REVIEW ARTICLE

Ubiquitin-proteasome pathway-mediated regulation of the Bcl-2 family: effects and therapeutic approaches

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Abstract

Proteasomal degradation of proteins represents an important regulatory mechanism in maintaining healthy homeostasis in cells. Deregulation of the ubiquitin-proteasome system is associated with various diseases as it controls protein abundance and turnover in cells. Furthermore, proteasomal regulation of protein turnover rate can determine a cell's response to external stimuli. The Bcl-2 family of proteins is an important group of proteins involved in mediating cell survival or cell death in response to external stimuli. Aberrant overexpression of anti-apoptotic proteins or deletion of pro-apoptotic proteins can lead to the development of cancer. Unsurprisingly, proteasomal degradation of Bcl-2 proteins also serves as an important factor regulating the level of Bcl-2 proteins and thereby affecting the functional outcome of cell death. This review aims to highlight the regulation of the Bcl-2 family of proteins with particular emphasis on proteasomal-mediated degradation pathways and the current literature on the therapeutic approaches targeting the proteasome system.

Introduction

Cancer is mostly a disease of aging, with mutations accumulating over time. The first step of cancer development, known as tumor initiation, is thought to be due to mutations leading to abnormal proliferation.[1] These mutations confer additional selective advantages to the cells, such as avoiding cell death, and those cells that have such mutations (including their progeny) will consequently become the bulk population. This clonal selection resembles the process of Darwinian evolution, with the caveat that it occurs microscopically instead of in the wild. With each clonal selection, the population will gain advanced neoplastic characteristics, ultimately forming a highly aggressive malignant tumor that threatens the patient.[2] In general, mutations that promote oncogenic traits and/or repress tumor-suppressive traits are highly favorable towards tumor progression. Such mutations may occur in the ubiquitination process which affects the stability of onco- and tumor-suppressive proteins. In this review, we discuss how the various elements of the ubiquitination process, more specifically the E3 ubiquitin ligases and deubiquitinating enzymes (DUB) affect Bcl-2 family proteins and their functions in regulating apoptosis.

Apoptosis and the Bcl-2 family of proteins

Physiologically, cell death and cell proliferation must be balanced to ensure normal cellular function. Dysregulated cell death may lead to disorders such as Parkinson disease, while dysregulated cell proliferation may lead to cancer. Therefore, a dynamic balance between cell death and cell proliferation is important and is highly regulated by anti-apoptotic and pro-apoptotic proteins such as Bcl-2 family proteins.

Bcl-2 family members are categorized into three distinct subsets, but all members contain one Bcl-2 homologous (BH) domain, which is the BH3 domain. The distinct subsets are the anti-apoptotic proteins, pro-apoptotic proteins, and BH3-only proteins. With regards to the anti-apoptotic Bcl-2 family proteins, they suppress apoptosis and are upregulated in cancer cells.[3,4] These proteins include Bcl-2, Bcl-xL, and Mcl-1. Besides their canonical anti-apoptotic function, these proteins can also exert non-canonical effects such as redox regulation, thus highlighting their multifunctional role in maintaining cellular homeostasis.[5] On the other hand, the pro-apoptotic proteins, namely Bax and Bak, serve to negate the effect of

the anti-apoptotic proteins of triggering apoptosis and are downregulated in cancer cells.[4] They are important in the permeabilization of the mitochondrial membrane, resulting in the release of cytochrome c. Lastly, BH3-only proteins, namely BIM, PUMA, and NOXA, are responsible for either (i) directly binding to anti-apoptotic proteins and inactivating them (also known as sensitizers), or (ii) directly activating mitochondrial outer membrane permeabilization (MOMP) by activating the pro-apoptotic proteins (also known as activators). Activators can also bind to anti-apoptotic proteins and trigger their inactivation. Therefore, activators are sensitizers that have an added role, which is to bind and activate pro-apoptotic proteins. A list of binding partners of Bcl-2 family proteins can be found in Table 1.

Mechanistically, apoptosis involves many protein interactions and signaling cascades, and is primarily divided into two pathways, the extrinsic and intrinsic pathways. The extrinsic pathway involves receptors and external ligands,[6] which are not be covered in this review. The intrinsic pathway promotes downstream pathways such as the release of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi from mitochondria into the cytosol to trigger caspase-dependent apoptosis.[7,8] Regardless, these pathways converge into the execution pathway, through which activated caspase 3 triggers downstream signaling cascades, leading to the degradation of chromosomal DNA, protease activation and formation of apoptotic bodies.[9,10]

Delving deeper into the intrinsic pathway, this pathway involves stimuli that directly target proteins or molecules within the cells. For example, radiation causes DNA damage, which in turn upregulates tumor suppressors such as p53.[11] p53 promotes transcription of pro-apoptotic proteins such as NOXA and PUMA.[12,13] NOXA can then bind directly to the anti-apoptotic Mcl-1 and displace the pro-apoptotic activator, Bak, allowing Bak to oligomerize with Bax to induce MOMP.[12,14] Besides acting on NOXA transcription, p53 has also been shown to activate Bax.[15] Subsequently, Bax can be inserted into the mitochondrial membrane to promote MOMP (this process is not p53-dependent), which induces cytochrome *c* release. As a result, an apoptosome complex can be formed with cytochrome *c*, APAF-1, and procaspase 9. This apoptosome complex then activates caspase 9 which subsequently activates caspase 3 and the downstream signaling cascade resulting in apoptosis. The dysregulation of these proteins can be detrimental, as observed in tumor samples.[16] Therefore, understanding the regulation of these proteins (summarized in Table 2), more specifically ubiquitination and deubiquitination (summarized in Figure 1), is important for future research and identification of potential therapeutic strategies for cancer.

Effect of ubiquitination on Bcl-2 family proteins

Mcl-1 (Myeloid cell leukemia sequence-1)

Mcl-1 is an anti-apoptotic protein and serves as a proto-oncogene. Canonically, Mcl-1 localizes to the outer mitochondrial membrane and exerts its function by

Table 1. Ligands of Bcl-2 family proteins.

Bcl-2 family protein	Ligand	Summary	Reference
Mcl-1	Bak	Mcl-1 displaces Bak from the outer mitochondrial membrane, preventing apoptosis.	14
	NOXA	NOXA enhances the MULE/Mcl-1 interaction which promotes Mcl-1 ubiquitination and degradation.	17, 18, 26, 27
	BIM	BIM/Mcl-1 complex stabilizes both Mcl-1 and BIM.	19
Bcl-2	Bak	Binding of Bcl-2 to Bak prevents Bax/Bak activation.	35
	BIM	Binding of BIM to Bcl-2 prevents Bcl-2 activation and promotes apoptosis.	36
	ARTS	ARTS binds to Bcl-2 and XIAP, promoting XIAP-dependent ubiquitination of Bcl-2.	46
BIM	Bax	BIM targets Bax for oligomerization and activation.	36
	Mcl-1	Reciprocal protection when BIM and Mcl-1 form a bi-complex.	19, 53
	PP2A	Dephosphorylation of BIM by PP2A reduces βTrCP binding to BIM for ubiquitination.	57
	Rsk1/2, Aurora A	Phosphorylation of BIM by Rsk1/2 and Aurora A promotes βTrCP binding and ubiquitination.	56, 57
Bax	Bcl-2	Bax binds to Bcl-2 to inhibit its activity.	39
	VDAC2	Responsible for Bax recruitment and activation to the mitochondrial membrane.	65
	Bcl-xL	Bcl-xL suppresses Bax activity through a direct interaction.	67

Table 2. Regulatory proteins of Bcl-2 family proteins.

Bcl-2 family protein	Regulatory proteins	Effect	Reference
Mcl-1	Fbw7	Direct ubiquitination and destabilization of Mcl-1 by Fbw7 is inhibited by GSK3β-dependent Mcl-1 phosphorylation.	22
	MULE	Direct ubiquitination and destabilization of Mcl-1 by MULE.	25
	USP9x	Deubiquitination of Mcl-1 by USP9x prolongs its half-life.	29
	Ku70	Deubiquitination and stabilization of Mcl-1 by Ku70 is dependent on the C-terminus of Ku70.	32
Bcl-2	PPARα	PPARα induces poly-ubiquitination on Bcl-2 and causes its degradation.	40
	Parkin	Parkin induces mono-ubiquitination on Bcl-2 and stabilizes Bcl-2.	41
	XIAP	XIAP expression positively correlates with Bcl-2 expression.	44
	USP1, USP8, USP 22	No direct interaction concluded. Downregulation of these USP caused downregulation of Bcl-2 expression.	47-50
BIM	βTrCP	Direct ubiquitination of BIM by βTrCP.	56, 57
	APC/Cdc20	Direct ubiquitination of BIM by APC/Cdc20.	58
	USP27x	Deubiqutination and stabilization of BIM by USP27x.	59
Bax	TRAIL	TRAIL promotes ubiquitination of Bax, leading to its activation.	60
	USP30	Deubiquitination and stabilization of Bax by USP30.	62
	Parkin	Direct ubiquitination and destabilization of Bax by Parkin.	63
	IBRDC2	Direct ubiquitination and destabilization of Bax by IBRDC2.	66

sequestering pro-apoptotic proteins such as Bak from the membrane.[14] This prevents the dimerization of Bax and Bak, thereby suppressing MOMP and apoptosis. NOXA has also been shown to bind and reduce Mcl-1 anti-apoptotic activity.[17,18] Short interfering (siRNA) knockdown of NOXA reduced camptothecin-induced cell death, possibly due to the increase in Mcl-1 upon campthotecin treatment.[17] By forming a complex with Mcl-1, NOXA destabilizes Mcl-1 by promoting Mcl-1 phosphorylation and ubiquitination.[18] BIM is another BH3-only protein that negatively regulates Mcl-1 activity. However, the BIM/Mcl-1 complex has also been shown to stabilize both BIM and Mcl-1.[19] Hence, the net result of such binding, pro- or anti-apoptotic, is dependent on the relative ratio and affinity of these proteins.

To date, many E3 ubiquitin ligases and DUB have been documented to regulate Mcl-1 stability, some of which are discussed here. Prominently, Fbw7 ubiquitinates many glycogen synthase kinase (GSK3β)-phosphorylated substrates, including Mcl-1.[20] GSK3β-dependent phosphorylation of Mcl-1 at both Ser159 and Thr163 of Mcl-1 is observed to promote its degradation.[21] Notably, inhibition of AKT (a negative regulator of GSK3β) through the small molecule API-1 resulted in increased Ser159 and Thr163 phosphorylation and prompted Fbw7-dependent degradation of Mcl-1.[22] Such findings suggest that phosphorylation at these sites is essential in promoting Mcl-1 stability. However, this might only be true in cells that are dependent on the GSK3β pathway. A study by Nifoussi *et al.* concluded that phospho-Thr163 resulted in increased Mcl-1 protein expression in cells that are not dependent on the GSK3β pathway, although no ubiquitin ligases were found to be involved.[23] Hence, it is logical to hypothesize that Fbw7 was not involved as these cell lines are not dependent on GSK3β. Instead of GSK3β, ERK was the kinase reported to be responsible for Thr163 phosphorylation of Mcl-1, with such phosphorylation being upregulated upon 12-O-tetradecanoylphorbol 13-acetate-induced ERK activation in cells[23] and downregulated in cells treated with the ERK inhibitor, PD98059.[24] Taken together, these findings suggest that GSK3β-mediated phosphorylation of Mcl-1 at both Ser159 and Thr163 is a target of Fbw7 for ubiquitination and degradation, while ERK-induced Thr163 phosphorylation of Mcl-1 enhances Mcl-1 stability. Nonetheless, more studies should be carried out to determine whether these events are independent or whether they interact with one another.

Another E3 ubiquitin ligase involved in Mcl-1 ubiquitination is Mcl-1 ubiquitin ligase E3 (MULE). MULE contains a well-conserved BH3 domain, which bind specifically to Mcl-1 but not to other Bcl-2 family protein members.[25] A synthetic MULE mutant (mutation in the MULE BH3 domain) prevented binding to Mcl-1 and thus downregulated Mcl-1 ubiquitination and its subsequent degradation.[25] Additionally, reduction of MULE expression through RNA interference stabilized Mcl-1 protein while impairing tetracycline-induced apoptosis.[25] Etoposide-induced degradation of Mcl-1 was abrogated by MULE deficiency, although basal Mcl-1 expression level was not affected by the lack of MULE in these cells.[25] These results suggest that there may be other E3 ubiquitin ligases concomitantly involved in the regulation of Mcl-1 stability. A report by Gomez-Bougie *et al.* described that NOXA enhances the MULE/Mcl-1 interaction, while promoting ubiquitination of

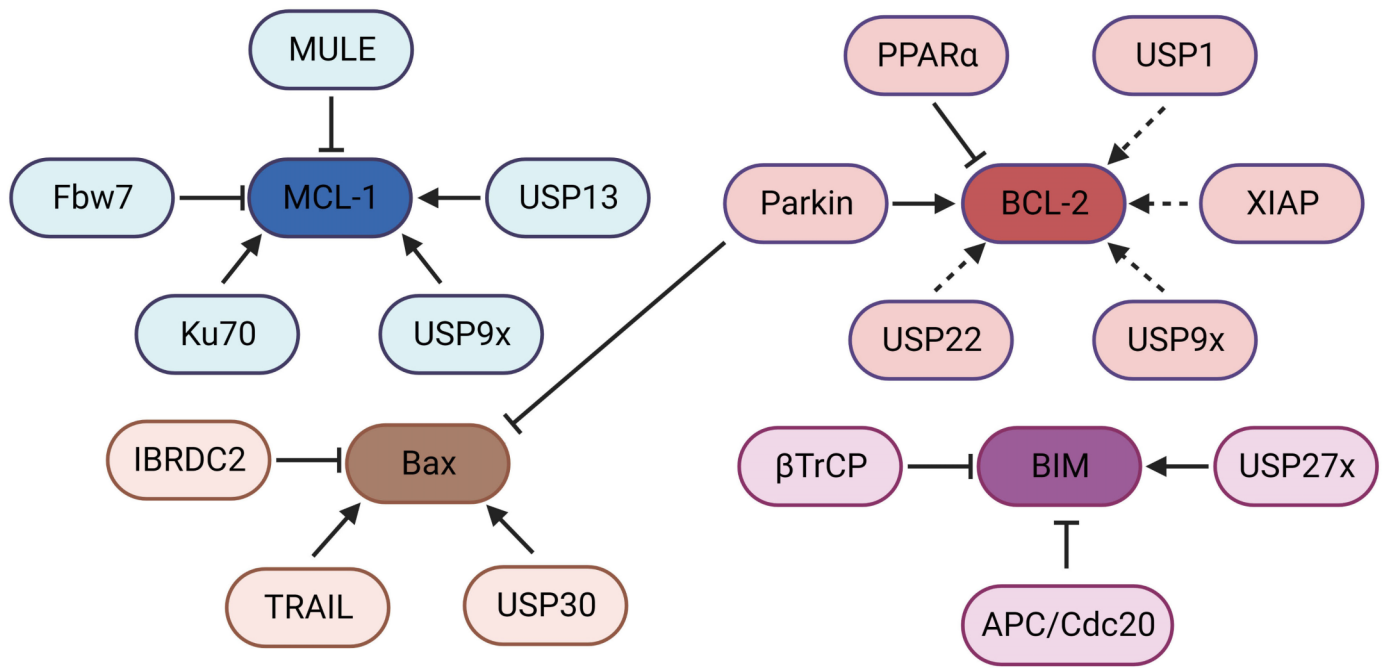

Figure 1. Schematic diagram of Bcl-2 ubiquitin ligases and deubiquitinases. Schematic diagram of the network of ubiquitin ligases and deubiquitinases that are involved in regulating the expression of Bcl-2 proteins (Mcl-1, Bcl-2, Bax and BIM). Solid lines indicate that a direct interaction was reported whereas dotted arrow lines indicate an association with the protein of interest (i.e., no direct binding was observed). Figure created using Biorender.com

Mcl-1, although no mechanism of action was described. Overexpression of NOXA was also observed to displace USP9x, a DUB that targets Mcl-1 for deubiquitination.[26,27] This is supported by the observation that siRNA-mediated knockdown of NOXA promotes USP9x availability to Mcl-1, leading to a lower rate of apoptosis.[28] Another study showed that phosphorylated Mcl-1 at several sites displaces USP9x.[29] Hence, it is possible that MULE- and USP9x-binding to Mcl-1 are phosphorylation-dependent, such that NOXA-mediated phosphorylation of Mcl-1 promotes the interaction with MULE, while suppressing USP9x binding.[18]

Deubiquitination is the reverse process of ubiquitination and is catalyzed by specific DUB. USP9x is one of the many DUB that deubiquitinate Mcl-1 to prolong its half-life. USP9x expression correlates positively with Mcl-1 in human follicular lymphoma and diffuse large B-cell lymphoma. In multiple myeloma patients, high expression of USP9x is associated with poor prognosis.[29] Hence the interaction between USP9x and Mcl-1 has prognostic relevance in these human malignancies. Like many DUB, USP9x stabilizes Mcl-1 through cleaving its K48-linked polyubiquitin chain that normally promotes degradation. Knockdown of USP9x increased Mcl-1 turnover but did not alter *Mcl-1* mRNA levels, suggesting that the USP9x modifies Mcl-1 post-translationally. The C1566 mutant (lacking DUB activity) of USP9x also failed to stabilize Mcl-1, which further suggests that Mcl-1 stability is dependent on USP9x DUB activity.[29] Similarly, the decrease in the Mcl-1/USP9x interaction through knockdown of USP9x[29] or WP1130 treatment,[30] a USP9x inhibitor, sensitized cells to ABT737-induced cell death.

Lupus Ku autoantigen p70 (Ku70) is a protein that has dual functions as a tumor suppressor. Ku70 is known as a DNA double-strand break repair protein that is important for non-homologous end joining recombination,[31] thus preventing apoptosis. At the same time, it serves as a Mcl-1-specific DUB, while having no effect on other Bcl-2 anti-apoptotic family proteins.[32] Structurally, Ku70 interacts with Mcl-1 through its C terminus, while carrying out its DSB repair via its N terminus.[32] With regards to the role of Ku70 in stabilizing Mcl-1, deletion of Ku70 caused an accumulation of K48-linked polyubiquitin chains on Mcl-1, together with a decrease in Mcl-1 expression and half-life. Overexpression of Ku70 in Ku70$^{-/-}$ cells rescued Mcl-1 expression.[32] As for USP9x, knockdown of Ku70 was reported to augment the effect of ABT737 on apoptosis,[32] and in a separate study, caused accumulated DNA damage and triggered the p53-induced apoptotic pathway.[33] Considering the role Ku70 plays in Mcl-1 stability and the DNA damage response, Ku70 inhibition appears to be a promising approach for therapy. However, the feasibility of inhibition of Ku70 as a therapeutic strategy remains to be determined as Ku70 fulfills many other cellular processes.

Bcl-2 (B-cell lymphoma-2)

Bcl-2, an anti-apoptotic protein, is highly expressed in tumor samples in various cancers, in which its expression correlates positively with poor prognosis.[34] Similarly to Mcl-1, Bcl-2 is reported to bind to Bak and prevent Bak and Bax allosteric activation.[35] Bcl-2 also interacts with BIM, thereby preventing Bcl-2 from exerting its anti-apoptotic function.[36] Interestingly, Bcl-2 has a relatively longer half-life as compared to Mcl-1, possibly due to fewer ubiquitin ligases regulating Bcl-2.[37,38] Hence, unlike Mcl-1, there is limited evidence of Bcl-2 ubiquitination, with contrasting

findings. It was first thought that stabilization of Bcl-2 was not dependent on the ubiquitin proteasome system, as treatment with the proteasome inhibitor, MG132, did not alter Bcl-2 levels.[39] However, some studies showed otherwise that ubiquitination of Bcl-2 has both stabilizing and destabilizing effects. For example, PPARα induces the transfer of polyubiquitin to Bcl-2 and causes its degradation,[40] while Parkin promotes mono-ubiquitination on Bcl-2 which stabilizes Bcl-2.[41] With regards to the former, PPARα was described to play an E3 ubiquitin ligase role by binding directly to Bcl-2 and transferring K48-linked polyubiquitin chains to the K22 site on Bcl-2, governing its stability.[40] Short hairpin (shRNA) silencing of PPARα resulted in the inhibition of Bcl-2 ubiquitination, while overexpression of PPARα augmented chemotherapeutic effects through decreased Bcl-2 levels.[40] Hence, it is evident that PPARα negatively regulates Bcl-2 protein stability. On the other hand, Parkin, an E3 ubiquitin ligase known to destabilize Mcl-1,[42,43] was reported to mono-ubiquitinate Bcl-2. Interestingly, such mono-ubiquitination was associated with Bcl-2 stability and increases in its steady-state levels.[41] This increase in Bcl-2 mediated by Parkin inhibits autophagy, as the conversion of LC3-II (a marker for autophagy) was reduced under both normal and starvation conditions.[41] Furthermore, it was found that mutation in the RING1 domain, but not the RING2 domain, of Parkin impairs mono-ubiquitination on Bcl-2, suggesting that the RING1 domain is important for the Parkin and Bcl-2 interaction.[41] This suggests a potential combinatorial therapy targeting the RING1 domain of Parkin to destabilize Bcl-2, alongside Bcl-2 inhibitors, such as ABT737, to inhibit autophagy and promote apoptosis. However, as mentioned above, Parkin also sensitizes cells to apoptosis by ubiquitinating and destabilizing Mcl-1,[42,43] indicating that Parkin can both stabilize and destabilize anti-apoptotic proteins under certain conditions. Hence, targeting Parkin for cancer therapy might not be as straightforward as it seems.

Like Parkin, XIAP is another E3 ubiquitin ligase. Its expression was observed to correlate positively with that of Bcl-2. In cells with XIAP knockdown, Bcl-2, and Bcl-xL expression decrease significantly, with an increase in efflux of cytochrome *c* from mitochondria into cytoplasm to trigger apoptosis.[44] Interestingly, Bax was not affected by such knockdown, further implying that XIAP possibly mediates apoptosis through Bcl-2 and Bcl-xL.[44] Another study delved deeper by performing a drug screen, followed by *in vivo* validations. It was concluded that XIAP and Bcl-2 are among the top five targets for combinatorial therapy in acute myeloid leukemia patients, and that inhibition of both Bcl-2 and XIAP eradicated established acute myeloid leukemia in *in vivo* patient-derived xenografts.[45] It would be intriguing to establish the mechanism behind the interaction between these two anti-apoptotic proteins, Bcl-2 and XIAP. Surprisingly, Edison *et al.* described a mechanism through which XIAP and Bcl-2 do interact, in a complex formed of ARTS, Bcl-2, and XIAP.[46] ARTS is a pro-apoptotic tumor suppressor, contrary to XIAP and Bcl-2. Edison *et al.* demonstrated that ARTS achieves its tumor suppressive role by binding to Bcl-2 through its BH3 domain, and recruits XIAP to ubiquitinate Bcl-2. This ubiquitination event at K17 of Bcl-2 promoted the destabilization of Bcl-2.[46] Consistently, the K17A mutant increased Bcl-2 stability and was able to protect cells from apoptosis.[46] This begs the questions of whether XIAP is a *bona fide* ubiquitin ligase for Bcl-2, or whether the binding of ARTS alters the conformation of either Bcl-2 or XIAP, causing an increase in affinity towards each other for Bcl-2 ubiquitination and destabilization. In either case, answering these questions will improve our understanding and facilitate the development of combinatorial therapy for cancer.

Canonically, DUB bind to and promote deubiquitination of their substrate proteins, ultimately promoting their stabilization. Such interactions require direct binding between the proteins. However, reported research on DUB regulating Bcl-2 expression did not investigate specific DUB and/or their interactions, either direct or indirect. The downregulation of USP1, USP8 and USP22 through their respective siRNA decreased Bcl-2 protein, as shown by western blot analysis.[47-50] Kuo *et al.* demonstrated that the non-specific DUB inhibitor, PR619, could downregulate Bcl-2 expression to enhance anti-tumor effects and augment cisplatin cytotoxicity.[51] Furthermore, Xu *et al.* observed that transfection of si*USP1* decreased *Bcl-2* mRNA levels as well, which might explain the results observed in the western blot analysis.[52] Hence, future research should include ubiquitination and/or co-immunoprecipitation assays to investigate the interaction between DUB and Bcl-2.

BIM (Bcl-2 interacting mediator of cell death)

Besides negating the anti-apoptotic effects of anti-apoptotic Bcl-2 family proteins, BIM also activates Bax by facilitating Bax oligomerization.[36] Knockdown of BIM was observed to attenuate the ability of Bax to trigger MOMP, suggesting that BIM plays an 'activator' role in triggering apoptosis.[36] Interestingly, the BIM and Mcl-1 interaction provides reciprocal protection and potentially balances out their apoptotic effects.[19,53] In other words, BIM binding to Mcl-1 prevents the recruitment of ubiquitin ligases to both of these proteins. Disruption of such binding through a mutation in the BH3 domain of BIM was reported to cause an increase in the turnover rates of both proteins.[54] Interestingly, ERK1/2-dependent phosphorylation of BIM at Ser65 (for mice)[54] and Ser69 (for humans)[55,56] also disrupted the BIM-Mcl-1 interaction and promoted the dissociation of BIM. Therefore, BIM becomes a target for ubiquitination and its subsequent destabilization. BIM phosphorylation at Ser69, regulated by ERK1/2, is important for its subsequent phosphorylation at Ser93, Ser94,

and Ser98 by kinases such as Rsk1/2 and Aurora A.[56,57] Inhibition of Rsk or Aurora A reduced phosphorylation of BIM on Ser93, Ser94 and Ser98. These phosphorylation sites are negatively regulated by phosphatases such as protein phosphatase 2A (PP2A).[57] Such phosphorylation events are important for the binding of BIM to beta-transducin repeat containing E3 ubiquitin protein ligase (βTrCP) for βTrCP-mediated degradation of BIM.[56,57] An inhibitor of MEK, UO126, strongly reversed phorbol 12-myristate 13-acetate (PMA)-induced ERK activation and reduced binding of BIM to βTrCP.[56] Phosphorylation at Ser93, Ser94, and Ser98 was consequently reduced as well. Consistently, an alanine mutation at these sites drastically reduced binding to βTrCP,[57] while siβTrCP,[56] mutant βTrCP (delta Fbox),[56] and MLN8054, an Aurora A inhibitor,[57] failed to induce *in vitro* ubiquitination on BIM, resulting in BIM stabilization. Taken together, the investigations showed that ERK1/2-mediated phosphorylation of BIM at Ser69 creates a binding site for Rsk1/2 and Aurora A, which in turn phosphorylate BIM at Ser93, Ser94, and Ser98 to allow βTrCP binding and ubiquitination of BIM.

Besides βTrCP, APC/Cdc20 has also been linked to the ubiquitination of BIM. Wan *et al.* revealed that the E3 ubiquitin ligase responsible is cell-cycle dependent, with βTrCP regulating BIM during the S phase, and APC/Cdc20 regulating BIM during the M phase.[58] Consistently, the level of BIM was reduced in the late S phase, even when APC/Cdc20 activity was low, and such downregulation of BIM expression could be reversed with βTrCP depletion. In contrast, BIM expression was upregulated upon depletion or knockdown of Cdc20. This regulation was specific to BIM and no other BH3-only proteins.[58] Since βTrCP and APC/Cdc20 can directly regulate BIM ubiquitination, combinatorial therapies might emerge from these studies to sensitize tumour cells to apoptotic cell death.

In opposition to ubiquitin ligases, DUB such as USP27x, stabilize BIM by removing ubiquitin added by ubiquitin ligases, specifically K48- and K63-linked ubiquitin chains.[59] Overexpression of USP27x reduced ERK-dependent BIM phosphorylation and ubiquitination in PMA-stimulated cells as well as tumor cells with a constitutively active Raf/ERK pathway. Binding of USP27x to BIM was dependent on the 42-101 amino acid region on BIM, as mutation of these sites on BIM disrupted binding to USP27x.[59] Interestingly, binding of USP27x and βTrCP to BIM is independent, as knockdown of βTrCP did not reduce the association of BIM with USP27x. Concomitantly, phosphorylation of BIM at Ser69 is required for USP27x binding,[59] which is similar to βTrCP although some further research established the need for Ser93, Ser94 and Ser98 on BIM to be phosphorylated as well.[56,57] Nevertheless, it is possible that both USP27x and βTrCP are simultaneously recruited by BIM upon its phosphorylation to regulate its stability.

Bax (BCL2-associated X, apoptosis regulator)

Bax, a member of the pro-apoptotic Bcl-2 family of proteins, oligomerizes and promotes cytochrome *c* release from mitochondria, resulting in apoptosis. Degradation of Bax prevents cytochrome *c* release and is widely observed in cancer cells.[39] Inhibition of proteasome through MG132 treatment increased the accumulation of ubiquitinated Bax without altering its mRNA levels.[39] Moreover, inhibition of proteasome in Bcl-2-overexpressing cells led to accumulation of Bax thus allowing Bax to inhibit Bcl-2 activity.[39] Additionally, Peng *et al.* demonstrated that K21 and K123 of Bax are required for ubiquitin binding on Bax, where K21R and K123R mutations have reduced ubiquitinated Bax levels with prolonged half-lives.[60] Interestingly, Bax ubiquitination was observed to activate Bax as well.[60,61] Previous studies demonstrated that the TNF-related apoptosis-inducing ligand (TRAIL) cytokine can activate Bax while promoting its degradation in malignant B cells.[61] Consistently, K21R and K123R mutants decreased ubiquitination of Bax, with decreased pro-apoptotic activity as compared to wild-type Bax upon TRAIL treatment.[60] This is concordant with the hypothesis that ubiquitination is required for Bax activation, given that overexpression of USP30, a DUB that binds directly to Bax, was observed to negatively regulate Bax-mediated apoptosis.[62] Overexpression of USP30 alleviated Poly ADP-ribose polymerase (PARP) cleavage and apoptosis, whereas siRNA depletion of USP30 increased PARP cleavage and sensitized cells to drug-induced apoptosis.[62] Treatment with an USP30 inhibitor, aumdubin, led to higher levels of ubiquitinated Bax, and Bax-mediated apoptosis, possibly due to ubiquitination on Bax, enhances its mitochondrial localization and activation.[62] Collectively, these findings suggest that Bax activation is dependent on ubiquitination of the protein, and can be targeted for degradation after activation.

Parkin is another E3 ubiquitin ligase reported to interact with Bax. It was shown to destabilize and inhibit Bax activity by binding directly to the BH3 domain on Bax.[63] Binding of Parkin to Bax promoted ubiquitination and subsequent degradation on Bax. Parkin-deficient cells had lower ubiquitinated Bax as compared to wild-type cells, consistent with the notion that Parkin is the ubiquitin ligase responsible for Bax ubiquitination.[64] Moreover, activated Parkin promoted the dissociation of Bax from mitochondria.[63,65] Interestingly, K21 and K64 of Bax are critical for Parkin-dependent regulation of Bax activity, and K21R and K64R mutants increased Bax mitochondrial translocation without affecting the Parkin-Bax physical interaction.[63] Double mutant K21R/K63R had the highest rate of mitochondrial translocation as compared to individual K21R and K63R mutants and wild-type Bax, suggesting that these mutations are additive.[63] Nevertheless, this suggests that K21 and K64 on Bax are important for Parkin-dependent translocation of Bax away from mitochondria. On the other hand, Parkinson disease-linked Parkin mutations R275W and W453X both re-

sulted in loss of function of Parkin. Both mutants co-immunoprecipitated with Bax and were observed to have higher affinity with Bax than with wild-type Parkin.[64] These mutants failed to inhibit Bax translocation to mitochondria, which resulted in apoptosis and cell death, one of the hallmarks of Parkinson disease.[64] In addition, Parkin also negatively regulates Bax activity without ubiquitinating Bax, as suppression of Bax activity by Parkin was recorded in the absence of Bax ubiquitination.[65] Mechanistically, it was found that Parkin promoted the destabilization of VDAC2, a known Bax activator, potentially altering the conformation of VDAC2 and preventing Bax activation. These reports suggest a dual role of Parkin in regulating Bax activity by both (i) directly ubiquitinating and destabilizing Bax, and (ii) promoting the degradation of Bax activator.

IBR-type RING-finger E3 ubiquitin ligase, IBRDC2, is another E3 ubiquitin ligase that was shown to protect cells from unregulated Bax activation and cell death. A direct interaction between IBRDC2 and Bax was documented by Benard and colleagues and was described to destabilize Bax, but not other Bcl-2 family proteins including Bak, through ubiquitination.[66] Downregulation of IBRDC2 through shRNA induced accumulation of active Bax, while IBRDC2 overexpression stimulated Bax ubiquitination. Interestingly, IBRDC2, usually localized in the cytosol, accumulated in mitochondria alongside activated Bax upon induction of apoptosis by staurosporine and actinomycin D, suggesting that IBRDC2 localization to Bax on mitochondria is dependent on Bax activation.[66] Bcl-xL, which suppresses Bax activation,[67] was also shown to inhibit mitochondrial accumulation of IBRDC2, supporting such a hypothesis.[66] Taken together, loss of IBRDC2 failed to promote Bax ubiquitination and ultimately sensitizes cells to Bax-mediated MOMP and cell death.

Targeting Bcl-2 family proteins through regulating ubiquitination

Ubiquitination of tumour suppressors and proto-oncoproteins is important in regulating their stability and function. As a result, these ubiquitinated proteins are often targeted by the ubiquitin proteasome system for their degradation. Hence, it is not surprising that researchers have investigated inhibiting the ubiquitin proteasome system to prevent the degradation of tumor suppressors as an anti-cancer strategy (summarized in Table 3). Bortezomib, a reversible inhibitor of 26S proteasome, is the first proteasome inhibitor approved by the Food and Drug Administration to treat multiple myeloma patients.[68] Mechanistically, bortezomib was shown to inhibit the NFκB signaling pathway by preventing the degradation of IκB, a negative regulator of NFκB.[69] Normally, upon activation of the NFκB signaling pathway, IκB is phosphorylated by IKK and such phosphorylation primes IκB for its ubiquitination and subsequent degradation. IκB dissociates from NFκB complexes, releasing its inhibitory effect. Hence, downstream target genes of NFκB, such as the anti-apoptotic genes XIAP, Bcl-2, and Bcl-xL, can be transcribed.[70-72] As a result, upon bortezomib treatment, IκB is thought to be protected from the ubiquitin proteasome system, allowing IκB to exert its inhibitory effect on NFκB and attenuate the aforementioned anti-apoptotic gene expression. Another possible mechanism to promote apoptosis employed by bortezomib is through Myc-dependent upregulation of *NOXA* gene expression.[73] Such upregulation mediates the downregulation of the anti-apoptotic activity of Bcl-2 and Bcl-xL proteins through direct binding of NOXA. Consistently, RNA interference of *Myc* blocks NOXA induction by bortezomib in tumor cells.[73] However, because of the effect of bortezomib on general proteasome inhibition, researchers have also investigated targeting the upstream pathways, such as DUB and ubiquitin ligases. In theory, this direct method might produce fewer side effects and toxicities because of it higher specificity for its target protein, such as inhibiting DUB that stabilize oncogenes or ubiquitin ligases that destabilize tumor suppressors. Recruiting oncogenes for ubiquitination through proteolysis-targeting chimeras (PROTAC) is another viable option, and examples are discussed below.

Table 3. Inhibitors targeting the ubiquitin proteasome system.

Drug	Summary	Reference
Bortezomib	Proteasome inhibitor targeting the ubiquitin proteasome system.	68, 83
Pimozide, GW7647, ML323	USP1-specific inhibitors.	74, 75
KSQ4279	USP1 inhibitor currently in a phase I study, proposed to eliminate resistance tp PARP inhibitors.	76
WP1130	Non-selective inhibitor of USP5, USP9x, USP14, USP24 and UCH37.	77, 78
DT2216	A dual-inhibitor PROTAC targeting Bcl-xL and Bcl-2.	80
753b	Improved version of DT2216.	81
XZ424	PROTAC specifically targeting Bcl-xL for ubiquitination and degradation.	82

USP: ubiquitin-specific peptidase; PARP: poly ADP-ribose polymerase; UCH: ubiquitin C-terminal hydrolase; PROTAC: proteolysis-targeting chimera.

With regard to DUB inhibitors, pimozide, GW7647 and ML323 are USP1 inhibitors that selectively bind to USP1.[74,75] Pimozide and GW7647 were identified by Chen and colleagues to be USP1/UAF1 inhibitors, with both inhibitors binding reversibly to the allosteric site instead of its active site.[75] Consistently, treatment of these inhibitors caused the accumulation of ubiquitinated substrates downstream of USP1, such as PCNA and FANCD2, thus regulating the DNA damage response. In addition, the accumulation of ubiquitinated substrates sensitized cells to cisplatin treatment.[75] However, these USP1 inhibitors can also bind to and inhibit USP7, albeit with approximately 24-fold higher IC_{50} for USP7.[75] Hence, the use of these inhibitors are limited by off-target specificity. These limitations led to the development of ML323, which is highly selective towards USP1 and does not have any activity against other ubiquitin-specific peptidases.[74] ML323 also augmented cisplatin sensitivity in tumor samples and was shown to increase endogenous mono-ubiquitination of PCNA and FANDC2, similarly to pimozide and GW7647.[74] Recently, another highly selective inhibitor of USP1, KSQ4279 was discovered by KSQ Therapeutics.[76] KSQ4279 is currently being tested in a phase I clinical trial as a monotherapy or in combination with an oral PARP inhibitor in patients with advanced solid tumors. This small molecule inhibitor (i.e., KSQ4279) serves to eliminate resistance to PARP inhibitors in cases in which resistance is common in tumors with homologous recombination repair deficiencies, such as BRCA1. Unfortunately, not much research has been performed on the effect of USP1 inhibitors on other USP1 target genes such as Bcl-2 family proteins. Since USP1 may potentially regulate Bcl-2, it would be interesting to investigate whether Bcl-2 plays a role in regulating apoptosis induced by the USP1 inhibitors. WP1130, a cell-permeable DUB inhibitor, is known to upregulate pro-apoptotic proteins while suppressing anti-apoptotic proteins, such as p53 and Mcl-1. However, WP1130 is not selective towards USP9x but can also inhibit USP5, USP14, USP24 and UCH37.[77,78] These DUB are known to regulate survival pathways and 26S proteasome function. Upon WP1130 treatment, p53 and Mcl-1 were observed to be up- and down-regulated respectively.[77] Interestingly, it was revealed that the upregulation of p53 was dependent on USP5 inhibition, while the downregulation of Mcl-1 was dependent on USP9x inhibition.[77] However, the downregulation of Mcl-1 was also concluded to be dependent on inhibition of USP24 in different cell lines.[78] These contradictory results suggest that the effect of WP1130 on Mcl-1 might not be solely dependent on one DUB. At the same time, the exact mechanism by which WP1130 achieves its inhibitory effect on these DUB is still unknown. It is possible that WP1130 targets the electrophilic cysteine residue on the active site and does not recognize specific conformations of the various DUB, which may explain the ability of WP1130 to target multiple DUB.

More progress has been made recently in the development of targeted anti-cancer therapies. PROTAC have emerged as adapter proteins to bring two proteins together. PROTAC have two binding regions, with one end holding/inhibiting the disease-causing protein, also known as the protein of interest, and the other end recruiting the E3 ubiquitin ligase.[79] These two binding regions are joined together by a linker peptide. The purpose of PROTAC is to target the protein of interest for degradation instead of solely inhibiting it, unlike many small molecule inhibitors. This allows PROTAC to overcome resistance caused by mutation of the substrates, which reduces the effectiveness of small molecule inhibitors. In the context of Bcl-2 family proteins, one example of an effective PROTAC is DT2216. DT2216 consists of ABT263, a dual inhibitor of Bcl-xL and Bcl-2, a linker peptide, and a von Hippel-Lindau (VHL) ligand.[80] The use of ABT263 allows the recruitment of both Bcl-xL and Bcl-2 to DT2216 and their inactivation. At the same time, VHL, an E3 ubiquitin ligase, is recruited to DT2216 through its ligand. The Bcl-xL and Bcl-2 that are recruited to DT2216 are targeted for ubiquitination by VHL and are degraded by the ubiquitin proteasome system.[80] DT2216 was documented to bind to both Bcl-xL and Bcl-2 with strong affinities. However, only Bcl-xL was potently targeted for degradation, possibly because of the lack of accessible lysine residues on Bcl-2 upon binding to DT2216.[80] Therefore, 753b was developed to ensure that both Bcl-2 and Bcl-xL can be targeted for degradation.[81] The only difference between DT2216 and 753b is the linker site on ABT263. This alteration allows both proteins of interest to be recruited for ubiquitination by VHL and subsequently degraded by the proteasome.[81] It was concluded that 753b was more effective in inducing apoptosis in acute myeloid leukemia cells than DT2216 while resulting in low toxicity similar to DT2216. This low toxicity of DT2216 and 753b is due to the low level of VHL ligase present in human platelets, which limits the degradation of the protein of interest in these cells.[80,81] In addition to DT2216 and 753b, XZ424 is another PROTAC that is intended to target Bcl-xL for ubiquitination. XZ424 utilizes A-1155463, another Bcl-xL inhibitor with the Cereblon E3 ubiquitin ligase ligand, pomalidomide.[82] The introduction of PROTAC as anti-cancer therapeutics is relatively recent, but these drugs have incredible potential.

Concluding remarks

Ubiquitination plays an essential role in maintaining cellular homeostasis and deregulation of this cellular process can lead to cancer progression. Notably, deregulation of the Bcl-2 family of proteins is associated with cancer progression. In line with this, while not all Bcl-2 proteins are susceptible to ubiquitin-mediated degradation, this review has highlighted the available data on ubiquitin-mediated degrada-

tion of Bcl-2 proteins such as Mcl-1, Bcl-2, Bim and Bax. Advancements in understanding the regulation of these proteins would certainly lead to improved therapeutic options, but there are still many nuances in the regulation of these proteins which require further investigations.

Disclosures

No conflicts of interest to disclose.

Contributions

SP conceptualized the work. GLQT wrote the first draft of the manuscript and generated the tables and figures. JXHL and SP reviewed, edited, and revised the manuscript.

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