domain. We have shown that embelin inhibits cell growth, induces apoptosis, and activates caspase 9 in prostate cancer cells with high levels of XIAP, but has a minimal effect on normal prostate epithelial cells with low levels of XIAP[80]. Furthermore, embelin combined with radiation potently suppressed prostate cancer cell proliferation that was associated with S and G2/M cell cycle arrest[81]. Moreover, the combination treatment promoted caspase-independent apoptosis. *In vivo*, embelin significantly improved tumor response to x-ray radiation in PC-3 xenograft model. Combination therapy resulted in tumor growth delay and prolonged time to tumor progression, with minimal systemic toxicity. These findings demonstrate the potential to utilize embelin as a novel adjuvant therapeutic candidate for the treatment of hormone-refractory prostate cancer that is resistant to radiation therapy[81].

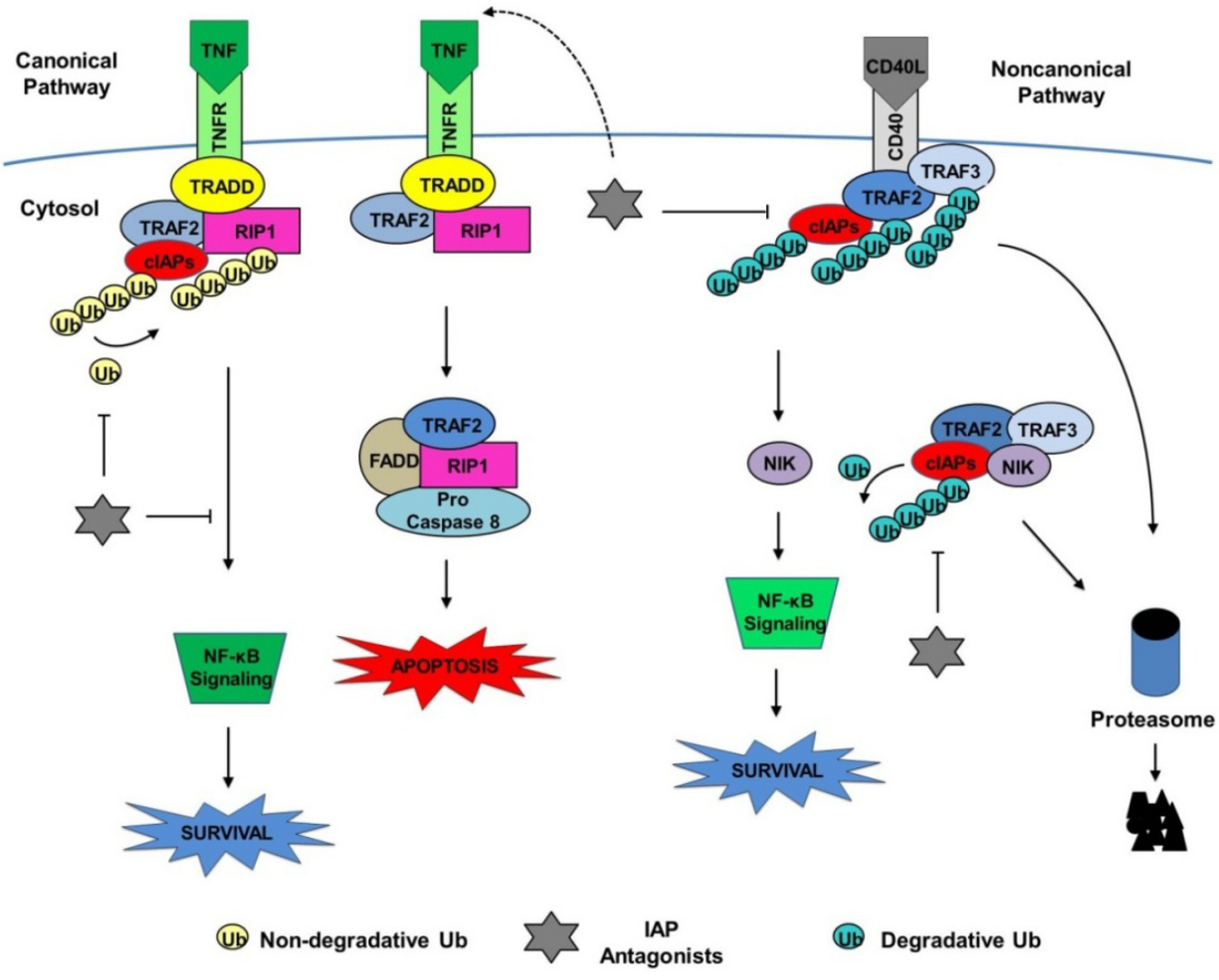


**Figure 2. Canonical and non-canonical prosurvival NF-κB pathways**. cIAPs are required for stimulus-dependent activation of NF-κB canonical pathway and alternatively for constitutive suppression of the non-canonical NF-κB pathway. TNF-mediated activation of the canonical NF-κB pathway requires the assembly of an ubiquitin-dependent signaling complex comprised of TRADD, TRAF2, RIP1, and cIAPs. cIAPs induce non-degradative ubiquitylation of both RIP1 as well as themselves which leads to activation of downstream pro-survival NF-κB signaling. IAP antagonists can inhibit NF-κB canonical pathway by preventing cIAP ubiquitylation of RIP1 which leads to recruitment of pro-caspase 8, thereby inducing apoptosis. Alternatively, in the non-canonical pathway, cIAPs negatively regulate NF-κB transcription by ubiquitylating and targeting NIK for proteasomal degradation. In unstimulated cells, a cytoplasmic complex composed of cIAPs; TRAF2, TRAF3 and NIK, maintains constitutive ubiquitin-dependent proteasomal degradation of NIK, thereby preventing activation of NF-κB pathway. Upon ligand binding, receptors of the TNFR family, such as CD40, recruit TRAF2, TRAF3 and the cIAP proteins into their respective signaling complexes. This results in cIAP ubiquitylation and subsequent degradation of cIAPs, TRAF2, and TRAF3. Degradation of this complex leads to stabilization and accumulation of NIK and downstream activation of NF-κB anti-apoptotic target genes. Interestingly, IAP antagonists can switch the non-canonical NF-κB signaling pathway from pro-survival to pro-apoptotic pathway (dashed arrow). IAP antagonists induce activation of this pathway by blocking cIAP inhibition, which leads to TNF$\alpha$ secretion. The autocrine TNF$\alpha$ signaling in turn induces caspase 8 activation and cancer cell death.

IAP antagonists have proven to be effective in overcoming apoptosis resistance in cancer cells. Clinical trials are currently underway to test the applicability of small-molecule IAP antagonists in single and combined anti-cancer therapies. Preliminary results suggest that IAP antagonists are well tolerated and effective in inhibiting IAPs (Table I)[6]. In addition, these molecules are providing insight into additional regulatory networks that exist in cancer cells, thereby providing new understanding of apoptosis resistance.

### 6.5. Inhibition of IAPs through RNA interference

Inhibition of IAPs using RNAi has further demonstrated the role of IAPs in drug resistance. Esophageal cancer cell lines transfected with XIAP siRNA demonstrated increased cell apoptosis[82]. Another study demonstrated that RNAi targeting of XIAP increased breast and pancreatic cancer cell susceptibility to functionally diverse chemotherapeutic agents, including TRAIL and taxanes and therefore increasing the effectiveness of chemotherapeutic agents[83]. Furthermore, *in vivo* studies also demonstrated that inhibition of XIAP by RNAi radiosensitized lung cancer cells by up-regulating apoptotic signaling and down-regulating cell survival[84]. We have also shown that combination treatment using RNAi silencing of IAPs and SH122 SMAC mimetic shows a greater sensitization of cells to apoptosis, than SMAC mimetic alone[76].

Clinical trials using anti-sense oligonucleotide AEG35156 is proving to be successful. The first-in-human study with AEG35156 in patients with advanced refractory cancers demonstrated that the compound was well tolerated and showed some anti-tumor activity[83]. However, AEG35156 was less effective in Phase I clinical trials with pancreatic cancer patients[85, 86]. Phase II trials treating primary refractory AML patients with both chemotherapy and AEG35156 demonstrated a 91% rate of complete remission[87]. Therefore, RNAi therapy shows significant promise in treating apoptosis-resistant cancers. While AEG35156 demonstrates promise in treating primary refractive disease, it is important to identify the patients that express high levels of IAPs in order to gain the most therapeutic benefit. Again, this demonstrates the need for molecular marker screening in order to develop personalized therapies.

## 7. BCL-2 family proteins regulate the intrinsic apoptotic pathway

In addition to XIAPs, the BCL-2 protein family members are essential regulators of the intrinsic apoptotic pathway, also known as the BCL-2-regulated pathway, and significant contributors to apoptosis-resistance during chemo/radiotherapies[88]. BCL-2 family members are characterized by their BCL-2 homology (BH) domain and can be categorized into three classes: the anti-apoptotic multi-domain proteins, such as BCL-2, BCL-xL, and MCL-1, are essential for cell survival, the pro-apoptotic BH3-only proteins, such as BID, BIM, BAD, and PUMA, initiate apoptosis signaling; and the pro-apoptotic multi-domain effector proteins, such as BAX and BAK, are required for MOMP and activation of caspases that leads to cell death[89, 90]. Both the anti-apoptotic and pro-apoptotic functions of BCL-2 family members are regulated through their BH domains [91, 92]. Furthermore, the BH1-

BH3 domains of anti-apoptotic proteins form a hydrophobic binding pocket that binds the $\alpha$-helix of the BH3-only pro-apoptotic proteins [93, 94]. BCL-2 proteins are typically found at the outer mitochondrial membrane (OMM), however, they can also be localized to the endoplasmic reticulum (ER) and in the cytosol[95].

Death signals induced by DNA damage, growth factor deprivation, or chemotherapies induce apoptosis via the mitochondrial pathway (Figure 1) by transcriptional or post-translational activation of BH3-only proteins[96-98]. After activation, the pro-apoptotic BH3-only proteins prompt a conformational change of monomeric BAX and BAK resulting in homo-oligomerization and activation[99-101]. Activated BAX and BAK cause MOMP, followed by release of cytochrome *c* and other pro-apoptotic factors, such as SMAC, from the mitochondria. BAX and BAK are essential for the pro-apoptotic function of BH3-only proteins, therefore, loss of BAX and BAK prevents apoptotic cell death[101, 102]. Cancer cells that display overexpression of anti-apoptotic proteins and/or down-regulation of pro-apoptotic proteins, have the potential to evade chemotherapeutic cell death resulting in drug resistance.

While it is generally accepted that activation of BAX and BAK is required to induce permeabilization of the mitochondria, there are multiple models that describe the mechanisms used in the activation/inhibition of BAK/BAX. One model suggests that BH3-only activating proteins, such as Bid or Bim, directly bind to BAX/BAK to induce oligomerization and subsequent activation [65, 103-106]. Another model describes an indirect mechanism. Anti-apoptotic proteins, such as BCL-2 and BCL-xL, inhibit cell death by binding to and sequestering activating BH3-only proteins thereby preventing their activation of BAX/BAK[107-110]. The indirect mechanism involves a subset of BH3-only proteins, called sensitizers, which induce BAX/BAK oligomerization indirectly, by binding anti-apoptotic proteins, thereby displacing the activating BH3-only proteins allowing them to bind to BAX/BAK[111, 112].

Anti-apoptotic proteins, BCL-2 and BCL-xL, are also capable of heterodimerizing with BAX or BAK, thereby inhibiting BAX or BAK[113-115]. It has been shown that BCL-2 undergoes a conformational change to bind to and inhibit oligomerization of mitochondrial membrane bound Bax. However, if BAX is in excess, apoptosis resumes due to the availability of free BAX able to activate the apoptotic pathway [115].

The activation models, as described in the previous paragraphs, are simplified examples of the complex interactions required to carry out the intrinsic apoptosis pathway. Dysregulation of intrinsic apoptosis pathways, due to altered ratios of antiapoptotic members to proapoptotic members, leads to apoptotic blocks. Identifying the proteins involved in these blocks is essential for designing more effective rational therapies. Studies called “BH3 profiling” used BH3 peptides that selectively antagonize BCL-2 family members to identify apoptotic blocks in cancer cells[107, 116]. It was demonstrated that BH3-only proteins show distinct binding preferences to anti-apoptotic BCL-2 family members[107, 116]. Identifying differential BH3-only protein binding affinities for anti-apoptotic BCL-2 protein family members has led to the development of specific small

molecule inhibitors of anti-apoptotic BCL-2 proteins which are designed to overcome apoptosis resistance in cancer cells and induce cell death.

## 7.1. Dysregulation of the BCL-2 family of proteins augments chemo/radioresistance

The dysregulation of BCL-2 family members, such as overexpression of anti-apoptotic genes or silencing of pro-apoptotic genes, is a key determinant for apoptosis-resistance during tumorigenesis and chemotherapy. BCL-2 was initially discovered to be overexpressed in human B-cell lymphomas and is located near chromosomal translocation break points frequently found in B-cell lymphomas[118]. Additional studies have demonstrated that BCL-2 protein levels in cancers are enhanced due to promoter hypomethylation, loss of inhibitory microRNA expression, and gene amplifications, signifying that up-regulation of BCL-2 expression is often found in a variety of cancers[119, 120].

Expression of anti-apoptotic BCL-2 family members has a significant effect on chemoresistance and prognosis[120, 121]. BCL-2, BCL-xL, and MCL-1 expression increases during prostate cancer progression[122]. Furthermore, BCL-2/BCL-xL expression levels correlate with resistance to a wide spectrum of chemotherapeutic agents[123, 124]. Alternatively, the pro-apoptotic BCL-2 family members can be down-regulated resulting in suppressed apoptosis. Spontaneous deletions or mutations of BAX have been observed in colorectal tumors, which results in significant reduction of apoptosis in response to anticancer agents[125, 126]. The BH3-only protein PUMA is also down-regulated in melanoma and Burkitt lymphomas[127, 128].

Dysregulation of BCL-2 family of proteins also occurs in cancer cells due to a loss of p53 tumor suppressor expression or function. p53 expression is lost in a majority of cancers. p53 can activate transcription of BAX, BID, PUMA and NOXA (Figure 1)[97, 129-132]. Cytosolic accumulation of p53 results in activation of BAX similarly to the BH3-only activating BCL-2 proteins, thereby inducing apoptosis[133]. Interestingly, p53 has also been shown to inhibit anti-apoptotic BCL-2 family members as well. DNA damage induces p53-Bcl-2 binding, thereby sequestering BCL-2 from inhibiting BAX/BAK oligomerization resulting in apoptotic cell death in cancer cells[134]. Inhibiting apoptosis via p53-associated regulation of the BCL-2 family displays another level of complexity in inducing cell death of cancer cells.

## 7.2. BH3-mimetics as a therapeutic strategy to overcome apoptosis resistance

Due to the dysregulation and importance of BCL-2 family members for inhibiting apoptosis in cancer cells, attempts aimed at developing novel drugs that can inhibit anti-apoptotic BCL-2 proteins. Crystal structure analysis of BCL-xL revealed that the BH1-BH3 domains formed a hydrophobic groove[93]. Further studies demonstrated that this BCL-xL hydrophobic groove could bind to a BAK BH3 peptide indicating the ability to design small molecules that could bind to BCL-xL and inhibit its anti-apoptotic function[94]. Indeed, numerous small molecule BH3-mimetics have been identified or designed to bind to this

BH3 binding pocket with the potential to block BCL-2/xL binding to pro-apoptotic BCL-2 proteins. The BH3 mimetics have demonstrated diverse binding specificity and efficacy in inducing apoptosis (Figure 3)[135-137].

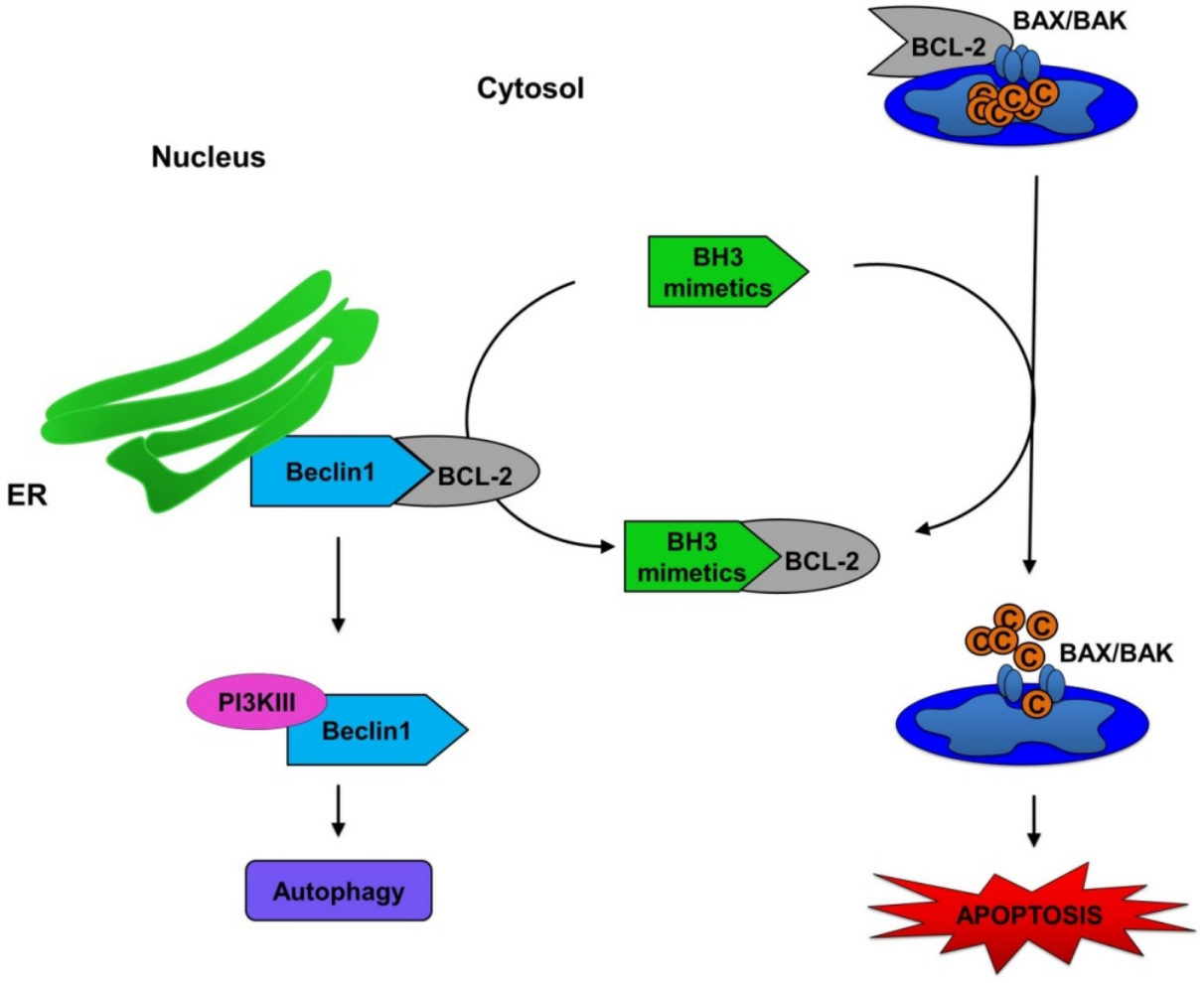


**Figure 3. BH3 mimetics inhibit anti-apoptotic BCL-2 proteins therefore inducing both apoptosis and autophagy.** BH3 mimetics are designed to bind to anti-apoptotic BCL-2 proteins and induce apoptosis. BH3 mimetics also induce autophagy-associated cell death by preventing BCL-2 proteins from binding to the autophagy activating protein, Beclin1.

One of the first small molecules developed via *in silico* screens was HA14-1[136]. HA14-1 was initially demonstrated to induce the activation of Apaf-1 and caspases in human acute myeloid leukemia cells. HA14-1 was subsequently found to prevent BCL-2 binding to BAK[138]. In addition, treatment with HA14-1 caused cytosolic Ca(2+) increase, change in mitochondrial membrane potential, BAX translocation, and reactive oxygen species (ROS) generation prior to cytochrome c release[139]. Obatoclax (GX15-070MS) was one of the first pan anti-apoptotic BCL-2 protein inhibitors capable of inhibiting BCL-2, BCL-XL, and MCL-1[140]. Clinical trials using obatoclax treatment have demonstrated success across many cancer types both independently as well as in combined therapies. Representative clinical trials are listed in Table 2.

| Drug | Cancer type(s) | Clinical Trial | Co-therapy | Outcome |
|---|---|---|---|---|
| AT-101/ Gossypol | SCLC | Phase 2 | None | Not active in patients with recurrent chemosensitive SCLC.[226] |
| | NSCLC | Phase 2 | Docetaxel | AT-101 plus docetaxel was well tolerated with an adverse event profile indistinguishable from the base docetaxel regimen.[227] |
| | SCLC | Phase 1/2 | Topotecan | Relapsed progression - 17.4 weeks, refractory progression - 11.7 weeks.[228] |
| | CRPC | Phase 1/2 | None | Evidence of single-agent clinical activity was observed with prostate-specific antigen declines in some patients.[229] |
| | Metastatic Breast Cancer | Phase 1/2 | None | Gossypol appears to affect the expression of Rb protein and cyclin D1; negligible antitumor activity against anthracycline and taxane refractory metastatic breast cancer.[230] |
| ABT-263/ Navitoclax | CLL | Phase 1 | None | Low MCL1 expression and high BIM:MCL1 or BIM:BCL-2 ratios in leukemic cells correlated with response.[143] |
| | SCLC | Phase 1 | None | Changes in a surrogate marker of BCL-2 amplification (pro-gastrin releasing peptide) correlated with changes in tumor volume.[144] |
| | Lymphoma | Phase 1 | None | Navitoclax has a novel mechanism of peripheral thrombocytopenia and T-cell lymphopenia, attributable to high-affinity inhibition of BCL-XL and BCL-2, respectively.[231] |
| GX15-070MS/ Obatoclax mesylate | Leukemia | Phase I | None | Well tolerated and these results support its further investigation in patients with leukemia and myelodysplasia.[232] |
| | Solid tumors | Phase I | Topotecan | Safe and well tolerated when given in combination with topotecan.[233] |
| | CLL | Phase I | None | Activation of Bax and Bak was demonstrated in peripheral blood mononuclear cells, and apoptosis induction was related to obatoclax exposure, as monitored by the plasma concentration of oligonucleosomal DNA/histone complexes.[234] |

| Drug | Cancer type(s) | Clinical Trial | Co-therapy | Outcome |
|---|---|---|---|---|
| Oblimersen (Genasense) | Breast Cancer | Phase I | TAC | Two of 13 patients showed a decrease of BCL-2 transcripts after 4 days of treatment with oblimersen.[235] |
| | CRPC | Phase II | Docetaxel | The primary end points of the study were not met: PSA response rate >30% and a major toxic event rate <45% were not observed with docetaxel-oblimersen.[236] |
| | Breast Cancer | Phase II | TAC | Oblimersen up to a dose of 7 mg/kg/day administered as a 24-h infusion on days 1-7 can be safely administered in combination with standard TAC on day 5.[196] |
| | HRPC | Phase II | Docetaxel | Oblimersen combined with docetaxel is an active combination demonstrating both an encouraging response rate and an overall median survival. [237[ |

HRPC – Hormone Refractory Prostate Cancer; Chronic lymphocytic leukemia – CLL; Small Cell Lung Cancer- SCLC; Non-small cell lung cancer – NSCLC; Castrate-resistant prostate cancer – CRPC; TAC – docetaxel, adriamycin and cyclophosphamide.

**Table 2.** Selective list of published BH3 mimetics clinical trials with and without combination therapy.

Using nuclear magnetic resonance-based screening and structure-based design, the BH3 mimetic, ABT-737, was developed and shown to possess greater affinity and ability to inhibit BCL-2, BCL-xL and BCL-w, than MCL-1[141]. ABT-737 was initially developed by screening a library of BH-3 like analogues with high binding efficiency to the hydrophobic groove of BCL-xL. ABT-737 has been shown to synergistically enhance cell death in combined treatments with chemotherapeutics and radiation[141]. An oral form of ABT-737, called ABT-263 (Navitoclax), has also been developed and is also undergoing clinical trials for lymphoma, leukemia, and small cell lung cancer[142-144].

The BH3 mimetic (-)-gossypol is a natural polyphenol purified from the cottonseed. We previously demonstrated that the (-)-gossypol significantly enhances the antitumor activity of docetaxel chemotherapy in hormone-refractory prostate cancer patients with BCL-2/BCL-xL/MCL-1 overexpression[145]. Mechanistically, we demonstrated that (-)-gossypol blocked the interactions of BCL-2/Bcl-xL with Bax or Bad in cancer cells. (-)-Gossypol (AT-101) is the first BCL-2/BCL-xL inhibitor entered clinical trial and is now in Phase IIb clinical trials for hormone-refractory prostate cancer and many other types of cancer at multiple centers in the United States. In addition, more potent and less toxic gossypol derivatives, such as Apogossypolone and TW-37, are being developed[146-148].

BH3 mimetics are designed to inhibit anti-apoptotic BCL-2 proteins and demonstrate significant therapeutic potential in clinical trials. Interestingly, BH3 mimetics induced

toxicity independent of Bax/Bak suggesting the existence of an alternative route of cell death induction[149]. BCL-2 has also been linked to a non-apoptotic cell death mechanism associated with autophagy, usually known as a cell survival mechanism[150]. It was later determined that BCL-2 and BCL-xL can bind the BH3 domain of tumor suppressor Beclin1 (BECN1) and inhibit autophagy (Figure 3)[151, 152]. This discovery revealed a new role for anti-apoptotic BCL-2 protein family as anti-autophagic proteins. The following sections will discuss autophagy and the role played by the BCL-2:Beclin 1 interaction for inducing/inhibiting autophagy, and the mechanism of novel therapies, such as BH3 mimetics, aimed at disrupting the interaction in order to induce autophagy-associated cell death.

## 8. Autophagy and autophagic cell death: background

Autophagy is a highly regulated catabolic process that functions as a cell survival mechanism activated upon cellular stresses such as nutrient deprivation, starvation, hypoxia and chemo/radiotherapy[153]. There are three primary types of autophagy, chaperone-mediate autophagy, microautophagy and macroautophagy[154]. This chapter will focus on macroautophagy, referred as autophagy further in the text. Activation of autophagy induces the formation of autophagosomes that engulf damaged organelles or particles. Eventually, the autophagsome fuses with the lysosome and degrades its interiors to provide cells the nutrients such as amino acids or fatty acids necessary for cell metabolism[155]. Defective autophagy machinery can lead to diseases such as neurodegenerative, liver, cardiac, and muscle diseases, as well as a variety of cancers. Recent studies have reported that apoptosis-resistant cancer cells can avoid chemo/radiotherapeutic-induced cell death by activating autophagy [156-159]. Furthermore, apoptosis-associated proteins, such as NF-κB, p53, UVRAG and the above-discussed BCL-2, have been shown to play dual regulatory roles in both apoptosis and autophagy [160-162]. Paradoxically, activation of autophagy upon drug treatments can induce cell death independent of or in parallel with apoptosis and necrosis.[163]. Therefore, researchers are actively developing novel cancer therapies that aim to promote cell death by modulating autophagy pathways.

### 8.1. Autophagy pathways

Autophagy can be activated by a variety of stimuli and signaling pathways. The classical induction of autophagy occurs upon nutrient deprivation; however, autophagy can also be induced by other factors, such as hypoxia, cytokines, hormones, genotoxic stress, p53 activation, and chemo/radiotherapy. Autophagy has also been attributed to tumor suppression. This was first demonstrated in mice with allelic loss of Beclin1, a key protein involved in inducing autophagy. Complete loss of the Beclin1 resulted in death during early embryogenesis whereas heterozygous loss of Beclin1 resulted in formations of spontaneous tumors [164, 165]. Autophagy involves a conserved family of proteins known as the autophagy-related gene families (ATGs). The canonical autophagy pathway in mammals occurs in a series of stages: initiation, nucleation, elongation, and degradation. All stages are regulated by a core molecular machinery (Figure 4).

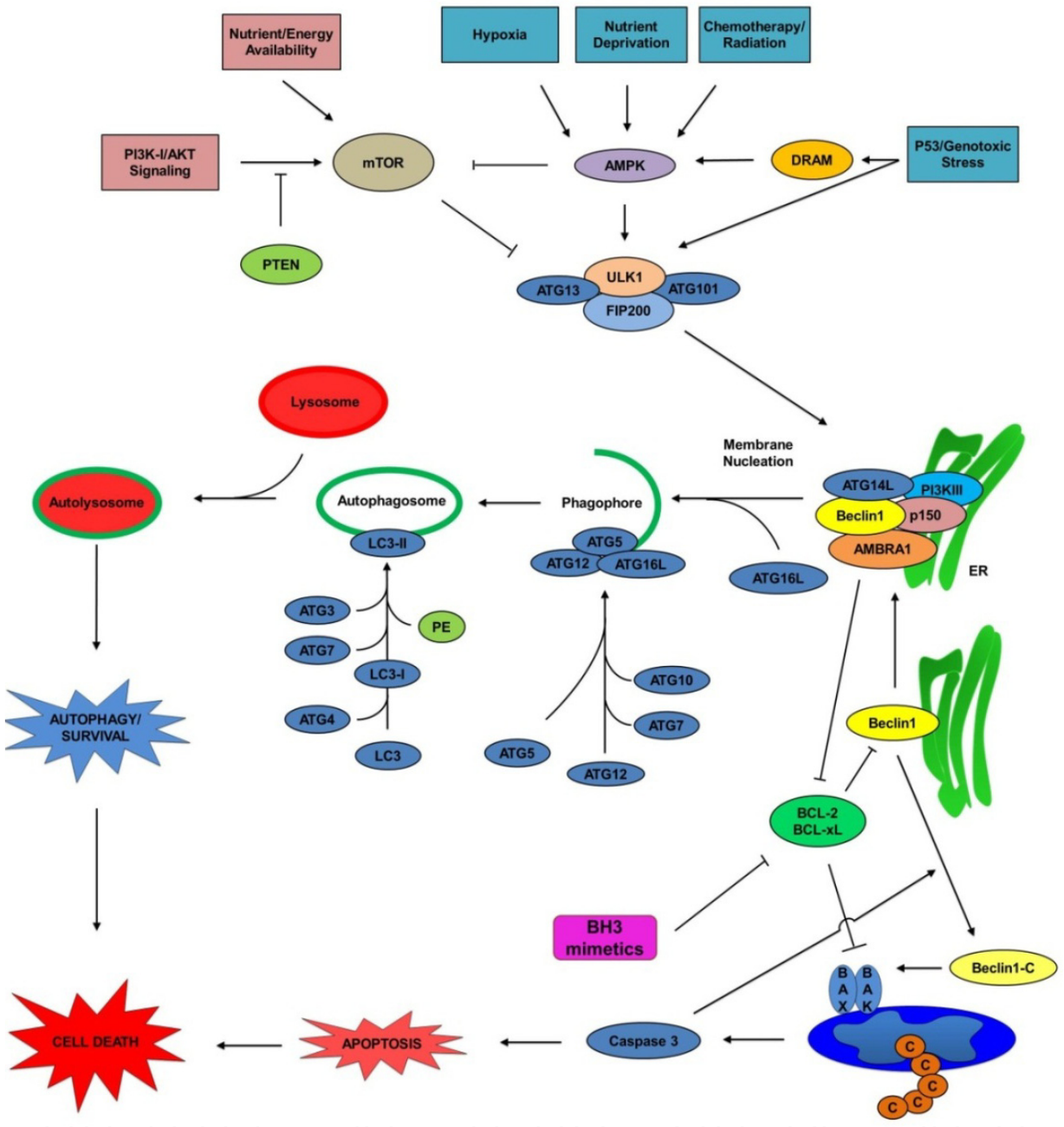


**Figure 4. Cross-talk between apoptosis and autophagy.** Autophagy takes place in a series of stages; initiation, nucleation, elongation, and degradation. Autophagy can be activated by a variety of stimuli and signaling pathways, including nutrient deprivation, hypoxia, p53 genotoxic stress, suppression of mTOR, or chemo/radiotherapy, followed by activation of AMPK. ULK1, ATG13, ATG101, FIP200 protein complex forms and mediates autophagy initiation. ATG13 mediates ULK1 phosphorylation of FIP200 and activates the ULK complex. Subsequently, the ULK complex localizes to the ER and initiates pre-autophagosome formation. The vesicle nucleation involves the core complex consisting of PI3KIII, p150, ATG14L, Beclin1 and AMBRA1. ATG14L induces a translocation of the PI3KIII complex to the site of autophagosome formation and initiates the formation of the phagophore. Phagophore elongation into an autophagosome requires ATG12, LC3-I, and two ubiquitin-like protein conjugation systems. The first system involves ATG7 and ATG10 conjugation of ATG12 into the ATG16L-ATG12-ATG5 complex. The second conjugation system involves LC3-I modification by ATG7 and ATG3 into LC3-II and inserts

into the autophagosome membrane. Finally, the autophagosome fuses with the lysosome and contents within the autophagosome are degraded. Beclin1 can interact with autophagy machinery at the ER and induce autophagy. In addition, Beclin1 can bind to anti-apoptotic BCL-2 family of proteins, preventing BCL-2 binding to BAX or BAK monomers, therefore inducing apoptosis. Beclin1 can also be cleaved by caspase 3 to form Beclin1-C which inhibits autophagy. However, Beclin1-C can induce apoptosis by localizing to the mitochondria facilitiating the release of apoptotic factors.

A protein complex consisting of unc-51-like kinase 1(ULK1, homolog of yeast ATG1), ATG13, ATG101 and a scaffolding protein FIP200 (ortholog of yeast ATG16) mediates autophagy initiation (Figure 4)[166]. In nutrient-rich environment, an upstream regulator called mammalian target of rapamycin (mTOR) phosphorylates ATG13 and ULK1 to inhibit the initiation of autophagy. Following starvation or cellular stress, mTOR is inhibited and dissociates from the ULK1 complex. Then, ATG13 mediates ULK1 to phosphorylate FIP200 and activates the ULK complex[167]. Subsequently, the ULK complex localizes to the endoplasmic reticulum (ER) and initiates pre-autophagosome formation [168]. The vesicle nucleation involves the core complex consisting of Class III phosphatidylinositol 3-kinase (PI3KIII/ homolog of yeast Vps34), p150 (Vps15), ATG14L, Beclin1 (ATG6), and activating molecule in Beclin 1-regulated autophagy (AMBRA1)[165, 169]. ATG14L induces a translocation of the PI3KIII complex to the site of autophagosome formation and initiates the formation of an isolated membrane, also known as the phagophore[170]. A recent study revealed that PI3KIII lipid kinase activity produces and accumulates phosphatidylinositol 3-phosphate (PI3P) at the ER to induce a high membrane curvature that attracts ATG14L binding. Bound to the ER, ATG14L produces more PI3Ps and recruits other parts of the core complex. Recruitment of these proteins induces phagophore elongation [171, 172]. Phagophore elongation into an autophagosome requires ATG12, ATG8/LC3-I, and two ubiquitin-like protein conjugation systems. The first system involves an E1-like ATG7 and an E2-like ATG10 conjugation of ATG12 to ATG12-ATG5 that interacts with ATG16L to form the ATG16L-ATG12-ATG5 complex[173-176]. The second conjugation system involves the cytosolic protein isoform known as the LC3-I (ATG8) to undergo modification by ATG7 and E2-like ATG3 into LC3-phosphatidylethanolamine (LC3-II), an important biomarker for autophagy[177, 178]. The ATG16L complex acts as an E3-like enzyme to promote lipidation of cytosolic LC3-I into LC3-II and correctly localizes LC3-II onto the autophagosome formation site to help form the membrane [179]. Finally, the autophagosome fuses with the lysosome and contents within the autophagosome are degraded. This final step requires the endosome marker, RAB7, and a lysosomal membrane protein, LAMP2, however, the exact mechanism involved in the fusion of autophagosome and lysosomes is still unclear [180, 181].

## 8.2. Autophagy induction

Activation of autophagy is regulated by multiple molecular pathways depending upon the stimuli (Figure 4). As mentioned above, mTOR is activated under nutrient-rich environment thereby suppressing autophagy. Starvation of growth factors and certain amino acids represses class I PI3K signaling to promote cell survival via autophagy induction [182, 183]. PI3KI forms the substrate PI3P which leads to activation of the PKB/AKT protein that

inhibits a heterodimer complex involving the tuberous sclerosis complexes 1 and 2 (TSC1 and TSC2). The TSC2 protein suppresses mTOR activity via activation of a Ras family small GTPase called Ras homolog enriched in brain (Rheb) [184]. Tumor suppressor phosphatase

and tensin homolog (PTEN) dephosphorylates the PI3K product PI3P, thereby suppressing AKT signaling. Loss of PTEN occurs in multiple cancers including brain, breast, and prostate cancer [185]. Additional aberrant signaling of PI3KI can result in cancers that exhibit mutated amplification of upstream receptor tyrosine kinase, such as HER2 in gastric cancer or PDGFR and EGFR in glioblastoma [186, 187]. Under metabolic stress, such as high AMP level, hypoxia and cytosolic calcium level increase, AMP-activated protein kinase (AMPK) can mediate autophagy by negatively regulating mTOR and inducing the dephosphorylation of ATG13 and ULK1 [188-190]. Alternatively, AMPK has been found to activate autophagy by direction phosphorylation of ULK1 [191].

The tumor suppressor protein p53 plays a more complicated role and can induce as well as inhibit autophagy, based upon subcellular location and cellular context. Upon exposure to DNA-damaging agents, nuclear p53 can induce autophagy by transcriptionally activating damage-regulated autophagy modulator (DRAM)[192]. DRAM activates target proteins Sestrin1 and Sestrin2, which subsequently activate AMPK thereby inhibiting mTOR and inducing autophagy [193]. In addition, nuclear p53 can up-regulate ULK1 transcriptionally and directly activate autophagy[194]. Cytoplasmic p53 has the opposite effect and can actually inhibit autophagy[195]. High mobility group box 1 (HMGB1) is a Beclin1-interacting accessory protein that assists in autophagy activation. p53 has been discovered to form a complex with HMGB1 in the cytoplasm resulting in the inhibition of autophagy and induction of cell death (22345153)[87]. Loss or knockdown of p53 increases the binding of HMGB1 to Beclin1 and mediates cytosolic localization of the complex to the ER [87]. Subsequently, HMGB1 mediates the Beclin1-PI3KIII complex formation and initiates autophagosome production.

### 8.3. Beclin1:BCL2 interaction regulates autophagy/apoptosis switch

As discussed above, Beclin1 is a critical inducer of autophagy. Interestingly, Beclin1 is also a BH3-only protein and therefore interacts with anti-apoptotic BCL-2 family members via its BH3 domain [151, 196]. BCL-2 binding of Beclin1 at the ER prevents Beclin1 from assembling the pre-autophagosomal structure mediated by the Beclin1/PI3KIII complex (Figure 3 and 4)[196, 197]. Therefore, BCL-2 anti-apoptotic proteins have dual pro-survival roles by preventing both apoptosis and autophagy-associated cell death that makes these proteins ideal chemotherapeutic targets.

The expression of BCL-2 and/or Beclin1 is critical for regulating the switch between autophagy and apoptosis. Down-regulation of Beclin1 also contributes to tumorigenesis, evident in hepatocellular carcinoma, brain, colorectal, and gastric cancer [198-200]. Low expression of Beclin1 results in insufficient removal of damaged organelles. Deficient Beclin1 causes cell transformation through the accumulation of reactive oxygen species and

genotoxic stress, [165]. Furthermore, it was shown that inhibiting BCL-2 in breast cancer cells via siRNA knockdown did not induce apoptosis as expected but observed a form of autophagic cell death [201]. The autophagic cell death was the result of combinatorial treatment with doxorubicin that lead to increased expression of Beclin1[201]. Therefore, maintaining adequate levels of Beclin1 is important to override BCL-2 inhibition of autophagy-related cell death. Evidently, BCL-2 and Beclin1 expressions are important determinants for identifying the proper chemotherapy or combination treatments that would provide the greatest therapeutic benefit.

Autophagy regulatory proteins can promote or inhibit the BCL-2:Beclin1 interactions. As previously mentioned, AMBRA1 is a key regulator in initiating autophagy by binding to Beclin1. In presence of autophagic stimulus, ULK1 phosphorylates AMBRA1, which results in AMBRA1 dissociation from the Dynein motor complex [202]. After dissociation, AMBRA1 translocates to the ER, binds to Beclin1 in the autophagy initiation complex and results in the induction of autophagy [202]. Moreover, a recent study demonstrated that BCL-2 localized to the mitochondria can also bind AMBRA1whereas ER-localized BCL-2 does not [203]. The BCL-2:AMBRA1 interaction at the mitochondria is down-regulated during autophagy and apoptosis. Therefore, BCL-2 can regulate Beclin1-induced autophagy by directly binding to Beclin1, as well as by sequestering AMBRA1, the activator of Beclin1 at the mitochondrion [203].

BCL-2/Beclin1 complex can be disrupted by otherBCL-2 and Beclin1 binding partners. As discussed above, HMGB1 can bind to Beclin1 and initiate autophagy. Inhibition of HMGB1 decreases autophagy and increases apoptosis[204]. For example, a study has shown that deletion or deactivation of HMGB1 in mouse embryonic fibroblasts reduces LC3-I expression. In response to starvation, cells lacking HMGB1 cannot initiate autophagy and undergo apoptotic cell death[205]. Additionally, HMGB1 bound to Beclin1 has also been found to induce the phosphorylation of BCL-2 which disrupts the BCL-2:Beclin1 complex.

Autophagy can also be inhibited by Beclin1 cleavage. Chemotherapy-induced and mitochondria-mediated apoptosis was shown to induce Beclin1 cleavage by caspase 8 to form Beclin1-C. This event renders defective Beclin1 activity and autophagy pathway [206]. Furthermore, the C-terminus of cleaved Beclin1 can acquire pro-apoptotic ability by translocation to the mitochondria and inducing release of apoptotic factors [207]. This demonstrates a novel therapeutic approach to induce apoptosis by inhibiting autophagy.

## 8.4. Inducing autophagy-associated cell death using BH3 mimetics

This chapter has previously discussed the therapeutic benefits of using BH3 mimetics to induce apoptosis by preventing anti-apoptotic BCL-2 proteins from binding to pro-apoptotic proteins, BAX/BAK. Upon the discovery that Beclin1 was a novel autophagic BH3-only protein, BH3 mimetics have been utilized to induce autophagy[208]. ABT737 was the first BH3 mimetic reported to induce apoptosis and autophagy by inhibiting anti-apoptotic action of BCL-2 or BCL-xL[208]. At first the findings were counterintuitive; how could a

drug induce both apoptotic cell death and autophagic cell survival? As discussed above, BH3 mimetics appeared to kill cells in a BAK/BAX-independent manner suggesting that apoptotic cell death was not the only mechanism for BH3 mimetic-induced cell death[149]. It was later determined that BH3 mimetics could induce autophagy-associated cell death, especially in apoptosis-resistant cells [209].

We recently investigated the effect of the natural BH3-mimetic (-)-gossypol in apoptosis-resistant prostate cancer cells with high levels of BCL-2 versus prostate cancer cells with low BCL-2 expression[210]. (-)-Gossypol induced similar levels of total cell death in both prostate cancer cell lines. However, the dominant mode of cell death depended upon the expression of the anti-apoptotic BCL-2 family of proteins[210]. BH3 mimetics induced apoptotic cell death in prostate cancer cells with low BCL-2 expression. Conversely, prostate cancer cells with high BCL-2 expression died via modulation of the autophagy pathway [210]. Furthermore, overexpressing BCL-2 decreased the level of (-)-gossypol-induced autophagy, possibly due to the stoichiometric abundance of BCL-2 sequestering Beclin1 and inhibiting autophagy induction. The data demonstrate that BH3 mimetics can be utilized to kill cells with both high and low BCL-2, therefore, enhancing the ability to overcome chemo/radioresistance.

BH3 mimetics induce autophagy by disrupting the BCL-2:Beclin1 inhibitory complex as well as additional autophagy pathways. BH3 mimetics, ABT-737 and HA14-1, also stimulate other pro-autophagic pathways and hence activate the nutrient sensors Sirtuin1 and AMPK, inhibit mTOR, deplete cytoplasmic p53, and trigger the IKK Kinase[211]. Activation of autophagy was independent of reduced oxidative phosphorylation or reduced cellular ATP concentrations. Furthermore, induction of autophagy by ABT-737 and HA14-1 was completely inhibited by knockdown of Beclin1 or PI3KIII. This suggests that BH3 mimetics can interfere with multiple pathways, eliciting a coordinated effort to induce autophagy-associated cell death.

## 9. The role of autophagy in therapy resistance

A number of therapeutic strategies have been developed to target autophagy in cancer cells. Similarly with apoptosis-resistance, autophagy-associated resistance to chemotherapy has become a challenging variable in the successful treatment of patients. For example, in human lung cancer cells treated with EGFR tyrosine kinase inhibitors (TKI), gefitinib and erlotinib, autophagy contributed to cell survival[212]. Inhibition of EGFR suppresses PI3KI activity and results in downstream activation of the ULK complex[212]. Other studies have shown that autophagy contributes to chemotherapy resistance through its cytoprotective mechanism. For example, chronic myeloid leukemia treated with imatinib, glioblastoma multiforme treated with temozolomide, colorectal cancer treated with 5-FU, and breast cancer treated with both tamoxifen and trastuzumab have all shown resistance that is associated with increased autophagy[213]. Recent studies have shown that cytotoxic agents and starvation may play a role in activating autophagy via HMGB1[214]. Increased

expression of HMGB1 during treatment with doxorubicin, cisplatin, and methotrexate in osteosarcoma patients has been found to facilitate chemotherapy resistance by promoting the formation Beclin1/PI3KIII complex. In addition, HMGB1 also antagonizes drug-induced cell death in leukemia, colon cancer, and prostate cancer by up-regulating autophagy but the exact mechanism remains unclear[214].

Not only does autophagy contribute to chemotherapy resistance, it also plays a role in radiotherapy resistance. Investigators exposed radioresistant MDA-MB-231 cells to ionizing radiation at different doses and found increasing levels of LC3-II, a hallmark of autophagy activation. This indicates that activation of autophagy may protect these cells from radiation-induced cell death[215]. In addition, researchers found upregulation of autophagy in radiosensitive HBL-100 cells after inhibition of mTOR by rapamycin. In further experiments, inhibition of autophagy by 3-methyladenine (3-MA) resulted in reduced cell survival and displayed a radiosensitizing effect[215]. From these experiments, researchers deduced that cancer cells use autophagy as an escape mechanism from apoptosis to overcome radiotherapeutic stress via degradation of IR-induced cellular damage.

## 10. Re-sensitization of cancer cells to treatment by autophagy inhibition

To counter autophagy in cancer resistance, novel cancer therapies uses target inhibition of autophagy for re-sensitizing cancer cells to drug treatments. Researchers have used autophagy inhibitors such as 3-MA, LY294002, wortmannin to inhibit the PI3K [158, 216]. 3-MA contributes to autophagy suppression by down-regulating the PI3KI/Akt/mTOR signaling pathway. Surprisingly, the autophagy inhibitor 3-MA has been found to induce autophagy and contribute to cell survival when used for a prolonged period[217]. This controversial phenomenon is most likely due to the dual effect of 3-MA on PI3KI and PI3KIII. 3-MA blocks Class I permanently, but only temporarily Class III PI3K. Thus, treatment with 3-MA should only be considered under specific conditions such as limited treatment periods. Other types of autophagy inhibitors include LC3 knockdown by siRNA, which decreased breast cancer resistance to trastuzumab and increased cell death in CML in combination with imatinib[156, 218]. Chloroquine (CQ) and Hydroxychloroquine (HCQ) are the most successful autophagy inhibitors that suppress the autophagic lysosomal protease activity to promote the accumulation of autophagic vacuoles that often leads to apoptotic and necrotic cell death[219, 220]. Phase I and II clinical trials are ongoing using HCQ or CQ in combination with treatment such as docetaxel in prostate cancer, tamoxifen in breast cancer, and gemcitabine in pancreatic cancer[221].

Inhibiting autophagy poses another potential problem since anti-autophagic therapeutic drugs reduce tumor-specific immune response thereby limiting the therapeutic success[222]. Activated autophagy in glioblastoma cells treated with EGF toxin has been found to release HMGB1 that binds to and activates Toll-like receptor 4 (TLR4). Activated TLR4 increases T-cell mediated anti-tumor response to eliminate the malignant cells[223]. Deactivating autophagy decreases the release of HMGB1, leaves tumor cells unattended by the host

immune system, and results in increased resistance[224]. Although inhibiting autophagy is effective, researchers must take its adverse side effects into consideration.

## 11. Concluding remarks

As this chapter has outlined, chemo/radioresistance is a key contributor to decreased patient survival. In order to develop more effective cancer therapies and improve treatment outcome, more research is required to delineate this complicated biological mechanism. Furthermore, the ability of cancer cells to acquire heterogeneous genetic and epigenetic alterations across tumors elicits deregulation of cell death-associated signaling pathways in a variety of ways. Cancer cells are smart to quickly figure out ways to overcome a treatment that targets any particular cellular signaling pathway. Therefore, designing novel drugs and enhancing therapeutic strategies must simultaneously target multiple pathways and mechanisms. Using IAP antagonists that target multiple cell survival pathways, as well as BH3 mimetics that can overcome anti-apoptotic BCL-2 proteins to induce both apoptosis and autophagy-related cell death can improve survival and quality of life for cancer patients. The complexity of tumor biology and drug resistance suggests that we need to design treatment strategy based on the genetic/signaling profiles of the patient in order to provide the safest and most effective cancer therapies tailored to a particular patient, the ultimate goal of the personalized medicine.

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