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Targeting Mcl-1 for the therapy of cancer

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Abstract

Introduction—Human cancers are genetically and epigenetically heterogeneous and have the capacity to commandeer a variety of cellular processes to aid in their survival, growth and resistance to therapy. One strategy is to overexpress proteins that suppress apoptosis, such as the Bcl-2 family protein Mcl-1. The Mcl-1 protein plays a pivotal role in protecting cells from apoptosis and is overexpressed in a variety of human cancers.

Areas covered—Targeting Mcl-1 for extinction in these cancers, using genetic and pharmacological approaches, represents a potentially effectual means of developing new efficacious cancer therapeutics. Here we review the multiple strategies that have been employed in targeting this fundamental protein, as well as the significant potential these targeting agents provide in not only suppressing cancer growth, but also in reversing resistance to conventional cancer treatments.

Expert Opinion—We discuss the potential issues that arise in targeting Mcl-1 and other Bcl-2 anti-apoptotic proteins, as well problems with acquired resistance. The application of combinatorial approaches that involve inhibiting Mcl-1 and manipulation of additional signaling pathways to enhance therapeutic outcomes is also highlighted. The ability to specifically inhibit key genetic/epigenetic elements and biochemical pathways that maintain the tumor state represent a viable approach for developing rationally based, effective cancer therapies.

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1. Apoptosis and the Bcl-2 family of proteins

Apoptosis is a biological process that is integral to normal physiological functions and maintenance of homeostasis in an organism. The cell's ability to undergo apoptosis is a consequence of a vast array of complex cellular processes that involve multiple proteins. Apoptosis can occur through two distinct, but interrelated, pathways: the extrinsic pathway of apoptosis or the intrinsic/mitochondrial pathway of apoptosis (Figure 1). The extrinsic pathway involves activation of cell surface death receptors (Fas, TNFR) by extracellular ligands such as FasL or TNF. Activation of any of the death receptors results in cleavage and activation of caspase-8, leading to a signaling cascade that culminates in death of the cell. The intrinsic pathway, which can be initiated by a variety of stress signals, involves permeabilization of the outer membrane of the mitochondria, which leads to cytochrome c release. Once released, cytochrome c binds to Apaf-1 and forms the apoptosome, which results in cleavage and activation of caspase-9 and, ultimately, cell death (1). This mitochondrial pathway is controlled primarily by the complex interactions of the Bcl-2 family of proteins.

Bcl-2 is the founding member of this family of proteins and was discovered in studies of B-cell lymphoma. The proteins in this family share certain sequence homology via the presence of Bcl-2 homology (BH) domains. There are four BH domains that exist in this family and each member has at least one. The family is divided into two groups: one group that has pro-apoptotic effects and one group that has anti-apoptotic effects. The pro-apoptotic group is further divided into two subgroups: one group containing proteins such as Bax and Bak and a second group containing proteins including Noxa, PUMA, Bim, and Bid. The latter group is often referred to as the BH3 only proteins, as the members of this subgroup share sequence similarity to the rest of the family only through their BH3 domain. The anti-apoptotic group includes the proteins Bcl-2, Mcl-1, Bcl-X_L, Bfl-1/A1 and Bcl-w (2).

Apoptosis through the intrinsic pathway is imminent when mitochondrial outer membrane permeabilization (MOMP) occurs. This process arises as the result of the formation of homo/heterodimers of the pro-apoptotic proteins Bax and Bak. The other two groups of proteins in this family ultimately regulate apoptosis by either promoting or inhibiting this dimerization. The Bcl-2 family of proteins does this through physical interactions with each other. Two different models have been proposed to describe precisely how this process might occur (Figure 1).

The first scheme is an 'indirect activation model'. In this model, the anti-apoptotic proteins bind to Bax/Bak and block dimerization. The BH3 only proteins exert their pro-apoptotic actions by binding to the anti-apoptotic proteins, thereby displacing Bax and Bak. Free Bax and Bak are now free to form dimers resulting in MOMP. The second theory is a 'direct activation model' that is somewhat more complicated. In this model, the BH3 only proteins are divided into sensitizer and activator proteins. In this model, Bax/Bak oligomerization occurs when the activator BH3 only proteins bind to and activate Bax/Bak. The goal of the anti-apoptotic proteins is to bind the activator proteins and prevent them from binding and activating Bax/Bak. Accordingly, the sensitizer BH3 only proteins bind the anti-apoptotic proteins and prevent them from interacting with the activator BH3 only proteins (3). Ultimately, it is likely that these two models may both occur, since apoptosis regulation is all about balance. At a given point in the life of a cell, the ratio of pro-apoptotic to anti-apoptotic proteins dictates whether the cell will survive or undergo apoptosis. When the pro-apoptotic proteins are expressed in greater quantity, the balance is pushed toward apoptosis. However, when the ratio favors the anti-apoptotic proteins, cell survival will be the net consequence.

2. Mcl-1 (myeloid cell leukemia-1)

Mcl-1 was the second member of the Bcl-2 family discovered. Kozopas *et al.* (4) were investigating the human myeloid leukemia cell line, ML-1. Their goal was to differentiate ML-1 cells into monocytes/macrophages and identify genes whose expression was increased during this differentiation process. One of the early induction genes identified through this strategy was named Mcl-1 (myeloid cell leukemia-1) and was shown to have sequence similarity to the previously identified protein, Bcl-2. Based on this similarity and its mode of isolation, Mcl-1 was predicted to play significant roles in cell differentiation and death (4). Future studies showed that c-myc-induced apoptosis in CHO cells was delayed by Mcl-1 overexpression, illustrating that Mcl-1 indeed contributed to the cell death process (5).

Since its discovery in 1992, studies of Mcl-1 have revealed this to be an intriguing protein, which performs a fundamental role in cell physiology. Mcl-1 is an anti-apoptotic member of the Bcl-2 family of proteins and contains three BH domains. This is unlike the other anti-apoptotic members, which contain four BH domains. Despite this fact, it is the largest of the proteins, containing 350 amino acid residues (6). Mcl-1 is also different than other pro-survival proteins due to its N-terminus, which is larger than that of other Bcl-2 family proteins and has been shown to affect Mcl-1's function and localization (7). It also contains two proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) sequences (4) which may account for its relatively short half life (i.e., 2–3 hr), as these regions have been shown to target proteins for degradation (8).

The structural differences seen between Mcl-1 and the other prosurvival Bcl-2 proteins account for some of the differences seen in binding partners among these proteins. The anti-apoptotic proteins all generally function in the same way as described above, but the pro-apoptotic proteins with which they specifically interact differ. For example, Mcl-1 binds with high affinity to the BH3-only protein Noxa, but does not bind to Bad. However, Bcl-2 binds well with Bad, but not as well with Noxa (6).

Mcl-1 retains an alpha helical fold, similar to the other proteins, but differs in the exposed surface of its binding groove. In addition to simply having different residues, this groove is more electropositive than the other anti-apoptotic proteins. It is flanked on either side by positive electrostatic potential and contains multiple histidine residues. Proteins like Bcl-X_L, however, have grooves that are almost completely uncharged. Furthermore, there are differences in the positions of some surface helices of Mcl-1, adding to the differences between this protein and its other family members (9). These structural differences become very important in the development of inhibitors of these proteins, which is discussed later in this review.

Mcl-1 is regulated at multiple levels, including transcriptional and translational. Transcriptionally, Mcl-1 expression can be induced by a variety of cytokines and signaling pathways, including the PI3K/AKT, Stat-3, and p38/MAPK pathways (10). Additionally, alternative splicing of Mcl-1 results in two protein isoforms, Mcl-1_L and Mcl-1_S. Interestingly, the Mcl-1_S isoform behaves in a manner opposite to that of the normal protein, functioning to promote apoptosis, much like the BH3 only proteins (11). At the translational level, many studies have highlighted the role of microRNAs in Mcl-1 regulation. Mott *et al.* (12) identified mir29b as targeting Mcl-1 in studies comparing cholangiocarcinoma cells to normal cholangiocytes. Additional studies have implicated this microRNA in Mcl-1 regulation in other cell types as well (13, 14).

Mcl-1 also contains multiple phosphorylation sites in its PEST region. Multiple proteins resulting in different outcomes mediate the phosphorylation of these sites. Thr¹⁶³ can be phosphorylated by ERK leading to the increased half-life of the protein (15). Conversely,

GSK-3 mediated phosphorylation of Ser¹⁵⁹ leads to increased ubiquitination and degradation of Mcl-1 (16). As previously noted, Mcl-1 has a very short half-life. This is due to its rapid degradation in the cell, primarily through the proteasome. Proteasome-mediated degradation of Mcl-1 can occur through a variety of mechanisms, GSK-3-mediated phosphorylation representing the most highly investigated of these. Mcl-1 Ubiquitin Ligase E3 (MULE) has been shown to ubiquitinate Mcl-1 on 5 different lysine residues and be the primary mediator of normal Mcl-1 degradation (17). Additionally, Mcl-1 can be targeted for degradation through interactions with additional proteins such as Noxa and the tumor suppressor SCF^{FBW7} (10, 18).

3. Role of Mcl-1 in Cancer

Mcl-1 is highly expressed in a variety of human cancer cell lines including breast, CNS, colon, lung, ovarian, prostate, renal, and melanoma (Table 1)(19). Human melanoma cell lines express Mcl-1 protein, though they do not show increased expression when compared to normal melanocyte controls. However, studies done in paraffin sections of benign nevi, primary melanoma, and metastatic melanoma showed an increase in Mcl-1 expression that correlated with melanoma progression (20, 21).

Mcl-1 overexpression is exploited by cancer cells to evade cell death, i.e. to survive, and as a mechanism for developing resistance to diverse chemotherapeutic agents. The other anti-apoptotic members of the Bcl-2 family of proteins are also frequently overexpressed in cancers, although they will not be discussed in this review. Although overexpression of Mcl-1 does not result in increased cell proliferation, its ability to suppress apoptosis is an important contributor to the transformed state. As noted earlier, apoptosis regulation is based on the balance between pro-and anti-apoptotic proteins within cells. The ability of a cancer cell to shift the balance toward survival provides it with a major advantage in protecting itself from toxic factors. The clinical implications of this phenomenon may be manifested by chemoresistance. For example, while chemotherapeutic agents operate through a variety of mechanisms, many of them are believed to act through initiation of apoptosis in a cell. Consequently, protection from apoptosis via overexpression of Mcl-1 may represent a significant barrier to the effectiveness of chemotherapeutic agents.

Dysregulation of Mcl-1 is an important genomic change in diverse cancers, many of which become dependent upon this protein for survival as well as resistance to chemotherapy. Indeed, numerous reports have documented the importance of this protein in transformed cell survival. Thallinger *et al.* (22) reported that Mcl-1 antisense oligonucleotides (ASO) resulted in decreased Mcl-1 expression in cell lines *in vitro* and in tumors *in vivo*, and that Mcl-1 ASO treatment of SCID mice with melanoma subcutaneous tumors resulted in tumor sensitization to the chemotherapeutic drug, DTIC, accompanied by increased levels of apoptosis in tumor cells (22). In a study of head and neck cancer, twenty-six tumor samples from patients with locally advanced head and neck cancer showed a 92% increase in Mcl-1 expression (23). Mcl-1 ASO treatment of a head and neck squamous cell carcinoma cell line promoted cytotoxic synergy when combined with chemotherapeutic agents, paclitaxel and cetuximab (24). Additionally, Mcl-1 is overexpressed in hepatocellular carcinoma (HCC). Sieghart *et al.* (25) showed that human tumor specimens overexpressed Mcl-1. They went on to show that the use of Mcl-1 ASO induced apoptosis in HCC cells and sensitized them to cisplatin (25). Additionally, Mcl-1 ASO decreased cell viability and induced apoptosis in multiple human myeloma cell lines as well as two primary myeloma cell lines. This result was not seen following Bcl-2 or Bcl-X_L ASO treatment (26). Pancreatic adenocarcinoma is another cancer that overexpresses Mcl-1. Studies have shown that knockdown of Mcl-1 in a pancreatic cancer cell line leads to decreased cell viability and colony formation *in vitro* and decreased tumor size and weight in xenograft models. Furthermore, loss of Mcl-1 sensitized

these resistant cells to the current first line treatment for pancreatic cancer, Gemcitabine (27).

Mcl-1 has been shown to play important roles in lymphomas and leukemias as well. In mantle cell lymphoma, Mcl-1 overexpression correlated with high-grade morphology, high levels of proliferation, and p53 overexpression (28). Additionally, mice expressing a Mcl-1 transgene were shown to develop B cell lymphomas at a frequency of 85% over a period of 2 years (29). In both acute and chronic leukemias, Mcl-1 has been demonstrated to be extremely important for cancer survival, with siRNA knockdown of Mcl-1 leading to apoptosis in cell lines. Furthermore, patients who show resistance to Rituximab, an anti-CD20 antibody used to treat B-cell malignancies, exhibit increased levels of Mcl-1. Mcl-1 knockdown has also been demonstrated to sensitize cells to Rituximab treatment (30).

4. Strategies employed in targeting Mcl-1

There have been numerous approaches designed to target Mcl-1. Some of these schemes focus primarily on Mcl-1, but the majority of strategies target multiple anti-apoptotic proteins in the Bcl-2 family. Here we review the most significant inhibitors developed to date.

4.1 Drugs that result in Mcl-1 Downregulation

There are multiple drugs that, despite not being designed to specifically target Mcl-1, display a mechanism of action that involves downregulation of Mcl-1. Studies with these drugs provide continued evidence of the importance of this protein in maintenance and progression of the cancer phenotype.

Cyclin-dependent kinase inhibitors

4.1.1 Flavopiridol—Flavopiridol is a semisynthetic flavonoid that functions as a cyclin-dependent kinase inhibitor by competing for the kinase active site. It is able to inhibit the activity of multiple CDKs and results in G₁/S and G₂/S cell cycle arrest (31). Flavopiridol also globally decreases transcription levels in the cell *via* inhibition of P-TEFb and it can also bind double stranded DNA (32). However, one of the more interesting observations seen with Flavopiridol was its ability to downregulate Mcl-1 protein levels in B-cell chronic lymphocytic leukemia cells (33). Notably, significant activity was observed in patients with high-risk CLL treated with a hybrid infusional flavopiridol schedule. This new dosing regimen included a 30-minute loading dose prior to 4 hours of infusion given weekly for 4–6 weeks. A group of 42 patients with refractory CLL were studied and of those 42, 45% showed a partial response. Importantly, genetically high-risk patients showed a 42% response rate in patients with del(17p13.1) and a 72% response rate in patients with del(11q22.3)(34).

Further studies in breast cancer showed that Flavopiridol treatment synergistically enhanced the toxicity of the ERBB1/ERBB2 inhibitor Lapatinib in a Mcl-1-dependent manner. The combination of Lapatinib and Flavopiridol increased the rate of reduction of Mcl-1 protein compared to Flavopiridol alone. Moreover, overexpression of Mcl-1 induced resistance to this combination (35). In studies involving human leukemia cells, flavopiridol interacted synergistically with the HDAC inhibitor vorinostat through a mechanism involving Mcl-1 down-regulation (36).

Flavopiridol is the first cyclin-dependent kinase inhibitor to enter clinical trials. It has been studied in a variety of cancer types and has shown moderate effects in some neoplastic diseases. Dose-related toxicities, which include neutropenia and diarrhea, have limited some studies (32, 37). Interestingly, one study looking at a combination of Flavopiridol and

Cisplatin/Carboplatin in solid tumors suggested that the lack of significant activity seen may be the result of the failure to achieve decreases in Mcl-1 levels in tumors (32). Nevertheless, there is preclinical evidence of synergism between flavopiridol and proteasome inhibitors associated with Mcl-1 down-regulation (38, 39).

4.1.2 SNS-032—Another cyclin-dependent kinase inhibitor, SNS-032 was originally identified in a screen developed to identify specific inhibitors of Cdk2. This compound has since been shown to also inhibit Cdk7 and Cdk9 as well. SNS-032, like Flavopiridol, also shows strong cytotoxic effects *in vitro* and results in decreases in anti-apoptotic proteins such as Mcl-1 (40). Unfortunately, this compound exhibited disappointing results when evaluated in clinical trials (41).

4.1.3 Sorafenib—Sorafenib is a multi-kinase inhibitor that was originally developed as an inhibitor of B-Raf, but was subsequently shown to inhibit multiple other kinases, including PDGFR, FLT, Kit, and VEGFR. In human leukemia cells, pharmacologically achievable concentrations of sorafenib induced apoptosis through a mechanism involving Mcl-1 down-regulation (42, 43). This has been found to be due to inhibition of translation resulting from sorafenib-induced ER stress (44).

Deubiquitinase Inhibitors

4.1.4 WP1130—As discussed previously in this review, Mcl-1 is degraded rapidly in the cell via a proteasome-dependent pathway. Blocking the proteasome can lead to increased levels of Mcl-1 protein. Mcl-1 can also be rescued from degradation by deubiquitinases (DUBs), which are able to remove ubiquitin from ubiquitinated Mcl-1. In a study investigating interactions between Mcl-1 and other proteins in the cell, Schwickart *et al.* (45) found that one of the proteins that co-immunoprecipitates with Mcl-1 is the DUB USP9X. This group hypothesized that this interaction might lead to increased stability of Mcl-1 and play an important role in cancers that overexpress Mcl-1. Indeed, they showed that USP9X, in addition to Mcl-1, was overexpressed in samples of ductal adenocarcinoma, colon adenocarcinoma, and small cell lung cancer. Additionally, USP9X overexpression was found to correlate with poor prognosis in multiple myeloma. Knockdown of USP9X via siRNA resulted in decreased Mcl-1 protein levels, but not RNA levels, supporting the suggestion that USP9X knockdown results in increased Mcl-1 degradation. This knockdown also led to increased sensitivity of colon carcinoma and leukemia cells lines to ABT-737, a compound discussed later in this review, which are usually resistant to this agent (45).

WP1130 is a small molecule that has multiple anti-cancer effects. It has been shown to induce removal of Bcr-Abl from the cytoplasm into aggresomes, where it is not able to facilitate oncogenic signaling. Additionally and most interesting for this review, it directly inhibits USP9X. When used as a treatment in CML, WP1130 results in decreased levels of Mcl-1, compartmentalization of Bcr-Abl, and the induction of apoptosis (46).

4.2 Antisense oligonucleotide (ASO) treatment

Despite the fact that there are many studies looking at the effects of Mcl-1 ASO treatment on cancer *in vitro* and in animal models *in vivo*, there is no report of a Mcl-1 ASO treatment being effectively translated into the clinic. This is likely due to the issues that can arise with the use of ASO treatment in patients. In the human body, ASO have an extremely short half-life, which makes achieving sufficient blood concentrations difficult. Additionally, DNases can degrade them, further contributing to problems with stability. Delivery of ASO is also an issue, as it is difficult to direct ASO to their specific cellular target (47).

4.3 BH3 Mimetics

The concept of BH3 mimetics has been one of the most promising translational strategies. As discussed, pro-apoptotic or anti-apoptotic effects arise, ultimately, as a result of physical interactions between the anti-apoptotic Bcl-2 protein and the BH3 proteins. The anti-apoptotic proteins contain a hydrophobic binding pocket made from the folding of their BH1, BH2, and BH3 domains. The BH3 domain of the BH3 only proteins fits into and binds this hydrophobic pocket (48). Based on this modeling prediction, small molecules have been developed that mimic the BH3 domain and therefore are able to fit into the hydrophobic pocket of the anti-apoptotic proteins and block their ability to bind pro-apoptotic proteins, inhibiting their function. Some of these mimetics have been developed through structural studies and others through screening studies. Regardless of how these drugs were discovered, they represent a novel and exciting new strategy in cancer therapeutic development.

4.3.1 ABT-737—One of the most successful and well studied BH3 mimetics, ABT-737, was discovered using NMR-based screening and demonstrated strong binding affinity to the Bcl-2 family anti-apoptotic proteins Bcl-2, Bcl-XL, and Bcl-w. ABT-737 does not bind to Mcl-1 or Bfl-1. This difference in affinity is due to differences in the structures of these proteins as was described previously in this review. Initial studies showed that ABT-737 was effective in inducing cytotoxicity as a single agent in follicular lymphoma and small cell lung carcinoma (SCLC) cell lines. Additionally, it was found to enhance the lethality of paclitaxel in NSCLC (non small cell lung carcinoma) (49). Subsequently, ABT-737 has been widely studied in a variety of cancers. It has been shown to induce cytotoxicity both *in vitro* and *in vivo* in leukemia and lymphoma, glioblastoma, multiple myeloma, and SCLC (50–54). Despite many promising studies in these cancer types, there remain a large percentage of cancers that are resistant to ABT-737. This resistance has been shown to stem from an overexpression of Mcl-1, one of the anti-apoptotic proteins to which ABT-737 does not bind. Even within cancers that are sensitive to ABT-737, specific cell lines that express higher levels of Mcl-1 display increased resistance to this compound (50). Mcl-1-dependent ABT-737 resistance has been consistently shown in the literature and multiple studies document that downregulation of Mcl-1 through a variety of mechanisms is able to induce sensitivity to ABT-737 (55–58). Notably, the CDK inhibitor roscovitine dramatically increased ABT-737 lethality in human leukemia cells through a mechanism involving Mcl-1 down-regulation (59).

Phase I/II clinical trials with ABT-263, an orally available form of ABT-737 with a longer half-life, are in progress in patients with SCLC, leukemia, and lymphoma. Thus far, studies in chronic lymphocytic leukemia (CLL) and SCLC have shown this drug to be well tolerated, with some potentially manageable toxicities including back pain, nausea/vomiting, diarrhea, and thrombocytopenia (60, 61). Although trials are ongoing, initial results look promising, with some CLL patients showing response to treatment and possible nodal regression (60).

4.3.2. Obatoclax (GX15)—Obatoclax is another example of a BH3 mimetic. This mimetic differs from ABT-737 in that in addition to targeting Bcl-2, Bcl-XL, and Bcl-w, it also binds to and inhibits Mcl-1 and Bfl-1. Studies have shown that this compound induces cytotoxicity in a number of cancer types including, but not limited to, NSCLC and a variety of leukemias. Additionally, it has shown synergy when combined with various conventional chemotherapeutic agents (35, 62–65). Obatoclax also serves to again illustrate the importance of Mcl-1 in cancer. Studies have shown that this compound's cytotoxic effects are partially mediated through specific effects on Mcl-1. Obatoclax can disrupt the constitutive Bak-Mcl-1 interaction on the outer mitochondrial membrane (66). This

compound has advanced to clinical trials and Phase I studies in a variety of cancer types have shown it to be fairly well tolerated, with neurologic toxicities being the most common adverse event seen. Some evidence of efficacy was seen in these studies, though ongoing Phase II trials will ultimately determine the effectiveness of this agent (67–69).

4.3.3 Gossypol—Gossypol is a polyphenolic aldehyde derived from cottonseed. Original studies with this extract focused on its ability to function as a male contraceptive (70). However, it has also been shown to have potent anti-cancer activity. Gossypol functions as a typical BH3 mimetic and binds to and inhibits the Bcl-2 anti-apoptotic proteins Bcl-2, Bcl-X_L, and Mcl-1 (71). Many groups have shown Gossypol to exhibit anti-carcinogenic effects toward a wide variety of cancer types both *in vitro* and *in vivo*, including, but not limited to, breast, prostate, glioma, and colon cancer (70, 72–75). LeBlanc *et al.* (76) also demonstrated Gossypol's ability to inhibit growth of adrenal gland carcinoma cells, medullary thyroid carcinoma cells, ovarian carcinoma cells, and endometrial carcinoma cells. Compared to ABT-737, Gossypol displays toxicity against a much wider array of cancer types, possibly due to its ability to target Mcl-1. This compound is currently in Phase II clinical trials as a single-agent in patients with advanced malignancies (71). Clinical trials are also ongoing in evaluating Gossypol in combination therapy with conventional chemotherapeutics (77).

The presence of two reactive aldehyde groups on Gossypol, combined with initial clinical trial results that showed difficulty in achieving sufficient Gossypol blood concentrations, prompted groups to create Gossypol derivatives. Apogossypol was the first derivative designed and showed better efficacy and less toxicity than its parent compound (78). To date, there have been many such derivatives created with varying binding affinities to the Bcl-2 anti-apoptotic proteins.

4.3.4 Sabutoclax (BI-97C1)—Sabutoclax (BI-97C1) is one of the newest Apogossypol derivatives developed by Wei *et al.* (71, 79). This novel compound binds to the Bcl-2 anti-apoptotic proteins Bcl-2, Mcl-1, Bcl-X_L, and Bfl-1. It was originally identified for its ability to bind Bcl-X_L with low to submicromolar binding affinity. Competitive fluorescence polarization assays showed that BI-97C1 inhibited Bcl-X_L with an IC₅₀ value of .31 μM. Further assays showed that this compound also had displacement activity against Bcl-2 (IC₅₀ = 0.32 μM), Mcl-1 (IC₅₀ = 0.20 μM), and Bfl-1 (IC₅₀ = 0.62 μM) (79).

BI-97C1 (Sabutoclax) induced apoptosis in the large B-cell lymphoma cell line, BP3, which expresses high levels of Mcl-1 and Bfl-1 and is resistant to ABT-737. Furthermore, while toxic to wild type mouse embryonic fibroblasts, this compound showed only slight toxicity against BAX/BAK double knockout MEF cells. This provided evidence that BI-97C1 (Sabutoclax) only has minimal off-target effects. Additionally, as BI-97C1 (Sabutoclax) showed efficacy against a cell line that overexpressed Mcl-1, some earlier compounds like ABT-737 did not, it was evaluated in the context of a prostate cancer xenograft involving M2182 cells, which rely on Mcl-1 for survival. A dose of 3 mg/kg induced nearly 60% tumor inhibition, while a dose of 5 mg/kg nearly eliminated the tumors (79).

BH3-M6

This BH3 mimetic is a very new compound designed to inhibit Bcl-2, Bcl-X_L, and Mcl-1. It has been shown to disrupt the interactions between these proteins and the pro-apoptotic BH3 only proteins. In the lung adenocarcinoma cell line, A549, this compound induced apoptosis through cytochrome c release from the mitochondria. Preliminary studies, however, showed high doses of this compound (doses of 25 and 50 μM) were needed to induce apoptosis. Additional studies with this compound in other cancer types will be useful in determining the efficacy of this new BH3 mimetic (80).

5. Combination therapies

Ultimately, the real potential of these compounds may lie in combination therapies. Many of these Bcl-2 family inhibitors show effectiveness as single agents, albeit only moderate efficacy. Therefore, understanding the mechanism of action of these agents provides an entry point for developing successful combinations with other anti-cancer agents. A major impediment to successful cancer therapy is inherent and acquired drug resistance. There are some very good drugs, like Gleevec and Cisplatin, being used in patients that have shown great success. However, many patients unfortunately develop resistance to these cancer agents, rendering them ineffective.

Many conventional chemotherapeutic drugs, despite having different mechanisms of action, ultimately result in apoptotic cell death (81). In many cases of resistance, both intrinsic and acquired, cancer cells rely on the overexpression or upregulation of pro survival genes, like the anti-apoptotic members of the Bcl-2 family of proteins. Thus, the potential for a group of compounds, like BH3 mimetics, that target this group of proteins, is significant. If one is able to use such compounds to relieve the block in cancer cell apoptosis that the anti-apoptotic proteins convey, there is now the clinical potential for cancers to become sensitized to additional novel therapeutics or have their sensitivity restored to conventional chemotherapies. Additionally, a potential problem with combination treatment is that these compounds also exhibit single agent toxicities, which may augment the cytotoxic effects seen in combination therapies.

One of the major limitations of ABT-737 is that in ABT-737 resistance cells it caused the levels of Mcl-1 expression to rapidly increase through protein stabilization (82), probably by stabilizing the interaction between USP9X and Mcl-1 (83). Accordingly, combination strategies have now been designed by suppressing Mcl-1 with an agent that synergizes with ABT-737 to sensitize the resistant cells. In ABT-737-resistant cancer cells, the interaction between USP9X and Mcl-1 (83), which was increased by ABT-737 treatment, could be disrupted by gemcitabine, thus resulting in enhanced ubiquitination and the subsequent degradation of Mcl-1 ultimately provoking synergism of this two drug combination (83). Similarly, in HCC the combination of Sorafenib (84), in small lung cancer cell lines Actinomycin D (85), and in prostate cancer cells bortezomib (86) (which are known inhibitors of Mcl-1) and ABT-737 synergize in ABT-737 resistant cells.

A successful application of combination therapy was shown recently in a study using the combination of BI-97C1 (Sabutoclax) and the anti-cancer cytokine *mda-7/IL-24* (87). *mda-7/IL-24* a unique member of the IL-10-related cytokine gene family, exhibiting nearly ubiquitous antitumor properties *in vitro* and *in vivo* (88–90), which culminated in its successful entry into the clinic where safety and clinical efficacy was achieved (91). Unique therapeutic aspects of adenovirus delivered *mda-7/IL-24* (Ad.*mda-7*) lie in its selective induction of apoptosis in cancer cells, profound antitumor “bystander” effect exerted by the secreted MDA-7/IL-24 protein, and the ability of this unique cytokine to inhibit angiogenesis and provoke a potent antitumor immune response (92, 93). Upon ectopic expression of *mda-7/IL-24* by an adenovirus, MDA-7/IL-24 interacts with the endoplasmic reticulum (ER) chaperone protein BiP/GRP78 and initiates an unfolded protein response in tumor cells that promotes apoptosis (94). Recently, we reported that in PC, ovarian, and malignant gliomas, *mda-7/IL-24*-induced ER stress response produced apoptosis by suppressing expression of the antiapoptotic protein Mcl-1 (87, 95, 96) (Figure 2). Consequently, the reliance of this cytokine’s mode of action on Mcl-1 provides a unique opportunity for prostate cancer treatment with a combination of *mda-7/IL-24* and a BH3 mimetic. Indeed, studies that combined *mda-7/IL-24* and BI-97C1 (Sabutoclax) showed that this BH3 mimetic sensitized prostate cancer cells to *mda-7/IL-24*-induced lethality (87).

Initial studies in PC-3 prostate cancer cells showed that siRNA mediated Mcl-1 reduction resulted in sensitization of these cells to viral infection with Ad.5/3-*mda-7* (a chimeric adenovirus between serotype 5 and 3 that infects cancer cells in a Coxsackie-adenovirus (CAR)-independent manner), again providing evidence of Mcl-1's role in *mda-7/IL-24* function. As a single agent, BI-97C1 (Sabutoclax) was shown to have an IC₅₀ value in PC-3 cells of 750 nM, compared to a value of 12μM seen with ABT-737 treatment (Figure 3). Profound induction of apoptosis was seen upon combination treatment of BI-97C1 (Sabutoclax) and Ad.5/3-*mda-7* in PC-3, DU-145, and M2182 prostate cancer cells. Notably, treatment of normal immortal P69 prostate epithelial cells showed no toxicity (87). DU-145 cells, which are sensitive to *mda-7/IL-24*, become resistant when they overexpress Bcl-2, Bcl-X_L, or Mcl-1. Interestingly, these stable cell lines showed restored sensitivity to *mda-7/IL-24* when treated with BI-97C1 (Sabutoclax). Perhaps the most compelling aspect of these studies, however, are results obtained *in vivo*. Initial *in vivo* studies were done with xenografts of the human prostate cancer cell line, M2182. (Figure 4). Further studies were conducted in an immune competent Hi-Myc mouse model that develops prostate cancer spontaneously. These Hi-Myc mice were treated with *mda-7/IL-24* using a novel microbubble delivery approach (87, 97, 98), wherein the Ad.5/3-*mda-7* virus was packaged inside of microbubbles, which were treated with complement and were then intravenously injected into the mouse. Ultrasound of the prostate resulted in release of the virus in the prostate area, providing a systemic targeted viral delivery approach. BI-97C1 (Sabutoclax) was administered via intraperitoneal (IP) injection throughout the study. Again, tumor size was very much diminished in the combination therapy group and examination of tumor sections revealed decreases in Ki-67 expression and increases in apoptosis via TUNEL staining (Figure 4) (87).

6. Expert Opinion

From a clinical perspective, there is significant potential in targeting Mcl-1 for suppression. The Bcl-2 anti-apoptotic proteins continue to present roadblocks in the treatment of cancers when using conventional chemotherapeutics. Their overexpression becomes vital to the survival of many types of tumors. Mcl-1, in particular, plays a significant role in the ability of cancers not only to survive, but also to resist treatment with chemotherapy and radiation. *In vitro* experiments using Mcl-1 ASO emphasize the anti-tumor potential of targeting Mcl-1 both alone and in combination with other therapeutic agents.

Developing an optimal cancer therapeutic for any tumor indication is clearly an imprecise process and very difficult. To achieve this objective, a number of issues must be considered one of which is the best approach to use. What technique would be most appropriate to target Mcl-1? Since the ultimate aim is to develop a viable clinical therapy, it is important to consider the translational potential of any strategy. Some stratagems, like those that employ ASO, may be very effective in *in vitro* and preclinical experimental settings, but are not easily translated into the clinic. In contrast, other methodologies, like the use of small molecule antagonists, may hold greater potential for clinical translation if an effective drug level can be attained without unacceptable toxicity.

Another important consideration in developing an appropriate cancer therapeutic is the target itself. There is abundant evidence in almost every cancer type that anti-apoptotic proteins play a significant role to some extent in that cancer's survival. Many studies provide evidence that targeting these proteins individually might be a promising treatment strategy. What is only recently becoming abundantly evident is the importance of interactions between these proteins in both normal and cancer cells. Protein:protein interactions are complex and the interplay between multiple proteins in these complexes and their consequences on cellular phenotype are active areas of investigation. It is also

important that when targeting these proteins to block their interactions off-target toxicity in normal cells and tissues is not promoted.

As discussed previously, the majority of compounds that target Mcl-1 also affect other members of the anti-apoptotic Bcl-2 family of proteins. This reflects structural and functional redundancy in many protein members of this gene family. While some cancers rely more heavily on Mcl-1 (26), others rely on alternate anti-apoptotic members of the family for survival and progression (99). Accordingly, one might try to identify and employ therapies for a particular cancer based on such reliance. Indeed, there have been compounds, such as ABT-737, that do not target all of the anti-apoptotic Bcl-2 proteins. This compound is most effective in cancers that rely on proteins targeted by ABT-737. However, the limitations observed with ABT-737 highlight why targeting of specific anti-apoptotic proteins alone may not promote optimal therapeutic outcome.

In addition to a lack of efficacy against cancers reliant on Mcl-1 and Bfl-1, multiple studies have now shown that, over time, cells sensitive to ABT-737 begin to develop resistance to the drug by upregulating the anti-apoptotic proteins that it does not target, i.e., Mcl-1 and Bfl-1. Yecies *et al.* (82) showed that lymphoma cells subjected to long-term exposure of ABT-737 developed resistance via transcriptional upregulation of Mcl-1 and Bfl-1. They further demonstrated that decreasing Mcl-1 protein levels via multiple mechanisms resulted in restored sensitivity to ABT-737 (82). Previously, a similar phenomenon was also seen in acute myeloid leukemia (AML) cells. This group noticed a positive correlation between Mcl-1 expression and ABT-737 resistance (100). These studies indicate that the Bcl-2 family of anti-apoptotic proteins may provide overlapping compensatory functions when one of the proteins is lost. This supports strategies involving compounds that target all of the anti-apoptotic proteins, as this approach may help prevent the development of resistance mechanisms to BH3 mimetics.

In continuing efforts to develop new and better BH3 mimetics, understanding potential mechanisms of resistance is critical. Resistance remains a significant problem in cancer therapy in general, and understanding how it develops may be of great value in designing approaches to circumvent this major barrier to cure. Considering that the Bcl-2 family of anti-apoptotic proteins plays a critical role in cancer maintenance and resistance, these proteins represent high-priority targets for the next generation of combinatorial therapies for neoplastic diseases.

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Article highlights box

- Mcl-1 is an anti-apoptotic member of the Bcl-2 family of proteins, and plays an integral role in cell survival and apoptosis
- Mcl-1 is frequently overexpressed in a variety of cancers and contributes to cancer cell survival and apoptosis resistance
- Targeting Mcl-1 in cancer therapy has become a promising strategy due to the vast amount of in vitro evidence documenting the pro-apoptotic and chemosensitization effects of Mcl-1 knockdown
- A number of different strategies have been employed to target Mcl-1 from a therapeutic standpoint, including genetic and pharmacological approaches
- BH3 mimetics stand as the most promising tactic to date in targeting Mcl-1 and other Bcl-2 anti-apoptotic proteins and offer an exciting new opportunity in cancer therapeutics
- Combinatorial approaches targeting Mcl-1 and modifying specific signaling pathways holds promise for enhancing therapeutic efficacy in multiple cancers

Figure 1.

Two proposed hypothetical models of the mechanism of action of the Bcl-2 family of proteins. The “indirect activation model” describes a scenario in which the binding of anti-apoptotic proteins inhibits Bax/Bak oligomerization. Displacement of these anti-apoptotic proteins by a BH3 only protein allows dimers to form and apoptosis to occur. In contrast, the “direct activation model” holds that BH3 only proteins are divided into two classes: activators and sensitizers. The activator proteins bind to Bax or Bak, activating them and leading to apoptosis. The anti-apoptotic proteins function in this model by binding to these activator and sensitizing proteins and sequestering them. The BH3 only sensitizers bind to anti-apoptotic proteins in an attempt to displace the activator BH3 proteins. When enough activator BH3 proteins are free, they are able to activate Bax/Bak and induce apoptosis.

Figure 2.
Hypothetical model depicting *mda-7/IL-24*-induced lethality through induction of an ER stress response. [Adapted from Dash et al. (95)]

Figure 3.

The combination of BI-97C1 (Sabutoclax) and *mda-7*/IL-24 cooperate to induce death in prostate carcinoma cells. **A**) PC3 cells were infected with the indicated doses of Ad.5/3-*mda-7* for 6 h and then treated with DMSO or BI-97C1 (Sabutoclax) for 48 h. **B**) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-*mda-7* for 6 h and then treated with indicated concentrations of BI-97C1 (Sabutoclax) for 48 h. **C**) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-*mda-7* for 6 h and then treated with DMSO or 520 nM of BI-97C1 (Sabutoclax) for the indicated time. In **A**, **B** and **C**, % of apoptosis was evaluated. Bars represent S.D. (n = 3). **D**) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-*mda-7* for 6 h and then exposed to the indicated concentrations of BI-97C1 (Sabutoclax) for 48 h after and Western blotting was performed with the indicated antibodies. [Adapted from Dash et al. (87)]

Figure 4.

A combination regimen of *mda-7*/IL-24 and BI-97C1 (Sabutoclax) additively inhibits prostate tumor growth in athymic and immunocompetent mice. **A)** Effect of *mda-7*/IL-24 plus BI-97C1 (Sabutoclax) on the growth of subcutaneous implanted prostate tumor cells in nude mice. M2182-Luc (1×10^6) cells were injected s.c. in the left and right flank of male athymic nude mice. After establishing visible tumors of $\sim 100\text{-mm}^3$, intratumoral injections of Ad.5/3-vec or Ad.5/3-*mda-7* were given to the tumors on the left flank at a dose of 0.5×10^8 pfu in 100 μ l PBS. The injections were given 3X the first week and then 2X a week for two more weeks for a total of seven injections. BI-97C1 (Sabutoclax) (dissolved in ethanol/ Cremophor EL/saline = 10:10:80) was injected i.p. at a sub-toxic level either at 1 mg/kg or 3 mg/kg 3X a week throughout the study (total 9 injections). Tumor growth was visualized by bioluminescence measured by the Xenogen imaging system. **B)** Suppression of tumor development and growth in Hi-Myc mice using a novel ultrasound targeted microbubble destruction (UTMD) approach with complement-treated microbubbles containing Ad.5/3-*mda-7* and administration of BI-97C1 (Sabutoclax) intraperitoneally. The prostatic region of male Hi-Myc mice was sonoporated for 10 min following tail-vein injection of the indicated complement-treated microbubble/Ad complexes. BI-97C1 (Sabutoclax) was administered intraperitoneally at a dose of 3 mg/kg 3X a week for the duration of the study (total 9 injections). At the end of the experiment the mice were sacrificed and the prostates were harvested, weighed and photographed. **C)** Immunohistochemistry of tumors from combination therapy treated Hi-Myc mice. Hi-Myc mice were treated as described in panel B, paraffin-embedded sections obtained from the prostate were analyzed by immunohistochemistry using anti-Ki-67 and anti-MDA-7/IL-24 antibodies. Apoptosis in the prostate sections was detected by TUNEL assay.[From Dash et al. (87)]

Table 1

Human cancers that overexpress the Mcl-1 protein

Mcl-1 Expression in Human Cancer	
Cancer Type	Reference
Breast	(19)
Ovarian	(19)
Renal	(19)
Prostate	(19)
Melanoma	(19, 20, 21)
Pancreatic	(27)
Hepatocellular Carcinoma	(25)
Head and Neck	(23, 24)
Multiple myeloma	(26)
Colon	(19)
Lung	(19)
Leukemia (moderate)	(19)
Lymphoma (moderate)	(19)

Table 2

Current drugs that target the Bcl-2 family of proteins

Drug Name	Drug Description	Drug Targets	Stage of Study
Flavopiridol	Cyclin-dependent kinase inhibitor	Multiple cyclin-dependent kinases (leads to Mcl-1 downregulation)	Clinical trials
SNS-032	Cyclin-dependent kinase inhibitor	Cdk2, 7, 9 (leads to Mcl-1 downregulation)	Clinical trials
Sorafenib	Multi-kinase inhibitor	Includes B-raf, PDGFR, FLT, Kit, and VEGFR (leads to Mcl-1 downregulation)	Clinical trials
WP1130	Deubiquitinase inhibitor (DUB)	USP9X (Mcl-1 stabilizing protein); induces compartmentalization of Bcr-Abl	Pre-clinical
ABT-737	BH3 Mimetic	Bcl-2, Bcl-X _L , and Bcl-w	Clinical trials
GX15	BH3 Mimetic	Bcl-2, Mcl-1, Bcl-X _L , and Bcl-w	Clinical trials
Gossypol	BH3 Mimetic	Bcl-2, Mcl-1, and Bcl-X _L	Clinical trials
BI-97C1 (Sabutoclax)	BH3 Mimetic	Bcl-2, Mcl-1, Bcl-X _L , and Bfl-1	Pre-clinical
BH3-M6	BH3 Mimetic	Bcl-2, Bcl-X _L , and Mcl-1	Pre-clinical