Published in final edited form as:
Expert Opin Ther Targets. 2013 January ; 17(1): 61–75. doi:10.1517/14728222.2013.733001.

Targeting the Bcl-2 Family for Cancer Therapy

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Abstract

Introduction—Programmed cell death is well-orchestrated process regulated by multiple pro-apoptotic and anti-apoptotic genes, particularly those of the Bcl-2 gene family. These genes are well documented in cancer with aberrant expression being strongly associated with resistance to chemotherapy and radiation.

Areas covered—This review focuses on the resistance induced by the Bcl-2 family of anti-apoptotic proteins and current therapeutic interventions currently in preclinical or clinical trials that target this pathway. Major resistance mechanisms that are regulated by Bcl-2 family proteins and potential strategies to circumvent resistance are also examined. Although antisense and gene therapy strategies are used to nullify Bcl-2 family proteins, recent approaches use small molecule inhibitors and peptides. Structural similarity of the Bcl-2 family of proteins greatly favors development of inhibitors that target the BH3 domain, called BH3 mimetics.

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Conflict of interests: None of the authors have any financial conflict of interest.

Expert opinion—Strategies to specifically identify and inhibit critical determinants that promote therapy-resistance and tumor progression represent viable approaches for developing effective cancer therapies. From a clinical perspective, pretreatment with novel, potent Bcl-2 inhibitors either alone or in combination with conventional therapies hold significant promise for providing beneficial clinical outcomes. Identifying small molecule inhibitors with broader and higher affinities for inhibiting all of the Bcl-2 pro-survival proteins will facilitate development of superior cancer therapies.

Keywords

BH3 domain; apoptosis; Mcl-1; radiation resistance; chemotherapy resistance

1. Introduction

Continuous programmed cell death is essential for maintaining tissue homeostasis, and this process is positively or negatively regulated by specific genes [1]. An imbalance can lead to severe tissue disturbances that can ultimately culminate in cancer. The *BCL-2* (B-cell lymphoma-2) [2–4] gene was first discovered at the t (14; 18) chromosome translocation breakpoint in B-cell lymphomas. As a result of this translocation, immunoglobulin heavy chain gene promoter and enhancer in chromosome 14 drives the transcription of *BCL-2*, subsequently leading to constitutive expression of Bcl-2 in B-cell clones [3]. Unlike previously identified oncogenes, Bcl-2 does not promote cell proliferation. Instead, overexpression of Bcl-2 inhibits cell death [5].

Over the years, the Bcl-2 family of proteins has expanded and now includes at least 12 predominantly expressed members including Bcl-2 itself. Functionally these molecules differ by either promoting or inhibiting apoptosis, thus establishing these molecules as pivotal determinants of whether a cell lives or dies. Based on their structure and function, the Bcl-2 family of proteins is further divided into three groups as listed in Figure 1. There are several pro-survival proteins, but 5 are well characterized including, Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1, and three pro-apoptotic proteins, BAK, BAX and BOK, of which the first two are predominant and localized on the mitochondrial membrane. Upon receiving a death signal, oligomerization of BAK, BAX and BOK leads to formation of mitochondrial pores subsequently resulting in increased permeability of the mitochondrial membrane releasing cytochrome *c* (cyt c) into the cytosol ultimately leading to cell death. Both anti-apoptotic and pro-apoptotic proteins have a similar C-terminal membrane localization domain, three or four Bcl-2 homology domains (BH1, BH2, BH3 and BH4), and similar three-dimensional structures [6]. However, the structural differences that apparently decide their mutually opposing roles are attributed to a few amino acids. There are eight members of another class of BH3-only pro-apoptotic proteins that lack all other Bcl-2 homology domains except BH3, named BIM, BID, BIK, BAD, BMF, HRK, PUMA and NOXA. All BH-3 only proteins also play pivotal roles by regulating the core Bcl-2 family proteins to promote apoptosis through binding via its BH-3 domain. The intrinsic apoptosis pathway starts with BH3-only protein induction or post-translational activation, which results in the inactivation of some BCL-2 family members. This relieves inhibition of BAX and BAK activation, which in turn promotes apoptosis. Some BH3-only proteins, such as BIM and PUMA, may also activate BAX and/or BAK [6].

Apoptosis can be operationally divided into three stages. In the first stage, or initiation phase, the cells undergoing stress or DNA damage initiate a signaling cascade either through an intrinsic or extrinsic pathway. This is followed by the regulatory phase, where a sum of all of these signals is integrated to make the decision whether to undergo apoptosis or not. The third and final phase is the execution phase where caspases are cleaved and the cells are

further engulfed by neighboring phagocytic cells [7]. The Bcl-2 family of pro-apoptotic and anti-apoptotic proteins regulates the intrinsic pathway in the initiation phase leading to caspase-9 activation (Figure 2). BIM and PUMA bind to all five anti-apoptotic Bcl-2 family members. By contrast, NOXA only binds to Mcl-11 and A1, and BAD binds selectively to Bcl-w, Bcl-2 and Bcl-X_L. BID binds avidly to Bcl-X_L, BCL-w, Mcl-11 and A1, but only weakly to BCL-2. These binding specificities recapitulate the ability of these proteins to activate apoptosis. For example, BIM, BID or PUMA alone can induce apoptosis, whereas a combination of NOXA and BAD is required [6]. On the other hand, the extrinsic pathway does not involve Bcl-2. Instead, the extrinsic pathway is triggered by ligation of death receptors, that are members of the tumor necrosis factor family (TNF) containing an intracellular death domain that can recruit and activate caspase-8 through the adapter protein Fas-associated death domain (FADD) at the cell surface [6]. It is the interaction between the pro- and anti-apoptotic proteins mainly via their BH3 domains that determines cell fate. In a model suggested by Willis et al., Bcl-X_L antagonizes the pro-apoptotic action of BAK or BAX by binding to their BH3 domains, and any member of the BH3-only proteins can relieve this interaction by binding to Bcl-X_L [8]. Also, BH3-only proteins can interact directly with BAX and BAK leading to apoptosis induction [9]. Bcl-X_L and Mcl-1 directly target BAK, while BAX regulation also requires Bcl-2 [10]. Bcl-2 and Bcl-X_L were shown to be involved in non-apoptotic cell death via their interaction with another BH3 domain containing protein Beclin-1 (BECN1). Binding of Bcl-2 or Bcl-X_L with BECN1 inhibits autophagy, while release of this interaction induces autophagy [11, 12].

Although both pro-apoptotic and anti-apoptotic proteins share similar three-dimensional structure and BH domains, the specific difference in their amino acid sequences that determines their mutually opposing role is remains elusive. However, from a therapeutic standpoint, a BH3 mimetic can bind to pro-survival proteins to induce apoptosis and may also induce autophagy [13].

2. Role of the Bcl-2 family in mediating resistance of cancer cells to therapy

Impaired apoptosis is pivotal for tumor development, and altered expression of molecular determinants of apoptosis, such as the Bcl-2 family of proteins, has a detrimental outcome. Apoptosis resistance can lead to escape of tumor cells from immune-surveillance, and can eventually result in the expansion of a population of resistant neoplastic cells. Ranger *et al.* suggest that at least one Bcl-2 family pro-survival protein is required for the survival of every cell type [14]. Other studies provide evidence that an appropriate balance between anti-apoptotic and pro-apoptotic molecules are required for tissue homeostasis [15]. Although Bcl-2 was originally discovered as a gene translocated in B-cell malignancies, later studies showed that Bcl-2 and other Bcl-2 family pro-survival genes are transcriptionally upregulated by alternate mechanisms that do not involve chromosomal translocations. These observations expand the significance of Bcl-2 family pro-survival genes in multiple cancer indications [16, 17].

3. Resistance Mechanisms

Treatment of cancer with chemotherapy or radiation kills target cells primarily by induction of apoptosis. Anticancer drugs are classified as DNA-damaging agents, antimetabolites, mitotic inhibitors, nucleotide analogues or inhibitors of topoisomerases [18]. Radiotherapy can induce cellular oxidative stress that culminates in tumor cell death [19]. These approaches are effective in some patients; however, they are not very effective for others, mostly due to failure to activate apoptosis resulting in inherent resistance of some tumors to therapy, which represents a major clinical challenge. Tumor cells can acquire resistance to apoptosis

by various mechanisms, including overexpression of anti-apoptotic genes, down-regulation or mutation of pro-apoptotic genes and alteration of p53 or the PI3K/AKT pathways [20].

3.1 Altered expression of pro-survival proteins

3.1.1 Bcl-2—Multiple studies have shown that high levels of *BCL2* gene expression correlate with severity of malignancy of human tumors [21–23]. Elevated expression of Bcl-2 in acute myeloid leukemia (AML) or increased Bcl-2/BAX ratio was shown to be associated with poor clinical response [22, 24, 25]. Additionally, several studies have demonstrated a correlation between elevated Bcl-2 expression and poor prognosis in melanoma [26], breast [27], prostate [28], small cell lung [29], colorectal [30] and bladder cancers [31]. Further studies using both *in vitro* and *in vivo* models have proven that higher Bcl-2 expression leads to resistance to chemotherapy and radiation [21, 22, 32]. In follicular B-cell lymphoma, chromosomal translocation t (14, 18) places the *BCL2* gene next to enhancer elements of the immunoglobulin heavy chain promoter locus leading to enhanced *BCL2* gene expression [4, 33–35]. This translocation is evident in 90% of follicular cell lymphomas and about 30% of diffuse large B cell lymphomas [36]. Furthermore, overexpression of Bcl-2 has been demonstrated as a result of gene amplification [37], hyper-methylation of the *BCL2* gene [38] or chromosomal deletions leading to loss of *BCL2* targeting miRNAs such as miR-195, miR-24-2 and miR-365-2 [39, 40]. In addition, tumor associated viruses, such as Epstein-Barr virus (EBV) and human herpes virus 8 (HHV8 or Kaposi's sarcoma-associated herpes virus), encode proteins that are homologues of Bcl-2, and elicit similar anti-apoptotic functions [41–43]. Overexpression of the Bcl-2 family of pro-survival proteins by itself has not proven to be highly tumorigenic; however, in combination with additional synergistic mutation(s) this overexpression is profoundly detrimental to the host [53]. Many follicular lymphomas with *BCL2* gene translocations are relatively indolent [53, 54]. Nevertheless, 8% of follicular lymphomas can progress to aggressive disease and those were shown to be associated with an additional *MYC* translocation [55]. This correlation was first suggested using *in vitro* studies [5] and later confirmed in mice expressing both *BCL2* and *MYC* transgenes [56]. Further studies have provided convincing evidence that co-expression of Bcl-2 and c-myc makes cells resistant to therapy in multiple malignancies including lymphomas [57], breast [58] and pancreatic [59] cancers.

3.1.2 Bcl-X_L—Besides Bcl-2, Bcl-X_L has also been shown to confer resistance to multiple apoptosis-inducing pathways [44]. Advanced and relapsed multiple myeloma (MM) are characterized by higher levels of Bcl-X_L [45]. Nagane *et al.* showed that constitutive activation of epidermal growth factor receptor (EGFR) tends to increase BCL-X_L expression thereby leading to apoptosis resistance [46]. Both Bcl-2 and Bcl-X_L are overexpressed in close to 100% of hormone-refractory prostate cancers and are associated with therapy resistance leading to a very poor clinical outcome [47]. Enhanced proliferation and survival of pancreatic cancers were also found to be due to Bcl-X_L overexpression in conjunction with c-myc [59, 60]. Studies also demonstrated that, lymphoid tumors with increased levels of Bcl-X_L formed in *MYC* transgenic mice carried mutations in the p53 gene [61]. Hence, mutations that enhance tumor growth along with suppression of apoptosis by overexpression of the Bcl-2 family of pro-survival genes lead to unfavorable clinical outcome.

Michaud *et al.* analyzed the levels of Bcl-2 and Bcl-X_L in a panel of oropharyngeal squamous cell carcinomas (OPSCC) to determine any relationship with platinum-based therapy resistance [62]. In OPSCC, Bcl-2 expression was associated with increased cisplatin resistance, while Bcl-X_L expression failed to show any correlation. However, this association of Bcl-2 expression with cisplatin resistance was not observed in an H69 SCLC cell line [63].

3.1.3 Mcl-1—In leukemia patients, Mcl-1 expression was shown to be upregulated, particularly at relapse, indicating a possible enrichment of chemotherapy-resistant Mcl-1 expressing cells following a certain chemotherapy regimen [48]. Expression of Mcl-1, but not of Bcl-2 or Bcl-X_L, was shown to be associated with poor survival outcome in ovarian cancer patients [49] and in human cervical neoplasms IL-6 regulates the expression of Mcl-1 via a PI3K/Akt-dependent pathway and facilitates oncogenesis of cervical cancer by inhibiting cellular apoptosis [50]. Additionally, Schwickart *et al.* demonstrated that in hematologic malignancies [51], the expression of deubiquitinase USP9X expression correlates with that of Mcl-1, and USP9X binds to Mcl-1, stabilizes it and thereby promotes Mcl-1 overexpression and cell survival. Recent studies showed that in B-cell malignancies, patients treated with the anti-CD20 antibody rituximab exhibited an increased level of Mcl-1 [52]. Mcl-1 downregulation using RNA interference sensitized these cells to rituximab treatment [52], underscoring the importance of Mcl-1 as a mediator of chemotherapy resistance.

Additionally, studies in a panel of lymphoma cell lines treated with protease inhibitors identified Mcl-1 increase and accumulation as an unwanted molecular consequence leading to apoptosis inhibition [64].

3.2 Inactivation of pro-apoptotic proteins

Besides overexpression of pro-survival genes, tumors acquire apoptosis resistance by down regulation or mutation of pro-apoptotic molecules. Two key events known to induce cancer cell survival and progression are loss of function of tumor suppressor genes such as p53 [65] and mutation of pro-apoptotic proteins such as BAX, which are common in hematologic malignancies [66, 67]. Since BH3 only proteins NOXA and PUMA are transcriptional targets of p53, loss of p53 by itself suppresses apoptosis induced by these proteins [68–70]. Frame shift mutations causing loss of expression and mutation of BH domains of pro-apoptotic proteins that lead to loss of their function are common in colon cancers and result in apoptosis resistance [67].

To evade apoptosis, cancer cells are selected based on which specific group of Bcl-2 family proteins is altered. As suggested in a recent study, there are three types of apoptosis blockade [71]. Class A blockade is due to a loss of function of the BH3-only activator proteins. Class B blockade constitutes failure to activate effectors due to loss or inactivation of BAX and BAK. Class C blockade is mainly due to increased expression of Bcl-2/Mcl-1. Based on this classification, BH3 profiling can be used to predict drug sensitivity and has been a useful tool in diffuse large B-cell lymphomas for stratifying drug sensitivity in patients [72].

3.3 Radiation-resistance mechanism

Tumor resistance to radiation is defined by the percentage of cells that are resistant after administering a total dose of radiation that can be safely delivered. Radiation induces oxidative stress and DNA damage resulting in apoptosis via p53- and BAX-dependent mechanisms. Bcl-2 expression has been implicated in radiation-resistance. Strasser and colleagues showed that Bcl-2 strongly induces clonogenic survival of lymphoma cells after irradiation [73]. In contrast, Bcl-2 overexpressing PC-3 and LNCaP prostate cancer cells failed to induce radiation-resistance [74]. However, both *in vitro* and *in vivo* approaches showed that cells exposed to lower levels of radiation induce Bcl-2 expression, as an adaptive measure to withstand environmental stress [75]. Follow-up *in vitro* studies in pancreatic cancer cells proved that knockdown of Bcl-2 in these cells sensitized them to radiation resulting in effective cell death [75]. Lee *et al.* investigated the role of Bcl-2 family of proteins in eliciting radiation-resistance in PANC-1 and AsPC-1 pancreatic cancer cells with mutated p53.

Radiation resistant clones showed no change in Bcl-2, but displayed upregulation of Bcl-X_L, emphasizing the importance of the Bcl-2 family proteins in inducing radiation-resistance. Elevated expression of Bcl-X_L was detected in bladder carcinomas and squamous cell carcinomas of the oropharynx and it was shown to be associated with radiation-resistance [76,77].

3.4 Additional chemo-resistance and radiation-resistance mechanisms involving Bcl-2

Besides the mitochondrial pathway of apoptosis, Bcl-2 may block apoptosis via other mechanisms including inhibition of calcium mobilization [78], acting as an antioxidant to prevent free radical damage [79] and elevation of glutathione [63] (GSH [3]) levels by preventing its depletion [80]. Studies by Wright *et al.* showed that elevated Bcl-2 levels by virtue of increased GSH levels can prevent the activation of serine protease AP24 following treatment with TNF or UV light, which is capable of inducing nuclear fragmentation [81]. This effect confers apoptosis resistance by GSH-induced elevated Bcl-2 expression.

4. Reversing resistance to chemotherapy and radiation by genetic and pharmacological inhibition of Bcl-2 family members

From a therapeutic standpoint, it is critical to understand that the process of apoptosis is intricately balanced, and perturbing the balance to induce apoptosis should be the goal of therapy. Accordingly, Bcl-2 interfering strategies have been accepted as a potential approach to induce apoptosis in cancer cells. Later studies shed light on a new concept that the apoptotic signal itself is induced in cancer cells even in the absence of chemotherapy, and abnormally higher expression of Bcl-2 proteins neutralize that apoptotic signal, making those cancer cells addicted to expression of Bcl-2 pro-survival proteins. Therefore, Bcl-2 inhibition strategies also present a form of ‘synthetic lethality’ that selectively kills cancer cells addicted to Bcl-2 expression for survival. For radio-sensitization as well, modifying the activity of cell survival genes such as Bcl-2 will be very beneficial [75].

Several strategies have been formulated over the years to target the Bcl-2 family of proteins including antisense oligonucleotides [82]; peptides and small molecules inhibitors (SMIs) targeted toward apoptosis mediators. They are classified in Table 1.

4.1 Bcl-2 inhibition strategies

Recombinant adenovirus encoding BAX was the first to be introduced in its class. However, this approach was not successful due to toxicity affecting healthy cells [83]. Recently, microRNAs, a novel class of gene regulators, regulating Bcl-2 expression have been identified. Using computational and experimental approaches, Sing *et al.* showed that miR-195, miR-24-2 and miR-365-2 act as negative regulators of Bcl-2 by binding to the 3’-UTR of the *BCL2* gene [40]. Overexpression of these miRNAs alone resulted in increased apoptosis and also augmented etoposide-induced apoptosis in MCF-7 breast cancer cells, suggesting that miRNA-based Bcl-2 inhibition strategies hold promise in the future.

4.2 Mcl-1 targeting

Recently, many researchers have recognized Mcl-1 as a critical player in apoptosis-resistance in multiple cancers. Despite the fact that Mcl-1 is structurally similar to other Bcl-2 family of proteins, there are significant differences in its BH3 binding groove, which weakens the affinity of other BH3 mimetics to target Mcl-1. Previous studies using phage display libraries have analyzed the binding requirements of the Mcl-1 binding groove [84]. Cyclin dependent kinase inhibitors including Flavopiridol, and SNS-032 were shown to transcriptionally suppress the expression of several genes including Mcl-1. Based on that

premise, Flavopiridol has been used for treating high-risk CLL patients demonstrating promising results [85] and synergism when treated with a proteasome inhibitor. Sorafenib, originally developed as a B-raf inhibitor, is suggested to decrease Mcl-1 translation, resulting in increased apoptosis in leukemia cells [86]. A unique characteristic of Mcl-1 is that it has an extremely short half-life due to ubiquitin-mediated proteosomal degradation as compared to other Bcl-2 family proteins. This gives an added advantage for therapeutic exploitation [87]. A recent study showed that USP9X, a deubiquitinase, modified the expression of Mcl-1, and its knockdown lead to rapid degradation of Mcl-1 [51]. WP1130, a small molecule inhibitor for Bcr-Abl, has shown inhibitory effects on USP9X. When used to treat CML, WP1130 led to decreased levels of Mcl-1 and removal of Bcr-Abl eventually leading to apoptosis [88]. In total, these data highlight the value of Mcl-1 inhibition as a potential tumor inhibition strategy.

5. Targeting Bcl-2 family members for therapy

Targeting the mitochondrial-mediated apoptosis pathway might facilitate the cytotoxic effects of chemotherapy as well as radiotherapy. Tumors that are resistant to apoptosis due to aberrant expression of Bcl-2 family proteins, when treated with DNA intercalating agents or radiotherapy, may survive and induce further genetic instability instead of undergoing apoptosis [89]. Therefore, treating cells with drugs modulating the Bcl-2 pathway is critical in achieving desired clinical outcomes. Multiple strategies have been established to target the Bcl-2 pathway, including antisense-mediated inhibition, peptide inhibitors and small molecule inhibitors.

5.1 Antisense oligonucleotides (ASOs) and antibodies

The principle of antisense strategy is that introduction of a single oligonucleotide strand complementary to the target sequence of a chosen mRNA leads to formation of a DNA heteroduplex, which is vulnerable to destruction by RNAse H, ultimately resulting in reduced levels of the target mRNA [90].

5.1.1 Oblimersen (G-3139)—One of the first drugs developed for inhibiting the Bcl-2 family was Oblimersen sodium; an antisense modified 18-mer oligonucleotide with a phosphorothioate backbone complementary to the *BCL2* gene. Although trials initiated by Gentra for this agent showed moderate success in treating low-grade lymphoid malignancies, it failed to get FDA approval, since it did not show substantial survival advantage in clinical trials in melanoma, multiple myeloma and chronic myelocytic leukemia [91]. A Phase II study in myeloid leukemia showed robust intracellular accumulation of G-3139 in bone marrow leading to Bcl-2 downregulation [92]. A recent report showed that a combinatorial approach with dacarbazene in melanoma patients improved survival at 24 months and significantly increased progression-free survival [13]. Use of ASOs is limited due to disadvantages such as DNAse-mediated degradation, which is resolved by phosphorothioate modifications, and non-specific binding. Also, with the relatively short half-life of ASOs, a complete suppression of the target gene is less likely to be achieved.

A similar strategy was employed in developing Bcl-X_L ASOs [93]. As a single agent, Bcl-X_L ASOs also suffered a similar inconclusive fate as Bcl-2 ASOs. Nevertheless, an antisense approach that targets both Bcl-2 and Bcl-X_L had augmented therapeutic value [94]. Mcl-1 antisense strategy also showed promising results *in vitro* in multiple cancer indications, and hepatocellular carcinomas (HCC) displayed increased sensitivity to cisplatin treatment [82].

Besides ASOs, other approaches were also employed to inhibit the expression of Bcl-2. An antibody targeting Bcl-2 was developed and was shown to increase drug-induced cytotoxicity in breast and other cancers [95]. Furthermore Bcl-2 targeting ribozymes were

developed that showed promise in inhibiting myeloid leukemia growth [96]. Like ASOs, antibodies and ribozymes suffered from a lack of stability thus limiting its use in the clinic.

5.2 Peptide and peptidomimetics

Given the limited success of approaches to reduce the expression of the Bcl-2 family of anti-apoptotic proteins, a different approach was introduced to antagonize Bcl-2 function, rather than changing its levels. Understanding the crystal structure of all eight BH3-only proteins revealed the relationship of each of these proteins with its pro-survival counterparts. Based on the mechanism of apoptosis induction explained earlier, BAX and BAK need to be activated as a result of either BH3-only proteins binding directly to them or BH3-only proteins binding with anti-apoptotic proteins resulting in release of pro-apoptotic proteins. BH1-BH3 domains of Bcl-X_L form a hydrophobic groove [97] where the α-helix of a BH3-only protein can bind. Sattler *et al.* [98] first tested the concept of whether a BAX-BH3 peptide that can bind to the hydrophobic groove of Bcl-X_L could inhibit its anti-apoptotic function. Results from this study provided the proof-of-concept for designing numerous specific antagonists that are referred to as BH3 mimetics, which, when bound to the hydrophobic crevice on Bcl-2 or Bcl-X_L, impair their function and induce apoptosis. Over the years several short peptides representing the BH3 domain of BH3-only proteins have been designed.. Recently, Zhang and colleagues discovered that nuclear receptor Nur77 is capable of binding between the BH3 and BH4 domains, unmasking the BH3 domain and leading to a functional switch of Bcl-2 from an anti-apoptotic to a pro-apoptotic protein [101]. This strategy might help construct newer derivatives holding promise. BIM-BH3 peptide is a novel hydrocarbon labeled-peptide modeled to target the BH3 domain of BIM, inhibiting Bcl-2-BIM interactions. This stapled peptide is highlighted for its selective activation of cell death in hematologic tumors and AML xenografts [102].

5.3 Small molecule inhibitors

Small molecules are organic molecules of low molecular weight (less than 750 Daltons). Compared to ASOs and peptides, their relatively smaller size and lower manufacturing costs make small molecule inhibitors (SMIs) a more attractive treatment strategy. SMIs are developed based on the fact that the BH3 binding hydrophobic cleft in Bcl-2 is critical for its anti-apoptotic function, and blocking that groove, using SMIs might inhibit its heterodimerization, thus disarming the anti-apoptotic function of Bcl-2 and tipping the balance towards apoptosis. Various screening strategies have been undertaken over the years in an attempt to find SMIs against the bcl-2 family of proteins. Natural products such as actimycin A, celerythrin and purpurogallin, and some compounds derived from tea and cotton extracts have demonstrated efficacy as antitumor agents by modulating the Bcl-2 family of anti-apoptotic proteins [99, 103]. Although the advent of SMIs resolved most of the problems associated with antisense therapy, newer problems arose, such as non-specific binding to catalytic subunits of other oncoproteins leading to off-target effects. Following are some of the SMIs in clinical and preclinical stages of development.

5.3.1 Gossypol—Gossypol is a natural polyphenol isolated from cottonseeds [104]. Gossypol, also known as BL-193, was used as a contraceptive and for anticancer studies since 1980s. Subsequently, more effective isoforms such as (−)-BL-193, (+)-BL-193 and (±)-BL-193, were developed, with the -(−) form being the more potent one [105]. Using nuclear magnetic resonance (NMR), the mechanism of (−)-BL-193 action was resolved showing that BL-193 binds to the hydrophobic groove of Bcl-2 and Bcl-X_L [106]. Moreover, gossypol was shown to induce DNA breakage in the presence of metal ions such as copper [104]. Gossypol is presently in clinical trials as a single agent and in combination (Table 2).

5.3.2 TW37—TW37 is a second-generation benzenesulphonyl derivative of gossypol [107]. TW37 has an added advantage that it shows higher affinity to Bcl-2, Bcl-X_L and also Mcl-1, as compared to other BH-3 binding SMIs. TW-37 induced apoptosis in a *de novo* chemoresistant WSU-$DLCL_2$ lymphoma cell line [108]. Moreover, pre-exposure of lymphoma cells to TW-37 significantly enhanced the killing effect of the cyclophosphamide-doxorubicin-vincristine-prednisone (CHOP) regimen.[108, 109].

5.3.3 ApoG2—Apogossypolone is a third generation gossypol derivative, which is also effective in targeting Mcl-1. ApoG2 was developed by Ascenta to reduce toxicity and non-specific reactivity. In preclinical studies, ApoG2 induced apoptosis and displayed promising results in different types of lymphomas [110, 111].

5.3.4 ABT-737—The first in class of SMIs eliciting cancer-specific killing is ABT-737, developed by Abbott Laboratories. Combining structure-activity relationship (SAR) by NMR with structure-based drug design and chemical synthesis led to the discovery of the lead compound ABT-737 that mimicked the BH3 domain of BAD and bound selectively to Bcl-2, Bcl-X_L and Bcl-w. ABT-737 was ineffective at activating apoptosis in cells doubly deficient in BAX and BAK, suggesting its activity is mediated through Bcl-2 [112]. ABT-737 was tested in multiple myeloma (MM) and was shown to abrogate the viability of bortezomib, dexamethasone and thalidomide-refractory patients [113]. Acquired resistance to ABT-737 was shown to be associated with increased Mcl-1 expression and reduced expression of its primary target Bcl-2 or Bcl-2 heterodimers [114].

5.3.5 ABT-263—ABT-263 is an orally available derivative of ABT-737 that can bind to serum proteins, resulting in longer half-life, and inhibit Bcl-2, Bcl-XL and Bcl-w. Given its effectiveness as a single agent in preclinical studies, ABT-263 is currently being evaluated in clinical trials for SCLCs and leukemia [115]. Using a systems biology approach, Tahir *et al*. correlated Bcl-2 expression as a function of ABT-263 sensitivity in 36 SCLC and 31 leukemia/ lymphoma cell lines [116], and generated an ABT-263 sensitivity signature based on this data.

5.3.6. AT-101—AT-101 is the negative (−) enantiomer of gossypol and a potent inhibitor of the antiapoptotic Bcl-2 family members, Bcl-2, Bcl-X_L and Mcl-1, with an IC_{50} of 1-10 μM. AT-101 was effective against B-cell lymphomas [117]. In mantle cell lymphoma, AT-101 was synergistic when sequentially combined with carfilzomib, etoposide, oxorubicin, and 4-hydroxycyclophosphamide [117]. In diffuse large B-cell lymphoma, AT-101 displayed a synergistic effect when sequentially combined with 4-HC [117]. AT-101 is currently in phase II clinical trials as a single agent.

5.3.7 Obatoclax—Obatoclax is a synthetic derivative of prodiginines developed by Gemin X (GX015-070), presently Cephalon. Although it is a pan Bcl-2 inhibitor, its binding affinity to Bcl-2 family is less than ABT-737 [118, 119]. However, Obatoclax was shown to inhibit Mcl-1 and antagonize Mcl-1-mediated resistance, a characteristic not displayed by ABT-737, by interfering with direct interaction between Mcl-1 and BAK [120]. Unlike other BH3 mimetics, Obatoclax does not entirely depend on BAX and BAK for apoptosis induction [121]. A recent study showed that Obatoclax could induce Bax-mediated apoptosis in cholangiocarcinoma [122]. At a lower concentration, Obatoclax induced a S/G2 cell-cycle block, while at a higher concentration it induced apoptosis in CD34+ AML progenitor cells [121], and its effect was more pronounced when combined with AraC. Similarly, in esophageal cancers, Obatoclax showed synergism with carboplatin and 5-flurouracil (5-FU) [123]. Another recent study showed that Obatoclax could overcome glucocorticoid resistance in acute lymphoblastic leukemia (ALL) via induction of apoptosis and

autophagy [124]. However, toxicity may restrict the use of Obatoclax in humans. In a phase I dose escalation study, Obatoclax showed neuropsychiatric toxicities with 1-hour infusions, although 3-hour infusions improved the clinical efficacy and considerably reduced toxicity, suggesting 3-hour infusions as clinically accepted standard of care[125]. Furthermore, a phase III trial (NCT01563601) for Obatoclax in combination with Carboplatin and Etoposide compared with chemotherapy arm alone is underway in naïve patients with advanced-stage small cell lung cancer.

5.3.8 HA14-1—Wang *et al*, using *in silico* screens, developed a new compound HA14-1 as a small molecule antagonist for Bcl-2 proteins [96]. It was shown to disrupt the interaction of BAK-BH3-domain peptides with Bcl-2 and Bcl-X_L proteins, and strongly inhibit Bcl-2-BAX interactions [126]. In MDA-MB-231 breast cancer cells, HA14-1 was shown to act as a chemosensitizer by enhancing the apoptotic effects of cisplatin [127].

5.3.9 Sabutoclax (BI-97C1)—Sabutoclax is a new gossypol derivative originally identified to bind Bcl-X_L with low binding affinity. Additionally, it was shown to bind Bcl-2, Mcl-1 and Bfl-1 [128] and failed to display toxicity against BAX/BAK double knock out mouse embryonic fibroblasts (MEF), suggesting lack of significant off-target effects compared to its predecessors. Sabutoclax induced apoptosis in diffuse large B-cell lymphoma cells, which were resistant to ABT-737 [129]. Further testing showed affinities for specific proteins such as Bcl-2 (IC_{50}=0.32 μM), Mcl-1 (IC_{50}=0.20 μM) and Bfl (IC_{50}=0.32 μM) [130]. Wei *et al.* tested the efficacy of BI-97C1 in Mcl-1 overexpressing M2182 prostate cancer cells and observed 60% tumor xenograft inhibition [105].

Our previous results showed that melanoma differentiation associated gene-7/interleukin-24 (*mda-7*/IL-24) [131] induces cancer-specific apoptosis in multiple cancers and suppression of Mcl-1 augments its cell killing effects [132–134]. In a recent study, we showed that Sabutoclax, but not ABT-737, sensitizes *mda-7*/IL-24-induced apoptosis in prostate cancer cells, confirming the Mcl-1 suppressing role of Sabutoclax [132, 133]. Also, a combinatorial approach of Sabutoclax and an adenovirus delivering *mda-7*/IL-24 induced autophagy that facilitated NOXA and Bim-induced and Bax/BAK-mediated apoptosis.

5.3.10 BI-97D6—In a recent study, we reported the synthesis of a series of ApoG2 derivatives, particularly a chiral compound (±) BI-97D6 that can potently inhibit Bcl-X_L, Bcl-2, Mcl-1and Bfl-1 with IC_{50} values of 76 ± 5, 31 ± 2, 25 ± 8, and 122 ± 28 nM, respectively [129]. In the same study we report that, BI97D6 could induce apoptosis in a panel of prostate, lung cancer and lymphoma cell lines, while displaying little toxicity in BAX/BAK deficient cells, suggesting its mode of action is predominantly dependent on the Bcl-2 pathway.

5.3.11 BH3-M6—This is the most recent BH3 mimetic that was designed as a pan Bcl-2 antagonist that inhibits binding of Bcl-X_L, Bcl-2 and Mcl-1 to multi-protein domains of BAX or BAK [135]. In a recent study Kazi and colleagues showed that, at higher concentrations (25–50 μM), BH3-M6 induced apoptosis in the lung adenocarcinoma cell line A549, and also sensitized cells to apoptosis induced by proteasome inhibitor CEP-1612 [135].

6. Combination therapies

Both chemotherapy and radiation-therapy agents are intended to induce apoptosis in cancer cells. However, studies over time proved that these approaches alone exert limited success in the clinic. This is primarily due to the innate ability of cancer cells to acquire resistance based on the mechanisms already discussed. Therefore, tailoring cancer treatment by

combining strategies to inhibit anti-apoptotic mediators along with conventional therapies would in principle be a promising approach. Bcl-2 family targeting strategies sensitize cancer cells to be vulnerable to conventional chemotherapeutics, especially those cancers that are resistant due to genetic complexity Furthermore, the effect of Bcl-2 inhibitors in combination with immunotherapy has been evaluated [136]. Farsi et al. showed a significant difference in tumor burden in preclinical studies when Obatoclax was treated in combination with recombinant vaccine [137, 138]. Several trials that employ this strategy are ongoing and completed studies are listed in Table 2.

7. Expert opinion

Studies over the years in multiple cancer subtypes have provided compelling evidence of the importance of overexpression of anti-apoptotic proteins Bcl-1, Bcl-X_L and Mcl-1 in regulating apoptotic-resistance following chemotherapy or radiation-therapy. Overexpression of these proteins or loss of function of pro-apoptotic proteins augments therapy-resistance and further generates genetically unstable cells. There is a pressing mandate to develop novel strategies and drugs using existing tactics to circumvent this observed resistance in multiple cancers. Understanding the regulation of the Bcl-2 family of pro-survival genes and underlying resistance mechanisms and their tissue dynamics will greatly facilitate the development of effective strategies to inhibit Bcl-2-mediated resistance. By inhibiting the Bcl-2 family of pro-survival proteins, the ultimate goal is to eliminate the surviving cancer cells that are primarily apoptosis-resistant or make these cells more vulnerable to conventional therapies when pretreated or used in combination with these agents. Additional critical questions remain that need to be answered in this field and generating a clearer picture of apoptosis-resistance will be useful for developing more efficacious approaches. Although both pro-apoptotic and anti-apoptotic proteins share similar 3-dimensional structure and BH domains, the clear difference in their amino acid sequence that determines mutually opposing roles remains elusive. Better understanding of these amino acid residues and how these proteins interact will aid in developing strategies to modify pro-survival proteins to assume the function of pro-apoptotic proteins.

A major weakness of current Bcl-2 family targeting therapy strategies is their inability to target the complete repertoire of anti-apoptotic proteins with the same affinity, making them effective in killing only those cells that depend on the primary target of the drug as the driver of resistance. Although the significance of Mcl-1 as an oncogene and anti-apoptotic gene has received increasing experimental support, most of the Bcl-2 inhibitors to date are weak Mcl-1 inhibitors. Therefore, treating with those agents may not provide any therapeutic advantage for cancers whose main driver is Mcl-1. Pancreatic cancer is a typical example and conventional chemotherapy and radiotherapy along with Bcl-2 drugs are ineffective in inducing apoptosis in pancreatic cancers. Therefore, we have been employing novel SMIs that can target Mcl-1 as well as Bcl-2 and Bcl-X_L to treat therapy-resistant cancers such as pancreatic cancer. Besides Obatoclax, using Sabutoclax or BI-97D6 with their broader spectrum of activity holds promise as a single agent, and more robustly, in combination with other conventional and non-conventional therapeutic agents. Given the heterogeneity of cancers, researchers are recognizing the importance of developing targeting strategies that cover all the Bcl-2 family pro-survival proteins to avoid developing resistance to this targeted therapy. Since Mcl-1 is a major driver of resistance, more potent Mcl-1 targeting strategies could become a mainstay therapy in complex tumors that are resistant to most forms of chemotherapy and radiotherapy such as pancreatic cancers that overexpress Mcl-1 [132, 139]. Besides Mcl-1, other potential mediators in the apoptotic machinery need to be explored and targeted. Moreover, use of Bcl-2 inhibitors as neoadjuvant therapies in radiation-resistant tumors such as pancreatic cancers, could yield clinical advantage and benefit. High throughput screening technologies coupled with bioinformatic approaches will

continue to be employed in the coming years to develop novel drugs against crucial players in cancer development and progression, such as Mcl-1 [139]. In our opinion, future development of more targeted inhibitors that have higher and broader affinities for all the major players of the Bcl-2 family, combined with personalized approaches targeting other cancer-regulated pathways, will greatly improve patient survival and limit or prevent tumor recurrence

Acknowledgments

Support was provided by National Institutes of Health grants 1R01 CA097318, 1R01 CA127641 and the National Foundation for Cancer Research (NFCR) to PBF; 1R01 CA108520 (PD); National Institutes of Health Grant R01 CA138540 and the James S. McDonnell Foundation; National Foundation for Cancer Research (NFCR) (PBF); Samuel Waxman Cancer Research Foundation (SWCRF) (PBF and DS); Army DoD W81XWH-11-1-0480 (PBF and XYW); National Institutes of Health grant R01 CA149668 (J.C.R., M.P.); National Institutes of Health grant R01 CA168517 (MP); and California Institute for Regenerative Medicine grant TR2-01789 (M.P.). DS is a Harrison Scholar and a Blick Scholar in the VCU Massey Cancer Center (MCC) and VCU School of Medicine (SOM), respectively. PBF holds the Thelma Newmeyer Corman Chair in Cancer Research in the VCU MCC and VCU SOM. DS and PBF are SWCRF Investigators.

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Article Highlight Box

- Bcl-2 family of proteins are functionally involved in either promoting or inhibiting apoptosis, thereby establishing these molecules as pivotal determinants of whether a cell lives or dies.
- An appropriate balance between anti-apoptotic and pro-apoptotic molecules is required for tissue homeostasis.
- Tumors acquire apoptosis resistance by aberrant expression of bcl-2 family of proteins, mainly by upregulation of pro-survival molecules and down regulation or mutation of pro-apoptotic molecules.
- Genetic or pharmacological targeting of Bcl-2 family proteins is a potential strategy to reverse apoptosis resistance to radiation and/or chemotherapy.
- Among the strategies developed, BH-3 mimetics hold the most promise and provide an exciting opportunity for cancer therapeutics.
- Combining Bcl-2 family targeting strategies has been shown to sensitize cancer cells to conventional chemotherapies and immunotherapies, especially in those cancers that are resistant due to genetic complexity.

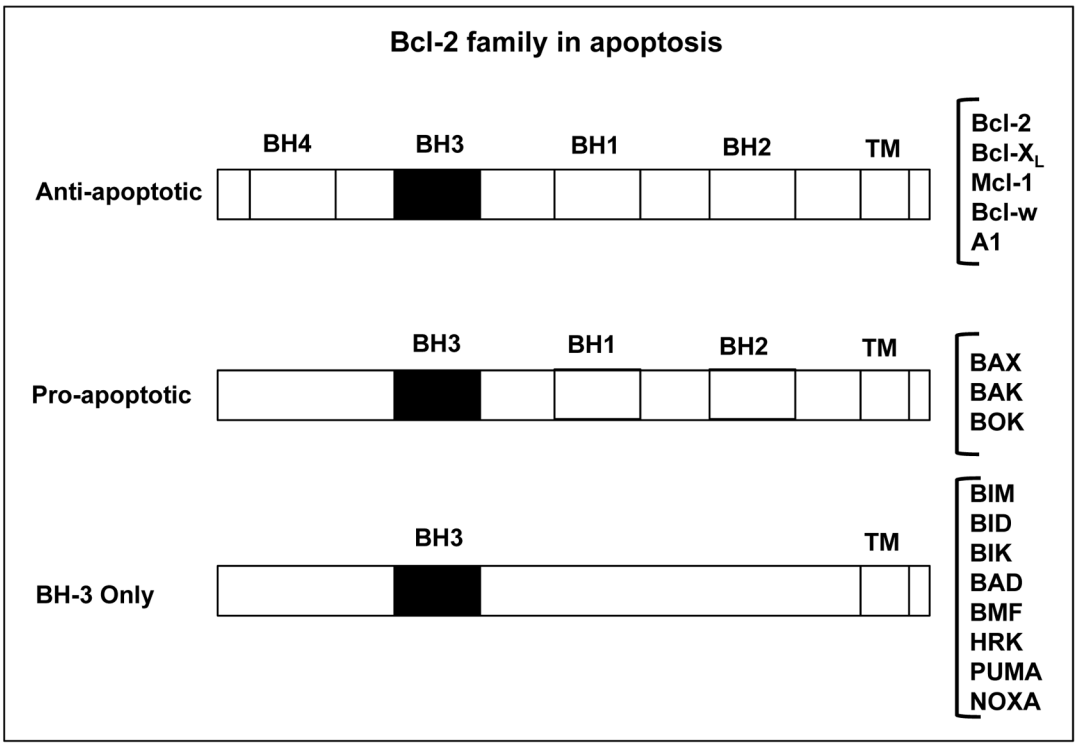

Figure 1. Three subfamilies of Bcl-2 related proteins

Family members sharing four bcl-2 homology (BH) domains are the multidomain proteins. These proteins share a common three-dimensional fold. Anti-apoptotic proteins are antagonists of BAX and BAK, in part by directly binding to them. BH-3 only proteins only have BH3 domain. They respond to stress and are natural antagonists of anti-apoptotic proteins.

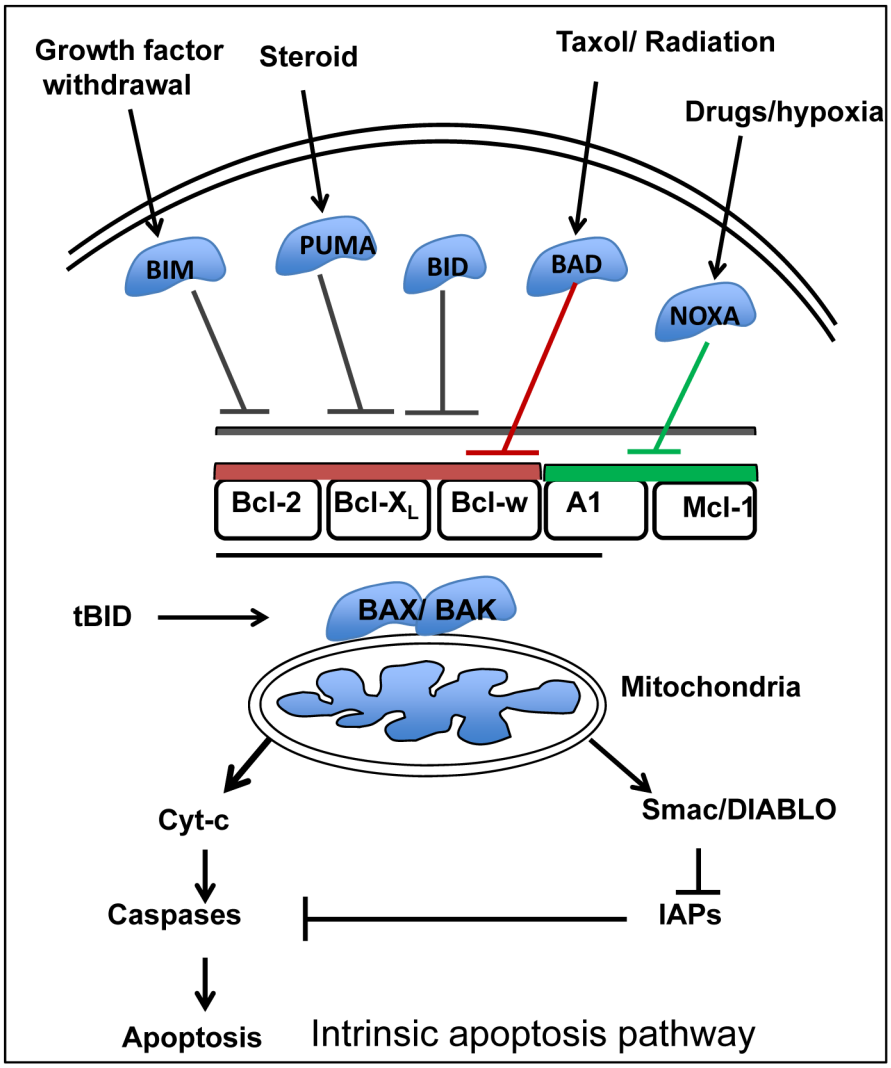

Figure 2. Pathway to apoptosis following various cellular insults

Chemotherapy, radiation and other insults can initiate apoptosis through the mitochondrial intrinsic pathway. Pro-apoptotic proteins (BAD, BIM, BID) are activated and upon its activation mitochondria release cytochrome c into the cytosol. Cytochrome c binds with apoptotic protease activating factor-1 (APAF1) to activate initiator caspase-9 subsequently leading to apoptosis.

Table 1

Bcl-2-family targeting drugs

Drug name	Drug class-mechanism of action	Drug targets	Stage of development	Ref (PMID #)
Cyclin-dependent kinase inhibitor	Flavopiridol	Cyclin dependent kinases and Mcl-1	Clinical trials	22374332
	SN-032	CdKs 2,7,9 and MCL-1		22966018
	Sorafenib	B-RAF, PDGF, FLT, KIT, VEGF and Mcl-1		22698419
Deubiquitinase inhibitor [78]	WP1130	Bcr-Abl compartmentalization, inhibits USP9X (stabilize Mcl-1)	Pre-clinical	17202319
Antisense inhibitors	Oblimersen sodium	Bcl-2	Phase III	19738118
BH3 mimetics	ABT-737 (AB-263)	Bcl-2, Bcl-X_L, Bcl-w	Clinical trials	22821746
	Gossypol (AT-101)	Bcl-2, Bcl-X_L, Bcl-w, Mcl-1		21918390
	Apogossypol (ApoG2)	Bcl-2, Bcl-X_L, Mcl-1		18769131
	Obatoclax (GX-15-070)	Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 Bcl-2		22333598
	HA-14	Bcl-X_L		19228717
	BH3Is	Bcl-2, Bcl-X_L, Mcl-1		16951185
	TW-37	Bcl-2, Bcl-X_L, Mcl-1, Bfl-1		21780116
	Sabutoclax (BI-97C1)	Bcl-2, Bcl-X_L, Mcl-1, Bfl-1	Preclinical	22655238
	BI-97D6	Bcl-2, Bcl-X_L, Mcl-1		22931411 22655238
	BH-3 M6	Bcl-2, Bcl-X_L, Mcl-1		21148306

Abbreviations: CdK- cyclin-dependent kinase; PDGF: platelet derived growth factor; VEGF-Vascular endothelial growth factor

Table 2

Completed Combination clinical trials

Bcl-2 inhibitor	Other drug	Tumor type	Phase
Flavopriridol	Cytarabine, Mitoxantrone	AML	Phase II
	Vironostat	Advanced adult solid tumors	Phase I
	Oxaliplatin, Fluorouracil, Leucovorin	Advanced adult solid tumors	Phase I
Oblimersen	Dacarbazine	Advanced Melanoma	Phase III
	Fludarabine/Rituximab, Cyclophosphamide	CLL	Phase III
	Dexamethasone	MM	Phase III
	Ara C/ Daunorubicin	AML	Phase II
	Carboplatin/ etoposide	SCLC	Phase II
	Docetaxel	HR-Prostate cancer	Phase II
	IFNα	Renal cancer	Phase I/II
	Doxorubicin	Hepatocellular	Phase I/II
AT101	Temozolomide	Brain and CNS tumors	Phase I
	Lenalidomide	ALL, chronic B-cell Leukemia	Phase I/II
	Rituximab	CLL, Follicular lymphoma	Phase II
	Docetaxel	Prostate Cancer, NSCLC	Phase II
	Erlotinib	EGFR mutant lung cancer	Phase II
	Topotecan	SCLC	Phase II
Gossypol	Paclitaxel, Carboplatin	Lymphoma	Phase I
	Cisplatin, Etoposide	SCLC	Phase I/II
	Docetaxel, Prednisone	HR-Prostate cancer	Phase I
ABT-737	Platinum	Ovarian cancer	
Obatoclax	Etoposide	Extensive stage-SCLC MCL, Hodgkin's or Non	Phase I/II
	Bortezomib	Hodgkins lymphoma, MM	Phase II
	Docetaxel	NSCLC	Phase II
	Rituximab, Bendamustine	Lymphoma	Phase II

This data is adopted from www.clinicaltrials.gov.

Abbreviations: AML Acute Myelocytic leukemia; CLL Chronic Lymphocytic leukemia; MM Multiple Myeloma; SCLC small cell lung cancer; HR hormone refractory; NSCLC- Non-Small cell Lung cancer; CNS-Central Nervous System; MCL-Mantle cell lymphoma.