Rational Strategies for Combining Bcl-2 Inhibition with Targeted Drugs for Anti-Tumor Synergy

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ABSTRACT

Based on the well-established importance of dysregulated apoptosis as a hallmark of cancer, there has been robust interest in development of targeted drugs to promote cancer cell apoptosis. A promising target for promoting apoptosis is the Bcl-2 family of proteins. Bcl-2 family proteins are crucial in maintaining balance between cell survival and death through regulation of apoptotic signaling pathways via pro-survival and pro-apoptotic family members. To date, there has been limited efficacy with Bcl-2 inhibition alone in clinical development with benefit restricted to hematologic malignancies. However, combination approaches to inhibition of Bcl-2 and other oncogenic signaling pathways have provided evidence for potential anti-tumor synergy. We review herein the current evidence for targeting Bcl-2 family proteins as a cancer therapeutic strategy across both hematologic and solid organ malignancies.

Introduction

B-cell lymphoma 2 (Bcl-2) family proteins are an integral player in intrinsic cellular apoptosis. The extrinsic apoptotic pathway is mediated by cell surface receptors, while intrinsic apoptosis involves mitochondria. The Bcl-2 family balance of cell survival and death regulates mitochondrial apoptotic signaling pathways which influence mitochondrial permeability. This family of proteins contain Bcl-2 homology (BH) domains that are imperative for heterodimeric interactions between Bcl-2 family members1.

Bcl-2 family members are categorized into three groups: pro-survival Bcl-2 proteins, death effector proteins, and pro-apoptotic BH3-only proteins. Pro-survival, anti-apoptotic members include Bcl-2, Bcl-xL, Bcl-w, Bcl-b, Mcl-1, and Bcl-2A1 and share four regions of sequence homology (BH1-4)2,3. Overexpression of any of the pro-survival members leads to cellular protection against apoptosis, even in the presence of cytotoxic stimuli4. Death effector proteins which lack the BH4 domain include Bax, Bak, and Bok. These are sequestered by pro-survival Bcl-2 proteins under normal conditions. Pro-apoptotic BH3-only proteins include BIM, BIK, PUMA, BAD, NOXA, and BID; they remain inactive until a cytotoxic stimulus5. When a stimulus occurs, BH3-only proteins insert the BH3 domains into the BH3-binding grooves of the pro-survival Bcl-2 proteins causing release of Bax and Bak. These death effector proteins induce pore formation in the mitochondrial outer membrane which releases second mitochondria-derived activator of caspase (Smac, Diablo) and cytochrome c. This event activates the caspase cascade to ultimately trigger apoptosis5,6. One mechanism of apoptotic escape and tumorigenesis is through the overexpression of pro-survival Bcl-2 family members5,6.
Role of Bcl-2 in tumorigenesis

Overexpression of Bcl-2 has been classically demonstrated in B-cell and follicular lymphomas via a translocation between chromosome 14 and 18 leading to a fused immunoglobulin heavy chain and Bcl-2 loci. Bcl-2 expression has been suggested to have prognostic value in solid organ malignancies. In prostate cancer, Bcl-2 expression has been correlated to castration resistance and disease progression. In breast cancer, Bcl-2 positivity on expression has been correlated to 10-year overall survival. Expression of Bcl-2 is regulated by methylation and NF-kB of the promotor region, with hypomethylation having been demonstrated in lymphoproliferative neoplasms.

Pre-clinical models of have suggested that overexpression of Bcl-2 may be more permissive than causative in tumorigenesis. Transgenic mice with t(14;18) were initially observed to express a lower grade indolent lymphoma; however, after a prolonged latency period, disease often progressed to aggressive variants associated with additional genetic abnormalities such as c-myc rearrangement. Similar findings of prolonged latency but eventual development of aggressive B-cell lymphomas have been seen with Mcl-1 transgenic mice. Bcl-2 family proteins are additionally implicated in actively promoting tumor survival, as Mcl-1 is known to be critically important in survival of multiple myeloma cell lines. Though t(14;18) is not seen outside of hematologic malignancies, Bcl-2 overexpression is seen in other cancers including solid organ malignancies.

Similarly to overexpression of Bcl-2, suppression of pro-apoptotic genes leads to a permissive rather than causative effects on tumorigenesis. TP53 is a well-known tumor suppressor gene that is also implicated in regulation of apoptosis. Cytotoxic stimuli typically upregulate TP53 to induce apoptosis; however, this regulatory pathway is often lost in malignancies with mutated TP53. PUMA, or p53 upregulated mediator of apoptosis, is a BH3-only pro-apoptotic protein that is a downstream effector of p53-mediated apoptosis. PUMA expression has been implicated in the progression of malignant melanoma, with lower expression corresponding to more advanced disease and worse prognosis.

Targeting Bcl-2 for cancer therapy

Strategies for targeting Bcl-2-associated pathways in cancer include antisense oligonucleotides which neutralizes gene expression at the mRNA level and BH3 mimetics to promote apoptosis. The latter category typically includes small molecular inhibitors against anti-apoptotic proteins.

Antisense oligonucleotides such as oblimersen have been evaluated in chronic lymphocytic leukemia (CLL) up through phase III clinical trials either as monotherapy or in combination with chemotherapy. Modest responses were seen with monotherapy and improved upon with various combinations of fludarabine, cyclophosphamide, and rituximab. Unfortunately, toxicity even in monotherapy limits its use with the most common adverse events being infusion reactions with or without tumor lysis, fever, fatigue, cytopenias, cough, and hypotension.

Peptide sequences mimicking BH3-only proteins have been developed and found to bind to Bcl-2 with higher affinity than pro-apoptotic proteins, affecting their interaction. Those pro-apoptotic proteins then homogenize, triggering apoptotic cascades with mitochondrial pore formation. One of these peptides was derived from the BH3 domain on BAX, inhibiting the BAX-Bcl-2 formation. Unfortunately, these proteins were limited in scope due to degradation by cellular proteases and poor cell permeability and metabolic instability. Several studies have tried to overcome the instability of the protein by methods such as adding hydrocarbons to form a stable alpha-helix of BLC2 domains. This addition was shown to inhibit growth of leukemia xenografts, but these molecules have not progressed to clinical trials.

The third and most successful strategy in drug development has been the development of Bcl-2 small molecule inhibitors. As with the peptides, these inhibitors have a high affinity to pro-survival proteins like Bcl-2, Bcl-xl, and Bcl-w. Thus, BH3 mimicking small molecule inhibitors work by blocking the Bcl-2 and BAK/BAX interaction. Homology modeling predicted Bcl-2 protein structure by employing the Bcl-XL in complex with Bak BH3 peptide as a template. This method led to the development of HA14-1, the first small molecule Bcl-2 inhibitor developed by computer-based modeling. Since then, many other Bcl-2 inhibitors, including gossypol and ABT-199, have been developed.

BH3 mimetics as mono-therapy

As mentioned above, small molecular inhibitors have had to the most success with anti-cancer effects in clinical trials. Gossypol is a naturally occurring polyphenolic compound found in cotton plants and had shown to inhibit apoptosis in mouse models. It acts as a pan-Bcl-2 inhibitor and comes in two enantiomers; the negative enantiomer, referred to as AT-101, was found to be more cytotoxic via Bcl-2 inhibition, reactive oxygen species (ROS) generation and DNA cleavage. It was shown to be efficacious in a wide variety of tumor types including breast, prostate, and non-small cell lung cancer (NSCLC). Unfortunately, this benefit did not extend to phase II clinical trials. Low binding affinity and toxic side effects limited this option.

Obatoclax is another pan-Bcl-2 inhibitor that has not demonstrated efficacy in phase I clinical trials for hematologic malignancies and is associated with neurologic
ABT-199 with tamoxifen showed similar cell viability and seen with tamoxifen therapy, the selective Bcl-2 inhibitor with tamoxifen therapy. Despite higher levels of Bcl-xl also had higher levels of cleaved caspase-3 and combinatorial therapy mitigated the Bcl-2 and BIM upregulation seen ultimately converges with the PI3K/AKT/mTOR pathway on Bcl-2-mediated apoptosis to suppress the pro-apoptotic BH3 protein BIM. MEK inhibitors increase BIM expression but its apoptotic activity can be inhibited by Mcl-1 and Bcl-xl. Therefore, combined MEK and Mcl-1 inhibition is a promising strategy that has been examined in in vitro and in vivo experiments.

Combination with MEK inhibitors in KRAS-mutated NSCLC

KRAS is a frequent driver mutation in multiple solid organ malignancies including non-small cell lung cancer. KRAS exerts oncogenic effects via the MAPK pathway (also known as the RAS/RAF/MEK/ERK pathway) which ultimately converges with the PI3K/AKT pathway on Bcl-2-mediated apoptosis to suppress the pro-apoptotic BH3 protein BIM. MEK inhibitors increase BIM expression but its apoptotic activity can be inhibited by Mcl-1 and Bcl-xl. Therefore, combined MEK and Mcl-1 inhibition is a promising strategy that has been examined in in vitro and in vivo experiments.

Combination with Endocrine Therapy in Estrogen Receptor (ER)-positive Breast Cancer

The protein product Bcl-2 is overexpressed in 85% of cases of ER-positive early breast cancers and nearly 75% of cases in all early breast cancers. Pre-clinical studies examining Bcl-2 inhibition with endocrine therapy have shown promise. ER-positive xenograft mouse models were treated with either ABT-737 alone, tamoxifen alone, or combined ABT-737 and tamoxifen. ABT-737 alone had minimal effect but combined therapy demonstrated slowed tumor progression and complete response in a subset of xenografts. The mechanism of synergy was attributed to increased apoptosis – responsive xenografts had higher levels of cleaved caspase-3 and combinatorial therapy mitigated the Bcl-2 and BIM upregulation seen with tamoxifen therapy. Despite higher levels of Bcl-xl also seen with tamoxifen therapy, the selective Bcl-2 inhibitor ABT-199 with tamoxifen showed similar cell viability and tumor growth delay compared to ABT-737 with tamoxifen in ER-positive cell lines and xenografts. Thrombocytopenia was seen more in ABT-737 than ABT-199 therapy owing to Bcl-xl sparing in the latter. Resistant xenograft models to combined tamoxifen and Bcl-2 inhibition demonstrated higher pAKT levels suggesting a role for the PI3K/AKT/mTOR pathway. Similar to tamoxifen, mTOR inhibition upregulated Bcl-2 expression. Cell lines treated with ABT-737 or ABT-199 and a PI3K/mTOR inhibitor AZD8055 resulted in synergistic, caspase-dependent cell death. Triple therapy of Bcl-2 inhibition, PI3K/mTOR inhibition, and tamoxifen in xenografts demonstrated synergistic reduction of tumor growth and prolonged survival. Interestingly, ABT-737 reduced endometrial thickness whereas ABT-199 did not, suggesting a role of Bcl-xl in tamoxifen-induced endometrial hyperplasia. This is an important finding given known pro-estrogenic side effects of tamoxifen on the endometrium with tamoxifen monotherapy.

A phase Ib dose escalation study of 33 patients has examined the role of Bcl-2 inhibitor ABT-199 (venetoclax) combined with tamoxifen in ER-positive, Bcl-2-positive metastatic breast cancer. Venetoclax and tamoxifen therapy resulted in a clinical benefit rate of 70% time to objective response of 12 weeks, and median duration of response 42 weeks. The expansion phase recruited 24 patients and resulted in a clinical benefit rate of 75% with an objective response rate (ORR) of 54%. Correlatives included measurement of circulating tumor DNA (ctDNA) at baseline and during therapy, with serial ctDNA measurements corresponding to clinical response. At baseline, PIK3CA (42%) and ESR1 (30%) were the most commonly detected abnormalities. Although no particular mutations seemed predictive of this treatment strategy, response was present in patients with an ESR1 mutation - a mutation which is an important mechanism for endocrine resistance. The most common adverse events included grade 1-2 nausea (67%) and cytopenias, predominantly lymphopenia (88%; grade ≥3 30%).

Combimatrix approaches to BH3 mimetic therapy

Exploiting apoptosis through Bcl-2 family proteins with other modalities of systemic therapy is an active area of research across a wide variety of malignancies. Other oncogenic signaling pathways such as MEK-ERK, PI3K-AKT, BRAF, KRAS, and EGFR have been associated with the Bcl-2-mediated apoptotic cascade, thus combinatorial approaches are an attractive strategy to augment responses to Bcl-2 family-directed therapies.

Venetoclax (ABT-199) is a selective Bcl-2 inhibitor without activity against Bcl-xl and Bcl-w. By sparing Bcl-xL, venetoclax is thought to cause less thrombocytopenia compared to navitoclax. A phase I clinical trial of venetoclax monotherapy proved efficacy in relapsed/refractory CLL with a 79% response rate, 20% complete remission rate, and 5% minimal residual disease-negative rate along with a 15-month progression-free survival of 69% in patients receiving 400 mg daily. This led to FDA approval of venetoclax for CLL as single agent for relapsed CLL in April 2016.

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Combinations of the MEK inhibitor trametinib with either the Mcl-1 inhibitor AM8621, navitoclax, or Bcl-xl inhibitor A1331852 have been shown to decrease cell viability. Venetoclax, however, did not demonstrate this level of synergy suspected due to its Bcl-2 selectivity. Therefore, exploiting pro-apoptotic pathways with MEK inhibition likely depends on Mcl-1 and Bcl-xl, and not Bcl-2. Sensitivity to MEK and Mcl-1 or Bcl-xl inhibition in cell lines was not clearly dependent on baseline levels of Mcl-1 or Bcl-xl, respectively. Patient-derived xenografts of KRAS-mutated NSCLC models treated with trametinib and Mcl-1 inhibitors showed greater tumor response than trametinib alone or trametinib with navitoclax. Interestingly, when KRAS-mutated cell lines were treated first with a Bcl-xl inhibitor, this increased BIM-Mcl-1 binding while decreasing levels of Bcl-xl. Sequential treatment using a Bcl-xl inhibitor and then adding Mcl-1 and MEK inhibition yielded reduced cell viability and enhanced tumor regression of xenografts when compared to non-pre-treated specimens. By targeting Bcl-xl first, tumor survival becomes increasingly dependent on Mcl-1 and therefore are primed against Mcl-1-directed therapy.

Combination with hypomethylating agents in acute myeloid leukemia (AML)

Venetoclax monotherapy has been trialed in AML to modest efficacy. Hypomethylating agents (HMAs) such as decitabine and azacitidine are thought to additionally inhibit Mcl-1 thus providing synergism between venetoclax and HMAs.

HMAs plus venetoclax have been examined in the first-line setting for AML in elderly or frail patients unfit for standard induction. Complete remission (CR) and complete remission with incomplete hematologic recovery (CRI) rates were 37% and 30%, respectively, with ORR 68% and leukemia response rate (CR + CRI + partial response + morphologic hematologic free state) 83%. Of patients with CR and CRI, median duration of response was 11.3 months (95% CI 8.9 m-NR). Overall median overall survival (OS) was 17.5 months (95% CI 12.3 m-NR). Responses were seen across multiple cytogenetic and mutation subgroups although patients with adverse risk cytogenetics and TP53 had lower rates of response. Adverse events were mostly attributed to hematologic abnormalities, many of which were present at enrollment.

Prior to the development of this treatment strategy, single agent HMAs were standard-of-care in elderly or unfit newly-diagnosed AML patients. Previously reported efficacy outcomes have included median OS of 10 months, CR + CRI rates of 25-30%, and median duration of CR + CRI 10 months. The Bcl-2 selective inhibitor venetoclax in combination with hypomethylating agents has therefore been a practice-changing therapeutic strategy which received accelerated FDA approval for first-line treatment of AML in elderly patients or those unfit for standard induction.

Combination with XPO1 in Glioblastoma

Chromosome region maintenance 1 (CRM1) also known as exportin 1 (XPO1) is a transporter of tumor suppressor proteins and its role in oncogenesis have been described in both hematologic and solid organ malignancies. Increased expression of XPO1 has been postulated to move tumor suppressor proteins from the nucleus to cytoplasm where they cannot regulate the cell cycle. Selinexor or KPT-330 is an oral selective inhibitor of XPO1 that crosses the blood-brain barrier and therefore has been tested as monotherapy for recurrent glioblastomas.

BAG3 or BIS is a Bcl-2 interacting protein that synergizes with Bcl-2 to prevent apoptosis. Its expression typically portends a worse prognosis in glioblastomas. Preclinical glioblastoma models have demonstrated improved apoptotic response to BH3 mimetics with concurrent BAG3 silencing or deletion. Selinexor with a variety of BH3 mimetics including navitoclax, venetoclax, WEHI-539 (Bcl-xl inhibitor), and A1210477 (Mcl-1 inhibitor) has been shown to be effective in glioblastoma cell lines and patient-derived xenografts. Combination therapy reduces cellular proliferation and increases sensitivity to selinexor via enhanced apoptosis as demonstrated through DNA fragmentation, annexin staining, mitochondrial membrane disruption, and cleaved caspas. Navitoclax alone was seen to increase Mcl-1 levels; by adding selinexor, Mcl-1 protein levels were then suppressed. Similar effects were noted in PDX models of glioblastoma with combined therapy of selinexor and navitoclax demonstrating greater growth inhibition than either therapy alone likely due to broad inhibition of pro-apoptotic proteins.

Combination with BRAF inhibitor

BRAFV600E-mutated malignancies generally respond to the BRAFV600E-specific inhibitors, and the combination of dabrafenib and trametinib has moved into the first-line setting for advanced BRAFV600E-mutated melanoma. In melanoma, median progression-free survival to single-agent vemurafenib tends to be brief at around 7 months. This is improved with the addition of MEK inhibition to 11 months. While the addition of MEK inhibition prolongs response duration, resistant pathways inevitably develop thus limiting the efficacy of BRAF-targeted treatment.

Selinexor has been evaluated with vemurafenib in BRAFV600E-mutated thyroid cancer cell lines. Navitoclax alone had no effect on cellular survival. Vemurafenib alone reduced cell viability by 10.4% and also increased expression of Bcl-2 and Bcl-xl 24 hours after exposure. In contrast, the combination reduced cell viability by...
combination therapy in CLL

Ibrutinib is a Bruton's tyrosine kinase inhibitor approved for front-line therapy of CLL. Mechanistically, ibrutinib is thought to mobilize CLL cells thus removing them from a pro-survival microenvironment. Additional effects on the apoptotic pathway includes reduced levels of Mcl-1 and Bcl-xl59. Thus the combination of ibrutinib and venetoclax is a potential strategy in CLL that has now been tested in a phase II clinical trial for first-line therapy of CLL60. Of 80 evaluable patients, 74% of patients were able to achieve CR or CRi. Of the 33 patients that received 12 cycles of combination therapy, 29 patients (88%) were in CR or CRi and 20 patients (69%) had no minimal residual disease in the bone marrow. One-year PFS and OS were 98% and 99%, respectively. Responses were seen across high-risk subgroups. Adverse events included atrial fibrillation, neutropenia, and neutropenic fever at similar rates to prior published data with ibrutinib monotherapy61. Laboratory tumor lysis was seen in only 3 patients. While the combination is a promising front-line option, longer follow-up and randomized trials examining this strategy compared to either in monotherapy are warranted.

Combination with Aurora Kinase Inhibitor

The aurora kinases are a family of serine/threonine kinases that include Aurora Kinase A (AKA), B, and C. While all three have the potential for tumorigenesis, AKA has been the most well-studied, and its overexpression is associated with genomic instability, abnormal G2/M checkpoint transition, mitotic disruption, and aberrant activation of other oncogenic signaling pathways62,63. Alisertib or MLN 8237 is an AKA inhibitor that has had little success as monotherapy and is therefore being increasingly examined in combination therapy. Alisertib has been observed to reduce Mcl-1 levels, and since Mcl-1 is a potential resistance mechanism to single agent BH3 mimic therapy58, the addition of alisertib to BH3 mimetics is an attractive combinatorial therapy strategy. Pancreatic cancer cell lines, organoids, and xenografts exposed to either alisertib or navitoclax alone had variable sensitivity to each; however, the combination yielded increased growth inhibition. Apoptotic activity with this approach was confirmed with higher levels of cleaved caspase-3, cleaved caspase-7, cleaved PARP, and annexin V. Furthermore, pancreatic cancer organoids undergoing treatment with MLN 8237 alone demonstrated reduced Mcl-1 levels while treatment with navitoclax alone demonstrated increased Mcl-1 levels and to a lesser extent Bcl-xl levels. The addition of MLN 8237 to navitoclax counteracted the Mcl-1 upregulation seen with navitoclax only59. Therefore, the synergistic effect of this combined therapy may be attributable to AKA inhibition effects on Mcl-1.
For patients with baseline renal dysfunction or other medical co-morbidities as determined by the Cumulative Illness Rating Score, venetoclax plus obinutuzumab has been compared to chlorambucil plus obinutuzumab in the front-line setting in a phase III clinical trial. The venetoclax/obinutuzumab arm had a 2-year median PFS of 88.2% compared to 64.1% for chlorambucil/obinutuzumab, and again the benefit was seen across poor-risk features. Toxicity profile between the two arms was similar. Therefore, the addition of anti-CD20 therapy to venetoclax is synergistic in its clinical effect and mechanistically mitigates potential resistance to venetoclax alone by promoting both apoptotic and lysosomal cell death.

**Conclusion**

The Bcl-2 family of proteins is an important regulator of intrinsic apoptosis and thus represents an attractive target for cancer-directed therapy. BH3 mimetics and small molecular inhibitors of pro-apoptotic proteins are now increasingly exploited therapeutic strategy that has moved into the first-line setting for hematologic malignancies including AML and CLL. Combinatorial approaches with other agents such as chemotherapy, inhibitors of other oncologic driver mutations, or monoclonal antibodies offers synergistic anti-tumor effects that likely offset resistant mechanisms seen with BH3 mimetic therapy alone. These agents exhibit significant potential to improve current therapies in hematologic and non-hematologic malignancies.

**References**


